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Cell Death Regulation in Pathology

Edited by Vincenzo Carafa

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Biochemistry

Volume 67

Aims and Scope of the Series

Biochemistry, the study of chemical transformations occurring within living organisms, impacts all of the life sciences, from molecular crystallography and genetics, to ecology, medicine and population biology. Biochemistry studies macromolecules - proteins, nucleic acids, carbohydrates and lipids –their building blocks, structures, functions and interactions. Much of biochemistry is devoted to enzymes, proteins that catalyze chemical reactions, enzyme structures, mechanisms of action and their roles within cells. Biochemistry also studies small signaling molecules, coenzymes, inhibitors, vitamins and hormones, which play roles in the life process. Biochemical experimentation, besides coopting the methods of classical chemistry, e.g., chromatography, adopted new techniques, e.g., X-ray diffraction, electron microscopy, NMR, radioisotopes, and developed sophisticated microbial genetic tools, e.g., auxotroph mutants and their revertants, fermentation, etc. More recently, biochemistry embraced the 'big data' omics systems. Initial biochemical studies have been exclusively analytic: dissecting, purifying and examining individual components of a biological system; in exemplary words of Efraim Racker, (1913 –1991) "Don't waste clean thinking on dirty enzymes." Today, however, biochemistry is becoming more agglomerative and comprehensive, setting out to integrate and describe fully a particular biological system. The 'big data' metabolomics can define the complement of small molecules, e.g., in a soil or biofilm sample; proteomics can distinguish all the proteins comprising e.g., serum; metagenomics can identify all the genes in a complex environment e.g., the bovine rumen.

This Biochemistry Series will address both the current research on biomolecules, and the emerging trends with great promise.

Meet the Series Editor

Ana Maria Carmona-Ribeiro has been a full professor of Biochemistry at the University of São Paulo (USP), Brazil, since 2001. She founded the Biocolloids Laboratory at USP in 1993, and since then she has been developing novel important assemblies aimed at drug and vaccine delivery. Her background in Physics, Chemistry, Biology, and Pharmaceutics has been useful for the development of biomolecular assemblies with potential for novel biomedical applications.

Meet the Volume Editor

Vincenzo Carafa is an Associate Professor of General Pathology at Università degli Studi della Campania "L. Vanvitelli". He holds a Ph.D. in Signal Transduction Pathology and a Master's in Forensic Genetics. His research focuses on epigenetics and programmed cell death (PCD) mechanisms in cancer, leading to the identification of a novel SIRT inhibitor with tumor-selective properties. He has authored over 70 publications (H-index: 31) and participated in different EU and national projects. Prof. Carafa is active in teaching, mentoring, and editorial roles, serving on the board of Cells (MDPI). He has received multiple research grants and awards for his scientific contributions.

Contents

Preface

Regulated cell death (RCD) constitutes a highly conserved and intricately controlled set of molecular processes that are essential for tissue homeostasis, organismal development, and adaptive responses to a wide range of cellular stimuli. Dysregulation of RCD is a defining feature of diverse pathological conditions, including malignancies, chronic inflammation, neurodegenerative disorders, and musculoskeletal degeneration. Recent advances in the molecular characterization of non-canonical cell death pathways have significantly expanded our understanding of cell fate regulation and revealed novel therapeutic opportunities. Among the various modalities of RCD, apoptosis remains the most extensively characterized, featuring a tightly orchestrated cascade involving initiator and effector caspases, BCL-2 family proteins, and mitochondrial signaling. Apoptosis ensures the immunologically silent elimination of damaged, infected, or oncogenically transformed cells. However, evasion of apoptosis is a hallmark of cancer, contributing to therapeutic resistance and disease progression. Restoring apoptotic sensitivity through pharmacologic modulation—such as nitric oxide donors, pterin-based agents, or statins—has emerged as a promising anticancer strategy. Beyond apoptosis, increasing attention has been directed toward non-apoptotic forms of RCD. Among these, ferroptosis, a distinct form of iron-dependent cell death driven by lipid peroxidation, is particularly relevant in therapy-resistant cancers such as acute myeloid leukemia (AML). The transcription factor p53, classically known for its role in DNA damage responses and apoptosis, also exerts pivotal control over ferroptotic pathways, thereby linking genomic integrity to metabolic regulation of cell death. More recently, the discovery of cuproptosis, a copper-induced and mitochondria-mediated cell death mechanism, has provided novel insights into metal ion toxicity and its pathological role in conditions such as skeletal muscle atrophy. These findings underscore the emerging paradigm that perturbations in trace metal homeostasis can serve as proximal triggers for regulated cytotoxicity and tissue degeneration. The interplay between RCD and the immune system represents a critical axis in both health and disease. Pattern recognition receptors, notably the NOD-like receptor (NLR) family, govern inflammasome activation and modulate the immunological context of cell death, influencing downstream cytokine profiles and immune cell recruitment. The balance between immunogenic and tolerogenic cell death is crucial for preventing autoimmunity while facilitating effective immune surveillance in cancer and infectious diseases. Master transcriptional regulators such as MYC and p53 integrate proliferative and death signals to finely tune cellular responses to stress. MYC not only drives oncogenic proliferation but also sensitizes cells to specific RCD pathways, thereby modulating therapeutic susceptibility and tumor progression. Simultaneously, heat shock proteins such as HSP70 confer cytoprotective functions by stabilizing unfolded proteins and modulating apoptosis, particularly in the context of maintaining cancer stem cells, as observed in breast cancer. Targeting cell death pathways is now considered a cornerstone of innovative therapeutic design. In oncology, combination strategies involving radiotherapy and agents that induce ferroptosis or restore apoptosis hold promise in overcoming resistance. Radiotherapy, in particular, elicits a spectrum of RCD mechanisms—ranging from apoptosis to mitotic catastrophe and necroptosis—necessitating a deeper understanding of its multifaceted

cellular effects. In the neurological domain, emerging evidence suggests that the endocannabinoid system, in conjunction with autophagy, plays a neuroprotective role in neuropathic pain and neuroinflammatory states. Cannabinoid-based compounds thus represent a promising class of multitarget agents capable of modulating neuroimmune responses and cellular stress resilience. Moreover, the role of mitochondrial dysfunction as a central hub linking metabolic stress to apoptotic and non-apoptotic death further highlights the organelle's integral role in disease pathogenesis and therapy response. Mitochondria orchestrate redox balance, bioenergetic signaling, and pro-death factor release, serving as both sensors and effectors of cellular demise. In summary, regulated cell death is a nexus of molecular, metabolic, and immunological signaling with profound relevance across human pathology. A refined understanding of its mechanistic diversity, context-dependent roles, and therapeutic tractability will pave the way for the development of more effective and personalized interventions in cancer, neurodegeneration, inflammatory diseases, and other conditions. The following chapters delve into the specific pathways, molecular regulators, and translational implications of RCD, offering an integrated perspective on its role as both a driver of disease and a therapeutic target.

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Section 1

Role of Cell Death in Pathogenesis

Chapter 1

The Relationship between Basic Cell Death Pathways and Diseases

Ayşe Usta

Abstract

The activation of cell death mechanisms, which play critical roles in biological processes such as development, immune response, and tissue homeostasis, results in cell death under both physiological and pathological conditions. Cell death pathways are biological processes that determine the mode of cell death. These mechanisms are primarily classified into two main categories. Programmed cell death refers to mechanisms that actively induce cell death in a genetically regulated manner. The major types include apoptosis, necroptosis, pyroptosis, ferroptosis, and autophagic cell death. On the other hand, unregulated cell death typically occurs due to external factors and is uncontrolled. This category includes necrosis, which is generally detrimental to the body. Dysregulation of cell death mechanisms is critical in the pathogenesis of various diseases. Excessive cell death is linked to neurodegenerative diseases and the depletion of immune cells. On the other hand, inhibition of cell death can lead to pathological conditions like cancer, where cell death is suppressed in areas where it would normally occur, resulting in the accumulation of abnormal cells. The regulation or disruption of cell death pathways affects the onset, progression, and severity of diseases. Understanding cell death mechanisms provides a foundation for developing new therapeutic approaches to treat these conditions.

Keywords: apoptosis, autophagy, cell death, necrosis, pathological

1. Introduction

In the cellular life cycle, cell death is an inevitable and essential process. Cell death is a fundamental biological process that ensures the growth and homeostasis of organisms by removing damaged or unnecessary cells. It can be triggered by a variety of factors, including intracellular and extracellular stimuli such as genes, drugs, and other environmental factors. There are three primary forms of cell death based on morphology [1]. Apoptosis (type I cell death), which is genetically controlled and programmed; autophagy (type II cell death), which occurs particularly when caspases are inactive; and necrosis (type III cell death), an unprogrammed and accidental form of cell death [2].

In different forms of cell death, cells exhibit distinct physiological and morphological behaviors. When cells are damaged, certain metabolic processes may cease, cell structures may become disrupted, or other irreversible changes may occur, ultimately leading to cell death [3]. In multicellular organisms, tissue homeostasis depends on a

delicate balance between cell proliferation, differentiation, and death [4]. Cell death is a hallmark of many inflammatory diseases and a common side effect of bacterial or viral infections [5]. It is not merely a byproduct of inflammation but a dynamic process closely linked to the onset and progression of inflammatory diseases [6]. Active or programmed cell death is essential in the selective elimination of potentially harmful or infected cells. High levels of oxidative damage can induce cell death. Severe and prolonged oxidative stress can trigger autophagy, apoptosis, and necrosis. While cell death is a critical and active process that removes potentially harmful cells, excessive cell death is undesirable [2]. Dysregulation of cell death often underlies the development of cancer [7].

In conclusion, this review aims to provide an in-depth analysis of the molecular pathways involved in cell death while highlighting their physiological and pathological implications.

2. Programmed cell death (regulated cell death)

These processes are genetically controlled, and cells are mediated by specific biochemical mechanisms. This type of cell death is genetically controlled and generally necessary for the health of the organism. The main types include

3. Apoptosis

3.1 Definition

It is the most well-known mechanism of programmed cell death. It requires energy and is a regulated process. The cell shrinks, chromatin condenses, membrane budding (blebbing) occurs, and it is cleared by phagocytes. Apoptosis is a biological process in which a cell ceases to grow and divide, entering a controlled death phase without the need for spilling its contents. Apoptosis is also known as a genetically regulated cell suicide program [8].

3.2 Importance

Apoptosis is crucial for maintaining cellular homeostasis and development [9]. The significance of apoptosis lies in its ability to eliminate unnecessary, damaged, or harmful cells without triggering an inflammatory response, thereby contributing to the organism's internal balance [10]. In summary, the intrinsic and extrinsic pathways of apoptosis are complex and tightly regulated pathways essential for preserving cellular stability and the general health of the organism. By elucidating the pathways, it makes it possible to treat diseases such as cancer, which are seen in impaired apoptosis.

3.3 Causes

Cells that do not overcome adaptive stress responses sustain damage beyond repair and must be eliminated because they may have lost their function or could pose a threat to the entire organism. For instance, cells with unrepaired DNA are prone to accumulating somatic mutations during division and are therefore at risk of malignant

transformation [11]. Both stressed and dying cells release a broad spectrum of signals, including various cytokines and "damage-associated molecular patterns," which alert other cells to danger [12]. The majority of cellular stressors, including endoplasmic reticulum (ER) stress (caused by the buildup of misfolded proteins) and DNA damage (caused by genotoxic chemicals or flaws in DNA repair), cause apoptosis when the damage is irreversible [13]. Numerous types of cellular stress, such as oxidative stress, growth factor deficiency, and DNA damage, usually trigger apoptosis [14].

3.4 Types

In the apoptotic process, two main signaling pathways trigger apoptotic cell death [15]. These are (1) the extrinsic apoptosis pathway and (2) the intrinsic apoptosis pathway.

3.5 Mechanism

It is initiated intrinsically when pro-apoptotic proteins start the permeabilization of the outer membrane of the mitochondria and extrinsically when ligands attach to cell surface death receptors. The caspase protease family is activated by both mechanisms, which are ultimately responsible for cellular disassembly [13].

3.5.1 Extrinsic apoptosis pathway

The binding of extracellular death ligands to cell surface death receptors starts the extrinsic pathway (**Figure 1**). The immune system's process of eliminating contaminated cells is essential [16].

3.5.1.1 Death receptors

The extrinsic apoptosis pathway, also referred to as the death receptor pathway, is regulated by the interaction between cell surface death receptors and specific ligands. Tumor necrosis factor receptor 1 (TNFR1), CD95, TRAIL-R1, and TRAIL-R2, which belong to the tumor necrosis factor superfamily, transmit death signals by binding to ligands through conserved cytoplasmic death domains. Hence, they are termed "death receptors" (DRs) [17]. In the extrinsic apoptosis pathway, the binding of death ligands (e.g., FasL, TRAIL) to their corresponding death receptors on the cell surface (e.g., Fas, DR4, DR5) promotes receptor trimerization and activates the adapter protein FADD (Fas-associated death domain). Members of the tumor necrosis factor (TNF) receptor superfamily, including death receptors such as Fas (CD95) and TRAIL receptors (DR4 and DR5), contain a critical intracellular death domain (DD) necessary for transmitting apoptosis-inducing signals. The ligand binds to the cell surface receptor. By expressing TNF death receptor ligands, cytotoxic lymphocytes can kill infected cells. These ligands cause target cells to undergo apoptosis. Cell death induced by death receptors is generally critical for immune system function and homeostasis [18].

3.5.1.2 Caspase-8 activation

Caspase-8, an aspartate-specific enzyme, is essential for controlling and initiating programmed cell death mediated by death receptors. It triggers apoptosis by

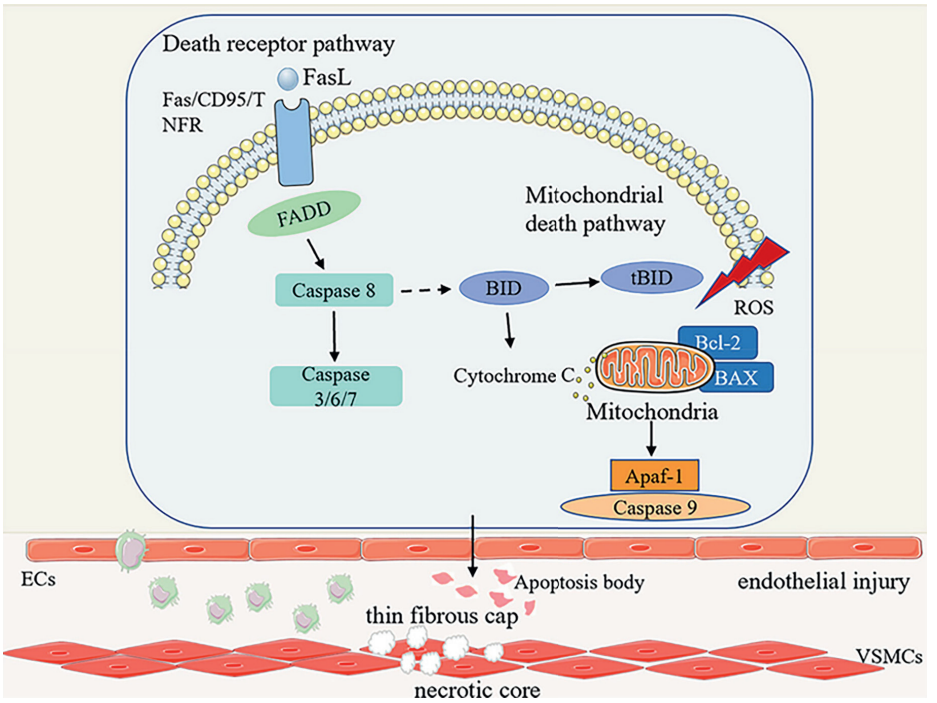

Figure 1.
Extrinsic and intrinsic pathways [16]. BAX: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; FAS: Soluble Fas ligand; FADD: Fas-associated protein with a novel death domain.

activating the caspase cascade downstream in two distinct ways [19]. The first mechanism involves death receptors (DRs), such as tumor necrosis factor, FASL/CD95L, or TRAIL ligands. Together with the Fas-associated death domain (FADD) protein and pro-caspase-8, they form the death-inducing signaling complex (DISC). Incorporating FADD into the death receptor complex facilitates the binding of pro-caspase-8 through its death effector domain, aiding in the assembly of the DISC. Dimerization and activation of pro-caspase-8 occur within the DISC. Caspase-8 initiates the apoptotic process by activating caspase-3. This mechanism involves the dimerization and auto-activation of the initiator caspase, which subsequently cleaves and activates the executioner caspases-3/6/7, ultimately resulting in cell apoptosis. The second mechanism activates apoptosis by cleaving BID (BH3-interacting domain death agonist), altering its conformation, and promoting its translocation to the mitochondria [20]. Additionally, caspase-8 can cleave Bid to generate truncated Bid (tBid), which translocates to the mitochondria to enhance apoptotic signaling along the intrinsic pathway, involving the pro-apoptotic BH3-only protein [21].

3.5.2 Intrinsic apoptosis pathway

The balance between pro-apoptotic and anti-apoptotic Bcl-2 protein members is essential for the intrinsic apoptosis pathway, which is controlled by mitochondrial signals. The mitochondrial mechanism of apoptosis, also known as the intrinsic pathway, involves mitochondrial dysfunction. It is distinguished by the activation of pro-apoptotic proteins, particularly cytochrome C, from the mitochondria into the cytosol, as well

as the activation of initiator caspase-9. This ultimately leads to the activation of effector caspases (caspase-3, caspase-6, and caspase-7), which execute cellular apoptosis. The mitochondrial apoptotic pathway is typically initiated in a cell-autonomous manner [22].

3.5.2.1 The role of Bcl-2 family proteins

The Bcl-2 protein family plays a critical role in regulating the intrinsic pathway by determining cell fate through its pro-apoptotic and anti-apoptotic members [23]. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL preserve mitochondrial function by inhibiting pro-apoptotic proteins [24]. BH3 proteins are ones that can activate pro-apoptotic proteins such as Bax, Bak, Bid, Bim, and Puma in response to apoptotic stimuli. This oligomerization causes the outer mitochondrial membrane to permeabilize [25].

3.5.2.2 Release of cytochrome C and activation of caspase

When Bax and Bak permeabilize the outer mitochondrial membrane, proteins from the mitochondrial intermembrane gap, such as cytochrome c, are released. The intrinsic pathway of apoptosis, also known as the mitochondrial apoptotic pathway, can be triggered by numerous factors such as DNA damage, nutrient deprivation, growth factor withdrawal, reactive oxygen species (ROS), and free radicals. These stress signals induce the activation of pro-apoptotic BH3-only proteins (e.g., Bid, Bim, Puma), which in turn activate Bax and Bak. Cytochrome c is released into the cytosol when these proteins oligomerize to create holes in the outer mitochondrial membrane. Cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) in the presence of dATP, forming a polymer. Through the caspase recruitment domain at the N-terminal of Apaf-1, caspase-9 in the cytoplasm binds to it, forming apoptosomes. This complex activates pro-caspase-9. Activated caspase-9, in turn, activates downstream effector caspases such as caspase-3 and caspase-7, leading to the cleavage of cellular substrates and ultimately resulting in cell fragmentation through apoptosis [22, 26–28].

The Bcl-2 family and its Role in Macrophage Apoptosis. The Bcl-2 family includes Bcl-2, Bcl-xL, and Bax proteins, which play a crucial role in regulating macrophage apoptosis. Bcl-2 is a particularly significant survival molecule, as it has been proven to prevent macrophage apoptosis, especially in more advanced lesions. The Bcl-2 protein is pivotal in controlling the permeability of the mitochondrial membrane. Recent studies have shown that vascular cells mediate the apoptotic response to oxidation and inflammation through stimulation or inhibition mechanisms [29]. Bax, a pro-apoptotic protein, responds to mitochondrial dysfunction and cell death, playing a critical role in the death of macrophages within atherosclerotic plaques [30]. The apoptosis process is regulated by a balance between pro-apoptotic and anti-apoptotic proteins. P53, a tumor suppressor protein, typically remains inactive and degraded under normal conditions. However, proatherogenic stimuli such as DNA damage, oxidative stress, and oxidized lipoproteins induce p53 expression [31].

4. Autophagy

4.1 Definition

The term autophagy, derived from the Greek words for "self" and "eating," translates to "self-eating" [32]. Autophagy is a "survival mechanism" that protects

cells by degrading intracellular proteins and organelles within the cytoplasm, facilitating the generation of new and healthier cells. However, in some instances, components of the autophagic signaling pathway actively promote cell death, a process known as autophagic cell death. In this case, the cell dies by digesting its contents via autophagosomes [13, 33]. Excessive autophagy can lead to cellular degradation and, under certain conditions, result in cell death, such as during self-destruction of cells under stress [34]. Functioning as a cytoprotective mechanism, autophagy breaks down damaged or unnecessary cellular components, such as cytoplasmic contents, proteins, and organelles, into fundamental biomolecules via lysosomal degradation. These components are subsequently utilized as nutrients and building blocks for organelles, aiding in cellular survival. In this context, autophagy directs a continuous degradation-regeneration cycle, maintaining a flow of biomolecules [2, 35].

4.2 Importance

Autophagy is activated as a cellular process for the degradation of modified cellular components to prevent the accumulation of altered molecules that could compromise cellular survival under stress [36]. It is an evolutionarily conserved cellular degradation mechanism typically induced under various cellular stress conditions, including nutrient deprivation and infection, particularly during stress or nutrient scarcity [37]. Autophagy plays a critical role in the degradation of protein aggregates, pathogens, lipids, and aging/damaged subcellular organelles, such as mitochondria [38]. Macroautophagy happens at a fundamental level in eukaryotic cells. It helps to maintain bioenergetic balance by degrading molecules and turning over organelles. Furthermore, autophagy influences the survival rate of cells exposed to stress. In this context, activated autophagy serves as a stress-adaptation pathway that supports cellular survival [39]. Autophagy also contributes to the removal of toxic substances. For example, it enhances neutrophil phagocytosis during sepsis [40]. Autophagy plays a vital part in the host's defense against infections by removing non-self-pathogens in addition to its own organelles and proteins. The pathogen-lytic process known as xenophagy is the intracellular breakdown of pathogens, which lowers the pathogen load. A particular kind of selective macroautophagy called xenophagy is used to isolate and break down pathogens inside autophagosomes. Cells can recognize, separate, and break down pathogens by xenophagy, stopping their growth and distribution [41]. Increased oxidative stress and nitric oxide production are two factors that contribute to the marked impairment of mitochondrial activity during sepsis. Autophagy serves to preserve the integrity of mitochondria, which are vital organelles for cellular life [42].

4.3 Causes

Autophagy is triggered by adverse conditions such as starvation, low oxygen levels, growth factor deprivation, low adenosine triphosphate (ATP) levels, and nutrient or amino acid deficiency. Other signals, including hypoxia, reactive oxygen species (ROS), elevated intracellular calcium levels, and mitochondria with low membrane potential, can also influence autophagic activity [2]. Autophagy is primarily activated in response to a metabolic crisis or to remove damaged organelles and protein aggregates [43]. Additionally, autophagy plays an essential role in responding to stress caused by infection. In summary, autophagy plays a crucial role in the degradation

of misfolded or aggregated proteins, the clearance of damaged organelles, and the elimination of intracellular pathogens, thereby maintaining cellular homeostasis [44].

4.4 Types

In mammals, three general types of autophagy have been identified:

1. Macroautophagy

2. Microautophagy

3. Chaperone-mediated autophagy

Each type depends on a specific mechanism by which cytoplasmic cargo, organelles, or components within the cell are degraded through lysosomes [35, 36].

4.5 Mechanism

4.5.1 Macroautophagy

Macroautophagy is the primary mechanism underlying autophagic cell death. It involves the sequestration of larger particles and organelles into autophagosomes, which subsequently fuse with lysosomes to facilitate degradation. Macroautophagy is the most extensively studied type of autophagy and is often referred to simply as "autophagy." This process entails the formation of a double-membrane structure called the autophagosome, which non-selectively engulfs cytoplasmic components and then fuses with lysosomes to degrade the internalized content [45]. Autophagy, mediated by complex signaling pathways, is characterized by significant cytoplasmic vacuolization [46]. Similar to apoptosis, it involves the phagocytic uptake of cellular components, which are then degraded within lysosomes [47].

ATG is an abbreviation for "AuTophaGy" and refers to both the genes and proteins involved in the biological process of autophagy, which is orchestrated by ATG genes [48]. This multi-step process encompasses nucleation, elongation, and the formation of autophagosomes and autolysosomes, all of which are regulated by a conserved set of genes known as autophagy-related genes (ATGs) [49]. Macroautophagy occurs in four main stages (**Figure 2**):

1. *Initiation*: The activation of the ULK1 complex, which comprises ATG101, FIP200, and ATG13, leads to autophagy. This activation is controlled by energy sensors like mTOR (mechanistic target of rapamycin) and AMP-activated protein kinase (AMPK). Under stress conditions, mTOR is inhibited, leading to the activation of the ULK1 (Unc-51-like kinase 1) complex (**Figure 2**).

2. *Nucleation*: The activated ULK1 complex triggers the formation of a double-membrane structure known as the phagophore, a process facilitated by the PI3K complex comprising ATG14L, Beclin-1, and VPS34. This complex produces phosphatidylinositol 3-phosphate to activate other proteins associated with autophagy [51]. Autophagy induction is highly sensitive to cellular stress and involves the formation of double-membrane phagophores, which gradually elongate to enclose damaged or modified proteins, ultimately forming autophagosomes [52].

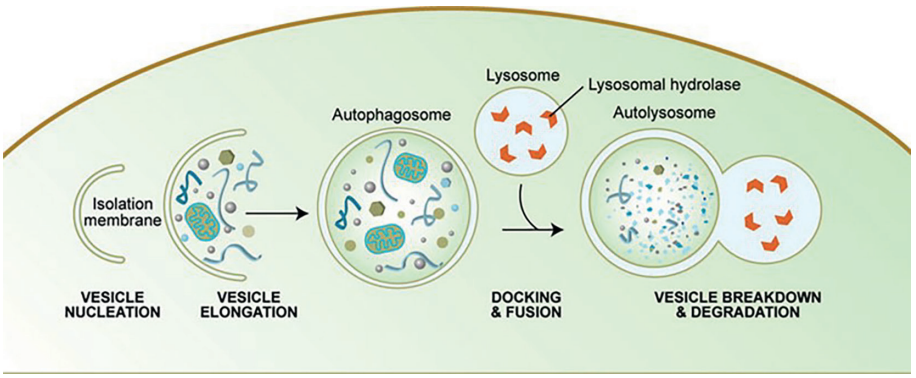

Figure 2.
Molecular mechanism of autophagy [50].

Following ULK1 complex activation, phagophore formation is initiated [53]. A collection of autophagy-related proteins (Atgs), such as Atg5, Atg12, and Atg16L1, helps the phagophore membrane lengthen [54].

3. *Elongation and maturation*: With the aid of the LC3-PE conjugation system and the ATG12-ATG5-ATG16L complex, the phagophore enlarges to absorb cytoplasmic particles. LC3-II plays a crucial role in membrane expansion and cargo recognition [55]. Following this, a sequence of events incorporates microtubule-associated protein 1 light chain 3 (LC3) into autophagosomes. The cysteine protease Atg4 cleaves LC3 to create LC3-I. LC3-II is then produced by processing Atg3 and Atg7 with phosphatidylethanolamine. LC3-II is then embedded in the autophagosomal membrane [56]. A double-membraned autophagosome is created when the phagophore membrane shuts around the cargo.

4. *Fusion with lysosomes*: When lysosomes and autophagosomes combine to produce an autolysosome, lysosomal hydrolases convert the material that has been sequestered into basic biomolecules that can be recycled and used again in the cytoplasm (**Figure 3**) [57, 58]. This fusion allows for the degradation of trapped molecules by lysosomal enzymes [52]. A double-membraned structure known as the autophagosome engulfs and eventually degrades portions of the cytosol and particular organelles during the catabolic process of autophagy [13]. Lysosomal hydrolases degrade the autolysosome's inner membrane and its contents, returning macromolecules like sugars, fatty acids, and amino acids to the cytoplasm for the cell to use again [59]. Growth variables that alter mTOR activity, energy state, and nutrition levels all influence autophagy. The AMPK pathway detects the energy levels of cells and can trigger autophagy by phosphorylating ULK1 directly and blocking mTOR [60].

4.5.2 Microautophagy

Microautophagy involves the direct engulfment and degradation of cytoplasmic material by lysosomes through invagination of the lysosomal membrane, allowing small particles to enter and undergo degradation [61]. ATG proteins are not directly responsible for the microautophagic uptake of soluble components [62].

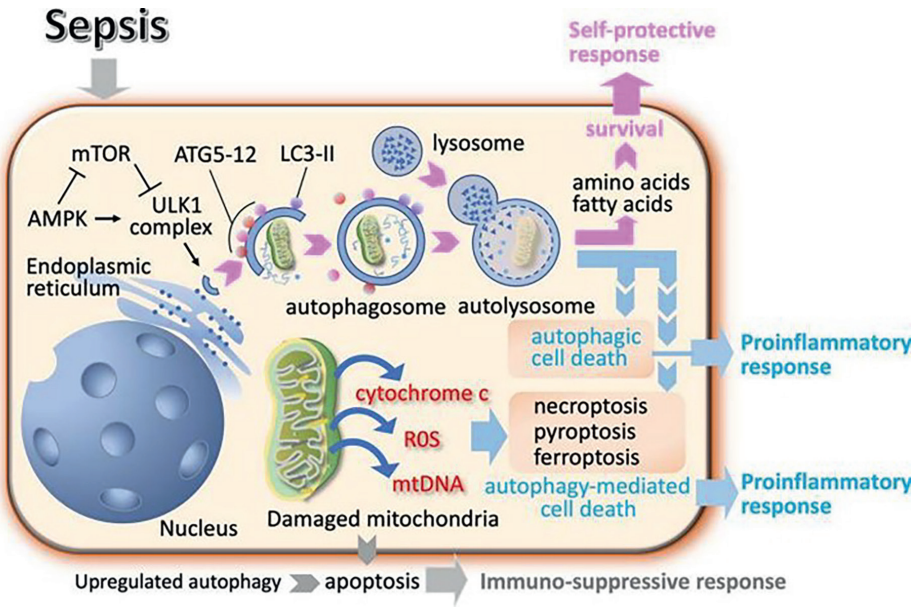

Figure 3.
Mechanism of autophagosome formation [40].

4.5.2.1 Selective autophagy (mitophagy)

Mitophagy is the process by which certain substrates, like mitochondria, are broken down via selective autophagy, targeting damaged organelles like mitochondria [63]. When mitochondria become damaged or dysfunctional, they produce excessive reactive oxygen species (ROS), leading to cellular damage and death [64]. One type of autophagy that specifically targets damaged or superfluous mitochondria for destruction is called mitophagy. This mechanism ensures mitochondrial quality control by removing defective mitochondria and preventing their accumulation. Additionally, it renews mitochondrial components through biogenesis by incorporating new proteins and lipids, resulting in mitochondrial turnover [65]. Multiple mechanisms can induce mitophagy, with the ubiquitin-dependent pathway regulated by PTEN-induced kinase 1 (PINK1) and Parkin being the most well-known [66]. PINK1 is typically destroyed by the ubiquitin-proteasome system after being delivered to the inner mitochondrial membrane [67]. However, when mitochondria are injured, PINK1 remains in the outer mitochondrial membrane. It is activated by autophosphorylation and encourages Parkin recruitment. Other outer membrane proteins are subsequently polyubiquitinated by Parkin, and PINK1 phosphorylates them. This ubiquitin-dependent pathway, along with other mechanisms, mediates the elimination of damaged mitochondria [68].

4.5.2.2 Chaperone-mediated autophagy

Chaperone-mediated autophagy (CMA) involves the selective degradation of specific proteins recognized by molecular chaperones, such as the 70 kDa heat shock cognate protein (HSC70) (**Figure 4**). This process identifies cytosolic proteins with specific peptide sequences, directing them to the lysosome for translocation and

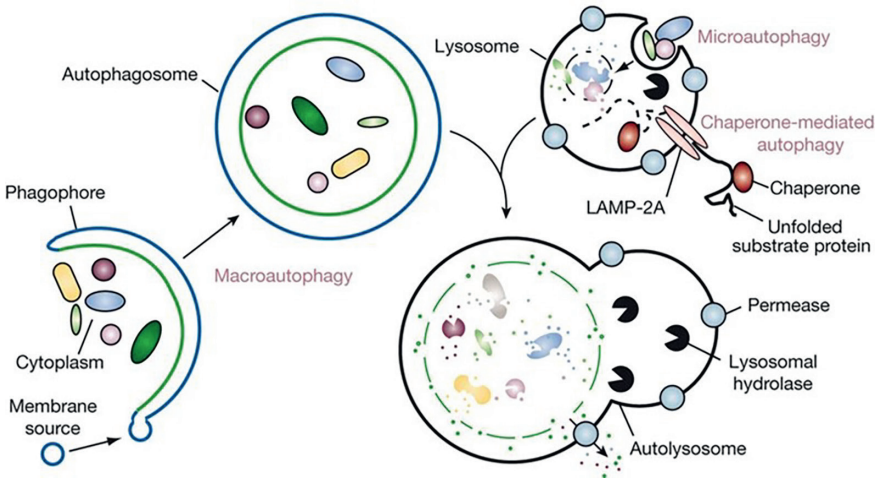

Figure 4.
Types of autophagy [69].

degradation [70]. Unlike other autophagic pathways, CMA does not require vesicular trafficking [71]. CMA functions exclusively to degrade a specific subset of misfolded or aberrantly formed cytosolic proteins. It is the only autophagic pathway that allows the selective breakdown of soluble proteins within lysosomes [72]. This pathway is highly selective in determining which proteins cross the lysosomal barrier, requiring neither vesicle formation nor significant changes to the lysosomal membrane [73].

5. Necroptosis

5.1 Definition

Necroptosis is a form of programmed cell death that shares morphological features with necrosis. It is a designed kind of necrosis that shares the physical characteristics of necrosis but is carried out by controlled signaling pathways. As a fallback method for cell death, this procedure can be initiated in situations where apoptosis is suppressed [74].

5.2 Importance

Necroptosis is essential in many physiological and pathological settings, such as immunological responses, illness, and development. This type of cell death is implicated in several pathophysiological disorders, including inflammatory, neurodegenerative, infectious, and malignant diseases [75]. The molecular mechanism governing necroptosis involves a well-coordinated interaction between RIPKs and MLKL (mixed lineage kinase domain-like protein) [76]. Two distinct forms of cell death, necroptosis and necrosis, are connected. Necrosis is caused by abrupt cell injury, whereas necroptosis is a regulated kind of cell death that involves RIPK1, MLKL, and RIPK3 [77]. Elucidation of molecular mechanisms can enhance our knowledge of their impact on health and disease and help identify potential therapeutic targets for disorders characterized by abnormal cell death.

5.3 Causes

Multiple signaling pathways stimulate receptor-interacting protein kinase 3 (RIP3), which causes necroptosis to occur. When RIP3 is drawn to macromolecular complexes downstream of different cell surface receptors, including death receptors (DRs), Toll-like receptors (TLRs), and the T-cell receptor (TCR), it becomes active. Furthermore, without the need for cell surface receptor ligation, DNA damage can directly trigger the creation of a RIP3 activation platform. Lastly, upon viral infection, the cytosolic sensor might operate as a DNA-dependent activator of interferon-regulating factors (DAI). RIP3-dependent necrosis may result from viral DNA present in the cytosol [13].

5.4 Types

Although there are no distinct subtypes of necroptosis, it can manifest in various forms due to its activation through different signaling pathways in different contexts. When classified by their triggering mechanisms, different types of necroptosis can be

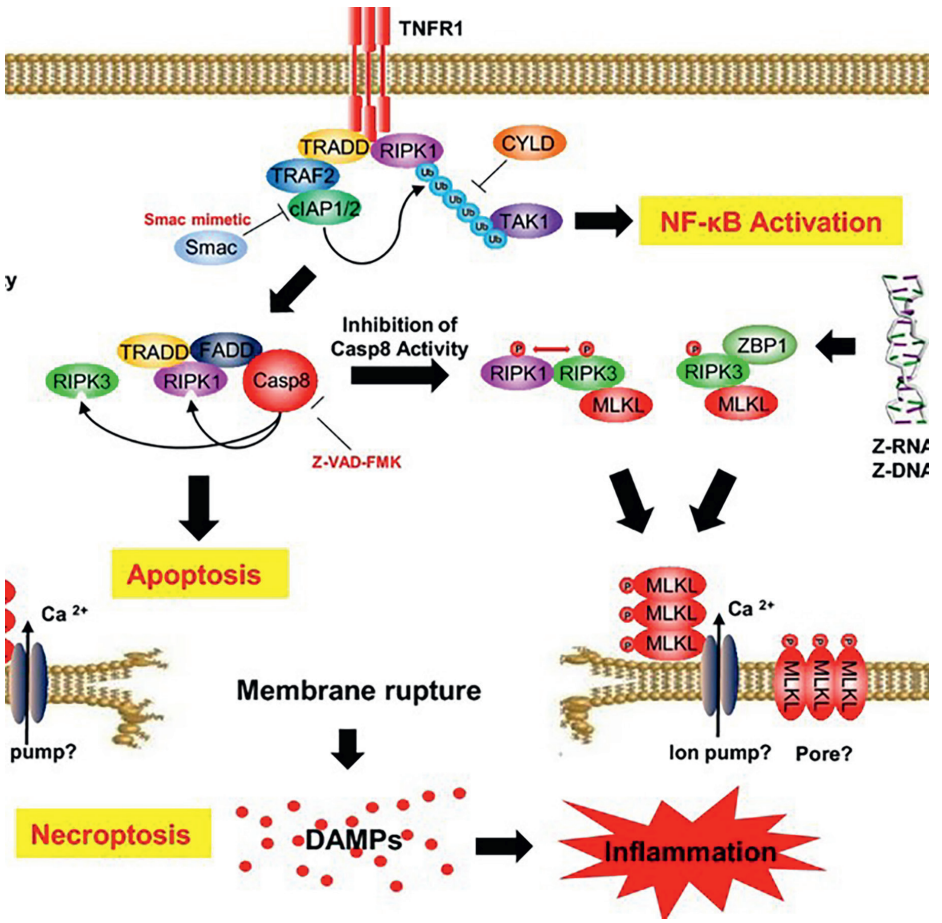

Figure 5.
Necrotic cell death [26].

identified, including classical TNF-α-mediated necroptosis, TLR-mediated necroptosis, viral necroptosis, inflammation-related necroptosis, cancer-associated necroptosis, and ischemia/reperfusion injury-related necroptosis. Given that this area is still the subject of active research, more specific subtypes of necroptosis may be defined in the future.

5.5 Mechanisms

RIPK1 Activation: The activation of death receptors, such as tumor necrosis factor receptor 1 (TNFR1), usually triggers necroptosis. The adapter protein TRADD activates RIPK1. Together with RIPK3, RIPK1 forms a complex known as the necrosome [78].

RIPK3 Activation: RIPK1 phosphorylates RIPK3 inside the necrosome. The downstream signaling cascade that causes necroptosis depends on this phosphorylation event. Subsequently, RIPK3 activates and phosphorylates MLKL [79].

MLKL Activation and Membrane Disruption: MLKL, which has been phosphorylated, changes its structure, enabling it to oligomerize and go to the plasma membrane. MLKL creates pores in the membrane as it integrates, impairing its integrity and causing cell enlargement, membrane rupture, and necrotic cell death (**Figure 5**) [80].

Different from apoptosis, necroptosis is a type of planned cell death marked by inflammation, membrane rupture, and cellular swelling. Important proteins, including MLKL, RIPK1, and RIPK3, control it [81]. Necroptosis has two sides in disease contexts because, in contrast to apoptosis, it is frequently linked to inflammatory reactions.

6. Pyroptosis

6.1 Definition

Pyroptosis is a type of programmed cell death observed in immune cells, occurring as a defense mechanism against infections. It involves an inflammatory process similar to necrosis but is regulated by specific enzymes. This process is controlled by Caspase-1 and Caspase-4/5/11, and is often triggered as an immune response to pathogens. Example: Macrophage response to bacterial infections. Pyroptosis is characterized by cell swelling, membrane rupture, and the release of pro-inflammatory cytokines. It is primarily mediated by the gasdermin family of proteins and is initiated by inflammasome activation. Pyroptosis plays a dual role in various diseases, including cancer and inflammatory conditions, making it a significant area of research for therapeutic interventions [82].

6.2 Importance

Targeting Pyroptosis: Research is exploring small-molecule inhibitors and other strategies to modulate pyroptosis for therapeutic benefits in cancer and inflammatory diseases [83]. While pyroptosis is often considered a detrimental process due to its inflammatory nature, it also holds promise as a therapeutic target. In particular, in cancer treatment, pyroptosis can be harnessed to enhance anti-tumor immunity.

6.3 Causes

Tumors: The most prevalent cause, accounting for approximately 45% of cases, includes both benign and malignant neoplasms. Infectious Causes: Represent about 25% of pyroptosis cases, often linked to conditions like orbital cellulitis. Inflammatory Conditions: Conditions such as thyroid eye disease contribute to around 16.6% of pyroptosis cases. Vascular Issues: Vascular malformations or hemorrhages account for approximately 6.66% [84]. Nutritional Deficiencies: Rarely, conditions like iron deficiency anemia can lead to extramedullary hematopoiesis, causing pyroptosis [85].

6.4 Types

Each type of pyroptosis plays a crucial role in different stages of immune responses and various disease conditions. Pyroptosis holds particular significance in infection and inflammation research. There are three main types of pyroptosis:

1. Classical Pyroptosis

2. Non-canonical Pyroptosis

3. Gasdermin-mediated Pyroptosis

6.4.1 Classical pyroptosis

This is the most common type of pyroptosis and is initiated by the activation of inflammasomes, particularly the NLRP3 inflammasome. In this process, caspase-1 is activated, increasing cell membrane permeability, leading to cell rupture and the release of inflammatory molecules. It is typically observed as an immune response against pathogens such as viruses and bacteria.

6.4.2 Non-canonical pyroptosis

Non-canonical pyroptosis involves distinct caspases, such as caspase-4 and caspase-5 in humans or caspase-11 in mice. This mechanism is specifically activated in response to lipopolysaccharides (LPS) from gram-negative bacteria. Unlike classical pyroptosis, this type can function independently of the inflammasome, yet it typically induces a stronger inflammatory response.

6.4.3 Gasdermin-mediated pyroptosis

Gasdermin proteins play a critical role in the execution of pyroptosis. When caspases cleave gasdermin proteins, pores are formed in the cell membrane, causing the release of cellular contents. This type of pyroptosis is associated with inflammatory cell death, one of the most prominent features of inflammation, and can also intensify the severity of the inflammatory response.

6.5 Mechanisms

Inflammasome Activation: Pyroptosis is triggered by inflammasomes, which detect cellular stress and damage, leading to the activation of caspase-1 and the

subsequent cleavage of gasdermin proteins. Cytokine Release: The rupture of the cell membrane during pyroptosis results in the release of inflammatory cytokines, such as IL-1β and IL-18, which amplify the immune response [83, 86].

7. Ferroptosis

7.1 Definition

Iron-dependent lipid peroxidation, which causes cellular damage and death, is a characteristic of ferroptosis, a unique type of controlled cell death. Glutathione peroxidase 4 (GPX4) enzyme inhibition plays a crucial role in this process.

7.2 Importance

Since its discovery, ferroptosis has been implicated in various diseases, including neurodegenerative disorders, cancers, and fibrotic diseases. Understanding the mechanisms of ferroptosis opens new avenues for therapeutic interventions, particularly in conditions where traditional treatments are inadequate. Classical ferroptosis is a cell death process that occurs due to iron-dependent lipid peroxidation, triggered by the inhibition of GPX4. This process causes irreversible oxidative damage to the cell membrane and is associated with various diseases. It can be targeted in cancer therapy or inhibited in neurodegenerative diseases.

7.3 Causes

Cancer Treatment: Ferroptosis is being investigated as a potential target for the destruction of some cancer cells. Cancer cells may become susceptible to ferroptosis under certain stress conditions [87]. Neurodegenerative Diseases: Ferroptosis is thought to play a role among the cell death mechanisms in neurodegenerative diseases such as Parkinson's and Alzheimer's [88]. Drug Development: Agents that trigger ferroptosis (e.g., erastin, RSL3) and molecules that modulate this process are important in treatment strategies [89].

7.4 Types

Although distinct varieties of ferroptosis have not yet been clearly defined, this process can operate in different ways through various modifications. Currently, there is only one known type, which is classical ferroptosis.

7.4.1 Classical ferroptosis

Impairment of antioxidant defense: Under normal conditions, cells reduce lipid hydroperoxides via the enzyme glutathione peroxidase 4 (GPX4). This process protects the cell membrane from oxidative damage. Impairment of GPX4 function (for example, under the influence of GPX4 inhibitors) or depletion of glutathione in the cell causes this protective mechanism to cease functioning.

Increased lipid peroxidation: With the loss of GPX4 protection, lipids in the cell membrane begin to undergo oxidation. The accumulation of lipid peroxides disrupts the structural integrity of the cell membrane, and harmful oxidative products are formed.

The role of iron: Free iron in the cell supports the production of reactive oxygen species (ROS) through chemical reactions such as the Fenton reaction. This ROS production accelerates lipid peroxidation and causes further damage to the cell membrane.

Cell death: Increased lipid peroxidation and iron-catalyzed oxidative stress severely damage the cell membrane, triggering ferroptosis [90]. As a result, an irreversible process occurs, leading to cell death.

7.5 Mechanisms

Iron (Fe^{2+}) accumulation: An increase in free iron within the cell is one of the key factors triggering ferroptosis. Through the Fenton reaction, Fe^{2+} reacts with hydrogen peroxide (H_2O_2) to generate hydroxyl radicals (•OH).

Lipid Peroxidation (Membrane Damage): Multiple polyunsaturated fatty acids (PUFAs) in the cell membrane are oxidized by reactive oxygen species (ROS), leading to the formation of lipid peroxides (L-OOH). This oxidation disrupts the structure of the cell membrane and causes damage to the membrane.

GPX4 Enzyme Inhibition: GPX4 is a critical antioxidant enzyme that prevents lipid peroxidation. Classical ferroptosis begins with the inhibition or reduction of GPX4. When GPX4 is inactive, accumulated lipid peroxides kill the cell.

Cell Death (Membrane Damage and Loss of Cellular Integrity): Increased lipid peroxidation and ROS lead to irreversible damage to the cell membrane. The cell undergoes controlled death, but like necrosis, membrane integrity is lost.

8. Unprogrammed cell death

This type of cell death is usually uncontrolled and is typically associated with pathological events.

9. Necrosis

9.1 Definition

Necrosis occurs when the cell swells and breaks down due to external factors (trauma, toxins, lack of oxygen), causing inflammation. Example: Tissue loss as a result of burns or infarction. Necrosis refers to irreversible cell damage and ultimate cell death caused by pathological processes, typically triggered by external stimuli or injury. It occurs due to various factors and develops randomly, leading to localized and sudden death of tissues and organelles within a living organism. Unlike apoptosis, necrosis is an unregulated process not governed by genetic control [91]. This devastating type of cell death is brought on by chemical or mechanical damage, such as a shift in pH or extremely high or low temperatures [92]. Necrosis is characterized by the abrupt and uncontrolled death of cells due to acute damage [93]. It involves swelling of cell organelles, rupture of the plasma membrane, and subsequent disintegration of the cell, leading to the release of intracellular contents into surrounding tissues and causing tissue damage [94]. The rupture of the cell membrane triggers the release of cellular components, which can provoke an inflammatory response. Necrosis is usually caused by trauma, infection, or ischemia and does not entail particular signaling pathways like

apoptosis does. Necrotic cells show signs of organelle breakdown, swelling (oncosis), and finally rupture of the plasma membrane. These events lead to the extracellular release of intracellular components, activating the immune system and triggering an inflammatory response. Due to the uncontrolled nature of necrosis and the resulting inflammation, tissue damage and disease progression are often exacerbated [95, 96]. Necrosis typically arises in response to acute injury, ischemia, infection, or chemical exposure, distinguishing it from apoptosis and autophagy, as it lacks the characteristic features of both. It is generally viewed negatively, characterized by plasma membrane disruption and an absence of typical apoptotic markers such as pyknosis, karyorrhexis, cell shrinkage, and apoptotic body formation. Moreover, necrosis does not involve significant autophagic vacuolization, setting it apart as a distinct form of cell death [97].

9.2 Importance

As an inflammatory form of cell death, necrosis not only results in organ damage but also triggers immune stress responses [98].

9.3 Causes

Both internal anomalies and external traumas can cause cell harm. The most common causes of harmful stimuli include [99]. Hypoxia: This may result from shock, respiratory failure, or ischemia. Physical agents include things like trauma, high temperatures, radiation exposure, and electric shock, among other exterior damage. Chemical agents: These include harmful substances, industrial exposures, recreational drugs, and poisons. Examples of biological agents include viruses, fungi, and bacteria. Autoimmune responses are immunity-related reactions.

9.4 Types

(1) Coagulative Necrosis, (2) Liquefactive Necrosis, (3) Caseous Necrosis, (4) Fat Necrosis, (5) Fibrinoid Necrosis, (6) Gangrenous Necrosis, (7) Hemorrhagic Necrosis, (8) Chemical Necrosis.

9.4.1 Coagulative necrosis

The cell architecture is unaltered in this kind of necrosis. Cells seem anucleate, eosinophilic, and structurally intact under a microscope. Leukocytes participate in the process of phagocytosis, which eventually eliminates dead cells [100].

9.4.2 Liquefactive necrosis

The central nervous system is where this morphology is most frequently seen. Hydrolytic enzymes break down dead cells, causing them to lose their structural integrity and produce a viscous mass. This shape is also seen in the majority of bacterial infections, where pus is the term used to describe the buildup of such necrotic material [101].

9.4.3 Caseous necrosis

The word “caseous” describes the necrotic area’s cheese-like look. The necrotic area is known as a granuloma, and this kind of necrosis happens in tuberculosis infections.

9.4.4 Fat necrosis

Acute pancreatitis causes this kind of necrosis. The peritoneal cavity's fat cells liquefy as a result of the release of pancreatic enzymes. Saponification is the process by which these liquid fat cells mix with calcium to create chalky white patches. Under a microscope, this manifests as basophilic calcium deposits inside necrotic fat cell outlines. Fat saponification in breast tissue can potentially result in fat necrosis [102].

9.4.5 Fibrinoid necrosis

The accumulation of immune complexes and fibrin seeping into the vessel walls causes this kind of necrosis in blood vessels. Under a microscope, this manifests as the damaged tissues staining with a bright pink, amorphous substance [103].

9.4.6 Gangrenous necrosis

It is a medical term for limb ischemia necrosis. Two categories exist: Dry Gangrene: ischemia-induced coagulative necrosis. Wet Gangrene: liquefactive necrosis brought on by a bacterial infection that develops on top of ischemia [104].

9.4.7 Hemorrhagic necrosis

Blood arteries becoming blocked and allowing blood to seep into the tissue causes necrosis. Usually, it is seen in organs with a restricted blood supply. Features of Morphology: The tissue appears bruised and bloodied because it has hemorrhagic (bleeding) regions and blood leaking. Examples include the kidneys, intestines, and lungs.

9.4.8 Chemical necrosis

Rapid cell death can result from toxic substances. Morphological Features: Usually, blood vessels coagulate, tissue hardens, and cells enlarge. Alkaline substance-induced chemical burns are one example.

9.5 Mechanism

The triggering factors of necrosis are tumor necrosis factor receptor 1 (TNFR1) and Ripk3. Once Ripk3 is activated, Ripk1 is also activated, leading to necrosis. Ripk1 and Ripk3 are genes that induce the necrosis process [2]. Necrosis results from excessively damaging external stimuli and is nearly always linked to inflammatory reactions brought on by the release of nuclear proteins, ATP, heat shock proteins, uric acid, and DNA. This leads to the activation and secretion of the pro-inflammatory cytokine interleukin-1 beta (IL-1β). The cellular mechanism leading to necrosis involves the loss of cell membrane integrity due to exposure to harmful stimuli. This allows extracellular ions to flow into the cell, causing swelling of the cell and its organelles. Proteolytic enzymes, including proteases, RNases, DNases, and phosphatases, are released into the cell through a different method that includes the rupture of the lysosomal membrane. Once activated in the cytosol, these enzymes damage DNA, RNA, and proteins [105]. These enzymes aid in the breakdown of biological constituents, which results in the death of cells. Both mechanisms result in the disruption of the plasma membrane, causing the release of intracellular contents into the surrounding tissue. This

process is characterized by swelling of organelles, rupture of the plasma membrane, and disintegration of the cell [106]. Such changes make the cells appear more vacuolated, eosinophilic, and glassy. Damage to organelles and plasma membranes causes these changes. The first metabolic alteration seen upon injury is ATP depletion. In the presence of oxygen, oxidative phosphorylation in the mitochondria produces ATP. In necrosis associated with hypoxia or chemical injury, cells experience a lack of oxygen, leading to reduced ATP production. A lack of ATP causes disruptions in the plasma membrane's energy-dependent sodium pump. The cell swells, and the ribosomes separate from the endoplasmic reticulum as a result of calcium and water entering the cell. Mitochondrial damage results from oxidative stress and elevated cytosolic calcium. Cytosolic calcium also activates various cytosolic enzymes, including phospholipases and proteases, causing the degradation of both membranes (including lysosomal membranes) and proteins [94]. Rapid breakdown of plasma membrane integrity is a hallmark of necrotic cell death. Active signaling pathways that rely on the activity of the best-characterized protein kinase, RIP3, may cause necrosis.

10. The relationship between cell death mechanisms and diseases

An uncontrolled increase in apoptosis plays a critical role in the development of many diseases. Cell loss, particularly in neurodegenerative, autoimmune, cardiovascular, and degenerative diseases, is a significant pathological factor. A decrease in apoptosis is especially associated with cancer, autoimmune diseases, infectious diseases, and fibrotic disorders. Failure of cells to undergo timely death triggers disease processes, leading to uncontrolled cell growth and tissue damage," for smoother flow. Targeting apoptosis mechanisms may provide opportunities for developing new strategies in the treatment of these diseases.

The increase in necrosis is generally the result of factors such as cellular damage, oxygen deprivation, infections, or exposure to toxins. Numerous diseases, including cardiovascular diseases, infections, liver diseases, and neurological disorders, can cause necrosis in tissues, leading to severe damage in the body. A reduction in necrosis may lead to the abnormal survival of cells, which can worsen tissue damage. This condition can trigger a range of health issues, which include cancer, autoimmune diseases, fibrosis, and neurological disorders. Proper tissue healing and regulation of cell death are essential for maintaining healthy body functions.

Increased autophagy may lead to the development of certain diseases and is generally associated with conditions such as cancer, neurological diseases, infections, autoimmune disorders, and cardiovascular diseases. While autophagy plays a vital role in maintaining cell health, excessive or unbalanced autophagy can disrupt cellular functions and contribute to disease progression. A decrease in autophagy impairs the cells' ability to clear and repair damaged components, leading to a range of diseases. Some examples of diseases related to reduced autophagy include neurological disorders, cancer, autoimmune diseases, heart diseases, liver diseases, and muscle diseases. Autophagy is a critical process for cell health, and thus, its proper functioning is essential for the overall health of the body.

Autophagy is widely seen as a protective mechanism. However, in certain specific situations, it may cause tissue damage and lead to cell death via necrosis or apoptosis [107]. Apoptosis is another consequence of excessive autophagy. The imbalance between apoptosis and autophagy may lead to diabetic complications [108].

Exposure to various stimuli, such as heat, radiation, toxins, hypoxia, and anticancer drugs, can induce different forms of cell death depending on the dose. At lower doses, these stimuli may trigger apoptosis, a regulated form of cell death characterized by specific morphological and biochemical features. In contrast, higher doses of the same stimuli can lead to necrosis, which is often considered an uncontrolled and accidental form of cell death. The type of cell death induced is influenced by factors such as the intensity and duration of the stimulus, as well as the specific cellular context. The relationship between apoptotic, autophagic, and necrotic cell deaths and 65 diseases is examined in **Table 1**.

	Apoptosis	Necrosis	Autophagy
Neurodegenerative disorders			
Parkinson's disease [109–111]	+	+	+
Alzheimer's disease [112–114]	+	+	+
Huntington's disease [115–117]	+	+	+
Amyotrophic lateral sclerosis [118–120]	+	+	—
Creutzfeldt-Jacob disease [121–123]	+	+	+
Retinitis pigmentosa [124–126]	+	+	+
Spinal muscular atrophy [127–129]	+	+	+/–
Digestive system diseases			
Inflammatory Bowel Diseases [130]	+	+	+
Crohn's disease [131–133]	+	+	—
Ulcerative colitis [134–136]	+	+	+
Pancreatitis [137–139]		+	—
Kidney diseases			
Acute Renal Failure [140–142]	+	+	+
Chronic Kidney Disease [143–145]	+	+	+
Infections			
Fungal infection [146–148]	+	+	+
Parasites [149–151]	+	+	—
Viral infections			
Adenoviruses [152–154]	—	+	+
Baculoviruses [155–157]	+	+	+
Herpesviruses [158–160]	+	—	—
Influenza virus [161–163]	+	+	+
HIV virus [164–166]	+	+	+
Ebola virus [167–169]	+	+	+
HPV virus [170–172]	—	+	—
Hepatitis B and C Viruses [173–175]	+	+	+
COVID-19 [176–178]	+	+	—

	Apoptosis	Necrosis	Autophagy
Bacterial infections			
Helicobacter pylori [179–181]	+	+	—
Neisseria meningitis [182–184]	—	+	+
Salmonella typhimurium [185–187]	+	+	—
Mycobacterium tuberculosis [188–190]	—	+	—
Autoinflammatory diseases [191–193]	+	+	+
Cancer			
Neuroblastoma [194–196]	—	—	—
Renal carcinoma [197–199]	—	—	—
Colorectal cancer [200–202]	—	—	—
Prostatic cancer [203–205]	—	—	—
Breast cancer [80, 206, 207]	—	+	+
Small cell lung cancer [208–210]	—	+	—
Cervical cancer [211–213]	—	+	+
Premalignant diseases			
Ataxia telangiectasia [214–216]	+	+	+
Paroxysmal nocturnal hemoglobinuria [217–219]	—	+	+
Myeloblastic syndromes [220–222]	+	+	+
Xeroderma pigmentosum [223–225]	+	+	+
Autoimmune disorders			
Rheumatoid arthritis [226–228]	+	—	—
Fulminant hepatitis [229–231]	+	+	—
Graft-versus-host disease [232–234]	+	+	+
Hashimoto's thyroiditis [235–237]	+	+	—
Insulin-dependent diabetes [238–240]	+	+	+
Multiple sclerosis [241–243]	+	+	+
Sjogren syndrome [244–246]	+	+	+
Systemic lupus erythematosus [247–249]	—	+	+
Psoriasis [250–252]	+	+	—
Hematologic disorders			
Aplastic anemia [253–255]	+	+	—
Fanconi anemia [256–258]	+	+	—
Polycythemia vera [259–261]	—	+	+
Ischemic injury			
Ischemia and reperfusion [262–264]	+	+	+
Myocardial infarction [265–267]	+	+	+
Brain ischemia [268–270]	+	+	+

	Apoptosis	Necrosis	Autophagy
Diseases caused by toxins			
Alcohol-induced hepatitis [271–273]	+	+	—
Pulmonary fibrosis [274–276]	+	+	—
Sepsis [277–279]	+	+	+
Metabolic disorders			
Osteoporosis [280–282]	+	+	—
Wilson's disease [283–285]	+	+	+
In Premature and Physiological Aging			
Down syndrome [286–288]	—	+	—
Premature aging (progeria) [289–291]	+	+	+
Xeroderma pigmentosum [292–294]	—	+	+
Other diseases			
Atherosclerosis [295–297]	+	+	+
Traumatic spinal cord injury [298–300]	+	+	+

Disease name (x, y, z) description: x: apoptosis, y: necrosis, z: autophagy, x, y, z: indicates the added reference number.

Table 1.
The relationship between cell death and diseases.

11. Conclusion

The association of cell death disorders with numerous diseases, such as cancer, infectious diseases, neurodegenerative disorders, and ischemic diseases (stroke, myocardial infarction), underscores the importance of researching cell death pathways for human health. In this context, studies on how cell death mechanisms function, how they are regulated, and which signaling pathways control them are rapidly progressing. A deeper molecular understanding of these pathways will pave the way for the discovery of new drugs, diagnostic tools, monitoring methods, and treatment options, providing novel, informed, and molecularly based solutions to major diseases threatening human health. Gaining more detailed knowledge about the molecular mechanisms of cell death, particularly in clarifying how cells die, is essential. Understanding the physiological and pathological roles of this critical process, which governs both physiological functions and disease, is of utmost importance.

Conflict of interest

No conflicts of interest are disclosed by the author.

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Chapter 2

Regulation of Cell Death: Therapeutic Strategies for Cancer Treatment

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Abstract

This chapter explores how to regulate programmed cell death and its relevance in cancer therapy. It points to the need of investigate mechanisms of cell death, like apoptosis, necroptosis, and, more recently, exposed ferroptosis, while creating approaches for the treatment of malignant diseases. In spite of progress in the field of targeted therapies, cancer is still a main cause of death due to the incapability of the cancer cells to undergo apoptosis. The chapter highlights the significance of adopting complex approaches that encompass multiple types of cell death occurring within both the cells and the tumor microenvironment. Of note are the functions of oncogenes and tumor suppressor genes in the regulation of cellular death processes, the specific role of apoptosis in the tumor formation process, and the possibility of treatment to increase cell death in neoplastic cells. There is metastasis of genes that are critical for apoptosis such as the TP53 tumor suppressor gene, along with the presence of an over expression of anti-apoptotic proteins. Examples of this would include Bcl-2 and IAPs, which protect multiple types of cancer cells against stimuli required for cell death. The role of immunotherapy and combination therapies is described as emerging strategies to enhance the efficacy of treatments. The document also deals with the problem of resistance to the induction of cell death and the necessity for further clinical studies of new therapeutic agents in practice. In summary, it highlights the diversity in cancer biology and the need for a paradigm shift to achieve tumor cell death.

Keywords: cell death, cancer therapy, apoptosis, combination therapies, necroptosis

1. Introduction

Recent knowledge has improved cancer classification and targeted therapies, yet it remains a leading cause of death globally. The failure of several drug candidates in clinical trials has uncovered a novel perspective on cancer development: the lack of appropriate cell death execution. It is more evident than ever that cell death regulation needs an integral understanding in oncology. In addition, a major issue is the management of apoptosis-signaling resistance leading to cancer, since widely used

anticancer agents trigger apoptosis in cancer cells. The availability of a comprehensive toolset for curation, identification, and hierarchical classification of the molecular interactions driving the cell death network may focus on future cross-disciplinary drug discovery trials. In this review, we provide an overview of the major cell death pathways and contemporary knowledge on their regulation and highlight hubs of the potential regulatory role of multiple pathways in cell death. As apoptosis is emerging as an early event in cancer development, the relevance of targeting apoptosis-signaling components for cancer therapy is also discussed [1, 2].

A considerable amount of pioneering work and the development of transgenic models that mimic human diseases have led to an impressive expansion in our understanding of oncology. These studies have revealed the profound roles of proto-oncogenes, tumor suppressors, biological outcomes, signaling networks, and molecules; founded molecular principles for cell transformation; and provided a combination of preventive, diagnostic, and targeted therapies. These advances also urged the refinement of the conventional classification of hematologic and solid cancers. The three primary human cancer classes (self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death) were completed by another three categories: limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Further research into cell death reviews the new, original roles of oncogenes and tumor suppressor genes and the interactions they mediate in numerous cell death pathways. These studies, alongside the discovery that numerous widely used chemotherapeutics trigger apoptosis to impose their cytotoxicity in cancer cells, make cell death drugs a priority in experimental and translational anticancer research [3].

1.1 Significance of cell death in biology

The growth and development of an organism, a prerequisite for the evolution of unique traits, must involve a balance of cellular production and elimination. The elimination of undesirable or improperly formed cells, either in programmed cell death or cell suicide, may appear to be a rather wasteful process. It is now clear, however, that the elimination of cells is crucial in determining the selective advantage of a living organism, and for the adaptation of an individual, it is absolutely, essential to have an organized or programmed method for the removal of cells. The ability to eliminate cells at an appropriate time during development or during the cellular restoration or repair process is important because, if a cell is not removed, it could remain a source of inflammation or transformation. Furthermore, uncontrolled or unregulated proliferation may lead to tumor formation, while continued cell elimination can prevent tumorigenesis [4]. In the absence of cell death, there would be excess cell production and wear of cells and tissues at higher eukaryotic masses, and evolution has been possible only because of the elimination of unwanted cells and tissues. It is obvious from the above discussion that cell death is a key factor in the maintenance of desired numbers of cells and tissues to perform specific functions in multicellular organisms. Since a clarified relationship exists between physical space and the functions of cells within a multicellular system, the prevention of excessive cell density in space is an important part of controlling the normal state of a tissue cell population and its size. Control of cell number in organs is a very precise process and is regulated by several complex intercellular and cell autoregulatory mechanisms. These may be chemical or mechanical signals or regulatory proteins that influence cell division and the number of cells present [5].

2. Intrinsic cell death

Two main pathways of cell death have been identified in metazoans: the extrinsic and intrinsic pathways. The extrinsic pathway, also called apoptosis, is triggered by extracellular ligands, which activate cognate death receptors on target cells. The intrinsic pathway, also mediating apoptosis, is triggered by physical, chemical, or radiation-induced intracellular damage. In response to these insults, certain intracellular molecules are either activated or inactivated, inducing cell death. These pathways mediate the controlled destruction of damaged, aged, or surplus cells, functioning as barriers to neoplastic transformation and uncontrolled proliferation [6]. The molecular mechanisms governing each of these pathways and the interactions between them have been the subject of extensive research in the last few decades. Regarding the intrinsic pathway, the process in which cells undergo an operational cell death program without the participation of death ligands and their receptors has been called 'intrinsic cell death', and some have proposed to use this term instead of death by resistome genes or non-apoptotic cell death. Indeed, the term 'intrinsic' contrasts with extrinsic, connoting the essential role of intracellular control mechanisms, their resistomes, at work in driving cells to death. These resistomes are activated in order to eliminate survivors that are genomically unstable. As a matter of convention, the term 'intrinsic cell death' will be used within this manuscript, acknowledging that it gathers different cell death processes, some of which are highly conserved across a wide range of evolutionary divergent organisms. These exhibit distinct morphological and biochemical characteristics, despite sharing common molecular and biochemical features, which are used to distinguish the process called 'intrinsic apoptosis' (**Figure 1**) [7].

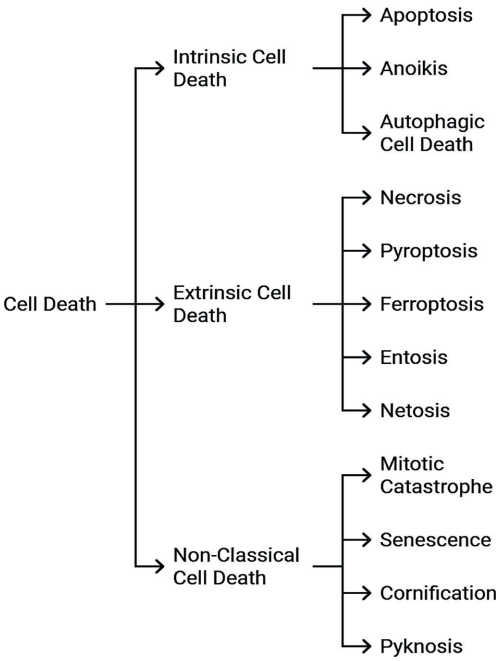

Figure 1.
Cell death types.

2.1 Apoptosis

The two most well-known types of cell death are apoptosis and necrosis. Apoptosis is a morphological term that in recent years has become understood at the molecular level. The major event in apoptosis is a non-lytic, energy-dependent, cysteine protease-mediated explosion of a cell into membrane-bound apoptotic bodies containing shrunken, condensed, and/or fragmented organelles and a nucleus that is itself highly condensed and fragmented. Many factors, including hormones, growth factors, and cytokines, regulate apoptosis, and several of these, including members of the tumor necrosis factor receptor superfamily, can either engage or antagonize the apoptotic program.

Apoptosis plays an enormously important role during development and morphogenesis. By this means, and also by taking part in tissue homeostasis and renewal, apoptosis transiently removes cells, such as neurons or germ cells, whose presence is no longer required, or which have otherwise become damaged. This is vastly removed from the idea that the only purpose of a cell is to produce more cells, and despite the irruption of cells killed by apoptosis being detected in the developing nervous system of different species of animals, the idea is still popular in developmental neurobiology [8, 9].

2.2 Autophagy

Autophagy is the process of self-digestion by a cell through the action of its own lysosomes. It is an intracellular scavenging process in which cells use their lysosomes to degrade damaged organelles and long-lived proteins. As part of the normal turnover of intracellular components, a double membrane-bound vesicle, or autophagosome, sequesters a portion of cytoplasm and then fuses with a lysosome or vacuole to deliver its cargo to the interior of the lysosome coat. The morphological hallmark of autophagy is the formation of double or multimembrane structures that engulf cytoplasmic contents. When an additional membrane engulfs the autophagosomes, it will become an autolysosome, a vacuole filled with vesicles and large bodies of degraded material, and autophagic bodies and lysosomal enzymes. During nutrient deprivation or stress, macromolecular components are sequestered within double-membrane vehicles called autophagosomes, which typically bud from the endoplasmic reticulum, and are then delivered to lysosomes for degradation. In the lower eukaryotes, autophagy provides a mechanism for survival during starvation. In plants and mammalian development, autophagy appears to play a role in both the turnover of cellular components and in the removal of superfluous or damaged organelles. In addition, autophagy may act to destroy intracellular pathogens [10, 11].

2.3 Anoikis

As a special case of apoptosis, anoikis represents the passive yet programmed form of detached cells from the extracellular matrix and cell–cell contact. The triggering of the anoikis process occurs when a given cell is genetically prone to undergo a detaching process. The notable phenotypic features associated with anoikis include cell shape transition that encompasses an increase in rounding, membrane ruffling, and apoptosis induction. Given the relationship that exists between altered anoikis and the onset of cancer metastasis, this type of cell death has received significant attention in the research community. Normally, most adherent cells will attach to

neighboring cells or the extracellular matrix, and their survival would be highly threatened in their absence. A logical question to be asked here is what would be the fate of such cells when they detach from their neighbors under normal physiological conditions? Indeed, it is possible that cells could be removed by the immune system and be considered dead cells. Under normal conditions, cell detachment would result in the activation of the cell death program which is known as anoikis, instigated first by the cell-surface receptors and followed by the activation of the intrinsic and extrinsic apoptosis pathways. Considering the role of the tight cell–matrix and cell–cell interaction in tissue homeostasis, anoikis involvement in the normal physiological state can be understood [12].

3. Extrinsic cell death

Extracellular signals through death receptors activate the external apoptotic pathway. Ligands such as FasL, TNF-α, and TRAIL bind to their death receptors, resulting in the oligomerization of the intracellular death domain in the cytoplasmic tail of the receptor. This death domain then recruits the adapter molecule Fas-associating death domain-containing protein, causes the formation of the death-inducing signaling complex, and activates caspase-8 by dimerization, leading to the activation of executioner caspases, such as caspase-3, caspase-7, caspase-6, and initiates apoptosis. The extrinsic apoptotic pathway can also interact with the intrinsic apoptotic pathway, like the cleavage of Bid by caspase-8. Finally, this increases the permeabilization of the mitochondrial outer membrane and releases pro-apoptotic molecules such as cytochrome C, AIF, SMAC/DIABLO, and Omi/HtrA, provoking apoptosis. There is also the process of necroptosis that occurs in the extrinsic apoptotic pathway when caspase-8 is inactive or insufficient. When caspase-8 is not sufficiently activated, RIP1 and RIP3 are dephosphorylated through FADD deubiquitylation, and then necrosome assembly occurs, and subsequently, necroptosis proceeds [7].

3.1 Necrosis

Necrosis of an organ causes severe damage to an organism due to inflammation and subsequent autolysis of tissue. Necrosis occurs due to many stimuli, such as infection, ischemia, and physical or chemical injury, and is biochemically accompanied by dephosphorylation of cellular substrates and activation of hydrolyzing enzymes, such as proteases. This form of cell death is believed not to be programmed because of its acute and accidental emergence and gross disruption of cell membranes. However, dephosphorylation by dephosphatase is strictly regulated in necrosis induced by several apoptotic stimuli and plays a crucial role in cell death signaling. Poly(ADP-ribose) polymerase is a representative molecule that undergoes degradation and/or dephosphorylation in the early stage of necrosis downstream of various stress signals, and recent investigations have revealed characteristic roles of dephosphorylated poly (ADP-ribose) polymerase (PARP) in cellular physiological and pathological pathways [13, 14].

3.2 Pyroptosis

Pyroptosis is a distinctive pro-inflammatory form of cell death that is triggered by inflammasome protease-containing protein complexes. Pyroptosis can occur

following canonical or noncanonical inflammasome activation. Canonical activation involves the formation of an inflammasome containing a nucleotide-binding oligomerization domain (NOD)-like receptor, the adapter, and pro-caspase-1. The adapter molecule apoptosis-associated Speck-like protein containing a CARD (ASC) allows for the recruitment of pro-caspase-1 to the NOD-like receptor, allowing for autocleavage and activation of the caspase. This main form of canonical inflammasome activation can occur as a result of recognition of microbial molecular patterns or cell death-associated molecular patterns. Microbial molecular patterns are pathogen-associated molecular patterns, and these are recognized by pattern recognition receptors. PRRs can be membrane-bound, such as the Toll-like receptors, C-type lectins, or something called the LRRP, or they can be cytosolic, such as the NLRs. All of these PRRs are able to recognize pathogen-associated molecular patterns, which can come from bacteria, viruses, proteins, parasites, or fungi. This in turn allows sterile inflammation by DAMP-triggered inflammasome activation. Canonical inflammasome-mediated death relies on the activation of caspase-1, and caspase-1 substrates are involved in the processing and release of pro-inflammatory cytokines, such as IL-1β and IL-18. In addition to cytokine release, pyroptosis includes pore formation and cytolysis, resulting in the release of intracellular contents, extracellular labeling of nucleic acids, and the release of danger signals. These, in turn, boost pro-inflammatory signaling and propagation of the immune response. Noncanonical inflammasome-mediated pyroptosis does not require the activation of the inflammasome but also leads to caspase-1-mediated lysis of the cell and release of pro-inflammatory cytokines [15, 16].

3.3 Ferroptosis

Ferroptosis in 2019 marks the seventh type of regulated cell death. Ferroptosis is a newly identified mode of non-apoptotic cell death driven by iron-dependent lipotoxic damage. As demonstrated by the pioneering studies, ferroptosis is caused by the perturbation of glutathione (GSH)-dependent antioxidant defenses orchestrated by two key enzymes: the lipid repair enzyme glutathione peroxidase 4 (GPX4) and the rate-limiting enzyme of glutathione synthesis, system xc-. As such, direct inhibition of GPX4 could cause an overwhelming accumulation of cytotoxic lipid reactive oxygen species (L-ROS) to execute ferroptotic death. Similarly, the functional inhibition of GPX4 by a highly selective GPX4 ligand represents the most common method to induce ferroptosis due to high biosafety and controllability. Inhibition of system xc- by its derivatives indirectly leads to the inactivation of the lipid repair GPX4, which is crucial for ferroptosis too. Dysregulated metabolic reprogramming of excessive lipid biosynthesis and suppressed lipid catabolism, specifically monophosphorylation of pantothenate by pantothenate kinase 1 (PANK1), has been reported as triggers of ectopic lipid peroxidation in several malignancies. The typical pathognomonic features of ferroptosis, such as ultrastructural condensation of the mitochondrial membrane, resolution of the mitochondrial crista, mitochondrial rupture, increase in cytoplasmic density, and rupture or "ballooning" of the outer mitochondrial membrane, have all been delineated. Given that there are morphological differences among the RIPK1/RIPK3 MLKL-triggered necroptosis, the pannexin1/pyroptosome-induced pyroptosis, and the GPX4-mediated ferroptosis, reliable cooperation of an immunity-related GTPase subfamily M protein has been reported to be required for effective lytic podosome-induced plasma membrane destruction during some specific

contexts of GPX4-mediated ferroptosis in cellular coculture models. Such findings consolidate our understanding of ferroptotic procedures and have implications for therapeutic intervention. The recent findings on the genetic and epigenetic regulation of GPX4-mediated ferroptosis further reveal the mechanism of action of GPX4 and will facilitate subsequent applications of ferroptosis in diseases including cancer and neurodegenerative diseases [17, 18].

3.4 Entosis

Entosis is a cell death process initiated by one cell type, the entotic cell, in the process of internalizing another cell, the entosed cell. Entosis is a complex and still poorly understood process. Despite the increased entotic cell deaths seen in certain carcinomas, it was observed that entosis was not influenced by the apoptotic state of the entotic cell. In studies outside of U-373 cell lines, both Bram and others observed mitotic and non-mitotic elimination of entundles, by which entotic cells are ejected back out into the tissue. It was shown that most entotic cells were mitotic non-mitoses above the normal frequency at this cell line, supporting the observation that mitotic entosis is frequent in the tissues from which these cells are derived. Together, the literature suggests that entosis has a variety of cell death and survival modes, and that the tissue environment dictates the response of the surrounding cells [19].

3.5 Netosis

Netosis is the process by which activated neutrophils can trap and kill microbes by actively releasing meshworks of extracellular DNA decorated with microbicidal proteins and histones. In the process, netosis can kill not only bacteria but also fungi and even enveloped viruses and extracellular protozoan parasites, some of which may become entrapped in neutrophil extracellular traps (NETs). Although considered a form of cell death, unlike apoptosis and necrosis, the process of netosis is unique among cell death pathways in that it is a type of suicidal mechanism by which viable neutrophils, having exhausted their phagocytic, degranulation, and superoxide defenses, can undergo their own programmed self-destruct that costs them their lives but, in turn, benefits the host by releasing meshworks of DNA and cytotoxic proteins. Netosis may more closely resemble necroptosis than apoptosis, as much as it seems to be triggered only under conditions where cells are critically stressed, such that inflammasome activation or ATP depletion in stressed neutrophils might lead to the release of their curated DNA [20]. NET release can also be triggered in cell-free preparations, such as by exposing soluble neutrophil DNA to microcrystals or upon addition of calcium ionophore to isolated nuclei. NETs can also entangle and eventually kill platelets or erythrocytes, thereby contributing to thrombosis. However, as in all forms of cell death, together with the perceived benefits of activating netosis, there are also associated pathologic liabilities, namely anaphylaxis in the case of IgE-mediated netosis, vessel occlusion in the case of thromboinflammation, vascular and tissue damage in autoimmune diseases, and chronic inflammation related to elevated NET levels in cancer or cardiovascular diseases. Therefore, even though several netosis-inhibiting strategies have been proposed, the clinical exploration of NET degradation as a form of therapeutic intervention warrants further investigation [21].

4. Nonclassical cell death

Cell death is critical for removing unnecessary or unwanted cells from organisms. It is also essential for maintaining homeostasis by balancing cell loss and cell production. Non-apoptotic, non-necrotic cell death research has broadened our understanding of how this type of cell death, which is not easily identified by conventional methods, can be regulated and how the process can be manipulated. This review focuses on recent research on cell death that attempts to unveil the existence of nonclassical cell death and some of its functions, as well as the relationship between non-apoptotic, non-necrotic cell death and inflammatory processes in animals. This research suggests that recognizing the diversity of cell death can increase our understanding of basic biological phenomena and provide some new opportunities for translational research. To monitor the cells that undergo non-apoptotic, non-necrotic cell death, several reporters have been utilized, such as lactate dehydrogenase in Drosophila. Drosophila Ldh is expressed in apoptotic cells and is not an apoptotic executioner, rather it is a p53-independent dominant pro-survival factor. LDH is necessary for the death of various cell types. The Hippo pathway is a crucial signaling pathway regulating cell fate. Its core kinases are the large tumor suppressor 1/2 and neurofibromatosis type 2, which phosphorylate and deactivate the transcriptional coactivators, Yap, and Taz. When the Hippo pathway is on, it inhibits the activity of Yap and Taz, leading to cell death. However, the depletion of Drosophila Ldh in the wing disk suppresses the apoptosis induced by activated Ras in GOF clones.

4.1 Mitotic catastrophe: Cell death during mitosis

Mitotic spindle perturbations may hamper segregation of sister chromatids or cytokinesis, leading to the formation of cells with a slowly ballooning, sick morphology and highly condensed chromosomes. One of the most common blockades of the mitotic program involves the inhibition of spindle structure oligomerization. The cells try to progress through prophase, but neither their nuclear envelopes break down nor their chromosomes change shape. They gradually swell up, do not elongate, and are frequently connected by a microscopic cytoplasmic mass that becomes filamentous upon treatment. Because these cells cannot divide, they die at a later stage by apoptosis. Other spindle poisons get the affected cells arrested transiently at prometaphase in mitosis, but if the poisoning conditions are acute, the cells undergo instant apoptosis. Once these apoptotic cells reach anaphase, progression is awarded by a prematurely condensed morphology, the formation of chromosomal rosettes, and excessive chromosomal fragmentation. During the process of programmed anaphase development of spindle checkpoint-insensitive cells, a couple of hundred apoptotic cells accumulated in the cultures. The majority of the apoptotic cells are anucleate when their apoptotic fate is decided, that is, they are blasted to bits after DNA synthesis has been completed. Topoisomerases, during late S phase of progression of DNA-denatured chromosomes, make up the great majority of early apoptotic cells. The lack of anaphase is proposed to be the first cellular malfunction that precedes apoptotic death [22, 23].

4.2 Senescence: Dormant state due to DNA damage or telomere shortening

The occurrence of senescence, a type of nonproliferation, can no longer be refuted. Once regarded as an artifact due to massive amounts of DNA damage or as a clinical phenomenon *in vitro* because of continued cell activation, recent research

has revived classics for incalculable reasons of cell behavior. The key to re-evaluation was the identification and functional application of specific types of senescent cells *in vivo*. Senescence-like phenomena that occur under various circumstances suggest that senescence plays a role in various life phenomena, from development to certain crises in life that are closely related to senescent phenotypes, such as tumors or aging. In a process known as replicative senescence, normal cells reach a telomere-dependent proliferative limit that later transforms them into a senescence-like state, even without exogenous stress, and typically activates the p53-p21 or p16-Rb axis to restrict cell cycle progression. Exacerbating telomere erosion induces a signal that is, similar to a DNA damage harbinger. This signal is amplified by a 53BP1 axis, which tackles leading and lagging telomeres and is an active source of dysfunctional telomeres. Large chromatin fragments resulting from dephosphorylation of the cohesin subunit protect broken chromosome ends from harmful effects, thus providing growth-restrictive signaling. However, this is not a general picture of senescence caused by damage. In an experiment using the thickest particle, GM-CSF R is elevated compared to Ra, leading to the formation of micronuclei, and senescence is inactivated in a manner dependent on the presence of the long intergenic non-coding RNA (LINC) complex or Lamin A/C, suggesting that cells that require signaling from the nuclear envelope need to be transmitted. Such questions still remain, and the specific signals that trigger role retraction still need to be tested, so the study of various senescent subtypes progresses [24, 25].

4.3 Cornification: Cell death in skin

When a cell nucleates, it becomes denser and more opaque due to the presence of numerous organelles, especially mitochondria, which contain ribonucleases and proteases. This is especially true for cells in the outer epithelial layers, in the skin, and the digestive or respiratory apparatus. When the nucleated cell is surrounded by several layers of cells and keratin filaments, it eventually dies, establishing a compact mass of cells in which no "live" replicating cells are present. The dying process of the cells in the upper layers of the skin is called cornification. At a given moment, the plasma membrane of a cell becomes impermeable, complex lipids are released, the cell becomes dry, and the contents increase in concentration. Some small cytoplasmic organelles are then shed, leaving a cell that is only a case and a nucleus. The cell is usually genetically programmed to remain for a certain period, some functions being maintained in this phase, such as the synthesis of filaggrin. The K1 and K10 filaments are at that stage hyperactivated to grow in size and to prepare for a later phase of hyperactivation. Cells outside the nucleus degrade the DNA and are anucleated. The keratin of corneocytes can have a protective function for the cell membrane and can protect the time capsule from tissue alterations around the cells, permitting the storage of transglutaminases in its cytoplasm. It can also be a source of energy and carbon in the skin [26].

4.4 Pyknosis: Nuclear shrinkage and chromatin condensation

The nucleus of a cell undergoing apoptosis exhibits condensation of the chromatin, involving shrinkage and additional chromatin aggregation toward the inner nuclear envelope. However, the nucleus retains a polygonal outline, and the chromatin condensation is irregular in distribution, resulting in adjacent nuclear regions with different densities. Nuclear fragmentation into multiple, equal-sized, non-abutting,

uniformly dense chromatin bodies or karyorrhexis ensues, and eventually, fragments of the chromatin bodies are extruded from the nucleus, enveloped by single, thin stretches of physical continuity. Nuclear fragmentation proceeds by lysis of the peripheral and internal nuclear envelope structures, including the nuclear pore complexes and the lamin network, without evidence of disassembly of the nuclear envelope. Most cells examined during apoptosis undergo chromatin condensation, which is the earliest ultrastructural marker, and some cells undergo nuclear fragmentation and chromatin clumping. These cells show characteristic apoptosis-associated biochemical changes, including DNA fragmentation into nucleosomal repeats, activation of endonucleases, nicking of the nuclear matrix protein, indicating apoptosis-specific caspase activity, and membrane-associated cellular changes. Some studies suggest that the nuclear pseudo-environment remains intact during apoptosis. The chromatin condensation displays topoisomerase II and mitotic kinase activity that are blocked by known non-apoptotic stimuli. Upon inhibition, the histones as well as topoisomerase II reach the nucleus but are not involved in repressing DNA replication. Summatively, microscopy of nuclei from cells undergoing apoptosis demonstrates DNA shattering into nucleosomal repeats and the morphological counterparts of condensed and clumped chromatin, as well as nuclear envelope lysis and the transport of the apoptosis-specific nuclear alteration [27, 28].

5. Role of cell death in cancer development

Cancer is a group of diseases characterized by the inability of cells to control their proliferation. This behavior stems from alterations in intracellular signaling pathways that control cell division and the mechanisms regulating the cell cycle. These alterations can be either gene mutations that lead to constitutive activation of signaling pathways or the inactivation of suppressor genes that counteract the activity of these pathways, leading to alterations in cellular behavior. The primary drawback of the excessive number of cells in a certain area is that it overburdens the organism with resources, thereby compromising the functionality of organs. In fact, cancer is a life-threatening condition due simply to the accumulation of damaged cells and the formation of masses of these cells, which we call tumors. Researchers have identified several strategies through which cancer cells escape death, thereby ensuring an advantage over their neighbors that reduces competition for resources. In the last 20 years, the field of cell death, particularly apoptosis, has been explored for therapeutic targets in cancer treatment. This was based on the fact that most chemotherapeutic agents act by modulating cell death and were generally accepted without a deep understanding of the molecular architecture that explains the ability of multiple insults to cause cell death. Today, with more and more researchers interested in cancer, we have a much deeper understanding of the genes and the molecular phenotype of the cell involved in cell death escape in the initiation and development of cancer. This has opened new therapeutic horizons in cancer research [29, 30].

5.1 Tumor suppression: Apoptosis eliminates precancerous cells, preventing tumor formation

The genetic damage or oncogenic events turn precancerous cells independent from the exogenous growth stimuli, which is a crucial event facilitating the development

of the tumor. Following the oncogenic activation, oncogene-dependent tumor suppressors sense the increase in proliferation signals and transmit the message to mitochondria to induce apoptosis by increasing the permeability of the mitochondrial membrane. The increase in the permeability of the mitochondria liberates apoptogenic factors leading to the activation of the BCL-2 dependent apoptosis cascade that is activated by its pro-apoptotic members. After they are activated, the pro-apoptotic proteins commit their inhibitory effects on the anti-apoptotic proteins, and by doing so, they prevent them from arresting the action of their pro-apoptotic counterparts. This action will lead to caspase activation, which will act on a number of proteins involved in the regulation of the cell cycle, cytoskeletal organization, and cellular interaction mechanisms of the cell leading to its death. The most known oncogenic events in colitis-associated cancer are the mutations in the K-Ras, TP53, and Adenomatous Polyposis Coli genes. When mutated, the mutant protein of K-Ras or Apc leads to increased proliferation signals, while the mutated form of TP53 leads to apoptosis inactivation. Similar to hereditary polyposis or sporadic CRC, disruption of the BCL-2-dependent apoptosis cascade contributes to the initiation of the oncogene-dependent apoptosis when the BAX pathway is silenced by methylation, and this leads to the initiation and development of colitis and the onset of the early cancer stem phenotype [31, 32].

5.2 Tumor progression: Dysregulated cell death pathways enable cancer cell survival and proliferation

Neoangiogenesis provides the necessary growth factors to initiate rapid proliferation. In addition, surviving cells secrete signals preventing apoptosis of their type in the developing tumor; however, the untreated surrounding endothelial cells undergo an injury signal cascade mediated by hypoxic stress. In the absence of appropriate new signaling or survival factors, a selection of cells from the genetically unstable pool is made to avoid growth signal dependence, and they progressively grow using the remaining carcass of the dying tumor. The dying cells ultimately undergo a cell-death-related procedure since they sustain genetic modification as a consequence of the epigenetic stress response [33]. The relatively high number of incompletely reproduced daughter cells and associated genetic mutations offer the ideal opportunity to the cells with reduced growth signal dependence to conceptualize a new clone evolving to reduce their apoptotic response. This recurring process detected in the *in vivo* evolution of tumors, whether neoplasms, primary cells developing spontaneous transformation, chemically induced tumors, or transplants in animals, mainly depends on the standard cellular reaction to stress damage, which induces both the main stress-protective anti-apoptotic response and the genetically deregulated BCL-2 family protein level. This modified response improves the apoptotic block. It has been increasingly recognized that the inability of a damaged cell to die contributes to tumor development [31, 32]. What is still unclear is the correct identity of the damaged tumor cell that sustains the genetic mutations. The immune system identifies those cells directly damaged by apoptosis. However, since, under constant immuno-surveillance, the dying cells are not yet recognized by innate mechanisms, genetic mutations are less likely to originate in mature cancer cells but need to be thought of as initiated in normal stem cells or early differentiating cells. If apoptosis also occurs in such rescuing tumor cells, both the lack of apoptotic signal components and the low mutations carried by

their daughters would make it harder to generate the complete disruption of growth signals needed for growth/survival autonomy [33].

5.3 Cancer stem cell maintenance: Autophagy promotes stemness, maintaining cancer-initiating cells

It is known that autophagy plays a role in both maintaining the cancer stem cell phenotype and resistance to therapy. In glioblastoma, the cancer stem-like cell subpopulation showed a great dependency on autophagy to retain their stemness characteristics. This can be seen in the fact that it is the resistant cancer stem cells that depend on autophagy, which is actually inhibited by caloric restriction in the sensitive cells, while caloric restriction was necessary for driving autophagy in the resistant population. The use of the autophagy inhibitor reduced sphere formation in the resistant population alone, with resistant subpopulations showing high basal levels of LC3 and low levels of SQSTM1. Similar high glucose stem-like cancers in squamous cell carcinomas have also shown differences in glucose utilization between the stem-like and non-stem-like populations. These differences in metabolism likely promote chemoresistance through the process of autophagy. Autophagy in cancer stem cells is tightly regulated by hypoxia-inducible factors. In non-stem-like cells, HIF1α will block the process of autophagy by upregulating mTOR, as illustrated by the stabilization of autonomous macropinocytosis through this relationship. Meanwhile, in the cancer stem-like cells, hypoxia-mediated autophagy protections promote stemness, along with pro-invasive and pro-migratory behavior. The molecular targeting of HIF1α has, in fact, been demonstrated to stimulate a process of mitophagy in a HIF1α-positive manner [34, 35].

5.4 Immune evasion: Cancer cells evade immune-mediated cell death, facilitating tumor growth

The ability of cancer cells to avoid immune surveillance has been considered a hallmark of cancer. Cancers arise and develop when growing malignant cells acquire alterations that enable them to avoid immune injury. These immunologic sculptors enable cancer cells to circumvent the normal checks and balances that innate and adaptive immunity impose. Quite simply, tumors are a classic example of clonal selection. It makes sense, then, that a defective cell death program would be adaptive in cancers; the tumor mass shields cancerous cells from immunologic recognition, thereby enhancing growth and evolution. Immune suppression may also be involved in the enhanced ability of premalignant and malignant cells, which would otherwise be killed, to evade immunosurveillance and grow, even in the absence of growth factor stimulation. Thus, immune evasion is not merely a philosophical oncology issue; it has significant clinical implications. Cancer is a consequence of dysregulation in a handful of signal transduction pathways that typically control immune and defense decisions. In addition to growth and death, these pathways connect with other normal cells and tissues throughout the body, producing angiogenesis, tissue remodeling, suppression of the host's immune responses, and all of the other capabilities unique to cancer. These immune responses can, nevertheless, be harnessed and tailored to target and destroy cancer cells without the need for chronic pharmacologic intervention or toxicity. Establishment of this concept and proven clinical protocols lie at the heart of the work of multiple groups in the field [36].

5.5 Genomic instability: Defective cell death pathways contribute to genetic mutations, driving cancer progression

The function of different cellular oncogenes or tumor suppressor genes implicated in cellular growth and proliferation has been well studied. Other gene alterations can be mutator genes, a new class of genes implicated in driving tumorigenesis. The proliferation of poorly proliferative cells can result in the induction of single-strand crosslink repair, reckless abandon, and p53. The inhibition of these factors and the absence of a functioning p53 lead to the introduction of mutations and a high level of genetic instability. Regularly, inhibition results in the accumulation of cells in the G2 phase; the cells appear to escape G2 arrest and proceed into mitosis with their aberrantly processed DNA. The damage then propagates through the S phase, allowing the replication of abnormal chromatids. Tumor suppressor genes can cause genetic instability not only through the regulation of cell cycle checkpoints and the integration of DNA repair but also by interfering with the activation of the apoptotic pathways. Defective apoptotic pathways can promote the survival of cells with unresolved genotoxic lesions, which can further replicate and incorporate mutations. The intrinsic apoptotic pathways promote cell death after the occurrence of DNA lesions that result in more dangerous damage to cell survivability. p53 is the key activator of the extrinsic apoptotic pathway and the first response to DNA damage. Cells with nonfunctional p53 would be sensed by the extrinsic apoptotic pathway and likely survive with unrepaired DNA damage, leading to genomic instability [37]. The resultant mutation accumulation contributes to tumor initiation and progression by increasing the accumulation of tumorigenic mutations and loss of heterozygosity. Indeed, autoimmune lymphoproliferative syndrome patients are at a 10,000-fold increased risk of NHL, mostly due to the downregulation of the extrinsic pathway-dependent apoptotic response and decreased immune contact cell-mediated apoptosis. Tumor suppressor p53 transcriptionally activates TRAIL receptor 1 and TRAIL receptor 2, which then activates the caspase 8 heterodimers and apoptotic cell death. Typically, the mutual amplification of the "death signal" between the extrinsic and intrinsic pathways requires apoptosis through the activation of caspase 8 and tBID cleavage. However, when caspase 8 is removed, the tBID cleavage is reduced and, consequently, the "amplification of the death switches" is turned off. Such a defect in the extrinsic pathway of cells prior to defective mitochondrial pathways will not be protected from apoptosis, which leads to caspase 8 and p53-mediated apoptosis and tumor suppression [37, 38].

6. Cell death mechanisms in cancer development

Resistance to cell death is a central cancer hallmark, as resistance to cell death enables the outgrowth of primary tumors and facilitates resistance to both chemotherapy and radiotherapy-induced cell death. The two major apoptotic pathways, the intrinsic or mitochondrial and the extrinsic or cell-surface agonist pathways, play important roles in cancer development and responses to both cancer and immunotherapy. The intrinsic apoptotic pathway is controlled by B-cell lymphoma-2 (BCL-2) proteins, which are either pro-apoptotic or anti-apoptotic. Apoptotic pathways are also coordinately regulated by other cell-intrinsic factors such as the cellular FADD-Like IL-1β-Converting Enzyme (FLICE is also known

as Caspase-8)-inhibitory protein, caspase-8 homolog, and inhibitors of apoptosis. Mutations that result in hyperactivation or loss of death receptor expression can disable the extrinsic apoptotic pathway in tumors. In most cases, multiple cell death pathways are inhibited or resistant in cancer patient cells. Non-apoptotic forms of cell death such as necroptosis and ferroptosis can also control oncogenesis and cancer therapy eradication as well as patient immune surveillance. The ubiquitin-proteasome system also plays many key regulatory roles in apoptosis and can be accelerated by tumor transformation, both by mutating key regulatory components or increasing the rate of protein synthesis in cell death-inhibitory proteins. A control pool of the pro-apoptotic protein BAX can be eliminated through proteasome/ubiquitin-mediated regulatory steps. BAX interacts with a protein that tags BAX for proteasome-mediated destruction. Targeting this interaction may increase BAX levels, and this can enhance apoptotic sensitivity in tumors. Defects in apoptosis are commonly selected during cancer development because they provide cancer cells that replicate with a transformed competitive edge [39]. During treatment, defects in apoptotic cell death are likely to provide cancer cells with a therapeutic advantage, and as a result, many anticancer treatments act through the activation of apoptosis. Small molecules trigger the extrinsic and intrinsic apoptotic pathways, and chimeric antigen receptor T cells target the extrinsic apoptotic pathway, efficiently clearing tumor cells in certain patient clusters. Since treatment often results in immunosuppressive side effects, several antigens have been targeted to eradicate CAR cells from the treatment site. In many cases, combined treatment strategies have been developed to enhance anti-tumor activity, which may include the targeting of non-apoptotic death pathways [40].

6.1 Apoptosis: Programmed cell death, inhibited in cancer cells

Since cancer cells grow out of control, thinking of cancer in terms of cell death often may seem counterintuitive. However, it is excessive cell growth rather than excessive cell death that is truly maladaptive. When cells divert from the standard program of cell death, as occurs in cancer, they not only continue to propagate to form a tumor, but they also develop changes that make them less compatible with their tissue and surrounding environment, ultimately causing harm to the macro-organism in which the tumor is forming [2, 9, 41]. Apoptosis, also known as programmed cell death, is the process by which cells are efficiently guided to death, and defective apoptosis is classic in cancer. In order to further dissect the importance of the elevation of apoptosis efficiency in cancer, we will briefly discuss apoptosis and its role outside of cancer. Then we will discuss its effects on cancer initiation and progression in greater detail. Apoptosis is a desirable process for multicellular organisms at multiple levels. At the simplest level, it allows for the appropriate number of cells needed in tissues to be present; if the entire organism consisted of nothing but fast-producing cells without a corresponding rate of cell destruction, the organism as a whole would grow at an unchecked rate and its growth would not be controlled. As a result, certain distinguishing phenotypes of apoptosis include the relatively sedate process of cellular breakdown and disposal. Furthermore, as chemotherapy follows the logic of regulated cell death, by pushing cells over into apoptosis, apoptosis is particularly attractive in cancer treatment, as apoptotic cells are less likely to spill pro-inflammatory molecules, undergo destructive lysis, or evoke inflammation than necrotic cells [2, 9, 41].

6.2 Necrosis: Unprogrammed cell death, contributing to tumor progression

Necrosis is generally regarded as an unprogrammed mode of cell death. This type of cell death is inappropriate from an evolutionary point of view. Unlike programmed cell death, such as apoptosis, necrotic cells swell, and plasma membrane integrity is disrupted. This results in the accidental massive release of pro-inflammatory intracellular components from the dead cells, which can contribute to an inflammatory response. In some cases, inflammation can be a strong promoter of tumor development, possibly by facilitating tumor formation and proliferation. Necrotic cell death might enhance tumor formation and growth just by stimulating obligatory immunological clearance of cells in very early pre-oncogenic developmental stages, prior to any tumor dormancy establishment [42]. On the other hand, the increase in the number of necrotic cells at the tumor core could contribute to larger necrotic areas and to the subsequent hypoxia, which is known to trigger adaptive mechanisms that boost tumor progression. Clearly, but in principle, the manipulation of necrosis should be independently determined in context. Though many efforts have been made to understand the intricate nature of necrotic cell death, the mechanistic insight is lagging far behind what is known for apoptosis. In the context of cancer, some patients might show increased resistance to necrotic cell death. On the other hand, cancer therapy can trigger necrotic cell death in patient tumor cells, inducing anti-tumor immune responses inside the patient's body. Such opposing roles of necrosis in a cancer scenario could provide a promising direction for tumor-specific therapeutic strategies. Especially, the ability of tumor cells to escape the cytotoxic effect of necrotic cell death could be exploited [43, 44].

6.3 Autophagy: Self-digestion, promoting cancer cell survival or death

Autophagy: Self-digestion, promoting cancer cell survival or death several hallmarks of cancer, in particular regarding therapy resistance and the malignant phenotype, could be related to an abnormal cancer cell capacity to activate protective autophagy, often also contributing to an increased necrotic cell death. Tumor cells exposed to hypoxia or nutrient-deprived conditions often endure survival *via* the activation of the ATM signaling pathway that promotes cell cycle arrest and resistance to apoptotic cell death. The main functions of protective autophagy include: the acquisition of metabolic substrates such as amino acids, fatty acids, and nucleotides, and the removal of molecules for lysosomal degradation when other forms of cell death programs are inhibited. The lysosomal cathepsins promote direct non-apoptotic pATM-mediated phosphorylation events of cytoplasmic proteins that help cancer cells adapt to hypoxia or nutrient starvation, initiating protective autophagy [45, 46]. The crosstalk between autophagy and apoptosis is intricate and part of the complex scenario also involves the autophagic degradation of pro-apoptotic proteins and the inhibition of apoptosis through the activation of anti-apoptotic proteins. Reactive oxygen species and p62 play important roles in ensuring the balance between autophagic survival and apoptotic cell death. Autophagy can inhibit apoptosis, providing cancer cells new chances to promote tumorigenesis or develop therapy resistance. Under prostate cancer hypoxic conditions, high mobility group box 1 protein and poly(ADP-ribose) polymerase 1 activities stimulate p62-related activation of the NRF2 pathway to promote PCSC survival *via* protective autophagy function. GTPases are key players in autophagy, and their activities can also lead to

pro-survival benefits for cancer cells, including increased chemoresistance. ATG5 overexpression/mTORC1 inhibition also renders lymphoma cells less sensitive to apoptosis, and ATG5 is associated with worse clinical outcomes in at least one lymphoma subtype. Other less expected autophagy-related gene signals counteract tumor-suppressive signals, such as the TET1/2 DNMT methylation regulation-mediated PANX1-associated autophagy pathway that allows breast cancer cells to escape from senescence as PANX1 silencing or rapamycin induces a cell cycle arrest in the G0/G1 phase of breast adenocarcinoma cells. In contrast, some downsides of autophagy activation are apparent. For example, BCL2-mediated protection of the beclin 1-BCN signalosome has an essential role in chemotherapy resistance of melanoma cells, and silencing of both BCL2 and ARRB2 genes activates protective autophagy. The inhibition of these reroutes chemotherapy-induced death of melanoma cells toward autophagic cell death. It should be noted that although this is a form of programmed cell death, the reduced amount of caspase-independent regulated cell death did not express immune-stimulatory signals when 'eat-me' signals were not properly switched on. Therefore, to activate protective autophagy in combination with other therapies, further exploration of the signaling pathways based on the characteristics of individual types of cancer cells is required [47, 48].

6.4 Pyroptosis: Inflammatory cell death, influencing tumor microenvironment

Inflammatory responses in the tumor microenvironment have emerged as an important factor in stimulating the advancement and immunosuppression of cancer. Pyroptosis, both a pattern of cell death and an effector of inflammation, offers an insightful link between cell injury and inflammation. Pyroptosis may directly result from damage-associated molecular patterns that appear in the extracellular environment or from damage signals that bypass inflammasome activation, releasing cytokines into the surroundings. These patterns modulate the tumor immune microenvironment and interact with tumor growth, invasion, and metastasis. Specifically, gasdermin D, which metamorphoses in the process of pyroptosis, contributes to ferroptosis in some settings and participates in a subset of inflammatory events linked to neutrophil recruitment [49, 50]. Nonetheless, the role of pyroptosis in cancer is far from clear because of contradictions in previous findings. Some of these studies argue that inducing pyroptosis in neoplastic cells ordinarily does not act on inflammation-borne keratinocytes and alters macrophage-cytotoxic T lymphocyte communication to minimize the immunosuppressive tumor microenvironment. These aspects underscore the complexity and potential context-dependent effect of pyroptosis in cancer and suggest that understanding the intricate regulatory interaction between neoplastic cells and immune cells in each of these processes can help manage pyroptosis for efficient cancer treatment. Although the prevalent stimulants and paths of pyroptosis have been established, the related medicinal significance within the context of cancer is still uncertain, suggesting further investigations are mandatory [51, 52].

6.5 Ferroptosis: Iron-dependent cell death, linked to cancer therapy resistance

Leukemia cells treated with buthionine sulfoximine, a blocker of glutathione synthesis, and with an inhibitor of cystine-glutamate antiporter, system Xc-, acquired resistance to erastin. These resistant cells also showed a decrease in the level of ROS, and inhibition of xCT expression suppressed the ROS level and cell death induced

by erastin in parental Jurkat cells. Furthermore, melanoma, breast cancer, and osteosarcoma cells treated with cisplatin, a cysteine analog, showed resistance to erastin-induced cytotoxicity associated with decreased intracellular ROS levels. Thus, the level of intracellular cystine regulates the sensitivity of cancer cells to ferroptosis inducers, such as erastin and cisplatin. However, glutathione peroxidase 4, a glutathione-dependent lipid hydroperoxidase that protects membranes from lipid peroxide damage, has been shown to be a negative regulator of this death process. Knockdown of GPX4 expression results in sensitivity to ferroptosis in erastin- or cisplatin-treated cancer cells. Therefore, depletion of intracellular glutathione levels or inhibition of GPX4 activity is strongly related to the ferroptotic mechanism. Furthermore, mammalian targets of rapamycin and p53, both of which are involved in inhibiting GPX4 expression or activity to enhance lipid peroxidation, have been shown to modulate ferroptosis. Therefore, ferroptosis is considered a death signaling pathway that can be utilized to eradicate molecularly targeted therapy-resistant cancer stem cells [17, 53, 54].

7. Cancer treatments targeting cell death

As the hallmark of cancer cells is to be resistant to cell death, an efficient way to kill cancer cells is to reactivate one or several cell death pathways. Cells become resistant to apoptosis in many ways. Cancer research on cell survival regulation mainly focuses on the intrinsic, or mitochondrial pathway, since it is inhibited in a significant percentage of aggressive cancers. There are two aspects of cancer cells. The first is to overcome the blockage of the DNA damage checkpoint of p53, usually solved by deletions or missense mutations of TP53. Therefore, reactivation of the tumor suppressor p53 is quite complex, and no promising therapy exhibits clinical efficacy to date. Anyway, new therapies aim to elevate the wild-type p53 protein level for eradicating cancer cells, and in a different strategy, to prevent its degradation. These suppressor drugs, Fas ligand, and TRAIL, are called super killer drugs. The problem is that they have undesirable side effects on healthy cells. This new therapeutic approach is studied in combined therapies at subtoxic concentrations of both agents. However, resistance to Fas ligand and TRAIL is also possible with defects in the extrinsic apoptotic pathway. Another objective is then to correct the capability of cells to initiate apoptosis through death receptors. Optimally, the response to targeted therapy for cancer cell elimination is increased. In this regard, arsenicals, such as an agent from Traditional Chinese Medicine, active against acute promyelocytic leukemia (APL), have been shown to revert TRAIL resistance in different cancers [55, 56].

7.1 Chemotherapy: Induces apoptosis, necrosis, or autophagy

The main aim of chemotherapy is to kill cancer cells, thereby shrinking or completely eradicating tumors and mitigating the effects of most types of advanced cancer. However, chemotherapy mainly targets rapid-replicating cells by activating apoptosis and/or necrosis as the cells undergo mitosis. Unfortunately, this method not only affects cancer cells but also all rapidly proliferating normal cells such as hair, blood cells, and the stomach lining. Consequently, the appearance of side effects in these tissues is known. It is known that when a certain proportion of cell death is induced, it results in a secondary phase of apoptosis. Numerous studies have reported

the relationship between the levels of p53 and chemosensitivity; when the expression level of p53 is low, the resistance to these treatments is enhanced. The naked truth is that only 10% of tumors express wild-type p53, which could be attributed to factors such as the technique used to assess the levels of the protein or the systems involved in regulating the TRP53 gene. Moreover, the excessive cell death in these patients intensifies the pressure faced by oncologists who come under enormous scrutiny for the treatment regime and their decisions. Unfortunately, in recent years, the reported resistance to certain chemotherapeutic agents is attributed to the activation of the autophagic process, which can set apart micro-metastases or depleted tumor cells from the effects of treatment administration. On this note, combinative treatments are aimed at autophagy inhibitors [57, 58].

7.2 Targeted therapies: Inhibit anti-apoptotic proteins (e.g., Bcl-2 inhibitors) or activate pro-apoptotic pathways

Targeted therapies: Inhibit anti-apoptotic proteins or activate pro-apoptotic pathways. Bcl-2 forms heterodimers with pro-apoptotic molecules to prevent their activity. Inhibitors of Bcl-2 are promising to treat chronic lymphocytic leukemia, which could be extended to other blood cancers that depend on Bcl-2 expression. Hdm2 proteins bind the transcription factor p53, inactivating its pro-apoptotic function. An inhibitor of Hdm2 could restore p53-dependent apoptosis in cancer cells with wild-type p53. Proteasomes play a key role in the regulation of apoptotic pathways by degrading key components associated with cell death. Inhibitors of the proteasome have been approved for multiple myeloma, and these may also restore sensitivity to standard agents that induce apoptotic pathways. Epigenetic regulation of genes involved in apoptotic pathways could offer new opportunities to explore, including the downregulation of Bcl-2 by hypomethylating agents or targeting the histone deacetylases associated with poor response to chemo-radiotherapy through the promotion of acetylated histones involved in cell death pathways [59–61]. Tyrosine kinase inhibitors induce not only cytostasis and differentiation but can also induce apoptosis in the leukemic cells and those of other tumors expressing high levels of anti-apoptotic proteins. Their inductive potential of apoptosis has been associated with the presence of mutations of the anti-apoptotic proteins, with their ability to reduce pathway activity, as well as with the inhibitory effect on BCR-ABL. The newly tested FLT3-TKI increases apoptosis in FLT3-AML both in monotherapy and in association with other compounds. Reactivation of pro-apoptotic pathways was also obtained with agents such as Imatinib and Trastuzumab. Imatinib has been shown to induce apoptosis in K562 cell lines and in primary cells from CML patients because of its ability to inhibit BCR-ABL through the activation of caspases. Trastuzumab adds the antibody-dependent cellular cytotoxicity property to the rational therapy since Her-2/Neu proteins are displayed on the extracellular portion of the plasma membrane. Preclinical studies have shown that combined therapy with anti-HER2 agents and PARP inhibitors induces apoptosis in acute lymphocytic leukemia cells. Adenosine triphosphate competitive dual inhibitors prevent phosphorylation of the downstream molecule AKT; hence, AH-TORKs lead to apoptosis in lymphoma cells, including those resistant to rapamycin and bortezomib. AH-TORKs may also be excellent candidates for the treatment of leukemias and lymphomas with high mTOR activity. Finally, a compound shows potent preclinical activity in non-Hodgkin lymphoma, causing tumor regression in a large fraction of mice by inducing massive apoptosis due to deregulation [59–61].

7.3 Immunotherapy: Enhances immune-mediated cell death (e.g., checkpoint inhibitors)

CTLA-4, PD-1, and PD-L1 inhibitors are the most widely investigated immune checkpoint antibodies. Two are already approved, ipilimumab and nivolumab, and are prescribed for several types of cancer. In particular, nivolumab is of particular interest in NSCLC treatment. Nivolumab was well tolerated and demonstrated long-term clinical response with a long-term survival benefit in treated patients as a single agent. In the case of PD-1/PD-L1 inhibitors and the need for PFS and not OS data before regulatory approval, the opinion of survival as a primary endpoint for NSCLC is a landmark in regulatory history, with the recommendation for nivolumab approval based on overall survival data. Recently, tremelimumab administered as a single agent or in combination with durvalumab has shown encouraging activity in a phase Ib trial aimed at patients with a non-resectable, relapsed, metastatic small cell lung cancer (SCLC). The immunotherapy arm showed better response rates compared to conventional therapies. Since the regulatory body allows crossover-arm study design, the enrollment for the chemotherapy arm was stopped and a single-arm investigation for the ICI arm was opened, offering this possibly more promising, modern approach with promising results for control patients [62–64].

7.4 Radiotherapy: Induces DNA damage, leading to cell death

It is a more standard treatment for patients with early-stage diseases or having certain unfavorable clinical features after surgery; however, through primary or acquired resistance to this treatment, cancer cells will undergo rapid evolution over a long time, leading to reoccurrence afterward. It is known that within the tumor mass, hypoxic cell populations are relatively radioresistant through activation of HIF-1, which in turn significantly increases the potential of stem cells and promotes cancer progression and resistance. Therefore, a better understanding of hypoxia and the associated signaling pathways can improve the effectiveness of radiotherapy. Thus, targeting the key signaling pathways that are activated by hypoxia can be used as a radiosensitization strategy to combine with the current standard cancer treatment to increase tumor killing, preventing tumor recurrence and treatment resistance. In contrast to cycles of chemotherapy, radiation therapy is often given daily for several weeks, and a full course of radiation therapy usually requires multiple treatment fractions. Radiotherapy works by damaging the DNA inside the tumor cells, leading to cell death. I. Radiotherapy: Induces DNA damage, leading to cell death. It is a more standard treatment for early-stage diseases or for patients having certain unfavorable clinical features after surgery. However, through primary or acquired resistance to this treatment, the cancer cells will undergo rapid evolution over a long time, leading to reoccurrence afterward. It is known that within the tumor mass, hypoxic cell populations are relatively radioresistant through the activation of HIF-1, which in turn significantly increases the potential of stem cells and promotes cancer progression and resistance. Therefore, a better understanding of hypoxia and the associated signaling pathways can improve the effectiveness of radiotherapy. Thus, targeting the key signaling pathways that are activated by hypoxia can be used as a radiosensitization strategy to combine with the current standard cancer treatment to increase tumor killing, preventing tumor recurrence and treatment resistance. In contrast to cycles of chemotherapy, radiation therapy is often given daily for several weeks, and a full course of radiation therapy usually

requires multiple treatment fractions. Radiotherapy works by damaging the DNA inside the tumor cells, leading to cell death [65–67].

7.5 Small molecule inhibitors: Targeting cell death regulators (e.g., caspase inhibitors)

Caspases are intracellular cysteine aspartate-specific proteases that play a key execution role during the induction of apoptosis. Generally, upon apoptosis induction, caspases in the cell are activated either by interactions with extracellular molecules or by recruitment through adapter proteins. These zymogen proteases cleave downstream substrates upon self-activation and are mainly responsible for breaking cells into apoptotic bodies so that the cell debris does not initiate a chronic immunogenic response. In this way, the body is able to efficiently scavenge and clean up the cell debris and remove it from the environment. This could be considered to be why most of our cells are programmed to die upon various provocations that cause cancers. The process of caspase activation has been a major interest for researchers in the field of cell death, and ongoing investigations are generating more novel discoveries about how the process occurs. Before we go further, we need to be reminded that pathologies such as cancers represent the ultimate failure of apoptosis because the disease is usually associated with the failure to eliminate abnormal cells that otherwise should be eliminated by apoptosis. Data from cell death research already indicates this phenomenon. If we briefly consider a hallmark cancer gene such as p53, which is known to have functions in apoptosis regulation, we see that nearly half of human cancer pathologies possess inactivating mutations in p53. In this way, cancerous cells are able to bypass the function of p53, promote survival, and evade the normal process of apoptosis. Because of such findings, pharmaceuticals have been developed to increase p53 function with the hopes of killing off the cancer cell before it propagates. Also, since doxorubicin directly encourages cells to undergo apoptosis, researchers and physicians are utilizing the capabilities of triggering programmed cell death to aid in the assistance of cancer therapy [9, 68–70].

8. Emerging strategies

Emerging strategies for drug design to induce cancer cell death over the past several years, novel strategies have been proposed. Inhibitors of the BCL2 family protein have been proposed to selectively induce the death of cancer cells. A strategy that has also gained relevance in the scientific community is associated with second mitochondrial-derived activator of caspases inhibitors, and it should be noted that Smac is a protein involved in caspase-9 activity. The carrier protein BIRC, associated with the BIR domain or inhibitor of the apoptosis domain, binds to the protein cIAP, the E3 ligase, and this blockage inhibits ubiquitylation that occurs in the proteins that would undergo caspase-9/3-dependent apoptosis. As a result of the low levels of Smac, cIAPs, caspase-9, and caspase-3, apoptosis will not be driven, and there are other issues, such as amplified caspase-8 inhibitors, resulting in decreased effectiveness [9, 68–70]. Another approach involves blocking inhibitors of apoptosis proteins by targeting other proteins that interfere with caspases, thereby rescuing cells from apoptosis. Members of the X-linked inhibitor of apoptosis protein family are known to function by switching the caspase activating scaffolding E3 ligase to activate caspase-9 and caspase-3 and trigger the effector cell apoptosis pathway, such as the

family of second mitochondria-derived activators. This leads to an earlier wave of caspase-8/9 activation, resulting in caspase engagement with the consequences of that view. As a result, their activation will attenuate caspase-dependent cell death. With the lower levels of Smac and XIAPs, the block occurs in caspase-9 and caspase-3, as previously mentioned [71].

8.1 Combination therapies: Synergizing cell death mechanisms

Recent insights highlight the existence of interconnections between the cell death pathways described in this chapter. Understanding how these pathways are related, providing that a drug produces a specific type of cell death, may guide us in the development of combination therapies that synergize their pro-death effects. For example, synergizing apoptotic death with therapies that increase the glucotoxic/ER-stressed state of beta cells should be avoided, although the combination of incretin hormone-based therapies, which promote beta cell survival, and glucose-lowering therapies that stimulate beta cell apoptosis in an insulinopenic T2DM, such as sulfonylureas, may have benefits in combination. Today, both activation of cell death programs and blockage of cell death pathways are considered important strategies for developing novel and better anticancer therapies. B-RAF inhibition reestablishes the appropriate signalization level in the MAPK pathway and leads to cell cycle arrest in the G1 phase and apoptosis in HRASV12-transformed NIH3T3 cells. However, the presence of the mutant and hyperactive HRASV12 might redirect cells that would die following the oncosuppressor function of p53/INK4A through a compensatory autism spectrum disorder (ASD) program toward an AIF-mediated non-apoptotic cell death route. Co-targeting mutant HRASV12 and the p53-INK4A cell death pathway might be required for greater pharmacological success through a combinatorial approach using MEK and B-RAF inhibitors for HRASV12-dependent tumors and MEK and IMCs for p53-defective HRASV12-transformed tumors [72, 73].

8.2 Personalized medicine: Tailoring therapies based on tumor cell death profiles

Research in cell death and related signaling has been supported by major advancements in recent years, particularly concerning immune and inflammatory regulation, which has given rise to the burgeoning field of cell death in cancer. Tumor immunology has become an area of study that is at the forefront of cancer research, being directly associated with increasingly used therapies in the clinic that seek to reverse the immune escape mechanisms developed by cancer cells. Curiously, some of the main basics of such mechanisms, such as the release of damage-associated molecular patterns by apoptotic and necrotic cells, have long been described. Cell death signaling and the development of immune responses to dead cells remain areas that, although under intensive study, still have many missing links in their general comprehension. Certainly, patient-based screenings should result in a better definition of cell death signatures, allowing one to identify expression levels of genes that code for key cell death proteins and, thereafter, stratify patients into specific groups amenable to better treatments with novel drugs or drug combinations. In this context, personalized medicine may be the transition from patient-oriented medicine to the setting where patients are treated with the discovery of therapies that are better fitted for their specific disease conditions. This becomes particularly important when one envisions conditions that can be treated with the induction of tumor mass cell death that may need complementation to minimize toxicity to normal organs [74, 75].

8.3 Cancer biomarkers: Monitoring cell death-related biomarkers for diagnosis and prognosis

Cell death plays crucial roles in a variety of physiological and pathological events, particularly cancer initiation, progression, metastasis, and therapy failure. Therefore, monitoring cell death-related biomarkers and evaluating their activity might be useful in both the diagnosis and prognosis of cancer, as well as in the development of efficient anticancer treatment strategies. To achieve the high goals for the clinical use of cell death-related biomarkers in cancer therapy and the industrialization of these potential tools, suitable technology platforms and continuous interaction among fields such as biology, engineering, and clinical medicine are highly required [76]. Evidence has demonstrated that the development of a cell death-resistant tumor microenvironment is an essential determinant for tumor initiation and progression. Tumor cells possess a great survival ability and are able to continue to grow and form macroscopic tumors by evading the effects of several cell death-inducing factors. In addition, substantial cell death occurs during chemotherapy, but the tumor is not effectively eradicated due to cell death-resistant properties. This arises from the fact that tumor cells have developed different pathways to escape cell death induced by cytotoxic agents. To date, several cell death-related biomarkers have been discovered and developed to evaluate the effectiveness of different treatments and could even be used to provide an early warning or prognosis for cancer [77, 78].

8.4 Immunogenic cell death: Stimulating anti-tumor immunity

Stimulating anti-tumor immunity certain types of apoptotic cell death stimulate an immune response against the dying cell, and such immunogenic cell death presents new and exciting clinical possibilities for cancer therapy. We call a death immunogenic if it results in an immune response against the dying cell [79, 80]. We usually detect this response by monitoring the induction of immunity to tumors or to a pathogen. Dying cells emit signals that indicate that they should be rapidly taken up and degraded by a scavenger macrophage. A sense of urgency is conferred on the scavenger by further signals that report on the presence of transmembrane phosphatidylserine and on other "eat me" signals associated with the surface of the dying cell. The "eat me" signals attract and activate the scavenging macrophages. Such macrophages have acted as professional pallbearers during eumetazoan evolution. Chemically equivalent cells fulfill the function even in the seemingly simple sponges. Scientists have been working with macrophages for a long time and understand a great deal about the cell biology of the normal and membrane-attached populations. For example, macrophages are activated and stabilized in their anti-inflammatory mode by CD47 on the dying cell; therapeutic antibodies that block CD47 cause tumor cells to be ingested and degraded. Therapies that promote CD47, but not CD95, expression by a tumor can reduce its ability to promote new blood vessels and, eventually, prevent it from growing [79, 80].

8.5 Senolytic therapy: Targeting senescent cells

Senescent cells that express high levels of cyclin-dependent kinase inhibitor (CDKIs) can be very resistant to death stimuli. These cells generally have active DNA damage responses, and the inhibition of this signaling pathway is the only factor able to eliminate these cells. However, this is also associated with death and growth

suppression for nearby cancer cells. The activation of two pro-apoptotic factors induced by senescence, such as pro-apoptotic factor Bcl-2 and FOXO-dependent p21, results in the tumor suppression of neighboring cancer cells. Another pathway has been proposed that enhances the apoptotic potential of senescent cells as a unique and specific possibility for inducing their death, which is the activity of SMAC-mimetic that allows for the induction of an autocrine loop to attract cytotoxic factors from outside the cell. Furthermore, data revealed that inhibitors of the mTOR complex and active inhibitors can stop this autocrine loop, allowing SMAC-mimetic to directly affect cell death. Thus, a pro-senescent therapy can reduce local recurrence after traditional treatments *via* this non-cell-autonomous mechanism. The activity of these senescent cells is also very important for dampening the possible metastatic potential of surviving cancer cells. It is proposed that senescent cells represent a phenotype rather than a static endpoint. If we consider senescent cell biology in this view, we can effectively manipulate this therapy according to the needs of the surrounding cells and microenvironment. Furthermore, considering that apoptosis and senescence can prevent each other, becoming cautious about using inhibitors of Bcl-2, pro-apoptotic agents, or inhibitors is also mandatory [81, 82].

9. Key regulators of cell death in cancer

A large number of genes have been implicated in different forms of cell death, which also form an intricate network. We illustrate a snapshot of the association network through a selected number of cell death key regulators. The death domain superfamily consists of proteins with so-called death domains, through which they interact with each other, and this association with the death domain superfamily. A large number of genes are implicated in promoting apoptosis, a representative cell death type. This large number of genes should help cells decide on life and death, meet multiple requirements, and simplify a signaling system to be less robust [18, 83, 84]. The promoters of apoptosis can be divided into several categories, including Bcl-2 family members, receptor-mediated extrinsic apoptotic pathway-related proteins, p53-targeting surveillance target genes, and inhibitors such as caspase activating or inactivating proteins. Bcl-2 family proteins contribute to the change of mitochondrial outer membrane permeabilization to induce the release of proteins from the mitochondrial intermembrane space into the cytoplasm. BAX and BAK are key promoters for permeabilization that determine life or death. Upon auto-activation, BAX or BAK is integrated into its homooligomer, which forms a pore in the mitochondrial outer membrane allowing the release of cytochrome C and the subsequent activation of caspase in the cytoplasm, eventually resulting in cell demolition. These permeabilization-inducing molecules are regulated by multiple mechanisms, wherein mainly the pro-apoptotic BH3-only proteins are activated by multiple damage signals, interact with anti-apoptotic Bcl-2 molecules to release BAX or BAK, and localize and activate BAX to form pores in the mitochondrial outer membrane, initiating apoptosis [18, 83, 84].

9.1 p53: Tumor suppressor regulating apoptosis

The p53 gene codifies for a 393 amino acid protein and it functions as a homotetrameric transcription factor. Normally, the high rate of p53 is held low through continuous degradation achieved due to the lack of posttranslational modifications, causing

the proteasomal degradation of p53 because of the action of MDM2 and COP1 [85]. Some exogenous and endogenous aggression provokes the modifications, preventing p53 degradation. p53 triggers cell cycle arrest and apoptosis, which contributes to restraining neoplasia progression. Withdrawal of p53 from cells causes losses in almost all steps that lead to cell death initiation. This fact shows the importance of p53 in response to genotoxic events. Inactivating mutations in the cells exert a negative effect and increase accidents in the cells, causing tumors. The characterization of compound mutant mice has been essential to clarify the events that p53 regulates. This gene has a negative role in angiogenesis, inflammation, self-renewal, metastasis, invasion, and cancer cell survival. Despite that, the major anti-tumor activity of p53 comes from the fact that its controlled genes are actively responsible for causing cell cycle arrest or regulated cell death. These events are responsible for trying to preserve genomic stability in normal and cancer cells and avoiding the natural selective advantage that tumor cells acquire with loss of p53 apoptotic activity [86, 87].

9.2 Bcl-2 family: Regulating apoptosis

The Bcl-2 family has received extensive attention in the context of cell death pathways. They include both anti-apoptotic and pro-apoptotic members. The Bcl-2 family members contain conserved homology regions termed Bcl-2 homology 1–4. However, whether these homology regions are conserved in the amino acid sequence or they form similar structures is still not completely clear. Anti-apoptotic Bcl-2 family members collectively contain all four BH motifs, while pro-apoptotic members contain at least a single motif [88]. Bax and Bak are both directly responsible for MOMP induction, while BH3-only proteins initiate apoptosis by either inhibiting Bcl-2 or activating pro-apoptotic Bax and Bak. Although the anti-apoptotic and pro-apoptotic Bcl-2 families mediate apoptosis, they are involved in different stages of the apoptosis pathway. The anti-apoptotic members Bcl-2, Bcl-xl, and Mcl-1 are responsible for preventing leakage of cytochrome c from mitochondria, while pro-apoptotic members mainly target Bax and Bak to the mitochondrial membrane, which is indispensable for MOMP induction. Some anti-apoptotic Bcl-2 family members employ similar binding strategies to suppress Bax and Bak, while others use different protections. Bcl-2 and Bcl-xl are both able to bind Bax and Bak directly through the hydrophobic groove located at the BH3 domain of Bax/Bak. On the other hand, Mcl-1 might suppress Bak through the interaction of its flexible loop, instead of the BH3-binding groove. In addition to interacting with Bcl-2 pro-survival proteins to release Bax and Bak, BH3-only proteins have been revealed to activate the process of mitochondrial apoptosis through a different mechanism. TBid has been shown to bilaterally neutralize Bcl-xl to release Bax, while Bim has been revealed to possess the ability to directly activate Bax. Clearly, different BH3-only proteins targeting different pro-survival and/or pro-apoptotic proteins mobilize Bax and Bak to initiate the apoptotic cascade [89].

9.3 PI3K/AKT: Regulating autophagy and apoptosis

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) cascade is often dysregulated in cancer. PI3K class I phosphorylates the lipid second messenger PtdIns(4,5)P2 to generate PtdIns(3,4,5)P3 for localization of the kinase AKT/PKB to the plasma membrane. Phosphoinositide-dependent kinase-1 (PDK1) activates AKT by phosphorylation at T308 while mTOR complex 2 activates AKT by

phosphorylation at S473 [90–92]. AKT is a key regulator of cell survival, associated with resistance to chemotherapy drugs. PI3K/AKT can suppress apoptosis through direct phosphorylation of BCL-2, CASPASE-9, BID, and BAD and can inhibit GSK3 from phosphorylating MCL-1. PI3K/AKT is a major negative regulator of the pro-apoptotic protein BIM. In addition, the interaction of AKT with the second mitochondria-derived activator of caspase can suppress apoptosis. AKT can also inhibit autophagy by promoting the phosphorylation of serine-757 of the pro-autophagic factor ULK1. Beclin-1 is a key regulator of the initiation of autophagy, and its activity can be suppressed by AKT. AKT has a linkage to the autolysosome complex through TU-CATD and VPS34, which increases the activity of CD38, which in turn suppresses autophagy. In addition, AKT has a linkage to the autolysosome complex through mTOR, regulating ULK1, VPS34 gene, and ATG13. The PtdIns(3)P protein binds to the Atg14L-containing class III phosphatidylinositol 3-kinase complex and enhances the nucleation of autophagosomes. Due to the functional link between AKT and the class III phosphatidylinositol 3-kinase complex, ablation of the latter results in an increase in AKT activity, which then suppresses autophagy [90, 91].

9.4 Caspases: Executing cell death programs

Following identification and pioneering studies, a crucial role of inhibiting apoptosis in cancer has been indicated and further characterized. Anti-apoptotic Bcl-2, which also promotes cancer development, inhibits the function of pro-apoptotic macromolecules, some of which were discovered in studies focused on programmed cell death. One such protein is Syk, which is also expressed in immune system cells and is likely associated with the oncogenesis of leukocytes. These proteins interact with both anti-apoptotic proteins and another group of important proteins located in mitochondria: pro-apoptotic Bax and Bak, which gate the release of cytochrome c by forming pore structures in mitochondrial membranes, and the so-called Apoptosis Inducing Factor, which migrates from the mitochondria to the nucleus. Mechanisms regulating the activity of caspases are complex. Apoptosis depends on the action of 'executioner caspases.' These are cysteine protease enzymes. Their activity leads to the death of the cell. Activation of caspases must be tightly regulated. The caspase enzymes are normally present in the cell environment as inactive precursors, and their unwanted premature activation must never occur [93, 94]. Apart from the intracellular factors, many of which are usually called 'initiators' that activate the caspases, the inactive pro-caspase forms themselves assure a safe mode of activation. The enzymes exert their killing function only in the so-called DD or Death-Inducing Signaling Complex, where the active parts of two or more caspase enzymes are effectively sequestered and protected from uncontrolled premature activation. A cardinal element to start the caspase activation is the proteolytic activity of proteins termed caspase-8. If two distinct possibilities exist for the programmatic removal of important 'suppressive' inhibitory factors, one embodiment is the sequestration of inhibitory proteins during the formation of the silhouette-like DD Complex. The caspase-killing function must be short-lasting and strictly sequestered [93, 94].

9.5 Apoptosis-related proteins: Bax, Bak, Bcl-xL

Bax is a pro-apoptotic protein from the Bcl-2 family that promotes cell death by damaging mitochondrial walls. With the appearance of a signal for the initiation of apoptosis in a cell, p53 begins to stimulate the synthesis of Bax and simultaneously

inhibits the generation of Bcl-xL. Newly synthesized Bax finds its partner at the mitochondrial membrane—proteins of the Bcl-xL family—and when bound to them, it forms channels through the mitochondrial membrane; as a result, molecules from the intermembrane space are transported to the cytoplasm, molecules that are necessary for cell respiration and the generation of energy. As soon as these small molecules leave the mitochondria, a collapse of the mitochondrial membrane potential occurs and the expression of different proteins begins. Bak is another protein from the Bcl-2 family, which is called a "partner" for Bax. Apoptosis does not occur when either Bax or Bak is lacking. The quantitative balance between these two proteins determines the direction of cells. It is known that an increase in the concentration of Bax can stimulate a breach in the mitochondrial membrane even from the outside of a cell. These properties of Bax could be used in the therapy of tumors; it might be possible to regulate apoptotic activity by artificially stimulating the synthesis of Bax in apoptotic cells [95, 96].

10. Conclusion

Chapter concludes by emphasizing the critical role of programmed cell death (PCD) in cancer development and therapy, highlighting the complexity of its regulation and the challenges it presents in clinical applications. Despite significant advancements in understanding cell death mechanisms such as apoptosis, necroptosis, and ferroptosis, cancer remains a leading cause of mortality due to the ability of malignant cells to evade these processes. The dysregulation of apoptosis, in particular, is a pivotal factor in tumorigenesis, therapy resistance, and metastasis, underscoring the need for innovative therapeutic strategies.

Combination therapies have emerged as a promising approach to overcome the limitations of monotherapies by targeting multiple pathways involved in tumor progression and resistance. These strategies aim to enhance the efficacy of treatments by sensitizing cancer cells to cell death while minimizing damage to normal tissues. Advances in molecular targeting, immunotherapy, and the development of pro-apoptotic agents offer new opportunities to exploit the unique vulnerabilities of cancer cells. Furthermore, the integration of emerging technologies and a deeper understanding of the tumor microenvironment and immune system interactions provides a foundation for next-generation therapies. Ultimately, the chapter underscores the necessity of a paradigm shift in cancer treatment, moving beyond traditional approaches to embrace the complexity of cancer biology. By leveraging the interplay of various cell death pathways and refining therapeutic strategies, future research holds the potential to improve patient outcomes and achieve more effective and durable cancer treatments.

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Chapter 3

Mitochondrial Dysfunction and Cell Death: Mechanisms and Implications in Pathology

Jaeeun Lee

Abstract

Mitochondria are crucial regulators of both cellular life and death, influencing ATP production, calcium homeostasis, and multiple forms of regulated cell death (RCD), including apoptosis, necroptosis, mitochondrial permeability transition (MPT)-driven necrosis, pyroptosis, and autophagy-dependent cell death. Dysregulated mitochondrial function not only amplifies oxidative stress and perturbs energy balance but also activates pathogenic cell death pathways that drive neurodegenerative diseases, cancer, and metabolic disorders. This chapter examines key mechanisms by which mitochondria orchestrate regulated cell death and highlights their centrality in disease pathogenesis. We discuss how mitochondrial dysfunction, through the accumulation of reactive oxygen species (ROS), the loss of membrane potential, and the release of pro-death factors, precipitates pathological cell demise. We then explore emerging therapeutic strategies, including mitochondrial antioxidants, inducers of mitochondrial biogenesis and mitophagy, modulation of the mitochondrial unfolded protein response (UPR^{mt}), and the regulation of mitochondrial dynamics. By focusing on both mechanistic insights and translational developments, this chapter aims to provide a comprehensive view of how restoring mitochondrial health may halt disease progression and improve clinical outcomes.

Keywords: mitochondrial dysfunction, regulated cell death, apoptosis and necroptosis, mitochondrial unfolded protein response (UPR^{mt}), mitophagy, mitochondrial dynamics, oxidative stress

1. Introduction

Historically described as the "powerhouses" of the cell, mitochondria are now recognized as dynamic organelles essential for ATP production, calcium homeostasis, and the regulation of multiple cell death pathways [1–3]. Their dysfunction is increasingly recognized as a unifying factor in the development of neurodegenerative disorders and metabolic syndromes [4, 5]. Rather than simply functioning as ATP generators, mitochondria function as critical signaling hubs that coordinate metabolic inputs with cell survival or death decisions.

Dysregulated mitochondrial function can trigger a cascade of harmful processes, such as excessive reactive oxygen species (ROS) generation, energy depletion, and the release of pro-apoptotic signals, all of which can drive cellular demise. Through pathological events such as the mitochondrial permeability transition (MPT), mitochondrial outer membrane permeabilization (MOMP), and disrupted fission-fusion cycles, dysfunctional mitochondria can push cells from a survival state toward irreversible death pathways [6, 7]. Because these mitochondrial processes are vital for normal cellular function, elucidating how mitochondrial defects contribute to disease pathogenesis is of paramount importance.

In this chapter, we examine how mitochondrial dysfunction intersects with multiple types of regulated cell death (RCD). Although apoptosis remains a principal example, recent evidence shows that mitochondria also play critical roles in necroptosis, MPT-driven necrosis, pyroptosis, and autophagy-dependent cell death. We explore how these pathways converge on dysfunctional mitochondria to drive disease processes and discuss both established and emerging mitochondrial-focused therapies, including interventions aimed at modulating the mitochondrial unfolded protein response (UPR^{mt}). By integrating current findings, this chapter highlights the therapeutic potential of restoring mitochondrial fitness to counteract pathologies originating from mitochondrial damage.

2. Mitochondria and multiple forms of regulated cell death

2.1 Apoptosis

Apoptosis is among the most extensively characterized types of regulated cell death (RCD) and is predominantly controlled by mitochondria through mitochondrial outer membrane permeabilization (MOMP). Under stress conditions, pro-apoptotic Bcl-2 family proteins such as Bax and Bak oligomerize within the outer mitochondrial membrane, creating pores that allow cytochrome c and other apoptogenic factors to escape into the cytosol [8]. Once released, cytochrome c facilitates the activation of initiator caspase-9, which subsequently triggers executioner caspases, most notably caspase-3, thus orchestrating the orderly dismantling of cellular components [9].

Mitochondrial integrity plays a decisive role in determining whether MOMP occurs. Disruptions in the balance of pro- and anti-apoptotic Bcl-2 proteins can be aggravated by increased ROS levels, reduced ATP production, or abnormal mitochondrial dynamics [10, 11]. In particular, excessive fission mediated by dynamin-related protein 1 (Drp1) often enhances apoptotic signaling, whereas promoting mitochondrial fusion through mitofusins (Mfn1/2) or OPA1 can help stave off cell death [12]. Additionally, metabolites such as NAD^+ play a regulatory role in apoptosis; when NAD^+ is depleted, cells become more vulnerable to pro-apoptotic stimuli in both cancer and neurodegenerative conditions [13, 14]. Collectively, these observations underscore how mitochondrial health is pivotal for deciding whether apoptotic pathways remain in check or proceed to full activation.

2.2 Necroptosis

Necroptosis is a tightly controlled, caspase-independent form of necrosis that commonly arises when apoptosis is blocked or insufficient to manage cellular stress. The receptor-interacting protein kinases RIPK1 and RIPK3 drive this process by

phosphorylating mixed-lineage kinase domain-like protein (MLKL), which in turn compromises membrane integrity [15]. Mitochondria contribute to necroptosis by amplifying ROS production and undergoing depolarization following RIPK3 activation [16]. The resultant oxidative stress intensifies necroptotic damage and triggers inflammatory responses *via* NF-κB or MAPK signaling pathways.

Excessive mitochondrial fission, typically orchestrated by Drp1, can precede or aggravate necroptosis, while the mitochondrial permeability transition pore (mPTP) may open under necroptotic conditions, leading to mitochondrial swelling and membrane rupture [17]. Various disorders, including ischemia-reperfusion injuries, inflammatory diseases, and neurodegenerative conditions, are characterized by elevated necroptosis linked to mitochondrial dysfunction [18, 19]. Studies show that inhibiting the mPTP or Drp1 decreases necroptosis in experimental models, underscoring the pivotal role of mitochondria in governing this cell death pathway [19].

2.3 Mitochondrial permeability transition (MPT)-driven necrosis

Distinct from necroptosis, MPT-driven necrosis refers to pathological cell death resulting from sustained opening of the mPTP [20]. Under extreme stress, such as calcium overload, acute ischemia-reperfusion, or severe oxidative damage, the mPTP can open persistently, leading to mitochondrial depolarization, ATP depletion, and rapid necrotic cell death [21]. Unlike necroptosis, this form of necrosis may or may not require RIPK3 signaling but relies heavily on mitochondrial dysfunction as the triggering event.

MPT-driven necrosis is particularly relevant in acute pathologies such as myocardial infarction and stroke, where sudden mitochondrial calcium overload triggers pore opening [22]. Pharmacological inhibitors of mPTP, such as cyclosporine A and sanglifehrin A, have shown promise in reducing ischemia-reperfusion injury in cardiac and neurologic models [23, 24]. Including this process in the RCD spectrum further highlights how mitochondrial integrity can distinguish survival from irreversible necrotic demise.

2.4 Pyroptosis

Pyroptosis is an inflammatory variant of regulated cell death involving the gasdermin protein family, generally triggered by caspase-1 or other inflammatory caspases in response to microbial threats or sterile danger signals [25]. Although this process is typically associated with cytosolic inflammasomes, mitochondrial dysfunction, especially heightened ROS production and the release of mitochondrial DNA, can initiate or exacerbate inflammasome activation [26]. This interplay reveals how mitochondria, even in pyroptotic scenarios, can profoundly affect both cell death and inflammatory responses.

2.5 Autophagy-dependent cell death

Autophagy typically functions as a protective mechanism, removing damaged proteins and organelles through lysosomal degradation. Under specific circumstances, however, its overactivation or misregulation can result in autophagy-dependent cell death. A key subset is mitophagy, a targeted form of autophagy responsible for clearing defective mitochondria. When mitophagy is disrupted, for instance, by mutations in PINK1/Parkin or by BNIP3 deregulation, malfunctioning mitochondria

accumulate, contributing to conditions such as Parkinson's disease, Alzheimer's disease, and various cardiomyopathies [27].

Paradoxically, an overabundance of mitophagy can lead to the elimination of even healthy mitochondria, depleting ATP and triggering cell death [28]. Proteins involved in mitochondrial dynamics, like Drp1 and Mfn2, also modulate mitophagy by regulating mitochondrial fission and fusion, thus determining which mitochondria are retained or removed [29]. These observations highlight the delicate equilibrium required: while moderate mitophagy safeguards mitochondrial function, excessive or impaired mitophagy may promote disease.

3. Mitochondrial dysfunction in disease pathogenesis

3.1 Neurodegenerative diseases

Neurons depend heavily on ATP generated by mitochondria to sustain ion gradients and support neuronal transmission [30]. In disorders such as Parkinson's disease, Alzheimer's disease, and Huntington's disease, faulty oxidative phosphorylation, excessive ROS, and disrupted mitophagy commonly signify underlying mitochondrial dysfunction [31]. Depending on the severity and nature of cellular stress, these mitochondrial deficits can activate diverse RCD pathways, such as apoptosis, necroptosis, MPT-driven necrosis, or pyroptosis. Interventions aimed at preserving mitochondrial quality, through augmenting mitophagy, stabilizing the mPTP, or inducing UPR^{mt}, show promise for alleviating neurodegeneration and represent a rapidly evolving therapeutic frontier [32, 33].

3.2 Cancer

In oncology, mitochondria occupy a paradoxical position. On the one hand, cancer cells frequently adopt mitochondrial metabolic reprogramming (known as the Warburg effect) to support their high metabolic demands, yet they must also circumvent mitochondria-mediated apoptosis [34]. Tumor cell survival can thus be bolstered by altered pro- and anti-apoptotic Bcl-2 family protein expression, increased mitophagy, and imbalances in fission-fusion dynamics [35]. While certain cancer treatments specifically target mitochondria to induce ROS overload or disrupt membrane potential, a deeper understanding of how mitochondrial stress responses, particularly the UPR^{mt}, are exploited by cancer cells is vital for advancing more effective therapeutic strategies [36].

3.3 Metabolic disorders

Metabolic conditions such as type 2 diabetes (T2D) and metabolic dysfunction-associated steatotic liver disease (MASLD) frequently exhibit persistent oxidative stress and mild inflammation, both of which stem from mitochondrial dysfunction. Elevated ROS can harm insulin signaling pathways, exacerbate hepatic steatosis, and trigger inflammatory cell death [37]. Therapeutic strategies that restore mitochondrial health, through antioxidants, mitophagy stimulators (like urolithin A), or interventions that enhance UPR^{mt}, have shown potential in alleviating insulin resistance and liver fibrosis, underscoring the pivotal contribution of mitochondria to the progression of metabolic diseases [38–40].

3.4 Cardiovascular disease

Mitochondria serve as crucial determinants of cell fate in ischemic heart disease and heart failure. During ischemia-reperfusion injury, sudden calcium overload and oxidative stress can lead to mPTP opening, necroptosis, or MPT-driven necrosis [41]. Experimental work indicates that stabilizing mitochondrial membranes or supporting oxidative phosphorylation *via* agents such as mPTP or Drp1 inhibitors diminishes myocardial infarct size and safeguards cardiac function [41, 42]. Additionally, therapeutic strategies that modulate UPR^{mt} may bolster cardiomyocytes' capacity to handle the heightened protein-folding demands imposed by ischemic stress [43].

4. Therapeutic strategies targeting mitochondrial dysfunction

4.1 Mitochondrial antioxidants

Mitochondrial antioxidants specifically target and neutralize ROS generated during oxidative phosphorylation. Among these, MitoQ, a ubiquinone derivative modified with a lipophilic cation, accumulates within mitochondria to reduce oxidative stress and preserve membrane integrity [44]. In preclinical models of Alzheimer's disease, MitoQ has been shown to mitigate amyloid-beta-induced mitochondrial dysfunction and improve cognitive outcomes [45]. In patients with type 2 diabetes (T2D), MitoQ has been shown to decrease mitochondrial ROS production, boost glutathione peroxidase 1 activity, and reduce both NF-κB signaling and TNFα release, thereby suppressing leukocyte-endothelium interactions and systemic inflammation [46]. Because oxidative stress also drives nonalcoholic steatohepatitis (NASH), MitoQ could similarly help curb liver fibrosis and inflammation in this setting [47]. Further clinical studies are needed to validate MitoQ's therapeutic utility and optimize dosing in metabolic disorders characterized by mitochondrial dysfunction.

4.2 Mitochondrial biogenesis and mitophagy

4.2.1 Mitochondrial biogenesis

Mitochondrial biogenesis is regulated predominantly by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which drives genes essential for mitochondrial replication and oxidative metabolism [48]. Compounds such as resveratrol and spermidine can activate PGC-1α, thereby increasing mitochondrial content, reducing oxidative stress, and enhancing cellular homeostasis. However, the extent of their efficacy in stimulating mitochondrial biogenesis appears to differ. Spermidine supplementation in aged hearts has been shown to restore polyamine levels, elevate SIRT1/PGC-1α activity, and alleviate mitochondrial dysfunction, collectively improving cardiac function [49]. In contrast, research into resveratrol's impact on PGC-1α and mitochondrial biogenesis has produced mixed results. Although some studies report beneficial effects, others suggest minimal or even negative influences on PGC-1α coactivator function under certain experimental conditions [50]. These discrepancies indicate that while both resveratrol and spermidine act on overlapping molecular pathways, their net effects may depend on factors such as tissue specificity, dosage, and experimental design. Further investigation is warranted to clarify each compound's precise mechanisms of action in promoting mitochondrial health.

4.2.2 Mitophagy

Mitophagy, the selective autophagic removal of damaged mitochondria, maintains mitochondrial quality [51]. Impairment of this process increases oxidative stress and inflammation, as seen in Parkinson's disease, where PINK1 and Parkin mutations thwart the clearance of dysfunctional mitochondria [52]. Stimulating mitophagy with compounds such as urolithin A (UA), rapamycin, and spermidine can help reduce the buildup of harmful organelles and byproducts, potentially improving health outcomes in a range of conditions. UA promotes mitochondrial respiration and muscle regeneration in both Duchenne muscular dystrophy and age-related muscle decline [53, 54]. It also alleviates mitochondrial and synaptic toxicities in Alzheimer's disease models [55], enhances mitochondrial function in joint tissues to lessen cartilage breakdown in osteoarthritis [56], and has demonstrated safety, bioavailability, and benefits for mitochondrial and cellular health in clinical trials [57]. Rapamycin, an mTOR inhibitor, fosters mitophagy and mitochondrial fission in disorders such as glioblastoma [58], and it delays aging while improving cardiac function by inducing autophagy, including mitophagy [59]. Spermidine similarly induces autophagy, including mitophagy, thereby supporting longevity and healthspan [59]. Further exploration of receptors like BNIP3 and other mitophagy modulators offers new therapeutic avenues for tissue-specific regulation of mitochondrial turnover. By concurrently activating mitochondrial biogenesis and mitophagy, it may be possible to restore energy balance, protect neurons, and alleviate metabolic dysfunction across diverse pathologies.

4.3 Mitochondrial unfolded protein response (UPR^{mt})

In contrast to the more generalized cytosolic and endoplasmic reticulum (ER) stress pathways, the mitochondrial unfolded protein response (UPR^{mt}) is specifically triggered by an accumulation of misfolded or damaged proteins in the mitochondrial matrix or inner membrane. In mammals, this process involves transcription factors such as ATF5 and CHOP, whereas in *Caenorhabditis elegans*, it is primarily mediated by ATFS-1 [60, 61]. Once activated, the UPR^{mt} pathway induces a group of mitochondrial chaperones (e.g., HSP60 and GRP75) and proteases (e.g., LONP1 and CLPP) to refold or degrade misfolded proteins, thereby restoring proteostasis within mitochondria.

Engaging UPR^{mt} has significant ramifications for maintaining mitochondrial quality and can intersect with regulated cell death (RCD) pathways. By reducing protein aggregates and supporting the electron transport chain, UPR^{mt} curbs excess ROS production and preserves the mitochondrial membrane potential [62]. In neurodegenerative disorders characterized by protein misfolding (e.g., Alzheimer's disease and Parkinson's disease), pharmacological or genetic activation of UPR^{mt} has been shown to alleviate mitochondrial damage and decelerate disease progression [63].

Similarly, upregulating UPR^{mt} in metabolic conditions can boost oxidative phosphorylation and dampen inflammatory signals arising from dysfunctional mitochondria, thus potentially alleviating insulin resistance, steatosis, and other metabolic syndrome indicators [60, 64]. In cardiac and ischemic injuries, a robust UPR^{mt} response could facilitate the clearance of damaged proteins that accumulate under stress, thereby preserving mitochondrial respiration and delaying cell death [65].

Therapeutic strategies aiming to enhance UPR^{mt} include small-molecule activators of ATF5/CHOP or other upstream sensors of mitochondrial stress. Pairing these

UPR^{mt} modulators with antioxidants or mitophagy inducers could synergistically improve mitochondrial health in scenarios where persistent proteotoxic stress intersects with oxidative damage. As such, UPR^{mt} stands at the forefront of interventions intended to maintain mitochondrial proteostasis and mitigate pathologic cell death.

4.4 Targeting mitochondrial dynamics

Mitochondrial fission and fusion dictate the organelle's morphology, distribution, and overall quality control. Imbalances in these processes can lead to either fragmented mitochondria or excessive fusion, ultimately contributing to disease. Fission, largely driven by Drp1, produces mitochondrial fragments that can release pro-apoptotic factors and exacerbate cellular damage [66]. In contrast, fusion, mediated by proteins such as Mfn1/2 and OPA1, merges mitochondrial membranes to dilute harmful mutations and bolster bioenergetic capacity [67].

Numerous studies have linked pathological elevations in mitochondrial fission to neurodegenerative diseases, like Alzheimer's, Parkinson's, and Huntington's, where excessive fragmentation correlates with energy deficits and neuronal loss. Preclinical evidence shows that inhibiting Drp1 can reduce oxidative stress, protect synaptic function, and mitigate neuron death [67]. Conversely, enhancing mitochondrial fusion through Mfn2 activation supports mitochondrial integrity and cardiomyocyte survival in heart failure models that exhibit excess fission and disrupted ATP production [68, 69].

In metabolic diseases, including obesity and T2D, a shift toward fission promotes insulin resistance and inflammation. Experimental interventions aimed at reducing Drp1 activity or increasing Mfn2 expression have demonstrated improved mitochondrial function, reduced metabolic dysregulation, and decreased inflammatory markers [70, 71]. In parallel, emerging compounds such as spermidine, which encourages mitochondrial fusion, are under investigation for their ability to foster healthy aging and bolster cellular resilience. Taken together, these approaches underscore the therapeutic potential of modulating mitochondrial dynamics across diverse pathological contexts.

5. Conclusions and future perspectives

Mitochondria lie at the core of numerous regulated cell death mechanisms, including apoptosis, necroptosis, pyroptosis, MPT-driven necrosis, and autophagy-dependent cell death. Their dysfunction consistently emerges as a key factor in the onset and progression of neurodegenerative diseases, malignancies, metabolic disorders, and cardiovascular conditions. As our knowledge of mitochondrial biology broadens, so do the potential therapeutic avenues. Strategies such as using mitochondria-targeted antioxidants, boosting biogenesis, optimizing mitophagy, inducing the UPR^{mt}, or recalibrating mitochondrial dynamics offer encouraging prospects for decelerating or reversing disease.

Continuing research will aim to fine-tune these interventions by enhancing their specificity, minimizing off-target consequences, and identifying biomarkers for patient stratification. In particular, exploiting UPR^{mt} pathways may give cells the means to better handle mitochondrial stress, thereby preserving organelle integrity and deterring harmful RCD activation. Ultimately, by safeguarding or restoring mitochondrial function, therapeutic efforts may more directly address pathogenic processes and substantially improve clinical outcomes.

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Section 2

Apoptosis and Disease: Regulation and Cell Signaling

Chapter 4

Apoptosis-Mechanisms, Regulation in Pathology, and Therapeutic Potential

Irshad Ahmad Bhat, Aalim Maqsood Bhat and Sheikh Tasduq Abdullah

Abstract

Apoptosis, a highly regulated form of programmed cell death (PCD), is essential for development, tissue homeostasis, and the immune response. This self-destructive process is characterized by distinct cellular changes, including membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies. Apoptosis can be triggered by two primary signaling pathways: the intrinsic (mitochondrial) pathway, responding to internal cellular stress, and the extrinsic (death receptor) pathway, initiated by external signals. Both pathways ultimately activate caspases, proteolytic enzymes that dismantle the cell in an orderly manner, preventing inflammation. The intrinsic pathway is regulated by the Bcl-2 protein family, balancing pro-apoptotic and anti-apoptotic signals to maintain cellular integrity, while mitochondrial disruptions lead to the release of cytochrome c and activation of downstream apoptotic machinery. Dysregulation of apoptosis is linked to various diseases, including cancer, where defective apoptotic processes allow uncontrolled cell proliferation, and neurodegenerative disorders, where excessive apoptosis leads to cell loss. This review offers an in-depth understanding of apoptosis, and its regulatory mechanisms offer crucial insights for therapeutic approaches targeting apoptosis in diseases characterized by abnormal cell survival or death.

Keywords: apoptosis programmed cell death (PCD), intrinsic pathway, extrinsic pathway, caspases, Bcl-2 family, cellular homeostasis, cancer, membrane blebbing, DNA fragmentation, apoptotic bodies

1. Introduction to apoptosis

The concept of apoptosis has deep historical roots, though early observations of programmed cell death predate its modern understanding. The term "apoptosis" itself was formally introduced in 1972 by John F. R. Kerr, Andrew H. Wyllie, and Alastair R. Currie in their seminal paper, which described the distinct morphological changes observed in cells undergoing this regulated death process. They choose the term "apoptosis" from the Greek word for "falling off," as in the falling of leaves from trees, symbolizing the natural and orderly process of cell removal [1]. This was a pivotal

moment that distinguished apoptosis from necrosis and paved the way for understanding cell death as an active, regulated process rather than a passive consequence of injury. Even before this formal introduction, researchers observed the phenomenon of controlled cell death in various biological contexts [2]. In the early nineteenth century, scientists noted the natural process of tissue sculpting and cell removal during embryonic development, such as the disappearance of the tail in tadpole metamorphosis and the formation of individual fingers and toes in human embryos [3]. However, these observations lacked a mechanistic framework and were often seen as isolated phenomena. In the mid-twentieth century, advancements in microscopy and cell biology allowed for more detailed observation of cellular processes. Researchers began to note consistent patterns of cell death that were not attributable to injury or infection by the 1980s and 1990s. Research in the field had exploded, with scientists identifying caspases, the Bcl-2 family of proteins, and various other regulators of apoptosis. This period also highlighted the importance of apoptosis in disease contexts, particularly cancer, where defective apoptotic pathways allow cells to evade death and proliferate uncontrollably [4].

Apoptosis is a form of programmed cell death (PCD) characterized by an organized and tightly regulated sequence of biochemical events, leading to distinct morphological changes and, ultimately, cell death without causing inflammation or damage to surrounding tissues [5]. This process is critical for maintaining cellular homeostasis by eliminating damaged, aged, or unnecessary cells. Unlike necrosis, which is typically a response to acute injury and results in cell rupture and inflammation, apoptosis proceeds in an orderly manner [6]. Apoptotic cells undergo specific changes, including cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing, and the formation of apoptotic bodies, which are then phagocytosed by neighboring cells or immune cells, preventing inflammatory response [7].

Two primary pathways mediate apoptosis: the intrinsic (mitochondrial) pathway, triggered by internal stress signals, and the extrinsic pathway, which is activated by external signals from the cellular environment [8]. Both pathways ultimately lead to the activation of caspases, a family of cysteine proteases that systematically dismantle the cell [9]. This self-destructive process is essential in numerous physiological contexts, including development, immune response, and tissue remodeling, as well as in the prevention of cancer and other diseases associated with uncontrolled cell growth [10].

2. Understanding the importance of apoptosis

Apoptosis is fundamental to the survival and health of multicellular organisms [11]. It is an essential, highly regulated process that helps organisms manage cellular turnover, tissue remodeling, immune response, and the elimination of potentially dangerous cells [5]. Here are the key aspects of apoptosis and why it is critical for biological systems. Apoptosis plays a central role in maintaining cellular balance within tissues [5]. In most adult tissues, the rate of cell death is closely matched by the rate of cell division. Apoptosis removes aged, damaged, or dysfunctional cells that could otherwise accumulate and impair tissue function [12]. By preventing the over-accumulation of cells, apoptosis maintains a healthy cellular environment, ensuring tissues and organs operate efficiently. For example, epithelial cells in the intestines and skin continuously regenerate, with older cells being replaced by

new ones [5]. Apoptosis is responsible for clearing away these old cells, allowing tissues to remain functional and preventing overcrowding, which could lead to structural abnormalities [13]. During embryonic development, apoptosis is crucial for shaping the developing organism. It helps to sculpt tissues, forming organs and structures by removing cells in specific patterns [14]. Apoptosis enables the precise removal of cells, ensuring that organs and limbs form correctly. Examples include formation of fingers and toes: In human and vertebrate embryonic development, apoptosis eliminates the webbing between fingers and toes, leading to the formation of distinct digits. Neural development: In the developing brain, excess neurons are produced initially [15]. Apoptosis selectively removes neurons that do not make proper connections, refining neural circuits and ensuring only properly connected neurons survive, which is critical for proper brain function [16]. Immune System Maturation: Apoptosis is also involved in eliminating self-reactive immune cells during immune system development, which helps prevent autoimmune diseases [14]. Without apoptosis during development, organisms could have structural malformations, impaired organ function, or dysfunctional immune responses [17]. Defense against disease and cellular damage: Apoptosis acts as a protective mechanism, eliminating cells that may be harmful to the organism [18]. Cells with irreparable DNA damage, oxidative stress, or exposure to harmful agents like radiation or toxins are often targeted for apoptosis [19]. This selective elimination helps prevent the accumulation of potentially cancerous or dysfunctional cells. Prevention of cancer: Apoptosis acts as a defense against cancer by eliminating cells with damaged DNA or other oncogenic mutations [20]. When cells accumulate mutations that could lead to uncontrolled proliferation, apoptosis mechanisms typically remove these cells before they become malignant [21]. In many cancers, apoptosis is dysregulated, allowing abnormal cells to evade death, leading to tumor development [22]. Response to infection: During infection, apoptosis plays a role in containing pathogens [23]. Infected cells often undergo apoptosis to prevent the spread of viruses or bacteria. For example, cytotoxic T cells and natural killer cells can induce apoptosis in infected cells, limiting pathogen proliferation [24]. By destroying infected cells, apoptosis helps control infections and minimize the damage to surrounding tissues. It helps in the regulation of immune system function. The immune system relies on apoptosis for both its development and its regulated response to infections and foreign invaders [25]. Apoptosis shapes immune cell populations and prevents autoimmunity by eliminating immune cells that might attack the body's own tissues [26]. In the thymus, apoptosis helps remove self-reactive T cells, a process known as clonal deletion. This process is critical for establishing immune tolerance, preventing the immune system from attacking the body's own cells. Without apoptosis in immune cell maturation, autoimmune diseases would become more common [27]. Apoptosis is also known to cause resolution of immune responses: After an infection is cleared, apoptosis reduces the number of active immune cells to return the immune system to its baseline state, preventing excessive inflammation or tissue damage. If immune cells persist inappropriately, they can cause chronic inflammation, contributing to diseases like rheumatoid arthritis [27]. The role of apoptosis has been identified in neurodegeneration and aging. In neurons, which are highly specialized and often non-dividing cells, apoptosis is carefully regulated, as excessive apoptosis in the brain can lead to neurodegenerative diseases. During aging, an imbalance in apoptosis can lead to cell loss in tissues with limited regenerative capacity [28]. Excessive apoptosis is implicated in neurodegenerative conditions such as Alzheimer's, Parkinson's, and

Huntington's diseases. In these diseases, neurons in specific brain regions undergo excessive apoptosis, contributing to the progressive loss of brain function [29]. The other role of apoptosis includes aging and tissue atrophy. As organisms age, apoptosis can contribute to tissue and organ atrophy if cellular replenishment cannot keep up with cell loss. For instance, excessive apoptosis in muscle or bone cells contributes to conditions like sarcopenia (muscle wasting) and osteoporosis [30]. Unlike necrosis, which typically results from injury and leads to cell rupture and inflammation, apoptosis is a controlled, contained process. The cell's contents are packaged into apoptotic bodies and phagocytosed by neighboring cells or immune cells, preventing the release of potentially harmful intracellular substances that could trigger inflammation. This aspect of apoptosis is particularly important in preventing inflammatory diseases and maintaining tissue integrity. For example, when heart cells die from ischemia (lack of blood flow), apoptosis limits inflammatory damage compared to necrosis, which can exacerbate injury to surrounding cells [31]. The controlled nature of apoptosis makes it a promising target for therapies in diseases where abnormal cell survival or cell death occurs. In cancer, for example, where apoptosis is often impaired, therapeutic agents that can restore apoptotic signaling pathways are under development to trigger death in cancer cells selectively. Apoptosis modulation also holds potential for treating autoimmune diseases, neurodegenerative diseases, and infections by targeting pathways that either enhance or inhibit apoptotic mechanisms as needed. Examples of therapeutic approaches include cancer therapy. Drugs like BH3 mimetics and proteasome inhibitors are designed to stimulate apoptosis selectively in cancer cells, exploiting the apoptotic machinery to overcome resistance. In neurodegenerative diseases, strategies to inhibit excessive apoptosis are being explored to protect neurons and slow disease progression [32].

3. Decoding apoptosis: A comparison with other cell death pathways

Apoptosis is a tightly regulated, programmed process of cell death. It is orchestrated by specific signaling pathways that ensure cell death occurs in an orderly, controlled manner. This control prevents damage to surrounding cells and allows efficient removal of the dying cell by immune cells without inflammation [12]. Necrosis, in contrast, is typically an uncontrolled, accidental form of cell death caused by external injury or trauma, such as physical damage, lack of oxygen, or exposure to toxins. Unlike apoptosis, necrosis lacks any regulation and generally occurs due to severe cellular stress or injury that the cell cannot repair [33].

Autophagy is primarily a survival mechanism. Unlike apoptosis and necrosis, it is an intracellular recycling process, allowing cells to break down and reuse damaged components under stress [34]. However, excessive or dysfunctional autophagy can lead to a self-destructive process known as autophagic cell death, although this process is less organized and regulated than apoptosis [30, 35].

Pyroptosis is a highly inflammatory form of programmed cell death that plays a critical role in the immune response. Unlike apoptosis, which is non-inflammatory, pyroptosis results in the release of pro-inflammatory cytokines such as IL-1β and IL-18, along with cellular contents, leading to localized inflammation. This process is primarily triggered by infections or cellular stress and involves the activation of inflammasomes, which are intracellular multiprotein complexes. Key molecular players in pyroptosis include caspase-1 and the gasdermin family, particularly

gasdermin D (GSDMD). Upon activation by inflammasomes, caspase-1 cleaves GSDMD, releasing its N-terminal fragment. This fragment forms pores in the plasma membrane, leading to cell lysis and the release of inflammatory mediators. Pyroptosis is critical for combating infections by eliminating infected cells and recruiting immune cells to the site of infection. However, dysregulated pyroptosis is implicated in various diseases, including chronic inflammation, autoimmune disorders, and sepsis. As a result, it is a significant focus of research in immunology and therapeutic development [36]. Cells respond to death-inducing stimuli by activating distinct pathways critical for maintaining balance in multicellular organisms illustrated in **Figure 1**.

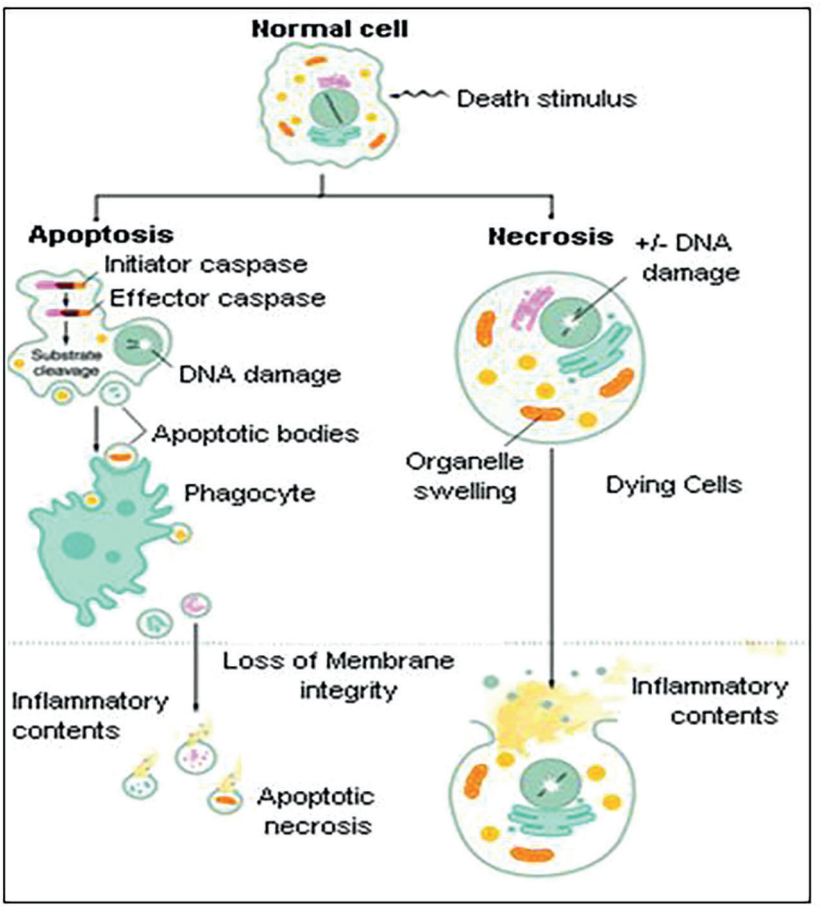

Figure 1.
This figure illustrates that how cells respond to death-inducing stimuli by activating distinct pathways critical for maintaining balance in multicellular organisms. Failure to execute these pathways can result in developmental defects, organ dysfunction, cancer, or inappropriate immune responses. Apoptosis involves caspase activation, nuclear condensation, apoptotic body formation, and phagocytic clearance; without clearance, secondary necrosis may occur. Autophagy degrades cellular components within autophagic vacuoles, characterized by vacuolization and slight chromatin condensation. Oncosis is marked by cellular swelling, membrane breakdown, and the release of inflammatory contents [37]. Pyroptosis, a caspase-1-mediated process, activates proinflammatory cytokines IL-1β and IL-18, causing cell lysis and inflammation. Susa. Fink et al. [2] ... https://www.ncbi.nlm.nih.gov/core/lw/2.0/html/tileshop_pmc/tileshop_pmc_inline.html?title=Click%20on%20image%20to%20zoom&p=PMC3&id=1087413_zii00405473000002.jpg.

4. Summary comparison table

Table 1 provides a detailed comparison of apoptosis, necrosis, and autophagy based on key characteristics, including the type of cell death, effects on surrounding tissue, underlying mechanisms, triggers, and their roles in health and disease. Apoptosis is a regulated form of programmed cell death essential for tissue homeostasis, while necrosis is an uncontrolled, pathological process often associated with inflammation and tissue damage. Autophagy primarily functions as a survival mechanism by degrading and recycling cellular components but can also contribute to cell death under certain conditions. Understanding these processes is critical for deciphering their implications in various physiological and pathological contexts, including cancer, neurodegeneration, and immune responses.

Feature	Apoptosis	Necrosis	Autophagy
Type of cell death	Programmed cell death (regulated)	Unregulated cell death (often pathological)	Survival mechanism, but can lead to cell death
Trigger	Internal/external signals (e.g., DNA damage, death signals)	Physical injury, toxins, lack of oxygen	Nutrient deprivation, cellular stress
Key mechanism	Caspase activation, mitochondrial and death receptor pathways	Loss of plasma membrane integrity	Formation of autophagosomes, lysosomal degradation
Morphology	Cell shrinkage, chromatin condensation, apoptotic bodies	Cell swelling, membrane rupture, release of contents	Double-membrane autophagosomes, increased lysosomes
Effect on tissue	Non-inflammatory, clean removal by phagocytes	Inflammatory, damaging to surrounding cells	Non-inflammatory, generally contained
Role in health	Development, immune function, homeostasis	No physiological role (response to injury/ pathology)	Survival, cellular quality control, metabolic balance
Role in disease	Cancer, autoimmune diseases, neurodegeneration	Stroke, infections, chronic inflammation	Cancer, neurodegeneration, metabolic diseases

Table 1.
Comprehensive comparison of apoptosis with necrosis, and autophagy: Mechanisms, triggers, and their roles in health and disease.

5. Morphological and structural changes in apoptosis

Light and electron microscopy have revealed various morphological changes characteristic of apoptosis [38]. In the initial stages of apoptosis, light microscopy reveals cell shrinkage and pyknosis [39]. Shrinking cells appear smaller, with a dense cytoplasm and more tightly packed organelles. Pyknosis, caused by chromatin condensation, is a hallmark of apoptosis. On histological examination with hematoxylin and eosin stain, apoptotic cells are observed as single cells or small clusters [40]. They typically appear as round or oval structures with dark eosinophilic cytoplasm and dense purple nuclear chromatin fragments. Electron microscopy provides a more detailed view of subcellular changes. During early chromatin condensation, electron-dense nuclear material aggregates near the nuclear membrane, though nuclei may

also appear uniformly dense [40]. Plasma membrane blebbing intensifies, leading to karyorrhexis and the formation of apoptotic bodies through a process called "budding." These apoptotic bodies, containing cytoplasm with tightly packed organelles and sometimes nuclear fragments, maintain organelle integrity and are enclosed within an intact plasma membrane. Macrophages, parenchymal cells, or neoplastic cells subsequently phagocytose and degrade these bodies in phagolysosomes [41]. Macrophages that engulf apoptotic cells, termed "tingible body macrophages," are often found in the reactive germinal centres of lymphoid follicles or occasionally in the thymic cortex. Tingible bodies represent nuclear debris from apoptotic cells [5].

Notably, apoptosis is not associated with inflammation or tissue damage, as apoptotic cells do not release their contents into surrounding tissue. They are rapidly engulfed, preventing secondary necrosis. Furthermore, phagocytic cells do not release pro-inflammatory cytokines [42]. Apoptosis is characterized by cell shrinkage, chromatin condensation, and nuclear fragmentation, leading to the formation of membrane-bound apoptotic bodies that sequester cellular components. In contrast, necrosis involves cell swelling (oncosis), membrane rupture, and uncontrolled release of cellular contents, which can trigger inflammation and damage nearby cells. Autophagy, distinct from both, features the formation of autophagosomes—double-membrane vesicles that encapsulate and degrade cellular components.

Autophagy lacks the characteristic nuclear changes of apoptosis and the membrane rupture seen in necrosis and instead involves extensive cytoplasmic vacuolization [2]. Apoptosis, a highly regulated form of programmed cell death, is marked by specific and distinctive morphological changes that distinguish it from other forms of cell death, such as necrosis. These changes include membrane blebbing, chromatin condensation, nuclear fragmentation, and the formation of apoptotic bodies. Such features are essential not only for recognizing apoptosis under a microscope but

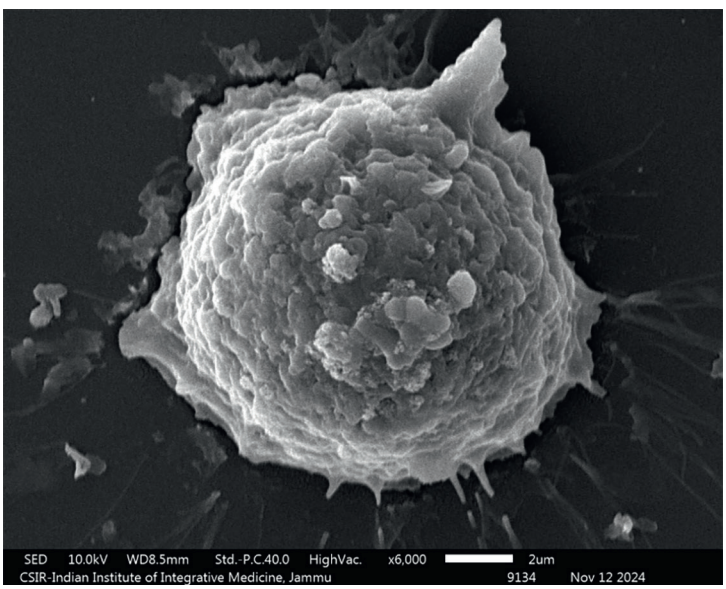

Figure 2.
Scanning electron microscopy (SEM) image performed in our study illustrating membrane blebbing in a cell. The image highlights the formation of spherical protrusions on the cell surface, characteristic of dynamic cytoskeletal changes. These blebs are indicative of cellular stress or a programmed cell death process such as apoptosis, where membrane integrity is maintained during early stages.

also for understanding the sequence and significance of events leading to cellular disassembly.

5.1 Membrane blebbing

Membrane blebbing is one of the most characteristic early morphological changes in apoptosis leading to the formation of spherical protrusions on the cell surface, characteristic of dynamic cytoskeletal changes represented in **Figure 2**. This process involves the formation of dynamic, bulging protrusions on the plasma membrane. Membrane blebbing begins as the cytoskeleton underneath the cell membrane reorganizes and becomes destabilized. The actin-myosin network contracts, driven by the activity of enzymes like rho-associated protein kinase (ROCK), which phosphorylates myosin light chains, promoting actin-myosin interactions. This contraction exerts tension on the plasma membrane, causing it to protrude outwards, forming "blebs. Blebbing plays a role in the later steps of apoptosis, contributing to cell disassembly [43]. By fragmenting into smaller parts, the cell prepares for the formation of apoptotic bodies. This blebbing helps prevent the release of cellular contents into the surrounding environment, thereby reducing the risk of inflammation, a key distinction between apoptosis and necrosis [44].

5.2 Chromatin condensation

Chromatin condensation (pyknosis), where chromatin undergoes profound changes in structure as shown in **Figure 3**. Chromatin condensation is initiated by the activation of caspases, particularly caspase-3 and caspase-6, which target proteins involved in nuclear integrity and chromatin structure. These caspases activate endonucleases, such as caspase-activated DNase (CAD), which cleaves DNA at specific internucleosomal sites. Consequently, chromatin condenses and aggregates at the periphery of the nuclear membrane. Under a microscope, chromatin condensation appears as a dense, dark staining of nuclear material in apoptotic cells, often forming crescent-shaped patches against the nuclear envelope [47]. Eventually, the condensed chromatin fragments, a feature that is easily distinguishable from the nuclear swelling and random DNA fragmentation typically observed in necrosis. Chromatin

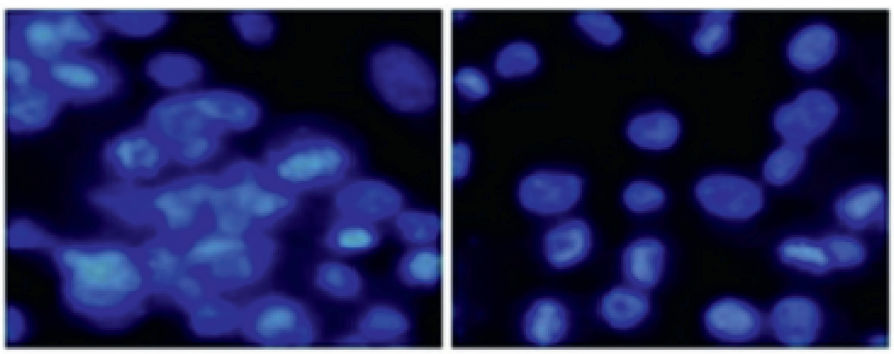

Figure 3.
Apoptosis (chromatin condensation) showing fragmented and condensed nuclei. Source: Md A Rahman [45] and Yan et al. [46] (https://www.researchgate.net/publication/311423220_Evaluation_of_anticancer_activity_of_Cordia_dichotoma_leaves_against_a_human_prostate_carcinoma_cell_line_PC3).

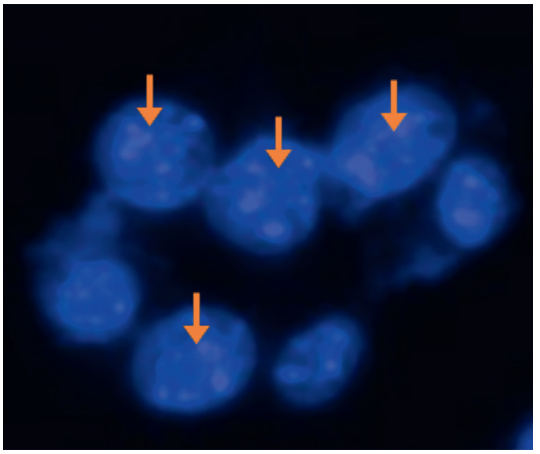

Figure 4.
Nuclear structure alterations during apoptosis visualized with DAPI staining. The figure shows the progressive distortion of nuclear architecture during apoptosis, visualized using DAPI (4′,6-diamidino-2-phenylindole) staining. DAPI binds to DNA and emits blue fluorescence under UV light. In healthy cells, the nuclei appear intact and uniformly stained. During early apoptosis, chromatin condensation is observed as intense, localized fluorescence [45].

condensation serves two main purposes. First, it compacts the DNA and prepares it for packaging into apoptotic bodies, aiding in the non-inflammatory disposal of nuclear material. Second, chromatin condensation signals to phagocytic cells that the apoptotic cell is ready for engulfment, ensuring that cellular remnants are cleared without spurring an immune response.

5.3 Nuclear fragmentation

Nuclear fragmentation (karyorrhexis) is the process by which the nucleus breaks down into smaller, distinct fragments, further aiding in cellular dismantling represented in **Figure 4**. Following chromatin condensation, CAD and other nucleases continue to break down nuclear DNA, fragmenting the nucleus itself. Caspase-activated nucleases degrade nuclear Lamins, proteins that maintain the nuclear envelope's structure, leading to the nuclear envelope's disintegration. The nuclear contents fragment into smaller pieces, each enveloped by a segment of the nuclear membrane [48]. Nuclear fragmentation ensures that the cell's genetic material is partitioned into manageable units, which can be engulfed by phagocytes. This compartmentalization of DNA prevents the exposure of nuclear material to the extracellular environment, thus reducing the risk of autoimmunity or inflammatory responses.

5.4 Formation of apoptotic bodies

The final stage of apoptosis involves the formation of apoptotic bodies, small membrane-bound vesicles that contain cellular components, including cytoplasm, organelles, and nuclear fragments. Apoptotic bodies form as the cell undergoes further cytoskeletal contraction and membrane blebbing. Cytoskeletal elements continue to fragment the cell into discrete sections, each surrounded by plasma membrane. Apoptotic bodies are often heterogeneous in size and may contain intact organelles, portions of the nucleus, or cytoplasmic proteins. Apoptotic bodies expose specific "eat-me" signals on their surface, such as phosphatidylserine, which

is normally found on the inner leaflet of the plasma membrane but flips to the outer surface during apoptosis. This signal is recognized by receptors on phagocytes, allowing these immune cells to engulf and digest apoptotic bodies without releasing their contents into the surrounding tissue [49]. The formation of apoptotic bodies ensures that cellular debris is neatly packaged, allowing it to be efficiently cleared by phagocytes. This organized packaging minimizes the risk of inflammation, as it prevents the release of pro-inflammatory intracellular components, which would otherwise occur in cases of cell rupture, as seen in necrosis.

6. Biochemical hallmarks of apoptosis

In addition to its distinct morphological characteristics, apoptosis is also characterized by specific biochemical changes. These changes serve as crucial indicators of apoptosis and distinguish it from other forms of cell death, such as necrosis and autophagy. Two of the most significant biochemical hallmarks of apoptosis are DNA fragmentation and phosphatidylserine (PS) exposure [50]. These hallmarks reflect the regulated and orderly dismantling of cellular components during apoptosis, enabling apoptotic cells to be efficiently recognized and removed by phagocytes, thereby avoiding inflammation. Below is an in-depth look at these biochemical markers and their roles in apoptosis.

6.1 DNA fragmentation

DNA fragmentation is one of the most definitive biochemical indicators of apoptosis, marked by the cleavage of genomic DNA into characteristic fragments shown in **Figure 5**. DNA fragmentation in apoptosis is largely mediated by a family of enzymes known as caspases, specifically caspase-activated DNase (CAD). Under normal conditions, CAD is kept inactive by an inhibitor, ICAD (inhibitor of caspase-activated DNase). During apoptosis, caspase-3 cleaves ICAD, releasing CAD to enter the nucleus and cleave DNA at internucleosomal sites. This cleavage results in fragments that are multiples of approximately 180-200 base pairs, corresponding to the DNA wrapped around each nucleosome [52]. The fragmented DNA generated during apoptosis often forms a characteristic "DNA ladder" pattern when analyzed by gel electrophoresis. This laddering is due to the precise and regular cleavage by CAD, which is distinct from the random and extensive DNA degradation that occurs in necrosis. Additionally, DNA fragmentation can be detected in situ using techniques like the TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labelling) assay, which labels DNA breaks to visualize apoptotic cells [53].

DNA fragmentation is a key step in the irreversible progression of apoptosis, ensuring that the genetic material is non-functional and preparing the cell for complete disassembly. By condensing and fragmenting the DNA, the cell also facilitates the efficient packaging of nuclear material into apoptotic bodies, allowing for their safe engulfment by phagocytes without exposing intact DNA to the extracellular environment [46, 53].

6.2 Phosphatidylserine (PS) exposure

Phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane is another essential hallmark of apoptosis. This event serves as an "eat-me" signal

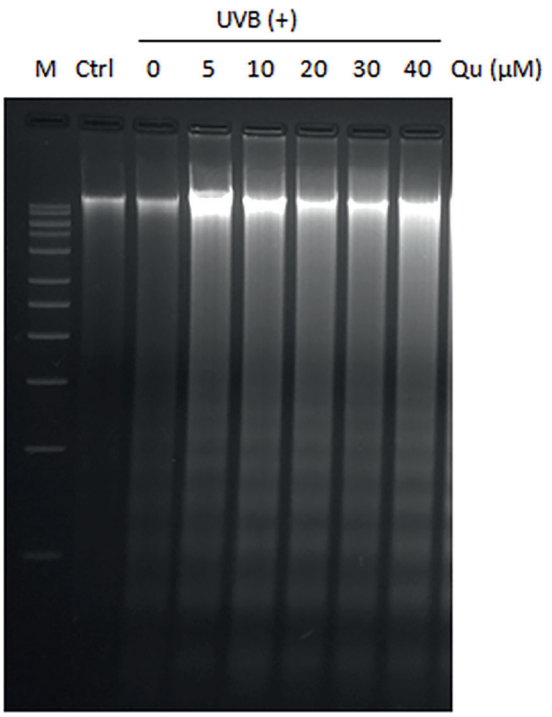

Figure 5.
DNA fragmentation during apoptosis assessed by agarose gel electrophoresis. DNA isolated from apoptotic cells was subjected to agarose gel electrophoresis to analyze the characteristic DNA fragmentation. The gel shows a DNA laddering pattern, indicative of internucleosomal cleavage, a hallmark of apoptosis. Lanes represent samples from untreated control cells, showing intact genomic DNA, and cells treated with an apoptosis-inducing agent, displaying the fragmented DNA pattern [51].

for phagocytes, marking the apoptotic cell for recognition and clearance as shown in **Figure 6**. Under normal circumstances, PS resides exclusively on the inner leaflet of the plasma membrane. During apoptosis, caspase activation leads to the inactivation of flippases, enzymes responsible for maintaining membrane asymmetry, and the activation of scramblases, which move PS from the inner to the outer membrane

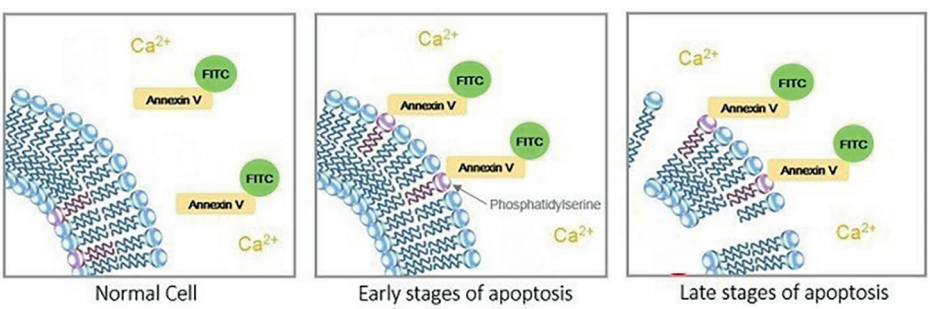

Figure 6.
This figure illustrates the binding of Annexin V conjugated with FITC (fluorescein isothiocyanate) to phosphatidylserine (PS) molecules translocated to the outer leaflet of the plasma membrane during apoptosis. Normal cells retain PS on the inner leaflet of their membrane and remain unstained (research gate by nacalai. com).

leaflet. One such scramblase, Xkr8, is directly activated by caspase-3, facilitating the exposure of PS on the cell surface [54]. PS exposure can be detected using annexin V, a protein that specifically binds to PS in the presence of calcium ions. Annexin V staining, in combination with propidium iodide (a dye that enters cells with compromised membranes), allows researchers to differentiate between early apoptotic cells (annexin V-positive, PI-negative) and late apoptotic or necrotic cells (annexin V-positive, PI-positive). The exposure of PS on the outer leaflet is crucial for the immunologically silent clearance of apoptotic cells. Phagocytic cells, such as macrophages, have receptors that recognize PS, facilitating the engulfment of apoptotic cells or apoptotic bodies. This process of "silent phagocytosis" prevents the release of inflammatory signals, distinguishing apoptosis from necrosis, where cellular contents are released and can stimulate an immune response. By enabling rapid and efficient clearance, PS exposure plays a key role in maintaining tissue homeostasis and preventing autoimmune reactions [55].

7. Additional biochemical changes in apoptosis

In addition to DNA fragmentation and PS exposure, apoptosis is accompanied by several other biochemical changes that contribute to its orderly execution: caspase activation. Caspases are a family of proteases that serve as central regulators of apoptosis. Initiator caspases (like caspase-8 and caspase-9) activate effector caspases (such as caspase-3, −6, and − 7), which then cleave various substrates in the cell, leading to the characteristic morphological and biochemical changes of apoptosis. Caspase activation is tightly regulated, ensuring that apoptosis proceeds in a controlled manner [56]. In addition to this, mitochondrial outer membrane permeabilization (MOMP) is a pivotal event in intrinsic apoptosis. The release of cytochrome c and other pro-apoptotic factors from the mitochondria into the cytosol activates apoptotic signaling pathways, including the formation of the apoptosome, a multiprotein complex that activates caspase-9. This leads to the downstream activation of effector caspases, amplifying the apoptotic cascade. MOMP finally leads to the loss of mitochondrial membrane potential. The mitochondrial membrane potential ($\Delta\Psi m$) is crucial for mitochondrial function, and its disruption is an early event in apoptosis. The loss of $\Delta\Psi m$ can be detected using specific dyes that respond to changes in mitochondrial potential, indicating the onset of apoptosis [57]. The biochemical hallmarks of apoptosis—specifically, DNA fragmentation and phosphatidylserine exposure—are fundamental to its role as a non-inflammatory form of programmed cell death. DNA fragmentation ensures the inactivation of genetic material and signals the cell's progression through apoptosis, while PS exposure facilitates efficient phagocytic recognition and clearance. These markers enable the precise identification of apoptotic cells and underscore the controlled, immunologically silent nature of apoptosis. This level of regulation distinguishes apoptosis from other cell death forms and highlights its essential role in maintaining tissue homeostasis and preventing disease [58].

Apoptosis is mediated by a cascade of caspases, especially executioner caspases like caspase-3 and caspase-7, which dismantle cellular structures. The Bcl-2 protein family plays a key role, balancing pro-apoptotic and anti-apoptotic signals, while proteins like cytochrome c initiate the cascade from the mitochondria. Necrosis lacks specific molecular regulation. However, certain types, like necroptosis (a regulated necrosis form), involve proteins like RIPK1 and RIPK3 that lead to cell rupture [59]. The loss of ATP, build-up of calcium ions, and production of reactive oxygen species

(ROS) are also characteristic of necrotic death. Autophagy relies on unique proteins, like LC3 (microtubule-associated protein 1A/1B-light chain 3) and Beclin-1, which form autophagosomes and recruit components for degradation. This pathway is regulated by nutrient-sensitive signaling cascades like mTOR and AMPK, which determine when cells need to enter survival mode [60]. The effect on surrounding tissue and inflammation apoptosis is non-inflammatory. The cell contents are enclosed in apoptotic bodies, which are then phagocytosed by immune cells, preventing any inflammatory response. This "clean" form of cell death minimizes harm to surrounding tissue. Necrosis, however, is highly inflammatory. The rupture of the cell membrane releases damage-associated molecular patterns (DAMPs) that activate the immune system, leading to an inflammatory response and often causing further tissue damage. This inflammatory environment can exacerbate diseases, such as during a heart attack or in chronic inflammatory conditions [61].

8. Molecular mechanisms and pathways of apoptosis

Apoptosis, or programmed cell death, is governed by two primary pathways: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. These pathways, though distinct in their initiation, are highly interconnected and converge to execute apoptosis, ensuring the elimination of damaged or unnecessary cells in a controlled and non-inflammatory manner.

8.1 The intrinsic pathway

The intrinsic pathway is primarily triggered by internal stress signals, such as DNA damage, oxidative stress, or metabolic dysfunction. These stressors lead to mitochondrial outer membrane permeabilization (MOMP), a crucial event mediated by the Bcl-2 protein family. Pro-apoptotic members of this family, such as Bax and Bak, oligomerize to form pores in the mitochondrial outer membrane, enabling the release of apoptotic factors, including cytochrome c, into the cytosol. Cytochrome c, upon release, binds to Apaf-1 and ATP, forming the apoptosome complex. This complex activates initiator caspase-9, which subsequently activates executioner caspases, such as caspase-3 and caspase-7 shown in **Figure** 7. These effector caspases dismantle the cell by cleaving structural proteins, enzymes, and other cellular components,

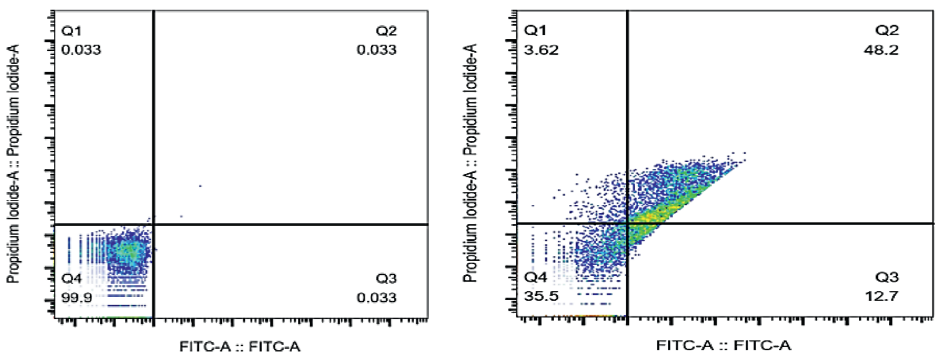

Figure 7.
Cell apoptosis determined by Annexin V/PI double-staining assay followed by flow cytometer analysis in our study. (a) Control cells without any treatment. (b) Induction of apoptosis in cells using capmptothecin.

resulting in chromatin condensation, DNA fragmentation, membrane blebbing, and cellular disassembly [62].

Anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL, counteract this process by sequestering Bax and Bak, thereby preventing MOMP and cytochrome c release. This balance between pro- and anti-apoptotic proteins is critical for determining cell fate and is tightly regulated [58]. Dysregulation of this balance can lead to pathological conditions, such as cancer, where anti-apoptotic proteins are often overexpressed, allowing cells to evade death.

8.2 The extrinsic pathway

The extrinsic pathway is initiated by external signals through the binding of death ligands, such as FasL (Fas ligand), TRAIL (TNF-related apoptosis-inducing ligand), or TNF-α, to their respective death receptors on the cell surface. These receptors, part of the tumor necrosis factor (TNF) receptor superfamily, include Fas (CD95), DR4, DR5, and TNFR1 shown in **Figure 8**. Ligand binding induces receptor oligomerization and the recruitment of adaptor proteins like FADD (Fas-associated death domain), leading to the formation of the death-inducing signaling complex (DISC). Within the DISC, procaspase-8 is cleaved to its active form, caspase-8, which either directly activates downstream executioner caspases or cleaves Bid, linking the extrinsic and intrinsic pathways. Caspases, a family of cysteine proteases, are central to the execution of apoptosis [64]. They exist as inactive precursors (zymogens) and are activated in response to apoptotic signals. Initiator caspases, such as caspase-8 and caspase-9, serve as the initial response elements, activating effector caspases, such as caspase-3 and caspase-7. Effector caspases dismantle the cell by cleaving substrates like PARP (poly ADP-ribose polymerase) and nuclear lamins, which are crucial for maintaining nuclear structure and DNA integrity. This cascade ensures the orderly dismantling of the cell while preserving surrounding tissue integrity. The regulation of caspase activity is essential for preventing excessive or premature apoptosis. Inhibitor of apoptosis proteins (IAPs), such as XIAP (X-linked inhibitor of apoptosis protein), suppresses caspase activation to maintain cellular survival under non-stress conditions. Additionally, SMAC/DIABLO, a mitochondrial protein released during apoptosis, counteracts IAPs to promote caspase activation. This intricate balance of pro- and anti-apoptotic regulators ensures that apoptosis occurs only under appropriate conditions [65].

8.3 Crosstalk between intrinsic and extrinsic pathways

Although the intrinsic and extrinsic pathways are triggered by different stimuli, they are highly interconnected. Bid, a member of the Bcl-2 family, serves as a key mediator of this crosstalk. Bid and tBid caspase-8, activated in the extrinsic pathway, cleaves Bid into its active form, tBid. tBid translocates to the mitochondria, where it activates Bax and Bak, promoting MOMP and integrating the extrinsic and intrinsic pathways. Amplification of apoptosis: this cross-talk ensures that signals from external stimuli are amplified through mitochondrial involvement, leading to a robust and irreversible apoptotic response [66].

8.4 Mechanisms of MOMP and the apoptosome

MOMP represents the point of no return in apoptosis, marking the release of mitochondrial proteins that drive the caspase cascade. Formation of the apoptosome

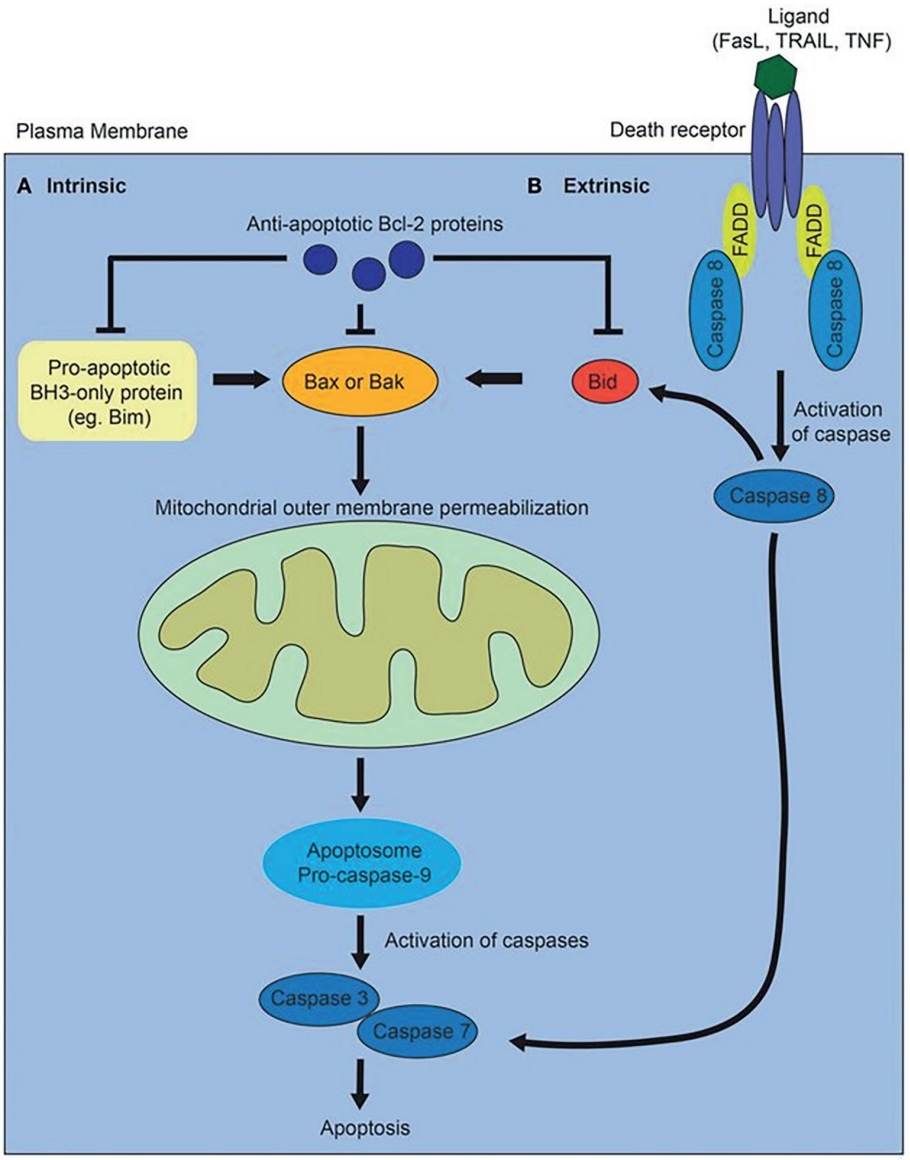

Figure 8.
Diagram of intrinsic and extrinsic pathways of apoptosis. (A) In the intrinsic pathway, the proapoptotic BH3-only family members activate Bax or Bak, leading to mitochrondrial outer membrane permeabilization, which drives formation of the apoptosome, activation of the executioner caspases, 3 and 7, and subsequent apoptosis. The proapoptotic BH3-only proteins are inhibited via interactions with the anti-apoptotic Bcl-2 family of proteins. (B) In the extrinsic pathway, ligands such as Fas, tumor necrosis factor (TNF), or tumor necrosis factor-related apoptosis-inducing (TRAIL) ligand bind to death receptors. This results in the recruitment of Fas-associated death domain protein (FADD) and activation of caspase 8. Caspase 8 directly activates caspase 3 and 7. The two pathways interact via caspase 8-mediated cleavage of Bid [63].

due to cytochrome c, released during MOMP, binds to Apaf-1 and dATP, forming the apoptosome. This complex recruits and activates procaspase-9, which in turn activates effector caspases. Effector caspases cleave numerous cellular components, including cytoskeletal proteins, nuclear Lamins, and enzymes like PARP, ultimately resulting in the orderly disassembly of the cell. The balance between pro-apoptotic

and anti-apoptotic members of the Bcl-2 family determines whether a cell will undergo apoptosis. Pro-apoptotic proteins: Bax and Bak form oligomers that create pores in the mitochondrial membrane, releasing apoptotic factors. Bid enhances this process by bridging extrinsic and intrinsic pathways' – apoptotic proteins: Bcl-2 and Bcl-xL prevent Bax and Bak activation, maintaining mitochondrial integrity and blocking the initiation of apoptosis [67].

Apoptosis is a highly regulated process involving the intrinsic and extrinsic pathways, both of which culminate in the activation of caspases that execute cell death. The intrinsic pathway is driven by mitochondrial changes, while the extrinsic pathway responds to external death signals. Crosstalk between these pathways ensures a robust apoptotic response. The Bcl-2 protein family serves as a crucial regulator, balancing pro-apoptotic and anti-apoptotic signals. This intricate regulation of apoptosis is critical for maintaining tissue homeostasis and has profound implications for understanding and treating diseases characterized by aberrant cell death, such as cancer, neurodegenerative disorders, and autoimmune conditions [68].

9. The role of apoptosis in pathogenesis regulation

Apoptosis is a vital process in the body that maintains cellular balance, prevents disease, and facilitates proper tissue development. Its precise control over cell death ensures that harmful, damaged, or excess cells are removed in a way that avoids inflammation or damage to surrounding cells. In health, apoptosis is essential for development, immune function, and tissue homeostasis. However, when dysregulated, apoptosis contributes significantly to numerous diseases, such as cancer, neurodegeneration, and autoimmune conditions. In this section, the importance of apoptosis in maintaining health, along with its pathological consequences when its regulatory mechanisms fail will be discussed. During development, apoptosis plays a pivotal role in sculpting tissues and organs by selectively removing unnecessary or redundant cells. One well-documented example is the elimination of cells in the webbing between the developing digits of embryos, which shapes fingers and toes. This process is tightly controlled and ensures that tissues achieve their proper form and function. Similarly, apoptosis is vital for neural development, where it removes surplus neurons to refine synaptic connections and optimize neural networks. This pruning ensures the brain's structural and functional integrity, enabling efficient information processing and adaptability. Studies have shown that insufficient or excessive neuronal apoptosis during development can lead to neurodevelopmental disorders such as autism and schizophrenia, underscoring the critical balance required in this process [69]. Apoptosis also underpins immune system development and regulation. Autoreactive immune cells, which have the potential to attack the body's own tissues, are eliminated during maturation in a process known as negative selection. This ensures self-tolerance and prevents autoimmune disorders. Following an immune response, apoptosis clears immune cells, such as neutrophils and lymphocytes, once they have fulfilled their function. This resolution of inflammation is critical to avoid prolonged immune activity, which could otherwise lead to tissue damage or chronic inflammation.

In adult tissues, apoptosis maintains tissue homeostasis by balancing cell turnover, particularly in high-turnover tissues such as the gut epithelium, skin, and blood. By removing aged, damaged, or dysfunctional cells, apoptosis prevents the accumulation of potentially harmful cells that could impair organ function or promote malignancy. For example, apoptotic pathways are activated in response to DNA damage or

oxidative stress, preventing the survival of cells with mutations that could progress to cancer [70]. The importance of apoptosis in maintaining cellular integrity is evident in its role in protecting tissues from hyperplasia or hypertrophy, which could disrupt normal organ function.

When apoptotic pathways are dysregulated, they contribute significantly to the onset and progression of various diseases. In this section, we will specifically look into the intricate role of apoptosis in disease pathogenesis like cancer neurodegeneration diseases, autoimmune disease, and inflammatory diseases, emphasizing the molecular mechanisms and pathological consequences of its dysregulation [12].

9.1 Role of apoptosis in cancer pathology

In normal tissues, apoptosis serves as a protective mechanism to eliminate cells with genetic abnormalities or oncogenic mutations. By doing so, it acts as a barrier to tumor initiation and progression. For example, elimination of mutated cells apoptosis removes cells that acquire DNA damage or undergo oncogene activation, thereby preventing their clonal expansion. Apoptotic processes are integral to immune system function, facilitating the removal of potentially malignant cells through phagocytosis by macrophages and other immune cells. In tissues with high turnover rates, apoptosis ensures a balance between cell proliferation and cell death, preventing hyperplasia and subsequent tumorigenesis [71].

Evasion of Apoptosis in Cancer Development.

In cancer, cells acquire the ability to evade apoptosis, contributing to uncontrolled growth and survival. This evasion is a hallmark of cancer and occurs through various mechanisms that collectively enable tumor progression [72]. Key consequences include the following:

- *Sustained proliferation*: The inability of cancer cells to undergo apoptosis allows them to continue dividing despite genetic aberrations and unfavorable microenvironmental conditions.

- *Increased resistance to cellular stress*: Cancer cells often face hypoxia, oxidative stress, and metabolic challenges in the tumor microenvironment. Resistance to apoptosis enables them to survive and adapt under these conditions.

- *Immune evasion*: By avoiding apoptosis, cancer cells may evade detection and destruction by the immune system. This is particularly significant in metastatic cancer, where immune evasion facilitates dissemination to distant sites [73].

9.2 Role in tumor heterogeneity and progression

Evasion of apoptosis contributes to the development of tumor heterogeneity—a critical factor in cancer progression and therapeutic resistance. Cancer cells that survive apoptotic triggers such as DNA damage or chemotherapy become the dominant population within the tumor. This selection pressure promotes the emergence of more aggressive and treatment-resistant clones. The failure of apoptosis to remove genetically unstable cells allows the accumulation of mutations, fostering a more heterogeneous tumor cell population with diverse phenotypic and genotypic trait. Apoptosis resistance is essential for cancer cells to survive during detachment, circulation, and colonization at secondary sites. Cells with enhanced survival capabilities are more

likely to establish metastases. The tumor microenvironment (TME) plays a significant role in modulating apoptosis in cancer cells. Interactions between cancer cells and their surrounding stroma, immune cells, and extracellular matrix can either suppress or promote apoptotic pathways. The hypoxic regions of tumors promote resistance to apoptosis by stabilizing hypoxia-inducible factors (HIFs), which regulate survival pathways. Pro-survival signals from cytokines and growth factors in the TME, such as interleukin-6 (IL-6) or vascular endothelial growth factor (VEGF), can inhibit apoptotic processes in cancer cells [74]. Tumor-associated immune cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), can suppress apoptosis-inducing immune responses, further promoting tumor cell survival [75]. Resistance to apoptosis is a significant contributor to cancer therapy failure and disease relapse. As cancer cells evade programmed cell death, they become less responsive to traditional treatments such as chemotherapy and radiation, which rely on inducing apoptosis to eradicate tumors. Furthermore, this resistance facilitates the survival of minimal residual disease and the re-establishment of tumors after treatment. Apoptosis plays a pivotal role in the pathology of cancer, with its disruption contributing to tumor initiation, progression, and resistance. Understanding the intricate relationship between apoptosis and cancer pathology highlights the complexity of tumor biology and underscores the importance of addressing apoptotic dysregulation in cancer research and treatment strategies [76].

In cancer, one of the hallmarks of tumorigenesis is the ability of cells to evade apoptosis, enabling unchecked proliferation and survival under otherwise lethal conditions. This evasion is achieved through several mechanisms such as mutations in tumor suppressor genes, overexpression of anti-apoptotic proteins, and the downregulation of pro-apoptotic factors. The TP53 gene, encoding the tumor suppressor protein p53, plays a vital role in initiating apoptosis in response to cellular stress, particularly DNA damage. Mutations in TP53, observed in many cancers, disrupt this function, allowing damaged cells to evade apoptosis. Proteins like BCL2 inhibit mitochondrial outer membrane permeabilization (MOMP), blocking the release of cytochrome c and subsequent activation of caspases. This anti-apoptotic shift promotes cancer cell survival under stress. Proto-oncogenes like c-MYC exhibit paradoxical roles, promoting proliferation in nutrient-rich environments while triggering apoptosis under growth-limiting conditions [77]. Cancer cells circumvent this apoptotic pressure through additional mutations or signaling alterations. Alterations in Fas/FasL signaling, critical for extrinsic apoptosis, allow cancer cells to escape immune-mediated apoptosis, contributing to immune evasion [78].

Spontaneous apoptosis occurs in untreated tumors and is influenced by factors such as mild ischemia, immune cell infiltration, and intrinsic tumor properties. While insufficient to control tumor growth, this process reflects the dynamic interplay between proliferation and cell death in the tumor microenvironment [79]. Understanding these mechanisms provides insights into tumor biology and therapeutic opportunities.

9.3 Apoptosis and neurodegenerative diseases

While insufficient apoptosis underlies cancer progression, excessive apoptosis is a hallmark of neurodegenerative diseases. In conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD), chronic neuronal apoptosis contributes to the progressive loss of neural populations and associated functional decline. The accumulation of amyloid-beta plaques and hyperphosphorylated

tau proteins in Alzheimer's disease induces oxidative stress and mitochondrial dysfunction, activating the intrinsic apoptotic pathway. Caspase-3, a key executioner caspase, is upregulated in affected brain regions such as the hippocampus and cortex, leading to synaptic dysfunction and neuronal loss [80]. In Parkinson's disease, misfolded alpha-synuclein proteins form toxic aggregates that disrupt mitochondrial function and initiate apoptosis in dopaminergic neurons of the substantia nigra, causing motor dysfunction. Similarly, the mutant huntingtin protein in Huntington's disease disrupts mitochondrial dynamics and induces oxidative stress, activating apoptotic cascades that result in striatal neuron loss and progressive motor and cognitive impairments. Therapeutic strategies in neurodegeneration focus on inhibiting excessive apoptosis, with interventions targeting caspases, mitochondrial integrity, and oxidative stress showing promise in preclinical models [81].

Apoptosis, is critical for maintaining homeostasis and eliminating damaged or dysfunctional cells in the nervous system. In neurodegenerative diseases, dysregulated apoptosis contributes significantly to neuronal loss, which underpins the progression of these conditions.

In neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS), excessive apoptosis leads to the loss of neurons in specific brain regions. Different neuronal populations show varying susceptibility to apoptosis. For instance, dopaminergic neurons in the substantia nigra are predominantly affected in PD, while hippocampal and cortical neurons are targeted in AD. Apoptotic neuronal loss disrupts neural circuits essential for cognitive, motor, and sensory functions, directly causing disease-specific symptoms. Neurodegenerative diseases are marked by persistent inflammation that exacerbates apoptosis [82]. Overactive microglia release inflammatory cytokines like TNF-α and IL-1β, which induce apoptosis in neurons and glial cells, amplifying neurodegeneration. Astrocytes, crucial for neuroprotection, may lose their supportive role and contribute to apoptosis through oxidative stress and inflammatory signaling. Misfolded and aggregated proteins characteristic of neurodegenerative diseases activate apoptotic pathways. Amyloid-β plaques and tau tangles cause synaptic dysfunction and neuronal apoptosis, leading to progressive memory loss. Aggregates of α-synuclein disrupt mitochondrial integrity and promote apoptosis in dopaminergic neurons. Mutant huntingtin protein induces transcriptional dysregulation and mitochondrial stress, triggering apoptosis in striatal and cortical neurons [83].

Oxidative stress, a common feature in neurodegenerative diseases, directly contributes to apoptosis. Neurons rely heavily on mitochondrial energy. Mitochondrial dysfunction leads to the release of pro-apoptotic factors like cytochrome c, promoting cell death. Oxidative damage to cellular components, including DNA and lipids, activates apoptotic signaling, further exacerbating neuronal loss. The failure to efficiently remove apoptotic cells intensifies neurodegeneration. Inefficient clearance by microglia results in the release of cellular debris and inflammatory mediators, perpetuating neuro-inflammation [84]. The accumulation of apoptotic remnants disrupts the extracellular environment, impairing neighboring cell functions and contributing to disease progression. Apoptosis disrupts synaptic integrity, leading to network-level impairments. Apoptotic loss of neurons and glial cells weakens synaptic connections, reducing the brain's ability to transmit and process information. Diminished ability to form new connections or repair damaged ones worsens cognitive and motor symptoms in neurodegenerative diseases. Apoptosis in the hippocampus and cortex leads to progressive memory impairment and cognitive decline. Parkinson's disease:

Loss of dopaminergic neurons in the substantia nigra due to apoptosis causes motor dysfunction, including tremors and rigidity. Apoptosis in the striatum disrupts movement coordination and contributes to psychiatric symptoms. Motor neuron apoptosis results in muscle weakness and eventual paralysis [16].

The role of apoptosis extends beyond the central nervous system in neurodegenerative diseases. Apoptosis in peripheral nerves may contribute to sensory and autonomic dysfunction. Endothelial cell apoptosis can compromise the blood-brain barrier, intensifying neuroinflammation and neuronal damage. Apoptosis is intricately linked to the pathology of neurodegenerative diseases, where its dysregulation contributes to neuronal loss, chronic inflammation, and systemic manifestations. Understanding how apoptosis shapes the course of these conditions provides critical insights into their progression and potential management strategies [85].

9.4 Apoptosis and autoimmune diseases

In autoimmune diseases, apoptosis plays a paradoxical role, contributing to both the prevention and exacerbation of immune-mediated damage. During immune development, apoptosis ensures the removal of self-reactive T and B cells in a process called negative selection. Defective apoptosis in this context allows autoreactive cells to persist, leading to autoimmune conditions such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Conversely, in affected tissues, excessive apoptosis can release intracellular antigens, perpetuating a self-amplifying cycle of immune activation and chronic inflammation. For instance, in RA, apoptotic debris can stimulate further immune responses, exacerbating joint destruction. Restoring apoptotic balance is critical in autoimmune diseases [86]. Approaches include promoting the apoptosis of autoreactive immune cells while minimizing excessive apoptotic activity in target tissues. In healthy individuals, apoptosis is critical for the establishment and maintenance of immune tolerance. It ensures the elimination of autoreactive lymphocytes during both central and peripheral immune tolerance processes. During the development of T and B cells in the thymus and bone marrow, apoptosis removes immature lymphocytes that strongly react to self-antigens. This prevents the release of self-reactive cells into the peripheral immune system. In the periphery, apoptosis eliminates autoreactive lymphocytes that escape central tolerance through mechanisms such as activation-induced cell death (AICD). This prevents inappropriate immune activation against self-antigens. In autoimmune diseases, the regulation of apoptosis is often impaired, leading to the survival of autoreactive immune cells or inappropriate death of essential immune-regulating cells. This dysregulation contributes to chronic inflammation and tissue damage. Key roles include the following: impaired apoptotic pathways allow autoreactive T and B cells to persist, leading to the production of autoantibodies and the activation of inflammatory cascades. For instance, in systemic lupus erythematosus (SLE), defective clearance of apoptotic cells results in the accumulation of cellular debris, which acts as a source of autoantigens. Regulatory T cells (Tregs) are crucial for suppressing autoimmune responses. Aberrant apoptosis leading to the depletion of Tregs can disrupt immune homeostasis and contribute to disease pathogenesis [87].

Apoptosis dysregulation in autoimmune diseases often results in chronic inflammation, which perpetuates tissue damage. This can occur as follows: when apoptotic cells are not efficiently cleared by phagocytes, they undergo secondary necrosis, releasing pro-inflammatory cytokines and autoantigens. This is a prominent feature in diseases such as rheumatoid arthritis (RA) and SLE. The

persistence of apoptotic cell-derived debris can activate dendritic cells and other antigen-presenting cells, leading to the stimulation of autoreactive T and B cells and amplifying the autoimmune response. The impact of apoptosis in autoimmune diseases often varies depending on the affected tissue: In RA, resistance to apoptosis in synovial fibroblasts leads to their hyperplasia and contributes to joint destruction. Simultaneously, defective clearance of apoptotic cells in the synovium enhances local inflammation. Apoptosis of insulin-producing beta cells in the pancreas, triggered by autoreactive T cells, is a hallmark of type 1 diabetes. This targeted cell death results in the loss of insulin production and the development of hyperglycemia [88]. Abnormal apoptosis in keratinocytes and immune cells contributes to the characteristic skin lesions of psoriasis, with hyperproliferation of keratinocytes and persistent inflammation. Beyond localized tissue effects, apoptosis dysregulation in autoimmune diseases can have systemic consequences [88]. In diseases like SLE, the failure to clear apoptotic debris leads to the generation of autoantibodies against nuclear antigens, exacerbating systemic inflammation. Chronic inflammation driven by apoptosis defects can result in widespread organ damage, as seen in lupus nephritis or autoimmune hepatitis. The microenvironment in autoimmune diseases influences apoptotic processes. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) can modulate apoptotic pathways, either promoting or inhibiting cell death in specific contexts. Interactions between dendritic cells, macrophages, and lymphocytes can either exacerbate or mitigate apoptosis-related dysregulation, depending on the signaling milieu [89].

9.5 Apoptosis and chronic inflammatory diseases

Chronic inflammatory diseases, such as chronic obstructive pulmonary disease (COPD) and Crohn's disease, are characterized by impaired apoptotic clearance, leading to persistent inflammation and tissue damage. The failure of macrophages to effectively clear apoptotic cells prolong inflammatory responses and contributes to the accumulation of necrotic debris, exacerbating tissue injury. Chronic inflammation can drive fibrotic changes in tissues, as seen in COPD, where prolonged immune cell activity damages alveolar structures, impairing lung function. Therapies aimed at enhancing efferocytosis—the phagocytic clearance of apoptotic cells—represent a promising avenue for reducing inflammation and tissue remodeling in these conditions [90]. One of the hallmark features of COPD is emphysema, characterized by the destruction of alveolar walls and loss of elastic recoil in the lungs. Apoptosis significantly contributes to this process. Increased apoptosis of alveolar epithelial cells and endothelial cells leads to the breakdown of alveolar walls. This disrupts the architecture of the gas exchange surface and contributes to airflow limitation. In COPD, excessive apoptosis is not adequately counterbalanced by tissue repair mechanisms, resulting in progressive loss of lung parenchyma. COPD is characterized by and persistent inflammation, which interacts with apoptosis to exacerbate tissue damage. Apoptosis is critical for regulating the lifespan of inflammatory cells such as neutrophils, macrophages, and T lymphocytes. In COPD, impaired clearance of apoptotic immune cells (efferocytosis) leads to secondary necrosis and the release of pro-inflammatory mediators, perpetuating inflammation. Chronic exposure to inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) promotes apoptosis in structural lung cells, contributing to tissue damage [91].

Small airway remodeling, another key feature of COPD, involves structural changes in the bronchioles, leading to airflow obstruction. Apoptosis contributes to these changes in several ways. Increased apoptosis of airway epithelial cells weakens the protective barrier, making the airways more susceptible to damage and inflammation. Dysregulated apoptosis of fibroblasts and myofibroblasts can lead to abnormal extracellular matrix deposition and airway wall thickening, contributing to remodeling and obstruction.

COPD is strongly associated with oxidative stress due to chronic exposure to cigarette smoke and environmental pollutants. Oxidative stress amplifies apoptosis through various pathways, exacerbating tissue damage. Oxidative stress increases the susceptibility of alveolar epithelial and endothelial cells to apoptosis, accelerating lung tissue destruction. Oxidative stress-induced mitochondrial damage in lung cells promotes apoptotic cell death, contributing to both emphysema and airway remodeling [92].

Cellular senescence is a prominent feature in COPD, where senescent cells accumulate in the lungs due to impaired apoptosis. Senescent cells secrete pro-inflammatory mediators, including interleukins and matrix metalloproteinases, which contribute to chronic inflammation and tissue degradation. The persistence of senescent cells in the lung microenvironment hinders regenerative processes, exacerbating the structural and functional decline in COPD [93].

The interplay between apoptosis and the lung microenvironment significantly influences COPD pathology. Inefficient clearance of apoptotic cells by macrophages in COPD leads to secondary necrosis, further amplifying inflammation and tissue injury. Apoptosis of lung structural cells contributes to ECM degradation and loss of lung elasticity, hallmarks of COPD progression. Dysregulated apoptosis alters the behavior of immune cells in the lung microenvironment, perpetuating a cycle of inflammation and cell death. COPD is a systemic disease, and apoptosis dysregulation in the lungs can have widespread effects. Apoptosis in skeletal muscle cells contributes to muscle weakness and reduced exercise capacity in COPD patients. Apoptosis of endothelial cells in systemic circulation may contribute to the increased risk of cardiovascular disease in COPD. Apoptosis plays a multifaceted role in the pathology of COPD, influencing alveolar destruction, chronic inflammation, airway remodeling, and systemic manifestations. The dysregulation of apoptotic processes is central to disease progression, highlighting its critical role in shaping the structural and functional decline observed in COPD [94].

Pathogens often exploit apoptotic pathways to enhance their survival and replication within the host. Viruses such as HIV and Epstein-Barr virus inhibit host cell apoptosis by upregulating anti-apoptotic proteins or directly interfering with caspase activation, prolonging the survival of infected cells. Conversely, pathogens like influenza virus and certain bacterial toxins actively induce apoptosis to disseminate infection and evade immune responses. For example, Shiga toxin-producing bacteria trigger endothelial cell apoptosis, contributing to vascular damage. Understanding pathogen-induced modulation of apoptosis has led to novel therapeutic strategies aimed at restoring host cell apoptotic pathways or blocking pathogen-driven apoptotic triggers [95].

9.6 Apoptosis and cardiovascular diseases

Dysregulated apoptosis is implicated not only in neurodegenerative diseases but also in disorders affecting the cardiovascular and immune systems, where both excessive and insufficient apoptosis can exacerbate disease progression.

In myocardial infarction, ischemia caused by blocked coronary arteries compromises oxygen supply to the heart, leading to mitochondrial dysfunction and increased production of reactive oxygen species (ROS). These events activate the intrinsic apoptotic pathway, resulting in cardiomyocyte apoptosis. The loss of heart muscle cells contributes to tissue damage and scar formation, impairing the heart's ability to contract and pump blood effectively. Therapeutic strategies to mitigate this damage focus on inhibiting apoptosis in cardiomyocytes through interventions targeting Bcl-2 family proteins, caspase activity, or oxidative stress.

In summary, dysregulation of apoptosis—whether through suppression or overactivation—is central to the pathogenesis of many diseases. While insufficient apoptosis drives cancer progression, excessive apoptosis underpins neurodegeneration and chronic inflammation. Therapeutic modulation of apoptotic pathways holds significant potential for addressing these pathological states, offering hope for improved outcomes in conditions ranging from malignancies to autoimmune and infectious diseases. As research continues, the ability to fine-tune apoptotic responses presents an opportunity to develop more targeted and effective treatments for a wide array of disorders, paving the way for advances in precision medicine and personalized therapeutic approaches.

10. Therapeutic targeting of apoptosis in disease

Small molecules and gene therapy approaches targeting apoptosis pathways offer promising strategies for treating cancer and diseases involving apoptosis dysregulation. The Bcl-2 family of proteins plays a critical role in regulating the intrinsic apoptosis pathway by balancing pro-apoptotic and anti-apoptotic signals at the mitochondria. Targeting these proteins with small molecules, such as BH3 mimetics, has shown therapeutic potential. BH3 mimetics mimic the function of pro-apoptotic BH3-only proteins like Bim, Bid, Puma, and Bad, which neutralize anti-apoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1. This interaction activates pro-apoptotic proteins like Bax and Bak, leading to mitochondrial outer membrane permeabilization (MOMP) and apoptosis. Venetoclax, a BH3 mimetic specifically targeting Bcl-2, has demonstrated efficacy in hematologic malignancies like chronic lymphocytic leukemia. Similarly, molecules like Navitoclax, which target multiple anti-apoptotic Bcl-2 family members, show promise in restoring apoptosis in cancer cells that evade death through overexpression of these proteins [96].

Caspases, central executioners of apoptosis, represent another therapeutic target. Activating caspases can promote apoptosis in cancer cells, while inhibiting caspases can prevent excessive apoptosis in conditions like neurodegenerative diseases. Caspase activators stimulate initiator caspases such as caspase-8 and caspase-9 or executioner caspases like caspase-3 and caspase-7, either directly or through upstream signaling pathways. For instance, Smac mimetics activate caspases by inhibiting inhibitor of apoptosis proteins (IAPs) that block caspase activity. Conversely, caspase inhibitors aim to protect neurons from apoptosis in diseases such as Alzheimer's and Parkinson's, though careful design is essential to avoid promoting cancer cell survival in other contexts [97].

Understanding the mechanisms of apoptosis provides insights into potential treatments for diseases where apoptosis is dysregulated. Cancer therapies aim to restore apoptotic pathways to kill cancer cells [98]. Drugs that inhibit anti-apoptotic proteins or activate pro-apoptotic signaling are designed to selectively induce

apoptosis in tumor cells, minimizing harm to healthy cells. In neurodegenerative conditions, inhibitors of apoptotic pathways, like caspase inhibitors, are explored to slow neuronal loss and potentially preserve cognitive and motor function in diseases like Alzheimer's and Parkinson's and for autoimmune diseases therapies focus on reducing autoreactive immune cells by promoting their apoptosis, helping to alleviate autoimmune symptoms by preventing attacks on the body's own tissues [99]. During chronic inflammatory conditions, regulating apoptosis in immune cells can resolve inflammation and could prevent tissue damage, offering therapeutic potential in conditions like chronic obstructive pulmonary disease (COPD) [100]. Gene therapy offers precise control over apoptotic pathways by delivering genes that either promote or inhibit apoptosis. In cancer therapy, reactivating or restoring mutant p53 function through gene delivery can re-enable apoptosis in tumor cells. Viral vectors, such as adenoviruses, have been engineered to deliver genes like TRAIL that activate the extrinsic apoptotic pathway, inducing tumor cell death. Gene therapy approaches can also introduce pro-apoptotic genes, such as Bax, Puma, or Bak, to promote apoptosis selectively in cancer cells. For example, adenovirus-mediated gene transfer of cytochrome c can activate caspases and enhance tumor cell apoptosis. In immunotherapy, genetically modified T cells expressing pro-apoptotic proteins represent another innovative approach to inducing apoptosis in cancer cells. These strategies collectively highlight the potential of targeting apoptosis pathways to treat a wide range of diseases [101].

11. Challenges and future directions in apoptosis-based therapies

Dysregulation of apoptosis contributes to a range of pathological conditions. In cancer, resistance to apoptosis enables tumor cells to evade immune surveillance and persist in the body. Conversely, excessive apoptosis in neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's, leads to neuronal loss, contributing to cognitive and motor impairments. In autoimmune diseases, inappropriate apoptosis regulation exacerbates immune responses, resulting in tissue damage [102]. Targeting apoptotic pathways for therapeutic intervention has emerged as a promising approach for treating these disorders. Strategies include the use of BH3 mimetics, caspase activators, gene therapies, and small molecules that modulate apoptotic regulators like the Bcl-2 family and death receptors. BH3 mimetics, for instance, mimic pro-apoptotic BH3-only proteins to neutralize anti-apoptotic Bcl-2 family members, promoting mitochondrial outer membrane permeabilization (MOMP) and subsequent apoptosis in cancer cells. While progress has been made, significant challenges persist in apoptosis-based therapies. Ensuring selective targeting of diseased cells, such as cancer cells, while sparing healthy tissue remains a critical hurdle. Resistance to apoptosis-targeting therapies is common in cancer cells, which can upregulate anti-apoptotic proteins or acquire mutations in pro-apoptotic genes. Overcoming this resistance is essential for improving therapeutic efficacy. Additionally, many apoptosis-inducing therapies have off-target effects that can lead to toxicity in healthy tissues. The challenge in neurodegenerative diseases lies in preventing excessive apoptosis in neurons without inadvertently promoting cancer cell survival [103].

Advancements in combination therapies, where apoptosis-inducing drugs are paired with chemotherapy, radiotherapy, or immunotherapy, offer a promising solution to overcome resistance and improve treatment outcomes. Personalized medicine is another key area of development, enabling tailored interventions based on the

genetic and molecular profiles of patients, particularly in cancers with mutations in apoptotic regulators like p53. Nanotechnology and novel drug delivery systems, including nanoparticles and liposomes, are also being explored to enhance the precision and bioavailability of apoptosis-targeting agents, minimizing off-target effects and improving therapeutic outcomes [104].

Future research in apoptosis-based therapies is poised to uncover novel apoptotic pathways, such as autophagic cell death and ferroptosis, which could offer new therapeutic targets. Furthermore, more advanced targeting of specific apoptotic molecules, like Mcl-1 or Bax, through protein-protein interactions, may lead to more selective and effective treatments. The integration of apoptosis modulation with other therapeutic approaches holds the potential for synergistic effects, particularly in treating cancers and chronic degenerative diseases. As advancements in genomic and proteomic technologies continue, personalized approaches to apoptosis-based therapies are expected to refine treatment strategies, enhancing their specificity and efficacy.

In conclusion, while significant challenges remain, apoptosis-based therapies hold immense potential for treating a wide array of diseases, from cancer to neurodegenerative and autoimmune disorders. Future developments in small molecules, gene therapy, combination treatments, and targeted delivery systems promise to overcome current limitations, offering new hope for patients. As our understanding of apoptosis deepens, these therapies are set to become a cornerstone of precision medicine, providing more effective and less toxic treatments for a range of conditions.

12. Detection and measurement of apoptosis

Identifying apoptotic cells and quantifying apoptosis are critical for understanding cell death processes in both physiological and pathological contexts. Various methods are used to detect apoptosis, each targeting specific biochemical or morphological hallmarks, such as DNA fragmentation, phosphatidylserine exposure, and nuclear changes. Below is an overview of some of the most widely used techniques in apoptosis detection: TUNEL assay, Annexin V staining, and DAPI staining.

12.1 TUNEL assay (terminal deoxynucleotidyl transferase dUTP Nick end labelling)

The TUNEL assay is a widely used technique for detecting DNA fragmentation, a key biochemical hallmark of apoptosis. The TUNEL assay detects DNA strand breaks by labelling the free 3'-OH ends of fragmented DNA. During apoptosis, CAD (caspase-activated DNase) cleaves DNA at internucleosomal regions, creating numerous 3'-OH termini. The enzyme terminal deoxynucleotidyl transferase (TdT) adds modified nucleotides, typically conjugated with a fluorophore or a chromogen, to these ends, allowing visualization of apoptotic cells under a microscope. Cells or tissue sections are fixed and incubated with TdT and labeled nucleotides. After labelling, apoptotic cells can be detected by fluorescence microscopy or flow cytometry. The presence of fluorescent or colored cells indicates DNA fragmentation typical of apoptosis. The TUNEL assay is sensitive and allows for the localization of apoptosis within tissue architecture, making it valuable for histological studies. However, it can also detect DNA damage unrelated to apoptosis, such as that caused by necrosis or autophagy, so results should be corroborated with additional assays [105].

12.2 Annexin V staining

Annexin V staining is one of the most specific and commonly used methods for identifying early apoptotic cells by detecting phosphatidylserine (PS) exposure on the cell membrane. During apoptosis, PS translocates from the inner to the outer leaflet of the plasma membrane, exposing it to the extracellular environment. Annexin V is a protein that binds specifically to PS in the presence of calcium, allowing for the identification of apoptotic cells. Annexin V is typically conjugated to a fluorescent dye (e.g., FITC or Alexa Fluor) for easy detection. Cells are incubated with labeled Annexin V and a viability dye, such as propidium iodide (PI), which is excluded by intact cell membranes. Cells that are Annexin V-positive and PI-negative are considered early apoptotic, while cells positive for both Annexin V and PI indicate late apoptosis or necrosis, as membrane integrity has been lost. Annexin V staining is highly specific for apoptosis and can be performed on live cells, allowing real-time analysis by flowcytometry or fluorescence microscopy shown in **Figure 7**. However, PS exposure is reversible in some contexts, and caution is needed to avoid false positives from cells undergoing reversible damage [106].

12.3 DAPI staining (4′,6-diamidino-2-phenylindole)

DAPI staining is commonly used to detect nuclear changes associated with apoptosis, such as chromatin condensation and nuclear fragmentation. DAPI is a fluorescent stain that binds specifically to A-T rich regions of double-stranded DNA, emitting blue fluorescence when bound. During apoptosis, nuclear chromatin condenses and becomes more densely packed, which intensifies DAPI staining. DAPI can also detect nuclear fragmentation, as fragmented apoptotic nuclei appear as multiple DAPI-stained bodies within a cell. Fixed cells or tissue sections are incubated with DAPI solution, followed by washing to remove excess stain. The stained cells are observed under a fluorescence microscope. Condensed or fragmented nuclei, indicative of apoptosis, appear brighter than normal nuclei. DAPI staining is quick, inexpensive, and effective for identifying apoptotic nuclei [58]. However, it is not specific to apoptosis; other types of cell death can also show chromatin changes, so DAPI is often used in combination with other assays (e.g., TUNEL or Annexin V staining) for accurate apoptosis detection. The accurate detection of apoptosis relies on recognizing its specific biochemical hallmarks. The TUNEL assay, Annexin V staining, and DAPI staining each provide unique insights into different aspects of apoptosis—DNA fragmentation, phosphatidylserine exposure, and nuclear morphology, respectively. When used in combination, these assays allow researchers to robustly and accurately identify apoptotic cells, distinguish apoptosis from other forms of cell death, and gain insights into cellular responses in both health and disease contexts [107].

13. Conclusion

Apoptosis presents transformative potential for therapeutic advancements. In cancer, therapies are being designed to restore apoptotic processes that tumors evade. Agents such as BH3 mimetics mimic the function of pro-apoptotic proteins, directly targeting cancer cells with overexpressed anti-apoptotic factors like Bcl-2. By reactivating apoptotic pathways, these therapies promote the selective destruction

of malignant cells while sparing healthy ones. Another promising approach involves the use of death receptor agonists, which activate the extrinsic apoptotic pathway, inducing cell death in tumors. These treatments are particularly effective in overcoming resistance to conventional therapies, such as chemotherapy and radiation, which often fail due to the cancer cells' ability to suppress apoptosis. In neurodegenerative diseases, apoptosis-modulating strategies focus on inhibiting excessive neuronal death. The gradual loss of neurons in conditions like Alzheimer's or Parkinson's is a direct result of oxidative stress, mitochondrial dysfunction, and protein aggregation, all of which activate apoptotic cascades. Therapeutic interventions aim to block key apoptotic players, such as caspases, or stabilize mitochondrial integrity to preserve neuronal populations. Although these approaches are still in experimental stages, they hold the potential to slow disease progression and maintain cognitive and motor functions for longer periods. Autoimmune disorders also benefit from apoptosis-centered therapies. Dysregulated apoptosis in immune cells leads to chronic inflammation and tissue destruction, as seen in diseases like systemic lupus erythematosus. Therapies are being developed to restore normal apoptotic processes, ensuring the elimination of autoreactive immune cells while preventing the accumulation of apoptotic debris that exacerbates autoimmune responses. Similar principles are applied in chronic inflammatory diseases, where regulating apoptosis in immune cells can resolve inflammation and prevent further tissue damage.

Despite these advances, challenges persist. Achieving therapeutic specificity is a significant hurdle. Therapies must selectively target diseased cells without inducing unwanted apoptosis in healthy tissues, which could lead to side effects such as immunosuppression or damage to normal tissues. Resistance to apoptosis-based therapies, particularly in cancer, is another obstacle. Tumors often adapt by mutating key apoptotic regulators or upregulating survival pathways, necessitating combination therapies that simultaneously target multiple apoptotic and survival mechanisms.

Future directions in apoptosis research promise to address these challenges. Advances in genomic and proteomic technologies pave the way for personalized medicine, allowing treatments to be tailored to the specific molecular profile of a patient's disease. For example, understanding mutations in apoptotic regulators like p53 or Bcl-2 can guide the use of targeted therapies in cancers with such alterations. Nanotechnology offers solutions for precise drug delivery, using nanoparticles to transport apoptosis-inducing agents directly to diseased tissues, minimizing off-target effects.

Moreover, the integration of apoptosis modulation with other therapeutic modalities, such as immunotherapy, is an exciting frontier. By combining apoptosis-based treatments with approaches that enhance the immune system's ability to recognize and attack diseased cells, it may be possible to achieve synergistic effects that improve therapeutic outcomes. In summary, apoptosis is more than a mechanism of cell death; it is a fundamental process that defines health and disease. Understanding its pathways and players has revolutionized our approach to treating diseases characterized by its dysregulation. From targeting cancer cells that evade death to protecting neurons from excessive apoptosis, therapeutic strategies are evolving to harness the power of apoptosis. As research continues to unravel its complexities, the potential for breakthroughs in treating some of the most challenging diseases of our time becomes ever more tangible. The future of apoptosis-based therapies lies in their precision, adaptability, and integration with cutting-edge medical advancements, offering renewed hope for improved patient outcomes and quality of life.

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Chapter 5

The Role of HSP70 in Regulation of Breast Cancer Stem Cells and Apoptotic Pathway

Gul Ozcan and Hasan Korkaya

Abstract

HSP70 is a molecular chaperone that plays a critical role in normal physiology of the cell and highly activated under pathological conditions such as cancer. It has been well established that HSP70 is implicated in breast cancer development and progression. Highly activated HSP70 has been linked to processes, such as cell proliferation, metastasis, drug resistance, and driving anti-apoptotic pathways. In the Luminal A subtype, HSP70 stabilizes the ESR1 (estrogen receptor 1) and PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha) pathways, supporting cell survival, while in the Luminal B subtype, its interaction with Cyclin D1 and TP53 contributes to treatment resistance. In the HER2 (+) subtype, HSP70 triggers aggressive tumor growth by increasing human epidermal growth factor receptor 2 (HER2) signaling via stabilizing the protein. In triple-negative breast cancer (TNBC), it supports stem cell-like properties by interacting with pathways, such as neurogenic locus notch homolog protein 1 (NOTCH1) and nuclear factor-kappa B (NF-κB) and suppressing anti-apoptotic pathways. The effect of HSP70 on cancer stem cells (CSCs) plays an important role in limiting therapeutic response as well as tumor initiating potential and metastasis. In turn, it inhibits apoptosis, preventing cell death through B-cell lymphoma 2 (BCL-2) stabilization and suppression of caspase activity. This review aims to provide an integrative view of breast cancer biology by addressing the functions of HSP70 in cancer subtypes, interactions with cancer stem cells and apoptosis.

Keywords: heat shock proteins, HSP70, cancer stem cells, apoptosis, breast cancer, HSP70 inhibitors, molecular subtype

1. Introduction

Breast cancer is the most prevalent cancer in women worldwide and encompasses multiple molecular subtypes. Hormonal factors, genetic predisposition, and environmental influences contribute to the development of breast cancer. According to recent data, Breast cancer is the most commonly diagnosed cancer type worldwide, and its burden has been increasing in recent years. Breast cancer, which has replaced lung

cancer as the most commonly diagnosed cancer worldwide, accounts for about 30% of all female cancers, reaching a total of 2.3 million new cases in both genders. In the United States, breast cancer cases have increased every year between 2012 and 2021. This increase is especially noticeable among women under the age of 50. However, according to two separate reports by the American Cancer Society, breast cancer-related death rates have decreased by approximately 10% in the last decade. It is also emphasized that black women have lower survival rates for all types of breast cancers, except localized disease. It has been stated that the increase in cases is largely "limited to localized stage and hormone receptor-positive diseases." The annual increase of 1.4% in women under the age of 50 was 0.7% in women aged 50 and over [1, 2].

HSP70 is a highly conserved molecular chaperone that plays an important role in maintaining cellular proteostasis under both physiological and stress conditions. Its primary functions include ensuring the folding, stabilization, and refolding of newly synthesized and misfolded proteins. In addition to these functions, HSP70 also plays a role in preventing protein aggregation and transporting proteins between cellular compartments. This versatile activity of HSP70 suggests that it is an important regulator of cellular homeostasis and survival, especially in cancer disease. In this context, HSP70 interacts with a wide range of client proteins, including many oncogenes or proteins involved in cancer progression. HSP70 increases the stability of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) receptors and prevents their destruction. This interaction is very important in HER2 (+) breast cancer, as increased HER2 signaling triggers aggressive tumor behavior. HSP70 supports the survival of cancer cells by protecting mutant forms of the tumor suppressor p53 from destruction and thereby increases apoptosis resistance. In addition, it prevents apoptosis by stabilizing anti-apoptotic proteins, such as BCL-2 and B-cell lymphoma-extra large (BCL-XL), thus not only promoting tumor progression but also supporting the formation of resistance to therapeutic treatment. In addition, HSP70 contributes to two fundamental features of cancer cells, namely sustainable cell proliferation and metabolic reprogramming, by stabilizing the Myc oncogene. Thus, it has been shown that the molecular chaperone role of HSP70 is closely related to the regulation of oncogenic pathways and stress responses. In summary, HSP70 stands out as a potential targeted therapeutic agent in cancers where HSP70 is overexpressed or dependent on this chaperone [3, 4].

Apoptotic pathways within breast cancer cells play a critical role in developing therapeutic strategies against tumors [5–7]. Apoptosis, also known as programmed cell death, is crucial for normal cellular development and tissue homeostasis. Increasing cell death through apoptosis in breast cancer has become a treatment target, making the identification of tumor-specific apoptotic signaling pathways essential for developing more specific therapeutic approaches. Furthermore, cancer stem cells (CSCs) contribute to tumor heterogeneity and therapeutic resistance, showing resistance to conventional chemotherapy and often leading to tumor recurrence [4]. Breast cancer is one of the most common cancer types in women with its heterogeneous structure and different biological subtypes. The molecular differences of these subtypes determine the clinical course of the disease, treatment response, and prognosis. HSP70 as a chaperone protein that plays an important role in the stress response is of central importance in maintaining cellular homeostasis and developing treatment resistance in breast cancer. In addition, its effects on processes, such as CSCs and apoptosis mechanisms, play a key role in regulating tumor formation, metastasis, and response to therapy [4, 8]. This review aims to evaluate the functions of HSP70 in different breast cancer subtypes, its relationships with cancer stem cells,

and apoptosis in detail and to evaluate the potential of targeting this molecule in developing new therapeutic approaches.

2. Breast cancer subtypes and HSP70

Types of breast cancers are classified into various subtypes, determined by cell type, growth rate, spread pattern, and prognosis. The most common breast cancer subtypes include invasive ductal carcinoma, invasive lobular carcinoma, ductal carcinoma in situ, and lobular carcinoma in situ. Treatment options vary depending on cancer subtype, growth rate, spread status, and the patient's health, and they may include surgery, radiation, chemotherapy, hormone therapy, and targeted therapies [9–11].

Breast cancer subtypes are categorized based on the unique characteristics of cells within the tumor [9, 12–14]. Breast cancer is commonly characterized by five primary molecular subtypes, often referred to as PAM50 (Prediction Analysis of Microarray 50) subtypes (**Table 1**).

Due to variations in gene expression and protein levels among these subtypes, the diagnosis and treatment of each breast cancer subtype also differ. Recently, with the advancement of new technologies like single-cell genomics, it has become possible to conduct more detailed molecular classifications of breast cancer. Single-cell genomics is a technology used to examine the genomic structures of individual cells [15–17]. This approach enables a deeper understanding of genomic diversity and heterogeneity within cancer cells, allowing for the identification of more precise therapeutic targets in cancer treatment [18, 19]. These techniques provide detailed insights into the gene expression profiles of tumor cells, facilitating the identification of more specific subtypes. With single-cell genomics, the genetic characteristics of each cell can be analyzed individually, enabling the identification of differences among distinct cell populations. This advancement aids in a more comprehensive understanding of breast cancer and the development of improved treatment options [9, 13, 20–23].

HSP70, a member of the heat shock protein (HSP) family, prevents protein misfolding and aggregation under cellular stress conditions. HSP70 plays a critical role in maintaining cellular homeostasis. Cancer cells overproduce HSP70, which enables them to survive under stress conditions. HSP70 is a significant factor in the growth and metastasis of cancer cells. HSP70 belongs to a family of proteins known as heat shock proteins and is expressed in response to cellular stress conditions.

Cancer subtypes	Clinical feature
Luminal A	Estrogen Receptor (ER) and/or Progesterone Receptor (PR) positive, HER2 negative, with low Antigen Kiel 67 (Ki-67) levels.
Luminal B	ER and/or PR positive, HER2 negative or positive, with high Ki-67 levels
HER2-enriched	ER and PR negative, HER2 positive.
Basal-like	ER, PR, and HER2 negative*
Claudin-low	ER, PR, and HER2 negative*

**With basal cell markers (cytokeratin 5/6 (CK5/6), EGFR) positive, showing immune response and epithelial-mesenchymal transition (EMT) features in the expression profile.*

Table 1.
Different breast cancer subtypes and their clinical features.

Increased expression of HSP70 in breast cancer cells has been observed, suggesting potential links between this protein and breast cancer subtypes. Breast cancer is divided into various subtypes that differ in molecular profile and clinical characteristics (**Figure 1**).

Investigating the expression of HSP70 across these subtypes and its relationship to cancer progression, invasion potential, and treatment response is essential [24, 25]. The most lethal aspect of breast cancer is its ability to spread to other organs through metastasis. The expression of HSP70 in metastatic breast cancer cells and its role in the metastasis process have yet to be fully elucidated. Particularly, HSP70's influence on metastasis initiation, apoptosis, cell migration, invasion, and changes in the tumor microenvironment is significant [26–29]. Resistance to treatments, such as chemotherapy and hormone therapy, is common in breast cancer, and HSP70 expression may be related to these resistance mechanisms. Understanding HSP70's role in the development of resistance to chemotherapy and hormone therapy could reveal its potential significance in breast cancer treatment [8, 30]. In recent years, immunotherapy has shown promising results in breast cancer treatment. The relationship between HSP70 expression in breast cancer cells and immunotherapy response is especially relevant, as HSP70 might affect mechanisms that modulate immunotherapy efficacy, presenting potential advantages when used in combination with immunotherapy [31–33]. HSP70 can exhibit anti-apoptotic effects in the apoptosis process by inhibiting apoptotic signaling pathways, preventing cells from entering apoptosis. In addition to these anti-apoptotic effects, HSP70 can also play pro-apoptotic roles by regulating the stability and activity of certain pro-apoptotic proteins, contributing to the apoptosis process. Thus, HSP70 inhibition could induce apoptosis in cancer cells.

Figure 1.
The roles of HSP70, CSCs characteristics, and apoptosis in different breast cancer subtypes.

Many studies have shown that most compounds with anticancer effects show this effect by reducing HSP70 expression and/or HSP70 inhibition [34]. Cancer cells often express higher levels of HSP70 than normal cells, which may help protect them under stress conditions and evade apoptosis. Studies have proposed HSP70 and apoptosis as potential targets for cancer treatment [7, 35, 36]. However, the mechanisms through which HSP70 regulates apoptosis are not yet fully understood and remain an active area of research [37–42].

HSP70 has been shown to promote tumor cell proliferation and metastasis by regulating Wnt/β-catenin, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR), and NF-κB signaling pathways in breast cancer [8]. Interestingly, the expressions of HSP70 heat shock proteins are significantly elevated in aggressive basal and triple-negative breast cancer (TNBC) subtypes and genetic downregulation or pharmacological inhibition of the HSP70 resulted in reduced tumor growth and invasion through induction of apoptotic cell death [24, 43–45].

Furthermore, the heat shock protein family A (Hsp70) member 2 (HSPA2) expression was shown to be higher in Luminal A and B subtypes, while heat shock protein family A (Hsp70) member 5 (HSPA5) and heat shock protein family A (Hsp70) member 6 (HSPA6) expressions were more common in Basal TNBC subtypes. In addition, Zhang and Bi demonstrated that some HSP70 members, such as heat shock protein family A (Hsp70) member 1A (HSPA1A), HSPA5, and heat shock protein family A (Hsp70) member 8 (HSPA8), were significantly increased in breast cancer tissues [46]. Therefore, the varying degrees of HSP70 expressions in breast cancer subtypes may be implicated in aggressive properties of the associated diseases in patients. It may also suggest that HSP70 provides survival advantages under stress conditions and accelerates tumor growth by increasing cell proliferation and suppressing apoptotic pathways as well as increasing therapeutic resistance [24]. In contrast, in hormone-sensitive breast cancers (ER+/PR+), the anti-apoptotic effects of HSP70 are dominant, and it was shown to promote resistance to hormone therapy by increasing tumor cell survival [3, 17].

The heat shock protein 70-2 (HSP70-2) expression was also shown to be highly elevated in breast cancer tumor samples, gradually increasing from ductal situ in carcinoma (DCIS) to invasive ductal carcinoma (IDC) with further increase in higher grades [43]. In this study, silencing of HSP70-2 resulted in a significant decrease in cell growth, motility, and invasiveness due to reversal of epithelial-mesenchymal transition (EMT), while apoptotic cell death is increased. These findings suggest that HSP70-2 may play a critical role in the progression of breast cancer and can be evaluated as a potential therapeutic target [43].

Yamaguchi-Tanaka et al. showed that chemotherapy increases the release of exogenous HSP70 from breast cancer cells, which leads to pro-tumorigenic effects via tumor-associated macrophages (TAMs). It was found that HSP70 release was increased in breast cancer cells treated with epirubicin (EPI), and this has activated TAMs and promoted tumor progression. There was a significant reduction of CD163 + TAMs and reduced expression of transforming growth factor beta (TGF-β) when treated with conditioned medium from HSP70 knockdown MDA-MB231 and MDA-MB453 cells. This study revealed that HSP70 may play a role in the generation of tumor microenvironment (TME) after chemotherapy and can be considered as a factor that promotes tumor growth [47].

Alternative treatment approaches also utilized HSP70 as a promising target for the treatment of breast cancer. Interestingly, onion-derived phytocompounds (e.g., quercetin, cyanidin-3-glucoside, and diosgenin) exhibit strong binding with HSP70

and have better binding scores compared to conventional agents such as Tamoxifen [34]. These findings suggest that herbal compounds can be evaluated as potential agents in the treatment of breast cancer through HSP70 inhibition.

In addition to being a therapeutic target, HSP70 has been utilized as an important biomarker in determining the functional severity of breast cancer type 1 susceptibility protein (BRCA1) mutations. Gracia et al. recently demonstrated that HSP70 preferentially (94%) binds to pathogenic variants of BRCA1, which is the C-terminal region of BRCA1 (BRCT) resulting in the loss of function of breast cancer susceptibility (BRCA) protein [48]. This study also emphasized that hypomorphic variants of BRCA1 maintain partial folding and function by binding moderately to HSP70 but may still predispose to cancer [48].

These findings suggest that HSP70 plays a critical role in the progression of breast cancer and has a significant effect, especially on tumors associated with BRCA1/BRCA2 (breast cancer type 2 susceptibility protein) mutations. Mutated BRCA1 and BRCA2 disrupt DNA repair mechanisms and increase cellular stress response. HSP70 may contribute to the survival of affected cells with accelerating tumor development by playing a protective role against such stress responses. Therefore, the above-mentioned results indicate that HSP70 is not only an indicator of folding disorders but can also function as a prognostic biomarker to determine the risk associated with BRCA1 mutations [48].

In conclusion, HSP70 plays a critical role in the pathogenesis of breast cancer and its effects are multifaceted. HSP70 facilitates cancer progression by increasing the survival of malignant cells and providing protection from chemotherapies as well as evading the immune system. Its association with BRCA1/2, regulation of apoptosis, and contribution to therapy resistance make it an important therapeutic target. Future studies on breast cancer should focus on determining subtype-specific sensitivities to HSP70 inhibition and developing combination therapies.

3. HSP70, cancer stem cells, and apoptosis

Cancer stem cells represent a unique and highly resilient subset of cells within tumors that are believed to drive cancer initiation, progression, and recurrence. CSCs were first identified in acute myeloid leukemia (AML) by Canadian scientist John Dick et al. in 1997, and they have since been found in various cancer types, including breast, colon, and brain cancers [49]. These cells share key characteristics with those of normal stem cells, especially the ability to self-renew and differentiate, allowing them to initiate and sustain tumor growth. This self-renewing capacity, coupled with their resistance to apoptosis, positions CSCs as central players in cancer biology, as they enable tumors to persist and recur even after aggressive treatment. Significant progress in cancer research has revolutionized our understanding of cancer pathogenesis and facilitated the development of innovative therapies that have substantially improved patient outcomes. Despite these advancements, many patients still face challenges, such as treatment resistance, disease recurrence, and metastasis, ultimately leading to disease progression and mortality. Research suggests that a specific subset of cancer cells, known as cancer stem cells, exhibit stem cell-like characteristics, including self-renewal, differentiation, and enhanced proliferative capacity. These stemness traits, regulated by pathways, such as signal transducer and activator of transcription 3 (STAT3), NANOG, NOTCH, WNT, and HEDGEHOG, are often disrupted in CSCs due to genetic and epigenetic alterations. Preclinical studies

targeting these stemness pathways, in conjunction with conventional therapies, have shown promising outcomes. As a result, several anti-CSC therapies are currently being evaluated in clinical trials across different stages of development. HSP70 has been shown to inhibit the apoptotic pathways at different levels (**Figure 2**) [2, 50, 51].

The significance of CSCs in cancer therapy has grown due to their unique ability to evade apoptosis, thereby enhancing their survival, promoting metastasis, and contributing to tumor relapse. Unlike most tumor cells, CSCs are highly adept at withstanding the effects of conventional treatments like chemotherapy and radiotherapy, as they can effectively suppress apoptotic pathways. For example, in cancers such as breast cancer and glioblastoma, CSCs have been shown to drive tumor recurrence and distant metastasis [51, 52]. A key molecule implicated in the survival of CSCs is HSP70, a chaperone protein involved in cellular defense mechanisms under stress. HSP70 is highly expressed in many cancer types and exerts a strong anti-apoptotic effect, particularly in CSC populations [51, 52]. This anti-apoptotic property of HSP70 allows CSCs to avoid treatment-induced cell death and maintain their stem cell-like qualities, even under intense therapeutic stress. By preventing apoptosis, Hsp70 enables CSCs to resist traditional therapies and contributes to treatment resistance [52]. The interplay between CSCs, HSP70, and apoptosis suppression has spurred interest in developing targeted therapies that disrupt these protective mechanisms. Research increasingly focuses on understanding the molecular pathways through which Hsp70 promotes CSC survival and apoptosis evasion [3]. By elucidating Hsp70's role within the tumor microenvironment and its influence on CSC biology, researchers aim to identify new therapeutic strategies that weaken CSC defenses, improve treatment efficacy, and reduce cancer recurrence. Targeting the interactions between CSCs, HSP70, and apoptotic pathways could represent a promising approach in developing more effective cancer therapies [53, 54]. Cancer stem cells are cells with the capacity for self-renewal and differentiation, closely linked to the initiation, progression, and recurrence of tumors (**Figure 2**). These cells can resist conventional therapies, potentially leading to tumor

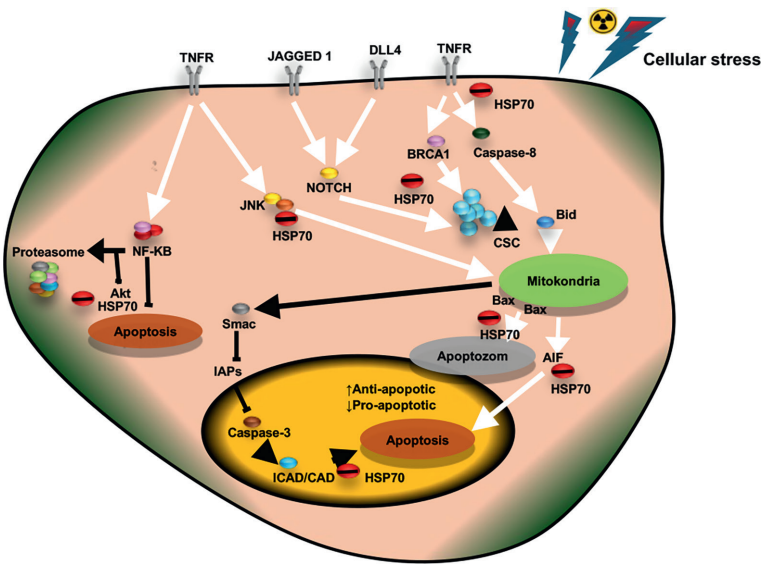

Figure 2.
The role of HSP70 in tumor initiation and interacting genes in cellular stress.

recurrence. CSCs are preserved within specialized niches in the tumor microenvironment and may be the main source of relapse after treatment. Proteins such as HSP70 play a role in maintaining and enabling resistance mechanisms in CSCs, and inhibiting these proteins could lead to more effective therapeutic strategies targeting CSCs [55].

Apoptosis, or programmed cell death, is critical in maintaining cellular homeostasis [55, 56]. Cancer cells survive by suppressing apoptotic mechanisms, and CSCs may have even more resistance to apoptosis. Proteins like HSP70 can interact with apoptosis-regulating proteins, preventing cell death. Inhibiting these proteins could reactivate apoptosis, potentially killing cancer cells. The inhibition of HSP70 offers promising strategies for effective therapies against CSCs. Future research aims to improve understanding and targeting of these proteins. Clinical trials are testing treatments targeting HSP70, which may pave the way for breakthroughs in CSC therapy [4]. Although targeting these proteins presents challenges, developing inhibitors may play a significant role in overcoming treatment resistance. Future studies should focus on understanding the molecular mechanisms of HSP70 in greater depth to develop new therapeutic strategies. HSP70 supports the resistance mechanism of CSCs, suppresses apoptosis, and increases treatment resistance in different breast cancer subtypes (**Figure 3**). HSP70 inhibits caspase activation by BCL-2 and since this pathway is stronger in CSCs, treatment efficacy decreases. In addition, Hsp70 promotes proliferation by supporting HER2 signaling [56–59]. It can cause resistance to immunotherapy by making CSC phenotypes resistant, especially in the TNBC subtype. Hsp70 has also been shown to play a role in resistance to hormonal treatment in luminal subtypes [60, 61].

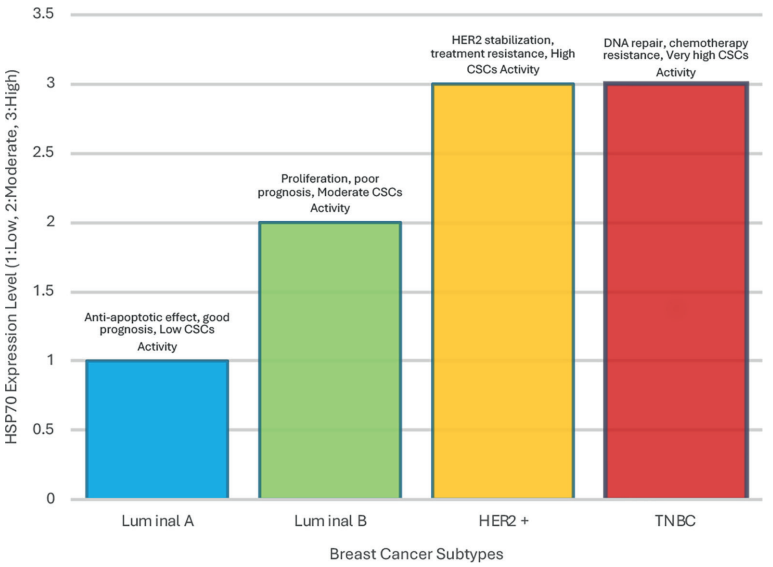

Figure 3.
The basic mechanisms of HSP70, apoptosis, and CSCs in breast cancer subtypes. Luminal A; HSP70 enhances estrogen receptor signaling by stabilizing ESR1. This contributes to cellular survival but is associated with a favorable prognosis due to the activation of apoptosis. Luminal B; HSP70 interacts with Cyclin D1 to promote proliferation and therapy resistance. Suppression of apoptosis further contributes to tumor aggressiveness. HER2(+); HSP70 helps amplify signaling through mitogen-activated protein kinase (MAPK) pathways by stabilizing the HER2 protein. This interaction drives aggressive tumor growth and resistance to targeted therapies. Triple-Negative Breast Cancer (TNBC); HSP70 protects TP53 mutants from degradation and interacts with the NF-κB pathway to promote inflammation and stem cell-like properties. This results in chemotherapy resistance and metastasis.

Research has explored the effects of HSP70 on apoptotic signaling pathways, cellular mechanisms, and the molecular regulation of apoptosis. One member of the HSP70 family, heat shock protein 90 (HSP90), is known to regulate intracellular signaling pathways in cancer cells, influencing cell growth, proliferation, and resistance to apoptosis [62, 63]. Beere et al. identified a mechanism by which HSP70 inhibits apoptosis by preventing the addition of procaspase-9 to the Apaf-1 (apoptotic protease-activating factor 1) apoptosome [64]. Additionally, Bagatell and Whitesell explored HSP90 inhibition as a therapeutic target, finding that it induced apoptosis in breast cancer and other cancer types, which suggests HSP90 inhibition may affect apoptosis through HSP70 interactions [65]. HSP70 has also been implicated in regulating immune cells' attack on cancer cells, contributing to the immune system's destruction of cancer cells. Calderwood and Neckers noted that HSP70 and HSP90 interaction plays a regulatory role in apoptosis within cancer cells and that HSP70 inhibition could increase apoptosis in breast cancer cells [62]. In a meta-analysis by Dimes et al., high HSP70 expression levels were associated with breast cancer progression, potentially affecting prognosis [66]. Furthermore, Du et al. reported that HSP70 inhibition enhances tumor cell sensitivity to radiotherapy by promoting apoptosis through interfering with mitochondrial integrity, blocking apoptotic complex formation, and impairing DNA damage repair mechanisms [67]. Anadon et al. tested a novel small molecule inhibitor, 2-phenylethynesulfonamide (PES), and found that targeting HSP70 with PES induced apoptosis in ovarian cancer cells, suggesting a potential treatment strategy [68]. Studies highlight that members of the HSP70 family promote cancer cell growth through various mechanisms and that HSP70 inhibitors might be promising therapeutic targets in breast cancer, with potential pro- and anti-apoptotic effects [69–72].

As mentioned above, Hsp70 is one of the molecules in the heat shock protein family, playing a critical role in cellular stress response while regulating correct protein folding and intracellular protein homeostasis. However, the relationship between apoptosis and HSP70 is not yet fully understood, and further research is needed. Detailed studies examining their molecular mechanisms and potential interactions will help us better understand the relationship between apoptosis and HSP70 and potentially aid in the development of new therapeutic strategies. HSP70 plays a dual role in CSCs and apoptosis, which are critical to breast cancer development and treatment resistance. HSP70 interacts with CSC-associated pathways, such as Wnt/β-catenin and NOTCH1, to promote stemness, self-renewal, and tumorigenic potential. These interactions contribute to tumor initiation, metastasis, and treatment resistance. HSP70 prevents apoptosis by stabilizing anti-apoptotic proteins, such as BCL2 and impairing caspase activation. In TP53 mutant cancers, HSP70 also promotes survival by preventing the degradation of dysfunctional p53 protein (**Figure 4**).

Today, the role of HSP70 in the apoptosis process is increasingly emphasized in in vitro studies using breast cancer cell line models, with a focus on their expression levels, localization, interactions, functions, and molecular mechanisms. The expression levels of HSP70 in breast cancer cells are evaluated concerning different treatment responses across various breast cancer subtypes. At the same time, intensive research is conducted on topics, such as HSP70 in breast cancer cells, modulation of intracellular signaling pathways, and apoptosis processes. Research on HSP70 in breast cancer cell line models today helps us understand their molecular mechanisms, potential interactions, and cellular functions in more detail. These studies provide valuable insights into breast cancer pathophysiology, treatment strategies, and the development of targeted therapies [3, 41, 66, 73]. Additionally, gene expression analyses conducted to examine the expression level changes of HSP70 in breast

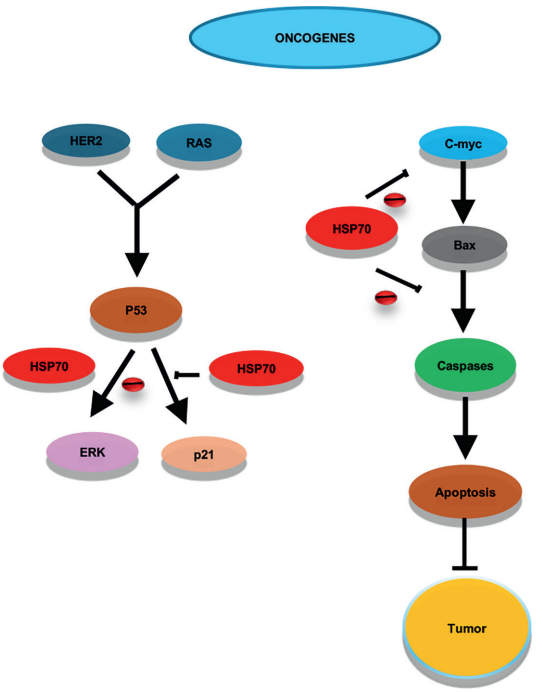

Figure 4.
The role of HSP70 in apoptosis and interacting oncogenes in tumor progression.

cancer cells shed light on how HSP70 gene expression varies in different breast cancer cell lines, treatment conditions, or clinical samples. A study by Mhaidat et al. focused on the protective effect of HSP70 against apoptosis induced by 5-fluorouracil (5-FU) in colorectal cancer cells. It was found that HSP70 reduces cell apoptosis induced by 5-FU by inhibiting the activity of Bcl-2-associated X protein (Bax), an apoptotic protein [74]. Another study investigated the effects of HSP70 inhibition on inducing apoptosis and inhibiting tumor growth in hepatocellular carcinoma [75]. They found that Hsp70 inhibition increases apoptosis and suppresses tumor growth in hepatocellular carcinoma cells, suggesting that HSP70 inhibitors could serve as a potential therapeutic strategy in cancer types like hepatocellular carcinoma. On the other hand, HSP70 is a chaperone protein synthesized in response to cellular stress conditions, helping cells cope with stress. HSP70 has also been shown to play an important role in processes, such as protein folding, cellular homeostasis, and apoptosis [73, 76].

Recent research has shown that single-cell genomics technology can also be used in breast cancer research. With single-cell genomics, it is possible to gain a better understanding of genomic differences and heterogeneity in breast cancer cells, classify cancer cells more effectively, and identify targets in cancer treatment. Studies using single-cell genomics technology aim to better understand genetic heterogeneity and various subtypes of cancer cells in breast cancer. In these studies, the genomes of cancer cells from patient samples were individually examined, and genomic characteristics of different subtypes of cancer cells were identified. Moreover, single-cell genomics technology enables better identification of breast cancer subtypes, more accurate diagnosis of the disease, and the development of treatment methods.

4. Therapeutic use and potential of HSP70 inhibitors

Considering the roles of HSP70 in suppressing apoptosis, promoting tumor progression, and providing resistance to chemotherapy, inhibitors targeting HSP70 are thought to be an important therapeutic tool in cancer treatment. We have previously mentioned that breast cancer is a complex disease with a heterogeneous structure and different biological subtypes. In breast cancer subtypes, overexpression of HSP70 is associated with suppression of apoptosis and resistance to treatment. Therefore, inhibitors targeting HSP70 stand out as a promising therapeutic strategy, especially in aggressive subtypes. TNBC is one of the breast cancer subtypes with one of the highest expression levels of HSP70. HSP70 suppresses apoptotic signals by stabilizing TP53 mutations and increases cancer stem cell-like properties. It is suggested that HSP70 inhibitors can be used to both activate apoptotic signals and increase sensitivity to chemotherapy in TNBC cells. In HER2 (+) tumors, HSP70 supports the overactivation of the HER2 receptor by increasing its stability. This may lead to accelerated cell proliferation and resistance to anti-HER2 treatments. HSP70 inhibitors may create a synergistic effect when combined with anti-HER2 treatments by disrupting HER2 stability and targeting this signaling pathway. In Luminal A and B subtypes, HSP70 supports cellular growth by keeping hormone receptors stable. It is suggested that HSP70 inhibitors can be used in these subtypes, especially in patients who have developed resistance to endocrine treatments. Combination treatments have a strategic importance to increase the effectiveness of HSP70 inhibitors in breast cancer. When combined with standard chemotherapeutic drugs, such as Doxorubicin or Paclitaxel, HSP70 inhibitors make tumor cells more susceptible to apoptosis. HSP70 inhibitors can also be effective in treatment-resistant tumors when used with targeted drugs, such as Trastuzumab (HER2+), Everolimus (mTOR inhibitor), or Palbociclib (cyclin-dependent kinase 4 and 6 (CDK4/6) inhibitor). The clinical use of HSP70 inhibitors requires the development of more specific and selective agents to minimize side effects in healthy tissues. Nanoparticle systems that will deliver HSP70 inhibitors directly to breast tumors will increase therapeutic efficacy while reducing side effects. It is thought that determining patient groups using HSP70 expression levels and subtype-specific biomarkers will increase the success of these treatments [3, 4, 9, 17, 45, 46, 77–79].

5. Conclusion

In summary, HSP70 stands out as a critical molecule in the complex landscape of cellular stress responses, apoptosis, and breast cancer. As a chaperone protein that helps maintain cellular stability under stress, HSP70 plays an important role in supporting cell survival, especially in cancer cells exposed to therapeutic stressors. In the context of breast cancer, the anti-apoptotic properties of HSP70 are particularly important because they help protect cancer cells from the effects of chemotherapy, thereby contributing to treatment resistance. HSP70 inhibitors are emerging as a powerful therapeutic key, especially in aggressive and treatment-resistant breast cancer subtypes. Preclinical and clinical studies suggest that these inhibitors may be effective both as monotherapy and in combination therapies. However, further research is needed to optimize these strategies and transition them to clinical use. Emerging research highlights the connection between HSP70 and CSCs, which are implicated in tumor initiation, metastasis, and recurrence. CSCs possess unique

mechanisms to survive and proliferate under adverse conditions, including an enhanced resistance to apoptosis. HSP70 may support CSCs by stabilizing their cellular machinery, thus preserving their "stemness" and resistance to treatments. This interaction suggests that HSP70 not only aids cancer cells in evading apoptosis but may also play a role in the persistence and resilience of CSC populations, which are often challenging targets in conventional therapies. Further investigation into the specific roles of HSP70 in CSCs and breast cancer subtypes can help elucidate the molecular pathways that underlie apoptosis evasion and therapeutic resistance. Insights gained from these studies could aid in developing innovative strategies targeting HSP70 to reduce CSC survival and overcome treatment barriers. By exploring HSP70's functions across different molecular profiles within the breast cancer microenvironment, we may open new avenues for personalized treatments aimed at effectively targeting CSCs and reducing recurrence.

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Chapter 6

Nitric Oxide-Mediated Apoptosis: The Role of Pterins and Statins for Cancer Therapeutics

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Abstract

In cancer biology, nitric oxide (NO) has emerged as an important signalling molecule, depending on its concentration and cellular context, exhibiting both pro- and anti-tumorigenic effects. Numerous cytotoxic and/or genotoxic effects, including inhibition of mitochondrial respiration, protein and DNA damage that results in gene mutation, loss of protein function, necrosis, and apoptosis, are mediated by NO and NO metabolites such as nitrite, nitrate, S-nitrosothiols, nitrosamines, and peroxynitrite. Several strategies are used in the study to increase the NO concentration, aiming at the target for cancer therapy. Pterins, such as tetrahydrobiopterin (BH4), are essential cofactors for nitric oxide synthase (NOS) enzymes, regulating their activity and NO production. Statins, widely prescribed for lowering cholesterol, have been shown to possess many pleiotropic effects like anti-cancer properties through various mechanisms, including the upregulation of NOS and increased NO production. The combination of pterins and statins may have a synergistic effect on NO production by stimulating the NOS pathway, leading to elevated intracellular NO concentrations in cancer cells. High levels of NO can trigger apoptosis through multiple mechanisms, including p53 activation, NF-κB inhibition, guanylyl cyclase activation, and phosphodiesterase inhibition. The complex interplay between these signalling pathways determines the fate of cancer cells exposed to large NO concentrations. Furthermore, NO-mediated cytotoxicity can suppress DNA synthesis and mitochondrial respiration in cancer cells, contributing to their demise. This review aims to elucidate the molecular mechanisms underlying the synergistic efficiency of pterins and statins in, inducing NO-mediated apoptosis, offering novel insights into potential cancer therapeutic strategies.

Keywords: nitric oxide (NO), synergistic efficiency, tetrahydrobiopterin (BH4), statins, NO-mediated cytotoxicity

1. Introduction

Cancer represents an international healthcare priority at the forefront of biomedical research as both prevention and therapeutic options continue to be developed. The World Health Organisation (WHO) has declared that cancers constitute the largest

worldwide disease burden, and the incidence rates significantly surpass the rates of ischaemic heart disease, stroke, and lower respiratory infections in both males and females. The incidence rates are predicted to grow substantially, with an expected 36% rise in the number of new cancer cases and a 60% increase in cancer mortality from 2020 to 2040, according to the Global Cancer Observatory. The need for preventative, "onco-protective" agents takes precedence in cell and molecular biology research for the development of anti-tumour agents over the existing maintenance therapies available for cancer. Newer avenues for cancer treatment have added to the conventional options of chemotherapy and radiotherapy, with the latest therapies including killer CAR-T-cell therapy, stem cell transplantation, and the use of immune checkpoint inhibitors in the form of small molecules, protein conjugates, and peptide therapeutics.

Oxidative stress is considered a significant regulatory factor in different metabolic disorders and pathological processes. Nitric oxide (NO) is a widely present free radical gas molecule with signalling roles that have been implicated in its modulation of physiological events associated with cancer development and progression, such as angiogenesis, cell cycle regulation, invasion, and metastasis. The elucidation of anti-oncogenic characteristics of endogenous *in vivo* NO production by nitric oxide synthase (NOS) enzymes has been correlated to genotypic and phenotypic changes in cancer cells, promoting tumour suppression through its interactions with cellular targets. The three isoforms of NOS – nNOS, iNOS, and eNOS – are differentially regulated at the transcriptional and posttranscriptional levels. In cancer cells, NO has been shown to have both pro-tumorigenic and anti-tumorigenic effects, depending on the concentration and context [1–3]. NO may also exhibit a dual response, indicating that apoptotic or growth arrest pathways are triggered when NO levels surpass a threshold that would be appropriate for tumour development and survival. NO triggers apoptosis in a variety of cell types, such as neurones, thymocytes, macrophages, and pancreatic islets [4]. There are many reports that NO induces apoptosis in various cell lines like HeLa [5], HT-29 [6], neuroblastoma [7], and HL-60 leukaemia [8]. A number of mechanisms of NO-induced apoptosis include the following: inhibition of mitochondrial Fe–S complexes I and II [9], activation of multiple kinases, inhibition of the proteasome pathway [4], inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) *via* S-nitrosylation, which suppresses glycolysis and ultimately ATP synthesis [10], activation cGMP which thereby inducing apoptosis [11, 12]. Thus, based on these properties of NO, it can be used therapeutically with remarkable results in preclinical cancer models, slowing the growth of tumours and enhancing the curative effect of radiotherapy and chemotherapy.

Recent studies have highlighted the potential of pterins and statins as therapeutic agents for cancer treatment. Pterins, such as tetrahydrobiopterin (BH4), play a crucial role in regulating NOS activity and NO production [13, 14]. Statins, on the other hand, have been shown to induce apoptosis in cancer cells through the upregulation of NOS and increased NO production [15]. Extensive study of NO regulation by different metabolic compounds and pharmacological agents offers new therapeutic potential in utilising these physicochemical mechanisms and interactions as a novel onco-protective or anti-oncogenic strategy in cancer prevention and treatment. The combination of pterins and statins may have a synergistic effect on NO production and cancer cell apoptosis. This study will explore the role of pterins and statins in NO-mediated apoptosis in cancer cells.

2. Induction of nitric oxide

Nitric oxide (NO) is a crucial signalling molecule involved in various physiological processes, including vasodilation, neurotransmission, and immune response. At the genomic level, NO is induced through the activation of nitric oxide synthase (NOS) enzymes, which convert the amino acid L-arginine into citrulline and NO [16]. By employing NADPH and O_2 as co-substrates, the nitric oxide synthase (NOS) family of enzymes catalyses the oxidation of L-arginine to produce both NO and L-citrulline [17]. Inducible NOS (NOS II/iNOS), endothelial NOS (NOS III/eNOS), and neuronal NOS (NOS I/nNOS) are the three isoforms of the NOS enzyme that are expressed. A variety of substrates and cofactors are necessary for all of these NOS isoforms to function properly. For instance, the NO-generating process requires the cofactors tetrahydrobiopterin (BH4), thiol, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), as well as the substrates L-arginine, NADPH, and oxygen. Citrulline and NADP are co-products of the NOS catalysing process in addition to NO.

The creation and maintenance of the low basal level of NO synthesis in brain and endothelial cells depends on the constitutively produced NOS I and NOS III. The enzymatic activity of these constitutive NOS isoforms is cofactor-dependent and Ca^{2+}/calmodulin (CaM)-dependent [18, 19]. Research has elucidated the complex roles of nitric oxide synthase (NOS) isoforms, particularly NOS I and NOS III, in cancer biology. While NOS II has been extensively studied in the context of cancer, the involvement of NOS I and NOS III in cancer cells has also garnered significant attention. NOS I, primarily expressed in neuronal tissues, has been implicated in cancer development and progression. Studies have shown that NOS I is overexpressed in certain types of cancer, including breast, lung, and colon cancers [20]. The overproduction of nitric oxide (NO) by NOS I can contribute to cancer cell proliferation, survival, and migration [21]. Additionally, NO generated by NOS I can induce epithelial-to-mesenchymal transition (EMT), a process that facilitates cancer cell invasion and metastasis [22]. NOS III, predominantly expressed in endothelial cells, has also been linked to cancer biology. NOS III-derived NO has been shown to promote angiogenesis, the formation of new blood vessels that supply nutrients and oxygen to growing tumours [23]. Furthermore, NOS III expression has been correlated with tumour progression and poor prognosis in various cancers, including breast, prostate, and gastric cancers. The NO produced by NOS III can also contribute to cancer cell survival and resistance to chemotherapy [24]. Numerous cell types, including macrophages, dendritic cells, fibroblasts, chondrocytes, osteocytes, astrocytes, epithelial cells, and numerous cancer cells, contain the Ca^{2+}/CaM-independent inducible isoform (NOS II/iNOS). In spite of very low or no expression in the surrounding normal tissues, iNOS is highly expressed in breast cancer, human adenomas (60%) and colon carcinomas (20–25%) [25]. Other tumours that have demonstrated iNOS gene expression are brain [26], head and neck [27], oesophagus [28], lung [25], prostrate [29], bladder [30], pancreatic [31] and kaposi's sarcoma [32]. Interferon (IFN), interleukin-1 (IL-1), tumour necrosis factor (TNF), bacterial endotoxin (LPS), and oxidative stress (such as hypoxic state) are examples of stimuli that might affect the transcriptional regulation of iNOS. MicroRNAs (miRNAs) also regulate NOS expression at the post-transcriptional level. For example, miR-155 has been shown to target eNOS mRNA, leading to its degradation and reduced NO production [33]. Conversely, miR-21 has been shown to increase iNOS expression by targeting the inhibitory protein Src homology 2 domain-containing phosphatase-1 (SHP-1) [34].

The post-transcriptional regulation of NOS by miRNAs adds an additional layer of complexity to the regulation of NO production.

3. Role of nitric oxide (NO) in cancer

NO is biologically synthesised by nitric oxide synthases (NOS). Several studies have demonstrated that cytokine-activated rodent macrophages can produce high levels of NO by up-regulating the expression of the inducible nitric oxide synthase gene (iNOS). Hibbs et al. [1] were the first to describe the role of NO in macrophage cytotoxicity. This mechanism produces NO, which can destroy a variety of cancer cells of different origin and grade [3]. Nitric oxide's precise function in cancer and tumour biology is still unknown. Both the pathophysiology and the physiology of nitric oxide in tumour cells have been linked to a wide range of activities. Numerous direct and indirect processes have been put up to explain NO's anti-tumour effects. Direct DNA damage, suppression of DNA synthesis, and inhibition of the rate-limiting enzyme ribonucleotide reductase are some of the mechanisms. As potential mechanisms, decreased cis-aconitase activity and the loss of a significant portion of the iron pool have also been proposed. Crucially, reduced O_2 consumption, damage to complexes I and II in the mitochondrial electron transport chain, reversible suppression of complex IV activity, and induction of apoptosis are all consequences of NO generation's influence on mitochondrial physiology [1, 3, 35–38]. Numerous factors, including NO formation and metabolism, the type of NOS enzymes present, the interaction between NO-utilising processes, and most importantly, the concentration of NO in the system, influence the wide range of distinct biological effects that result from exposure to NO. The quantity and sources of nitric oxide being produced are the first differences we can identify. Low nitric oxide output has been linked to increased blood flow and the formation of new blood vessels (angiogenesis) that feed the tumour [39]. Furthermore, the immune suppression seen in conjunction with tumour growth may be explained by the production of nitric oxide by tumour cells, which may prevent the activation and proliferation of nearby lymphocytes or enhance their death. High intratumoral nitric oxide generation may also deter caspase activation and hence counteract pro-apoptotic signals [40, 41]. Nevertheless, the reverse effect has also been noted in numerous different settings, wherein tumour development and metastasis are inhibited by the production of a high output of nitric oxide, either through iNOS activation or the use of NO donors [42]. As a result, the final result of NO-mediated effects will depend on a number of variables, such as the tissue's local nitric oxide content and sources, as well as the existence of reactive chemicals that could change the cell's redox state. The idea that NO controls the expression of certain genes involved in the signal pathway including regulatory cytokines that alter the cellular response to apoptotic stimuli is supported by multiple lines of evidences [43–49].

As a key signalling molecule, NO, is produced and increased in a variety of physiological and pathological circumstances. It has been tried in clinical trials as a viable medication to treat various cardiovascular conditions. Furthermore, the NO actively contributes to the relaxation of smooth muscle, neurotransmission, and prevention of platelet aggregation and cell-cell adhesion. The importance of NO as a new oncoprotective agent and, more recently, as a novel therapeutic agent to overcome cancer cell resistance is being supported by mounting evidence. According to Blaise et al. [50], the chemistry of NO and its derivatives is a highly reactive free radical that can mediate a variety of reaction chains. Because the free radical NO is an uncharged molecule with an unpaired electron in its outermost orbital, it can undergo a number

of processes in which it can either operate as a mild oxidant (e-donor) or an antioxidant (e-acceptor). Since Hibbs et al. first discussed the role of NO in macrophage cytotoxicity in 1987 [1], numerous studies have demonstrated that cytokine-activated rodent macrophages can produce more nitric oxide by up-regulating the expression of the inducible nitric oxide synthase gene (iNOS) [3]. This process produces NO, which has the ability to kill a variety of cancer cells of various origins and types. Numerous direct and indirect mechanisms for the anti-tumour effects of NO have been put forth. Direct DNA damage, DNA synthesis inhibition, and inhibition of the rate-limiting enzyme ribonucleotide reductase are some of the mechanisms. Another theory for how NO kills cancer cells is the decreased activity of cis-aconitase and the loss of a significant portion of the iron pool. The NO generation also has an impact on the mitochondrial physiology, which results in decreased oxygen consumption, damage to complexes I and II in the mitochondrial electron transport chain, reversible inhibition of complex IV activity, and induction of apoptosis [1, 3, 35–37].

It has been proposed that certain NO intermediate metabolites can act as mediators for the biological effects of NO. For instance, NO can react quickly in the intracellular setting to produce nitrite, nitrate, S-nitroso-thiols, or peroxynitrite. By targeting the sugar-phosphate backbones, these metabolites may be essential in mediating many of the major genotoxic consequences, such as DNA damage.

Reactive nitric oxide species (RNOS) generated from NO and ROS can cause direct damage to DNA. Wink and Mitchell (1998) [51] state that NO harms DNA in three different ways: RNOS directly interacts with DNA structure in (i), inhibits the DNA repair mechanism in (ii), and increases the production of genotoxic species such as hydrogen peroxide and alkylating chemicals. In human and bacterial cells, RNOS causes a single-stranded DNA break (Nick translation) in addition to the deamination of cytosine, adenine, and guanine [52]. Szabo and Ohshima [53] claim that peroxynitrite dramatically alters DNA by producing 8-nitroguanine and 8-oxoguanine in addition to DNA single-strand breaks. DNA strand breaks result in nuclear enzyme poly (ADP-ribose) polymerase (PARP) activation and secondary overexpression of the tumour suppressor gene p53, processes that may encourage apoptosis [54].

Although the source and concentration of NO can influence how a cell reacts to exposure to it, pro- and anti-apoptotic reactions to NO exposure appear to be cell-type-specific. Numerous of these NO-induced death reactions are cell type-specific, with many being apoptotic at low levels and necrotic at larger ones. For instance, sub-millimolar doses of NO donor cause fast cell death in macrophages but appear to have no effect on cultured hepatocytes [55]. Interesting investigations have revealed that all three isoforms can influence whether the aetiology of cancer is promoted or inhibited.

Numerous tumour cells with diverse histogenetic origins have been shown to have NOS activity, which has been connected to the tumour's grade, rate of proliferation, and expression of important signalling molecules which lead to the development of cancer, which includes the oestrogen receptor. Large amounts of NOS expression (generated by activated macrophages) may be cytostatic or cytotoxic for cancer cells, but modest levels of NOS activity appear to have the opposite effect and promote tumour growth. High but physiologically significant levels of NO can trigger apoptosis, depending on the kind of cancer cell and its sensitivity to NO [56].

Through a variety of apoptotic mechanisms, abnormally elevated NO generation by NOS II/iNOS may cause cancer cell cytotoxicity. In the Apc(Min/+) colon cancer mouse model, it has been discovered that iNOS knockout mice enhance intestinal tumorigenesis [57]. According to a study by Xie et al. [35], murine melanoma cells

overexpressing iNOS had poor tumour development and survival due to NO-induced apoptosis *in vitro*, which also affected their potential to spread. The buildup of p53, suppression of mitochondrial respiration, changes to the expression of Bcl-2 family members, activation of caspase signalling, and DNA damage are some of the mechanisms of NO-mediated apoptosis [58].

4. Pterins and nitric oxide (NO) production

The NOS isoforms are known to be regulated at the transcriptional, translation and posttranslational levels, and BH4 has come to light as an important factor that regulates the level and mode of NOS activity [13, 59, 60]. The affinity of the aromatic amino acid hydroxylases for BH4 is in the 10–30 micromolar range [61], whereas the equilibrium dissociation constant for BH4 binding to NOSs is two to three orders of magnitude less [62]. Nonetheless, BH4 availability has often been found to limit NOS activity and enzymes of the BH4 de novo biosynthetic and recycling/salvage pathways are coordinately induced with NOSs in endothelial cells [63], fibroblasts [64], vascular smooth muscle cells [65] and cardiac myocytes [66]. Additionally, it has been demonstrated that NO directly controls BH4 by inhibiting the feedback inhibition that BH4 mediates on GTPCH (GTP cyclohydrolase I), the enzyme that limits the rate of BH4 biosynthesis [67]. GTPCH overexpression in transgenic mice increases cytokine-induced serum NO production in comparison to cytokine-treated wild-type mice [68], and cytokine therapy in rodents also exhibits this coordinated regulation, up-regulating both iNOS and GTPCH simultaneously [69]. On the other hand, eNOS [70], nNOS [71], and iNOS activity [63] were demonstrated to be negatively impacted by BH4 depletion, either pharmacologically [63, 70] or genetically [71]. In each of these cases, NOS activity was restored by replenishing BH4 levels through treatment with either BH4 or its precursor sepiapterin. Aside from its structural roles in catalysis, BH4 also protects against proteolysis, increases arginine binding, and stabilises or promotes the formation of dimers. Though only fully reduced pterins can promote NOS catalysis [72], redox-silent tetrahydrobiopterins, such as 6(R,S)-methyl-5-deazatetrahydropterin [73] and 6R-H4-aminobiopterin [74], are unable to do so. BH2 also stabilises NOS dimers, just like BH4. These results imply an obligatory redox activity and show that structural stabilisation of NOS is not enough to explain the role of the BH4 cofactor. Interestingly, the lack of bound BH4 clearly changes the coordination state of heme-iron in NOSs. The recombinant enzyme's changed state is "rescued" by adding BH4, despite the dimer formation, proving that the cofactor affects the active site metal's chemistry [75]. The interaction between BH4's pterin ring and the NOS oxygenase domain starts the binding of BH4 to NOS [76]. A conserved glutamate residue (Glu) in the NOS oxygenase domain facilitates this interaction by forming a hydrogen bond with the pterin ring of BH4. The dihydroxypropyl side chain of BH4 interacts with a conserved arginine residue (Arg) in the NOS oxygenase domain to aid in the binding of BH4 to NOS [77]. Once BH4 is bound to NOS, it undergoes a conformational change that facilitates the formation of a stable complex between BH4 and the NOS heme group. This complex is essential for the catalytic activity of NOS, as it allows for the transfer of electrons from BH4 to the NOS heme group, facilitating the conversion of L-arginine to citrulline and NO [78]. The consequences of decreased intracellular BH4 concentration on NOS activity further demonstrate a clear requirement for BH4 as a NOS cofactor [70, 79–81].

5. Statins in cancer therapy

A class of aromatic compounds renowned for its ability to decrease low-density lipoprotein (LDL) levels, statins are commonly recommended to patients with atherosclerosis and cardiovascular disease (CVD). In 1973, *Penicillium citrinum* created compactin, the first of the natural secondary metabolites known as statins, which were found to be produced by fungus *via* the polyketide pathway [82]. *Aspergillus terreus* and *Monascus ruber* were used to make lovastatin, the first commercial statin [83]. Atorvastatin, fluvastatin, pitavastatin, pravastatin, lovastatin, simvastatin, and rosuvastatin are among the statins that are currently on the market.

Statins act on several regulatory pathways and through different mechanisms of action, including competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, induction of endothelial nitric oxide synthase (eNOS), inhibition of arterial smooth muscle cells, and demonstrates anti-inflammatory, antiproliferative, and immunomodulatory effects, senescence, and oxidative stress [84].

The mevalonate pathway is the major metabolic and signalling pathway through which cancer cells are affected by statins, which involves the production of isoprenoids, including cholesterol, vitamin D, lipoproteins, polyol, and ubiquinone [85]. Although functionality of this pathway is essential for all cells, cancer cells are found to exhibit increased pathway activity in which increased mevalonate production supports the nutritional needs of tumour cells, hence promoting both tumorigenesis and tumour adaptability. The synthesis of mevalonate by HMG-CoA reductase is blocked by statins for hypercholesterolemia treatment, where non-cholesterol-mediated pathways show anti-cancer activity in *in vitro* and *in vivo* studies. Statins are also considered as a potential treatment for osteoarthritis (OA) due to the roles of dyslipidemia and inflammation in atherosclerosis and OA, which are alleviated by the anti-inflammatory effect of statins through inhibition of isoprenoid synthesis along with their ability to improve endothelial cell function, increase bioavailable NO, and exhibit antioxidant properties [86, 87]. Statins induce a number of effects in both endothelial and cancer cells. With the exception of rosuvastatin and pravastatin, several commonly prescribed statins are lipophilic in nature and are thus able to penetrate the cell membranes of extrahepatic cells by diffusion, including atorvastatin, lovastatin, and simvastatin. They inhibit cholesterol synthesis and also downregulate the synthesis of non-sterol products, particularly the isoprenoids FPP and GGPP, by which prenylation of different protein groups is inhibited, namely the Rho, Ras, and Rab families of proteins [88].

NOS is also regulated by the proposed mechanism of caveolin-1 (Cav-1) mediation in inhibition of eNOS inactivation following statin treatment. Caveolae are plasma membrane regions with cholesterol and caveolin proteins that facilitate cell signalling [89]. When Cav-1 inactivates eNOS upon attachment, the cholesterol components affect its function, hence exposing cells to free cholesterol for Cav-1 upregulation and so, statins inhibit this downstream effect by inhibiting cholesterol biosynthesis. Therefore, eNOS/Cav-1 attachment does not occur and eNOS remains active; statins confer increased eNOS ability to synthesise NO in a concentration-dependent manner [90]. Cav-1 binds to the reductase domain of NOS, specifically within a region known as the oxygenase-reductase linker (ORL). The binding of Cav-1 to NOS has important implications for the regulation of nitric oxide (NO) production. Under basal conditions, Cav-1 binding to NOS inhibits its activity by preventing the binding of calmodulin (CaM), a calcium-binding protein that is essential for NOS activation.

However, upon stimulation, such as by increased intracellular calcium levels, CaM can displace Cav-1 from NOS, leading to enzyme activation and NO production [91].

Cav-1 has been shown to interact with endothelial nitric oxide synthase (eNOS), a key enzyme responsible for producing nitric oxide (NO) in endothelial cells. The binding of Cav-1 to eNOS occurs through a specific region of Cav-1, known as the caveolin scaffolding domain (CSD). The CSD is a conserved sequence of 20 amino acids that mediates the interaction between Cav-1 and various signalling molecules, including eNOS [92]. The binding of Cav-1 to eNOS involves the formation of a stable complex between the two proteins, which is mediated by hydrophobic interactions between the CSD of Cav-1 and the oxygenase domain of eNOS [93].

The binding of Cav-1 to eNOS has been shown to inhibit eNOS activity by reducing its ability to bind to calmodulin, a calcium-binding protein that is essential for eNOS activation. The inhibition of eNOS activity by Cav-1 has been implicated in various pathological conditions, including cardiovascular disease and cancer [94]. In addition to eNOS, Cav-1 has also been shown to interact with other NOS isoforms, including neuronal NOS (nNOS) and inducible NOS (iNOS) [95].

6. Synergism of statins and pterins

Apart from the lipogenic inhibition property, statins also conquer many alterations in the metabolic pathways in cancer cells, which possess a therapeutic tool for cancer therapy. One best example is the up-regulation of NOS thereby increasing the concentration of nitric oxide in the cell which opens up various apoptotic pathways in cancer cells. Similarly, Pterin compounds also can influence the NOS pathway which thereby causes various changes in the cellular metabolism. In this view, a combination of statins and pterin compounds in a synergistic manner will induce the activation of the NOS pathway by increasing the production of nitric oxide concentration. NOS activity has been found in tumour cells with a variety of histogenetic origins and has been linked to tumour grade, proliferation rate, and expression of key signalling molecules involved in the growth of cancer, such as the oestrogen receptor. It seems that while low levels of NOS activity can have the reverse effect and encourage tumour growth, large levels of NOS expression (produced by activated macrophages) may be cytostatic or cytotoxic for cancer cells. According to the kind of cancer cell and its sensitivity to NO, high yet physiologically relevant amounts of NO can cause apoptosis [56]. The combination of statins and pterin compounds will have the impact of stimulation of the NOS pathway which increases the NO concentration in cancer cells. This gradual hike of NO concentration in the cancer cells can activate numerous apoptotic pathways by p53 phosphorylation [96], NF-kB inhibition [97], activation of guanylyl cyclase [98], PDE inhibition [99], ROS activation, and so on.

One effect of NO-mediated DNA damage is the increase of p53, which has the ability to cause apoptosis. This is one method *via* which NO may lead cancer cells to die. As a result, an increase in NOS activity in cancer cells (due to either enhanced transcriptional activity or posttranscriptional/protein regulatory activity) may raise NO concentrations to the point where p53-mediated growth arrest and apoptosis are triggered [100, 101]. It is interesting to note that p53 accumulation has been shown to limit iNOS promoter activity, which in turn causes iNOS expression to be down-regulated [25]. As a result, there is a negative feedback loop between the production of NO and the accumulation of p53. This could be a physiological response to endogenous NO-induced DNA damage. Overall, a high selection pressure for mutant p53

expression in tumour cells may be anticipated as a result of this p53-mediated growth suppression. Additionally, NO-mediated cytotoxicity influences the suppression of DNA synthesis and mitochondrial respiration in target cells, such as breast cancer cells [102]. Important anti-apoptotic and anti-microbial survival mechanisms are blocked when NF-kB-mediated production of iNOS in epithelial cells is inhibited. This is the link between NF-κB and iNOS. The prolonged activation of NF-kB is countered by NO through a negative feedback loop, which limits the survival of cancer cells [97].

NF-κB, a transcription factor that can be activated by NO, leading to the expression of anti-apoptotic genes that inhibit NO-mediated apoptosis [103]. Conversely, p53 is a tumour suppressor protein that can be activated by NO, leading to the expression of pro-apoptotic genes that promote NO-mediated apoptosis [104]. The balance between NF-κB and p53 activity can therefore determine the outcome of NO-mediated apoptosis. Studies have shown that NO can activate p53 through the formation of S-nitrosothiols, which can modify p53 and enhance its activity [105]. Activated p53 can then induce the expression of pro-apoptotic genes, such as Bax and PUMA, which can promote NO-mediated apoptosis [104]. Conversely, NF-κB can inhibit p53 activity by inducing the expression of Mdm2, an E3 ubiquitin ligase that can degrade p53.

The interplay between NF-κB and p53 in NO-mediated apoptosis is complex and can be influenced by various factors, including the concentration and duration of NO exposure, the cell type and context, and the presence of other signalling molecules. However, studies suggest that the balance between NF-κB and p53 activity can determine the outcome of NO-mediated apoptosis, with NF-κB promoting cell survival and p53 promoting cell death. NF-κB can induce the expression of anti-apoptotic genes, such as Bcl-2 and Bcl-xL, which can inhibit NO-mediated apoptosis [106]. Conversely, p53 can induce the expression of pro-apoptotic genes, such as Bax and PUMA, which can promote NO-mediated apoptosis [107].

It has been demonstrated that NO has a complex and paradoxical role in cancer. Both cGMP-dependent and cGMP-independent effects of NO are possible. The former involves the generation of the second messenger, cGMP, after NO activates soluble guanylyl cyclase (sGC), while the latter is mediated by reactive nitrogen species that are created when NO interacts with oxygen (O_2), oxygen reactive species, or superoxide radicals. Guanylyl cyclases [98] are the enzymes that synthesise cGMP from intracellular GTP. They belong to two classes: soluble (sGC) and particulate (pGC). The active enzyme in sGC is made up of two subunits, β (α1 and α2) and β (β1 and β2), which form a heterodimer with heme. By producing cGMP, which directly affects downstream effectors such as cyclic nucleotide-gated channels (CNGs), cGMP-dependent protein kinases (PKGs), and cGMP-regulated phosphodiesterases (PDEs), sGC can have a variety of physiological consequences [98, 108–110].

Different extracellular signals trigger biological reactions, which are mediated by cAMP and cGMP. Cyclic nucleotide phosphodiesterases (PDEs) 1 control intracellular concentrations and actions of these second messengers by catalysing the hydrolysis of cyclic nucleotide 3′–5′-phosphodiester bond. A broad class of structurally related enzymes is referred to as phosphodiesterases PDEs. These enzymes are members of at least seven related gene families (PDEs 1–7), which vary in terms of their core structures, affinities for cAMP and cGMP, reactions to certain effectors, susceptibilities to particular inhibitors, and regulatory processes. In intact cells, hormones that raise cAMP phosphorylate and activate a PDE4 isoform, which is also triggered *in vitro* by protein kinase A. The desensitisation to hormone signals and intracellular processes

that control the amount and duration of cAMP signals and responses are thought to revolve around the feedback regulation of both PDE3 and PDE4 activity by cAMP-dependent phosphorylation [99].

In experimental PAH models, phosphodiesterase (PDE) isoenzymes, mostly PDE3 and PDE4 are upregulated and crucial coregulators of cAMP catabolism in various organs, including the lung. It has also been demonstrated that cAMP-elevating drugs improve angiogenesis and other endothelial cell activities. In contrast, direct stimulation of PKA by cAMP has been found to limit endothelial cell survival and angiogenesis. Endothelial degeneration is regulated by cAMP-elevating drugs or a combination PDE3/4 inhibitor, although little is known about the molecular mechanisms and signalling pathways involved [111].

Furthermore, PDE inhibitors can be effective targets for apoptosis induction and tumour cell growth suppression. The proliferation of several carcinoma cell lines is regulated by nonselective PDE inhibitors like theophylline or aminophylline, indicating a possible use of PDE inhibitors as anti-cancer medications. PDE3 activity and PDE3A and PDE3B isoform expression have been found in human squamous cell carcinoma and malignant melanoma cells [112]. The PDE3-specific Cilostamide, an inhibitor, inhibits these cells from proliferating. As a result of PDE overexpression, many cancer cells show noticeably lower cAMP levels. Remarkably and intriguingly, cAMP-specific PDE4 was in charge of the highest cAMP-hydrolysing activity in 41 out of 60 cancer cell lines from the CNS, lung, and breast that were obtained from the NCI [113]. PDE4 activity is also higher in mouse keratinocytes representing various phases of malignant transformation than in their nonmalignant counterparts. Additionally, PDE4 inhibitors prevent MCF-7 mammary cancer cells and B16 melanomas from growing. However, PDE1 and PDE5 inhibitors either slightly or not at all decrease the development of these cancer cells [114]. Furthermore, the PDE4 inhibitor DC-TA-46 caused G1/G0 arrest in a wide range of human and mouse lung cancer cell lines, indicating a potential biological target for anti-tumour therapy that needs to be investigated in preclinical models [113]. Remarkably, PDE4 inhibitors decrease cellular proliferation and promote apoptosis in a caspase-dependent manner in pancreatic cancer cell lines that are resistant to the majority of chemotherapeutic treatments [115].

Nitric oxide (NO) plays a crucial role in this process by activating soluble guanylyl cyclase (sGC), a heterodimeric enzyme that catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The binding of NO to sGC triggers a conformational change that activates the enzyme, leading to an increase in cGMP production. The increased cGMP levels then activate protein kinase G (PKG), a serine/threonine kinase that plays a key role in various cellular processes, including apoptosis. PKG is activated through the binding of cGMP to its regulatory domains, which triggers a conformational change that activates the kinase [115, 116].

Once activated, PKG phosphorylates and activates various downstream targets, including caspases, Bcl-2 family proteins, and p53, which ultimately lead to the induction of apoptosis in cancer cells. One of the key mechanisms involves the activation of caspases, a family of cysteine proteases that play a central role in the execution of apoptosis. PKG activates caspases by phosphorylating and activating caspase-9, which then activates downstream caspases, such as caspase-3 and caspase-7 [117]. The activation of caspases leads to the cleavage of various cellular substrates, including poly(ADP-ribose) polymerase (PARP), which ultimately leads to the induction of apoptosis [54]. Another key mechanism of NO-mediated apoptosis involves the inhibition of anti-apoptotic Bcl-2 family proteins. PKG phosphorylates and inhibits Bcl-2 and Bcl-xL, which are anti-apoptotic proteins that inhibit the release of

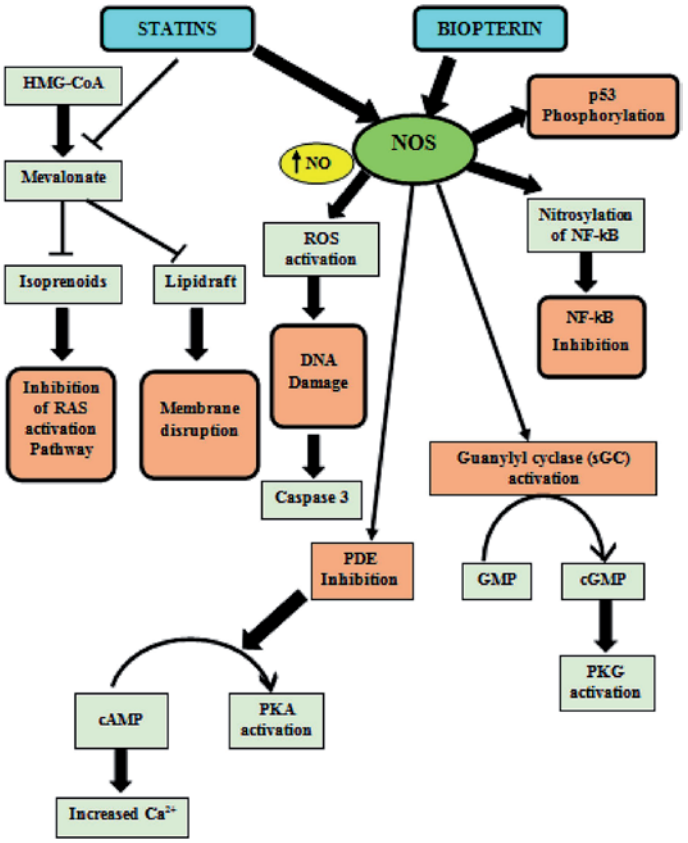

Figure 1.
Co-related pathway of statins and pterins. NOS, Nitric Oxide Synthase; NO, Nitric Oxide; ROS, Reactive Oxygen Species; PDE, Phosphodiesterases; cAMP, Cyclic Adenosine Monophosphate; PKA, Protein Kinase A; cGMP, Cyclic Guanosine 3′,5′-Monophosphate; PKG, Protein Kinase G; p53, tumour protein p53; HMG-CoA, Hydroxymethylglutaryl-CoA Reductase.

cytochrome c from the mitochondria. The inhibition of Bcl-2 and Bcl-xL allows for the release of cytochrome c, which then activates caspase-9 and triggers the apoptotic cascade [118].

Finally, NO-mediated apoptosis also involves the activation of p53, a tumour suppressor protein that plays a central role in regulating apoptosis. PKG activates p53 by phosphorylating and stabilising the protein, which then induces the expression of pro-apoptotic genes, such as Bax and PUMA. The activation of p53 ultimately leads to the induction of apoptosis in cancer cells (**Figure 1**) [119].

7. Conclusion

While the development of NO-releasing medications continues, many researchers have examined an alternative method for raising the concentration of NO in cancer cells. The NOS encoding sequence is typically present in cancer cells. There are disadvantages, such as transfectants dying soon since NOS is expressed constitutively, notwithstanding some successful cases. Another reason why NOS activation is not induced is the absence of substrates and cofactors necessary for complete NOS function.

For all NOS isoforms to produce NO, the cofactor BH4 is necessary. When BH4 is not present, NOS switches its activity from producing NO to superoxide generation. Sepiapterin, a BH4 precursor and a cofactor for NO, increased the statin-related increase in NO_{2^-}/NO_3-production. One promising strategy for regulating NO in cancer cells is the combination of statins and pharmacologic alteration of BH4 levels. By suppressing geranylgeranylation and supplementing with sepiapterin, statin-induced NO synthase iNOS/NO activity improves cancer cell death. Given this, we suggest that co-treating cancer cells with sepiapterin/pterin compounds (a precursor of BH4, a cofactor of NO) and statins (which raise NO) may boost the overall rate of NO generation and potentially treat and prevent cancer.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 7

MYC: Master Regulator of Cell Death and Tumor Progression

Lucia Capasso, Donato Mele, Fatima Fayyaz, Lucia Altucci and Angela Nebbioso

Abstract

MYC gene has become one of the most investigated oncogenes for regulating programmed cell death and tumor growth. MYC is a transcription factor that regulates the expression of numerous genes involved in critical cellular processes, such as metabolism, stress response, and proliferation. However, its dysfunction, often caused by gene amplifications or translocations, makes it a potent oncogenic driver, contributing to uncontrolled growth, angiogenesis, invasiveness, and metastasis. Paradoxically, MYC can promote both tumor cell survival and elimination through the activation of apoptotic mechanisms, creating a delicate balance between cell survival and death. This chapter explores the dual role of MYC as a regulator of cell life and death, analyzing the molecular mechanisms that determine its activity in different biological contexts. The main apoptotic pathways controlled by MYC, its contribution to tumor plasticity, and its interactions with other oncogenes and tumor suppressors will be discussed. Finally, emerging therapeutic strategies aimed at targeting MYC or its regulatory networks will be reviewed, along with the challenges of translating this knowledge into clinical interventions. A thorough understanding of MYC biology is crucial to develop innovative therapies and improve the treatment of aggressive and resistant tumors.

Keywords: MYC, cell death, tumorigenesis, signaling pathways, therapeutic target

1. Introduction

MYC gene, which locates on chromosome 8q24.21, is responsible for codifying the transcription factor MYC [1, 2]. MYC represents one of the more widely and influential oncogenes in cellular and tumor biology, because of its involvement in a multitude of vital processes [2]. The codified protein belongs to the bHLH-LZ (basic helix-loop-helix leucine zipper) family of transcription factors, including also N-MYC and L-MYC. It regulates the expression of an extraordinary number of genes, estimated to be approximately 15% of the human genome [3]. MYC activates or represses genes involved in vital activities such as cell proliferation, protein synthesis, energy metabolism, DNA repair, and the cell cycle by building complexes with the MAX

protein and binding to certain DNA regions known as E-boxes [4]. Under physiological conditions, MYC acts as a mediator of cellular responses to external stimuli, such as growth factors and mitogenic signals (**Figure 1**) [5, 6]. This function allows cells to adapt their growth and division to the needs of the surrounding tissue, maintaining a dynamic balance between proliferation and differentiation [5]. However, when MYC expression or activity is dysregulated, as frequently occurs in tumors, a cascade of pathological events occurs that favor uncontrolled growth and aberrant survival of cells (**Figure 1**) [7]. One of the many characteristics of MYC is its dual role in programmed cell death or apoptosis [8]. Under physiological conditions, MYC promotes cell survival and proliferation, but under stress conditions it can activate potent apoptotic pathways that lead to the elimination of damaged cells (**Figure 1**). This dual role makes MYC not only a growth promoter, but also a guardian against uncontrolled proliferation [9, 10]. At the molecular level, MYC activates apoptotic pathways by interacting with key proteins, such as p53, a major tumor suppressor [11]. Under conditions of cellular stress, excessive MYC activity can induce the expression of pro-apoptotic genes, such as BCL-2-associated X protein (BAX) and p53 upregulated modulator of apoptosis (PUMA), while inhibiting anti-apoptotic proteins, such as B-cell lymphoma 2 (BCL-2). This delicate balance represents an intrinsic safety strategy: if MYC activity exceeds a critical threshold, cells are induced to die to avoid pathological proliferation. However, in tumors, this mechanism is frequently bypassed [12, 13]. Mutations or alterations in apoptotic pathways, such as the loss of p53, allow tumor cells to use the proliferative activity of MYC without suffering its apoptotic consequences [14]. MYC is deregulated in the vast majority of tumors, indeed its overexpression, caused by several genetic alterations, such as gene amplifications, chromosomal translocations, or aberrant transcriptional activation, is associated with the uncontrolled proliferation of tumor cells [5, 15]. MYC promotes tumor progression through uncontrolled cell proliferation, activating genes involved in the synthesis of nucleotides and proteins, allowing tumor cells to replicate in an uncontrolled manner, or promoting the consumption of glucose, an essential element for tumor growth [16]. Furthermore, its hyperactivation makes cells insensitive to

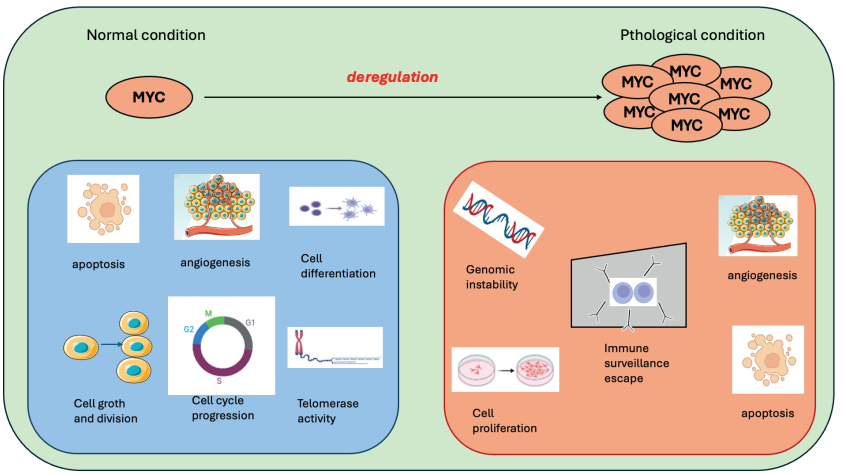

Figure 1.
Cellular processes regulated by MYC. MYC regulates several biological processes and their upregulation, in conditions of stress, or genetic alterations can cause the deregulation of genes involved in several pathologies.

antiproliferative drugs, also promoting the production of pro-angiogenic factors, such as vascular endothelial growth (VEGF), which support the development of new blood vessels [17]. These properties make MYC a central element in tumor progression, contributing not only to the primary growth of the tumor but also to the promotion of metastases and therapy resistance. The importance of MYC in tumorigenesis makes it a target of great interest for oncology research, but being a transcription factor and not having enzymatic pockets accessible to conventional drugs, its therapeutic targeting is extremely complex. However, there are innovative approaches, such as epigenetic therapies, that are opening new avenues for MYC targeting. In fact, direct inhibition of MYC could stop tumor growth and overcome resistance to existing treatments.

2. MYC structure and function

MYC, a proto-oncogene encoding a protein crucial in gene regulation, is considered one of the most important regulators of cell biology. It is a transcription factor that, by binding to specific DNA sequences called E-box: 5'-CACGTG-3′, activates or represses a lot of genes involved in different cellular processes [18]. MYC was originally identified in avian oncogenic viruses as a viral oncogene and named v-MYC. Its corresponding c-MYC was later recognized as essential for cell growth and proliferation in both normal and pathological conditions [19]. The MYC family consists of c-MYC, which plays a fundamental role in the regulation of the cell cycle and metabolism. It is the most studied and significantly expressed member of the family [20]; N-MYC, frequently amplified in neuroblastic tumors. It is the member mainly expressed in nervous tissues during embryonic development; L-MYC, involved in the regulation of cell proliferation in small cell lung cancer [21]. It is the member of the family whose functions are less understood than the others. The MYC genes, found in complex organisms, play a crucial role as a transcriptional regulator necessary for fundamental biological processes [22]. The three members of the MYC family exhibit significant similarities in regions that serve as docking points for various cofactors. These cofactors influence both the activity and stability of MYC, contributing to its oncogenic potential [23]. The conserved pattern known as the b-HLH-LZ motif, which is found in the C-terminal region of these proteins, is made up of a basic region (b), a helix-loop-helix (HLH), and a leucine zipper (LZ) domain. As seen in **Figure 2**, the b-region makes it easier to bind specific DNA sequences, whereas the HLH and LZ domains allow dimerization [24]. These motifs are crucial to create DNA-binding domain trough by MYC/MAX heterodimers [25–27]. Six domains known as MYC homology boxes (MBs) make up the N-terminal region of MYC proteins. The transactivation domain (TAD) contains the residues MB0 (residues 16–33), MBI (residues 45–65), and MBII (residues 128–144) (**Figure 2**). These domains are essential for preserving protein stability, allowing interactions between proteins, and regulating the transcriptional activation or repression of target genes [25, 28, 29]. MB0 interacts with transcription elongation factors and contributes to tumor progression, while MBI plays a role in regulating MYC degradation via the ubiquitin-proteasome pathway [29, 30]. MBII, the better characterized region within TAD, is essential for key MYC properties, including specific DNA binding, autoregulation, and transcriptional activity [28]. Transcription of MYC-bound genes can be activated through the recruitment of histone acetyltransferase (HAT) complexes by MBII binding to transformation/transcription domain-associated protein (TRRAP) [29, 31, 32]. In

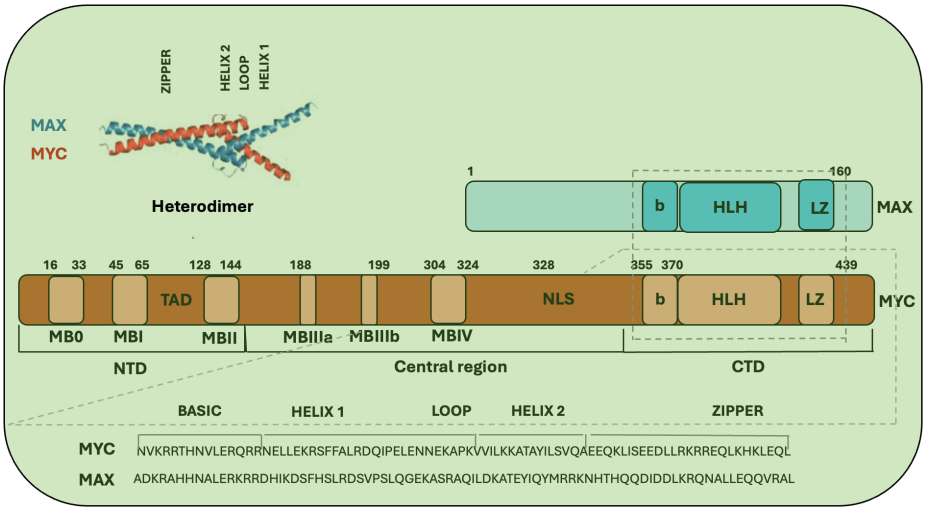

Figure 2.
Domain structure of MYC and MAX proteins. MYC and MAX domains. The N-terminal region of MYC engages with several interacting partners, while the C-terminal region associates with MAX through the b-HLH-LZ motif. The resulting MYC/MAX heterodimer recognizes and binds E-box sequences within the DNA of target genes.

the central region of MYC, there are three additional conserved domains: MBIIIa, MBIIIb, and MBIV (**Figure 2**). MBIIIa interacts with histone deacetylases (HDACs) and is involved in gene repression mediated by MYC, while MBIV contributes to DNA binding, though its precise mechanism remains unclear [33]. MBIIIb interacts with WD repeat-containing protein 5 (WDR5), a protein having WD-40 repeats, in order to connect MYC to chromatin and control the expression of genes involved in protein synthesis [34, 35]. Mutational analyses have revealed that mutations in MBI and MBII significantly impair MYC amplification, whereas mutations in MBIII enhance it. These data indicate that each MB domain interacts with distinct protein partners, affecting chromatin remodeling and transcription throughout the transcriptional cycle [36].

The structure of MYC is fundamental for its function as a transcriptional regulator and for the precise control of numerous cellular processes. In fact, MYC promotes the entry of cells into the S phase, regulating the cell cycle and activating genes, such as cyclin D, cyclin E, and cyclin-dependent kinase-4 (CDK4), essential for cell cycle progression, and simultaneously repressing cell cycle inhibitors, such as p21 and p27, ensuring continued proliferation [37, 38]. Furthermore, MYC modulates cellular metabolism to support the high energetic and biosynthetic demands of proliferation. It activates glycolysis enzymes, glutamine metabolism, and lipid and nucleotide biosynthesis, adapting the metabolism of tumor cells to the so-called Warburg effect [39]. MYC, under normal conditions, maintains cellular homeostasis, coordinating cell survival through the activation of proliferative pathways and promoting apoptosis after significant stresses, such as irreparable DNA damage [8]. Indeed, it can induce the activation of pro-apoptotic genes such as BAX and PUMA, while repressing anti-apoptotic genes such as BCL-2. However, in tumors, its regulation is subverted [5, 8, 40]. MYC overexpression, and in particular its hyperactivity, alters the correct balance between proliferation and apoptosis by enhancing growth signals and reducing sensitivity to programmed cell death signals [41, 42]. This "oncogenic addiction" makes MYC a crucial therapeutic target for the development of selective drugs.

3. MYC regulation between cell death and survival

Cell death is the irreversible arrest of cellular activity and an important physiological process in all organisms. It plays a crucial role in embryonic development, organ maintenance, and immune regulation [43]. In recent years, a greater understanding of the mechanism of programmed cell death or apoptosis has been gained and some key genes in this process have been identified such as MYC [44], which has a crucial role in the regulation of cell death through its ability to influence various pro-apoptotic pathways (BAX, PUMA, and NOXA (phorbol-12-myristate-13-acetate-induced protein 1) and anti-apoptotic pathways (BCL-2) [8, 45]. MYC regulates apoptosis through intrinsic and extrinsic death regulators: in particular, it stimulates the transcription of the intrinsic regulator BAX, which accumulates on the outer mitochondrial membrane facilitating its permeabilization [8, 46, 47]. This event triggers the movement of cytochrome c into the cytoplasm, promoting the formation of the apoptosome and subsequently the activation of caspases, triggering a cascade of events that culminate in DNA fragmentation and cell death [48–50]. MYC also has an involvement in the expression of two intrinsic targets: PUMA and NOXA, which neutralize anti-apoptotic proteins such as BCL-2 and B-cell lymphoma-extra large (BCL-XL), amplifying the apoptotic effect [51]. This function is enhanced by the ability of MYC to cooperate with p53, stabilizing and enhancing its transcriptional activity [52]. However, this balance can be disrupted in tumors, in which p53 is mutated or inactive, reducing the effectiveness of MYC-mediated apoptosis and promoting the survival of malignant cells [53, 54]. MYC also regulates the suppression of BCL-2, which plays a key role in protecting mitochondria from permeabilization. MYC-mediated suppression of BCL-2 controls the balance between pro- and anti-apoptotic factors, inducing programmed cell death in the absence of survival signals [55, 56]. In tumors, this balance is dysregulated, with an increase in the persistence of tumor cells and a reduction in their responsiveness to apoptotic stimuli [57]. Despite the complex dual role of MYC in cell growth and cell survival, in response to tumor microenvironment stimuli, the activation or inactivation of certain genes involved in these processes, aberrant MYC expression often exerts a dominant effect by suppressing apoptosis allowing cells to survive and proliferate in an uncontrolled manner (**Figure 3**) [58–60]. The paradoxical aspect of MYC biology is fully manifested in the concept of oncogene addiction, where tumor cells exploit its hyperactivity to sustain uncontrolled growth. Recent studies have reported the impact of MYC on several survival pathways, including the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway [61]. MYC, by positively regulating AKT signaling, indirectly promotes cell survival and inhibits apoptotic processes. In addition, AKT activation leads to the phosphorylation and inhibition of pro-apoptotic proteins, such as BCL-2-associated agonist of cell death (BAD), preventing cell death [62]. It has been reported that MYC enhances the anti-apoptotic gene expression, such as BCL-2 and BCL-XL. These proteins block the mitochondrial outer membrane permeabilization (MOMP), a critical step in the intrinsic apoptotic pathway [63]. By inhibiting MOMP, MYC effectively prevents the release of cytochrome c and the activation of caspases, which are key components of apoptosis [64]. MYC can suppress the pro-apoptotic genes' transcription, such as BAX, thus preventing apoptosis and promoting cell proliferation, even under stress conditions [65]. Dysregulation of MYC can affect the appropriate function of p53, which has an essential function in the apoptotic process. MYC-driven cells often find mechanisms to inactivate or bypass p53 signaling [66, 67]. For example, MYC can increase the levels of murine double

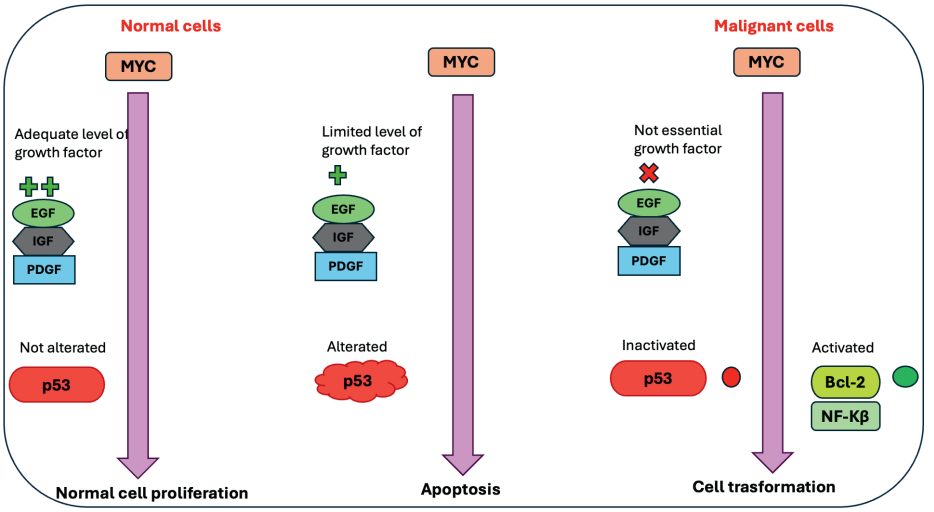

Figure 3.
Differences between apoptotic signaling pathways in normal and malignant cells. In the first representation (left), MYC is shown in normal cells and in the presence of a normal level of growth factor that led to normal cell proliferation without altering p53; in the center of the figure, when myc is associated with a limited level of growth factor, there is an alteration of the p53 level that causes apoptosis; in the last part of the image (right), there is the involvement of MYC in malignant cells, associated with the activation of some genes such as BCL-2 and nuclear factor kappa B (NF-κB) and the absence of growth factor, p53 is inactivated and the final result is cellular transformation.

minute (MDM2), a negative regulator of p53, leading to a decrease in p53-mediated apoptosis. This is essential to allow MYC-overexpressing cells to proliferate, despite DNA damage or other stressors that would normally activate p53 [68]. Dysregulation of MYC then leads to rapid cellular proliferation, which can overwhelm normal DNA repair mechanisms and increase genomic instability, leading to the accumulation of mutations that can further inhibit apoptosis pathways, strengthening the pro-survival role of MYC [69].

4. Role of MYC in regulating apoptosis

MYC plays a crucial role in the control of multiple cellular processes, including proliferation, metabolism, and programmed cell death [70]. Although, MYC is primarily known for its ability to promote cell growth and proliferation, it also acts as a potent sensitizer of apoptotic signals [71]. This dual role of MYC is particularly relevant in the context of cancer, where the balance between proliferation and apoptosis determines the fate of transformed cells [72]. MYC is able to regulate both the intrinsic (mitochondrial) and extrinsic (death receptor-mediated) pathways of apoptosis [73]. The intrinsic pathway is characterized by the permeabilization of the mitochondrial membrane and the release of cytochrome c into the cytoplasm, an event that activates a caspase cascade, culminating in the degradation of cellular components [74]. MYC stimulates the expression of pro-apoptotic genes, such as BAX, BID, and PUMA, which promote the opening of mitochondrial pores and the release of cytochrome c, activating caspase-9 and, subsequently, executioner caspases such as caspase-3 and caspase-7 [75]. However, in tumor cells, this pro-apoptotic

activity of MYC is often counteracted by the upregulation of anti-apoptotic proteins, such as BCL-2 and BCL-XL, which stabilize the mitochondrial membrane and prevent the initiation of the apoptotic cascade [76, 77]. In tumor contexts, MYC hyperactivity is associated with increased replicative stress and the production of reactive oxygen species (ROS), which can cause DNA damage and activate apoptosis [78]. However, mutations in tumor suppressor genes, such as p53, allow tumor cells to survive, despite high levels of stress. Indeed, p53 mutations, frequently observed in tumors, impair the ability of cells to respond to cellular damage by apoptosis [79, 80]. MYC modulates the balance between pro- and anti-apoptotic members of the BCL-2 family, both in a p53-dependent and -independent manner. Studies in Eμ-MYC transgenic mouse models of lymphoma have shown that MYC can regulate the expression of proteins such as BCL-2 and BCL-XL. In more than half of murine lymphomas, the levels of these anti-apoptotic proteins are elevated, even in the absence of functional p53, suggesting a p53-independent mechanism [76]. At the same time, MYC is able to indirectly suppress BCL-XL expression by reducing its RNA and protein levels. Such repression requires a new protein synthesis, suggesting an indirect transcriptional regulatory mechanism [77]. In parallel, MYC stimulates the expression of pro-apoptotic members such as BAX and BAK, which are essential for the activation of the mitochondrial apoptotic pathway. Indeed, MYC overexpression is associated with an increase in BAX expression at the transcriptional level, enhancing its functional activation [81]. The extrinsic apoptosis pathway is activated by the interaction between death receptors, such as FAS and TRAIL receptor (TRAIL-R, tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor), and their respective ligands. MYC is known to sensitize cells to these apoptotic signals by stimulating the expression of death receptors [82]. Activation of receptors such as FAS leads to the formation of the Death-Inducing Signaling Complex (DISC) and activation of caspase-8, which can act directly on executioner caspases or interact with the intrinsic pathway through the cleavage of BH3 interacting-domain death agonist (BID), an activator of mitochondrial permeabilization [83]. However, in tumors, the extrinsic apoptosis pathway is often deactivated by mechanisms that reduce the expression of death receptors or increase the levels of inhibitors, such as cellular FLICE-like inhibitory protein (c-FLIP), which block caspase-8 activation [84]. Furthermore, MYC counteracts survival signals associated with the death receptor pathway, such as activation of NF-κB, a known pro-survival factor [85]. Understanding the role of MYC in regulating apoptosis has important implications for the development of anticancer therapies. Drugs targeting anti-apoptotic proteins, such as venetoclax, a BCL-2 inhibitor, can reactivate the mitochondrial apoptotic cascade in tumor cells with overactive MYC, especially in hematological malignancies [86]. Similarly, restoring p53 function by compounds such as nutlin-3 could potentiate MYC-induced apoptosis [87]. Another promising strategy is the activation of the extrinsic apoptosis pathway by TRAIL ligands or agonist antibodies against death receptors [88]. These approaches exploit the predisposition of tumor cells with overactive MYC to succumb to apoptotic signals, offering therapeutic opportunities to treat aggressive tumors that are resistant to conventional therapies.

5. MYC in tumor progression

Cancer is a multifaceted process that needs the occurrence of multiple genetic alterations and the acquisition of distinct biological characteristics [89]. Notably,

MYC activation is involved in multiple genetic deregulation and in the development of specific biological characteristics, such as proliferation, cell survival, metabolic alterations, angiogenesis, metastasis, and relapse [90]. Early in vitro investigations of MYC demonstrated its transformative potential when combined with other oncogenes in embryonic fibroblasts [91]. These findings demonstrated the ability of MYC to independently induce tumors in multiple tissues, while revealing its dependence on additional genetic alterations for complete tumorigenic activity in vivo [92]. For example, breast cancer induced by the transgenic expression of MYC developed K-Ras mutations, resulting in more aggressive tumors [93]. Similarly, induction of MYC in normal cells activates checkpoints such as Arf or p53, resulting in cell cycle arrest or apoptosis. In MYC-induced transgenic lymphomas, however, the proper functioning of Arf and p53 is subverted [94]. MYC controls the expression of different genes that influence cell growth, survival, and metabolism and directly impact tumor biology and the tumor microenvironment [95]. The role of MYC extends to modulating immune responses and other extrinsic factors that shape cancer progression [25]. Initially, MYC members were thought to play a universal role in human cancers, both hematological and solid, with L-MYC thought to be linked only to small cell lung cancer and N-MYC to neuroblastoma [96]. However, N-MYC and L-MYC seem to be involved in the occurrence of other cancers [97]. Thus, when examining the entire MYC family, it is clear that genetic activation of at least one of its members is a recurrent event in the majority of human cancers [5]. Dysregulation of MYC and related pathways is among the most widespread in human cancers. MYC hyperactivation in cancer occurs through epigenetic, genomic, and post-translational mechanisms, including amplifications, translocations, and upstream regulatory changes of its gene [98]. A pan-cancer analysis of 33 different human cancers, as reported in The Cancer Genome Atlas dataset, found that in 28% of tumors all members of the MYC family are amplified (**Figure 4**) [99]. These amplifications can cause MYC overexpression, either directly or indirectly, by activating genes involved in the MYC pathway [100]. MYC undergoes genetic amplification in several solid tumors, such as breast and liver

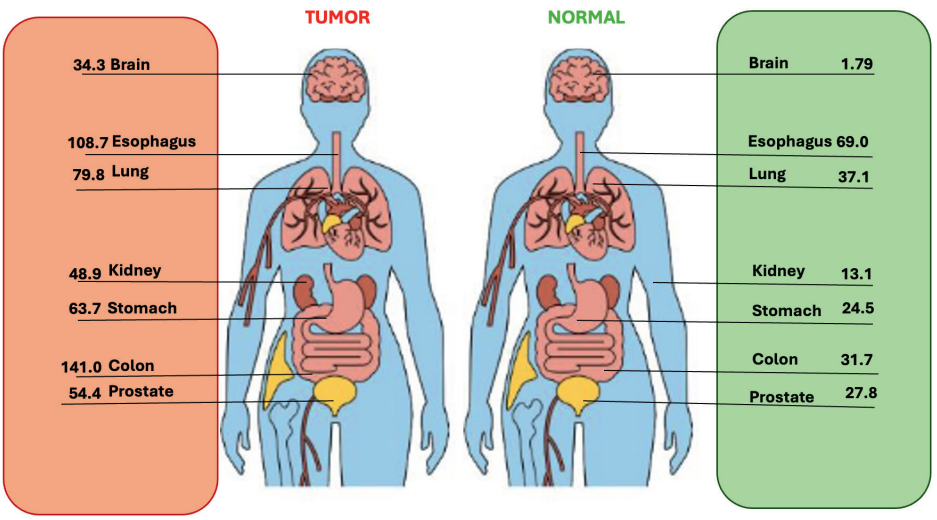

Figure 4.
Body map showing MYC expression in TPM (transcripts per million) different in tumor (left) and normal (right) human system.

cancers, and is often implicated in chromosomal translocations observed in B-cell and T-cell leukemias and lymphomas [101]. Retroviruses can enhance MYC expression through various mechanisms, including the insertion of enhancers upstream of the MYC gene and the activation of several oncogenic pathways such as SRC and Notch or loss of tumor suppressors such as adenomatous polyposis coli (APC) and transforming growth factor-beta (TGF-β) [102]. A lot of post-translational modifications determine MYC protein stability [103]. In normal conditions, a fine balance of phosphorylation events and proteasomal degradation determines the short half-life of MYC [104]. In tumors characterized by MYC overexpression, there is a notable increase in phospho-serine 62 (P-S62-MYC) levels and a corresponding decrease in phospho-threonine 58 (P-T58-MYC) levels. These alterations contribute to enhanced MYC stability and activity [105]. Rat sarcoma virus (RAS)-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling mitogenic pathway increases the levels of P-S62, destabilizing MYC. MYC T58-related mutations can cause constitutive phosphorylation of S62. Tumors can also decrease the expression of the serine/threonine protein phosphatase 2A (PP2A), that dephosphorylating P-S62 leads to an accumulated amount of MYC [106]. In addition, peptidyl-prolyl cis-trans isomerase, NIMA-interacting 1 (PIN1), a peptidyl-prolyl cis-trans isomerase (PPIase), can contribute to MYC regulation in several tumors, such as uterine cancer (18%), colon cancer (16%), and cervical cancer (13%), through deletion, mutation, or epigenetic changes. These events can enhance tumor growth by increasing MYC levels [107]. Although MYC plays a central role in tumorigenesis, its activation alone is typically insufficient to convert normal cells into malignant ones [108]. Apoptosis, senescence, or cell cycle arrest are physiological processes that frequently impair MYC carcinogenic function. MYC dysregulation can induce p53 activation [109], cyclin-dependent kinase inhibitor 2A (CDKN2A) upregulation [110], or BCL-2 modulation [111, 112]. An additionally protective mechanism against tumorigenesis is the shortening of telomeres during cell division cycles, which limits proliferation [113]. However, MYC can promote cellular immortality by stimulating telomerase activity, through the regulation of telomerase reverse transcriptase [114]. These multiple levels of MYC regulation and dysfunction underscore its critical role in driving cancer progression.

6. MYC, a therapeutic target

Targeting MYC has demonstrated the ability to induce tumor regression through both direct and indirect effects, but it remains a therapeutic challenge due to MYC's disordered structure and lack of a specific binding site or defined enzymatic activity [115]. Decades of research have highlighted several strategies to inhibit MYC activity that are emerging in preclinical models and clinical research [116]. Direct strategies include silencing MYC gene expression, disrupting MYC protein synthesis, or promoting its degradation via the proteasome (**Figure 5**) [117]. The MYC/MAX interaction is critical for the oncogenic function of MYC, and many approaches target this interaction [118]. For example, small compounds, such as MYCi361 and MYCi975, disrupt the MYC-MAX interface and promote phosphorylation of MYC on threonine-58, promoting proteasomal degradation of MYC [119]. Another study reported that KI-MS2-008 stabilizes MAX-MAX homodimers, reducing the levels of MYC and its transcriptional targets and suppressing tumor growth [120]. ME47, a minimalist small hybrid protein (MHP), disrupts the interaction with MAX by

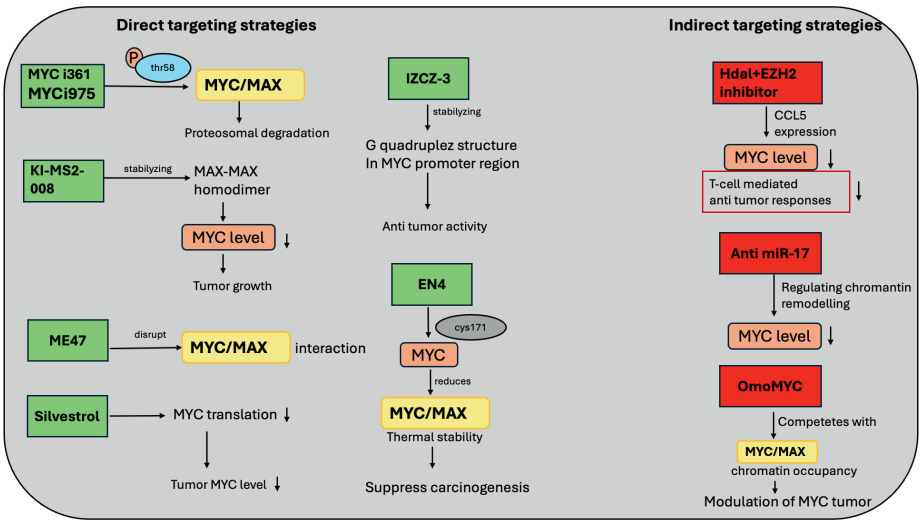

Figure 5.
Effects of several inhibitors on MYC levels. Direct effect (left) through some inhibitors such as IZCZ3 and EN4 that led to antitumor activity and suppression of carcinogenesis; on the right of the figure is shown the indirect effect of some epigenetic inhibitors and several others causing a "positive" modulation of MYC in tumor.

suppressing the transcriptional activity of MYC. These results suggest that MHPs offer an alternative therapeutic targeting method for transcription factors involved in human diseases, including cancer [121]. EN4, on the other hand, binds to an intrinsically disordered region of MYC (cysteine 171), reduces the thermal stability of MYC and MAX, inhibits MYC transcriptional activity, and suppresses carcinogenesis [122]. Another promising approach is to decrease MYC biosynthesis or enhance its degradation. Inhibitors targeting the PI3K-AKT-mTOR (mammalian target of rapamycin) signaling pathway have been shown to reduce MYC translation and lower MYC levels in tumors in mouse models [123]. Additionally, Silvestrol, an inhibitor of the translation initiator eukaryotic initiation factor-4A (eIF4A), suppresses MYC translation and prevents tumor growth in colon cancer mouse models [124]. Furthermore, Aurora kinase inhibitors can promote degradation of MYC protein and specifically reduce its overexpression in tumor cells without altering physiological expression of MYC [125]. Recently, substantial advancements have been achieved in developing specific protein degraders or chimeras, designed to directly induce the proteolysis of MYC (proteolysis targeting chimeras (PROTACs)) [126]. These approaches utilize the ubiquitin-proteasome system to degrade MYC, using bifunctional molecules with two components: a ligand that specifically binds to MYC and a ligand that targets an E3 ubiquitin ligase, such as cereblon or von Hippel-Lindau (VHL) [127]. Another potential strategy to reduce MYC levels is to target the stability of its messenger RNA (mRNA). Antisense oligonucleotides (ASOs), such as MYC-ASO, have been shown to reduce tumor development and stimulate antitumor immune responses in mouse models [128]. Another approach is to suppress MYC transcription. Bromodomain and extra-terminal motif (BET) inhibitors, epigenetic regulators, including IZCZ-3, that stabilize the G-quadruplex structure in the MYC promoter region, have shown antitumor activity [129]. Indirect targeting strategies exploit synthetic lethal interactions, targeting vulnerabilities in MYC-driven cells (**Figure 5**). For example, inhibiting MYC transcriptional activity through genetic approaches, such as the use of

OmoMYC, a dominant negative allele that competes with MYC/MAX for chromatin binding, reducing MYC/MAX occupancy at its targets and modulating gene expression in tumors with high MYC oncogenic activity [130]. Epigenetic modifiers, such as histone deacetylase inhibitor (HDACi) and histone methyltransferase (Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2)) inhibitors, have been used to treat MYC-induced malignancies. The combination of HDACi and EZH2 reduced MYC levels and activated immune transcriptional pathways, enhancing T-cell-mediated antitumor responses through C-C motif chemokine ligand 5 (CCL5) expression [131, 132]. Recent studies have shown that the use of epigenetic treatments can reduce MYC, reversing immune evasion. Similarly, MYC can cause cancer by regulating chromatin remodeling via miR17-92. Anti-miR-17 oligonucleotides inhibited tumor progression in a liver cancer mouse model [133]. Research continues to develop innovative approaches to target MYC, exploiting both its molecular dependencies and interactions with the immune system. These advances offer new perspectives for the treatment of MYC-induced malignancies.

7. Emerging strategies for targeting MYC in cancer therapy

Targeting MYC in cancer therapy is a promising but challenging frontier due to its critical role in cellular processes and its classification as a "difficult-to-drug" molecule [134]. Future directions to overcome these challenges and exploit MYC as a therapeutic target include several innovative approaches [135]. Studies are underway to design small molecules that disrupt MYC-MAX dimerization, which is essential for MYC transcriptional activity [136]. Additionally, PROTACs are being explored to selectively degrade MYC protein by exploiting the ubiquitin-proteasome system [137]. Clinical trials of OmoMYV a MYC inhibitory peptide, are expanding, along with investigations of its derivatives and other direct MYC inhibitors [138]. Tumors that overexpress MYC have specific vulnerabilities, such as dependence on cyclin-dependent kinases (CDKs), DNA repair enzymes (checkpoint kinase 1 (CHK1), ataxia-telangiectasia-mutated-and-Rad3-related kinase (ATR)), and anti-apoptotic proteins such as myeloid cell leukemia 1 (MCL1). Combining MYC inhibition with drugs that target these synthetic lethal pathways promises to improve therapeutic efficacy and mitigate resistance [5, 139]. HDACi are being explored to regulate MYC-driven transcriptional programs [140, 141]. Similarly, BET inhibitors, which block MYC transcription by interfering with chromatin regulators, offer another promising avenue for treatment [142]. Techniques such as antisense RNA are being developed to directly silence overexpressed MYC mRNA [143]. In addition, MYC inhibition is being combined with immune checkpoint inhibitors to exploit MYC's role in immune evasion [144]. These multifaceted approaches, individually and in combination, aim to address the complexity of MYC targeting and offer hope for more effective and personalized cancer therapies in the future.

8. Conclusions

MYC plays a pivotal and multifaceted role in tumorigenesis and cancer progression, regulating numerous cellular processes, such as the cell cycle, metabolism, and transcription [145]. It functions as a key determinant of the balance between cell survival and cell death through its transcriptional regulation of a wide range of genes [146].

This dual role makes MYC a critical factor in carcinogenesis. When functioning correctly, MYC can drive cells toward apoptosis, acting as a safeguard against uncontrolled proliferation [147]. However, dysregulation of MYC, often resulting from mutations or concurrent alterations in other oncogenes or tumor suppressor genes, can circumvent apoptosis and promote unchecked cell growth [148].

Despite its critical involvement in cancer, targeting MYC directly poses significant challenges. Its lack of enzymatic pockets and its pervasive overexpression in tumors make it a difficult therapeutic target [108]. Nevertheless, substantial progress has been made in developing strategies to inhibit MYC activity. However, clinical treatments that effectively target MYC remain elusive [149]. An understanding of the mechanisms by which MYC governs tumorigenesis versus apoptosis is essential [103]. Such insights could refine therapeutic approaches, enabling the precise targeting of MYC in cancer cells while minimizing adverse effects.

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Conflict of interest

The authors declare no conflict of interest.

Acronyms and abbreviations

ASO	antisense oligonucleotides
bHLH-LZ	basic helix-loop-helix leucine zipper
CDK	cyclin-dependent kinases
DISC	death-inducing signaling complex
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
HAT	histone acetyltransferase
HDACs	histone deacetylases
HDACi	histone deacetylase inhibitor
MBs	MYC homology boxes
MHP	minimalist small hybrid protein
MOMP	mitochondrial outer membrane permeabilization
PPIase	peptidyl-prolyl cis-trans isomerase
PROTAC	proteolysis targeting chimera
P-S62-MYC	phospho-serine 62
P-T58-MYC	phospho-threonine 58
ROS	reactive oxygen species
TRRAP	transformation/transcription domain-associated protein
VEGF	vascular endothelial growth factor

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Section 3

Cell Death and Immunity

Chapter 8

Inflammation and the Immune System: A Delicate Balance

Priyanka Mehta

Abstract

Inflammation and the immune system are fundamental components of human health, working in tandem to combat infections, repair tissue damage, and maintain homeostasis. The immune system comprises innate and adaptive immunity, orchestrating protective responses against pathogens while regulating inflammation. Acute inflammation, characterized by rapid immune responses involving neutrophils and cytokines, promotes tissue repair and pathogen clearance. However, chronic inflammation, driven by persistent stimuli, is associated with tissue damage and diseases such as cancer, cardiovascular disorders, and autoimmune conditions. Cell death mechanisms, including apoptosis, pyroptosis, and necroptosis, further regulate immune responses by eliminating infected or damaged cells. Immune checkpoints, such as PD-1 and CTLA-4, act as critical regulators to prevent excessive inflammation and maintain self-tolerance. Dysregulation of these pathways leads to pathological conditions, including chronic inflammation, autoimmunity, and tumorigenesis. Emerging therapeutic strategies targeting cytokines, inflammasomes, and cell death pathways offer promising approaches to modulate inflammation and restore immune balance. Understanding the intricate interplay between inflammation, immune regulation, and cell death is essential for developing targeted interventions against inflammation-related diseases.

Keywords: inflammation, immune response, innate immunity, adaptive immunity, inflammatory disorders

1. Introduction

Inflammation is a fundamental physiological response that enables the body to combat infection, repair tissue damage, and maintain homeostasis. Orchestrated by the immune system, this dynamic process is tightly regulated to ensure an appropriate and effective response to injury or pathogenic challenges while minimizing collateral damage to host tissues [1]. The interplay between inflammation and immunity is central to health, functioning as both a protective mechanism and a potential driver of disease when dysregulated.

The immune system, comprising innate and adaptive arms, plays a pivotal role in initiating and resolving inflammation. Innate immunity provides the first line of defense through rapid, non-specific responses involving immune cells such as macrophages, neutrophils, and dendritic cells. In contrast, adaptive immunity offers specificity and

memory mediated by lymphocytes, including T and B cells [2]. Cytokines and chemokines serve as critical mediators, coordinating cellular communication and guiding the inflammatory cascade. These processes are intricately regulated by signaling pathways, such as the nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which balance pro- and anti-inflammatory signals [3].

Homeostasis is achieved through a delicate equilibrium between immune activation and suppression. However, disruptions in this balance, driven by genetic predisposition, environmental factors, or chronic stressors, can lead to pathological conditions. Chronic inflammation is implicated in a spectrum of diseases, including autoimmune disorders, cancer, cardiovascular diseases, and neurodegenerative conditions. This chapter explores the molecular and cellular mechanisms underpinning inflammation, its integration with immune function, and its role in health and disease, emphasizing the importance of maintaining immune equilibrium.

1.1 Definition and overview of inflammation and immune response

Inflammation and immune response are fundamental processes that protect the body from harmful pathogens, toxins, and injuries. They represent the body's defense mechanisms, with inflammation serving as an immediate reaction to tissue damage or infection, and the immune response involving a more specific, longer-term defense against pathogens. Inflammation can occur due to various stimuli such as pathogens (bacteria, viruses, and fungi), injured cells, or irritants. The immune response is triggered when foreign substances, known as antigens, are detected by immune cells, leading to the activation of various immune mechanisms [4].

Inflammation and the immune response are highly interconnected. The innate immune system triggers inflammation to contain infection and tissue damage, while adaptive immunity is responsible for the specific elimination of pathogens. Once the pathogen is cleared, anti-inflammatory signals help resolve the inflammation and promote tissue repair [5].

Chronic or uncontrolled inflammation can contribute to various diseases, including autoimmune disorders, atherosclerosis, and cancer. Conversely, an impaired immune response can lead to increased susceptibility to infections and malignancies [6].

1.2 Importance of inflammation and immune system in human health

Inflammation and the immune system are critical components of human health, playing both protective and potentially harmful roles. Inflammation is a natural biological response to injury or infection, initiated by the immune system to remove harmful stimuli and begin tissue repair. This process involves the release of various signaling molecules, such as cytokines and chemokines, which recruit immune cells to the affected area. Acute inflammation is essential for healing, as it isolates and eliminates pathogens, removes dead cells, and stimulates repair mechanisms [7]. However, when inflammation becomes chronic or dysregulated, it can contribute to various diseases, including cardiovascular diseases, cancer, autoimmune conditions, and neurodegenerative disorders.

The immune system, comprising both innate and adaptive immunity, acts as a defense mechanism to protect the body against foreign pathogens like bacteria, viruses, and fungi. Innate immunity provides an immediate, non-specific response, while adaptive immunity creates long-term, antigen-specific protection through the activation of T and B lymphocytes [2]. This system also maintains immunological

memory, allowing the body to recognize and respond more rapidly to previously encountered pathogens. Together with the inflammatory response, the immune system ensures that the body can detect and neutralize harmful agents while also preserving healthy tissues [6].

An intricate balance between inflammation and immune response is essential for health; excessive or insufficient activity in either can lead to pathology. For instance, while inflammation helps control infections and promotes tissue repair, chronic inflammation has been linked to metabolic syndrome, diabetes, and obesity. Similarly, immune dysregulation, either through weakened immunity or autoimmunity, can make individuals vulnerable to infections or cause the immune system to target its own tissues, as seen in lupus and rheumatoid arthritis. Understanding the dual nature of inflammation and immunity is pivotal for developing therapeutic strategies that enhance immune function and modulate inflammation, potentially preventing or treating various chronic diseases.

2. Immune system components

Immune system is an extremely complex network of cells, tissues, and organs that constantly look out for invaders, and once it is spotted, an immune response is mounted to eliminate the invader from the body. The immune system is under tight regulation, and when the regulation is disrupted, it results in pathological conditions, allergic diseases, immunodeficiencies, and autoimmune disorders [8]. Although all components of the immune system interact with each other, the function of these components can be broadly divided into two categories: innate immune responses and adaptive immune responses. Innate immune responses are non-specific in nature and rely on cells that require no learning, whereas adaptive immune responses are specific in nature and require learning [9].

2.1 Innate immunity consists of four types of defense barriers

i. Anatomic: skin and mucous membrane.

ii. Physiological: temperature, low pH, and chemical radiators.

iii. Phagocytic/endocytic: monocytes, macrophages, and neutrophils.

iv. Inflammatory barriers.

2.2 Adaptive immunity

It is the second line of defense. It comes into action when innate immunity has been overwhelmed and is no longer effective in eliminating pathogens. It generates a specific and enhanced response and can store information about the invading pathogen, hence developing immunologic memory that can quickly eliminate the pathogen upon subsequent encounters. But the primary function of adaptive immunity is to discriminate between 'self' and 'non-self' antigens [10]. The key players of the adaptive immune system are antigen-specific T cells and B cells, which differentiate into plasma cells to produce antigen-specific antibodies [11].

2.2.1 Innate versus adaptive immunity: Nature's first responders versus specialist defenders

See **Figure 1**.

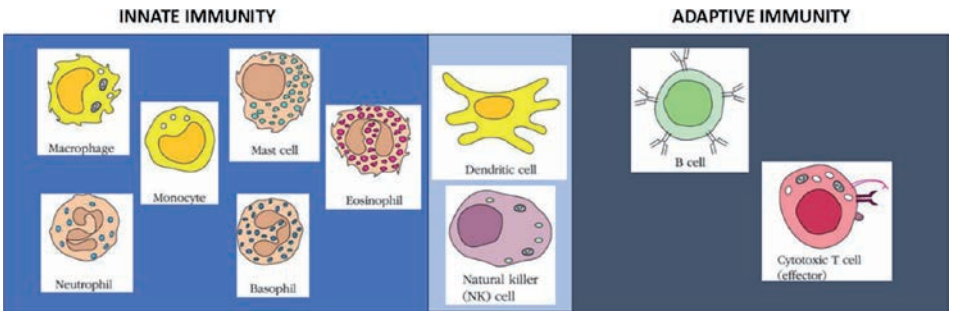

Figure 1.
The diagram illustrates the distinct features of innate and adaptive immunity, highlighting their respective components.

3. Organs of immune system

The innate immune system consists of (i) primary lymphoid organs which control the production and maturation of immune cells (B and T cells), that is, bone marrow and thymus. (ii) Secondary lymphoid organs where the immune cells perform their function by interacting with the antigen, that is, lymph nodes, spleen, tonsils, and mucosa-associated lymphoid tissue (MALT). Together, they are referred to as lymphoid organs of tissue [12].

4. Types of inflammation

Inflammation is broadly classified into two main types: acute and chronic inflammation. Both types represent fundamental responses of the immune system to harmful stimuli, yet they differ significantly in their duration, underlying mechanisms, and clinical implications. Acute inflammation is a rapid, short-term response to injury or infection, typically resolving within a few hours to days. It is characterized by the classic signs of redness, heat, swelling, pain, and loss of function, resulting from the vascular and cellular responses that aim to isolate and eliminate the offending agent and initiate repair [13]. During acute inflammation, immune cells, particularly neutrophils, are rapidly recruited to the site of injury. Pro-inflammatory mediators like cytokines (e.g., IL-1 and TNF-α) and prostaglandins are released, causing vasodilation and increased vascular permeability, which allows immune cells to enter the tissue more readily [14]. This process culminates in the clearance of pathogens, debris, and damaged cells, after which anti-inflammatory signals facilitate tissue repair and resolution of the inflammatory response.

Chronic inflammation, on the other hand, is a prolonged, often unresolved inflammatory response that can last from several weeks to years, resulting from the persistent presence of an inflammatory stimulus [15]. Unlike acute inflammation, which primarily involves neutrophils, chronic inflammation is dominated by

Feature	Acute inflammation	Chronic inflammation
Duration	Short-term (minutes to a few days)	Long-term (weeks, months, or years)
Onset	Rapid onset (immediate or within hours)	Slow, progressive onset
Primary Cells Involved	Neutrophils predominate	Lymphocytes, macrophages, and plasma cells
Symptoms	Pronounced and noticeable (redness, swelling, pain, heat)	Less noticeable or asymptomatic; persistent tissue damage
Outcome	Resolution with minimal tissue damage, or progression to chronic	Persistent inflammation; fibrosis or tissue damage common
Cause	Usually triggered by infection, injury, or irritants	Persistent infections, autoimmune diseases, or prolonged exposure to irritants

Table 1.
Comparison between acute and chronic inflammation.

mononuclear cells such as macrophages and lymphocytes. Chronic inflammation can arise due to a variety of factors, including persistent infections (e.g., tuberculosis), exposure to irritants (e.g., silica and asbestos), autoimmune reactions (where the immune system mistakenly attacks the body's tissues), or metabolic dysregulation as seen in obesity [16]. Chronic inflammation is marked by the simultaneous occurrence of tissue destruction and repair, mediated by pro-inflammatory cytokines, growth factors, and matrix-degrading enzymes that can lead to tissue remodeling and fibrosis [17]. This prolonged inflammatory state is implicated in the pathogenesis of many chronic diseases, including cardiovascular disease, type 2 diabetes, cancer, and neurodegenerative disorders. Unlike acute inflammation, chronic inflammation often lacks the overt signs of inflammation, making it difficult to detect in its early stages but posing a significant risk to long-term health (**Table 1**).

Another distinct form of inflammation, granulomatous inflammation, is often classified as a subset of chronic inflammation. It involves the formation of granulomas, which are small nodular aggregations of macrophages, often fused into giant cells, surrounded by lymphocytes and fibroblasts [18]. This type of inflammation occurs when the immune system attempts to contain a persistent irritant or pathogen that it cannot eradicate, such as tuberculosis, sarcoidosis, and certain fungal infections. Granulomatous inflammation represents a unique immune response where macrophages differentiate into specialized forms to encapsulate and isolate the offending agents [19]. This type of inflammation serves as a protective mechanism, but granulomas can also disrupt normal tissue function if they accumulate in large numbers [18]. Each type of inflammation—acute, chronic, and granulomatous—illustrates the complex, multifaceted nature of the immune response, which can range from a beneficial, targeted defense mechanism to a persistent, potentially damaging process depending on the context and duration of the stimulus. Understanding these types of inflammation is essential for developing targeted therapeutic interventions that address both the protective and pathological aspects of inflammation in various diseases.

5. Role of inflammation in immune surveillance and homeostasis

Inflammation is a critical component of the immune system, serving as a first-line defense mechanism to maintain tissue homeostasis and immune

surveillance [20]. It is initiated in response to infection, injury, or tissue damage and involves the recruitment of immune cells and the release of pro-inflammatory mediators. The primary purpose of inflammation is to eliminate harmful stimuli, clear damaged cells, and initiate tissue repair [21].

During immune surveillance, inflammation plays a key role in recognizing and responding to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) [22]. Activation of PRRs triggers signaling pathways that result in the production of pro-inflammatory cytokines (e.g., IL-1β, TNF-α, and IL-6) and chemokines, which recruit neutrophils, macrophages, and dendritic cells to the site of injury or infection. These cells phagocytose pathogens, present antigens to lymphocytes, and initiate adaptive immune responses [22].

In homeostasis, inflammation resolves through tightly regulated mechanisms to avoid excessive tissue damage. Anti-inflammatory cytokines (e.g., IL-10 and TGF-β), lipid mediators like resolvins and lipoxins, and apoptotic clearance of immune cells collectively promote the resolution of inflammation [23]. Dysregulation of this balance can lead to chronic inflammation, which underlies many pathological conditions such as autoimmune diseases, chronic infections, and cancer [16]. Importantly, inflammation also contributes to immune surveillance by recognizing and eliminating transformed cells through immunogenic cell death pathways [24]. Cytotoxic T cells and NK cells play critical roles in monitoring and removing abnormal cells, preventing tumorigenesis [25]. Thus, inflammation, when properly regulated, maintains immune surveillance and tissue homeostasis. However, persistent or uncontrolled inflammation disrupts this balance, leading to pathological outcomes [16, 20]. Understanding this delicate interplay is crucial for designing therapies targeting inflammation-related disorders.

6. Cell death and mechanisms

Cell death is a fundamental process in maintaining immune system balance, eliminating infected or damaged cells, and resolving inflammation [26]. Immune-mediated cell death occurs through multiple tightly regulated pathways, including apoptosis, pyroptosis, and necroptosis [27]. Each process has distinct molecular mechanisms and functional consequences, contributing to immune responses and disease pathology.

6.1 Apoptosis

Apoptosis is a programmed form of cell death characterized by cell shrinkage, chromatin condensation, membrane blebbing, and the formation of apoptotic bodies. It occurs without inducing inflammation [28]. Apoptosis is essential for immune homeostasis, eliminating autoreactive T cells, infected cells, and cancerous cells while avoiding inflammation [29]. Dysregulation of apoptosis can lead to autoimmune diseases or tumor survival [30]. Apoptosis is regulated by two main pathways:

- *Intrinsic pathway (mitochondrial)*: Triggered by cellular stress or DNA damage, this pathway involves activation of pro-apoptotic proteins (BAX and BAK) that disrupt mitochondrial outer membrane permeability, leading to cytochrome c

release. Cytochrome c binds Apaf-1, activating caspase-9, which subsequently activates executioner caspases (caspase-3/−7) [31].

- *Extrinsic pathway (death receptor-mediated)*: Triggered by extracellular signals *via* death receptors (e.g., Fas/CD95 and TNF-R), this pathway activates caspase-8, which directly cleaves executioner caspases [32].

6.2 Pyroptosis

Pyroptosis is a proinflammatory, lytic form of cell death triggered by intracellular infections or danger signals. It is mediated by inflammasomes, which activate caspase-1 [33]—activated caspase-1 cleaves pro-IL-1β, pro-IL-18, and gasdermin D (GSDMD). GSDMD forms pores in the plasma membrane, leading to cell swelling, membrane rupture, and release of inflammatory cytokines (IL-1β and IL-18) [34]. Pyroptosis enhances host defense by eliminating pathogen replication niches and activating inflammatory responses. However, excessive pyroptosis contributes to septic shock, chronic inflammation, and tissue damage [35].

6.3 Necroptosis

Necroptosis is a regulated necrotic cell death pathway that occurs when apoptosis is inhibited. It is mediated by receptor-interacting protein kinases (RIPK1 and RIPK3) and mixed lineage kinase domain-like protein (MLKL) [36]. Necroptosis is activated through TNF receptor signaling when caspase-8 is inhibited [37]. RIPK1 recruits RIPK3 to form the necrosome complex, which phosphorylates MLKL. Phosphorylated MLKL oligomerizes and disrupts the plasma membrane, causing cell lysis and release of DAMPs [36]. Necroptosis acts as a backup mechanism when apoptosis is blocked, playing a role in viral infections and immune defense. However, uncontrolled necroptosis can lead to chronic inflammation, neurodegeneration, and tissue injury [38].

7. Immune checkpoints and resolution of inflammation

Immune checkpoints are key regulators of the immune system that maintain self-tolerance and prevent excessive inflammation. These checkpoints, consisting of stimulatory and inhibitory pathways, play a pivotal role in controlling immune responses and facilitating the resolution of inflammation [39]. Dysregulation of immune checkpoints can lead to chronic inflammation, autoimmune diseases, and impaired tissue homeostasis.

During inflammation, immune checkpoints act as negative feedback mechanisms to prevent overactivation of immune cells and excessive tissue damage [40]. Inhibitory checkpoint molecules such as programmed cell death protein-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are expressed on activated T cells [41]. Upon engagement with their ligands (PD-L1/PD-L2 for PD-1; CD80/CD86 for CTLA-4), these molecules suppress T-cell activation, proliferation, and cytokine production, thus promoting resolution of inflammation [41]. In parallel, innate immune cells such as macrophages and dendritic cells undergo phenotypic changes during inflammation resolution, transitioning from proinflammatory (M1) to anti-inflammatory (M2) states [42]. This is mediated by anti-inflammatory cytokines

such as IL-10 and TGF-β, which downregulate inflammatory signaling pathways and promote tissue repair [43].

The resolution phase of inflammation also involves the clearance of apoptotic cells (a process called efferocytosis) by phagocytes [44]. This process is tightly regulated by molecules such as Annexin A1, lipoxins, and specialized pro-resolving mediators (SPMs) like resolvins and maresins [44]. These mediators inhibit neutrophil infiltration, promote macrophage-mediated clearance of cellular debris, and restore tissue homeostasis.

8. Therapeutic strategies targeting inflammatory pathways and cell death regulation

The interplay between inflammation and regulated cell death (apoptosis, pyroptosis, and necroptosis) plays a central role in the pathogenesis of many diseases, including autoimmune disorders, cancer, and chronic inflammatory conditions [45]. Therapeutic strategies targeting these processes focus on restoring the balance between inflammation and cell death, either by inhibiting excessive inflammation or promoting regulated cell clearance.

8.1 Targeting inflammatory pathways

- *Cytokine inhibition*: Proinflammatory cytokines like TNF-α, IL-1β, IL-6, and IFN-γ play key roles in driving inflammation and dysregulated cell death. Monoclonal antibodies such as infliximab and adalimumab (anti-TNF-α) are widely used in rheumatoid arthritis, Crohn's disease, and psoriasis to inhibit cytokine signaling and reduce inflammation [46]. Anti-IL-1β therapies like canakinumab are effective in autoinflammatory syndromes, while anti-IL-6 therapies (e.g., tocilizumab) have shown efficacy in rheumatoid arthritis and COVID-19 cytokine storms [47].

- *Inflammasome inhibition*: Inflammasomes, such as NLRP3, activate caspase-1, leading to pyroptosis and IL-1β release. Small molecule inhibitors like MCC950 and oltipraz block NLRP3 activation, reducing pyroptosis and inflammatory cytokine production in conditions such as gout, metabolic disorders, and neuroinflammation [48].

- *Resolution mediators*: Specialized pro-resolving mediators (SPMs) like resolvins, protectins, and maresins derived from omega-3 fatty acids actively resolve inflammation by inhibiting neutrophil infiltration and enhancing the macrophage-mediated clearance of cellular debris [49]. These agents are being explored in chronic inflammation and tissue repair therapies.

8.2 Regulating cell death pathways

- *Apoptosis modulation*: Promoting apoptosis of aberrant or inflammatory cells helps restore homeostasis. In cancer, BH3 mimetics (e.g., venetoclax) targets anti-apoptotic proteins like Bcl-2, triggering apoptosis in tumor cells [50]. In autoimmune diseases, therapies enhancing apoptosis in hyperactive immune cells help reduce inflammation [51].

- *Necroptosis inhibition*: Necroptosis contributes to tissue injury in diseases such as ischemia-reperfusion injury and neurodegeneration. Small molecule inhibitors like necrostatin-1 (Nec-1) inhibit RIPK1, a key mediator of necroptosis, preventing excessive cell death and inflammation [52].

- *Pyroptosis inhibition*: Strategies targeting caspase-1 or gasdermin D (GSDMD) aim to block pyroptotic cell death. Caspase-1 inhibitors, such as VX-765, have shown promise in reducing inflammation in sepsis and other inflammatory disorders [53].

9. Conclusion

Inflammation and the immune system are vital for maintaining tissue homeostasis and immune surveillance. However, dysregulated inflammation and immune responses contribute to chronic diseases and tissue damage. Targeted therapeutic approaches, including cytokine inhibitors and modulators of cell death pathways, hold significant potential in restoring immune balance and resolving inflammation-driven disorders. Addressing this delicate balance will be key to advancing treatment strategies for chronic inflammatory and immune-mediated diseases.

Conflict of interest

The author declares that no competing interest exists.

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Chapter 9

Assembly and Activation of the NLR Family in Innate Immune Regulation and Cell Death

Zhangfei Shen

Abstract

The nucleotide-binding oligomerization domain (NOD)-like receptor, also known as nucleotide-binding leucine-rich repeat receptors (NLRs), are evolutionarily conserved intracellular sensors critical for detecting pathogen- and damage-associated signals. As key mediators of innate immunity, NLRs orchestrate cellular responses through the assembly of higher-order signaling complexes. In humans, NLRs are central to initiating pyroptosis *via* inflammasome formation and caspase activation. Comparative studies across kingdoms reveal that although plant and bacterial NLRs do not form inflammasomes, they assemble analogous structures such as resistosomes and antiviral complexes, underscoring conserved principles of supramolecular immune signaling. This chapter provides a structural and mechanistic overview of representative NLRs in human, plant, and bacterial systems, highlighting their roles in immune surveillance and regulated cell death.

Keywords: NLR, innate immunity, cell death, PAMP, DAMP, pyroptosis, inflammasome, Caspase-1, CARD, NACHT, PYD, LRR, GSDMD, NLRP3, NAIP, NLRC4, resistosome, CNL, TNL, ZAR1, RPP1, AVAST3, AVAST4

1. Introduction

NLR family mediates a wide range of biological functions and induces distinct signaling mechanisms (**Figure 1**) [1]. Upon detecting pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), mammalian NLRs initiate signaling cascades that activate key inflammatory pathways, including NF-κB and MAPK, and often lead to the formation of multiprotein complexes known as inflammasomes [2]. Inflammasome assembly promotes the activation of caspase-1, the processing of pro-inflammatory cytokines such as IL-1β and IL-18, and a lytic form of cell death known as pyroptosis [3]. By integrating signals from intracellular pathogens and cellular stress, the NLR family functions as an early warning system, bridging innate and adaptive immunity to maintain immune homeostasis and protect the host from disease [4, 5].

Beyond classical pyroptosis, recent studies have revealed that NLR-initiated inflammatory cell death pathways are not isolated [1]. Substantial crosstalk exists

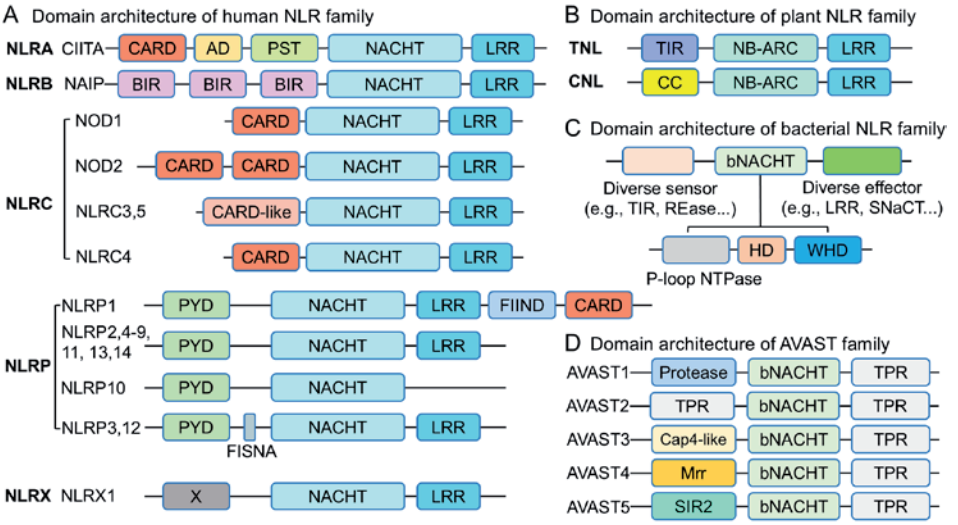

Figure 1.
Diversity and conservation of NLRs family are shown for human, plant and bacteria. (A) Domain architecture of human NLR family. NLRA has one founding member, also known as MHC class II transactivator (CIITA); NLRB containing N-terminal BIR domains [baculovirus inhibitor of apoptosis (IAP) protein repeat], also known as NAIPs; NLRC containing a N-terminal CARD domain or a CARD-like domain. NLRC1 and NLRC2 are more commonly known as NOD1 and NOD2; NLRP containing a N-terminal pyrin domain (PYD). NLRP1 has additional C-terminal FIIND domain and CARD domain. NLRP3 and NLRP12 are unique for having the fish-specific NACHT-associated (FISNA) domain. NLRX containing a domain with no similarity to known NLR subfamily members, currently assigned as "X" in the nomenclature. (B) Domain architecture of plant NLR family. Two representative members of the plant disease resistance proteins with either Toll-IL-1 receptor (TIR) or coiled-coiled (CC) domains are shown. (C) Domain architecture of bacterial NLR family. The sensor and effector domains of bacterial NLR family are all diverse. Bacterial NACHT contains a P-loop NTPas domain, a HD domain and a WHD domain, also called wHTH for winged helix-turn-helix winged helical domain. (D) Domain architecture of AVAST family. The domains are not shown to scale. Abbreviations: FIIND, domain with function to find, also called the autoproteolytic domain; LRR, leucine-rich repeat; NAIP, NLR family apoptosis inhibitory protein; PST, proline/serine/threonine; REase, Restriction endonuclease domain; SNaCT, short NACHT associated C-terminal domain; AVAST, prokaryotic antiviral STAND NTPases; TPR, tetratricopeptide repeat; SIR2, silent information regulator 2.

between pyroptosis, apoptosis, and necroptosis. In many cases, NLR-mediated inflammasome components also participate in larger multiprotein cell death complexes that integrate signals from multiple cell death pathways to drive a unified inflammatory response [1, 6–9]. The coordinated activation of these pathways is essential for effective host defense against infection. Human NLR activation benefits the host by reducing pathogen load, eliminating dysfunctional cells, and maintaining homeostasis [10, 11].

Additionally, plant NLR proteins can be classified into two main groups: TNL proteins, which contain a Toll/interleukin-1 receptor (TIR) domain, and CNL proteins, which feature a coiled coil (CC) domain (**Figure 1**). Upon ligand binding to the LRR domain, the nucleotide-binding domain (NBD) is released, allowing ADP to be exchanged for ATP [12]. ATP binding then triggers NLR oligomerization, forming various signaling assemblies known as signalosomes. Similar complexes include inflammasomes in humans [13, 14] and resistosomes in plants [15–18]. These multiprotein complexes activate defense-related genes, induce ion fluxes and reactive oxygen species, and initiate a hypersensitive response to limit pathogen spread. Furthermore, the structural diversity of resistosomes, which can function as either

ion channels or enzymes, reflects their dual role in immune activation and programmed cell death, thereby ensuring plant survival during infection [17, 19].

Notably, proteins containing a NACHT module, which is the central feature of the NLR family, are also found in bacteria, where they contribute to defense against phage infection (**Figure 1**) [20–22]. Recent studies have identified approximately 15,000 unique bacterial proteins with a NACHT domain, collectively referred to as the bacterial NACHT family. These proteins are present in roughly 9–10% of fully sequenced bacterial genomes [22]. Their expression has been associated with reduced bacterial growth following phage infection, suggesting a defense strategy that disrupts phage replication through abortive infection [21].

Therefore, understanding the precise molecular mechanisms by which specific NLRs trigger inflammasome or resistosome assembly is essential for developing new therapeutic strategies to regulate inflammatory cell death in disease contexts. In this section, I provide an overview of NLR architecture across human, plant, and bacteria, focusing on well-characterized examples such as human NLRP3 and NAIP-NLRC4 (which drive inflammasome formation) and plant ZAR1 and RPP1 (which assemble into two main types of plant resistosomes) [15, 17–19, 23]. Additionally, I introduce two members of the prokaryotic antiviral STAND family, which stands for signal transduction ATPases with numerous domains. These include *Salmonella enterica* AVAST3 and *Escherichia coli* AVAST4, both of which form similar supramolecular immune platforms (**Figure 1**). These proteins share conserved nucleotide-binding domain and sensor domain architecture with eukaryotic NLRs, recognize viral proteins through pattern recognition, and trigger bacterial abortive infection. This defense mechanism results in host cell death to prevent phage replication [21].

2. NLR family architecture

The architecture of NLRs is crucial for their ability to detect diverse stimuli and initiate immune responses. In mammals, NLRs typically exhibit a tripartite domain structure consisting of a central NACHT domain responsible for nucleotide binding and oligomerization, a C-terminal leucine rich repeat (LRR) region involved in ligand sensing, and a variable N-terminal domain that mediates downstream signaling. The N-terminal region may contain a pyrin domain, caspase recruitment domain, or other signaling motifs, which define subfamilies within the NLR family [24, 25]. The NACHT domain is composed of four subdomains: the nucleotide-binding domain (NBD), helical domain 1 (HD1), winged helix domain (WHD), and helical domain 2 (HD2), which is essential for ATPase activity. The term "NACHT" is derived from its presence in NAIP, CIITA, mammalian telomerase-associated protein 1 (TP1), and a predicted nucleoside triphosphatase from *Streptomyces coelicolor* (HET-E, a member of the WD40 repeat protein family) [26, 27], highlighting its evolutionary conservation. Oligomerization of the NACHT domain forms a scaffold that facilitates the assembly of signal-transducing molecules, ultimately initiating an immune response.

Human NLRs are classified into five subfamilies based on variations in their N-terminal domain structure (**Figure 1A**) [24]. These include: (1) the acidic transactivating domain containing NLR protein (NLRA), represented by a single member, CIITA, which functions as the major histocompatibility complex class II transcription activator; (2) the baculovirus inhibitor of apoptosis protein repeat containing NLR protein (NLRB), also with a single member, NAIP, known as the NLR family apoptosis inhibitory protein; (3) the caspase recruitment domain containing NLR proteins

(NLRC), which include five members (NOD1, NOD2, NLRC3, NLRC4, and NLRC5); (4) the pyrin domain containing NLR proteins (NLRP), the largest subfamily with 14 members (NLRP1-NLRP14); and (5) NLRX, a subfamily with one member, which contains an undefined N-terminal domain that does not resemble those found in other NLR subfamilies [1, 28, 29].

In plants, NLRs are similar to human NLRs but contain a shorter central domain composed of three subdomains: the nucleotide-binding domain (NBD), helical domain 1 (HD1), and winged helix domain (WHD) (**Figure 1B**). The central domain of plant NLRs, also referred to as the NB-ARC domain, is a highly conserved nucleotide-binding region shared with APAF1, various resistance proteins, and CED4. It is proposed to function as a molecular switch that cycles between ADP-bound (inactive) and ATP-bound (active) forms [30–32]. Upon effector recognition, plant NLRs oligomerize into resistosomes, which serve as immune signaling hubs and mediators of cell death. Recent studies have revealed three structural states of a CNL: the inactive, intermediate, and active oligomeric forms [28, 33, 34].

The tripartite domain architecture observed in bacterial NACHT proteins aligns with domain structures previously described in eukaryotic NLRs (**Figure 1C, D**) [22]. The N-terminal regions of bacterial NACHT proteins encode a variety of enzymatic domains involved in biological conflict, including nucleases, peptidases, degradative enzymes, and NADases. In contrast, the C-terminal regions can be broadly categorized into functional groups: those involved in antigen recognition or infection signal detection, those that contain transmembrane domains, those with short C-terminal extensions, or those that combine multiple features [22].

Thus, the functional similarity between bacterial and eukaryotic NLRs suggests a conserved inflammasome-like molecular defense strategy that spans all kingdoms of life.

3. Inflammasome overview

Inflammasomes are cytoplasmic supramolecular protein complexes that play a crucial role in innate immune responses [13]. These complexes are formed in response to intracellular detection of microbial components or cellular stress signals, enabling the host to mount a rapid defense. The innate immune system relies on multiple families of PRRs to detect intracellular or extracellular disturbances and initiate immune responses. PRRs recognize PAMPs, DAMPs, and other stimuli that disrupt cellular homeostasis. Upon activation, inflammasomes facilitate the maturation of pro-inflammatory cytokines and initiate pyroptosis in the cytoplasm to trigger innate immune activation [35, 36].

Activation of the inflammasome occurs in two steps: a priming phase and an activation phase [37]. Priming is driven by the recognition of PAMPs and DAMPs through Toll-like receptors (TLRs), resulting in the upregulation of inflammasome components, cytokines, and other inflammatory factors at both the transcriptional and post-translational levels. The activation step involves the assembly of the inflammasome in response to a wide array of triggers. Common PAMPs include microbiome-derived signals from bacteria, fungi, parasites, and viruses, while DAMPs consist of ion fluxes, mitochondrial dysfunction, reactive oxygen species (ROS), and metabolic factors [38, 39].

Inflammasome activation pathways can be broadly classified into canonical and non-canonical pathways. In canonical inflammasomes, the process begins with the

engagement of pro-caspase-1, which is converted into active caspase-1. Pro-caspase-1 is an inactive monomer, consisting of a CARD domain linker (CDL) that connects the N-terminal CARD domain to the C-terminal noncatalytic protease domain. The C-terminal domain of caspase-1 is further divided into a large (~20 kDa) subunit (p20) and a small (~10 kDa) subunit (p10) that are connected by an inter-domain linker (IDL). The IDL contains a self-cleavage site, leading to the formation of dimeric, active caspase-1, also known as caspase-1 p33/p10 [40–44]. Activated caspase-1 then processes the inactive precursors, interleukin-1β (IL-1β) and interleukin-18 (IL-18), to generate their active forms [45]. Mature IL-1β and IL-18 further cleave the inhibitory C-terminal domain of the pore-forming protein GSDMD, leading to the polymerization and insertion of the N-terminal fragment of GSDMD (GSDMD-NT) into membranes. This process initiates pore formation, resulting in gasdermin-mediated programmed lytic cell death, known as pyroptosis [39, 46, 47]. In contrast, non-canonical inflammasome activation relies on caspase-11 in mice or caspase-4/5 in humans, rather than caspase-1. Non-canonical inflammasomes are primarily activated by intracellular lipopolysaccharides (LPS) from the membranes of gram-negative bacteria. This activation leads to the direct cleavage of GSDMD, inducing pyroptosis without activating IL-1β or IL-18 [45, 48–50].

Canonical inflammasomes typically consist of a sensor protein, an adaptor protein, and the effector protein pro-caspase-1 [39]. Sensor proteins are responsible for recognizing PAMPs or DAMPs, and many of them belong to the NLR family [28]. Upon activation, the sensor protein recruits an adaptor protein [39]. NLR inflammasomes generally follow a similar activation mechanism, recruiting an essential adaptor protein named Apoptosis-associated Speck-like protein containing a CARD (ASC). ASC consists of two domains: a pyrin domain (PYD) and a CARD domain. The interactions between PYD-PYD and CARD-CARD facilitate the assembly of a highly oligomerized inflammasome, involving the sensor protein (NLR), adaptor protein (ASC), and effector protein (pro-caspase-1) [51, 52]. In contrast, non-canonical inflammasomes rely on caspases-4/5/11, which function as both the sensor and effector components to induce pyroptosis.

NLRs are involved in a wide range of activities beyond inflammasome activation and immune responses. For instance, significant evidence suggests that many NLRs play a role in the regulation of mammalian reproductive signaling [1]. To date, 22 members of the human NLR family have been identified, but only NLRP1, NLRP3, NAIPs with NLRC4, and NLRP6 have been shown to nucleate inflammasomes as sensor components. Among these, NLRP3 and NAIP NLRC4 are two of the best well-characterized examples of inflammasome assembly (**Figure 1A**) [1]. Notably, the NLRC4-mediated inflammasome, a well-known canonical inflammasome, does not recruit ASC. Instead, it recruits the NLR family of apoptosis inhibitory proteins (NAIPs), also known as NLRB, which are involved in activation upon sensing bacterial proteins [53, 54]. Other NLRs have been suggested as potential inflammasome sensors, but further investigation is needed to confirm their roles [1].

In fact, the activation and regulation of inflammasomes are highly diverse. In addition to NLR family members, proteins such as absent in melanoma 2 (AIM2) and pyrin can also nucleate inflammasomes. AIM2 recognizes cytosolic double-stranded DNA and assembles into another well-established canonical inflammasome [55]. Pyrin, on the other hand, detects bacterial toxin-induced inactivation of RhoA GTPases [56]. Furthermore, emerging evidence reveals crosstalk between the pathways of canonical and non-canonical inflammasomes. For instance, the NLRP3 inflammasome is activated downstream of non-canonical inflammasome caspase-11,

which promotes cytokine release [57]. Additionally, NLRP3 is also involved in the crosstalk between innate and adaptive immunity, influencing interferon-γ (IFN-γ) production by adaptive immune cells [58].

This chapter focuses exclusively on two representative well-characterized inflammasome assemblies, human NLRP3, and human NAIP-NLRC4, to exemplify the structural mechanisms underlying NLRs activation.

3.1 Activation and assembly of NLRP3 inflammasome

Among the NLR family members, NLRP3 is the most extensively studied due to its broad responsiveness to a wide array of stimuli and its involvement in numerous inflammatory diseases [59]. NLRP3 acts as a central molecular hub that integrates diverse danger signals, including microbial components, environmental irritants, and endogenous stressors, to initiate inflammasome assembly [59]. While its activation is primarily associated with the canonical inflammasome pathway, NLRP3 is also involved in non-canonical inflammasome activation [45, 48].

Structurally, NLRP3 consists of three essential domains: an N-terminal PYD domain that facilitates interactions with the adaptor protein ASC, a central NACHT domain responsible for ATP-dependent oligomerization and inflammasome assembly, and a C-terminal LRR domain implicated in autoinhibition and ligand sensing (**Figure 2A**) [59]. Additionally, there is a fish-specific NACHT-associated (FISNA) domain between PYD and NACHT domains, which conserved in NLRP3 and NLRP12 but absent in most other NLRPs (**Figure 2A**). FISNA domain stabilizes the active NACHT conformation and mediates key interactions in the activated inflammasome disc [60, 61]. The FISNA domain of NLRP3 has been implicated as a conformational switch in NLRP3 activation following induction of K^+ efflux [60].

The NLRP3 inflammasome signaling pathway follows a well-characterized two-step process: priming and activation [59]. The priming step is essential for upregulating the expression of NLRP3, as well as pro-IL-1β and pro-IL-18 [62–65]. The activation phase is triggered by a variety of stimuli, including potassium efflux, calcium flux, mitochondrial dysfunction, and lysosomal rupture. Additionally, organelle dysfunction, including lysosomal damage, mitochondrial stress, and ER stress, can also trigger NLRP3 activation [66–68]. Similar to other canonical inflammasome pathways, NLRP3 activation facilitates ASC recruitment, promotes caspase-1 cleavage, and leads to the maturation of IL-1β and IL-18. This process ultimately results in GSDMD-mediated pyroptosis [59].

Mammalian NIMA-related kinase 7 (NEK7) plays a crucial role in regulating and activating the NLRP3 inflammasome [46, 49]. A PYD domain-deleted human NLRP3 forms a complex with NEK7 at sub-micromolar binding affinity, with ADP and the NLRP3 inhibitor MCC950 further stabilizing the interaction. Structural analysis of the NLRP3(△PYD)-NEK7 complex revealed that the NACHT and LRR domains of NLRP3 tightly encase NEK7, highlighting a strong binding interaction (**Figure 2B**). Another full-length NLRP3 structure showed that the inactive form predominantly localizes to organelle membranes, assembling into an oligomeric double ring-like cage that utilizes the membrane as an oligomerization scaffold (**Figure 2C**) [69]. Structure-based mutagenesis studies demonstrated that the LRR domain and HD2 of NLRP3 are critical for NEK7 binding, supporting the idea that NEK7 is recruited after NLRP3 undergoes a conformational shift to its active state. NEK7 directly interacts with NLRP3, promoting its oligomerization and subsequent inflammasome assembly [70]. This interaction is necessary for transitioning NLRP3 from its inactive cage-like

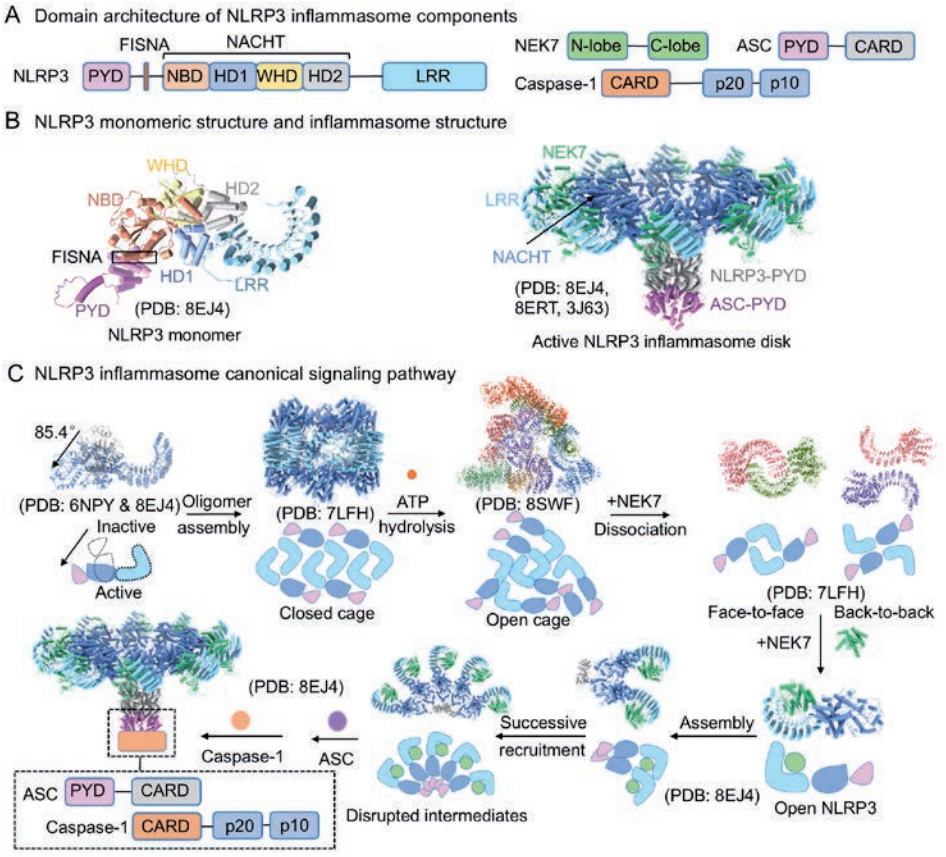

Figure 2.
Assembly process of NLRP3 inflammasome. (A) Schematic domain architectures of NLRP3, NEK7, ASC, and Caspase-1. (B) NLRP3 monomeric and inflammasome structures. Structures are colored by domains. (C) NLRP3 inflammasome canonical signaling pathway. The canonical activation of NLRP3 inflammasome occurs through oligomer assembly, ATP hydrolysis, structure reassembly, and forming an active octamer state by NEK7. Recruitment of ASC then helps to complete active NLRP3 disc by forming filament and transduce the downstream caspase-1 activation signaling.

conformation to its active disc-like structure [69]. Interestingly, NEK7 binding, rather than its kinase activity, is required to break the inactive NLRP3 cages, enabling reassembly into an active inflammasome [70]. Structural analysis of the active NLRP3-NEK7-ASC complex revealed that it is composed of approximately 10 to 11 individual NLRP3 subunits, with the C-terminal LRR domain and NEK7 not directly involved in the disc interfaces (**Figure 2B, C**) [61].

The regulation of NLRP3 canonical inflammasome activity follows a hierarchical activation pathway (**Figure 2C**). First, the priming process upregulates the expression of NLRP3 and other inflammatory factors. Second, NLRP3 assembles into a double ring-like closed cage structure on the trans-Golgi network (TGN) [69]. Third, the closed cage forms an open octamer and undergoes a ~ 90° hinge rotation at the NACHT domain [71]. Upon ATP or nigericin stimulation, NLRP3 undergoes a conformational change, leading to TGN dispersion. Fourth, the dispersed TGN vesicles are transported to the microtubule-organizing center (MTOC). Finally, NEK7 triggers the inactive NLRP3 cage into two halves, facilitating its transition into an active

disc-like state upon ASC recruitment (**Figures 2B** and **3C**) [61, 70]. Recruitment of ASC helps complete the active NLRP3 disc by forming filaments, transducing downstream caspase-1 activation signals, and facilitating the secretion of mature IL-1β and IL-18, which further trigger GSDMD-mediated cell pyroptosis.

In addition, post-translational modifications, including phosphorylation, ubiquitination, and SUMOylation, play key roles in modulating NLRP3 activity [63, 64]. For example, phosphorylation by kinases such as protein kinase A (PKA) and c-Jun N-terminal kinase 1 (JNK1) has been shown to enhance NLRP3 activation [65].

3.2 Activation and assembly of NAIP-NLRC4 inflammasome

The NAIP-NLRC4 inflammasome is a unique inflammasome composed of two different NLR members, in which NAIP proteins function as pathogen sensors, while NLRC4 serves as an adaptor for caspase activation [53, 54]. NAIP detects bacterial components in the cytosol and interact with NLRC4 to trigger inflammasome activation. The NAIP-NLRC4 inflammasome specifically responds to bacterial flagellin and type III secretion system (T3SS) proteins, including the "needle" and "rod" components, from several bacterial pathogens such as *Salmonella typhimurium*, enterohemorrhagic *Escherichia coli*, *Shigella flexneri*, and *Burkholderia* species [54, 72–74].

In humans, NAIP is encoded by a single gene, whereas mice possess a cluster of NAIP paralogs, including NAIP1–7, with NAIP3 identified as a pseudogene [1]. NLRC4 is composed of several conserved domains, including a caspase activation and recruitment domain (CARD) at its N-terminus, a central NACHT module that mediates ATP-driven oligomerization and contains the nucleotide-binding domain (NBD), HD1, WHD, and HD2 subdomains, as well as a C-terminal LRR region involved in autoregulation and protein interactions (**Figure 3A**). NAIPs share similar domain architecture but lack a CARD domain and instead contain three Baculovirus Inhibitor of Apoptosis Repeat (BIR) domains (**Figure 3A**) [14, 75–77]. ATP binding and hydrolysis are essential for NLRC4 conformational changes and subsequent canonical inflammasome activation. The assembly of the human NAIP-NLRC4 inflammasome begins when NAIP detect bacterial components [53, 54]. For example, human NAIP recognizes the T3SS Needle protein from *S. typhimurium*. In its inactive state, NLRC4 is bound to ADP, whereas ATP binding is required to transition into the active state and initiate inflammasome signaling [75].

Upon recognizing the bacterial T3SS Needle protein, apo human NAIP undergoes conformational tightening and stabilizes the Needle-NAIP complex (**Figure 3B**). This activated NAIP adopts a "key" conformation that engages the nucleotide-binding domain (NBD) of a "closed" NLRC4 subunit, inducing a ~ 90° rotation between its NBD and HD1 subdomains and repositioning the WHD, HD2, and LRR domains. This conformational rearrangement shifts NLRC4 into an "open" state, enabling ATPase-dependent oligomerization. Human NLRC4 uniquely harbors both the "key" and "open" conformational states, enabling it to sequentially activate adjacent "closed" NLRC4 subunits. In contrast, NAIP proteins cannot be directly activated by other "key" NAIP or "open" NLRC4 molecules (**Figure 3C**) [78].

The human Needle-NAIP complex thus initiates sequential recruitment and activation of additional NLRC4 monomers through NBD-NBD and LRR-LRR interfaces, assembling a disk-shaped inflammasome typically composed of 10–12 NLRC4 subunits [14, 76, 77]. Final closure of the disk may involve interaction between the last NLRC4 subunit and the backside of NAIP, potentially facilitating recycling of the Needle-NAIP complex or further stacking to form C11/C12 discs (**Figure 3C**)

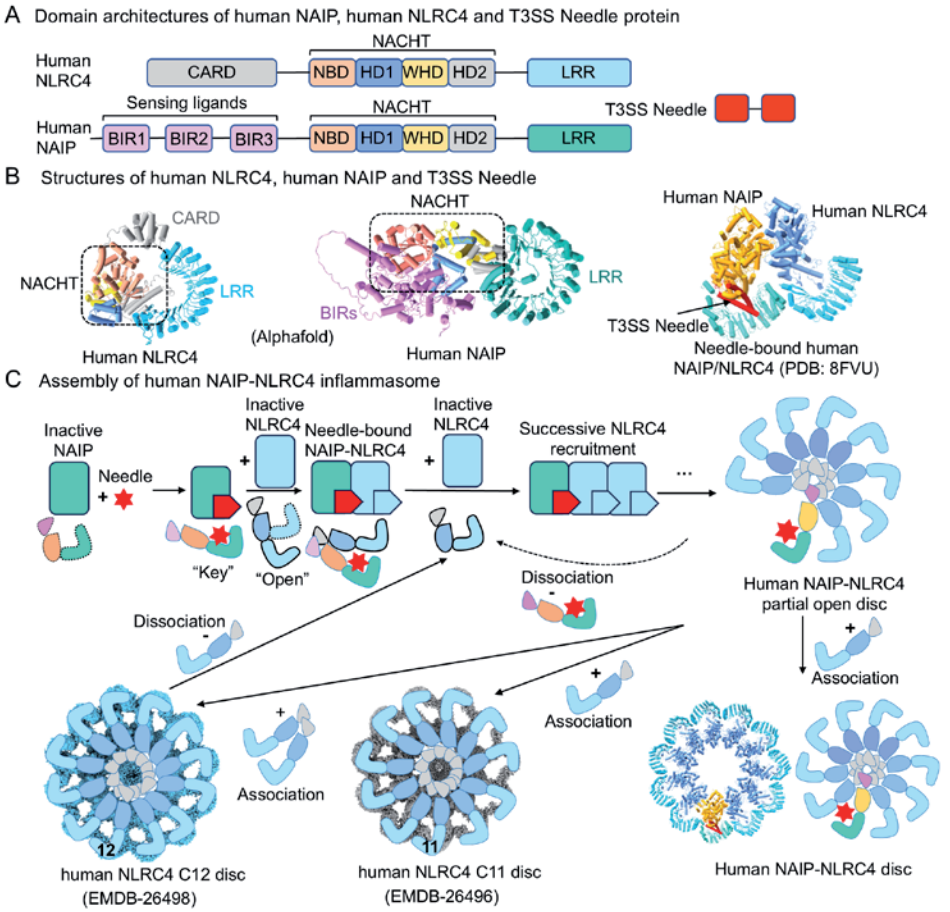

Figure 3.
Assembly of the human NAIP-NLRC4 inflammasome. (A) Schematic domain architectures of human NAIP, human NLRC4, and T3SS Needle protein. (B) Structures of human NAIP, human NLRC4, and human NAIP-NLRC4 heterodimer upon T3SS Needle binding. (C) Assembly model of human NAIP-NLRC4 inflammasome activation pathway. The apo human NAIP captures a bacterial T3SS Needle protein, triggering conformational tightening and stabilizing the Needle-NAIP complex. This activated Needle-NAIP further activate to open closed NLRC4 by engaging the interfaces between their NBD domains of NACHT. The LRR–LRR interface provides an additional docking site. Once activated, NLRC4 propagates the signal by sequentially activating additional NLRC4 subunits through the same mechanism, leading to the stepwise successive assembly of a disk-shaped inflammasome. Completion of the 10-subunit disk involves a final NLRC4 subunit interacting with the backside of NAIP, potentially inducing conformational changes that either release the Needle-NAIP complex for recycling or allow further continuous stacking of NLRC4 C11 disc or C12 disc. Human NLRC4 contains both the "key" and "open" states needed to activate other inactive NLRC4 molecules, whereas human NAIP adopts only the "key" state upon binding to the Needle protein.

[78]. Similar to NLRP3 inflammasome, assembly of the NAIP-NLRC4 inflammasome exposes the CARD domain of NLRC4, which further recruits pro-caspase-1 *via* CARD-CARD interactions as well, which further promotes caspase-1 autocatalysis, ultimately triggering GSDMD-mediated pyroptosis [40, 41].

Beyond pyroptosis, the human NAIP-NLRC4 inflammasome can also recruit caspase-8 through ASC, contributing to epigenetic regulation of the Nos2 gene, expulsion of infected intestinal epithelial cells (IECs), eicosanoid release, and caspase-7-induced cell death [79, 80]. The regulation of the NAIP-NLRC4 inflammasome

extends beyond bacterial infections and involves key post-translational modifications, such as phosphorylation and ubiquitination, which influence its activity and stability [81–84]. Phosphorylation of NLRC4 by kinases such as protein kinase C delta (PKCδ) and leucine-rich repeat kinase 2 (LRRK2) is essential for optimal inflammasome activation [81, 83].

3.3 Inflammasome in pathology

While inflammasomes are best known for their role in host defense, they also contribute to broader physiological processes and are implicated in various diseases. Dysregulated inflammasome activity is implicated in a spectrum of pathological conditions, ranging from autoinflammatory disorders, neurodegenerative disorders, chronic inflammatory bowel diseases, and infertility to cancers [1, 85, 86]. Understanding the molecular mechanisms that govern inflammasome assembly and regulation is therefore critical for developing targeted therapies. Below, I discuss the pathological roles of four NLR inflammasomes: NLRP1, NLRP3, NAIP-NLRC4, and NLRP6.

NLRP1 primarily expressed in epithelial cells and keratinocytes. NLRP1 mutations are associated with inflammatory disorders in the skin and throughout the body [1]. Skin disorders like vitiligo and familial keratosis lichenoides chronica, driven by unchecked IL-1β release [87, 88]. It also mediates host defense against pathogens such as Shigella and Semliki Forest virus, but excessive activation during infections can trigger tissue damage [89, 90].

The NLRP3 inflammasome is the most extensively studied and is linked to diverse pathologies due to its ability to sense cellular stress rather than specific pathogens. The NLRP3 inflammasome is implicated in diverse pathologies, including cryopyrin-associated periodic syndromes (CAPS), a group of autoinflammatory disorders caused by gain-of-function mutations [91]. It is also linked to chronic conditions such as obesity-driven insulin resistance, atherosclerosis, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [92, 93]. In cancer, NLRP3 exhibits dual roles: It promotes antitumor immunity through IL-18 while simultaneously fostering tumor progression *via* chronic inflammation [94].

Dysfunction of NAIPs-NLRC4 causes severe autoinflammatory syndromes, including macrophage activation syndrome (MAS) and neonatal-onset enterocolitis, marked by systemic inflammation and cytokine storms [95, 96]. NLRC4 promotes epithelial cell death (pyroptosis, apoptosis) in response to carcinogens or dysplastic cells, preventing tumor initiation. NLRC4 suppresses colorectal cancer by promoting epithelial cell death in colitis models, though its overactivation during chronic infections can drive pathological tissue destruction [97, 98].

Additionally, the NAIP-NLRC4 inflammasome plays a protective role in colorectal cancer by producing cytokines that maintain gut homeostasis and prevent tumor formation [99]. Emerging evidence suggests that dysregulated inflammasome activity, including NAIP-NLRC4, may contribute to neurodegenerative diseases such as Alzheimer's and multiple sclerosis [100–102]. The inflammatory environment driven by excessive IL-1β and IL-18 can exacerbate neuroinflammation and demyelination [103]. Additionally, NAIP-NLRC4 interacts with other NLRs, such as NLRP3, to enhance immune responses against bacterial infections [40, 41, 104, 105].

NLRP6 is primarily expressed in intestinal and hepatic cells. It forms a non-canonical inflammasome with caspase-11 to sense bacterial lipoteichoic acid (LTA) or viral dsRNA, while also suppressing NF-κB and MAPK pathways [106,

107]. NLRP6 deficiency exacerbates colitis and colitis-associated colorectal cancer due to dysbiosis and weakened epithelial integrity, though its influence on microbiome composition remains debated [108–110]. In the liver, NLRP6 protects against non-alcoholic fatty liver disease (NAFLD) by curbing TLR4-driven inflammation but may worsen damage during chronic viral hepatitis. Its dual role in antiviral defense (e.g., rotavirus) and inflammation underscores its context-dependent impact on pathology [111–113].

4. Plant resistosome overview

Plant resistosomes are sophisticated protein complexes that play a central role in plant innate immunity, acting as key defenders against pathogen invasion [114]. These structures are also composed of nucleotide-binding leucine-rich repeat receptors (NLRs), which detect pathogen-triggered effectors (PTI) and effector-triggered immunity (ETI) [115, 116]. Upon pathogen recognition, sensor NLRs oligomerize, often in coordination with helper NLRs, to form resistosomes with distinct functional architectures (**Figures 4** and **5**) [117, 118]. Some resistosomes assemble into calcium-permeable channels, facilitating ion flux that activates downstream immune responses of immune response by perturbing the plasma membrane integrity or ionic homeostasis [19].

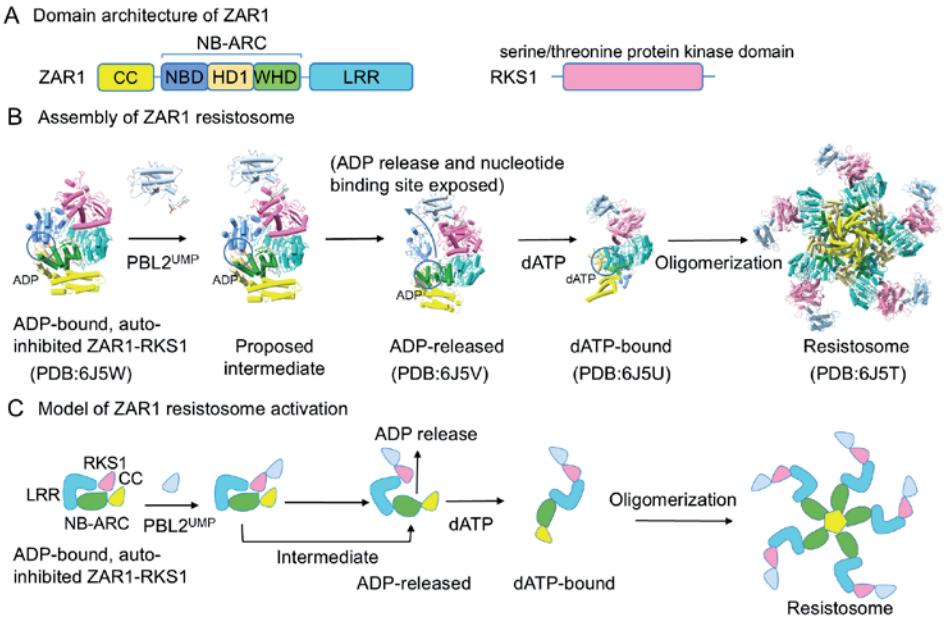

Figure 4.
Assembly of the ZAR1 resistosome. (A) Schematic domain architecture of ZAR1 and RKS1 (resistance-related kinase 1). (B) Assembly of ZAR1 resistosome. (C) Model of ZAR1 resistosome activation pathway. Upon pathogen attack, the RKS1 forms a resting complex with ADP-bound ZAR1. Effector-mediated uridylation of the kinase PBL2 produces PBL2^{UMP}, which binds RKS1 and triggers ADP release from ZAR1. This nucleotide exchange induces a major conformational shift, exposing oligomerization interfaces that drive ZAR1 assembly into a pentameric resistosome. In the final complex, the N-terminal α1 helices form a funnel-shaped structure that inserts into the plasma membrane, acting as a calcium-permeable pore to initiate immune signaling and hypersensitive cell death.

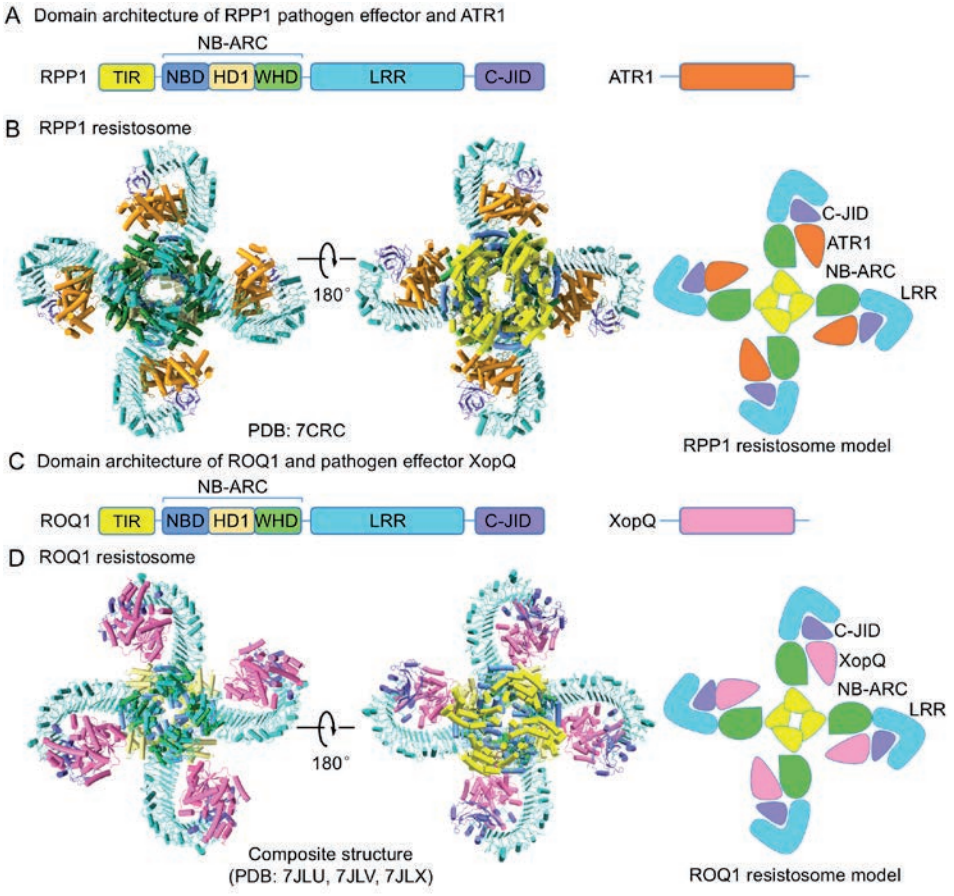

Figure 5.
Assembly of the RPP1 and ROQ1 resistosomes. (A) Domain architecture of RPP1 and pathogen effector ATR1. (B) Cryo-EM structure of RPP1 resistosome. (C) Domain architecture of ROQ1 and pathogen effector XopQ. (D) Cryo-EM structure of ROQ1 resistosome.

Plant resistosomes also display remarkable structural diversity that play their dual roles in innate immunity and programmed cell death. Recent structural studies reveal their diversity, with some acting as pore-forming entities, while others serve as enzymatic hubs [15, 17–19]. In coiled-coil NLRs (CNLs), such as the NLR hopz-activated resistance 1 (ZAR1) oligomerize into pentameric pores embedded in the plasma membrane, allowing $Ca2^{+}$ influx that activates calcium-dependent protein kinases and mitogen-activated protein kinase (MAPK) cascades. This activation leads to the phosphorylation of transcription factors and the upregulation of defense genes, including pathogenesis-related proteins, while also stimulating NADPH oxidases to generate reactive oxygen species (ROS) that inhibit pathogens and fortify cell wall defenses [17–19]. In contrast, Toll/interleukin-1 receptor NLRs (TNLs) such as RPP1 rely on helper proteins and NADase-active NLRs to propagate immune signals [15, 119, 120]. These helpers deplete cellular NAD^{+} and stabilize lipid-derived messengers, amplifying immune responses and triggering a hypersensitive response (HR) that restricts pathogen spread through the localized cell death, a form of programmed plant cell death [121].

4.1 CNL resistosome

The first cryo-electron microscopy (cryo-EM) structures of a plant NLR were resolved for *Arabidopsis* ZAR1 (HopZ-activated resistance). The ZAR1 resistosome assembly begins with ZAR1 in an inactive, the ZAR1-RKS1 complex (PDB: 6J5W) is stabilized by interactions between ADP-bound ZAR1 and resistance-related kinase 1 (RKS1). In its resting state, upon pathogen infection, AvrAC uridylates PBL2, generating $PBL2^{UMP}$, which binds RKS1 and stabilizes its activation segment. This enhances the nucleotide exchange activity of RKS1, triggering ADP release from ZAR1 and priming it to bind dATP or ATP (PDB: 6J5V). Subsequent structural rearrangements, including a CC domain fold switch (PDB: 6J5U), lead to oligomerization of ZAR1-RKS1 and the assembly of the pentameric ZAR1 resistosome (PDB: 6J5T) (**Figure 4B, C**) [17, 18].

This complex disrupts plasma membrane integrity, inducing immune responses and cell death [17, 18]. Upon activation, the ZAR1 resistosome translocates to the plasma membrane, where its coiled-coil (CC) domains form a calcium-permeable cation channel. This channel facilitates $Ca2^{+}$ influx and K^{+} efflux, by perturbing the PM integrity or disrupting ion homeostasis, leading to osmotic imbalance and organelle stress [19]. These actions initiate downstream immune responses, including the production of reactive oxygen species (ROS) and nitric oxide (NO) signaling [122]. Ultimately, these events trigger the hypersensitive response (HR), a programmed cell death mechanism that restricts pathogen spread [121]. Thus, CNL-type resistosome display its dual function as both a pathogen sensor and an ion channel effector [17, 18].

4.2 TNL resistosome

The structural assembly of TNL-type plant resistosomes, exemplified by RPP1 and ROQ1, begins with effector recognition. RPP1 directly binds the oomycete effector ATR1 through its C-terminal jelly roll/Ig-like domain (C-JID) and LRRs, enabling strain-specific detection *via* polymorphic residues. Similarly, ROQ1 recognizes the bacterial effector XopQ (*Xanthomonas* outer protein Q), though its precise binding mechanism remains less detailed (**Figure 5**). Effector binding disrupts the receptors' autoinhibited state, inducing conformational changes [15, 16].

Upon activation, both receptors oligomerize into tetrameric complexes. RPP1 forms a 4:4 RPP1:ATR1 resistosome stabilized by interactions between NBD-HD1-WHD domains (*via* β2-α2 loops) and asymmetric TIR domain homodimers created by BB-loop rearrangements. ROQ1 adopts a similar tetrameric architecture but retains ATP (unlike ADP-bound RPP1), suggesting differences in oligomer stability and activation dynamics. While RPP1 relies on C-JID and LRRs for effector recognition, assembly of ROQ1 may depend more on ATP-driven conformational changes, reflecting evolutionary divergence in NLR activation strategies (**Figure 5B, D**) [15, 16].

The final resistosome functions as a holoenzyme with NAD^{+} hydrolase activity. RPP1's asymmetric TIR homodimers form two catalytic sites that hydrolyze NAD^{+} in a $Mg2^{+}/Ca2^{+}$-dependent manner, generating immune signals like cyclic ADP-ribose to activate downstream components. ROQ1 likely employs analogous TIR interfaces for NAD^{+} cleavage but couples this with ATP binding for structural reinforcement. Both resistosomes mirror animal inflammasomes/apoptosomes in

their oligomeric architecture, underscoring conserved mechanisms for immune receptor activation across kingdoms. This integration of effector sensing, oligomerization, and enzymatic activity highlights how TNLs convert pathogen detection into robust plant immunity [15, 16].

4.3 AVAST3 oligomerization and AVAST4 oligomerization

The assembly and activation of AVAST family proteins in prokaryotic innate immunity follow a modular mechanism similar to eukaryotic NLRs [20, 21]. Currently, AVAST family has five members, named AVAST1-5 (**Figure 1D**). In their resting state, these proteins remain autoinhibited until viral triggers activate them. Their sensor domains, which are often composed of repeat motifs like tetratricopeptide repeat (TPRs), specifically recognize conserved structural or functional motifs in viral proteins. These motifs include key active sites of polymerases, proteases, or terminases, which are crucial for phage replication and are less likely to mutate [20, 21].

Recently, the assembly and activation process of AVAST3 and AVAST4 were determined and they play key prokaryotic innate immunity through conserved pattern recognition pattern as eukaryotes (**Figure 6**). Cryo-EM structures reveal that oligomerization relieves autoinhibition, positioning N-terminal effector domains (Cap4-like nuclease in AVAST3, Mrr-like nuclease in AVAST4) into catalytically active dimeric configurations (**Figure 6B, D**) [21, 22]. Both systems detect hallmark viral proteins through conserved mechanisms of innate immunity across domains of life. AVAST3 binds the phage terminase ATPase/nuclease domains (**Figure 6A, B**), while AVAST4 targets the portal protein, a structural component of viral capsids (**Figure 6C, D**). Recognition induces ATP/GTP-dependent conformational changes, driving oligomerization into tetrameric "dimer-of-dimers" complexes. For AVAST3, terminase binding exposes its STAND ATPase domain, enabling interactions between adjacent subunits to form a tetramer (**Figure 6B**). Similarly, AVAST4 tetramerizes *via* ATPase domain interactions upon portal binding, with its TPR sensor domains gripping the portal protein (**Figure 6D**) [20–22].

The activated oligomers function as $Mg2^{+}$-dependent DNA endonucleases, cleaving double-stranded DNA indiscriminately. AVAST3 directly targets the terminase's ATPase active site and bound ATP, while AVAST4 relies on shape complementarity with the portal's rigid fold. This enzymatic activity triggers abortive infection, by degrading both viral and chromosomal DNA, thereby halting phage replication and triggering an abortive infection response [21]. This is a form of programmed cell death that protects the entire microbial community by sacrificing the host cell [20, 21].

By targeting universal viral features rather than specific sequences, the AVAST systems provides broad-spectrum immunity, distinct from nucleic acid-targeting mechanisms like CRISPR-Cas or cyclic nucleotide-based signaling such as CBASS [123, 124]. For example, AVAST3 detects terminases with <5% sequence identity, while AVAST4 binds diverse portal variants. This "pattern recognition" similar to eukaryotic NLRs senses pathogen-associated molecular patterns (PAMPs) like flagellin or viral proteins. Evolutionarily, these protein-based pattern recognition strategies represent a convergent solution that links prokaryotic and eukaryotic immune principles, highlighting the ancient roots of NLRs-related innate immune defense and programmed cell death control.

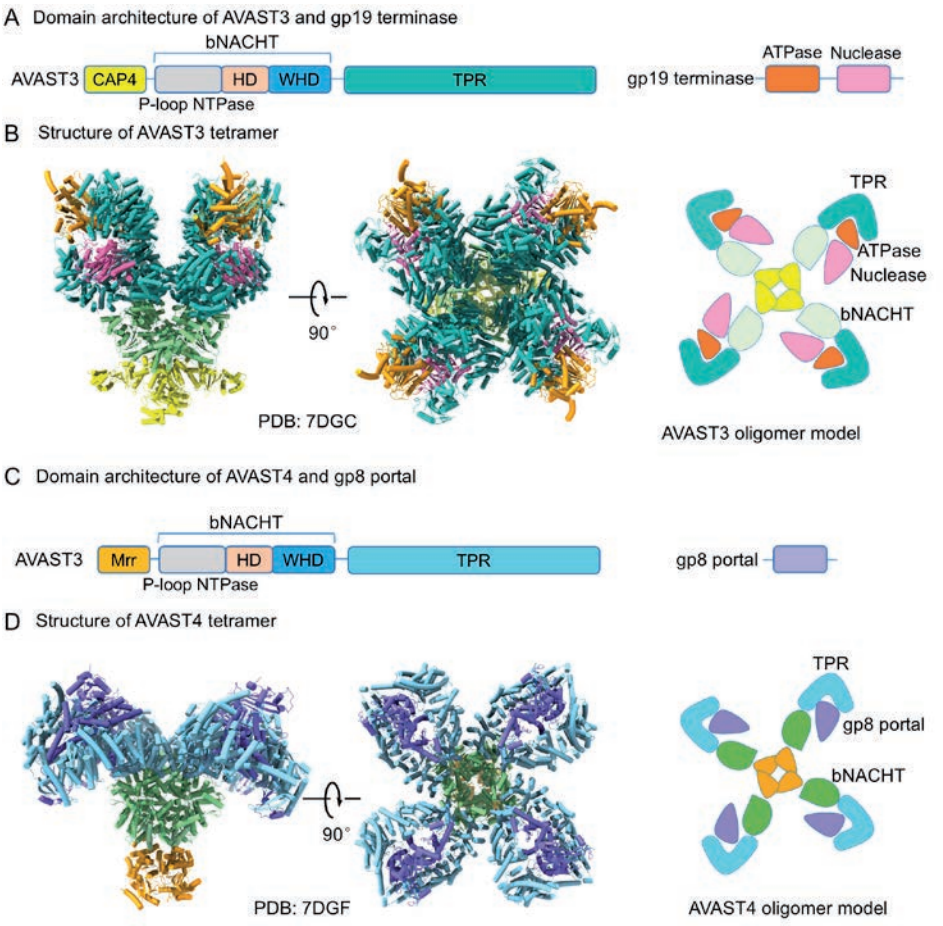

Figure 6.
Assembly of the AVAST3 and AVAST4 oligomers. (A) Domain architecture of S. ente *AVAST3 and gp19 terminase. (B) Cryo-EM structure of AVAST3 oligomer with gp19 terminase. (C) Domain architecture of* E. coli *AVAST4 and gp8 portal. (D) Cryo-EM structure of AVAST4 oligomer with gp8 portal.*

5. Summary

In eukaryotes, the NLR family orchestrates canonical inflammasome assembly, leading to caspase activation and pyroptosis, while also engaging in emerging non-canonical pathways and cross-talk with other biological and pathological processes. Inflammasomes are critical cytosolic immune sensors that, upon detecting pathogen- or danger-associated signals, undergo dramatic structural transitions from an autoinhibited state to form large oligomeric signaling platforms. These processes are increasingly recognized in both eukaryotic and prokaryotic systems. In plants, the coiled-coil NLR ZAR1 assembles into a wheel-like pentamer that functions as a calcium-permeable channel at the plasma membrane, triggering the hypersensitive response. Similarly, bacterial antiviral STAND protein, the AVAST oligomers use conserved sensor domains to recognize universal viral motifs. This recognition triggers ATP/GTP-dependent oligomerization into tetrameric complexes that activate

effector domains, such as nucleases or proteases, to induce abortive infection. While some NLRs are well studied, many others remain understudied. These supramolecular complexes maintain an autoinhibited conformation at rest and respond to cytosolic PAMPs or DAMPs by undergoing dramatic structural transitions, leading to the formation of large oligomeric signaling platforms. This convergence of molecular strategies across kingdoms highlights the ancient origins of innate immune surveillance and cell death.

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Section 4

Exploring New Cell Death Mechanisms: Ferroptosis and Cuproptosis

Chapter 10

Role of Ferroptosis in AML Pathophysiology and Therapeutic Strategies

Gregorio Favale, Vincenza Capone, Daniela Carannante, Giulia Verrilli, Antonio Beato, Fatima Fayyaz, Rosaria Benedetti, Lucia Altucci and Vincenzo Carafa

Abstract

Ferroptosis, an iron-dependent form of regulated cell death marked by lipid peroxidation, is critically implicated in the pathology of acute myeloid leukemia (AML). The dysregulation of iron metabolism and ferroptotic regulators, such as GPX4, the cystine/glutamate antiporter System Xc^-, and several iron homeostasis proteins, contributes to leukemic cell survival and therapy resistance. These disruptions not only facilitate the survival and proliferation of leukemic cells but also enable them to evade traditional apoptotic pathways, thereby increasing resistance to standard therapeutic interventions. Recent studies have focused on identifying specific targets within the ferroptosis pathway that are aberrantly expressed in AML, highlighting potential vulnerabilities that can be exploited for therapeutic benefit. Promising compounds such as Erastin and RSL3 have emerged as effective inducers of ferroptosis in AML cells, demonstrating the capacity to circumvent resistance mechanisms. These agents function by inhibiting GPX4 and disrupting cystine uptake, which culminates in enhanced lipid peroxidation and cell death. This chapter explores the therapeutic potential of targeting ferroptosis in AML, with a particular focus on modulating iron metabolism and key regulatory pathways. By exploiting the vulnerabilities in ferroptotic processes, these strategies offer a novel approach to enhancing therapeutic efficacy and addressing the critical challenge of drug resistance in AML.

Keywords: AML, ferroptosis, therapy, cell death, drug discovery

1. Introduction

Acute myeloid leukemia (AML) is a highly aggressive blood cancer defined by the clonal proliferation of myeloid progenitor cells that are either undifferentiated or poorly differentiated. This expansion disrupts normal hematopoiesis leading to significant bone marrow failure and various complications [1]. The pathogenesis of AML is driven by a complex interplay of genetic and epigenetic alterations, including chromosomal rearrangements such as translocations (e.g., t(8; 21), t(15; 17)), and

inversions (e.g., inv(16)), along with somatic mutations in key genes (*FLT3, NPM1, IDH1/2, DNMT3A*, and *TET2*) and dysregulation of transcriptional and epigenetic machinery [2]. These molecular aberrations orchestrate a pathogenic cascade that impairs differentiation, promotes uncontrolled proliferation, and ultimately leads to leukemic transformation. Despite significant advancements in the characterization of the molecular landscape of AML and the development of targeted therapies, clinical outcomes continue to be unsatisfactory. AML originates from the myeloid lineage of hematopoietic stem cells and is characterized by the rapid expansion of immature leukemic blasts that replace normal hematopoietic tissue within the bone marrow. This replacement leads to disruption of the bone marrow microenvironment and a blockage in progenitor cell differentiation and maturation at various developmental stages [3, 4]. The incidence of AML has been rising in the time, and despite recent advancements in therapy, the mortality rate remains high due to its aggressiveness and frequent relapses [3]. One of the hallmark features of AML is its capacity to escape programmed cell death, a pivotal mechanism for preserving cellular balance and removing cells with oncogenic potential. Evasion of cell death contributes to the aggressive nature of AML as well as facilitates disease progression and therapeutic resistance [3]. Apoptosis, the most extensively characterized form of regulated cell death, has been a central focus in AML research and therapeutic development. Apoptotic pathways are activated *via* intrinsic or extrinsic signals that converge on caspase-mediated cellular breaking down. However, AML frequently exhibits resistance to apoptosis through multiple mechanisms, including the overexpression of anti-apoptotic proteins, such as B-cell leukemia/lymphoma 2 protein (BCL-2), c-1 (MCL1), and B-cell lymphoma-extra large (BCL-XL), mutations in tumor suppressor genes like TP53, and dysregulated signaling in survival pathways such as phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [5, 6]. These adaptations limit the efficacy of chemotherapeutic agents and reduce the impact of apoptosis-targeted drugs, such as venetoclax, highlighting the necessity for alternative therapeutic approaches. The challenges associated with apoptosis-based therapies have prompted interest in investigating alternative forms of regulated cell death (RCD) [6]. Among these, ferroptosis has emerged as a particularly promising area of investigation in AML. Unlike apoptosis, ferroptosis is not mediated by caspases but is instead governed by disruptions in redox balance, iron metabolism, and lipid homeostasis. Mechanistically, ferroptosis is initiated by oxidative stress and the failure of the glutathione peroxidase 4 (GPX4)-dependent antioxidant defense system, which results in uncontrolled lipid peroxidation [3]. This process is further exacerbated by the intrinsic metabolic alterations of AML, which allow cells to adapt to increased levels of reactive oxygen species (ROS) by avoiding apoptosis. Additionally, the increased absorption and storage of iron enhance susceptibility to ferroptotic cell death, especially under conditions of oxidative stress [7]. This emergent cell death has also been implicated in modulating the tumor microenvironment, suggesting that its induction may not only eradicate leukemic cells but also reprogram the environment to favor immune-mediated clearance. Furthermore, iron metabolism plays a crucial role in the context of ferroptosis, as an essential element for ROS production through Fenton reactions [7]. In particular, AML cells exhibit increased iron uptake and storage, driven by overexpression of iron-regulatory players such as transferrin receptor 1 (TFR1) and ferritin [3, 8]. Inducers such as erastin (which inhibits cystine import via SLC7A11) and RSL3 (which directly inhibits GPX4) have shown promise in selectively inducing ferroptosis in AML cells while sparing normal hematopoietic stem cells [9, 10].

Inducers such as erastin (which inhibits cystine import *via* SLC7A11) and RSL3 (which directly inhibits GPX4) have shown promise in selectively inducing ferroptosis in AML cells while sparing normal hematopoietic stem cells. These agents have demonstrated synergism with traditional AML drugs such as anthracyclines (Anth) and cytarabine (Ara-C), enhancing cytotoxicity by exacerbating oxidative stress. Moreover, high expression of SLC7A11 and GPX4 has been identified as a poor prognostic marker in AML, highlighting their potential both as therapeutic targets and biomarkers [3]. These evidences highlight the therapeutic role of ferroptosis as a modality to eradicate minimal residual disease and reduce the risk of relapse in AML patients [11]. The unique metabolic profile of AML cells, including their reliance on aberrant lipid biosynthesis and oxidation, creates vulnerabilities that can be exploited through ferroptosis induction. Disruption of lipid signaling pathways, such as those involving acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), has been shown to enhance ferroptosis sensitivity. Similarly, targeting nuclear factor erythroid 2-related factor 2 (NRF2)-mediated antioxidant responses, which often confer resistance to ferroptosis in leukemic cells, represents another promising strategy [12, 13]. The intersection of these metabolic and signaling pathways offers a comprehensive framework for designing combination therapies that enhance ferroptosis while simultaneously addressing other hallmarks of AML pathogenesis. This chapter aims to deliver an analysis of the pathogenetic mechanisms underlying AML, with a focus on the dysregulation of cell death pathways and the therapeutic potential of ferroptosis. The elucidation of these interconnected processes not only advances our understanding of AML biology but also paves the way for novel therapeutic approaches to overcome treatment resistance and improve patient outcomes.

2. Ferroptosis: Molecular mechanisms and regulation

The maintenance of cellular homeostasis in multicellular organisms is orchestrated by intricate regulatory networks that control metabolic processes and mitigate the accumulation of reactive byproducts. Among these, ROS proteins, nucleic acids, and lipids are primary targets, with the latter being especially vulnerable due to their structural role in cellular membranes [14]. Polyunsaturated fatty acids (PUFAs) in phospholipids are particularly susceptible to lipid peroxidation, a chain reaction initiated by ROS and catalyzed by enzymes such as lipoxygenases (LOXs). These iron-dependent enzymes abstract hydrogen atoms from lipid C–H bonds, perpetuating ROS propagation and leading to the formation of lipid hydroperoxides. If unchecked, this process disrupts membrane integrity and culminates in ferroptosis, a unique, iron-dependent form of regulated cell death. Ferroptosis is intrinsically linked to iron metabolism [15]. The availability of free, catalytically active iron amplifies ROS generation through Fenton chemistry, wherein ferrous iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) to form hydroxyl radicals, potent inducers of lipid peroxidation [16]. To counteract this lethal cascade, cells trigger the activation of several antioxidant mechanisms. System Xc^-, a heterodimer consisting of SLC7A11 (xCT) and SLC3A2 (CD98), mediates the import of extracellular cystine in exchange for intracellular glutamate. Once inside the cell, cystine is reduced to cysteine, which is necessary for the GSH production, the primary cofactor for GPX4. This enzyme neutralizes lipid hydroperoxides by converting them into nontoxic lipid alcohols, thereby preserving membrane integrity and preventing ferroptosis [17]. Moreover, intracellular cystine

levels induce the mammalian target of rapamycin complex 1 (mTORC1), which enhances GPX4 synthesis, further improving cellular defenses against lipid peroxidation [18]. Recent findings have also highlighted the role of ferroptosis suppressor protein 1 (FSP1), an oxidoreductase that acts independently of GPX4 by reducing coenzyme Q10 (CoQ10) to ubiquinol, a lipophilic antioxidant that traps lipid radicals in membranes and prevents lipid peroxidation. This GPX4-independent mechanism adds another layer of protection against ferroptosis and may serve as a complementary or alternative therapeutic target in contexts where GPX4 is impaired [3]. Ferroptosis plays a critical role in several pathological conditions, such as neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis), ischemia-reperfusion injury (e.g., myocardial infarction and stroke), acute kidney injury, and liver disorders (nonalcoholic fatty liver disease and alcoholic liver disease). Additionally, targeting ferroptosis therapeutically is emerging as a potential strategy for cancer treatment [19]. Tumor cells frequently exhibit alterations in iron metabolism, including upregulated iron import and storage pathways, alongside impaired antioxidant mechanisms, which render them particularly susceptible to ferroptosis [20].

Ferroptosis-inducing agents such as erastin and RSL3 are currently under preclinical and clinical investigation, particularly for their ability to overcome chemoresistance in malignancies like AML. Moreover, combinatorial approaches that couple ferroptosis inducers with immunotherapies are being explored to amplify antitumor immune responses [3]. Selective targeting of key ferroptosis players, such as system Xc^- and GPX4, allows for the induction of cancer cell death while sparing normal tissues. This approach not only exploits the unique vulnerabilities of tumor cells but also enhances the efficacy of existing therapies, including chemotherapy and immunotherapy. By integrating ferroptosis in therapeutic strategies, it may be possible to overcome treatment resistance and improve clinical outcomes for patients with aggressive or refractory malignancies [21]. Elucidating the molecular interplay between iron metabolism, ROS generation, and antioxidant defenses could pave the way for novel interventions aimed at modulating ferroptosis in a context-specific manner, thus addressing a wide array of human diseases.

3. Dysregulation of ferroptosis in AML

Ferroptosis has emerged as a pivotal mechanism of cell death in AML. Leukemia cells exhibit distinctive metabolic and genetic adaptations that heighten their vulnerability to ferroptosis while simultaneously developing mechanisms to evade this process, thereby supporting their survival and progression [22]. Mitochondrial metabolism plays a central role in AML cell bioenergetics, serving as the primary source of ROS and oxidative phosphorylation (OXPHOS). AML cells rely heavily on mitochondrial metabolism and show a significant increase in mitochondrial mass when compared to normal hematopoietic stem cells [23]. While AML cells display limited metabolic flexibility, favoring glycolysis over fatty acid oxidation, their dependence on mitochondrial respiration renders them susceptible to ROS-induced damage and lipid peroxidation, key processes in ferroptosis (**Figure 1**). ROS, which are elevated in many malignancies, have a dual role in AML; high ROS levels can enhance chemotherapeutic efficacy by inducing apoptosis, whereas low levels support cell survival, proliferation, and drug resistance [23, 24].

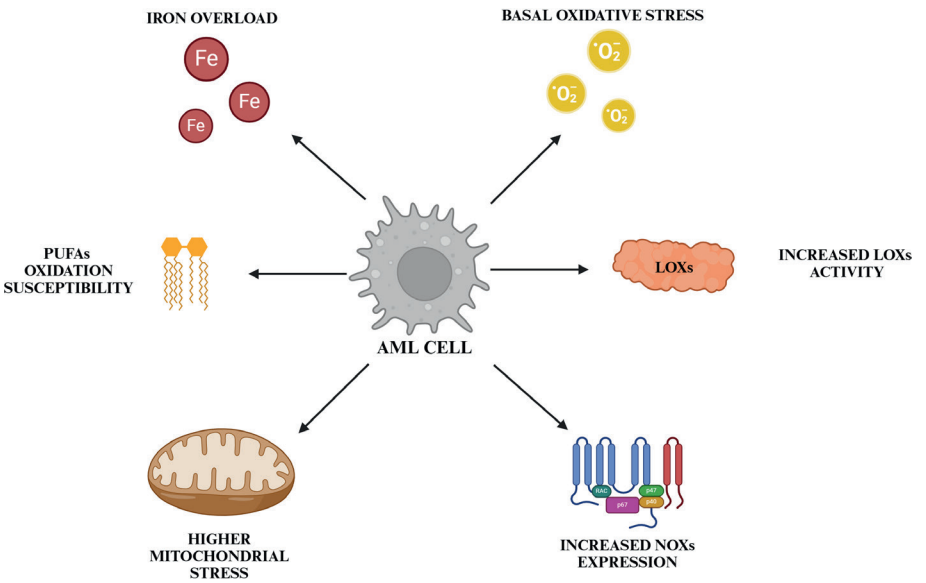

Figure 1.
Key dysregulated processes related to ferroptosis in AML cells. The image highlights major cellular pathways implicated in ferroptosis, including iron metabolism, lipid peroxidation, antioxidant defense, and mitochondrial function.

In AML, ROS overproduction arises not only from mitochondrial metabolism but also from altered enzymatic pathways, including xanthine oxidoreductase, nitric oxide synthase, and cytochrome P450 monooxygenase. Moreover, the NOX family of NADPH oxidases (NOXs), particularly NOX2 and NOX4, is frequently overexpressed, with NOX2 constitutively producing extracellular superoxide in approximately 60% of patients. This chronic ROS generation contributes to leukemic progression and ferroptosis susceptibility (**Figure 1**) [3].

Targeting NOX2 has been shown to improve the efficacy of oxidative stress-inducing agents, highlighting its potential as a therapeutic target [3]. AML cells have also evolved mechanisms to regulate ROS levels and mitigate oxidative stress, including the activation of antioxidant pathways such as the NRF2 pathway. Increased NRF2 expression is associated with drug resistance and poor clinical outcomes. A significant contributor to iron overload in AML is the frequent transfusion of red blood cells (RBCs) administered during chemotherapy. Transfused RBCs release iron, which is subsequently recycled and stored by macrophages in the liver and spleen, leading to the accumulation of unstable iron reserves [25]. At diagnosis, patients with AML often present with elevated serum ferritin levels, which correlate with tumor burden. These levels typically normalize following remission but may rise again during relapse. The transferrin (TF) system and TFR are critical for intracellular iron transport and represent the primary pathway through which tumor cells acquire iron. This mechanism is particularly important in inducing ferroptosis under conditions of glutamine depletion. AML cells demonstrate a strong affinity for TF and overexpress TFR, though the precise role of TFR in AML cells remains incompletely understood. TFR is essential for ferroptosis, as illustrated by findings that RAS-mutated cells with TFR overexpression accumulate intracellular iron and become more susceptible to ferroptosis induced by erastin [26]. Conversely, deletion of TFR reduces sensitivity to ferroptosis. Two isoforms of TFR exist: TFR1 and TFR2. TFR1

facilitates iron entry *via* receptor-mediated endocytosis through NCOA4 and serves as a validated marker of ferroptosis [27]. AML cells exhibit significantly higher TFR1 expression than normal cells, with levels increasing alongside malignancy differentiation. Elevated TFR1 expression has been linked to anemia, thrombocytopenia, and complex cytogenetics in AML, although it does not predict poor prognosis. In contrast, higher TFR2 mRNA expression in bone marrow samples of patients with AML or myelodysplastic syndromes (MDS) correlates with improved prognoses, suggesting a role for TFR2 in modulating iron metabolism and enhancing chemosensitivity [28]. The ferritin-ferroportin (FPN) axis serves as a key regulator of iron homeostasis and redox balance. Ferritin, composed of ferritin heavy chain (FTH) and ferritin light chain (FTL), stabilizes the labile plasma iron pool and inhibits ferroptosis by preventing iron efflux. Ferritin deficiency, however, reduces the regulatory activity of xCT and promotes ferroptosis. Both FTH and FTL are overexpressed in AML cells and leukemic stem cells (LSCs) compared to normal hematopoietic stem cells, with FTH overexpression often associated with NF-κB activation and decreased chemosensitivity. Consequently, elevated ferritin levels serve as markers of disease progression [3]. FPN, the primary axis governing intracellular iron efflux, inhibits tumor growth by lowering intracellular iron levels. In AML, FPN expression is strongly reduced respect to normal cells, and certain leukemic lines show severe downregulation. AML cells with low FPN expression accumulate more iron, rendering them more vulnerable to ROS. Studies using iron oxide nanoparticles in AML cells with low FPN expression have demonstrated enhanced antileukemic activity, further supporting the relationship between intracellular iron and chemotherapy sensitivity [29]. Additionally, reduced FPN levels in AML are frequently linked to elevated inflammatory cytokines, such as interleukin-6 (IL-6), contributing to dysfunctional iron metabolism and increased ferroptosis susceptibility [30]. Iron overload in AML and MDS patients has been evaluated using methods like biomagnetism, magnetic resonance imaging, and organ biopsies, revealing significant iron accumulation. This iron excess, along with heme, disrupts hematopoietic stem cell function in the bone marrow *via* ROS pathways, often necessitating additional blood transfusions and creating a pathological feedback loop (**Figure 1**) [31].

4. Ferroptosis as therapeutic strategy against AML

The modulation of ferroptosis-related processes in the context of AML represents one of the most challenging aspects of developing therapeutic alternatives to counteract the high rates of mortality, relapse, and resistance to standard treatments [32]. Identifying dysregulated metabolic mechanisms specific to AML neoplastic clones offers significant potential for ferroptosis-inducing strategies. These approaches aim to selectively activate molecular pathways of regulated cell death while restoring proper hematopoietic processes in the bone marrow and minimizing damage to healthy cells [33]. Although the antioxidant defenses of cancer cells are constitutively active to support survival and prevent cell death activation, the increased iron uptake observed in AML clones triggers lipid peroxidation mechanisms in cellular membranes. This suggests a heightened susceptibility to ferroptosis induction, paving the way for strategies targeting key nodes within the ferroptosis pathway, such as lipid metabolism, redox balance, and autophagy (**Figure 2**) [3]. The reported multitargeted approach not only improves the therapeutic effectiveness of ferroptosis induction but also holds promise for overcoming resistance to conventional therapies.

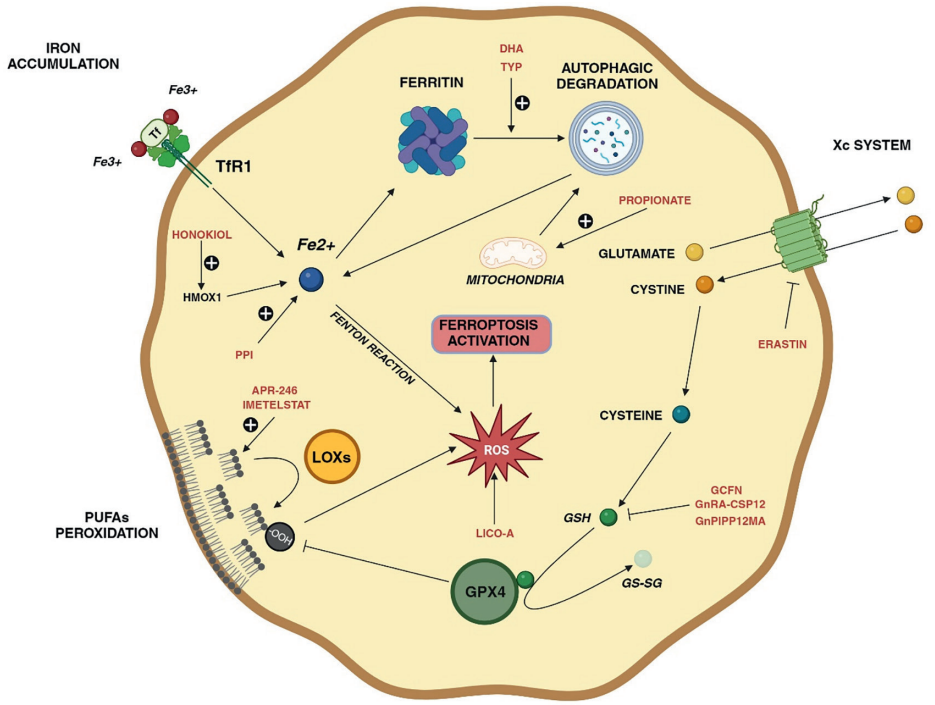

Figure 2.
Graphical overview illustrating key molecular mechanisms involved in ferroptosis. Pharmacological inducers of ferroptosis target critical regulators such as system Xc⁻, GPX4, and iron homeostasis pathways, promoting oxidative damage and cell death in AML cells.

4.1 Direct and indirect acting compounds on ferroptosis

Erastin, a well-characterized inhibitor of the system Xc⁻, exerts its biological effects by disrupting cystine and iron uptake and inhibiting GPX4 activity, ultimately triggering ferroptosis [34]. In the context of AML, erastin has been shown to activate the jun N-terminal kinase (JNK)/p38 molecular axis and promote the nuclear translocation of high mobility group box 1 protein (HMGB1), a key mediator in the production of ROS and the induction of ferroptosis. Furthermore, erastin demonstrates significant synergy with conventional chemotherapeutic agents such as Ara-C and doxorubicin (DXR), enhancing their antitumor efficacy [35]. Additionally, APR-246, initially known for reactivating mutant TP53, has been shown to induce ferroptosis by decreasing intracellular GSH and promoting lipid peroxidation, broadening its therapeutic potential beyond TP53-mutated AML cells [3, 36]. This therapeutic activity is evident both as monotherapy and when combined with GPX4 inhibitors, such as RSL3 and FINO2, further amplifying its ferroptotic effects. However, phase II clinical trials have reported significant neurological adverse events, with over one-third of patients experiencing these side effects [36].

The lipidomic analysis conducted on AML cells following treatment with the telomerase inhibitor imetelstat (GRN163L) demonstrates a marked increase in the levels of PUFAs, mediated by a fatty acid desaturase 2 (FADS2)-dependent mechanism [37]. FADS2 plays a crucial role by facilitating the desaturation of polyunsaturated fatty acids, particularly linoleic acid. This significant accumulation of

peroxidation substrates subsequently activates ferroptosis, a form of regulated cell death, both *in vitro* and in xenograft models. These findings highlight the potential for targeting lipid metabolism as a therapeutic strategy in AML, opening avenues for future clinical applications in patients with this aggressive hematological malignancy [37]. In the regulation of the ferroptosis process, NRF2 is known to induce the expression of the cystine antiporter subunit xCT, thereby inhibiting iron oxidation through GSH-dependent GPX4 activity and promoting resistance to cell death [38]. Notably, AML patients exhibit higher levels of NRF2 compared to healthy controls, suggesting the oncogenic role of this protein. The use of the specific NRF2 inhibitor, ML385, weakens the antioxidant defenses of leukemic clones, inducing ferroptosis in combination with the chemotherapeutic agent venetoclax [39]. Sulfasalazine, an anti-inflammatory drug, induces ferroptosis in cancer cells by inhibiting SLC7A11 and preventing GSH synthesis, and its combination with anthracyclines enhances antileukemic activity [3]. In addition, all-trans retinoic acid (ATRA), which is known to promote the differentiation of leukemic cells, induces ferroptosis by downregulating NRF2 [40]. Bithionol, a well-established anthelmintic drug, has also been employed as an antibacterial agent and in the treatment of Alzheimer's disease [41]. Recent studies have highlighted its potential for novel therapeutic applications, including the inhibition of NF-κB nuclear translocation and the induction of mitochondrial oxidative processes. These mechanisms simultaneously promote apoptosis and ferroptosis, both as a monotherapy and in combination with venetoclax in leukemia models [42]. Notably, the induction of ferroptosis by bithionol can be mitigated by the ferroptosis inhibitor ferrostatin-1 (Fer-1), further elucidating the mechanism of action and offering insights into potential therapeutic strategies (**Figure 2**) [42].

4.2 Nanocompounds inducing ferroptosis

Nanotherapy represents an emerging and innovative avenue in addressing complex diseases such as AML. Advanced research has led to the development of intricate nanocomposites capable of modulating intracellular GSH levels and suppressing the activity of GPX4, thereby facilitating ferroptosis and introducing new possibilities for AML treatment [43]. These approaches allow for highly targeted interventions at both cellular and molecular levels, offering significant potential to regulate critical biological pathways. A ferroptosis-inducing nanotherapeutic (GCFN) engineered using a GSH-reactive cysteine polymer has shown significant potential in preclinical models of aggressive AML. GCFN effectively triggered lipid peroxidation and ferroptosis by reducing intracellular GSH levels and suppressing GPX4 activity, highlighting its promise as a novel therapeutic approach for AML [44]. Additionally, gold nanorods (GnR) functionalized with chitosan and a 12-mer peptide (GnRA-CSP12), along with GCFN, have demonstrated the property to activate ferroptosis in AML cells by alternating the equilibrium between GSH and ROS while inhibiting GPX4 synthesis [45]. GNPIPP12MA is a nanocomposite that incorporates an FTO inhibitor and is bioengineered with GSH. Through the FTO/m6A signaling pathway, GNPIPP12MA depletes GSH, resulting in GPX4 inactivation, inhibition of lipid peroxidation (LPO) reduction, elevated intracellular iron accumulation, and selective induction of ferroptosis in AML cells [46]. This nanocomposite demonstrates a wide spectrum of anti-AML effects, even at relatively low doses. Additionally, GNPIPP12MA has the potential to enhance antileukemic immunity by promoting the infiltration of cytotoxic T cells (**Figure 2**) [46].

4.3 Natural compound activating ferroptosis

The discovery of bioactive compounds from natural sources has played a crucial role in advancing therapeutic options for numerous diseases, including autoimmune inflammatory conditions, neurological disorders, metabolic syndromes, and cancer [47]. Compared to synthetic drugs, these natural molecules often present fewer side effects, making them promising candidates for drug development. Recent investigations have focused on the ability of certain bioactive compounds to activate the ferroptosis pathway in AML cell models [48]. These studies underscore the therapeutic potential of these compounds and their ability to address challenges associated with standard treatments, such as drug resistance and off-target toxicity. Polyphyllin I (PPI), a steroidal saponin isolated from Paris polyphylla, has shown significant therapeutic efficacy in both *in vitro* and *in vivo* AML models, including improved survival in xenograft mouse models [49]. PPI operates through a dual mechanism: it suppresses GPX4 expression, compromising the antioxidant defenses of cancer cells, and inhibits the PI3K/mTOR pathway, which is essential for cellular proliferation. Interestingly, PPI demonstrates a stronger ability to induce ferroptosis compared to erastin, positioning it as a potentially superior anti-cancer agent [49]. Honokiol (HNK), derived from species of Magnolia, has emerged as a key modulator of oxidative stress through its regulation of heme oxygenase 1 (HMOX1) [49]. Unlike traditional ferroptosis inducers, HNK activates ferroptosis independently of GPX4, primarily by upregulating HMOX1. The enzymatic activity of HMOX1 increases intracellular iron levels and ROS, culminating in ferroptosis in AML models [50]. This effect is reversed when cells are co-treated with the HMOX1 inhibitor ZnPP, confirming the direct role of HMOX1 in HNK's mechanism of action. These findings highlight HNK as a therapeutic candidate with a distinct approach to inducing ferroptosis. Licochalcone A (Lico A), a flavonoid derived from the root of *Glycyrrhiza glabra*, exerts its effects by reducing the expression of insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3), a protein critical for stabilizing mouse double minute 2 homolog (MDM2) mRNA via m6A modifications [51]. By suppressing IGF2BP3, Lico A enhances the tumor-suppressive activity of p53, leading to increased ferroptosis both *in vitro* and *in vivo* (**Figure 2**) [51]. These findings emphasize the potential of natural compounds as Lico A to modulate epigenetic pathways, offering a novel strategy for ferroptosis-driven cancer therapies.

4.4 Ferroptosis inducers through autophagy regulation

Autophagy, a conserved cellular process involved in the degradation and recycling of intracellular components, plays a critical role in regulating ferroptosis [52]. In the context of AML, autophagy contributes to ferroptosis by facilitating ferritinophagy, a process that liberates intracellular iron and enhances lipid peroxidation [53]. Targeting autophagic pathways offers a unique approach to amplify ferroptosis in AML cells, creating opportunities for innovative therapeutic strategies.

Notably, ATPR, a novel all-trans retinoic acid derivative, induces ferroptosis in AML cells by promoting ROS accumulation that triggers autophagy activation and disrupts iron homeostasis. This cascade leads to AML cell differentiation and growth inhibition, illustrating the therapeutic potential of targeting autophagy-mediated ferroptosis [3]. Similarly, neratinib, a tyrosine kinase inhibitor, has been shown to induce autophagy-dependent ferroptosis; inhibition of autophagy significantly diminishes neratinib's ferroptosis-inducing effects, further underscoring

the essential role of autophagy in mediating ferroptotic cell death in AML [3]. The AMP-activated protein kinase (AMPK)/mTOR pathway plays a critical role in regulating autophagy by maintaining cellular energy homeostasis. Under low-energy conditions, AMPK activation inhibits mTOR, thereby promoting autophagy as a cellular survival mechanism. The antimalarial drug dihydroartemisinin (DHA) and the flavonoid glycoside plant extract Typaneoside (TYP) are closely associated with ferroptosis activation through ferritin degradation mediated by the AMPK/mTOR pathway. This process results in the release of iron, which triggers oxidation reactions and subsequent cellular damage [54, 55]. To support the critical role of autophagy activation in regulating iron homeostasis and ferroptosis, studies conducted in AML cellular models have demonstrated that the use of the autophagy inhibitor 3-methyladenine (3-MA) attenuates lipid peroxidation induced by the tyrosine kinase inhibitor (TKI) neratinib [56]. The disruption of redox homeostasis and ROS balance induced by propionate triggered mitochondrial fission and mitophagy, processes that enhanced both ferroptosis and apoptosis [56]. Additionally, propionate-induced ACSL4-mediated ferroptosis increased the immunogenicity of AML cells, facilitating the release of damage-associated molecular patterns (DAMPs) and promoting the maturation of dendritic cells (DCs) (**Figure 2**) [57].

4.5 Genetic and epigenetic activation of ferroptosis

Genetic and epigenetic mechanisms regulate ferroptosis, influencing processes such as redox balance, lipid metabolism, and iron homeostasis. Epigenetic modifications, such as changes in histone structure and the activity of noncoding RNAs (ncRNA), add complexity to ferroptosis regulation and offer promising therapeutic targets [58]. Gene knockdown studies have been particularly valuable in uncovering the functions of specific genes regulating ferroptosis. For instance, silencing GPX4 in AML cells induces ferroptosis, characterized by mitochondrial lipid peroxidation, and demonstrates significant anti-leukemic effects both *in vitro* and *in vivo* [59]. Recent studies have identified a novel ncRNA, CircKDM4C, which acts as a competitive endogenous RNA for the microRNA (miRNA) hsa-let-7b-5p, enhancing p53 activity and driving ferroptosis [60]. Elevated levels of brain and muscle Arnt-like protein-1 (BMAL1), commonly observed in AML patients, correlate with poorer outcomes. BMAL1 modulates ferroptosis resistance in AML cells *via* the BMAL1-HMGB1-GPX4 axis, while its depletion sensitizes these cells to first-line chemotherapeutic agents like venetoclax, dasatinib, and sorafenib [61]. Combination therapy involving the histone deacetylase (HDAC) inhibitor CS055 and the peroxisome proliferator-activated receptor (PPAR) pan-agonist chiglitazar has demonstrated selective efficacy against LSCs while sparing healthy hematopoietic progenitors. The non-thiazolidinedione small-molecule chiglitazar enhances the suppression of HDAC3 by CS055, leading to ferroptosis in LSCs through xCT downregulation [62]. This process involves increased H3K27 acetylation at the activating Transcription Factor 3 (ATF3) promoter, upregulating ATF3 expression, which suppresses xCT [62]. Additionally, targeting aldehyde dehydrogenase 3 family member A2 (ALDH3A2) in AML cells enhances the effects of GPX4 inhibitors, inducing ferroptosis without compromising normal hematopoietic cell function. This highlights the potential of combining ALDH3A2 depletion with ferroptosis-inducing strategies as a novel therapeutic approach for AML (**Figure 2**) [59].

5. Conclusions

In summary, ferroptosis represents a crucial mechanism of cell death in AML, influenced by a complex network of cellular and metabolic processes. AML cells are particularly vulnerable to ferroptosis cause of their heavy reliance on mitochondrial respiration, increased ROS production, and iron accumulation, all of which contribute to lipid peroxidation. However, these cells also employ various strategies to resist ferroptosis, including the activation of antioxidant pathways such as NRF2 and the modulation of iron homeostasis *via* the TFR system and ferritin. The intricate interplay between ferritin and FPN, as well as processes like ferritinophagy, further fine-tunes the susceptibility to ferroptosis in AML cells. These adaptive mechanisms suggest that targeting specific points within the ferroptosis pathway, such as lipid metabolism, redox balance, and autophagy, could improve therapeutic outcomes. Combining ferroptosis-inducing strategies with traditional chemotherapy holds promise for overcoming treatment resistance. Additionally, genetic and epigenetic factors that regulate ferroptosis, including GPX4 silencing or ALDH3A2 inhibition, present potential avenues for selective therapeutic interventions. The increasing understanding of ferroptosis regulation in AML provides valuable insights for developing novel treatments that selectively target leukemic cells while minimizing damage to healthy hematopoietic tissues.

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Conflicts of interest

The authors declare no conflict of interest.

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Chapter 11

The Effective Stress Response Role of p53 in Inducing Ferroptotic Cell Death

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Abstract

Ferroptosis, an iron-dependent regulated cell death (RCD) or programmed cell death (PCD), effectively occurs due to iron overload in the cells, thus depleting the glutathione-dependent antioxidant system, and eventually causes cellular lipid peroxidation. P53 is a well-established molecule involved in cellular stress response leading to the initiation of RCD/PCD. It is known to be involved in the onset of ferroptotic cell death through distinct diverse molecular pathways. Through this chapter, we want to elucidate a possible pathway of increased iron intake in the cell and a comprehensive analysis of the possible mechanisms on how p53-induced ferroptotic cell death occurs due to the disbalance in cellular iron homeostasis.

Keywords: ferroptosis, p53, cell death, stress response, cell death pathways

1. Introduction

Iron is necessary for a wide range of biological functions. However, iron in higher concentration is cytotoxic and causes a lot of illnesses. Since the discovery of ferroptosis, a type of necrosis that is regulated by iron, iron and iron-catalysed oxidative stress have garnered a lot of attention due to their complex cellular signalling system that causes cell death and is linked to a number of disorders. Lipid reactive oxygen species (ROS) are produced by both types of labile iron that is liberated from organelles under different stressors and iron that is incorporated into enzymes. Numerous signalling pathways, including mitogen-activated protein kinase (MAPK) signalling pathways like the apoptosis signal-regulating kinase 1 (ASK1)-p38/JNK pathway, are triggered by ROS generated by iron. These ROS-activated signalling pathways regulate senescence or cell death and are linked to cancer, ischaemia-reperfusion injury during transplantation, and ageing-related neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Thus, understanding the spatiotemporal availability of iron and the role of iron in generating ROS will provide clues for the suppression of ROS and cytotoxic redox-active iron. Moreover, elucidating the molecular mechanisms and signalling pathways of iron-dependent cytotoxicity will enable us to find novel therapeutic targets for various diseases. The role of iron in generating ROS

has been discussed. Iron may directly generate ROS, including lipid ROS, according to Fenton chemistry. Furthermore, lipid ROS, which may harm a variety of biomolecules including proteins and DNA, are produced by iron-incorporating enzymes such as LOXs. Iron-mediated ROS can cause cellular senescence that is mediated by mitochondrial malfunction or the DNA damage response. It is important to carefully examine how iron over exposure can cause cellular senescence, as several studies have demonstrated. Iron chelators may aid in mitigating iron toxicity–related symptoms such as ageing and increased human longevity. Conversely, if iron is a result, then understanding the cellular basis as to why iron builds up with age might offer hints for ways to prevent iron accumulation–related illnesses. Involvement of iron in cellular senescence is not completely understood in either scenario and more investigation is required. Iron has a well-established involvement in cell death as opposed to cellular senescence. The MAPK signalling pathway, which is a downstream signalling route of ROS, may be crucial for ROS detection and signal transduction. Additionally, the ASK1-p38 pathway is a potential signalling channel; however, the exact processes behind signalling downstream of lipid ROS are still mostly unclear. Yet, the activation of ASK1-p38 pathway varies depending on the kind of cell. Future research is thus required to ascertain why the ASK1-p38 pathway is cell type–specific and to uncover if other major pathways influence lipid ROS-induced cell death. Determining the spatiotemporal availability of labile iron will therefore help to decrease ROS and redox-active iron-mediated damage to cells and tissues. The identification of new treatment targets for a range of iron-related disorders will also be made possible by clarifying the molecular processes and signalling pathways of iron-dependent cytotoxicity [1].

Ferroptosis was first discovered in cells that expressed a mutant RAS oncogene, but it has since been discovered in normal cells after being treated with drugs (like sulfasalazine, sorafenib, and artesunate) or small molecules (like erastin and RSL3) that target antioxidant enzyme systems, particularly the glutathione peroxidase GPX4 and the amino acid antiporter system x_c^-. A number of transcription factors, including TP53, NFE2L2/NRF2, ATF3, ATF4, YAP1, TAZ, TFAP2C, SP1, HIF1A, EPAS1/HIF2A, BACH1, TFEB, JUN, HIC1, and HNF4A, influence ferroptosis sensitivity in a variety of ways. Numerous physiological and pathological processes (such as immunity, differentiation, and metabolism) are impacted by dysfunctional ferroptosis. Hence, targeting the ferroptotic network may aid in novel therapeutic approaches to treat illnesses such as cancer, neurodegeneration, and ischaemia reperfusion damage [2].

ROS-ferroptosis is caused by lipid oxidative degradation, which is mediated by metabolic dysfunctions that affect intracellular metabolic functioning and ROS generation. According to recent research, p53 promotes the formation of ROS, which functions as a positive regulator of ferroptosis. The p53 tumour suppressor is "the guardian of the genome" and plays a role in regulating cell division and survival under a variety of stressors. In addition to its effects on autophagy, apoptosis, and the cell cycle, p53 also controls ferroptosis, either transcriptionally or post-translationally. By promoting mitochondrial respiration, p53 directly controls the metabolic adaptability of cells, resulting in ROS-mediated ferroptosis. In human colorectal cancer, p53 inhibits ferroptosis by forming the DPP4-p53 complex; in moderate stress, p53 preserves cell life by removing ROS [3]. There are several approaches to control ferroptosis including modifying the quantity of transcription factors and the activity of antioxidant enzymes. p53 inhibits ferroptosis either by directly inhibiting the activity of dipeptidyl peptidase 4 (DPP4) or by inducing the expression of cyclin-dependent kinase inhibitor 1A (CDKN1A/p21). On the other hand, p53 might

promote ferroptosis either by increasing the expression of spermidine/spermine N1-acetyltransferase 1 (SAT1) and glutaminase 2 (GLS2) or by suppressing solute carrier family 7 member 11 (SLC7A11) [4].

2. Overview of the role of p53 and associated proteins in ferroptosis

Maintaining genome stability is crucial for cells since it ensures the reproduction of the entire organism. DNA damage from natural and non-natural stimuli, such as replication mistakes, UV radiation, and chemical agents, is identified by cellular machinery and repaired.

However, the accumulation of genetic defects triggers the activation of cell death to remove cells with a damaged genome. This characteristic is utilised to target rapidly reproducing tumour cells during chemotherapy, radiotherapy, and immunotherapy. Apoptosis is the most well researched kind of cell death caused by DNA damage. However, genotoxic stress triggers non-apoptotic cell death and adaptive stress responses, which impact the effectiveness of anticancer treatment. DNA damage is a common stressor that causes a brief cell cycle stop to repair the DNA. If a cell's genomic integrity is compromised, it can trigger molecular pathways that either freeze normal processes or cause cell death. Genotoxic stress can cause non-apoptotic cell death (necroptosis, MPTP-dependent regulated necrosis, or parthanatos) and non-lethal processes (senescence, mitotic catastrophe, or autophagy) to enable cells to survive or postpone death. The final three have strong tumour suppressive characteristics, albeit they cannot directly destroy cancer cells. To overcome tumour cell resistance to apoptosis-inducing medicines, targeting non-apoptotic pathways of cell death is a promising method for anticancer treatment. It's important to realise that the DNA damage–mediated cell response may cause negative side effects for individuals. SASP may have a detrimental impact on cancer treatment due to the pro-tumorigenic actions of secretory factors. Inhibiting senescence through non-apoptotic cell death pathways might be a potential technique to prevent tumour recurrence. Modulating stress-adaptive responses and non-apoptotic cell death pathways can improve therapy efficacy. Understanding these processes is important not only for academic purposes but also for creating safe and effective treatment methods addressing numerous molecular pathways [5].

Cancer cells and some normal cells (e.g., kidney tubule cells, neurons, fibroblasts, and T cells) can be triggered by experimental substances (erastin, RAS-selective lethal small molecule 3, and buthionine sulfoximine) or by pharmaceutical medications (sulfasalazine, sorafenib, and artesunate). Nuclear factor erythroid 2-related factor 2, glutathione peroxidase 4, and heat shock protein beta-1 all work as negative regulators of ferroptosis by lowering cellular iron absorption and regulating ROS generation. However, by encouraging the formation of ROS and suppressing the expression of SLC7A11 (a particular light-chain member of the cystine/glutamate antiporter), NADPH oxidase and p53, particularly acetylation-defective mutant p53, function as positive regulators of ferroptosis. Ferroptosis differs from other types of RCD in terms of appearance, metabolism, and genetic make-up. By directly or indirectly affecting iron metabolism and lipid peroxidation, a number of molecules (including VDAC2/3, glutathione peroxidase (GPX4), heat shock protein beta-1 (HSPB1), nuclear factor E2-related factor 2 (NRF2), NADPH oxidase (NOX), p53, and SLC7A11) control ferroptosis. Other forms of RCD are also linked to these so-called ferroptosis regulators. Finding the

downstream signalling pathways or executors of iron-dependent ROS metabolism is therefore the primary goal of ferroptosis research in order to differentiate ferroptosis from other forms of RCD. It has been demonstrated that medications that induce ferroptosis can both RAS dependently and RAS independently limit the development of cancer cells, indicating that the timing of the ferroptotic response varies genetically across cancer cells [6].

Mild and severe DNA damage are variably incorporated into the cellular signalling networks, resulting in distinct cell fate decisions. After mild damage, the tumour suppressor p53 guides the cellular response to cell cycle arrest, DNA repair, and cell survival; after severe damage, p53 leads the cell death response. One post-translational change of p53, phosphorylation at Serine 46, occurs specifically after substantial DNA damage and is thought to be a hallmark of the cell death response. The p53 Ser46 phospho-isomer's mechanism, timing, and effects on apoptosis and ferroptosis remain unknown. P53 influences cell fate decisions differently in response to mild and severe DNA damage. Upon moderate damage, p53 is phosphorylated at Ser15 and Ser20, preventing p53 degradation and resulting in p53 stability and transactivation of p53 target genes. When DNA is severely damaged, p53 Ser46 kinases phosphorylate p53 at Ser46, leading to the activation of cell death–stimulating genes and the induction of mitochondrial outer-membrane permeabilization (MOMP). p53 is phosphorylated at Ser46 and regulates simultaneously transcription-dependent and transcription-independent apoptosis. The kinases PKCδ, HIPK2, DYRK2, ATM, and p38α phosphorylate p53 at Ser46 in response to severe DNA damage. This post-translational alteration disrupts p53 from the antiapoptotic iASPP protein while also serving as a binding site for the prolyl-peptidyl cis/trans isomerase Pin1, which catalyses isomerization of the phospho-Ser46-Pro47 link. This conformational shift lets p53 to engage with the acetyltransferases CBP and p300 at PML nuclear bodies, resulting in acetylation of p53 and effective transactivation of p53-stimulated cell death genes. Furthermore, p53 phosphorylated at Ser46 promotes apoptosis in a transcription-independent way. Cytosolic p53, which has been isomerized by Pin1, causes a conformational shift in the pro-apoptotic protein BAX, enhancing BAX-mediated mitochondrial outer membrane polarisation, cytochrome C (Cyt C) release, and hence death. p53 Ser46 phosphorylation and isomerization have been demonstrated to occur in the nucleus, implying that phosphorylated and/or isomerized p53 moves from the nucleus to the cytoplasm. Furthermore, p53 phospho-Ser46 has been linked to controlling ferroptosis by depleting coenzyme A (CoA) and glutathione (GSH) [7].

Activating p53 by itself is not enough to cause ferroptosis directly, unlike apoptotic cell death. Instead, p53 can modify the ferroptosis response in the presence of ferroptosis inducers such GPX4 inhibitors or elevated ROS levels by targeting its metabolic targets. P53 dependent ferroptosis during tumour suppression is regulated by both canonical (GPX4-dependent) and non-canonical (GPX4-independent) ferroptosis pathways. Additionally, p53 affects the tumour microenvironment and its newly discovered unique roles, which include controlling ferroptosis and stemness. The antiferroptosis system, the executor of lipid peroxidation, and the substrate of lipid peroxidation are the three fundamental components of ferroptosis [8].

The immune system, maternal reproduction, tissue ischemia/reperfusion injuries, and neurodegenerative illnesses are just a few of the numerous physiologic and pathological processes that p53 is implicated in addition to tumour suppression. There have been reports linking ferroptosis to neurological illnesses, tissue ischemia/reperfusion damage, and cancer. Recent research has demonstrated that p53, its signalling system,

and tumour-associated mutant p53 may all control ferroptosis. MDM2 and MDM4 are essential for the cell's negative control of p53 [9].

It is unknown how p53's remaining functions are regulated, despite earlier research showing that the loss of p53-mediated cell cycle arrest, apoptosis, and senescence does not totally eliminate its tumour suppression role. Although p53-mediated transactivation is only slightly impacted by the loss of K98 acetylation (p53K98R), simultaneous mutations at all four acetylation sites (p534KR: K98R+ 3KR[K117R + K161R + K162R]) totally eliminate the protein's capacity to control metabolic substrates including TIGAR and SLC7A11. Notably, p534KR is significantly less effective than p533KR at inhibiting tumour development in mice xenograft models. Furthermore, although p53-dependent ferroptotic responses are much reduced, p534KR may still induce the p53-Mdm2 feedback loop. All of these findings point to the vital role that p53 acetylation plays in ferroptotic responses and its residual tumour-suppressive properties [10].

The iPLA2β is a crucial regulator of p53-driven ferroptosis in response to stress caused by reactive oxygen species (ROS). It is well known that the calcium-independent phospholipase iPLA2β may liberate oxidised fatty acids from phospholipids by cleaving acyl tails from the glycerol backbone of lipids. Even in GPX4-null cells, iPLA2β-mediated detoxification of peroxidized lipids is adequate to inhibit p53-driven ferroptosis during ROS-induced stress. Furthermore, iPLA2β is overexpressed in human malignancies; in xenograft mice models, inhibiting endogenous iPLA2β increases sensitivity of tumour cells to p53-driven ferroptosis and facilitates p53-dependent tumour suppression. These findings show that iPLA2β functions as a significant GPX4-independent ferroptosis repressor. Notably, in contrast to GPX4, deletion of iPLA2β has no discernible impact on normal cell survival or development in normal tissues, yet iPLA2β is crucial for controlling ferroptosis in response to ROS-induced stress. Therefore, without significant safety concerns, iPLA2β is a viable therapeutic target for triggering ferroptosis-mediated tumour reduction [11].

Ferroptosis, rather than apoptosis, necroptosis, or mitochondria-mediated necrosis, is the primary effect of oxidative stress in cardiomyocytes. It's interesting to note that oxidative stress brought on by organic oxidants like tert-butyl hydroperoxide (tBHP) and cumene hydroperoxide (CHP), but not hydrogen peroxide, increased lipid peroxidation by promoting glutathione depletion and glutathione peroxidase 4 (GPX4) degradation in cardiomyocytes. Furthermore, increased oxidative stress is associated with labile iron overload by upregulating the expression of heme oxygenase 1 (HO-1), downregulating the transcription suppressor BTB and CNC homology 1 (Bach1), and enhancing iron release through heme breakdown. Remarkably, oxidative stress also encouraged HO-1 translocation to the mitochondria, which resulted in the buildup of lipid reactive oxygen species (ROS) and mitochondrial iron overload. Oxidative stress–induced ferroptosis was significantly reduced by overexpressing mitochondrial ferritin (FTMT) or mitochondrial catalase (mCAT), respectively, which targeted the buildup of ROS or mitochondrial iron excess. Cardiomyocytes exposed to doxorubicin (DOX), a chemotherapeutic drug, or simulated ischaemia and reperfusion (sI/R) also showed a significant rise in mitochondrial iron and lipid peroxide levels. When combined, oxidative stress caused by organic oxidants but not H_2O_2 mostly causes ferroptotic cell death in cardiomyocytes via pathways that are reliant on GPX4 and Bach1/HO-1. The findings further identify HO-1 mitochondrial translocation as a major mechanism and a possible molecular target for ferroptosis in cardiomyocytes caused by oxidative stress [12].

The gene with the highest frequency of mutations in human cancer is still the p53 tumour suppressor. p53 plays a variety of intricate roles in ferroptosis and metabolic regulation. In both healthy and malignant cells, wild-type (WT) p53 positively controls oxidative phosphorylation and lipid catabolism while negatively controlling lipid synthesis and glycolysis. The opposite is true in tumour cells, where mutant p53 positively regulates glycolysis and lipid production. Even more intricately, WT p53 appears to positively control ferroptosis in normal tissues, and this mechanism may contribute to the capacity of basal, unstressed p53 to inhibit the genesis and growth of tumours. Mutant p53 sensitises tumour cells to ferroptosis, while WT p53 seems to have a limited involvement in tumours where other regulators of ferroptosis take precedence. The functions of WT and mutant p53 in metabolism and ferroptosis, as well as their involvement in tumour suppression, will be thoroughly clarified by future research, which might lead to novel treatment options for metabolic illnesses and cancer. WT p53 restricts glucose metabolism and lipid synthesis, but mutant p53 seems to have the opposite effect. This makes the function of p53 in metabolism quite evident, if not intuitive. It is still unclear if its metabolic function contributes to p53's tumour suppression or to mutant p53's capacity to promote tumour growth. It is even less evident how p53 regulates ferroptosis and how this activity contributes to tumour suppression. To further understand the function of p53 in ferroptosis and ferroptosis in tumour suppression, greater research in animal models is required, paying particular emphasis to ferroptosis in various tissues. Furthermore, it is necessary to have a better understanding of which p53-target genes contribute to ferroptosis sensitivity. The answers to these questions might open up much-needed new treatment options for p53-mutant tumours [13].

Even though p53-mediated cell cycle arrest, senescence, and apoptosis are essential obstacles to the development of cancer, p53's metabolic activities also play a significant role. Suppressing the expression of SLC7A11, a crucial part of the cystine/glutamate antiporter, p53 prevents cystine uptake and makes cells more susceptible to ferroptosis, a non-apoptotic kind of cell death. Notably, p533KR, an acetylation-defective mutant that is unable to trigger apoptosis, senescence, or cell cycle arrest, completely maintains its capacity to control SLC7A11 expression and trigger ferroptosis in response to stress generated by ROS. These non-canonical p53 actions are implicated in both the mortality linked to Mdm2 deletion and embryonic development, according to analysis of mutant mice. Furthermore, SLC7A11 is abundantly expressed in human tumours, and in xenograft models, its overexpression disables p533KR-mediated tumour growth suppression and suppresses ROS-induced ferroptosis. Based on p53 modulation of cystine metabolism, ROS responses, and ferroptosis, our findings reveal a novel mechanism of cancer suppression [14, 15].

All living things eventually die, and many cell components perish as a result of internal suicide mechanisms in reaction to various stimuli. Often referred to as programmed cell deaths (PCDs), these intrinsic cell death mechanisms are essential for tissue homeostasis, organism growth, and a number of illnesses. According to recent data, the majority of PCDs contain intra-mitochondria components and the tumour suppressor p53. Moreover, each PCD pathway's induction is influenced by the location and motion of p53 inside cells. Furthermore, by concentrating on its modified intracellular localization in response to various cellular stressors, p53 plays a function in each PCD pathway. A variety of cellular stressors cause p53 to become active. By blocking Mcl1 and promoting Bax/Bak oligomerization, activated p53 can be translocated to the mitochondria and is linked to mitochondrial outer membrane permeabilization (MOMP). Additionally, p53 triggered by cellular stress travels to the

nucleus, where it controls the transcription of proteins linked to apoptosis, including Puma, Bax, Bak, Noxa, p53AIP1, Bip, El24, REEP1, and REEP2. The mitochondria-associated ER membrane (MAM) is another location for p53. Through its interaction with SERCA2, p53 causes Ca^{2+} to flow from the ER into the mitochondria, which in turn triggers the apoptotic pathway via MOMP. PTP opening in the mitochondria is how p53 controls the necroptosis mechanism. Through RIPK1 and RIPK3, TNF-α causes ROS, which in turn causes the opening of mitochondrial PTP. The transcriptional activity of p53 indirectly controls the expression of RIPKs. Oxidative stress causes p53 to be translocated, accumulate at the mitochondrial matrix, and combine with CypD. Drp1 and MDM2 must interact for p53 to translocate into the mitochondria. Furthermore, p53 must translocate to the mitochondria and then interact with CypD in order for DAPK1 to phosphorylate p53 at serine-20 in humans [16].

Ferroptosis is important in human cancer, although it's unclear how it works in gliomas. It has been revealed that p62/SQSTM1 inhibits ferroptosis via activating the NRF2 signalling pathway. p62 has a dual function in glioblastoma (GBM) ferroptosis based on p53 status. In p53 mutant GBM, p62 overexpression enhances ferroptosis and suppresses SLC7A11 expression, whereas in p53 wild-type GBM, it attenuates ferroptosis and increases SLC7A11 expression. p62 binds to p53 and prevents it from being ubiquitinated. Depending on p53 state, p62 regulates the p53-NRF2 relationship and p53-mediated inhibition of NRF2 antioxidant activity in different ways. The dual regulation of p62 on ferroptosis requires the presence of a p53 mutation. The traditional p62-mediated NRF2 activation pathway is a key regulator of ferroptosis in wild-type p53 GBM. This leads to elevated production of SLC7A11, which in turn has an antiferroptosis function. Together with increased p53 transcriptional suppression on SLC7A11 by p62, which results in a decrease of SLC7A11 and a pro-ferroptosis role, stronger interaction of mutant-p53/NRF2 by p62 enhances the inhibitory effect of mutant p53 on NRF2 signalling, reversing the classical p62-mediated NRF2 activation pathway in mutant p53 GBM. p53 may bind to NRF2 and prevent the NRF2 signalling pathway's antioxidant action. The link between NRF2 and R273h mutant p53 may be strengthened by p62 overexpression, whereas the interaction between NRF2 and wild-type p53 may not be much impacted. Depending on the p53 state, p62 regulates the p53-NRF2 interaction and the p53-mediated inhibition of NRF2 antioxidant activity in different ways. p53 mutant GBM, the higher suppression of NRF2 antioxidant activity, was caused by the increased mutant-p53/NRF2 connection by p62 [17].

It is unknown how p53 uses several metabolic pathways to decrease tumours. Here, the spermidine/spermine N 1-acetyltransferase 1 (SAT1) gene is used as a p53 transcription target. SAT1 is a rate-limiting enzyme in polyamine catabolism that converts spermidine and spermine back to putrescine. Activating SAT1 expression leads to lipid peroxidation and ferroptosis during ROS-induced stress, resulting in tumour growth reduction in xenograft models. SAT1 expression is downregulated in human tumours, and CRISPR-cas9 deletion of SAT1 expression reduces p53-mediated ferroptosis. SAT1 induction correlates with ALOX15 expression levels. PD146176, a selective inhibitor of ALOX15, dramatically reduces SAT1-induced ferroptosis. The findings identify a metabolic target of p53 involved in ferroptotic cell death and shed light on the regulation of polyamine metabolism and ferroptosis-mediated tumour suppression. The molecular processes behind p53-mediated ferroptosis are not well known [18].

p53 in efferocytosis, which is the process of removing dead or dying cells, as well as its involvement in several non-canonical modes of cell death, such as ferroptosis, necroptosis, autophagic cell death, mitotic catastrophe, paraptosis, and pyroptosis,

has been reported. Aside from apoptosis, accumulating data suggests that mammalian species possess a variety of regulated cell death mechanisms. Research looked at seven different cell death mechanisms, as well as efferocytosis. p53 may, however, be involved in other types of cell death, such as keratinization-associated cell death (cornification), anoikis (anchorage-dependent cell death), NETosis (neutrophil extracellular traps/NETs release-associated pathogen-induced cell death), and entosis (cell-in-cell invasion). The aforementioned non-canonical cell death mechanisms can eventually be employed to eliminate undesired cells, depending on physical, physiological, pathogenic, pharmacological, or environmental factors. Certain pressures or triggers may cause many forms of cell death. Indeed, when caspases are blocked, some apoptosis-inducing stimuli, such as TNF, can generate necroptosis, whereas genotoxic stress can cause CIA, mitotic catastrophe, and paraptosis. However, each non-canonical cell death appears to have a favourable trigger. It is also unknown how p53 preferentially chooses target genes implicated in various cell death processes, as well as if there are any dominant pathways or stimuli that cause a certain kind of cell death in a p53-dependent or independent manner [19].

The most frequently utilised magnetic nanoparticles in biomedicine are Fe_3O_4. Most nanoparticles' biodistribution in vivo is dictated by macrophage capture; nevertheless, the effects of nanoparticles on macrophages are still poorly understood. Fe_3O_4 nanoparticles might lower macrophage survival after 48 hours of treatment and produce a change in macrophage polarisation towards the M1 phenotype; RNA sequencing confirmed activation of the ferroptosis pathway and p53 overexpression relative to the control group. The expression in p53, xCT, glutathione peroxidase 4 (GPX4), and transferrin receptor (TFR) in macrophages was similar to that in erastin-induced ferroptosis in macrophages, and the ultrastructural morphology of mitochondria was consistent with that of erastin-treated cells. DCFH-DA was used to estimate the intracellular reactive oxygen species content in Fe_3O_4 nanoparticles treated with Ana-1 and JC-1 fluorescent probes to detect the mitochondrial membrane potential change; both showed to be time-dependent. Fer-1 minimised the decrease of the glutathione/oxidised glutathione (GSH/GSSG) ratio and intracellular oxidative stress states; therefore, Fe_3O_4 nanoparticles caused ferroptosis in macrophages. Pifithrin-α hydrobromide (PFT) was used as a p53 inhibitor to determine if elevated p53 expression is responsible for this mechanism. PFT therapy decreased the live/dead cell rate, TFR, p53 expression, and GPX4 consumption while also mitigating the drop in GSH/GSSG ratio. This suggests that p53 may play a role in macrophage ferroptosis triggered by Fe_3O_4 nanoparticle exposure. A theoretical framework was established for the molecular processes of ferroptosis in macrophages, as well as the in vivo biotoxicity generated by Fe_3O_4 nanoparticles [20].

TP53 plays a crucial role in iron homeostasis and cancer cell survival by preventing ferroptosis, a kind of cell death caused by iron. Cell lines expressing either a tetracycline-inducible wild-type (WT) or a representative mutant TP53 gene from six "hotspot" mutations in the DNA-binding domain (R273H, R248Q, R282W, R175H, G245S, and R249S) were developed using H1299 cells that are TP53-null. TP53 mutants (R273H, R248Q, R175H, G245S, and R249S) showed higher susceptibility to ferroptosis than WT TP53-expressing cells. p53 mutations (R273H, R248Q, R175H, G245S, and R249S) showed more susceptibility to ferroptosis than WT TP53-expressing cells. Mutant TP53-expressing cells were expected to have elevated iron acquisition mechanisms due to the importance of iron-mediated lipid peroxidation in ferroptosis activation. Only cells with the R248Q, R175H, and G245S TP53 mutations showed substantial increases in iron regulatory protein (IRP) RNA-binding activity

after ferroptosis activation. The observed variations in ferroptosis sensitivity did not align with changes in the expression of downstream IRP targets. These findings suggest that ferroptotic cell death bypasses traditional iron regulatory mechanisms. Inducing ferroptosis might be a therapeutic method for tumour cells with specific TP53 mutations [21].

p53 prevents cyclin-dependent kinase (CDK) from phosphorylating cell cycle inhibitor retinoblastoma (RB) proteins via triggering transcription of the p21 gene. Cell cycle–promoting genes are transactivated by E2F transcription factor proteins, which are released when RB is phosphorylated. Research has shown this regulation's surprising intricacy. In a variety of inducible and isogenic settings, increased p53 levels promote ferroptosis. Ferroptosis was similarly decreased by increased CDK activity in situations where p21 repressed ferroptosis, indicating that p21's effects must go beyond CDK inhibition. Overexpression of E2F inhibits ferroptosis in part through a p21-dependent mechanism, which is in line with studies that this transcription factor can trigger p21 transcription. Ultimately, ferroptosis was boosted by RB gene deletion. When combined, these findings demonstrate that the signals influencing ferroptotic sensitivity originate from many locations along the p53 tumour suppressor mechanism. Ferroptosis is regulated by p53 without the assistance of p21. Ferroptosis is suppressed by E2F1 through both p21-dependent and p21-independent pathways. Nutlin-3a prevents ferroptosis without the need of p53. The RB family and CDKs both influence ferroptosis. Phosphorylated RB species may be produced by CDKs to control ferroptosis. It is not established how these proteins are connected by the canonical cell cycle route, which consists of p53-induced p21, p21-inhibited CDKs, CDK-phosphorylated RB, and E2F1 release. The final ferroptotic result is anticipated to be influenced by each of these regulatory connections [22].

The p21 protein used to be believed to mitigate the spread of cancer and encourage ageing. Depending on the type of stressor and the tissue of origin, however, p21 may limit tissue damage and increase cancer survival. Moreover, ferroptosis sensitivity is reversed in both resistant and sensitive human cancer cell lines when the quantity of p21 protein is experimentally changed. According to the research, p21 may also be a biomarker for cancer cells' susceptibility to ferroptosis. In addition to the quantity of p21 protein, the levels of p21 protein upon ferroptosis activation are highly correlated with the sensitivity of a variety of cancer cell types. It also makes it necessary to investigate how p21 mediates ferroptosis's capacity to harm organs. By preventing ferroptosis even under healthy settings, p21 may be able to enhance tissue regeneration if it is actually able to regulate cell differentiation through CDKs, as previously proposed. This offers a fresh viewpoint on the diverse range of roles that p21 may play in many circumstances, even if it goes against the conventional roles of p21 in ageing [23].

It is commonly known that glutathione peroxidase 4 (GPX4) is the primary regulator of ferroptosis. Remarkably, the ferroptotic responses are modulated by p53 activation without any discernible impact on GPX4 activity. Rather, ALOX12 ablation eliminates p53-dependent tumour growth inhibition in xenograft models and reduces p53-mediated ferroptosis brought on by reactive oxygen species stress, indicating that ALOX12 is essential for p53-mediated ferroptosis. Human chromosome 17p13.1, where the ALOX12 gene is located, is a hotspot for monoallelic deletion in human malignancies. In Eμ-Myc lymphoma models, carcinogenesis can be accelerated by the loss of a single Alox12 allele. Furthermore, ALOX12 missense mutations seen in human malignancies prevent it from inducing p53-mediated ferroptosis and oxygenating polyunsaturated fatty acids. Interestingly, ACSL4 is necessary for ferroptosis upon

GPX4 inhibition but dispensable for p53-mediated ferroptosis, whereas ALOX12 is dispensable for ferroptosis caused by erastin or GPX4 inhibitors. Therefore, the research finds a ferroptosis mechanism that is ALOX12-mediated and ACSL4-independent, which is essential for p53-dependent tumour suppression. The findings highlight the significance of ROS in this process, even if the exact mechanisms by which p53 causes ferroptosis still need to be clarified. Apart from TBH treatment, other ROS, such as low concentrations of hydrogen peroxide (H_2O_2) and paraquat, can also cause p53-mediated ferroptosis. Therefore, p53 sensitises cancer cells to ferroptotic death following oxidative stress, which at least partially enhances tumour suppression. Since elevated ROS production typically accompanies rapid cell growth, cancer cells are likely to choose different defence mechanisms, including overexpressing SLC7A11 or inactivating ALOX12, to fend against ROS-induced ferroptosis. Since human cancer frequently results in the loss of one copy of chromosome 17p13.1, it is probable that many human cancers have lost one ALOX12 allele. Remarkably, new research shows that a significant portion (31–36%) of human tumours with chromosome 17p deletion nonetheless have a wild-type TP53 allele. Monoallelic loss of ALOX12 may be an aetiological component in this subgroup of patients, showing that ALOX12 haploinsufficiency promotes Myc-induced carcinogenesis and suppresses p53-mediated ferroptosis without evident changes in the ARF-p53 pathway. Remarkably, deletion of the ALOX12 gene does not cause significant developmental abnormalities in mice, in contrast to the severe phenotypes linked to GPX4-mutant mice. As a result, ALOX12 is essentially unnecessary for the majority of embryonic events that include ferroptosis, much like p53. The p53-ALOX12 axis may serve as a possible barrier to the development of cancer as ALOX12 is mutated, downregulated, and deleted in human malignancies. To find out if ALOX12 activation is similarly influenced by other p53 metabolic targets, more research is required. The overall effects on the GSH/GSSG ratio and GSH levels appear to be neutralised, most likely by activating additional p53 targets such as TP53-inducible glycolysis and apoptosis regulator (TIGAR) and p21, even if p53-induced downregulation of SLC7A11 can partially block cystine uptake3. For instance, p53-mediated activation of p21 was reported to support GSH30 conservation, whereas p53-mediated activation of TIGAR was shown to decrease ROS levels and raise the GSH/GSSG ratio [24].

Using a time-lapse microscopy system to analyse cell death kinetics across time activating wild-type p53 reduces the beginning of ferroptosis in human cancer cells, but deleting p53 increases ferroptosis sensitivity. This function requires p53 transcriptional activity, which is achieved in part by transactivating CDKN1A (which encodes p21). The activation of the p53-p21 pathways inhibits the generation of lipid peroxides and increases the conservation of glutathione, a key antioxidant necessary for lipid peroxide reduction. Activation of the p53-p21 pathway reduces the production of the enzyme ribonucleotide reductase (RNR), which requires glutathione as a cofactor to reduce ribonucleotides to deoxyribonucleotides. RNR inhibition, both genetic and pharmacological, is sufficient to decrease ferroptosis and conserve glutathione, as are chemical activators of the p53-p21 pathway. These findings show that the p53-p21 pathway's capacity to downregulate RNR may allow cells to preserve glutathione and redirect it towards lipid peroxide management during ferroptosis [25].

Deprivation of cystine can cause ferroptosis, a non-apoptotic oxidative cell death. Cystine is transported into the cell and reduced to the amino acid cysteine. Cysteine is required to produce glutathione, a tripeptide antioxidant employed by the lipid hydroperoxidase GPX4 to prevent the formation of ROS.

Stabilising p53 causes increased expression of p21. p21 conserves glutathione during ferroptosis to prevent the generation of lipid ROS and increase cell survival [26].

Non-coding RNAs, namely microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), reportedly modulate ferroptosis by interacting with key genes such as SLC7A11, GPX4, FSP1, and NRF2 involved in lipid metabolism, iron homeostasis, and oxidative stress responses. SLC7A11, a key regulator of ferroptosis, is a target for several miRNAs, some of them being, miR-5096 (breast cancer), miR-378a-3p (nerve injury), miR-143-3p (renal cell carcinoma), and miRNA-375 (gastric cancer) [27]. Circ0097009 acts as a competing endogenous RNA and suppresses SLC7A11 by sponging miR-1261 in hepatocellular cancer [28]. GPX4 is a crucial antioxidant regulator in the ferroptosis network, which is a target for several other miRNA such as miR-15a-3p (colorectal cancer), miR-15a (prostate cancer), miR-1287-5p (osteosarcoma), and miR-324–3p (lung adenocarcinoma) [29]. A few lncRNAs have also been reported to induce ferroptosis via many tp53 downstream ferroptosis regulators; namely, LINC00472 (lung cancer), MT1DP (NSCLC), GABPB1-AS1 (HCC), and LINC00618 (leukaemia) [30]. Therefore, ncRNAs play a crucial role in ferroptosis regulation, impacting cancer progression, drug resistance, and tumour suppression with the scope for exploration of cancer therapeutics.

The response of cancer cells to dietary restriction is poorly known. Cysteine depletion causes non-apoptotic behaviour in certain cancer cells. Ferroptosis is a type of cell death that relies on iron. Research suggests that p53, a stress-responsive transcription factor and tumour suppressor, may influence ferroptosis sensitivity. Using CRISPR/Cas9 genome editing, small-molecule probes, and high-resolution imaging, researchers revealed that stabilising wild-type p53 prevents ferroptosis in response to cystine deprivation. This delay requires the p53 transcriptional target CDKN1A (encoding p21), resulting in delayed intracellular glutathione depletion and lower production of damaging lipid ROS. The p53-p21 axis may help cancer cells cope with metabolic stress produced by cystine deficiency by delaying non-apoptotic cell death [31]. **Table 1** briefly outlines the role of p53 in ferroptosis in cancer.

Pathway	p53 role	Effect on ferroptosis	Reference
NRF2 Suppression	Reduces antioxidant response	Promotes ferroptosis	Yuan et al. [17], Zhang et al. [3]
GPX4 Regulation	Modulates antioxidant defence	Suppresses ferroptosis	Kang et al. [4]
iPLA2β	p53 suppresses iPLA2β	Promotes ferroptosis	Chen et al. [11]
SLC7A11 Suppression	Downregulates cystine uptake	Increases ferroptosis sensitivity	Jiang et al. [14, 15]; Liu et al. [9]
SAT1 Activation	Promotes polyamine metabolism	Promotes ferroptosis	Ou et al. [18]
p21-dependent RB phosphorylation inhibition	Inhibits cell cycle targeting genes E2F1	Promotes ferroptosis	Kuganesan et al., [22]
Inactivation of ALOX12	Elevates ROS production	Suppresses ferroptosis	Chu et al., [24]

Table 1.
Role of p53 in ferroptosis in cancer.

3. Pathological perspectives of p53 regulated ferroptotic cell death

The TP53 gene encodes the p53 protein, which is a tumour suppressor with 393 amino acids and 4 primary functional domains. This protein reacts to numerous biological stressors by controlling the expression of target genes, resulting in DNA repair, cell cycle arrest, apoptosis, metabolic alterations, and ageing. Mutations in the TP53 gene and the actions of the wild-type p53 protein (wtp53) have been related to a number of human malignancies. Eight TP53 gene variants are found in codons, accounting for 28% of all p53 mutations. The p53 gene can be utilised as a biomarker for tumour progression and is an attractive target for developing cancer therapy techniques. In wild-type p53-carrying tumours, aberrant p53 pathway signalling is often caused by other atypical situations, such as elevated MDM2 expression. These distinctions between cancer cell p53 and normal cells have made p53 one of the most essential targets for cancer therapy [32].

p53 was identified 45 years ago as an SV40 substantial T antigen–binding protein encoded by the most frequently mutated TP53 gene in human malignancies. As a transcription factor, p53 is closely controlled by a complex network of post-translational modifications to carry out its many tasks in tumour suppression. Although early investigations identified p53-mediated cell cycle arrest, apoptosis, and senescence as the basic barriers to cancer formation, a rising number of additional p53 activities have been uncovered, and the breadth of p53-mediated antitumour action has greatly increased [33].

Ferroptosis, a novel type of cell death, differs from existing cell deaths including autophagy and senescence. Ferroptosis has a role in the pathophysiology of a variety of illnesses, including cancer, cardiovascular disease, nervous system disorders, and kidney injury. Because oxidative stress and iron accumulation are common clinical characteristics of neurological disorders, the function of ferroptosis has been extensively studied. Ferroptosis is primarily defined by alterations in iron homeostasis, iron-dependent lipid peroxidation, and glutamate toxicity buildup, all of which may be precisely reversed using ferroptosis inducers or inhibitors. Ferroptosis is primarily controlled by iron, lipid, and amino acid metabolism via System Xc−, voltage-dependent anion channels, p53, p62-Keap1-Nrf2, mevalonate, and other pathways. Current research on ferroptosis in neurological illnesses focuses mostly on ferroptosis's main mechanisms. At the same time, ferroptosis was discovered to have a bidirectional regulatory role in neurological disorders. As a result, the unique regulatory mechanisms of ferroptosis in neurological illnesses require additional investigation in order to give new possibilities for the use of ferroptosis in neurological disease therapy [34].

One well-known cellular protector of genomic integrity is the tumour suppressor p53, which, when exposed to cellular stressors, either stops cell cycle progression or triggers apoptosis. The regulation of p53's residual functions following the cessation of these regular functions is unknown, though. Ferroptosis, a kind of cell death caused by iron and lipid peroxide, is crucial for p53-mediated carcinogenesis and the prevention of related cancers. The tumour suppressor activity of p53 is clearly impacted by post-translational alterations [35].

The TP53 tumour suppressor is the most frequently mutated gene in human malignancies, and it has been the target of much oncology research. The p53 protein is a transcription factor that may trigger the expression of several target genes while also regulating the cell cycle, apoptosis, and genomic stability. It is often considered as the "guardian of the genome". Increasing data suggests that p53 also modulates cell metabolism, ferroptosis, the tumour microenvironment, autophagy, and other

processes that contribute to tumour suppression. Mutations in TP53 not only affect its tumour suppressor function but also bestow oncogenic features on p53 mutants. Because p53 is mutated and inactivated in the majority of malignant tumours, it has become a popular target for the development of novel anticancer therapies. Until recently, p53 was thought to be an "undruggable" target, and little progress had been achieved with p53-targeted medicines [36].

Survivors of subarachnoid haemorrhage (SAH) suffer from serious neurological disabilities. Previous investigations have suggested that ferroptosis is implicated in SAH. Ferroptosis is a type of controlled cell death characterised by the buildup of lipid peroxidation. However, the significance and mechanism of ferroptosis in SAH are yet unknown and require additional investigation. However, Ferrostatin-1 (Fer-1) might prevent ferroptosis. Fer-1 treatment increased SLc7a11 and GPx4 levels while decreasing damage-associated molecular pattern markers and inflammatory cytokines. Similarly, reducing ferroptosis improved the blood-brain barrier, decreased cerebral oedema, behavioural impairments, and neuronal damage. The p53 inhibitor pifithrin-α effectively prevented cortical SAH-induced ferroptosis. Overall, our findings suggested that ferroptosis exacerbated EBI following SAH, which was partially dependent on p53, and that decreasing ferroptosis might be a viable therapeutic target for EBI [37].

Ferroptosis has recently received a lot of attention because of its role in causing a variety of neurological issues. The primary cause of ferroptotic cell death is impaired iron homeostasis (mostly excess iron deposition). This sort of programmed cell death in neurons can cause neuropathological problems [38].

The most frequent cause of stroke with a high fatality rate and the most frequent cause of hippocampus neuronal loss is cerebral ischaemia. It has been proposed that ferroptosis alters the function of hippocampus neurons. Using simulations in August Copenhagen Irish rat models, this work investigates the impact of ferritin overexpression brought on by lentivirus infection on hippocampus neuronal damage and mortality. After 90 minutes of middle cerebral artery occlusion (MCAO), the rats were given a 24-hour cerebral ischemia–reperfusion injury. A lentivirus infection was used to cause ferritin overexpression. To create an MCAO model, hippocampus neurons were subjected to the tau hyperphosphorylation test and the Morris Water Maze (MWM) test. The impact of ferritin overexpression on hippocampus neuronal death was assessed by the use of annexin V/propidium iodide flow cytometry and haematoxylin-eosin staining. The MWM test showed that while ferritin overexpression somewhat counteracted the effect of MCAO, MCAO modelling reduced the rats' cognitive and locomotor performance. Ferritin also decreased the hyperphosphorylation of tau brought on by MCAO. Ferritin clearly restored pathogenic alterations, reduced viability, enhanced caspase-9 cleavage, and increased apoptosis in hippocampus neurons. Furthermore, ferritin reduced the strong generation of reactive oxygen species and glutathione consumption brought on by MCAO models. Additionally, it was shown that ferritin downregulated and MCAO modelling increased two important modulators of ferroptosis, p53, and SLC7A11. The p53 and SLC7A11-mediated hippocampus neuronal ferroptosis caused by cerebral ischaemia requires ferritin decrease [39].

Alzheimer's disease (AD) is one of the most common neurodegenerative illnesses worldwide, characterised by cognitive and behavioural abnormalities. Ferroptosis is a type of regulated cell death characterised by intracellular iron buildup and lipid peroxide production, which then promotes AD start and progression. AlCl3 was linked to disruptions in learning and memory parameters, neuronal degeneration

in the hippocampus, increased immunoreactivity of amyloid-β and tau proteins, a significant increase in iron, nitric oxide (NO), malondialdehyde (MDA), JNK, and p53 levels, and a significant decrease in glutathione peroxidase activity. Interestingly, treatment of CPX-O alone or in conjunction with SP600125 in the AlCl3-induced AD model improved the previously published test results. As a result, CPX-O may be a good option for AD therapy, and more clinical trials will be needed to corroborate these preclinical findings [40].

Ferroptosis, a method of cell death discovered in 2012, is characterised by iron-dependent lipid peroxidation and differs from other cell death processes such as autophagy and apoptosis. Ferroptosis is distinguished by changes in iron balance, iron-induced lipid peroxidation, and glutamate-induced cellular damage. Ferroptosis is regulated by iron, lipid, and amino acid metabolic pathways, including system Xc−, voltage-dependent anion channels, and p53. Neurodegenerative diseases cause slow neuronal loss, primarily in the central nervous system, and are classified as both sporadic and uncommon inherited illnesses. These disorders cause the gradual loss of certain neuron populations and their interactions. Recent studies have found a clear link between the onset and development of neurodegenerative disorders and ferroptosis. Pharmacological regulation of ferroptosis, whether by induction or inhibition, offers interesting treatment options for various illnesses [41].

Ferroptosis is a unique type of controlled cell death with iron-dependent features that differs from autophagy, necrosis, and apoptosis. Esteroxygenase alters the amount of unsaturated fatty acids and causes lipid peroxidation. GSH can also reduce GPX4, which can lead to ferroptosis. Ferroptosis is similarly regulated by p53 and its signalling pathways. According to recent research, ferroptosis accelerates the death of RGC. One of the clinical hallmarks of glaucoma is increasing RGC depletion, indicating that ferroptosis may be associated with glaucoma development. Downregulation of GPX4 causes nerve cell death, indicating that ferroptosis may be linked to disorders involving visual nerve injury. Ferroptosis is now being widely explored and progressed in systemic disorders such as cardiovascular disease, gastrointestinal malignancies of the stomach, liver, and pancreas, and brain ailments [42].

Unlike necrosis, autophagy, and apoptosis, ferroptosis is a form of controlled cell death marked by iron-mediated lipid peroxidation. Numerous pathogenic processes, such as ischemia-reperfusion injuries, tumours, neurological illnesses, cardiovascular diseases, and cellular metabolism, can cause it. It has recently been found that ferroptosis is linked to p53. p53 is a tumour suppressor protein that plays a variety of potent roles in mitophagy, cell cycle arrest, senescence, cell death, and DNA damage repair. Recent evidence indicates that ferroptosis is essential for p53-mediated tumour suppression. By modifying the metabolism of iron, lipids, glutathione peroxidase 4, reactive oxygen species, and amino acids through a conventional route, p53 serves as a crucial bidirectional regulator of ferroptosis. Furthermore, ferroptosis has been found to be regulated by a non-canonical p53 mechanism in recent years. More clarification is needed on the specifics. Translational investigations of ferroptosis have been conducted to treat a variety of disorders, and these pathways provide fresh concepts for therapeutic applications [43].

Pancreatic cancer cells undergo complex metabolic reprogramming to ensure their survival and multiplication. p53 plays a dual role in tumour cell ferroptosis. However, the specific function and methods of wild-type p53 activation in inducing ferroptosis in pancreatic cancer cells remain unknown. p53 plays a critical role in SLC35F2-mediated ferroptosis, both in vitro and in vivo. SLC35F2 prevents ferroptosis by promoting TRIM59-mediated p53 degradation. Further mechanistic studies

revealed that SLC35F2 interacts competitively with TRIM59's E3 ubiquitin ligase SYVN1, stabilising TRIM59 expression and, as a result, boosting p53 degradation. In the p53 mutant PDX model, however, irinotecan hydrochloride and lapatinib ditosylate had no effect on the tumour xenograft model's susceptibility to IKE-induced ferroptosis. In conclusion, our findings demonstrate a unique mechanism by which the SLC35F2-SYVN1-TRIM59 axis drives ferroptosis in pancreatic cancer cells by suppressing endogenous p53. Thus, SLC35F2 emerges as a possible therapeutic target for pancreatic cancer [44].

Ferroptosis is a key factor in the development of gastric cancer, one of the most common malignant tumours in the digestive tract. In gastric cancer, glutathione peroxidase 4 (GPX4), a crucial negative regulator of ferroptosis, is widely expressed and promotes the formation of tumours. A possible strategy to cause ferroptosis and provide a successful treatment for gastric cancer is to target the control of GPX4. Many researches were conducted using Western blotting, Co-IP, immunofluorescence, quantitative real-time PCR, Ub assay, and flow cytometry to verify that OTUD5 is a deubiquitinase of GPX4 and controls ferroptosis. A subcutaneous tumour model used CRISPR-Cas9 to knock out the Otud5 gene in the mouse stomach cancer cell line (MFC) in order to investigate the physiological role of OUTD5. An investigation of the pathological connection in human gastric cancer was conducted using immunohistochemistry (IHC) analysis. Here the interaction, deubiquitylation, and stabilisation of GPX4 by ovarian tumour domain-containing 5 (OTUD5). Depletion of OTUD5 causes GPX4 to become unstable, promotes lipid peroxidation, and makes gastric cancer cells more susceptible to ferroptosis. Additionally, OTUD5 transcription is suppressed by the p53 activator nutlin-3a, which causes gastric cancer cells to ferroptosis and degrades GPX4. Interestingly, p53 mutations or deficiencies correspond with increased OTUD5 expression, which promotes the development of gastric cancer. Only wild-type p53 can block OTUD5 transcription. Furthermore, nutlin-3a-induced GPX4 degradation and OTUD5 silencing increase the susceptibility of gastric cancer cells to ferroptosis in vivo. The p53/OTUD5/GPX4 axis is then verified in clinical samples of gastric cancer. All of these results point to a mechanism by which p53 inactivation increases the transcription of OTUD5 to deubiquitylate and stabilise GPX4, which inhibits ferroptosis and accelerates the growth of gastric cancer. This finding emphasises the possible therapeutic benefit of OTUD5 targeting to induce ferroptosis in gastric cancer that is p53-inactivated [45].

One of the most prevalent diseases in the world, bladder cancer is a malignant tumour of the urinary tract. The emergence of different types of cancer is directly linked to lipoxygenases. Nevertheless, there is little information on the connection between lipoxygenases and p53/SLC7A11-dependent ferroptosis in bladder cancer. The goal was to learn more about the functions and internal workings of p53/SLC7A11-dependent ferroptosis and lipid peroxidation in the initiation and spread of bladder cancer. First, the metabolite generation of lipid oxidation in the patients' plasma was measured using ultraperformance liquid chromatography-tandem mass spectrometry. Stevenin, melanin, and octyl butyrate were shown to be increased in bladder cancer patients' metabolic abnormalities. After that, candidates with notable alterations in bladder cancer tissues were eliminated by measuring the expressions of lipoxygenase family members. ALOX15B was considerably downregulated in bladder cancer tissues compared to other lipoxygenases. Additionally, bladder cancer tissues have lower levels of p53 and 4-hydroxynonenal (4-HNE). Subsequently, plasmids containing sh-ALOX15B, oe-ALOX15B, or oe-SLC7A11 were created and introduced into bladder cancer cells. Then, tert-butyl hydroperoxide, the iron chelator

deferoxamine, the selective ferroptosis inhibitor ferr1, and the p53 agonist Nutlin-3a were introduced. Through both in vitro and in vivo investigations, the effects of p53/SLC7A11 and ALOX15B on bladder cancer cells were assessed. The ALOX15B knockdown protected bladder cancer cells against p53-induced ferroptosis and increased bladder cancer cell proliferation. Additionally, via inhibiting SLC7A11, p53 increased the activity of ALOX15B lipoxygenase. When combined, p53 inhibited SLC7A11 to trigger ferroptosis in bladder cancer cells, activating the lipoxygenase activity of ALOX15B and shedding light on the molecular process behind the onset and progression of bladder cancer [46].

About 3% of all cancer patients have osteosarcoma, an uncommon illness. Its precise aetiology is still mostly unknown. It is yet unknown how p53 affects the up- and downregulation of conventional and atypical ferroptosis in osteosarcoma. Investigating how p53 controls both conventional and atypical ferroptosis in osteosarcoma is the main goal of this work. p53's regulatory functions in ferroptosis in osteosarcoma were downregulated when it was directly or indirectly activated or inactivated. The expression of genes linked to the formation of osteosarcoma was found to be responsible for increased tumorigenesis. Increased carcinogenesis was the outcome of altering target genes and protein interactions, particularly SLC7A11. p53 regulated both normal and atypical ferroptosis in osteosarcoma. Atypical ferroptosis was downregulated when MDM2 activated p53, but typical ferroptosis was increased when p53 was activated [47].

One consequence of hypertension is hypertensive nephropathy (HN). Although Taohong Siwu decoction (THSWD) is utilised in therapeutic settings, it is unclear how it may be used to prevent and cure HN. The components and targets of THSWD for the treatment of HN were predicted using a network pharmacology method. Verification of the network pharmacology results was done by animal trials. About 205 targets were found and are thought to be possible THSWD targets in the therapy of HN. After screening 17 hub genes, it was determined that TP53 was the most important one. According to KEGG enrichment analysis, the p53 signalling pathway may be important. Elevated salt diets have been linked to fibrosis, inflammation, renal damage, and elevated blood pressure, according to in vivo studies. Ferroptosis was also demonstrated by the changed levels of biomarkers (iron, malondialdehyde, catalase, ferritin, transferrin, superoxide dismutase, and glutathione peroxidase 4). By inhibiting ferroptosis, THSWD and the ferroptosis inhibitor ferrostatin-1 (Fer-1) may considerably reduce HN. High salt increased the protein and mRNA expression of p53, p21, RB, and CTNNB1, but THSWD and Fer-1 therapy decreased these expressions. Meanwhile, the downregulation of Nrf2 brought on by a high-salt diet was restored by THSWD and Fer-1. According to the findings, THSWD reduces HN brought on by a high-salt diet by blocking ferroptosis through the p53/Nrf2/p21 pathway [48].

Stroke is one of the most dangerous illnesses in the world, especially in nations with big populations; it is linked to high morbidity, death, and disability rates. As a result, substantial research is being conducted to address these concerns. Strokes can be haemorrhagic (blood vessel rupture) or ischaemic (artery obstruction). Stroke incidence is greater among the elderly (≥65), but it is growing in the younger population. Ischaemic stroke accounts for approximately 85% of all stroke cases. Cerebral ischaemic damage can be caused by inflammation, excitotoxic injury, mitochondrial dysfunction, oxidative stress, ion imbalance, or increased vascular permeability. All of the aforementioned mechanisms have been carefully examined, yielding valuable insights into the condition. Other clinical outcomes reported include cerebral

oedema, nerve damage, inflammation, motor impairments, and cognitive impairment, all of which produce disabilities that interfere with everyday living and raise death rates. Ferroptosis is a kind of cell death marked by increased iron buildup and lipid peroxidation in cells. Ferroptosis, in particular, has previously been linked to ischemia/reperfusion damage in the central nervous system. It has also been discovered as a mechanism of cerebral ischaemic damage. The tumour suppressor p53 has been shown to influence the ferroptotic signalling pathway, which has both favourable and negative effects on the prognosis of cerebral ischaemia damage. Understanding the p53/ferroptosis signalling system may help researchers discover approaches for better stroke diagnosis, treatment, and prevention [49].

TP53, which encodes the p53 transcription factor, is the most often altered tumour suppressor gene across all human cancer types. While p53 has long been known to induce antiproliferative cell cycle arrest, apoptosis, and senescence programs in response to various stress signals, recent research has revealed additional important functions for p53 that are likely to contribute to tumour suppression, including roles in tumour metabolism, ferroptosis, signalling in the tumour microenvironment, and stem cell self-renewal and differentiation. Not only can p53 depletion or mutation cause cancer, but also overactive p53 is responsible for a variety of diseases such as developmental abnormalities, accelerated ageing, neurodegeneration, and cancer therapy side effects [50].

Ferroptosis, a kind of controlled cell death caused by lipid peroxidation, has recently been found as a key process in radiotherapy (RT)-mediated tumour suppression and radio resistance, albeit the precise genetic circumstances in which to target ferroptosis in RT remain unknown. p53 is the most frequently altered gene in human malignancies and a primary effector of RT. p53 loss improves radioresistance in cancer cells or tumours, at least in part, by inhibiting ferroptosis via SLC7A11. Ferroptosis inducers (FINs) that inhibit SLC7A11 have a substantial radiosensitizing impact on tumour organoids and patient-derived xenografts with p53 mutations or deficiencies. It is demonstrated that RT-induced ferroptosis is associated with increased p53 activation and improved clinical outcomes in cancer patients. Findings reveal a previously unknown function for ferroptosis in p53-mediated radiosensitization and suggest employing FINs in conjunction with RT to treat p53-mutant malignancies [51].

Calcium oxalate (CaOx) kidney stones are prevalent and can cause tubular injury, interstitial fibrosis, and chronic kidney disease (CKD). The mechanism of CaOx crystal-induced kidney fibrosis is uncertain. Ferroptosis, a kind of controlled cell death, is distinguished by iron-dependent lipid peroxidation, and the tumour suppressor p53 is an important regulator of ferroptosis. In the current investigation, we found that ferroptosis was considerably activated in patients with nephrolithiasis and hyperoxaluric mice, as well as that inhibiting ferroptosis protected against CaOx crystal-induced renal fibrosis. The single-cell sequencing database, RNA-sequencing, and Western blot research showed that individuals with chronic kidney disease and the oxalate-stimulated human renal tubular epithelial cell line, HK-2, have elevated p53 expression. In HK-2 cells, oxalate stimulation also increased p53 acetylation. Pharmacologically inducing ferroptosis through sirtuin 1–mediated p53 deacetylation might prevent renal fibrosis in nephrolithiasis patients [52].

Dachshund homologue 1 (DACH1) is well known for its role in controlling a variety of cell fates, although its specific regulation mechanism in ferroptosis remains unknown. We explored whether DACH1 controls ferroptosis by influencing p53/solute carrier family 25 member 37 (SLC25A37) signalling in hepatic fibrogenesis. The CRISPR-Cas9 method was utilised to knock out DACH1 in HSCs in order to

investigate its influence on ferroptosis. Immunoprecipitation, pulldown, and a mouse model of hepatic fibrogenesis were employed to investigate the molecular basis of DACH1-mediated ferroptosis regulation. Surprisingly, upregulating DACH1 caused p53 to translocate to the mitochondria by triggering phosphorylation at serine 392. The serine 392 mutation can inhibit DACH1 and p53 from combining, p53's mitochondrial translocation, and DACH1-mediated ferroptosis. Furthermore, SLC25A37 has been identified as a potential target for mitochondrial p53. The binding of p53 to SLC25A37 can increase SLC25A37's iron absorption capability, potentially causing an iron excess in the mitochondria and a hyperactive mitochondrial electron transport chain. Knocking down SLC25A37 can affect p53-mediated mitochondrial iron excess and ferroptosis. Furthermore, erastin therapy can trigger HSC ferroptosis and reduce fibrotic lesion damage in a mouse model of hepatic fibrosis. HSC-specific knockdown of DACH1, p53, and SLC25A37 inhibits erastin-induced HSC ferroptosis and reverses hepatic fibrosis [53].

Although bone marrow mesenchymal stem cells (BMMSCs) have been shown to prevent animal models of chronic kidney disease (CKD), the precise processes involved need to be investigated further. The purpose of this work is to look into the molecular mechanisms by which BMMSCs suppress ferroptosis and reduce CKD damage caused by Adriamycin (ADR). Analyses of renal function and histological data revealed that BMMSC therapy alleviated ADR-mediated renal dysfunction, which was also adequate to mediate the partial recovery of renal damage and mitochondrial pathological alterations. BMMSCs reduced ferrous iron (Fe2+) and reactive oxygen species while increasing glutathione (GSH) and GSH peroxidase 4. Furthermore, BMMSC therapy increased the expression of the ferroptosis-related regulator NF-E2-related factor 2 (Nrf2) while inhibiting Keap1 and p53 in CKD rat kidney tissue. BMMSCs may relieve CKD by inhibiting renal ferroptosis through regulation of the Nrf2-Keap1/p53 pathway [54].

The cystine/glutamate antiporter xCT is a newly discovered tumour-associated antigen found in a variety of cancer forms. xCT shields cancer cells against oxidative stress and ferroptosis by engaging in glutathione production, as well as contributing to metabolic reprogramming, which promotes tumour development and chemoresistant behaviour. Furthermore, cancer stem cells overexpress xCT. Interestingly, research on the TP53 gene have demonstrated that both wild-type and mutant p53 cause post-transcriptional down regulation of xCT, which contributes to ferroptosis. Furthermore, APR-246, a small molecule medication that may restore wild-type p53 activity in cancer cells, has been characterised as an indirect regulator of xCT expression in tumours with mutant p53 accumulation, making it a prospective candidate for usage in conjunction with xCT suppression [55].

4. Conclusion

The function of p53 in tumour suppression has been widely acknowledged to be largely attributed to its regulation of cell cycle arrest, senescence, and apoptosis, and new research has suggested that p53 also suppresses tumours by regulating a wide range of other cellular processes, including ferroptosis, metabolism, and antioxidant defence. The distinct iron-dependent kind of regulated cell death known as ferroptosis is brought on by lipid peroxidation in cells. Cells collect and activate the p53 protein in response to different stress signals. After activation, p53 transcriptionally controls the expression of certain target genes to control a variety of cellular

functions, including as ferroptosis, senescence, apoptosis, metabolism, and cell cycle arrest. The classical and non-canonical ferroptosis processes are both master regulated by p53 through several different methods. p53 often encourages ferroptosis. However, p53 can prevent ferroptosis in some situations.

Excessive levels of iron buildup and lipid peroxidation are hallmarks of ferroptosis, a type of regulated cell death. It is generally accepted that p53's tumour suppressor properties are based on its capacity to cause senescence and regulated cell death. Recent results, however, firmly link p53 to tumour suppression in a variety of additional ways, especially in relation to its regulation of metabolism and ferroptosis (cell death caused by iron and lipid peroxide).

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Chapter 12

Revisiting Skeletal Muscle Atrophy: Links between Copper Overload, Cuproptosis, and Muscle Atropy

Zhen Shen, Sunfeng Pan, Fengjie Wu, Kaitao Luo and Yanbo Shi

Abstract

Skeletal muscle senescence is a significant biological process in the aging of the body, marked by a reduction in muscle mass and function. In recent years, there has been growing interest in understanding the role of copper in skeletal muscle aging. During aging and various pathological conditions, skeletal muscle often exhibits an accumulation of excess copper. This abnormal buildup can trigger specific molecular mechanisms that lead to programmed cell death pathways such as apoptosis, pyroptosis, ferroptosis, and cuproptosis, as well as promote the aggregation of α-synuclein. These effects set off a series of signal cascades that ultimately result in metabolic imbalances within aging muscle fibers, including protein, mitochondrial, and satellite cell dysfunction, leading to degeneration and abnormalities in neuromuscular junctions. This forms a new pathophysiological mechanism for skeletal muscle aging and atrophy. Here, we provide a comprehensive analysis of the molecular and biological functions of copper in the regulatory network of skeletal muscle aging and atrophy, exploring the potential mechanisms of copper overload in aging muscles and the novel roles of various cell death signaling pathways induced by copper overload. Our goal is to offer potential molecular targets and therapeutic options for improving and treating skeletal muscle aging and atrophy through copper chelation strategies in clinical settings.

Keywords: copper, cuproptosis, skeletal muscle atrophy, copper metabolism, copper overload

1. Introduction

Copper, an essential trace element for the human body, is of vital importance in ensuring the normal functioning of life activities. In adults, the amount of copper generally ranges from 50 to 120 mg. About 50–70% is distributed in the muscles and bones. As a transition metal with redox properties, copper can participate in various biological processes in the form of ions. It achieves this by accepting and transferring electrons, playing a critical role in life-sustaining activities like energy metabolism, active oxygen scavenging, iron absorption, and signal transduction [1].

The homeostasis of copper is strictly regulated by liver metabolism. Both copper deficiency and copper overload can have a significant impact on the body

and are closely associated with the onset and progression of various diseases. In recent years, diseases related to copper overload have attracted extensive attention. Research has indicated that under various pathological conditions, such as diabetes, obesity, heart failure, neurodegenerative diseases, and tumors, the copper levels in the body can increase significantly. Copper overload is a pathological process where the imbalance in intracellular copper metabolism leads to enhanced toxicity of copper ions. It has thus emerged as an important target for intervention in multiple diseases [2, 3].

Skeletal muscle, serving as a motor organ, is made up of amino acids and is rich in mitochondria. In the normal adult body, skeletal muscle constitutes approximately 40% of the total body mass. The stable maintenance of its mass forms the material foundation for skeletal muscle to carry out metabolic functions. Once skeletal muscle undergoes aging and atrophy, it poses a substantial threat to human health. Thus, exploring novel intervention targets for the treatment of skeletal muscle atrophy holds great significance.

Numerous studies have indicated that under various pathological conditions, copper overload and muscular atrophy occur concurrently [4–7]. This implies that there might be a close intrinsic connection between abnormal copper metabolism and skeletal muscle atrophy. Nevertheless, the underlying mechanism of copper-overload-induced skeletal muscle atrophy remains in the stage of in-depth exploration. Notably, currently, a variety of pharmacological techniques involving copper chelating, which aim to reduce copper ion levels in the body, have achieved remarkable progress.

"Cuproptosis" refers to a process where an excessive accumulation of copper ions or disruptions in copper metabolism lead to cell dysfunction, eventually leading to cell death. In 2022, the concept of cuproptosis was first reported in the prestigious journal Science, revealing for the first time how copper ions play a unique role in cell death. This groundbreaking finding highlights a new mechanism of cell death that is distinct from well-known processes like apoptosis, necrosis, and autophagy.

Recent studies have revealed a significant potential link between copper overload, cuproptosis, and skeletal muscle atrophy. Here, we thoroughly explore the mechanisms behind muscle atrophy caused by excess copper and develop a comprehensive mechanism map. Our goal is to provide valuable insights that can support the clinical application of copper chelation technology for treating copper-related conditions or alleviating skeletal muscle atrophy.

2. Regulation of copper homeostasis and cuproptosis

In nature, copper primarily exists in the form of monovalent cuprous ions (Cu^{+}, with reducing properties) and divalent cupric ions (Cu^{2+}, with oxidizing properties). Copper in our diet mostly appears as Cu^{2+} and often combines with various substances to form stable complexes. Copper absorption mainly takes place in the duodenum and small intestine of the human body. During this process, Cu^{2+} is first reduced to Cu^{+} by the metal reductase Six Transmembrane Prostate Epithelial Antigen 1 (STEAP1) or Cytochrome b Reductase 1 (DCYB). Subsequently, Cu^{+} rapidly and specifically binds to the copper transporter 1 (CTR1) and then enters the intestinal epithelium. The absorbed Cu^{+} is then transported *via* the portal circulation

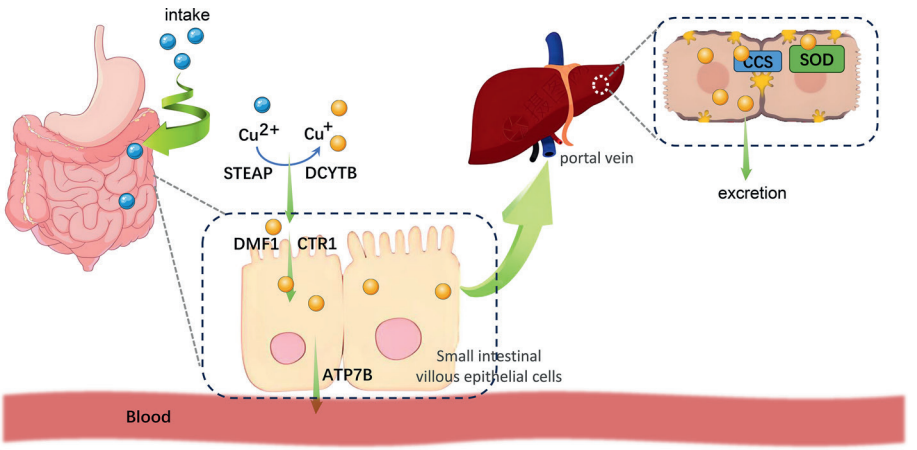

Figure 1.
Schematic diagram of human copper metabolism.

system to be stored in liver cells. After that, it is transported to specific tissues and organs to perform their corresponding physiological functions. Excess Cu^{+} maintains the balance of copper ions in the body by being excreted into the bile, and a small amount of excess Cu^{+} is directly excreted with the feces in the form of unabsorbed metal ions (**Figure 1**) [8].

Upon entering the circulatory system, Cu^{+} combines with copper transporters such as ceruloplasmin, albumin, and trans-cupric. These complexes are then transported to various organs and tissues of the body [9]. Once Cu^{+} reaches the target organs and tissues, it binds to different copper-chaperone proteins, thereby exerting its catalytic effects. The main copper-chaperone proteins include cytochrome C oxidase copper chaperone 17 (COX17), copper chaperone for superoxide dismutase (CCS), and antioxidant 1 copper chaperone (ATOX1).

COX17 is mainly distributed in the cytoplasm and mitochondrial membrane. When it binds with Cu^{+}, it is further transported to the secondary copper-binding proteins SCO 1/2 (synthesis of cytochrome C oxidase 1/2) and cytochrome C oxidase (CCO), playing a crucial role in regulating enzyme activity in the mitochondrial respiratory chain. CCS transfers Cu^{++} into superoxide dismutase 1 (SOD1), endowing SOD1 with the ability to combat oxidative stress and promote protein synthesis and secretion. ATOX1 transports Cu^{+} to the nucleus, where it combines with transcription factors to drive gene expression. Additionally, ATOX1 transfers Cu^{+} from the trans-Golgi network (TGN) to ATPase copper-transporting alpha/beta (ATP7A/B). Under physiological conditions, ATP7A/B transports Cu^{+} to the TGN. When the intracellular Cu^{+} content increases, ATP7A/B fuses with the plasma membrane, pumping Cu^{+} back into the blood circulation. The excess Cu^{+} is then transported *via* the portal vein and stored in the hepatocytes of the liver. If there is an excessive amount of copper in the body, the excess copper is excreted through bile and feces [10].

In conclusion, the equilibrium state of copper is maintained not only by duodenal absorption and bile excretion to achieve homeostasis but also by the synergistic action of copper-chaperone proteins and membrane transporter proteins. The main regulatory factors of copper metabolism homeostasis are summarized in **Table 1**, and the metabolic balance diagram is shown in **Figure 2**.

Molecule	Function	References
STEAP	Metalloreductase	[11]
CTR1	Transmembrane solute carrier, Cu importer	[12]
CP	Major exchangeable plasma Cu carrier	[13]
COX17	Cytochrome c oxidase copper-chaperone protein	[14]
CCS	Copper chaperone for superoxide dismutase	[15]
ATOX1	Cytosolic Cu metallochaperone	[16]
ATP7A/B	Cu exporter/Golgi apparatus Cu chaperone	[17]
MT1/2	Cu/Zn storage protein	
SOD1	Superoxide scavenger	

STEAP: six-transmembrane epithelial antigen of the prostate; CTR1: copper transporter 1; CP: ceruloplasmin; COX17: cytochrome C oxidase copper chaperone 17; CCS: copper chaperone for superoxide dismutase; ATOX1: antioxidant 1; ATP7A/B: Cu^{2+} transporting, alpha polypeptide/beta polypeptide; SOD1: superoxide dismutase 1.

Table 1.
Genes involved in copper homeostasis and cuproptosis.

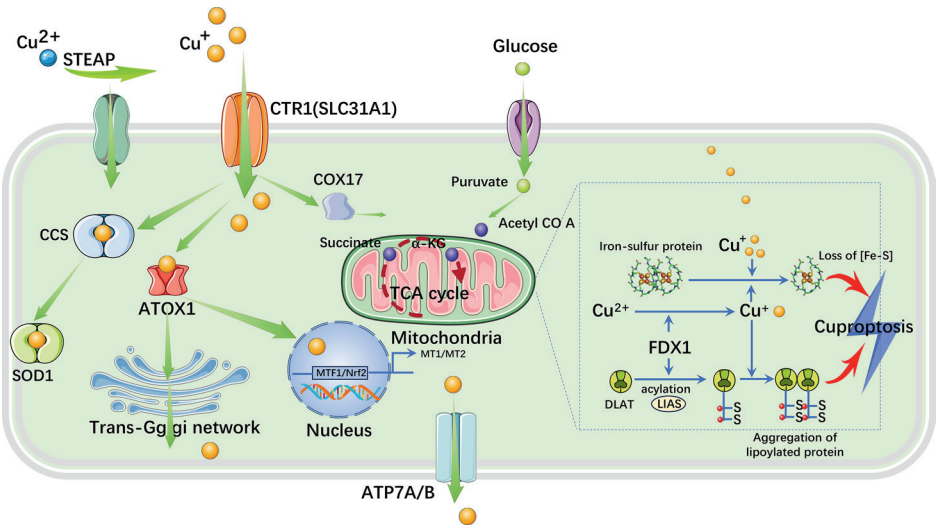

Figure 2.
Diagram of copper metabolic and cuproptosis mechanism.

3. Regulatory network of skeletal muscle atrophy

The pathological and physiological mechanisms of skeletal muscle atrophy are complex, primarily involving imbalances in protein and mitochondrial metabolism, a reduction in the number and function of satellite cells, the activation of cell death processes, and the degeneration of the neuromuscular junction (NMJ). Research has revealed that copper overload is a crucial biological factor that triggers oxidative stress. It promotes the accumulation of reactive oxygen species (ROS) through the Fenton reaction. This process inhibits the classical phosphatidylinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, ultimately resulting in a decreased protein synthesis capacity in skeletal muscle.

Furthermore, copper overload can also disrupt the homeostasis of proteins involved in mitochondrial dynamics regulation, such as mitofusin 1 and 2 (MFN1/2), optical atrophy protein 1 and 2 (OPA1/2), and dynamin-related protein 1 (DRP1). These changes are important factors contributing to mitochondrial abnormalities and the aging and atrophy of skeletal muscles. Further research indicates that reactive oxygen species (ROS) generated by copper overload have a negative regulatory effect on muscle satellite cells, hindering the maintenance of satellite cell numbers and the exertion of their functions. In the elderly body, muscle fibers damaged by normal physiological activities cannot be replenished in a timely manner, thus leading to the formation of muscle fiber atrophy phenotypes [9].

Cell death plays a crucial role in the loss of aging muscle fibers. Multiple forms of cell death, including apoptosis, pyroptosis, ferroptosis, and cuproptosis, are all involved. These different forms of cell death involve different intermediate proteins and have distinct signaling pathways. Current research shows that when there is copper overload, it may activate multiple cell death programs, promote the aggregation of α-synuclein, directly cause the degradation and loss of muscle cell contents, and simultaneously trigger the degeneration and abnormalities of the neuromuscular junction (NMJ), ultimately leading to the aging and atrophy of skeletal muscles.

Muscle atrophy is a common complication of various chronic diseases such as obesity, diabetes, heart failure, and drug addiction. Its pathological features are manifested as a decrease in muscle mass and strength decline, and its pathogenesis is complex, which may be closely related to factors such as protein metabolism imbalance, neuromuscular junction inactivation, and mitochondrial loss. Muscle atrophy not only affects normal gait and motor ability in humans, severely reducing the quality of life, but also is closely linked to the occurrence, progression, and prognosis of various diseases. In various chronic disease states, it is a key factor affecting mortality [18]. Current research shows that in pathological conditions, combating skeletal muscle atrophy is an indirectly effective method for treating certain diseases [19]. Therefore, it is particularly important to identify novel intervention targets for skeletal muscle atrophy (such as copper metabolism) to benefit its treatment.

Skeletal muscle is an important motor organ in the human body. It is composed of amino acids as the basic building blocks and is rich in mitochondria. In normal adults, it accounts for approximately 40% of the body mass. Maintaining the mass of skeletal muscle is the material basis for the metabolic function of skeletal muscle. When pathological stimulation signals from the outside world occur, skeletal muscle will respond and then develop an atrophic phenotype. The imbalance in the homeostasis of muscle proteins and mitochondria is the fundamental cause of skeletal muscle atrophy [20]. Under normal physiological conditions, skeletal muscle proteins are in a continuous dynamic balance of synthesis and degradation, and mitochondria also maintain their own quality control through continuous fusion and fission. However, once in a pathological condition, this metabolic balance is disrupted, leading to the degradation rate of muscle proteins exceeding the synthesis rate and mitochondrial fission occurring more frequently than fusion, ultimately triggering muscle atrophy.

The occurrence of skeletal muscle atrophy involves various metabolic processes and different regulatory mechanisms. In terms of protein metabolism, the synthesis of skeletal muscle proteins mainly depends on the pro-synthetic signals mediated by growth factors such as testosterone, growth hormone, and insulin-like growth factor. On the other hand, the degradation process mainly occurs through degradation pathways such as the ubiquitin-proteasome system, autophagy, apoptosis, and the activity of calpains. In terms of mitochondrial metabolism, the fusion and fission

of mitochondria are precisely regulated by the dynamic balance of their respective upstream target proteins, namely mitofusins and fission proteins [21]. A deep understanding of these regulatory mechanisms and signaling pathways is the key theoretical basis for the development of therapeutic drugs for skeletal muscle atrophy, providing an important research direction for overcoming this medical challenge.

4. Copper overload and skeletal muscle atrophy

4.1 The roles and physiological functions of copper in skeletal muscle

Copper serves as a cofactor for various essential enzymes in the body and participates in various biochemical reactions within cells in an ionic form, playing a crucial role in maintaining the normal metabolism of organs. The metabolic homeostasis of copper ions within cells is jointly regulated by copper transporters and chaperone proteins. Among them, mutations in the transporter genes copper-transporting ATPase 7A (ATP7A) and copper-transporting ATPase 7B (ATP7B) can lead to copper deficiency or copper overload, which in turn trigger Menkes disease and Wilson's disease (hepatolenticular degeneration). Exogenous copper is the primary source of copper in the body and is mainly obtained through food intake. Most of the copper in food exists in the form of organic copper bound to proteins, and this type of dietary organic copper can be absorbed by gastrointestinal cells and enter the body (**Figure 1**).

Copper metabolism mainly occurs in two stages: In the first stage, copper from food is absorbed in the human small intestine and enters the bloodstream, where it binds to small molecules in the serum, such as histidine, α2-macroglobulin, albumin, etc., thus forming a copper storage pool. In the second stage, most of the copper ions in the copper storage pool enter the liver *via* the portal vein. After being processed in the liver, they enter the bloodstream in the form of ceruloplasmin and are transported and distributed to various organs throughout the body that rely on copper, such as the brain and skeletal muscles [22]. The remaining part of the copper continues to exist in the serum in the form of binding to small molecules. The degradation of ceruloplasmin usually occurs in the liver as well. Hepatic endothelial cells can capture ceruloplasmin in the serum through transcytosis and completely remove the sialic acid residues at the N-terminus of its oligosaccharides. The desialylated ceruloplasmin, under the action of the asialoglycoprotein receptor, is then excreted into the space of Disse and subsequently endocytosed by hepatocytes. Under the degradation of hepatocyte lysosomes, ceruloplasmin is decomposed and releases copper ions. In order to maintain the metabolic balance of copper ions, excess copper ions are excreted from the liver into the bile and solidified by bile salts before being excreted from the body. This is the main pathway for copper excretion [23, 24]. However, during the electron transfer process, oxidative stress is generated. The key copper-containing enzyme in muscle cells that resists oxidative stress is Cu,Zn-superoxide dismutase (Cu,Zn-SOD) located in the cytoplasm. This enzyme can utilize a copper ion to catalyze the disproportionation reaction of superoxide and effectively inhibit the damage caused by oxidative stress to cells (**Figure 1**).

Copper ions are essential for myoblast proliferation and differentiation, as well as for maintaining normal metabolic homeostasis in differentiated myocytes [25]. As a key static cofactor, copper ions can participate in the construction and formation of various copper enzymes (such as oxidoreductases, oxygenases, hydroxylases,

and transferases) by virtue of their own redox properties. These enzymes have flexible active sites that can maximize the transfer and transmission of electrons. Among them, the most classic copper enzyme is cytochrome C oxidase (CCO). As the terminal enzyme of the electron transport chain, it can couple electron transfer through the oxidation of cytochrome C and the reduction of oxygen, thereby enhancing the shuttle activity of the proton pump across the mitochondrial membrane and promoting the generation of ATP [26]. However, oxidative stress is generated during the electron transfer process. The key copper enzyme in myocytes to resist oxidative stress is SOD1 located in the cytoplasm. This enzyme can use a copper ion to catalyze the disproportionation reaction of superoxide and effectively inhibit the damage caused by oxidative stress to cells.

Another important site of copper ions in cells is the pathways related to the copper secretion pathway, including the trans-Golgi network (TGN), endolysosomes, secretory granules, and copper storage vesicles. The copper secretion pathway can prompt different types of cells to activate copper-dependent kinases to perform their respective functions. Moreover, in certain copper-dependent cells, copper ions can also regulate the activities of kinases such as Unc-51-like protein kinase 1/2 (ULK1/2), mitogen-activated protein kinase kinase 1 (MEK1), and phosphodiesterase 3B (PDE3B), thereby influencing the expression of autophagy-related genes (ATGs), the formation of autophagosomes, as well as cell proliferation and metabolic activities [23]. It is worth noting that some of these kinases may be associated with the signaling pathways related to skeletal muscle cell atrophy induced by copper overload [27].

4.2 Regulation of copper metabolism in skeletal muscle

Skeletal muscle is not only an organ that highly depends on copper ions and has a high metabolic rate but also a major organ where copper is distributed [28]. Copper-dependent organs (including skeletal muscle) follow common physiological principles for the uptake of copper ions. This process is regulated by intracellular copper transporters (CTRs) and copper-chaperone proteins. Specifically, copper transporters (CTR1, CTR2, and CTR3) are responsible for the uptake of copper ions. They transport copper ions across the membrane into the cell. The other two copper transporters, ATP7A and ATP7B, show tissue-specific expression. Except in hepatocytes, ATP7A is more abundantly expressed than ATP7B in most cells [28]. These two mainly regulate the distribution, storage, and efflux of intracellular copper ions. Copper-chaperone proteins can bind to intracellular copper ions, neutralize their toxicity, and transfer them to specific target proteins. The three most important copper-chaperone proteins include cytochrome C oxidase 17 (COX17), antioxidant 1 copper chaperone (ATOX1), and copper chaperone for superoxide dismutase (CCS). Different copper-chaperone proteins mediate different copper ion transfer pathways within the cell. The metabolism of copper ions in skeletal muscle is regulated by copper transporters and copper-chaperone proteins. Among them, CTR1, ATP7A, and ATP7B have been proven to be widely expressed in skeletal muscle cells [29]. CTR1 is the most crucial copper uptake transporter protein. The expression of CTR2 is cell-specific and may be more involved in intracellular copper ion transport. However, its expression in muscle cells and the related mechanisms regulating copper ion uptake are still unclear. Human CTR1 consists of 190 amino acids and fragments containing specific metal-binding sequences. The methionine 43 and 45 regions are the key to mediating copper ion uptake [30]. CTR1 can regulate the expression and abundance of CTR1 on the membrane through endocytosis and extra-membrane position migration, thereby

controlling the cell's copper ion uptake. When the intracellular copper ion content increases, the cell will enhance clathrin-and dynamin-dependent endocytosis, reduce the abundance of CTR1 on the cell membrane, and simultaneously migrate away from the membrane edge. These two aspects work together to inhibit the cell's capture and uptake of copper ions [28], avoiding damage to the cell caused by copper overload. This automatic sensing mechanism may be mediated by specificity protein 1 (Sp1) as an intermediate [31].

After copper ions enter the cell through CTR1, they dissociate from it and then rely on different copper-chaperone proteins to mediate various copper ion transport pathways. Among these pathways, COX17 and the non-protein ligand CuL play a crucial linking role in the process of copper ion transport targeted to mitochondria. COX17 can bind copper ions by reducing its own cysteine residues and then transfer the copper ions to the mitochondrial intermembrane space, interacting with two cytochrome C oxidase synthesis proteins (SCO1 and SCO2) and participating in the CCO metabolic pathway [32]. At the same time, after CuL in the cytoplasm binds to copper ions, it can cross the outer mitochondrial membrane and enter the intermembrane space, participating in the assembly of CCO, the maturation of SOD1, and the further uptake and storage of mitochondria [32]. COX17 and CuL jointly regulate the copper ion metabolic balance in mitochondria. In addition, ATOX1 can transfer copper ions to the N-terminal regions of ATP7A and ATP7B at the membrane structures of the secretory pathway, enhancing the transport activity of the copper ion secretory pathway and thus regulating the distribution of copper ions within the cell [33]. CCS, on the other hand, transfers copper ions to SOD1 by forming a highly specific complex, and completes metal substitution and disulfide bond formation at specific sites, thereby antagonizing the damage of superoxide to cells [34]. The distribution of copper ions within the cell mainly depends on the transmembrane transport of ATP7A and ATP7B between different structures within the cell such as the trans-Golgi network (TGN), endosomes, and melanosomes. This process is crucial for the activation of copper enzymes, the storage of copper ions, and the excretion of excess copper ions and is a key link in the regulation of intracellular copper homeostasis [35].

4.3 Possible mechanisms of copper overload

Intracellular copper uptake and efflux are maintained in a precise state of homeostasis, regulated by proteins like Ctr1, ATP7A, and ATP7B. Once the uptake of copper ions surpasses the efflux, copper overload ensues. Copper overload is a biological phenomenon characterized by copper toxicity resulting from the malfunction of the copper ion metabolism mechanism. It is closely associated with neurodegenerative diseases, cardiovascular diseases, cancer, and numerous other human ailments. Currently, the exact regulatory mechanism of copper overload remains incompletely understood. This may involve multiple distinct signaling pathways and molecular mechanisms, and it is one of the key problems to be resolved in the field of copper metabolic physiology.

Aging, serving as a pathological model associated with the occurrence of copper overload, offers valuable clues for delving into the mechanism of intracellular copper overload under pathological conditions [36]. Under normal physiological circumstances, extracellular copper overload can activate the intracellular copper homeostasis regulation response mechanism. Firstly, claudin-dependent endocytosis is enhanced, which leads to a reduction in the abundance of the copper transporter CTR1 on the cell membrane. Additionally, CTR1 migrates away from the membrane

edge. These combined effects inhibit the cells' capture and uptake of copper ions, thus reducing the influx of copper ions into the cells. Secondly, the copper transporters ATP7A and ATP7B can enhance the excretion and metabolism of copper ions. Working in tandem, they jointly maintain intracellular copper homeostasis and prevent copper overload [28].

In various chronic disease conditions, the level of circulating ceruloplasmin increases significantly, leading to extracellular copper overload in skeletal muscle cells. Pathological states can disrupt the aforementioned copper homeostasis mechanism, causing metabolic disorders in copper ion uptake and efflux, and ultimately triggering intracellular copper overload. REDOX imbalance and autophagy dysfunction may also be involved in this process [36, 33]. Evidently, copper overload is not the outcome of single-factor regulation but the product of multiple signal cascade events. Further exploring and elucidating the relevant molecular mechanisms will be a crucial research direction in the field of copper metabolism physiology.

4.4 Signal involved in copper overload and muscle atrophy

Copper ions not only contribute to the formation of copper proteins but also play a profound role in regulating sugar and lipid metabolism within the body. Research has indicated that copper deficiency in the body can trigger insulin secretion inhibition and impaired glucose tolerance, which clearly demonstrates the crucial role of copper in maintaining glucose homeostasis [37]. As an endogenous regulator of lipolysis, copper ions can directly bind to the cysteine site of PDE3B, thus inhibiting its activity. Through this mechanism, copper ions further promote lipolysis *via* the cyclic adenylate-dependent pathway, exerting a significant impact on fat metabolism [38]. Moreover, copper ions are also vital in the mutual regulation of sugar and fat metabolism. In a high-sugar environment, ceruloplasmin can significantly enhance the oxidation of low-density lipoprotein [39]. Skeletal muscle is a key organ for carbohydrate and fat metabolism, and its health is of utmost importance for energy metabolism. The onset of skeletal muscle atrophy is often accompanied by energy metabolism disorders [40], and the regulatory function of copper ions in this process warrants further investigation and attention.

4.4.1 Copper overload inhibits skeletal muscle protein synthesis via the PI3K/PKB/Akt/mTOR signaling pathway

The PI3K/Akt/mTOR signaling pathway regulates muscle protein synthesis, and its signal transduction process is as follows: Upon stimulation by growth factors, PI3K can phosphorylate diphosphoinositol on the muscle membrane, converting it into phosphatidylinositol triphosphate. Subsequently, it phosphorylates and activates the downstream target Akt. Once Akt is activated, it promotes muscle protein synthesis through two distinct signaling pathways. Firstly, in an mTOR-independent manner, glycogen synthase kinase 3β (GSK3β) is directly inhibited, and at the same time, eukaryotic initiation factor 2B (eIF2B) is activated to promote muscle protein synthesis. Secondly, Akt phosphorylates and activates the downstream target mTOR, which mainly consists of mTOR complex 1 (mTORC1) and mTORC2.

Among them, mTORC1 is particularly sensitive to rapamycin. Rapamycin can phosphorylate and activate p70 S6 kinase, which in turn promotes the high phosphorylation of ribosomal S6 protein. This ultimately facilitates mRNA transcription and translation, leading to increased muscle protein synthesis. Additionally, activated

mTORC1 can phosphorylate eIF4E binding protein 1, inhibiting its activity and further promoting mRNA translation, thus enhancing muscle protein synthesis.

It is worth noting that copper overload significantly inhibits the PI3K/Akt/mTOR signaling pathway, thereby weakening the protein synthesis capacity of skeletal muscle. This effect may be mediated by reactive oxygen species (ROS), markers of oxidative stress. Copper overload is a potent inducer of ROS, which can directly activate NF-κB and promote the overexpression of myostatin. Myostatin inhibits the expression of miR-486 through an unknown mechanism, thereby activating the activities of phosphatase and tensin homolog (PTEN), and further inhibiting the recruitment and phosphorylation of Akt [41]. Therefore, ROS can form miR-486/PTEN/PI3K/Akt signaling cascade-mediated by myostatin and inhibit myosin synthesis, which may be one of the potential mechanisms of copper overload inducing skeletal muscle atrophy.

4.4.2 Copper overload disrupts mitochondrial homeostasis in skeletal muscle

Mitochondria are organelles that exhibit a unique dependence on copper. Each mitochondrion contains approximately 45,000 to 50,000 copper ions, which play an essential and irreplaceable role in maintaining the normal metabolic functions of cytochrome c oxidase (CCO) within mitochondria and copper-zinc superoxide dismutase (SOD1). The distribution of copper ions in mitochondria displays distinct regional characteristics, being primarily concentrated in the intermembrane space and the matrix.

In the intermembrane space, the acquisition and transport of copper ions mainly rely on copper-chaperone proteins COX17 and CCS, as well as the capture and transport of copper ions in the cytoplasm by the alternative ligand glutathione (GSH) and the non-protein ligand CuL. The copper ions in the matrix are exclusively derived from CuL. After binding to copper ions in the cytoplasm, CuL traverses the intermembrane space, enters the matrix, and is stored there under the mediation of a specific copper transport receptor, SLC25A3, on the inner mitochondrial membrane. When there is a deficiency of copper ions, the copper ions stored in the matrix will be transported and released to the intermembrane space and the cytoplasm to participate in the cyclic metabolism [26]. However, the copper transport receptor on the inner mitochondrial membrane that mediates the transfer of copper ions from the matrix to the intermembrane space has not yet been clearly identified.

Copper homeostasis is important in maintaining the normal fusion, fission, and metabolism of skeletal muscle mitochondria. Copper ions participate in the electron transfer process of cytochrome c oxidase (CCO) and the oxidative phosphorylation process within mitochondria, thereby facilitating the generation of adenosine triphosphate (ATP) [42]. Under pathological conditions, the total quantity, number, and function of mitochondria undergo remodeling and alterations, which indirectly impact muscle force output and mass maintenance. The regulatory role of copper overload in this process warrants significant attention.

Mitochondria rely on their continuous fusion and fission processes to respond to changes in physiological environments and stimuli in pathological environments. The dynamic balance between fusion and fission can construct a mitochondrial functional network that is most conducive to ATP production. Fusion refers to the interconnection of different mitochondria under the action of fusion proteins, which in turn promotes the mixing and redistribution of their contents, such as metabolites, proteins, and mtDNA. This process is mainly regulated by mitofusin 1/2 (MFN1/2)

and optic atrophy 1/2 (OPA1/2). During fusion, the mitochondrial membranes on both sides of the reaction are the same, which belongs to homologous fusion. Among them, MFN1/2 dominates the outer membrane fusion of mitochondria, while OPA1/2 dominates the inner membrane fusion [43]. Research has found that inhibiting MFN1/2 is sufficient to cause the accumulation of mtDNA with mitochondrial deficiency and muscle atrophy [44]. Thus, mitochondrial fusion is crucial for maintaining skeletal muscle mass, and fusion defects are important factors leading to skeletal muscle atrophy. Fission refers to the process in which mitochondria, under the action of fission proteins, grid and isolate the irreversibly damaged or unnecessary parts of mitochondria, so that they can be degraded and removed through the autophagy-lysosome pathway [45]. This process is mainly completed by the combination of dynamin-related protein 1 (DRP1) in the cytoplasm with the corresponding receptors on the outer mitochondrial membrane, such as mitochondrial fission factor 1 (MFF1), fission protein 1 (FIS1), and mitochondrial dynamics proteins of 49/51 kD (MiD49/51), and triggering the relevant signal cascade. The fission state of mitochondria is closely related to skeletal muscle mass. Excessive activation of fission can lead to excessive protein decomposition, mitochondrial functional defects, and muscle atrophy phenotypes [44].

The imbalance between fusion and fission is the main source of abnormal mitochondria and an important potential factor in inducing skeletal muscle atrophy. Copper overload can interfere with mitochondrial quality control through two pathways: Firstly, under the mediation of ROS, copper overload significantly upregulates the expression levels of DRP1 mRNA and protein in skeletal muscle, while inhibiting the expression of MFN1/2 and OPA1, thus leading to abnormal mitochondrial morphology. Secondly, copper ions can activate the MEK1/extracellular signal-regulated kinase (ERK) signaling pathway. The activated ERK can inhibit mitochondrial fusion and promote fission by regulating the oligomerization of MFN1. These evidences suggest that copper overload not only weakens mitochondrial fusion and exacerbates mitochondrial fission but also causes abnormal mitochondrial structure and functional defects, which is likely the internal mechanism of muscle atrophy.

4.4.3 Copper overload leads to excessive activation of the autophagy program in skeletal muscle through ULK1/2 mediation

Cell autophagy is a process through which cells encapsulate certain components of their cytoplasm, including mitochondria and toxic proteins, to form autophagosomes. These autophagosomes are subsequently transported to lysosomes, where hydrolytic enzymes break them down into metabolic products like amino acids. Moderate levels of cell autophagy play a beneficial role in the renewal of skeletal muscle proteins and mitochondria as well as in maintaining their homeostasis. Nevertheless, excessive activation of autophagy can result in muscle atrophy and loss.

ULK1 and ULK2 are proteins featuring highly conserved serine/threonine kinase activity. The Atg1 complex, which consists of the ULK1/2 protein kinases, the 200 kD focal adhesion kinase family interacting protein (FIP200), ATG13, and ATG101, serves as the initiator of autophagy in mammalian cells. This complex is capable of promoting the formation and expansion of the double-membrane structure of autophagosomes by activating the autophagy-related protein ubiquitin-like conjugation system. Notably, the phosphorylation and dephosphorylation of the threonine at position 180 of ULK1/2 represent a crucial step in this cellular process [23].

Copper overload plays a crucial role in inducing the autophagy program, and its action pathways may involve direct and indirect mechanisms. On the one hand, copper ions are important regulatory factors for ULK1/2 kinases. ULK1/2 contains an amino acid residue sequence for covalent copper binding, which can directly connect to copper ions. Once the copper-binding site of ULK1/2 mutates, ULK1/2 will be inactivated and lose its ability to activate the downstream substrate ATG13. Meanwhile, copper chelation treatment or disruption of the interaction between copper ions and ULK1/2 will lead to the inhibition of autophagy. These results indicate that copper overload can directly activate ULK1 and exacerbate autophagic activity. On the other hand, amino acid and energy status are important upstream regulatory factors of ULK1/2, which regulate the autophagy activity mediated by ULK1/2 through mTORC1 and AMP-activated protein kinase (AMPK), respectively. Research has found that copper overload can significantly inhibit Akt/mTORC1 in skeletal muscle cells, accompanied by the phosphorylation of beclin-1, a downstream substrate of autophagy-related genes, and autophagy activation [46]. The above evidence fully confirms the key role of ULK1/2 in copper overload-induced autophagy and skeletal muscle loss. Based on this research, further experimental verification work can be carried out using muscle cells as a model.

4.4.4 Copper overload promotes cell apoptosis, contributing to muscle atrophy

Apoptosis is a programmed cell death process that is coordinately regulated by regulatory proteins, endonucleases, protease inhibitors, and caspases. During this process, downstream signal transduction is triggered, followed by the formation of apoptotic bodies, ultimately leading to non-inflammatory self-destruction of the cell [47]. When cells are subjected to death stimuli, initiator caspases (i.e., caspase-8, −9, and −12) are mobilized and activated. They further activate effector caspases (i.e., caspase-3, −6, and −7), thereby inducing cell degradation and DNA fragmentation. Depending on the source of the stimulating signal, apoptosis can be classified into two types: extrinsic and intrinsic. Extrinsic apoptosis is triggered by the interaction between cell surface death receptors (such as tumor necrosis factor receptors) and their ligands (such as tumor necrosis factor α). Intrinsic apoptosis involves the participation of mitochondria or the endoplasmic reticulum, and its main inducers include DNA damage, hypoxia, and metabolic stress. It is worth noting that mitochondria can induce apoptosis through a caspase-independent activation mode, that is, releasing apoptosis-inducing factor (AIF) and endonuclease G to cleave and fragment DNA [48].

In skeletal muscle, muscle cells are multinucleated. When the apoptotic cascade signal is activated, individual myonuclei and a portion of the sarcoplasm are cleared, and this process is defined as myonuclear apoptosis. Significantly, myonuclear apoptosis does not result in cell death; however, it can trigger muscle fiber atrophy. Moreover, several studies have indicated that apoptotic signals may also activate the ubiquitin-proteasome system, initiating the muscle protein degradation program and thereby further promoting muscle fiber atrophy [49]. In reality, apoptosis and protein degradation often occur concurrently during muscle atrophy. Another characteristic of skeletal muscle fibers is the presence of two mitochondrial subpopulations that differ in bioenergetics and structure: subsarcolemmal mitochondria and mitochondria within muscle fibers. These two subpopulations exhibit different susceptibilities to apoptotic stimuli and may therefore be involved in different pathogenetic mechanisms underlying skeletal muscle atrophy [49].

Recently, scientists have limited understanding of the mechanisms behind copper overload and its impact on apoptosis. Studies using human cell models have revealed that exposure to high concentrations of copper ions leads to an unusual distribution of nucleophosmin and fibrillarin within the nucleoplasm, which in turn disrupts ribosomal RNA (rRNA) processing. Specifically, there is an increase in abnormal 34S rRNA due to cleavage at the A2 site. At the same time, a decrease was observed in downstream precursor rRNA (pre-rRNA) and insufficient accumulation of the 60S subunit. Moreover, transcriptome analysis shows that copper overload also affects the expression of genes involved in ribosome biogenesis. This disruption ultimately leads to apoptosis by causing nucleolar stress and through a p53-independent pathway. In studies of skeletal muscle samples, we find that copper overload not only directly triggers myolysis but also increases endoplasmic reticulum stress. This happens as a result of the simultaneous activation of initiator caspase-12, effector caspase-3, and several endoplasmic reticulum stress-related proteins, leading to cell death [50].

Based on these findings, it is hypothesized that the degradation and loss of muscle fibers induced by copper overload may predominantly occur *via* the intrinsic apoptotic pathway. Nevertheless, whether extrinsic apoptosis, myonuclear apoptosis, and differential disorders of mitochondrial subpopulations are implicated in this pathological process still necessitates further experimental validation.

4.4.5 The potential role of cuproptosis in muscle atrophy

Cuproptosis is a novel form of cell death distinct from apoptosis, pyroptosis, necrosis, and ferroptosis [51]. It is another metal ion-related cell death pathway following ferroptosis. Hypoxia can inhibit the cell death induced by copper ionophores, and this inhibitory effect can be blocked by hypoxia-inducible factor prolyl hydroxylase inhibitors. Meanwhile, under glycolytic conditions, cells are resistant to the killing effect of copper ions. These evidences strongly suggest that cellular respiration is a key factor mediating the occurrence of cuproptosis.

After analyzing the metabolites of ABC1 cells exposed to copper ions, researchers observed disruptions in multiple metabolic pathways related to the tricarboxylic acid cycle. However, A549 cells, which are resistant to copper ions, did not exhibit such changes. This result implies a close connection between cuproptosis and mitochondrial metabolism. Through analysis using the CRISPR technology for the whole genome, it was found that ferredoxin 1 (FDX1) and protein lipoylation are key regulatory factors in copper-overload-induced cell death. Among them, FDX1 encodes a reductase that can reduce divalent copper ions to more toxic monovalent copper ions. Protein lipoylation mainly occurs on four enzymes that regulate the tricarboxylic acid cycle and is a highly conserved post-translational modification of lysine.

In addition, since the knockout of the FDX1 gene can completely abolish protein lipoylation by inhibiting the tricarboxylic acid cycle of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, it indicates that FDX1 is an upstream regulator of protein lipoylation. Further research shows that copper ions can directly bind to and induce the oligomerization of lipoylated dihydrolipoamide transacetylase, while causing an imbalance in iron-sulfur proteins and triggering proteotoxic stress. In summary, cuproptosis mainly occurs through the direct binding of copper ions to the lipoylated components of the tricarboxylic acid cycle (TCA). This process promotes the aggregation of lipoylated proteins and the loss of iron-sulfur cluster proteins, thereby inducing proteotoxic stress and ultimately leading to cell death [52]. Currently, the mechanism of cuproptosis in skeletal muscle cells under copper overload conditions

remains unclear. Based on the existing research evidence, the possible mechanism by which copper overload mediates muscle atrophy is shown in **Figure 1**.

5. Conclusion and prospect

Copper plays an essential role as a cofactor for many enzymes, but excessive copper levels can lead to oxidative stress. Given the unique behavior of copper in various diseases, it is likely to become a significant research focus as a potential therapeutic target. The phenomenon of copper-induced cell death will also attract considerable attention in future studies. While there are currently no direct reports linking the mechanism of copper-induced cell death with breakthroughs in skeletal muscle atrophy treatments, progress has been made in this area. Continued in-depth research will provide a clearer scientific foundation for strategies aimed at preventing and treating copper-related diseases by targeting copper-induced cell death, thereby advancing their clinical applications.

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Conflict of interest

The authors declare no conflict of interest.

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Section 5

Modulation of Cell Death

Chapter 13

Cannabinoids as Potential Multitargeting Neuroprotectants in Neuropathic Pain: Exploring the Interplay between Cannabinoid System and Autophagy

Heba-Tallah Abd Elrahim Abd Elkader, Sara El Idrissi, Sana Sellami and Ahmed S. Al-Shami

Abstract

The dysregulation of autophagy plays a significant role in the onset of various pathologies, with emerging evidence indicating its potential involvement in chronic pain conditions. The cellular and molecular mechanisms underlying this pathology have been thoroughly investigated, with the endocannabinoid system (ECS) identified as a crucial factor in the progression of chronic neuropathic pain. This chapter highlights several plant-derived cannabinoids, including cannabidiol, cannabinol, Δ-9-tetrahydrocannabinol, which exhibit unique modulatory effects on the ECS. We discuss the roles of cannabinoid receptors, cannabinoid enzymes, and PPARγ as modulators in decreased levels of inflammatory cytokines, as well as a reduction in microglial activation, and inhibit the assembly of NLRP3 inflammasome complex, which potentially provides substantial neuroprotective effects in neuropathic pain. Since the discovery of the endocannabinoid system and the implications of mitochondrial dysfunction and autophagy impairment in neuropathic pain, there has been an increasing interest in the therapeutic potential of this system.

Keywords: neuropathic pain, autophagy, endocannabinoid, cannabinoid receptors, NLRP3 inflammasome, microglial activation

1. Introduction

Neuropathic pain (NeP) represents one of the most prevalent forms of chronic pain, resulting directly from lesions or diseases that impact the somatosensory system. The primary symptoms associated with neuropathic pain include allodynia, hyperalgesia, spontaneous pain, and abnormal sensory experiences [1].

The prevalence of NeP in the general population is estimated to be between 1 and 8%, which translates to approximately 100 to 560 million individuals globally. Furthermore, among patients experiencing pain in emergency departments, the occurrence of NeP is reported to be around 20% [2]. A growing body of research suggests that neuroinflammation and immune responses are critical in the onset and progression of NeP. Additionally, reactive oxygen species (ROS) have emerged as significant contributors to pain modulation. As neuromodulators, ROS activate calcium/calmodulin-dependent protein kinase II (CaMK2/CaMKII) in glutamatergic neurons within the spinal cord and facilitate disinhibition by suppressing GABAergic interneurons. Furthermore, ROS are known to activate members of the transient receptor potential (TRP) channel family, particularly TRPA1, TRPM2, and TRPV1, which are involved in processing various endogenous and exogenous sensory signals during pathological pain states [1]. Recent research has increasingly demonstrated that dysfunction in autophagy is a fundamental factor contributing to NeP. Furthermore, the modulation of autophagy has been shown to mitigate pain-related behaviors [3].

Autophagy is a fundamental cytoprotective mechanism that plays a pivotal role in the degradation and recycling of cellular components. The protein kinase mammalian target of rapamycin (mTOR) serves as a key regulator of autophagy initiation; its activation inhibits autophagy, while its downregulation facilitates the process [4]. The hallmark of autophagy is the formation of double-membraned structures known as autophagosomes or autophagic vacuoles, which subsequently merges with lysosomal membranes to transport their contents into the autolysosome for degradation [5]. The formation of autophagosomes is governed by two primary protein complexes. The first complex includes Vps-34, Beclin-1, and the activating molecule in Beclin-1-regulated autophagy (Ambra1). The second complex, comprising autophagy-related genes (ATG)12, ATG5, and ATG16, is essential for the recruitment of the microtubule-associated protein light chain 3 (LC3). The transformation of LC3-I (cytosolic form) into LC3-II (lipidated form) is widely regarded as a reliable indicator of the autophagic process [4, 5]. Prior research has established a close association between autophagy and the onset of NeP. Furthermore, autophagy is linked to inflammation induced by microglia in the context of NeP. Despite the acknowledged importance of autophagy in the nervous system's pathophysiology and immune inflammation regulation, there remains a paucity of research exploring the connection between autophagy and pain, with existing studies primarily focusing on the induction phase and the peripheral nervous system (PNS) [1].

Cannabinoids (CBs), endocannabinoids (eCBs), and synthetic cannabimimetic compounds have been thoroughly investigated for their efficacy in modulating pain sensitization and transmission at both central and peripheral levels, primarily through their influence on critical cellular pathways [6]. Studies have established that the endocannabinoid system (ECS) plays a significant role in spinal and supraspinal structures that govern pain perception, with the principal eCBs, N-arachidonoylethanolamine (anandamide, AEA), and 2-arachidonyl glycerol (2-AG), functioning as antinociceptive agents *via* mechanisms that involve CB1 and CB2 receptors [6]. In addition to their action on these receptors, both plant-derived CBs and eCBs are recognized for their ability to influence pain sensation and response through various other targets, including G protein-coupled receptor (GPCR) 55, GPR18, opioid and serotonin receptors, PPARs, cys loop ligand-gated ion channels, and TRP channels, specifically the TRPV1, TRPA, and TRPM subfamilies [7, 8]. Furthermore, recent advancements in inflammasome research indicate that the anti-inflammatory properties of CBs may be partially mediated through the modulation

of autophagy [9]. Consequently, this chapter aims to review the existing literature on the effects of CBs, eCBs, and their derivatives on the inflammasome, to elucidate the potential therapeutic applications of CBs in the management of chronic NeP.

2. The role of autophagy in neuropathic pain

Autophagy serves as a critical mechanism for the degradation and recycling of misfolded proteins and cellular debris, playing a vital role in preserving cellular architecture and functionality [10, 11]. Prior research has established that autophagy is integral to neurodevelopment and the maintenance of neuronal function and homeostasis within both the CNS and PNS. Throughout development, autophagy in myelin-forming glial cells, such as oligodendrocytes in the CNS and Schwann cells (SCs) in the PNS, is persistently involved in the removal of surplus cytoplasm and the facilitation of myelin sheath formation through the maturation of myelinating cells [12]. Following nerve injury, autophagy is reactivated in these glial cells, resulting in the uptake of myelin sheaths that contain substantial quantities of membrane proteins and lipids, which can subsequently be degraded or redistributed to generate new myelin sheaths. Consequently, autophagy in glial cells is crucial for processes such as myelination, demyelination, and remyelination [13]. Nevertheless, the role of autophagy extends beyond glial cells. After nerve injury, alterations in autophagic signaling are also observed in neurons and macrophages that participate in nerve injury and regeneration [12].

2.1 Autophagy in astrocytes

A growing body of research indicates that astrocytes play a crucial role in the onset of chronic pain by modulating the extracellular levels of GABA, which is released from both neurons and glial cells, through their uptake mechanisms. Conditions such as ischemia or hypoxia, along with gangliosides, can trigger the autophagic/lysosomal pathway in astrocytes when they are subjected to oxidative stress [14]. Tonic synaptic inhibition is observed in specific areas of the CNS and plays a significant role in the regulation of neuronal excitability. In the cerebellum, astrocytic GABA is recognized as the primary source of tonic inhibition. While it is established that peripheral nerve injury (PNI) modifies astrocyte activity, the precise mechanisms by which astrocytes operate as GABAergic and GABAceptive entities following PNI remain unclear. Collectively, the involvement of astrocytic GABA may contribute to the disinhibition of neuronal circuits within the spinal dorsal horn, thereby influencing pain regulation [15].

2.2 Autophagy in macrophages and microglia

Following a mechanical injury to peripheral nerves, the development of Wallerian degeneration (WD) distal to the site of injury is associated with the activation of an inflammatory response in neuronal bodies located proximal to the injury, such as those in the spinal ganglia and spinal gray matter. This inflammatory response may result in neuronal death, which plays a significant role in the initiation and persistence of NeP following the injury. In addition to the phagocytic clearance of myelin debris that accumulates in the WD region, macrophages participate in the inflammatory processes at sites proximal to the injury and contribute to the development

of NeP [12]. Beyond their role in engulfing extracellular materials, macrophages also engage in autophagy, and an increase in their autophagic activity facilitates their transition to the anti-inflammatory M2 phenotype [16].

In response to mechanical injury, microglia in the gray matter of the spinal cord's posterior horn undergo proliferation and trigger inflammatory processes, which play a significant role in the onset and persistence of NeP [12]. Notably, TLR4 has been identified as predominantly expressed in microglia, and it plays a crucial role in regulating the proliferation of neural precursor cells during processes such as axonal growth, adult neurogenesis, and neuronal plasticity. Consequently, it is plausible to assert that the release of proinflammatory cytokines from microglia, in conjunction with the impaired autophagy observed in neurons, collaboratively enhances sensory hypersensitivity to pain through TLR4 pathways, suggesting a potential indirect relationship between TLR4-mediated microglial activation and autophagy dysfunction [15]. The overexpression of miR-195 has been linked to the inhibition of autophagy and the upregulation of pro-inflammatory factors in microglia [17]. Furthermore, elevated levels of miR-195 were associated with heightened sensitivity to mechanical and cold pain following spinal nerve ligation. Additionally, ATG14 has been identified as a functional target of miR-195 in microglia, indicating that miR-195 may inhibit autophagy by downregulating ATG14. Overall, the evidence suggests that increased autophagy in spinal gray matter microglia promotes their differentiation toward an anti-inflammatory phenotype, thereby mitigating pain hypersensitivity following traumatic PNI [12].

Microglia in the gray matter of the spinal cord's posterior horn, along with macrophages in the dorsal root ganglia (DRG), play a significant role in modulating the inflammatory response and the pathogenesis of NeP through autophagic processes, as demonstrated by a reduction in p62 levels and an increase in the expression of Beclin-1 and LC3-II/LC3-I. Furthermore, this autophagic enhancement appears to be linked to the activation of the AMPK signaling pathway [18]. Recent investigations have also suggested that macrophages in peripheral nerves may influence SC autophagy in both trauma-induced and hereditary demyelinating peripheral neuropathies. Notably, a study revealed that the selective ablation of macrophages using the colony-stimulating factor 1 receptor inhibitor PLX5622 resulted in a significant increase in SCs autophagic flux in a CMT1 mouse model and an *in vitro* nerve injury model. This finding implies that effective myelin degradation following nerve injury may require collaborative interactions between SCs and macrophages [12, 19].

2.3 Autophagy in Schwann cells

Numerous ATGs have been identified as being expressed in developing SCs. Autophagy plays a critical role during the developmental and myelination phases, as it is highly active and essential for the maturation and structural adaptability of SCs. This process facilitates the compaction of myelin sheaths by eliminating excess cytoplasmic material. In fully matured SCs, autophagy activity significantly decreases; however, a baseline level persists to remove redundant cytoplasm and sustain myelin homeostasis [20]. Vps34 (Pik3c3), a vital component of the PI3K complex necessary for initiating autophagy pathways, catalyzes the transformation of membrane phospholipids into phosphatidylinositol 3-phosphate, a lipid integral to both endosomal membrane remodeling and macroautophagy [21]. The

selective deletion of Vps34 in developing SCs led to delayed radial sorting of axons and significant disruptions in myelin formation within peripheral nerves, alongside elevated levels of autophagy-related markers such as LC3 and p62. The deficiencies in myelin formation may stem from the necessity of autophagy in the turnover of the SC membrane-associated neuregulin-I receptor ErbB2/3 [22], which is essential for SC proliferation, migration, and myelination. Furthermore, in the context of inflammatory demyelinating peripheral neuropathy, SC autophagy contributes to the detachment of SCs from unstable myelin sheaths before the phagocytosis of myelin fragments by macrophages. These observations indicate that the autophagic lysosomal function of SCs facilitates macrophage access to myelin phospholipids for subsequent digestion [12].

3. The interplay between autophagy and Nod-like receptor protein 3 (NLRP3) inflammasome activation in NeP

Microglial pyroptosis represents a recently identified mechanism of cell death that is driven by inflammasomes and is linked to various immune and inflammation-related conditions within the CNS, such as NeP and spinal cord injuries [23]. Chronic NeP is marked by the infiltration of immune cells into the DRG and microglia activation within the spinal cord and brain, ultimately resulting in a neuroinflammatory response [24]. The NLRP3 inflammasome comprises several key elements, including the pattern recognition receptor NLRP3, the apoptosis-associated speck-like protein (ASC), and the pro-caspase-1 enzyme. Upon activation by diverse stimuli and ligands, NLRP3 facilitates the formation of the NLRP3-ASC inflammasome complex, activating caspase-1. The activated caspase-1 then cleaves pro-IL-1β and pro-IL-18, leading to the release of their mature forms, IL-1β and IL-18 [25]. Additionally, activated caspase-1 cleaves gasdermin D (GSDMD), resulting in the generation of an N-terminal fragment that creates pores in the cell membrane. This process not only promotes the secretion of IL-1β and IL-18 but also allows for the influx of water molecules, resulting in excessive inflammatory responses and cell pyroptosis. Moreover, both acute and chronic pain conditions have been linked to the pyroptosis of inflammatory cells [23]. Therefore, targeting the process of microglial pyroptosis holds the potential for mitigating inflammatory damage and alleviating NeP. Numerous studies have indicated that autophagy serves a negative regulatory function in the activation of the NLRP3 inflammasome, effectively suppressing the inflammatory response and averting pyroptosis that is dependent on the NLRP3 inflammasome [23]. The NLRP3 inflammasome can be triggered by potassium ion efflux, the release of ROS, and the disruption of lysosomal integrity [26]. Mitochondrial impairment serves as a primary catalyst for excessive ROS generation and lysosomal dysfunction, which may lead to significant activation of the NLRP3 inflammasome [27]. In response to mitochondrial damage, PINK1 accumulates on the outer mitochondrial membrane through the action of the translocase of the outer membrane. This accumulation leads to the activation of PINK1, which in turn activates Parkin for phosphorylation. This cascade of events enables the binding of adaptor proteins, such as p62, to LC3, thereby facilitating the autophagic degradation of ubiquitinated proteins [28]. Thus, enhancing mitochondrial autophagy may mitigate NLRP3 inflammasome activation, ultimately reducing the transmission of pain signals.

4. The endocannabinoid system (ECS)

The ECS is a neuromodulatory network present throughout the human body, recognized for its crucial involvement in the regulation of numerous physiological functions, such as appetite control, immune system modulation, motor function development, and pain perception [29]. The ECS is characterized as a distinctive lipid signaling system that comprises cannabinoid receptors, and endogenous ligands known as eCBs, along with the enzymes responsible for their synthesis and degradation (**Figure 1**). These components are distributed across both the CNS and PNS, as well as in various peripheral tissues, where they govern specific physiological processes [30].

4.1 Cannabinoid receptors

Cannabinoid receptors, specifically CB1-R and CB2-R, are primarily differentiated by the amino acid sequence within their polypeptide chains and their distribution across various tissues in the body [31]. The CB1-R is an integral membrane protein that is encoded by the CNR1 gene located on chromosome 6. It is characterized by seven transmembrane helices that are coupled to heterotrimeric G-proteins on the intracellular side, as illustrated in **Figure 2**. These receptors represent the most

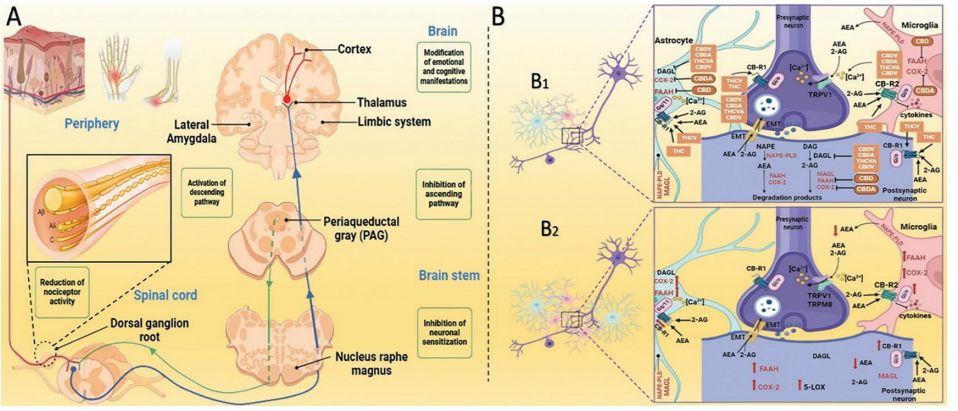

Figure 1.
(A) A schematic representation of the pathways involved in neuropathic pain sensation, illustrating the ascending pain pathways (depicted in blue) that transmit signals from peripheral nociceptors through sensory neurons located in the dorsal root ganglia (DRG), which primarily synapse in the dorsal spinal cord. These projection neurons establish connections with various brain regions, including the cortex and thalamus. The descending pathways (illustrated in green), which play a crucial role in pain modulation, encompass areas such as the amygdala and periaqueductal gray matter, ultimately terminating in the dorsal spinal cord. The transmission of signals from the DRG occurs via *afferent nerves classified into Aβ, Aδ, and C fibers. (B) The involvement of the endocannabinoid system (ECS) within the quadripartite synapse (B1) is highlighted, along with its modulation by phytocannabinoids and the alterations induced by neuropathic pain. The interaction among astrocytes, microglia, and neurons, along with endocannabinoid signaling through CB-R1 and CB-R2, results in distinct outcomes for each cell type. The presynaptic neuron is characterized by the expression of CB-R1, TRPV1, TRPM8, and the endocannabinoid membrane transporter (EMT). Endocannabinoids, such as AEA and 2-AG, target the ECS receptors. Modulation of CB-R1 initiates signaling cascades that inhibit the influx of Ca2+ into the cell, thereby reducing the fusion of intracellular vesicles with the neuron. (B2) In the context of neuropathic pain, both astrocytes and microglia are found near neurons, particularly within the dorsal spinal cord. The ECS undergoes modulation, leading to altered expression levels of its components. There is an observed increase in the expression of both CB-R1 and CB-R2 in glial cells and neurons. Additionally, the enzymatic activities of COX-2, FAAH, and 5-LOX are elevated, resulting in decreased levels of AEA and an increase in pro-inflammatory mediators.*

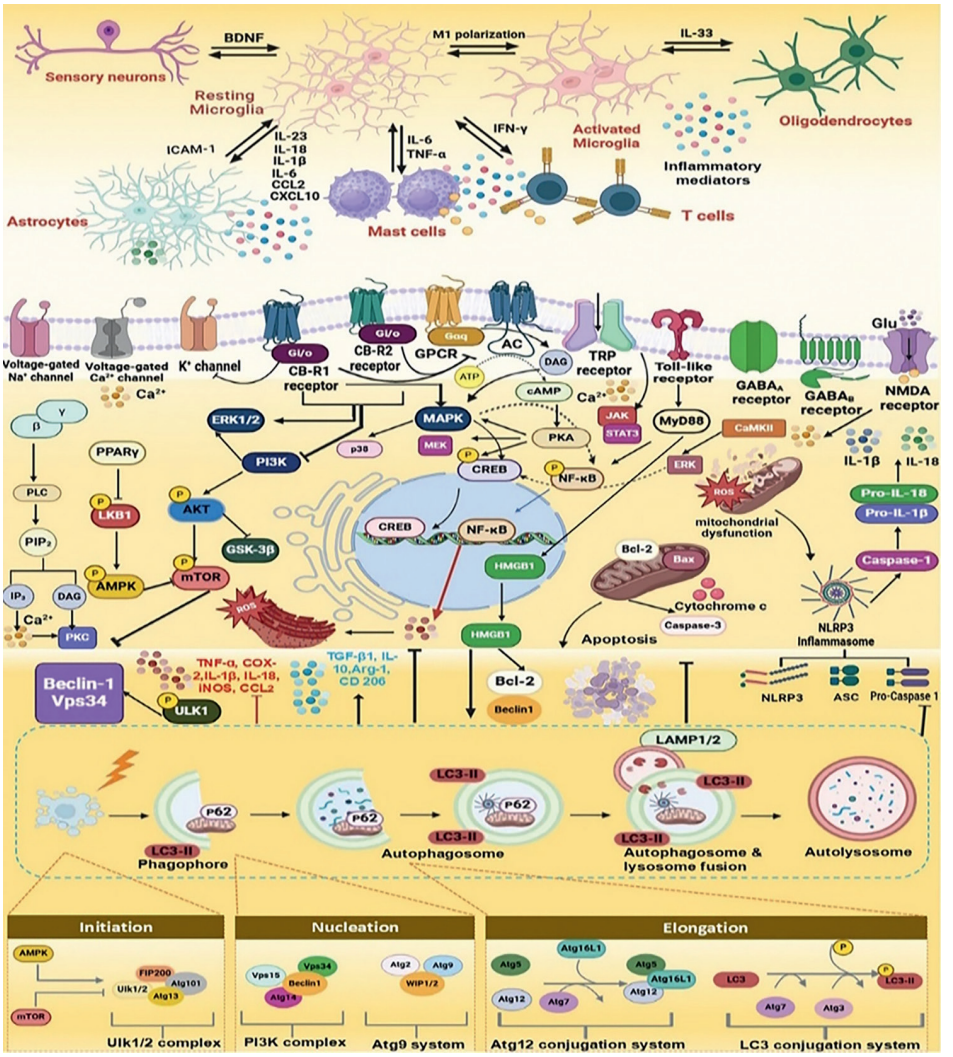

Figure 2.
Schematic representation depicting the molecular mechanisms underlying the interaction between neuroinflammation and autophagy in the context of neuropathic pain, highlighting the pathways that contribute to the advancement of neuropathic pain and the manifestation of its related symptoms.

prevalent type of G protein-coupled receptor in the CNS, predominantly found in neurons of brain regions such as the cerebral cortex, hippocampus, hypothalamus, and basal ganglia. Upon activation, the CB1-R decreases intracellular cAMP levels by modulating adenylate cyclase activity and enhancing the concentration of MAP kinases. This mechanism subsequently elevates presynaptic K^+ levels while reducing Ca^{2+} levels, thereby inhibiting neurotransmitter release in different regions of the brain [32].

CB2-Rs, which exhibit structural similarities to CB1-R, are G-protein coupled receptors encoded by the CNR2 gene located on chromosome 1. Both CB2 and CB1 receptors share a degree of similarity in their amino acid sequences. Notably, these proteins are closely related and interact with various ligands, with 2-AG serving as their primary endogenous ligand and full agonist [32]. CB2-Rs demonstrate less

evolutionary conservation across species and are predominantly expressed in cells involved in immune responses. They play a crucial role in modulating CNS activities by activating microglial cells, reducing oxidative stress, inhibiting neuroinflammation, promoting neuronal regeneration, and enhancing motor functions and memory. The regulatory mechanisms of these functions are similar to those of CB1-Rs, involving the inhibition of adenylate cyclase through the activation of $G_{i/o}$ subunits, as well as the activation of the MAPK-ERK signaling pathway [33].

4.2 eCBs and their synthesizing and degrading enzymes

eCBs function as lipid-based signaling molecules that operate through autocrine, paracrine, and potentially endocrine mechanisms, owing to their lipid solubility that facilitates membrane diffusion [30]. These compounds act as agonists for cannabinoid receptors CB1 and CB2, as well as for G protein-coupled receptor 55 (GPR55), TRPV1, and peroxisome proliferator-activated receptors (PPARs), as illustrated in **Figure 1** [34]. Unlike traditional neurotransmitters, eCBs are not stored in secretory vesicles; instead, they are synthesized in response to specific physiological or pathological stimuli and released into the extracellular environment in a phasic or tonic manner [35]. The primary eCBs, 2-AG and AEA, are derived from polyunsaturated fatty acids and are the most prevalent eCBs within the CNS [30]. 2-AG is integral to the retrograde signaling pathway dependent on CB1-Rs and serves as a precursor in the synthesis of arachidonic acid, which is essential for prostaglandin production [30]. The synthesis of 2-AG is initiated by depolarization of the neuronal membrane and the activation of Gq protein-coupled receptors (GPCRs) [36, 37]. The hydrolysis of membrane phosphatidylinositol by phospholipase C, either β or δ, generates diacylglycerol precursors that are subsequently degraded by diacylglycerol lipases (DAGL-α and DAGL-β), facilitating the production of 2-AG. Among these, the DAGLα isoform is responsible for most 2-AG synthesis, while DAGLβ contributes under specific conditions [38]. Monoacylglycerol lipase (MAGL), a serine hydrolase predominantly located in presynaptic terminals, hydrolyzes approximately 85% of brain-derived 2-AG into arachidonic acid and glycerol. In contrast, the contributions of α/β-hydrolase domain 6 (ABHD6) and domain 12 (ABHD12) to the hydrolysis of 2-AG are relatively minor, accounting for less than 20% of the total hydrolysis [30, 39]. Additionally, AEA is synthesized by the action of N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), which hydrolyzes N-arachidonoyl phosphatidylethanolamine located within the plasma membranes of cells [37]. The half-life of AEA is notably brief, primarily due to its rapid uptake by a high-affinity AEA membrane transporter that is present in glial cells and neurons [38]. Furthermore, fatty acid amide hydrolase (FAAH), a serine hydrolase enzyme associated with intracellular membranes, is found in various organs and at postsynaptic sites in the brain. FAAH catalyzes the conversion of AEA into arachidonic acid and ethanolamine, leading to the inactivation of AEA [36].

4.3 Physiological pain processing through the ECS

The ECS is characterized by its on-demand functionality and its heterogeneous presence across various structures within both the CNS and PNS. This includes critical areas involved in pain processing, such as the amygdala, thalamus, DRG, and spinal cord, among others (**Figure 1**) [40, 41]. In the context of pain modulation within the dorsal spinal cord, the ECS is instrumental in regulating synaptic transmission in

the DRG. CB1-R is found in the presynaptic sensory fibers of the DRG and trigeminal ganglion, as well as in the nerve endings of primary sensory neurons located in the dermis, which are responsible for nociceptive signaling [40]. Upon neurotransmitter release, glutamatergic receptors at the postsynaptic terminal become activated, leading to an influx of Ca^{2+} and an increase in intracellular calcium concentration. Elevated Ca^{2+} levels subsequently activate enzymes that synthesize endocannabinoids, primarily 2-AG and AEA, which are released into the synaptic cleft to bind to CBRs at the presynaptic terminal. The activation of CBRs results in the inhibition of voltage-gated Ca^{2+} channels presynaptically and suppresses adenylate cyclase activity, thereby reducing cAMP levels and initiating a signaling cascade that contributes to synaptic plasticity. This feedback mechanism also modulates sensory transmission within the dorsal horn (**Figure 1**) [40]. Additionally, the components of the ECS are found in astrocytes and microglia, with CB2-R being more prevalent than CB1-R in microglial cells. In microglia, the activation of CB2-R helps maintain the resting state or anti-inflammatory profile of these cells, while in astrocytes, signaling mediated by CB1-R leads to an increase in intracellular Ca^{2+} levels (**Figure 1**) [42].

5. Phytocannabinoids

Cannabis (*Cannabis sativa* L.) is a medicinal plant that has been cultivated and utilized for therapeutic purposes for many decades. It has been employed to address a diverse array of health issues and remains one of the most widely consumed psychoactive substances worldwide [43]. Beyond its recreational use, cannabis serves various medicinal purposes, including the management of anxiety, depressive disorders, epilepsy, chronic pain, multiple sclerosis, and nausea or vomiting associated with chemotherapy, among other medical conditions [44]. The cannabis plant encompasses several species, with the three most recognized being *C. indica*, *C. ruderalis*, and *C. sativa*. More than 500 compounds have been identified within these species, approximately 100 of which are classified as phytocannabinoids, characterized by their 21-carbon terpenophenolic structure [44, 45]. Notable compounds with significant therapeutic potential include cannabidiol (CBD), cannabinol (CBN), Δ-9-tetrahydrocannabinol (THC), as well as various flavonoids and terpenes [45]. CBD and CBN are non-toxic and do not produce euphoric effects; however, they exhibit strong pharmacological properties and are recognized as anxiolytics, anticonvulsants, anti-inflammatories, and muscle relaxants [46]. Conversely, THC is the primary psychoactive component of cannabis, responsible for its euphoric effects. Due to selective breeding and hybridization efforts, the concentration of THC in contemporary commercial strains has markedly increased over the past three decades, rising from an average of 2% in 1970 to over 20% in certain currently cultivated varieties [47].

5.1 The potential role of CBs in modulating autophagy in NeP

CBD may play a role in inducing autophagy, as evidenced by multiple studies. For instance, Shrivastava et al. [48] demonstrated that CBD can modulate autophagy by inhibiting the AKT and mTOR signaling pathways, leading to a downregulation of cyclin D1 and a reduction in the phosphorylation of mTOR and 4EBP1. In alignment with these findings, Giacoppo and colleagues [49] reported that CBD influences the PI3K/Akt/mTOR pathway and enhances neuroprotection by inhibiting JNK and p38 MAP kinases (**Figure 2**). Furthermore, Hosseinzadeh et al. [50]

found that repeated intracerebroventricular injections of 0.100 ng CBD in epileptic rats resulted in the activation of various autophagy markers, including the conjugation of Atg5/12, Atg7, Atg12, and the expression of LC3-II/LC3-I, particularly in hippocampal cells, thereby confirming the protective effects associated with the autophagy pathway.

The exact mechanisms through which CBD operates require further exploration; however, it is improbable that the activation of autophagy occurs *via* the CB1 receptor, which is found in lysosomal compartments [51]. A recent investigation indicated that CBD may inhibit BACH1, a known repressor of p62 expression [52], which plays a role in selective autophagy [53]. Giacoppo et al. [49] noted a significant increase in the phosphorylation of PI3K, Akt, and mTOR, alongside elevated BDNF expression, while also observing a decrease in the pro-inflammatory cytokines IFN-γ and IL-17 following CBD administration. Additionally, a recent study on cannabis-based therapies for NeP demonstrated that the transdermal application of CBD/THC (Sativex R) is effective in alleviating pain associated with multiple sclerosis [54].

5.1.1 NLRP3 inflammasome activation pathway

PNI leads to the release of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in the glial region of the spinal cord, which are recognized by microglia. PAMPs provide the initial priming signal for the NLRP3 inflammasome, whereas DAMPs act as a secondary signal that amplifies neuroinflammation and facilitates the transmission of pain signals from primary sites to higher brain centers. Inhibiting the activation of the NLRP3 inflammasome in spinal cord microglia may reduce pain hypersensitivity resulting from PNI [26].

Recent research has explored the impact of CBD on NLRP3 activation. Huang et al. [55] demonstrated that CBD markedly inhibits the nuclear translocation of NF-κB p65 and the activation of the NLRP3 inflammasome in both *in vivo* and *in vitro* studies, resulting in a decrease in the expression of factors associated with inflammation. In human gingival mesenchymal stem cells, CBD obstructs the activation of the NLRP3-inflammasome pathway by downregulating the expression of critical genes such as NLRP3 and caspase 1, while also inhibiting the subsequent production of IL-18. Collectively, these findings support the notion that CBD mitigates NLRP3 inflammasome activation through the inhibition of NF-κB, thereby reducing inflammasome priming [9].

5.1.2 Ion channel targeting

The role of ion channels in the transmission and signaling of pain is well-documented, positioning them as critical targets for the management of conditions such as chronic NeP. Ion channels can be categorized based on their ion selectivity (including Na^+, K^+, Cl, H^+, and non-selective channels), their gating mechanisms (such as voltage-gated, ligand-gated, cyclic nucleotide-gated, light-gated, and mechanosensitive channels), or their localization (whether they are found in the plasma membrane or intracellularly) [56]. Investigating the modulation of these ion channels is essential for exploring potential analgesic effects. This discussion will emphasize recent advancements in various ion channels that contribute to the hyperexcitability of sensory fibers, highlighting their potential as innovative targets for pain treatment [57].

5.1.2.1 Calcium channel modulation

Voltage-gated calcium channels (VGCCs), also referred to as Cav channels, are integral to the transmission of pain signals within nociceptive neurons at the spinal cord level [57]. These channels are primarily classified into three categories: Cav1, Cav2, and Cav3. Studies indicate that certain VGCCs are more significantly implicated in pain pathways than others, particularly the N-type (Cav2.2) and specific T-type channels (Cav3.2) [58, 59]. Notably, the overexpression of Cav2.2 in the dorsal horn's outermost layer, a critical area for nociception in the spinal cord, is associated with the onset of NeP [60]. The N-type calcium channels are crucial in pain development, and their activation can be effectively inhibited by reducing the release of pro-nociceptive neurotransmitters, such as glutamate and substance P, or through the application of mu-opioid receptor agonists like morphine, which serve to block these channels [61].

Cav3.2 is expressed in all parts of sensory neurons that participate in the transmission of nociceptive signals, encompassing peripheral nerve endings, axons, cell bodies, and synapses within the dorsal horn [62]. Studies indicate that T-type channels play a crucial role in modulating cellular excitability and rhythmic activity, and they are implicated in various pathophysiological conditions associated with neuronal hyperexcitability [57]. Cav3.2 may contribute to the nociceptive pathway by lowering the threshold for action potential generation [63] or by facilitating Ca^{2+}-dependent neurotransmitter release, which leads to synaptic enhancement [64]. The involvement of Cav3.2 in pain states is further corroborated by studies demonstrating that the antisense knockdown of Cav3.2, as opposed to Cav3.1 and Cav3.3 channels in DRG neurons, produced significant antinociceptive, anti-hyperalgesic, and anti-allodynic effects [57].

Both AEA and 2-AG are synthesized in postsynaptic terminals as a result of increased intracellular Ca^{2+} ion influx [65]. Following their synthesis, AEA and predominantly 2-AG move retrogradely due to their high lipophilicity, subsequently activating CB1 receptors located in presynaptic terminals. The activation of CB1 receptors leads to the inhibition of neurotransmitter release by decreasing Ca^{2+} influx and suppressing cAMP levels. Specifically, the activation of the CB1 receptor enhances the activity of G-protein-gated inwardly rectifying potassium (GIRK) channels while concurrently inhibiting voltage-gated (N-type) Ca^{2+} channels [9].

5.1.2.2 Potassium channel modulation

Potassium (K^+) channels are essential for regulating neuronal excitability, primarily through their role in hyperpolarization, which counteracts the continuous depolarization that can lead to neuronal hyperexcitability. Among these, the voltage-dependent Kv channels are particularly significant as they influence resting membrane potential, membrane repolarization, the shape of action potentials, and the firing frequency and adaptation of neurons in both the CNS and PNS [59]. A notable downregulation of K^+ channels has been observed in nociceptive signaling pathways. Different K^+ channel subunits exhibit predominant expression in various types of neurons; for instance, Kv1.1 and Kv1.2 are found in myelinated sensory axons, while Kv1.4 is present in C-fibers [66]. Kv7 channels are also of particular interest, as their activation can alleviate hyperexcitability and reduce pain. Furthermore, the interaction between Kv2.1 and Kv9.1 in A-fiber neurons suggests their involvement in pain signaling and direct participation in nociceptive pathways [59]. In addition to

their presence in the CNS, Kv7.2 and Kv7.3 are widely distributed in the PNS, where they are crucial for the nociceptive pathway. Alterations in their currents can lead to significant neuronal hyperexcitability, contributing to various pathophysiological conditions such as epilepsy, peripheral sensitization, and NeP [67].

5.1.2.3 Sodium channel modulation

Voltage-gated sodium (Na^+) channels (VGSCs) are crucial components in the mechanisms of pain, as they significantly influence cellular excitability and are integral to both the initiation and transmission of action potentials. These channels are recognized as essential factors in determining the excitability of nociceptors [59]. In humans, the voltage-gated sodium (NaV) channel family consists of nine closely related isoforms (NaV1.1–NaV1.9). Among these, four isoforms—Nav 1.3, Nav 1.7, Nav 1.8, and Nav 1.9—are particularly important in the development of NeP [68]. Nav 1.3 is often found to be upregulated in various pain conditions, and this increased expression may lead to hyperexcitability, facilitating its role in enabling peripheral nerves to generate high-frequency firing. The rapid kinetics associated with this channel further supports its function in pain signaling [69].

Nav 1.7 is predominantly expressed in peripheral neurons, especially in small-diameter DRG neurons that possess unmyelinated and slowly conducting axons (C-fibers) [59]. This channel plays a vital role in modulating the excitability of sensory neurons and is a significant contributor to pain disorders in humans [70]. Its characteristics include rapid activation and inactivation, coupled with a slower repriming phase, making it particularly effective for low-frequency firing in C-fibers. Additionally, Nav 1.7 channels may influence the release of neurotransmitters at the central terminals of nociceptors [71].

The Nav 1.8 isoform is a promising candidate for therapeutic intervention due to its selective expression in sensory neurons, particularly in small-diameter DRG neurons [59]. This isoform is noted for its slow activation and inactivation kinetics, alongside rapid repriming [72] and a depolarized voltage dependency for both activation and inactivation. It is responsible for generating the predominant Na^+ current during the action potential depolarization phase in the neurons where it is expressed. Furthermore, it facilitates repetitive firing in response to depolarizing stimuli [73]. Collectively, these biophysical characteristics, along with its localization in free nerve endings, indicate that the Nav 1.8 isoform plays a significant role in the excitability of nociceptors [74] and the transmission of nociceptive signals [73].

In contrast, the Nav 1.9 isoform is predominantly expressed in small-diameter DRG neurons, particularly those with unmyelinated and slowly conducting axons (C-fibers) [59]. However, its expression is downregulated in neurons following injury. This isoform is distinguished by its hyperpolarized voltage dependency for activation, which is close to the resting membrane potentials of neurons (approximately −60 to −70 mV), and it exhibits ultraslow inactivation [59]. These features enable it to generate a persistent Na^+ current, as its activation and inactivation profiles allow for activation at resting potentials [74]. A reduction in its expression, along with the associated decrease in persistent current, may result in more hyperpolarized membrane potentials, thereby facilitating the recovery of Na^+ channels from inactivation [59].

CBs are known to inhibit VGSCs and VGCCs, including specific subtypes such as Nav1.7–1.8 and CaV2.1–2 [75, 76]. CBD exerts its analgesic properties in part by modulating neuronal excitability through its strong affinity for the slow inactivated

state of Nav.1.8 channels, thereby preventing unnecessary depolarizations that could lead to repetitive neuronal firing [77].

5.1.2.4 Sodium-calcium exchanger (NCX) modulation

The NCX exchangers (Ca^{2+}/Na^{+}) belong to a broader category of transport proteins known as the CaCA (Ca^{2+}/cation antiporter) superfamily, which are crucial for regulating Ca^{2+} flux across the plasma membrane and within intracellular compartments [59]. Besides the previously mentioned forward mode, NCX can also facilitate Ca^{2+} entry into cells by functioning in a reverse mode, which links Ca^{2+} influx to Na^{+} efflux [78]. This reverse mode plays a significant role in the regulatory mechanisms of glutamatergic gliotransmission between astrocytes and neurons, as well as in the activity of N-methyl-D-aspartic acid (NMDA)/AMPA receptors that mediate Ca^{2+} entry [79]. In the PNS, NCX2 is found in the cell bodies of small-diameter DRG neurons and extends throughout the neurites and their tips. Specifically, NCX2 is present in epidermal free nerve endings and mechanosensory nerve endings, including nociceptors [80]. It is co-expressed with Na^{+} channels Nav 1.6, Nav 1.7, Nav 1.8, and Nav 1.9, which interacts physiologically under both normal and pathological conditions [59, 79]. Persson and colleagues have shown that the co-localization of NCX2 with Na^{+} channels in epidermal nociceptive terminals may enhance the sensitivity of these fibers to injury, particularly under energetic stress [80]. Overall, NCX2 plays a vital role not only in maintaining Ca^{2+} homeostasis but also in the transmission of noxious stimuli [79, 81]. Consequently, NCX2 has significant antinociceptive properties, and its dysfunction is closely associated with peripheral sensitization and NeP [79].

5.1.2.5 Nonselective cation channels

The superfamily of TRP channels in mammals consists of 28 recognized members that are permeable to Ca^{2+}, exhibiting a wide range of physiological roles and cellular localizations [56]. As non-selective cation channels, TRPs facilitate the movement of K^{+} and Na^{+}, although the role of Ca^{2+} has been the focus of extensive research.

Transient receptor potential ankyrin 1 (TRPA1) is a nonselective cation channel predominantly found in nerve terminals that innervate the skin, specifically within the neurons of the dorsal root and trigeminal ganglia [82]. Initially, TRPA1 was identified as a potential sensor for harmful cold temperatures (≤17°C); however, this function varies across species, with some species potentially utilizing it as a sensor for noxious heat. Consequently, TRPA1 has emerged as a significant target for investigating cold hypersensitivity in various animal pain models. Furthermore, a gain-of-function mutation in TRPA1 has been associated with familial episodic pain syndrome, as demonstrated in a human genetics' study [82]. Thus, TRPA1 antagonists may represent promising therapeutic options for these pathologies.

The nonselective cation channel transient receptor potential vanilloid type 1 (TRPV1) exhibits multiple modes of activation. It can be triggered by various chemical ligands, including capsaicin, AEA, and acidic conditions, as well as by thermal stimuli exceeding 42°C. Consequently, TRPV1 functions as an integrative channel for diverse noxious stimuli, playing a critical role in nociceptive processes [82]. In contrast, TRPV2 does not respond to heat or vanilloid compounds [56]. The activation of TRPV2 is facilitated by agents such as 2-aminoethoxydiphenyl borate, probenecid, and CBD, while its activity can be inhibited by compounds such as ruthenium red, gadolinium, and tranilast [83]. The translocation of TRPV2 from the endosomal

compartment to the plasma membrane, which significantly impacts both cell proliferation and apoptosis, is stimulated by various factors, including growth factors, cytokines, hormones, and eCBs [84]. Furthermore, the use of TRPV1 antagonists and the knockdown of TRPV1 protein have been shown to decrease nociceptive sensitivity in several preclinical models of pain, including neuropathic and arthritic pain [85].

The TRPM (melastatin) channels are characterized by a substantial cytosolic domain, rendering them the largest members of the TRP superfamily [59]. The mucolipin subgroup within the TRP superfamily (TRPML) comprises three distinct members: TRPML1, TRPML2, and TRPML3. Impairments in TRPML functionality are anticipated to significantly impact organelle acidification, vesicle fusion, endosome maturation, and signaling pathways, indicating that this protein family is crucial for both normal physiological and pathological processes [86].

Additionally, CBs interact with ligand-gated ion channels such as TRPV1, TRPV2, glycine (GlyR), and GABA-A receptors [87, 88]. THC functions as an agonist for TRPV2, whereas CBD acts as an agonist for TRPV1 [89]. The modulation of these TRP channels influences pain perception [90]. The activation of these channels, especially TRPV1, can induce analgesic effects (**Figure 2**). Furthermore, CBs such as THC and CBD serve as positive allosteric modulators for specific subtypes of these ion channels, enhancing pain relief [87]. In contrast, AEA also activates the TRPV1 receptor, inhibits L-type Ca^{2+} channels, and reduces the biosynthesis of 2-AG [65]. In trigeminal neurons, the activation of CB1 receptors contributes to pain relief by inhibiting the effects of TRPV1 through the activation of calcineurin [91]. The desensitization of TRPV1 by CBs may play a role in mitigating neuropathic alterations. Recent research has shown that the TRPV1 channel opens upon activation, facilitating the passage of ions across the membrane. Calcium ions enter the cell through the pore, activating various calcium-dependent pathways that ultimately result in the desensitization of the channel, thereby alleviating inflammatory pain. In a similar vein, CBD demonstrates anti-hyperalgesic properties that may stem from its ability to induce peripheral and spinal activation through TRPV1 desensitization [53]. Additionally, a study indicated that the induction of autophagy by CBD may be initiated through the activation of the TRPV2 [53, 83].

5.1.3 Receptor targeting

5.1.3.1 Nuclear peroxisome proliferator-activated receptors (PPARs)

PPARs play a significant role in various physiological processes. PPARγ serves as a crucial transcription factor that governs the differentiation of adipocytes and maintains lipid and glucose homeostasis [92]. Beyond its metabolic functions, PPAR ligands exhibit beneficial effects largely attributed to their anti-inflammatory properties, which include the suppression of pro-inflammatory cytokines such as interferon gamma (IFN-γ) and TNF-α [93]. This receptor is present in multiple immune cell types, including macrophages, dendritic cells, T cells, and B cells, and it modulates the transcriptional activity of NF-κB by inhibiting the inhibitor of kappa B (IκB) kinase and interfering with the DNA binding domains of NF-κB [92]. Various compounds, including eCBs, eCB-like substances, phytocannabinoids, and synthetic CBs, are known to bind to and activate PPARs [94].

CBs also target nuclear receptors such as PPAR-γ, which are involved in gene regulation and have been associated with NeP [7, 95]. The activation of PPAR-γ by CBs contributes to their anti-allodynic effects in models of NeP.

5.1.3.2 Serotonin receptor 1A

Serotonin receptor 1A (5-HT1A) is integral to the pathophysiology of conditions such as depression, aggression, and anxiety. CBD acts as an agonist for the 5-HT1A receptor, exhibiting micromolar affinity, and may produce anxiolytic effects by facilitating its post-synaptic interactions [92]. Within this receptor system, glutamate pyruvate transaminase (GTP)-binding proteins mediate the connection between the activation of 5-HT1A and its resultant physiological effects. Research indicates that CBD enhances GTP binding to the receptor-coupled G protein, Gi, a behavior typical of receptor agonists [92]. Furthermore, CBD promotes serotoninergic and glutamatergic transmission through positive allosteric modulation of the 5-HT1A receptor [95].

Beyond CBRs, CBs interact with other G protein-coupled receptors, including 5-HT1A and GPR55 [96]. The activation of these receptors, particularly 5-HT1A, has been demonstrated to exert anti-allodynic effects in NeP models [97, 98]. Moreover, CBs can interact with non-cannabinoid receptors, triggering analgesic and anti-inflammatory effects mediated by the 5-HT1A receptor [99].

5.1.3.3 GABA receptors

GABA is integral to the CNS of vertebrates, where it facilitates rapid inhibitory neurotransmission and contributes to brain hyperexcitability through its engagement with GABAA receptors. However, following an injury, this inhibitory mechanism may be compromised, resulting in hyperalgesia [92]. Furthermore, disinhibition can allow non-nociceptive myelinated A primary afferents to activate the pain transmission pathways, causing patients to experience pain in response to stimuli that are ordinarily non-painful. The death of GABAergic interneurons induced by spinal cord injury, along with a reduction in their population in the dorsal horn, leads to a decrease in GABAergic tone and contributes to NeP [15]. Moreover, the loss of GABAB receptor-mediated inhibition occurs after spinal nerve injury, particularly affecting the central terminals of primary afferents, which can lead to allodynia or spontaneous pain responses. Both the impairment of autophagy and endoplasmic reticulum stress collectively play a significant role in the dysfunction of GABAergic neurons located in the dorsal horn of patients suffering from NeP [15]. CBD functions as an allosteric modulator of these receptors, enhancing the currents elicited by low concentrations of GABA while having no significant effect on high concentrations. This modulation effectively increases the apparent affinity of GABA for its receptor.

5.1.3.4 NMDA receptors (NMDARs)

Excitatory glutamatergic NMDARs play a pivotal role in the modulation of spinal dorsal horn (SDH) plasticity and neuronal excitability [100]. The aberrant processing of nociceptive signals leads to pathological pain characterized by hyperexcitability of the SDH, a phenomenon that is contingent upon the activation of NMDARs [101]. Notably, NMDARs are present on both pre- and postsynaptic membranes; however, early investigations into their nociceptive functions have often overlooked this crucial differentiation between pre- and postsynaptic receptors within the SDH [100]. Additionally, there is evidence suggesting that CBs can activate the CB1 receptor, which may reduce NMDA receptor activation by inhibiting the release of glutamate, and they may also interact with opioid systems (**Figure 2**). These mechanisms are

present within the neuronal framework, highlighting the potential involvement of eCBs in the modulation of NeP [102]. Nevertheless, considerable evidence suggests that CBD functions as an antagonist for the chaperone protein σ1R, which represents a promising target for the treatment of NeP by diminishing the effects of glutamate NMDARs [53]. The σ1R antagonist also inhibits GPCRs, leading to a reduction in the activity of NMDARs [103]. These receptors generate secondary messengers and regulate Ca^{2+} homeostasis by activating PKA, which is crucial for the activation of Ca^{2+} channels [104].

6. Conclusions

NeP presents significant challenges for both clinicians and patients, primarily due to the limited advancement in treatment modalities. Although the role of autophagy in NeP has been explored for several years, credible data regarding the dysfunction of autophagy and its modulation in this context remain insufficient. As the prevalence of NeP continues to rise, there is an urgent imperative to explore and establish new multimodal treatment options. Emerging research indicates that CBs act as agonists at the CB1 receptor can provoke pro-inflammatory responses through the activation of inflammasomes *via* CB1 stimulation. Additionally, CBs interact with key proteins involved in inflammasome signaling pathways, such as NF-kB and IL-1β, which indicate that the anti-inflammatory properties of CBs may be partially mediated through the modulation of autophagy. Consequently, this chapter encompasses the roles of CBRs, cannabinoid-metabolizing enzymes, and PPARγ in mediating reductions in inflammatory cytokine levels, diminishing microglial activation, and inhibiting the formation of the NLRP3 inflammasome complex, which may yield significant neuroprotective benefits in the context of NeP. In summary, CBs present considerable promise as adjunctive therapies to enhance the management of NeP.

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Chapter 14

In-Depth Exploration of Radiation-Induced Cell Death Mechanisms in Tumor

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Abstract

Radiation therapy is a cornerstone of cancer treatment, targeting tumor cells through DNA damage and subsequent induction of various forms of cell death. This chapter explores the multifaceted biological effects of Radiation therapy (RT), highlighting its ability to trigger different lethal and non-lethal death mechanisms. The mechanisms underlying these responses involve complex interactions between radiation-induced DNA damage, reactive oxygen species production, and disruption of cellular homeostasis. RT therapeutic efficacy is influenced by factors such as tumor type, microenvironment, and the balance between cell death and survival pathways. Advances in understanding how RT impacts cell death mechanisms, including the modulation of ferroptosis and pyroptosis, have unveiled new opportunities to enhance radiosensitivity and overcome tumor resistance. Furthermore, non-lethal processes, such as senescence and mitotic catastrophe, underscore the potential of RT to suppress tumor progression through mechanisms beyond direct cytotoxicity. This chapter emphasizes the need for integrating molecular insights with clinical applications to optimize the efficacy of RT while minimizing damage to healthy tissues. By examining emerging strategies, such as the exploitation of immune responses and targeting tumor microenvironmental factors, this work provides a comprehensive foundation for advancing radiotherapy in oncology.

Keywords: radiotherapy, cell death, pyroptosis, ferroptosis, senescence, mitotic catastrophe, irradiation

1. Introduction

Cancer is the second greatest cause of death after heart disease and has long been a global health concern [1]. Radiation therapy (RT) is one of the main treatment modalities. It is used both as curative and palliative for almost all solid tumors. RT is frequently used in conjunction with immunotherapy, cytotoxic chemotherapy, and surgery as the first line for treating cancer [2]. Radiation kills cells in a way that is not specific to tumor cells, and the best way to deliver RT is to strike a balance between maximizing the dosage to the tumor and minimizing the damage to healthy tissue [3]. DNA is RT's main intracellular target; it is harmed by ionizing radiation (IR) either directly or indirectly via reactive oxygen species, which sets off a series of events that may result in cell death. DNA double-strand breaks (DSBs) are caused by radiation and can be repaired via non-homologous end joining (NHEJ) and homologous recombination (HR). NHEJ functions throughout the cell cycle, whereas HR is limited to the S and G2 phases. Furthermore, a third mechanism called alternative NHEJ was just identified: it repairs damaged DNA by utilizing tiny areas of microhomology within the break sites [4]. Therefore, HR and NHEJ pathways detect and effectively repair damaged DSBs, and NHEJ is the major mechanism for repairing RT-induced DNA damage [5]. A high radiation-associated deletion burden may indicate the sensitivity of recurring malignancy following RT and has been linked to poor survival. On the other hand, inadequate or ineffective DNA repair processes cause cell death [6]. Factors such as the rate of mitosis, the degree of differentiation, and the total and fractional radiation doses significantly affect the extent of cell death or resistance to treatment [7, 8]. Cell death is the cytological effect of radiation on the human body. In this context, it is known as proliferative death and interphase, which are further classified based on the molecular process [9, 10]. According to cell death classification, it is possible to distinguish between regulated cell death, generally known as programmed cell death (PCD), including: apoptosis, oncosis, necroptosis, eryptosis, ferroptosis, pyroptosis, parapoptosis, parthanatosis, mitoptosis, alkaliptosis, methuosis, oxeiptosis, sarmoptosis, Wallerian degeneration, transneuronal degeneration, NETosis, entosis, emperipolesis, anoikis, cornification, immunological cell death, mitotic cell death, autophagy-dependent cell death, autosis, and lysosome-dependent cell death, while the accidental cell death refers to the necrosis [11]. IR affects multiple biological targets and activates different pathways, resulting in diverse types of cell death depending on the radiation dose and environmental factors [9]. Due to the diversity of cell phenotypes, cell cycle phases, doses of radiation, and even cell subregions, different cell death types can occur. Furthermore, it is more difficult to differentiate and categorize the different types of cell death due to the presence of signaling and initial activation molecules shared by all of them, as well as due to complex crossovers in cellular molecules. This chapter serves as a critical foundation for understanding the biological basis of RT by exploring its essential role in oncology and examining its mechanisms, biological effects, and potential to drive advances in therapeutic approaches. An in-depth discussion is devoted to the fundamental impact of radiation at the cellular level, highlighting how various types of radiation induce damage to key cellular components such as DNA, proteins, and membranes. This chapter offers a comprehensive analysis of some cell deaths triggered by radiation exposure, including apoptosis, necroptosis, pyroptosis, and ferroptosis. Additionally, it explores non-lethal processes such as senescence and mitotic catastrophe. Furthermore, the chapter examines the diverse mechanisms through which radiation exerts its effects. It will also provide a thorough

understanding of the processes that underline the therapeutic and cytotoxic actions of radiation and highlight novel strategies intended to increase its effectiveness in the treatment of cancer. This comprehensive approach ensures a deeper understanding of the biological complexities of RT and paves the way for innovative strategies to overcome limitations, such as tumor radioresistance, thus refining its application in clinical oncology.

2. Principles of radiation therapy

RT is a cornerstone of cancer treatment in different solid tumors, using ionizing radiation (IR) as a physical agent to target and kill cancer cells [12–14]. IR generates electrically charged particles, ions, by transferring energy to the cells in the tissues it penetrates, thus directly destroying tumor cells or leading to genetic damage that ultimately leads to their elimination [15].

2.1 Types of radiation used in cancer treatment

The most common kind of radiation utilized in cancer treatment is photons, which include gamma and X-rays. Photon beams have a very low charge and mass. X-rays and γ-rays are both classified as low linear energy transfer (low-LET) and consist of massless energetic particles. Cathode ray tubes and linear accelerators, for example, emit X-rays when they excite electrons. γ- rays, on the other hand, come from radioactive compounds decaying. Photon radiation is routinely used for its versatility and ability to effectively penetrate tissue, making it suitable for treating various types of cancer. Particle radiation, on the other hand, involves the use of charged or neutral particles, which have unique physical and biological properties compared to photons [16]. Electron beams are commonly used in routine RT; these are particularly effective for treating tumors near the body surface because their penetration into deeper tissue is limited. Proton beams are a highly precise form of particle radiation [15]. Proton therapy exploits the Bragg peak phenomenon; this provides the maximum energy accumulation at the tumor location while sparing surrounding healthy tissue. Proton therapy is, therefore, particularly useful for pediatric cancers and for adult tumors located close to vital structures, including tumors of the skull base and spinal cord [17]. Neutron beams are produced by neutron generators, which direct proton beams onto a target. These show high-LET and induce more extensive DNA damage than photons (**Figure 1**). Despite their greater biological efficacy, their application is limited due to the technical complexity and cost of generating neutron particles and building treatment facilities. Synchrocyclotrons and synchrotrons create heavy ion beams such as helium, carbon, nitrogen, argon, and neon. These particles have a high-LET and are particularly effective against radioresistant tumors such as sarcomas, renal cell carcinomas, melanomas, and glioblastomas. Particle radiation has a higher LET than photons, offering greater biological efficacy, especially for tumors resistant to conventional photon therapy [18].

2.2 Types of cell damage induced by radiation

RT hit all the cellular components; indeed, proteins, lipids, the nucleus, mitochondrial DNA, and other constituents can all be impacted [19, 20]. However, the nuclear genome is the main target of radiation. IR destroys molecular bonds and induces

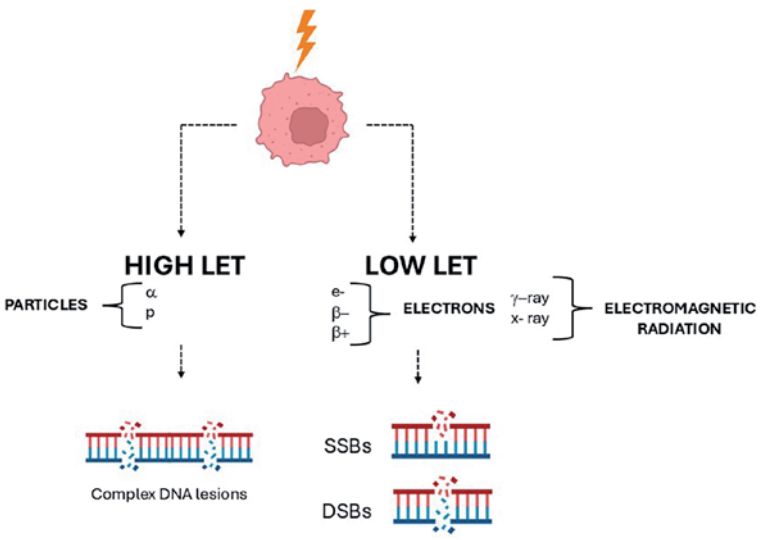

Figure 1.
Schematic representation of DNA damage caused by different types of ionizing radiation. High Linear Energy Transfer (LET) radiation, such as alpha particles (α) and protons (p), induces complex DNA lesions. Low-LET radiation, including electrons (e^-, β^-, and β^+) and electromagnetic radiation (γ-rays and X-rays), primarily causes single-strand breaks (SSBs) and double-strand breaks (DSBs).

numerous lesions caused through direct energy deposition and/or free radicals, commonly known as reactive oxygen species (ROS), including O_2 and OH generated during water hydrolysis [21–24]. The amount and severity of damage is determined by the radiation's quality, intensity, and absorbed dose [25]. In general, low-LET particle radiation, like photons, X-rays, and γ-rays, is less detrimental compared to high-LET particle radiation, which comes from protons, α-particles, and heavy ions. High-LET particles produce dense ionization by storing energy in the medium, exhibiting greater biological effects than low-LET radiation, which exhibits a uniform and sparse spatial distribution of ionization in cells [17, 26]. Single-strand breaks (SSBs), DSBs, oxidative base damage, and some clustered DNA lesions are caused by low-LET radiation, while complex clustered DNA lesions are induced by high-LET radiation [19]. Upon IR, SSBs and DSBs represent radiation-induced DNA damage, while clustered DNA damage, base damage, and cross-linking can also be distinguished, thus leading to DNA damage responses (DDRs). IR can also induce non-targeted effects, including those induced by bystander signals that occur in non-irradiated cells after receiving signals from nearby irradiated cells, whose trigger signals are very diverse, including ROS, reactive nitrogen species (RNS), and cytokines with proinflammatory action [27–29]. Signaling molecules can then cross gap junctions between cells, diffuse over long distances in the extracellular environment or bloodstream, and be transported by mediators like exosomes or another carrier [30]. IR can also trigger non-targeted effects by affecting some cellular organelles, such as mitochondria [31]. Regardless of the direct or indirect effects caused by RT, its main objective is to disable the proliferative ability of cancer cells and ultimately eliminate them: once the DNA is irreparably damaged, cells cease dividing and undergo death [15]. Following IR, cell death can be classified as interphase or proliferative death. Indeed, IR can cause a variety of cellular fates, including lethal and non-lethal processes (**Figure 2**) [3, 32].

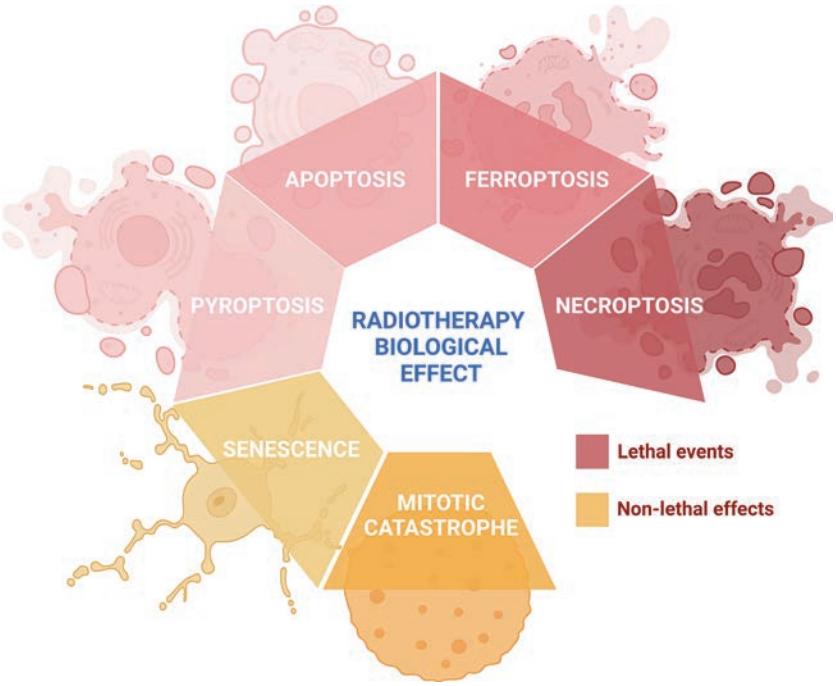

Figure 2.
Schematic representation of some biological effects induced by RT. Lethal events (apoptosis, ferroptosis, necroptosis, and pyroptosis) are shown in shades of red, while non-lethal effects (senescence and mitotic catastrophe) are highlighted in yellow.

3. RT inducing PCD

3.1 Apoptosis

Apoptosis is a vital homeostatic process involved in morphogenesis during early development that can also be activated in pathological conditions. It is one of the main mechanisms of cell death following anticancer therapy, particularly active upon RT. Morphologically, it presents with cellular shrinkage and the formation of apoptotic bodies [15]. In addition, the formation of vesicles on the membrane, compacting chromatin with nuclear edge localization, and fragmented DNA are frequently observed. Mitochondria are the cellular organelles primarily involved in this type of cell death [33]. In apoptosis, two different mechanisms can be distinguished: intrinsic or extrinsic mediated pathway; both pathways converge in the activation of caspase. These proteins are enzymes with cysteine protease activity considered essential in both inflammation and apoptotic pathways; they are classified as initiators: caspases-2, −8, −9, and, −10, and effectors: caspases-3, −6, and − 7, and oversee the primary catalytic events during the intrinsic and extrinsic pathways of apoptosis [33, 34]. The extrinsic apoptotic pathway is modulated by external signals via the activation of death receptors, including Fas receptors, DR4/DR5, tumor necrosis factor receptor (TNF-R), and TNF-related apoptosis-inducing ligand receptors (TRAIL-R) present on the surface of several cells [35, 36]. TNF receptors are composed of extracellular cysteine-rich domains and a cytoplasmic domain, the death domain, implicated in the transmission of the death signal from the outside to the inside of the cell [37]. On the cell surface, the death receptor

interacts with its specific ligands, recruiting adaptor proteins including Fas-associated protein with death domain (FADD), tumor necrosis factor receptor-associated death domain protein 1 (TRADD), and subsequently downstream factors such as caspase-8 [38, 39]. The death receptor, once it binds to specific ligands, recruits adaptor proteins such as Fas-associated protein with death domain such as FADD, TRADD, and then downstream factors such as caspase-8 [38, 39]. Crucially, ligand binding recruits and dimerizes death receptors, exposing the death domains and activating caspases-8 and 10 [40]. Finally, active caspase-8 and -10 trigger apoptosis by cleaving and activating caspases 3, 6, and 7 [41, 42]. DNA damage and p53 activation start the intrinsic apoptosis process, also referred to as the mitochondrial apoptosis pathway, which sets off a series of events that culminate in mitochondrial outer membrane permeabilization. The Bcl-2 protein family includes both pro- and anti-apoptotic proteins and is in charge of controlling the intrinsic pathway. Cell fate is ultimately determined by the heterodimeric connections between Bcl-2 proteins via the Bcl-2 homology domains: BH1, −2, −3, and − 4 [43]. Pro-apoptotic proteins such as Bax and Bak can initiate alterations in mitochondrial membrane potential after the death signal has been activated because BH3-only domain proteins can neutralize or activate the anti-apoptotic protein Bcl-2. Once cytochrome c, an intermembrane protein of mitochondria, is released into the cytosol, it assembles with APAF-1 and pro-caspase 9 to form the apoptosome. Within the apoptosome, activated caspase-9 cleaves and activates the apoptosis effector proteins caspase-3, −6, and − 7 [44, 45]. Cellular irradiation can affect mitochondrial membrane permeability via an intrinsic caspase-dependent pathway, resulting in greater release of pro-apoptotic proteins into the cytoplasm, therefore initiating a series of apoptotic cascades [3]. Indeed, several studies have indeed demonstrated that RT triggers the intrinsic pathway; in particular, the p53 protein has an important function in modulating the redox state in response to oxidative stress caused by IR. Following IR, cellular DNA is damaged, and in response, there is the activation of the proteins ATM and ATR. In turn, these proteins rapidly lead to the activation of the tumor suppressor protein, p53, in detail after IR, ATM protein, phosphorylates p53. The activation of p53 causes Bcl-2 proteins to release Bax, thus promoting apoptosis [12, 46, 47]. p53 regulates the transcriptional activation of apoptosis mediating proteins, particularly Bcl-2, Bax, Puma, and Noxa [48, 49]. Furthermore, p53 can induce the production of SOD2 and GPX1 by binding to their promoters, resulting in an antioxidant response [50, 51]. p53 plays a crucial role in modulating the cellular redox state in response to oxidative stress induced by IR. Crucially, alterations in apoptotic mediators can significantly impact the cellular response to RT. Mutations in p53, frequently observed in radioresistant tumors, impair the activation of the intrinsic apoptotic pathway, thus limiting the efficacy of RT. Alterations in p53 have been shown to be associated with liver cancer recurrence and radioresistance in patients. Mutations in the TP53 gene are also associated with the downregulation of BCL2 family proteins such as Bcl-xs or Bax proteins, leading to acquired resistance and poor efficacy of RT against this tumor [52]. In addition, under hypoxic conditions, the efficacy of RT is reduced due to the downregulation of pro-apoptotic members of the Bcl-2 family and the upregulation of anti-apoptotic ones, promoting radioresistance. However, targeting Bcl-2 with the inhibitor ABT-263 potentiates the RT effect, overcoming resistance both in vitro and in vivo [53]. Caspase modulation may also impact the outcome of RT. Indeed, it has been shown that activated caspase-3 promotes the release of several growth factors from irradiated tumor cells that stimulate the proliferation of adjacent cells, promoting post-irradiation angiogenesis. For this reason, the combination of RT with caspase-3 inhibitors could be a new and promising therapeutic strategy to substantially reduce tumor recurrence due

to post-irradiation angiogenesis [54, 55]. In contrast, tumor cells become more sensitive to RT due to the high production of pro-apoptotic proteins. Indeed, a crucial role of the Bax protein, which mediates the mitochondrial apoptotic pathway in prostate cancer cells within minutes after irradiation, has been reported [56].

3.2 Pyroptosis

Pyroptosis is a type of PCD, considered as an immunogenic cell death (ICD). It is modulated by both inflammatory caspases and the gasdermin superfamily proteins (GSDM). The GSDM proteins are inactivated because the C-terminal repressor domain masks their N-terminal pore-forming regions [57]. Once triggered, the caspase cleaves the GSD protein, releasing the N-terminal domain. Then, this domain binds to membrane lipids and perforates the membrane, causing membrane perforation and resulting in changes in osmotic pressure and swelling until the membrane ruptures [58]. Three pathways lead to pyroptosis: the first pathway is triggered by the caspase-1 pathway, which cleaves GSDMD into N- and C-terminal domains when the inflammasome forms and attaches to pro-caspase-1 via the adaptor protein the apoptosis-associated speck-like protein containing a CARD (ASC). As a result, the cytokines IL-1β and IL-18 are activated. After binding to the cell membrane, N-GSDMD leads to pore formation on the membrane, favoring the release of cellular contents. In the second pathway, bacterial LPS triggers the activation of caspase-4/5/11, which cleaves GSDMD to cause pyroptosis and simultaneously triggers the activation of caspase-1. In the Gasdermin C (GSDMC) caspase-8 cleavage pathway, apoptosis is converted to pyroptosis by PD-L1. Other pathways leading to pyroptosis involve distinct mechanisms, including the GSDMD caspase-3/8 cleavage pathway, the GSDME caspase-3/8/9 cleavage process, the GSDMB GZMA cleavage pathway, and the GSDME GZMB cleavage pathway [59]. Pyroptosis has attracted increasing interest in cancer RT due to its ability to both directly trigger cancer cell death and encourage the immune response to eliminate any remaining tumor cells after RT, possibly enhancing therapeutic effectiveness [60, 61]. The pyroptosis mechanism is more frequently activated following treatments with higher radiation doses [58, 59]. Indeed, in the literature, it has been reported that high-LET radiation can induce pyroptosis more than low-LET radiation [62–64]. The NLRP3 inflammasome has been shown to be activated after RT, causing pyroptosis in liver cells and bone marrow-derived cells [65, 66]. Furthermore, it has been demonstrated that upon IR, tumor cells generate damage-associated molecular patterns (DAMPs) triggering pyroptosis [59, 67]. IR activates pyroptosis and subsequently stimulates an antitumor immune response. Interestingly, the release of pyroptosis-specific signals could trigger the activation of immune cells that can target metastases far from the irradiated primary tumor [59]. An important player in the modulation of pyroptosis after RT treatment is undoubtedly the immune system: as already mentioned, this type of cell death is deeply linked to the immune system and closely related to its activation. Interestingly, NLRP3 has been demonstrated to greatly enhance antigen presentation, innate immune function, and T cell activation following RT [68]. Another crucial element in the induction of pyroptosis after RT is hypoxia. It is well known that high levels of hypoxia can interfere with RT, conferring radioresistant properties to the tumor [69]. IR can produce ROS in the TME due to water radiolysis. Then, ROS activates caspase-8 and GDSM with pyroptosis as a result [70]. Furthermore, free radicals can cause caspase 9/3-dependent GDSME activation; thus, low oxygen levels may limit ROS production in the tumor after RT by reducing the activation of pyroptosis [61].

RT can activate pyroptosis, exploiting the signal cascade of the canonical mechanism, the inflammasome-mediated pathway regulated by the activation of caspase-1 and inflammatory response. The canonical pyroptosis pathway is activated upon IR at different levels of the signal cascade and exploits different events that trigger the assembly of the inflammasome, direct activation of caspase-1, and ROS production. It has been shown that IR leads to the formation of inflammasomes containing NOD-like receptors, in particular NLRP1 and NLRP3 [71]. The formation of these cytosolic complexes triggers the activation of caspase-1, catalyzing the activation of proinflammatory cytokines. Subsequently, caspase-1 causes the cleavage of GSDMD, the final effector of pyroptosis. In addition, it has been shown that IR is able to trigger pyroptotic processes, directly activating the caspase-1 and downstream processes, in an inflammasome-independent manner [72]. ROS generated in the tumor microenvironment (TME) and within the cell are essential in the setting of pyroptosis upon RT. Indeed, studies have shown that RT can activate NLRP3 and the pyroptosis pathway once ROS related to glycolysis or those related to mitochondrial activity (mitoROS) accumulate [59, 73]. Also, the immune system is crucial for pyroptosis activation after RT: following irradiation, macrophages present in the TME can activate the p38 pathway MAPK-NLRC4-caspase-1 and trigger this process [74]. RT is also able to activate pyroptosis via a pathway that is not dependent on inflammasome formation or on caspase-1 activation. γ-radiation has been shown to cause cleavage of GSDM via the caspase-9/caspase-3 pathway in several solid tumors [72]. Following RT, antitumor immunity can also be activated by inducing GSDME-mediated pyroptosis, which transforms the tumor from "cold" to "hot," making it more susceptible to the immune response [61, 75]. On the other hand, pyroptotic tumor cells may secrete inflammatory modulators that encourage tumor cell repopulation and development [76]. In addition, pyroptotic cells can, in turn, release multiple inflammasomes to damage normal tissues [77]. It was reported that different pharmacological agents can modulate pyroptosis; for instance, the caspase 1 inhibitor Vx-765 used in combination with RT has shown promise in preventing pyroptosis and reducing radiation-induced damage [78]. In addition, key regulators of immune checkpoints, such as PD-1 and its ligand, PD-L1, are connected to pyroptosis. Under hypoxic conditions, PD-L1 undergoes nuclear translocation, which facilitates GSDMC transcriptional activation. Clinical studies indicate that individuals treated with radiation and PD-L1 inhibitors can undergo pyroptosis, which destroys tumor cells; these patients have a higher survival rate than those treated with PD-L1 inhibitors alone [79]. In another study, GSDME-high-expressing tumor cell lines, such as lung, liver, breast, and glioma, have been demonstrated to cleave GSDME in a dose- and time-dependent way following irradiation, and different forms of irradiation may cause pyroptosis. Moreover, pyroptosis was considerably activated when irradiation and DNA-damaging chemotherapeutic drugs like cisplatin or etoposide were combined [61].

3.3 Necroptosis

Necroptosis is triggered by the activation of a death receptor on the plasma membrane, the receptor-interacting protein kinase-1 (RIPK1). The TNF-α-TNFR complex recruits proteins such as RIPK1, TRADD, cIAP1, and TRAF2 to form the pro-survival complex I [80]. This complex can be deubiquitinated, resulting in subsequent complexes, such as complex II. These, in turn, can induce both apoptosis and necrosis; in particular, complex IIa, which consists of TRADD, FADD, and caspase-8, promotes apoptosis via caspase activation [81]. Apoptosis is promoted by

caspase-8's interaction with RIPK1, RIPK3, and FADD to create complex IIb, which cleaves RIPK1 and RIPK3 to render them inactive. Complex IIc, commonly referred to as the "necrosome," is generated when caspase-8 activity is blocked [82]. This complex is composed of RIPK1, RIPK3, and FADD. The necrosome develops after RIPK1 activates RIPK3, then RIPK3 recruits MLKL and promotes its phosphorylation. Once MLKL becomes phosphorylated, it creates oligomers that migrate to the cell membrane, altering its permeability and causing necrosis [3, 83]. RT can activate necroptosis mechanisms by stimulating different pathways [84]. In irradiated cancer cells, there is an abnormal accumulation of cytoplasmic DNA cells [85]. In addition, a correlation has been observed between the increase of cytosolic DNA and the activation of the ZBP1-MLKL pathway. Indeed, following RT, ZBP1-MLKL necroptotic signaling connects tumor cell damage to anticancer immune responses [86]. The cGAS-STING signaling, activated autonomously, can communicate with the ZBP1-MLKL pathway, thus generating a positive feedback effect between these two signaling pathways. Following this, irradiated cancer cells constantly maintain two useful pathways to drive inflammation processes, so the necroptotic pathway mediated by ZBP1-MLKL can increase antitumor immunity through communication *via* the STING pathway [87]. Interestingly, it has also been shown that cells subjected to IR increase ZBP1 expression after treatment, lowering the threshold for activation of the pathway RIPK1/RIPK3/MLKL [86]. In addition, the IR can activate the c-GAS-STING pathway: once DNA is damaged by ionizing radiation, it binds cGAS in the cytoplasm. This bond stimulates the production of cyclic guanosine monophosphate-adenosine monophosphate (cGAMP). The cGAMP increase induces the transcription of interferon genes such as IRF3 and NF-kB, causing the synthesis of type I interferons and other cytokines [88]. Also, mitoROS produced upon IR acts as a trigger and substrate for pro-necroptotic events. It has been shown that the increase in mitoROS causes an increase in the expression of crucial proteins in the necroptosis pathways, such as RIPK1 and RIPK3 [89]. Emerging results suggest that necroptosis modulation in combination with RT could improve patient outcomes and response. For instance, *in vitro,* it was shown that by deleting RIPK1, RIPK3, or MLKL genes from different breast cancer cell lines, it dramatically decreased their tumorigenicity and led to an increase in the RT sensitivity [90]. Another *in vitro* study reports that necroptosis is activated in non-small cell lung cancer (NSCLC), hypo-fractionated RT [91]. Furthermore, it was shown that patients with increased necroptosis-related gene expression had better overall survival [92]. On the other side, other evidence showed that necroptosis inhibition in irradiated cells drastically blocks the release of IL-8, which is a major factor in the repopulation of the tumor, thus resulting in a decrease of metastatic potential [93].

3.4 Ferroptosis

Ferroptosis is a cell death type triggered by iron accumulation and subsequent peroxidation of phospholipids. Indeed, it is considered an iron-dependent cell death, distinguished by an increase of lipid-derived ROS [94]. Ferroptotic cells exhibit physically tiny dysmorphic mitochondria with decreased cristae and condensed membranes. The identity of the executor proteins in ferroptosis is yet unclear, in contrast to classical Regulated cell death (RCD), which entails the involvement of proteins that carry out cell death, for instance, gasdermin D for pyroptosis, caspase for apoptosis, and the protein MLKL for necrosis [95]. Ferroptosis is mainly activated by the breakdown of cellular antioxidant defenses, particularly the glutathione

(GSH)-dependent glutathione peroxidase 4 (GPX4) pathway, which protects against lipid peroxidation [96, 97]. Iron-dependent lipid peroxidation is a hallmark of this type of PCD and is caused by redox imbalance. Under physiological conditions, the redox balance of lipids is necessary for cell viability. Lipids are essential because they act as buffers for ROS, thus preserving the dynamic equilibrium between reduced and oxidized states. The oxidation of polyunsaturated fatty acids (PUFA) present in the phospholipids of cell membranes, mediated by lipoxygenases, triggers ferroptosis. These enzymes catalyze the reaction between PUFA, susceptible to lipid peroxidation because of double bonds in their hydrocarbon chains, and ROS, converting them into chemically active toxic lipid peroxides. Furthermore, lipoxygenase activity and lipid peroxide accumulation also depend on the availability of intracellular iron, which acts as an essential cofactor for these reactions [98, 99]. Ferroptosis is regulated by several metabolic and molecular pathways: maintenance of redox homeostasis, concentration and accumulation of iron in cells and tissues, mitochondrial activity, and finally, metabolism of amino acids, lipids, and sugars. A crucial factor in the regulation of intracellular iron is dependent on phosphorylase kinase gamma 2 (PHKG2), which plays a role in modulating iron metabolism, influencing its availability in the cytoplasm. Once the intracellular iron pool increases, an environment favorable to lipid peroxidation is generated, pushing the cell toward ferroptosis. Ferroptosis is strongly related to tumor biology and has recently been identified as a target to prevent cancer development. On one side, ferroptosis seems to be an innate mechanism of tumor suppression [100–102]. Ferroptosis induction not only inhibits tumor development but can also boost immunotherapy responses and overcome resistance to current cancer treatments [102]. The damage induced by IR can be indirect, caused by water radiolysis and ROS accumulation. Therefore, there is a strong link between RT and ferroptosis; understanding the link between these two elements could open new avenues in the field of radiobiology and radiation oncology [103]. Ferroptosis can act in synergy with radiation to increase ROS production, compromising the antioxidant system and inhibiting tumor growth. ROS accumulation contributes to the decrease of GSH and activates genes related to DNA damage in tumor cells. Lipid peroxidation, a defining feature of ferroptosis, is triggered by RT via at least two parallel pathways. The generation of ROS by radiation encourages lipid peroxidation, which can strip PUFAs of their electrons to create fatty acid radicals. These are unstable carbon-centered radicals that react quickly with molecular oxygen to produce lipid peroxide radicals (PUFA-OO•). These radicals can then extract H• from other molecules through Fenton reactions, resulting in the formation of lipid hydroperoxides (PUFA-OOH). Furthermore, ACSL4, which is essential for mediating the manufacture of PUFA-PL, a class of lipids that are especially vulnerable to peroxidation, is expressed more when exposed to radiation [98]. In addition, ferroptosis can increase radiation sensitivity through iron overload, disruption of the antioxidant system, and lipid peroxidation [104]. After radiation exposure, iron increases in organs due to heme degradation in local tissues. Iron then accumulates and induces nonenzymatic phospholipid peroxidation through three different pathways:

1. It causes water radiolysis and the generation of products such as hydroxyl radicals, hydrogen peroxide, and hydrated electrons. Subsequently, hydroxyl radicals can generate lipoxide radicals and phospholipid hydroperoxides.

2. Ionizing radiation causes hemorrhage-dependent iron accumulation and an increase in phospholipid hydroperoxides [105].

3. Radiation-induced iron-dependent Fenton chain reaction with hydrogen peroxide, which further increases the level of hydroxyl radicals. All these processes generate nonenzymatic phospholipid peroxidation products that trigger ferroptosis [106].

The elevated expression of specific markers for lipid peroxidation, including MDA, 4-HNE, and prostaglandin-endoperoxide synthase 2, indicates the lipid peroxidation observed in tumor cell lines subjected to IR. This confirms the inference between radiotherapy and ferroptosis. Furthermore, mitochondria typical of ferroptosis show morphological changes in irradiated cells; these morphological features are strongly influenced by the dose of radiation administered [107]. To maintain redox balance and prevent ferroptosis, cells use the x_c system, which consists of two main subunits: a catalytic subunit, SLC7A11, and a regulatory subunit, SLC3A2, connected by disulfide bonds. These subunits work synergistically to ensure the import of extracellular cystine and the export of intracellular glutamate [108]. After importing cystine, it is converted to cysteine, an essential precursor to produce GSH. GSH is used by cells to prevent ferroptosis, as it has an antioxidant power that neutralizes free radicals and acts as a cofactor for the GPX4 enzyme, which is essential for reducing the peroxidation of phospholipids [109]. According to several studies, it was demonstrated that ionizing radiation reduces the levels of SLC7A11, GSH, and GPX4 in cells, compromising the cellular antioxidant protection system and promoting the lipid peroxides accumulation, thus inducing ferroptosis [106, 107]. Moreover, ROS generated after irradiation triggers ATM expression, which inhibits SLC7A11 and ACSL4, increasing fatal lipid peroxidation and then inducing ferroptosis. It has also been demonstrated that ACSL4 abrogation prevents ionizing radiation-induced ferroptosis, hence increasing radioresistance [98]. RT causes a significant increase in iron levels within target tissues [110]. Since iron serves as a catalyst for phospholipid peroxidation and the start of ferroptosis, IR significantly raises iron levels in target tissues. As a result, iron metabolism promotes ferroptosis and is essential to the body's reaction to IR. Transmembrane protein FPN1 is responsible for moving iron from the cell to the external environment. To preserve the tissue redox equilibrium, it has been demonstrated that IR damage can result in a localized upregulation of FPN1 expression in response to iron buildup [111]. Specifically, rats treated with IR showed a substantial rise in FPN1 in their livers following a 25 Gy X-ray treatment [112]. Another study reported that 4 Gy of radiation increases FPN1 expression in the bone marrow of irradiated mice [106]. Ionizing radiation has been shown to have the effect of increasing serum iron. Indeed, gamma radiation has been shown to cause an increase in serum iron levels in irradiated mice [113]. The increase of iron could be related to the oxidation of ferrous ions present in the blood by the radiolysis products of water caused by ionizing radiation [114]. Furthermore, upon IR, an increase of regulatory proteins was observed, including transferrin, which transports iron, transferrin receptor, and ferritin. In addition, by silencing transferrin, decreased radiation-induced cell death [115, 116].

4. RT-induced non-lethal processes: Mitotic catastrophe and cell senescence

4.1 Mitotic catastrophe

Mitotic catastrophe (MC) is an innate tumor suppressive mechanism that blocks cells from surviving. It is considered as incomplete mitosis, DNA damage, and

checkpoint errors [117]. MC morphological aspects include multiple centrosomes, misaligned chromosomes, abnormal mitotic spindles, micronuclei, and irregular nuclei [118]. This mechanism induces the formation of polyploid and aneuploid cells, which experience a variety of cellular stressors, such as elevated levels of ROS, and show increased sensitivity to treatments [119]. This mechanism is induced by genome damage and composed of mitotic death, interphase death, aberrant cell division, and genomic instability; it occurs as a mitotic arrest and non-immunogenic apoptosis dependent on Bax and Bak proteins [120]; instead of being a distinct type of PCD, mitotic catastrophe is a phase that comes before other forms of cell death. MC can block cell proliferation and induce cell death [118]. More appropriately, MC is caused by chemical or physical stressors that result in aberrant chromosome segregation. Both endogenous and external factors can result in mitotic abnormalities. Specifically, it is considered one of the key mechanisms for RT, chemotherapy drugs like doxorubicin, camptothecin, and paclitaxel, as well as other treatments with antitumor effects [119]. High levels of replicative stress and mitotic stress leading to abnormal ploidy are examples of endogenous sources. Cells that survive mitosis with chromosomal segregation abnormalities may initiate an inflammatory response by recognizing cytosolic DNA or RNA via cGAS or mitochondrial antiviral signaling [120]. Mechanistically, tumor cells are particularly vulnerable to mitotic aberrations because of their genetic instability, so they are vulnerable to the induction of MC. RT-induced DNA damage causes tumor cells, leading to MC. The G2/M checkpoint is inactivated, and consequently, the CDK1-cyclin A complex is not blocked, thus promoting mitosis. Furthermore, RT leads to hyper-amplification of the centrosome resulting from the inactivation of p53 and consequently also of p21, which activates the CDK2-cyclin E/A complex [3]. Bipolar mitotic spindles during mitosis can result from hyper-amplification of the centrosome. The formation of bipolar mitotic spindles leads to abnormal chromosome segregation, with the formation of giant cells with abnormal nuclear morphology and multiple nuclei ensuring MC. It has been shown that following treatment with BI2536, a compound with mitosis-regulating activity that induces MC, making oral cancer cells *in vitro* and *in vivo* more radiosensitive [121]. Furthermore, combining LB100 and protein phosphatase 2A triggers MC and enhances glioblastoma cells RT efficacy [122].

4.2 Cellular senescence

Cellular senescence is considered an irreversible arrest of the cell cycle [123]. Various stressors, such as DNA damage, oncogene activation or mutations linked to cancer, alterations in mitochondria, reactive metabolites, hyperoxia or hypoxia, proteotoxic stress, and extracellular signals, infections, leading to cell deformation, can trigger cell cycle arrest [124]. This process is characterized by increased senescence-associated β-galactosidase activity and senescence-associated secretory phenotype (SASP), and activation of cell cycle inhibitory pathways like p53/p21CIP, p16INK4A/pRB pathways, as well as the DNA damage response signaling [125]. This irreversible cell cycle arrest is typically triggered by oncogene activation or therapeutic interventions such as chemotherapy and RT. Proinflammatory cytokines, chemokines, growth factors, and extracellular matrix components or metalloproteases are among the soluble elements that make up the full SASP profile [126]. Senolytic drugs can be employed as adjuvant therapy; indeed, it has been observed that dasatinib with quercetin or ABT263 in conjunction with RT can considerably increase the *in vivo* survival time of gliomas and reduce the

relapse time [127]. In addition, it was demonstrated that palbociclib and other cyclin-dependent kinase 4/6 inhibitors may enhance the anticancer effect of RT in breast cancer. HR+, hormone receptor-positive breast cancer, the effectiveness of hypo-fractionated RT in conjunction with palbociclib is positively impacted by the removal of p16+ senescent cells [128]. In NSCLC cells, RT leads to the activation of the cGAS-STING pathway that increases the expression of LILRB2, also known as Immunoglobulin-like Transcript 4 or CD85d. This subsequently promotes p16- and SASP-dependent cellular senescence, which facilitates tumor progression and radiation resistance. Thus, blocking LILRB2 with RT may improve antitumor therapeutic outcomes and prolong survival [129].

5. Conclusions

In summary, the intricate interplay between RT and cellular mechanisms highlights the complexity of its biological effects and therapeutic potential. RT remains a cornerstone of oncology, targeting DNA and other cellular components to induce different types of cell death. In this chapter, some types of RT-induced PCD were discussed, including apoptosis, necroptosis, pyroptosis, and ferroptosis, as well as non-lethal processes such as senescence and mitotic catastrophe. Furthermore, it has been reported that some of these mechanisms, although distinct, often converge on shared molecular pathways, underscoring the need for deeper understanding to improve treatment outcomes. RT-induced processes such as mitotic catastrophe and cellular senescence further underscore its role in halting tumor progression. Despite its efficacy, challenges such as radioresistance and collateral damage to healthy tissues persist. Advances in targeting the tumor microenvironment, harnessing immune responses, and modulating non-lethal processes are paving the way for improved strategies. New insights into ferroptosis and pyroptosis as radiosensitization pathways show promise in overcoming limitations. Ultimately, this chapter highlights the importance of integrating molecular insights with clinical practice, fostering innovation to optimize the therapeutic impact of RT while minimizing adverse effects, thus advancing the frontiers of cancer treatment.

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Declaration of competing interest

The authors declare no conflict of interest.

Abbreviations and acronyms

RT	radiotherapy
IR	ionizing radiation
DSBs	double-strand breaks
NHEJ	non-homologous end joining
HR	homologous recombination
LET	linear energy transfer
ROS	reactive oxygen species
RNS	reactive nitrogen species
DAMPs	damage-associated molecular patterns
TME	tumor microenvironment
PCD	programmed cell death
ICD	immunogenic cell death
GSDM	gasdermin
FADD	fas-associated death domain
TRADD	tumor necrosis factor receptor-associated death domain
TNF	tumor necrosis factor
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
GSDMD	gasdermin D
MLKL	mixed lineage kinase domain-like protein
ZBP1	Z-DNA binding protein 1
cGAS	cyclic GMP-AMP synthase
STING	stimulator of interferon genes
NF-κB	nuclear factor Kappa-light-chain-enhancer of activated B cells
ATM	ataxia-telangiectasia mutated
GPX4	glutathione peroxidase 4
GSH	glutathione
PUFA	polyunsaturated fatty acids
ACSL4	acyl-CoA synthetase long-chain family member 4

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