

A molecular structure graphic featuring three dark, reflective spheres connected by thin, metallic rods, set against a blue gradient background.

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Edited by Mary C. Maj and Felicia Ikolo



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Biochemistry

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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 Pharmaceuticals has been useful for the development of biomolecular assemblies with potential for novel biomedical applications.

Meet the Topic Editor



Dr. Mary Maj is a Professor at the St. George's University School of Medicine. She has been teaching genetics and biochemistry to medical students since 2011 and is the school's genetics content expert. Dr. Maj has a strong publication history in protein biochemistry and in diagnosing patients with inborn errors of metabolism and other rare genetic disorders. She was the first to identify the molecular cause of pyruvate dehydrogenase phosphatase-1 deficiency and developed an assay to test for the disorder.

Meet the Associate Topic Editor



Dr. Felicia Ikolo is an Associate Professor in the Department of Biochemistry, St. George's University, School of Medicine, Grenada. Her doctoral research in Proteomics was conducted at Northumbria University in the United Kingdom. Her research interests include biomolecules, enzymes, and genetic diseases, including sickle cell disease. She is a fellow of the Institute of Biomedical Sciences, UK, and a member of the Caribbean Association of Medical Technologists. She is also a member of the Sickle Cell Association of Grenada.

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Preface

The origins of cellular and molecular biology begin with the zygote. The first cell of a new human being contains the blueprint for every cell in the body. The incredibly complex system that develops from a single cell at conception to the trillions of cells that make up the adult human body is remarkable. The majority of cells that differentiate into the major tissue types contain the same amount of genetic information and yet conduct vastly distinct functions, from epithelial to connective, muscle, and nervous tissue. Most mobile cells in the body similarly contain the same genetic information, yet exceptions exist, such as red blood cells, gametes, and some immune cells. All the cells in the human body are interconnected, forming a collaborative system that sustains life. The collaboration is also controlled by a precise balance among controlled cell differentiation, controlled cell division, and, lastly, controlled cell death. When this endogenous balance of molecular mechanisms falters, when DNA repair fails or when cell cycle checkpoints are bypassed, the consequences are devastating. When a cell or tissue is altered by the invasion of an external force, such as viral infection, exposure to toxins, or traumatic injury, the consequences can profoundly disrupt cellular homeostasis.

Investigators of cellular life work to decipher the intricacies of normal cellular function to understand not only the pathologies that can arise when the molecular processes are disrupted and lead to disease, but also to stimulate tissue repair after injury. This knowledge then becomes the foundation for therapeutic intervention. The strategies often aim to protect normal cells from infection, toxins, and injury. In cases of cancer or viral infection, the goal is often to disable the protective mechanisms of diseased or transformed cells, leading to their elimination.

In this annual volume of *Cell and Molecular Biology*, investigators have contributed collective knowledge in the field, some through original research and others through curated and synthesized recent discoveries. Each chapter reflects a deep engagement with emerging mechanisms and advances in understanding well-investigated pathways that continue to surprise, setting the stage for innovation in diagnosis and therapy.

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DNA Replication Stress and the Human Genome: Hurdles, Hijacks and Cell Health

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Abstract

During replication of the genetic material, cells often face hurdles that challenge DNA replication machinery, leading to replication stress. Multiple complex signaling pathways have evolved to counteract and overcome such challenges. However, DNA repair defects caused by inefficient functioning of the DNA damage response pathways (DDR) drive genomic instability, one of the hallmarks of cancer. Here, we review the known events in DNA replication that induce replication stress and the mechanisms deployed by DDR pathways to deal with and overcome replication stress. Since the defects in these molecular pathways are associated with tumour progression, we provide an update on the novel and promising druggable targets emerging from DDR pathways. Further, we discuss how viruses replicate their DNA by hijacking and exploiting the DDR pathways and the potential to target replication stress-associated molecules for the identification of new anticancer molecules.

Keywords: DNA replication, DNA damage response, replication stress, Okazaki fragment, origin licensing, virus replication

1. Introduction

Cells harbour inherited messages that dictate their functions on a millisecond basis [1]. The intricate processes operated by cells are susceptible to disruptions and challenges that can lead to irreversible damage or cell death. While we know the sequence of genomes required to create life forms, the precise role of each of the 3.9 billion letters in humans, the challenges cells face and the multiple displays of coordinated strategies they employ to overcome the hurdles in their lifetime largely remain a mystery. Proteins on the other hand, the real executors of cellular tasks, reside in specific locations or shuttles between compartments, interacting with various biological structures to regulate essential processes such as survival, adaptation, cell division and defence against microbial infections. DNA replication plays a central role in mammalian cell division in the context of intact transfer of genetic information and must be executed accurately to complete the cell cycle in a timely manner. However, the complexity of DNA replication processes can lead to significant

changes at the chromosome level due to minor imperfections, resulting in replication halting, defects in cell division and the development of diseases. Factors leading to such lesions in the DNA can be either exogenous or endogenous [1]. It is suggested that a single eukaryotic cell may incur as many as 10^5 lesions in the genome during each replication cycle [2]. Cellular defence mechanisms against these lesions include a series of responses collectively known as the DNA damage response (DDR). DDR involves multiple proteins that perform diverse functions such as damage sensing, recruitment of repair mediators, amplification of damage signalling, signal transduction and execution of cellular responses.

2. Eukaryotic DNA replication

The protein complexes that enable the regulation of dynamic genomic replication in eukaryotes are referred to as replication factories or replisomes, with the replication fork serving as a focal point. These proteins not only ensure fidelity but also coordinate with the chromatin for efficient DNA replication [3]. One notable feature of eukaryotic genomes is the presence of multiple origins of replication, many of which remain dormant and are passively replicated along with the rest of the genome. These dormant origins are kept in reserve and are activated only in the event of replication stress, ensuring the completion of replication [4]. In cases where replication stress occurs at regions lacking dormant origins, and two converging forks encounter the stress, cells restart one of the forks to enable genomic replication completion. Specific internal pathways facilitate this restart process, ensuring fork stability and timely resumption [4]. Moreover, external factors, such as induced lesions in the DNA, which can be caused by exposure to genotoxic agents or DNA-damaging conditions, introduce abnormalities and obstacles in the replication process. When DNA replication machinery encounters these lesions, it can lead to replication stress, further exacerbating the challenges faced during replication. Additionally, DNA replication forks frequently encounter hindrances and become stalled due to DNA lesions from both endogenous and exogenous sources [1]. Apart from DNA lesions, intrinsic disturbances to the replication fork, such as conflicts with RNA polymerases during transcription, DNA-protein complexes, abnormal DNA structures and oncogene expression, can also impede DNA replication fork progression [5]. When the cell faces challenging events that drastically slow down or completely halt replication fork progression, it is referred to as replication stress (RS). In cases where the replication forks fail to restart, it leads to fork collapse [5]. Fork collapsing involves the dismantling of replisome, fork reversal and/or breakage, resulting in the formation of double-strand breaks (DSBs), which can ultimately lead to gross chromosomal rearrangements and genomic instability [6].

3. Unchecked origin firing and unscheduled replication

In humans and many other eukaryotic organisms, it is estimated that there are approximately 50,000 DNA replication origins distributed throughout the genome. These origins are positioned at specific locations along the chromosomes, and their distribution is not random. In eukaryotic DNA replication, the origin licensing process begins with the binding of the origin recognition complex (ORC) and other essential replication regulatory proteins, including cell division cycle 6 (CDC6) and

chromatin licencing and DNA replication factor 1 (CDT1), to replication origins during the late mitosis (M) to early G1 phase. CDC6, in association with the 6-subunit ORC, binds to dsDNA through ATP utilisation. The ORC/CDC6 complex then recruits the hexamer minichromosome maintenance 2-7 (MCM 2-7) complex, along with CDT1, onto the dsDNA. This recruitment process occurs through ATP hydrolysis, leading to the release of CDC6 and CDT1 [7]. Subsequently, binding of the second CDC6 molecule forms the ORC/CDC6/MCM2-7 (OCM) complex, which actively recruits a second MCM2-7 hexamer in a CDT1-dependent manner. This results in the formation of a head-to-head MCM2-7 double hexamer that encircles the dsDNA, followed by the release of CDC6 along with the ORC/CDT1 complex. This double hexamer complex is also referred to as a pre-replication complex (pre-RC) or DNA licencing complex (**Figure 1**) [7–9].

The consequences of insufficient licenced origins and the subsequent under-regulated origin activation are noteworthy. This condition gives rise to replication stress, which manifests as a tolerable degree of stress caused by the partial depletion of the MCM2-7 complex. When cells experiencing such pre-existing replication stress are exposed to substances such as hydroxyurea (HU) and aphidicolin, DNA damage occurs, as observed in studies conducted on human cell lines [10, 11]. Further, investigations utilising mice with a homozygous mutant allele of *Mcm4*, which reduces the stability of the MCM2-7 complex, revealed an accumulation of stalled replication forks during the M phase, ultimately interfering with proper chromosome segregation [12]. Additionally, studies on *Mcm2-7* hypomorphic mice demonstrated a loss of genomic stability and tumorigenesis due to dysfunctional MCM complexes [13]. These findings suggest that the presence of dormant origins, licenced through the loading of MCM2-7 onto the replication fork, acts as a rescue mechanism, safeguarding cells from replication stress and maintaining genomic stability. Conversely, their

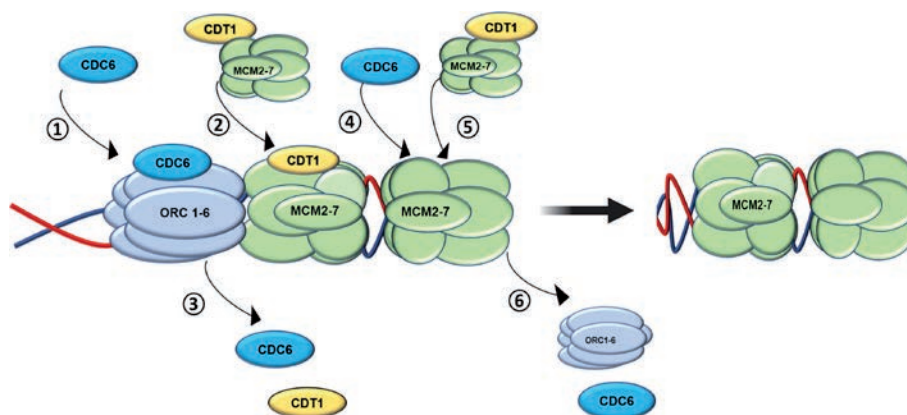


Figure 1. Origin licencing and DNA replication. The process begins with binding and encircling of the origin recognition complex (ORC1-ORC6) around the DNA, establishing the foundation for replication initiation. Subsequently, the cell division cycle 6 (CDC6) protein associates with the ORC complex, which comprises six subunits and forms a complex around the DNA (1). This ORC/CDC6 complex plays a crucial role in recruiting the MCM 2-7 helicase complex (2) for unwinding the DNA strands during replication. Together with the CDT1 protein, the MCM 2-7 helicase partially encircles the DNA, preparing it for replication (2). Following this crucial step, the CDC6 and CDT1 proteins are released from the origin site, allowing the replication process to proceed (3). A subsequent round of CDC6 binding occurs to the ORC/CDC6/MCM2-7 (OCM) complex (4). This binding event facilitates the recruitment of a second MCM2-7 hexamer, reinforcing the replication machinery at the origin site (5). CDC6 is eventually released, along with the ORC/CDT1 complex (6). The figure has been created using Biorender.com.

scarcity can lead to replication impediments and even tumorigenesis by facilitating oncogene-induced replication stress [13].

Re-replication, often associated with markers of replication stress, triggers the activation of the DDR pathway [14–16]. Unscheduled helicase loading during the S/M phase of the cell cycle, referred to as re-replication, results in DSBs and replication collisions [17]. This process, coupled with hallmarks of genome instability such as gene amplification, gross chromosomal rearrangements and aneuploidy, leads to adverse effects [18]. Induced replication during the G1 phase causes chromosome breaks and activates the DNA damage checkpoint during the subsequent S phase (**Figure 2**). Unscheduled replication also gives rise to head-to-tail replication collisions, resulting in an asymmetric accumulation of RPA-bound single-stranded DNA (ssDNA), a highly sensitive marker of acute over-replication [19]. Collectively, these events contribute to genome instability and highlight the detrimental effects of uncontrolled DNA replication.

Dysregulation of origin licensing, whether triggered by enhanced expression of oncogenes or other factors, can disrupt the normal replication process and contribute to replication stress [13]. Insufficient licensing of replication origins can lead to a reduced number of origins fired compared to the requirement, increasing the burden on individual origins and potentially compromising the efficiency of DNA replication. Furthermore, the premature firing of origins or re-replication of origins before

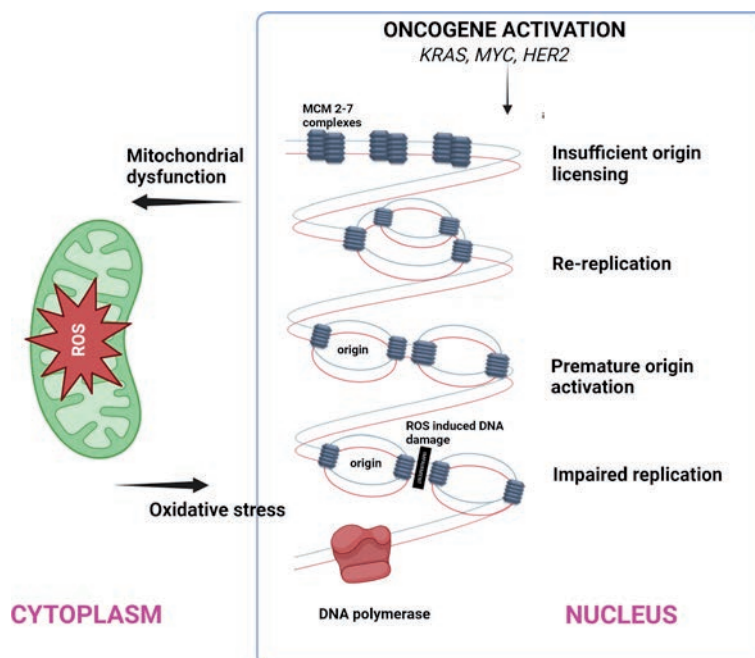


Figure 2.

Origin licensing and hurdles in replication. Enhanced expression of oncogenes can disrupt the regulation of replication leading to various routes towards replication stress. Oncogenes increase ROS production by enhancing cellular metabolism, dysregulating mitochondrial function and inducing the expression of ROS-generating enzymes, such as NADPH oxidases, promoting oxidative stress. A significant absence of active replication origins burdens the remaining origins excessively, leading to replication stress. In addition, re-replication of origins before the completion of replication can induce topological stress and further complicate the replication process. The figure has been created using Biorender.com.

the completion of replication by progressing forks can cause topological stress and further impede replication [10, 16].

4. Cell defence: stress detection and response

A well-established phenomenon associated with replication arrest is the formation of short single-stranded DNA segments at the junctions of replication forks in response to replication stress. This occurrence is thought to be a result of the uncoupling of the polymerase molecule from the actively unwinding helicase molecules [20]. In situations where the polymerase is halted due to factors, such as depletion of deoxynucleotide-triphosphates (dNTPs) or damage in the template strand, the helicase continues its unwinding process independently. Notably, even if the helicase encounters resistance, such as increased torsion, the polymerase remains tightly bound to it. The involvement of enzymes, including nucleases and helicases, in rectifying a stalled polymerase is underscored by the consistent observation of ssDNA at the replication fork junction under conditions of replication stress [21]. Upon the formation of ssDNA, replication protein A (RPA) rapidly stabilises DNA damage checkpoints. Subsequently, the activation of checkpoint pathways, including CHK2, as well as the involvement of transcriptional regulators, such as CDC25, occurs in a coordinated manner [22, 23]. This synchronised response helps orchestrate DNA damage repair mechanisms and, if necessary, initiates processes, such as apoptosis, to eliminate cells with irreparable DNA damage.

During DNA replication, the coordination between template unwinding and nascent strand synthesis at the replication fork junction is crucial. However, disruptions in this coordination can lead to the accumulation of ssDNA segments during the S phase [24]. Upon the binding of RPA to the ssDNA, the ssDNA-RPA complex recruits the ATR-ATRIP kinase complex. This complex activates the ATR protein, which then phosphorylates DNA topoisomerase binding protein 1 (DNA-ToBP1) (**Figure 3**). DNA-ToBP1 plays a critical role as it binds to the junctions between ssDNA and dsDNA, which commonly occur during lagging strand synthesis mediated by Okazaki fragments and at sites of fork reversal [24]. The presence of DNA-ToBP1 recruits various factors to resolve stalled replication forks. The fork can either bypass or remove the obstacle while maintaining fork integrity, allowing the polymerase to resume replication. DNA-ToBP1, along with RPA, ATR, and other proteins, play a crucial role in sensing and rectifying stalled forks, safeguarding the genome and ensuring survival under replication stress [19, 25].

5. Checkpoint dynamics in replication stress

Checkpoint mechanisms play a crucial role in orchestrating global DNA damage responses and maintaining genome stability [26]. In the event of replication fork stalling due to DNA lesions or other obstacles, the S phase checkpoint serves as a crucial machinery to ensure the completion of replication in a timely manner [27, 28]. Numerous studies have highlighted the significant impact of defective checkpoints on genome stability. For instance, cells with mutated checkpoint genes in *Saccharomyces cerevisiae* have been associated with induced and spontaneous chromosomal rearrangements [29, 30]. In higher organisms, such as vertebrates, loss of ATR function has been linked to chromosomal breakage, cell cycle arrest and embryonic lethality [31].

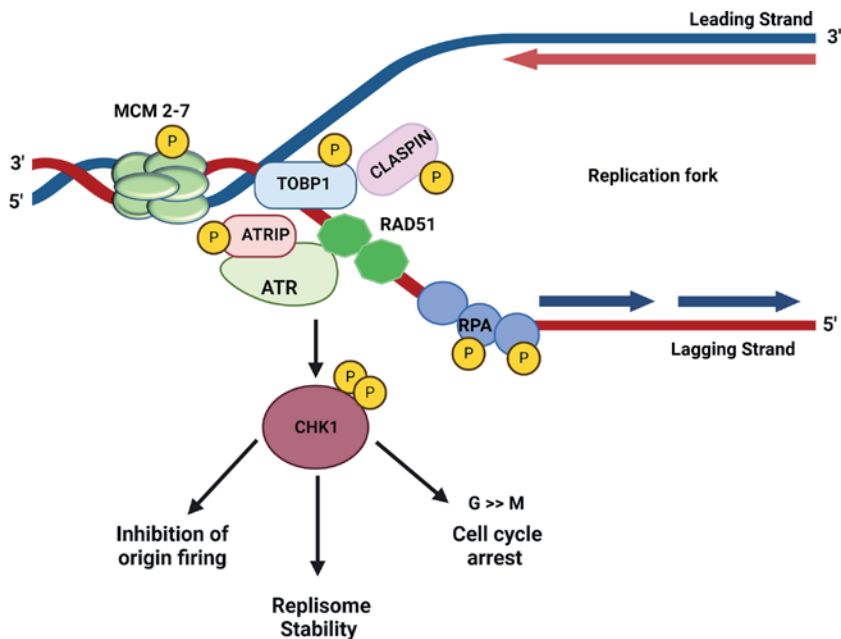


Figure 3.

Illustrates the recruitment and activation of the proteins in response to the persistence of ssDNA at the replication fork junction. The activation of ATR leads to the phosphorylation of numerous substrates, including MCM proteins, RPA, PCNA and ClaspIN. Therefore, the ATR signalling cascade is crucial for coordinating cellular response to replication stress and ensuring appropriate cellular outcomes, such as cell cycle arrest, DNA repair and maintenance of replisome stability.

Similarly, the absence of CHK1 has been shown to lead to DSBs and non-viability of embryos [32, 33]. Studies in *S. cerevisiae* have demonstrated that mutants lacking checkpoint genes, such as *mec1* and *rad53*, exhibit increased sensitivity to agents, such as HU, which induce replication stress. These mutants are also highly susceptible to alkylation by methyl methane sulfonate (MMS), ionising radiation or non-ionising radiation. Interestingly, while the loss of MEC1 and RAD53 does not induce lethality with an increase in dNTP levels [34, 35], it should be noted that checkpoints cannot be re-established by similar variations in dNTP concentrations [34]. In *mec1* and *rad53* mutant cells, prevention of mitosis becomes inevitable upon exposure to HU or MMS, irrespective of DNA replication stalling, which is a major cause of cell death. Additionally, it has been observed that restraining mitosis using microtubule inhibitors barely mitigates the lethality of *mec1* or *rad53* mutant cells treated with HU or MMS [34, 36].

6. Replication fork and sources of replication stalling

Replication stress arises from several sources, leading to disruptions in the replication process and potential genomic instability (**Figure 4**). During DNA replication, modifications on a single strand do not hinder the unwinding helicase, but they physically block the following polymerase. The recovery mechanisms employed by the cell to rectify the lesion partly depend on the nature of the induced lesion and its location on the leading or lagging strand [37–40]. For example, UV-induced

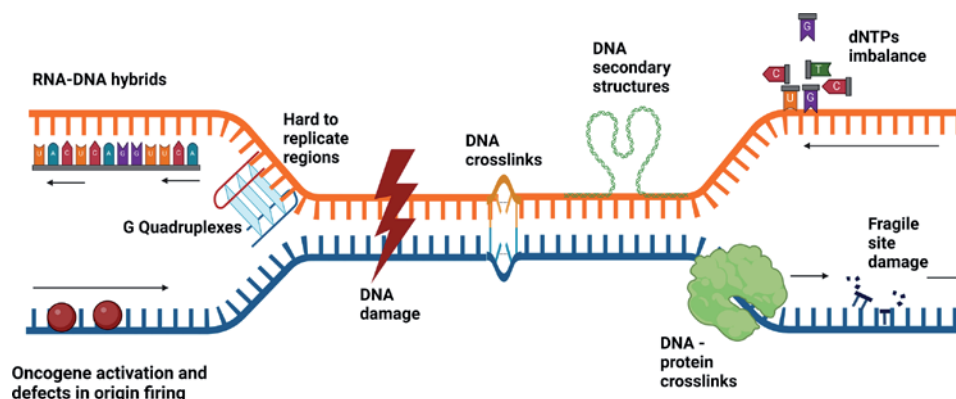


Figure 4. Overview of sources of DNA replication stress. Replication stress arises from various sources and can lead to the formation of DNA breaks and other aberrations. Single-strand breaks (SSBs) and double-strand breaks (DSBs) can occur when replication encounters obstacles. The formation of secondary structures or the incorporation of incorrect nucleotides due to nucleotide imbalance and exposure to chemicals can interfere with the replication process. The figure has been created using Biorender.com.

lesions encountered on a leading strand template result in the uncoupling of helicase and DNA polymerase ϵ , which abruptly stops DNA synthesis by polymerase ϵ . As the helicase progresses forward to unwind dsDNA, it leads to an exposure of the leading strand template coated with the RPA molecule. Unlike the leading strand template, UV lesions encountered on a lagging strand template arrest DNA synthesis only on the damaged Okazaki fragment and recommence upstream of the damaged Okazaki fragment through a scheduled re-priming event [39, 40]. To cope with these lesions, two strategies are employed, trans-lesion synthesis (TLS) by specialised polymerases and postponed repair. TLS polymerases allow elongation of the fork on lesion-rich templates, albeit with low fidelity [41]. Alternatively, postponed repair involves the fork skipping the lesion, leaving behind an ssDNA gap that is repaired after replication. Bacterial cells employ *de novo* priming and stalled DNA polymerase exchange to enable postponed repair and restart replication after a lesion [42, 43]. Eukaryotic cells also employ similar mechanisms, with specific proteins involved in re-priming DNA synthesis downstream of a lesion [43]. An example is the PrimPol protein in human cells, which can bypass UV-induced damage on the template and function under dNTP-depleted conditions. PrimPol exhibits TLS-like behaviour, raising the question of whether it primarily adopts TLS or postponed repair [44].

Studies indicate that cytoplasmic synthesis of dNTPs by ribonucleotide reductase (RNR) is the primary source. It is believed that the diffusion of dNTPs from the cytoplasm to the nucleus is sufficient for supporting nuclear DNA synthesis. Imbalanced nucleotide pools can impede replication fork progression and introduce errors in DNA synthesis. In addition, physical obstacles, such as DNA secondary structures or protein binding, can stall or collapse replication forks, while replication inhibitors and conflicts in replication timing also induce stress (Figure 4) [44].

Another mechanism is fork reversal, where a stalled replication fork moves in reverse, forming four-way structures to facilitate DNA damage repair [45–48]. Initially observed in prokaryotic cells, fork reversal was later proposed to explain replication over UV-damaged templates in mammalian cells. Studies on metazoans revealed that fork reversal serves as an adaptive mechanism, allowing the cell to pause and resume replication as needed while preventing chromosomal breakage [46, 48].

Fork reversal can be described as an emergency pausing mechanism that provides sufficient time for template repair, ensuring the completion of replication without significant mutations [47].

Nucleases are essential in the degradation of replication intermediates following exposure to genomic stress [49–51]. It is crucial to distinguish between the degradation of nascent DNA products and stalled replication forks [51, 52]. For instance, DNA2 degrades reversed forks in a controlled manner to facilitate restart while tolerating genotoxicity. However, this differs significantly from the pathological MRE11-dependent degradation observed in the absence of Fanconi anaemia (FA) factors and homology-directed repair (HDR) factors, such as BRCA2 and BRCA1 [49, 53]. Contrary to common belief, DSBs do not necessarily halt fork elongation, thanks to the presence of specialised mechanisms called break-induced replication (BIR) [54]. BIR involves strand invasion and the replication of several kilobases of DNA from the same donor. This process is particularly important near telomeres, where origin sites are absent. While previous studies suggested D loop formation through invasion of homologous regions, it is now understood that the loops form due to the unresolved nature of the structures, not rapid resolution into functional forks. DNA synthesis occurs through a migrating loop, while the lagging strand exhibits abnormal and conservative DNA synthesis, using the ssDNA of the bubble as the replication template [55, 56].

7. Replication stress as a hallmark of cancer

Replication stress, which leads to the manipulation of the standard replication process, is regarded as one of the primary factors responsible for the rise of cancers (Figure 5). Studies have shown that among the replication factors, CDT1 and CDT6, when over-expressed in cells can induce cell transformation and tumorigenesis in immunocompromised mice [57, 58]. In p-53 deficient mice, thymic lymphoblastic leukaemia was observed when subjected to T cell-specific CDT1 expression. Similarly, grafting of CDT1 overexpressing cells in immunocompromised mice leads to tumorigenesis [57, 58]. Studies have shown that the *Mcm2-7* complex also has similar effects. *Mcm2* and *Mcm4* hypomorphic alleles have resulted in lymphomas and mammary adenocarcinomas in mice models [59]. A study of the RPA1 protein shows that heterozygosity of the binding domain leads to DSB repair defects, which lead to chromosomal instability and even lymphoid tumours [25]. Similar trends of carcinogenesis were also observed in studies that manipulated the replication stress response pathways. RecQ helicases (RECQL4 and RECQL5) are essential proteins in the replication fork repair and restart machinery and loss of these proteins in mice increases susceptibility to cancer [60, 61].

Activation of DDR serves as a crucial barrier against cellular transformation and tumorigenesis by triggering pathways that induce apoptosis and cell cycle arrest [62]. The activation of DDR in response to replication stress caused by oncogene expression was one of the initial links explored, highlighting the role of replication stress in cell transformation [63]. Additionally, mutations in DDR genes are frequently observed in cancer cells [64]. The association between replication stress and tumorigenesis was further strengthened when studies demonstrated that the inactivation of DNA polymerases with aphidicolin led to an increase in microdeletions resembling those found in human cancers [65]. Moreover, the treatment of mice with HU, which depletes nucleotide pools, was shown to promote leukemogenesis and provide mutated cells with a survival advantage over non-mutated cells [66].

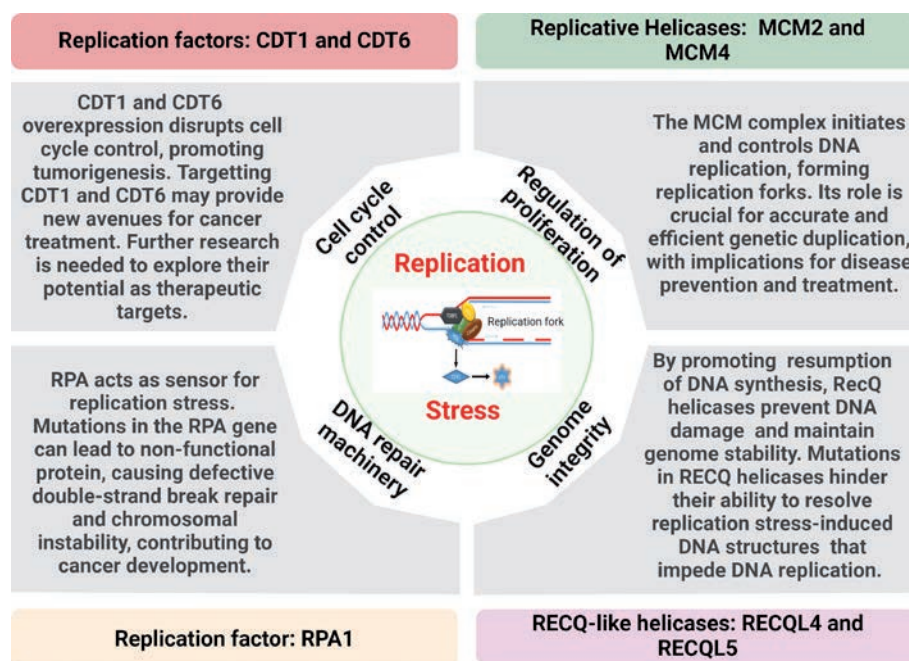


Figure 5.
 Replication stress as a hallmark of cancer. DNA replication is a stringent and tightly controlled process, which ensures generation of healthy cells. Failure in the control mechanisms of DNA replication allows mutated cells to proliferate and accumulate additional mutations, ultimately leading to tumour formation. Several genes involved in cell cycle regulation, proliferation control, DNA repair machinery and maintaining genome integrity are susceptible to mutations that induce replication stress, contributing to tumorigenesis. The figure has been created using Biorender.com.

8. Viruses and highjacking the DDR

The cellular programmes responsible for addressing replication fork stalling and DSBs play a crucial role in safeguarding the integrity of the cell's genome [21]. These programmes employ checkpoint proteins, transcription regulation and apoptosis to effectively detect, communicate and rectify such challenges. They are also capable of identifying unique structures, including DNA loops and other forms of damage, which pose a threat to DNA replication [36]. In addition to their surveillance functions, these programmes can recognise viral genomes that contain similar structures, thereby limiting the replication capability of viruses within host cells. However, in a battle for survival, viruses have evolved strategies to evade the surveillance mechanisms that detect and repair DNA damage and can also manipulate and exploit the DNA repair machinery to facilitate the replication of their genomes [67, 68].

Among the simplest viral forms are parvoviruses, which induce cell cycle arrest and exploit the cellular machinery to convert their ssDNA genomes into a dsDNA form compatible with cellular replication. For example, human parvovirus B19V activates and recruits all three phosphatidylinositol 3-kinase-like kinases (PI3KKs) involved in DDR along with their downstream substrates, to mediate B19V DNA replication [68]. Similarly, replication of minute virus of mice (MVM), another parvovirus, is facilitated by ATM signalling, although the ATR-CHK1 pathway is not extensively utilised [69]. Though connection between DDR signalling and

viral replication has been established the extent of the association between DDR components and viral-induced replication stress, as well as the impact of these factors on viral proliferative cycle, remains not fully understood [68, 69]. Certain viruses, such as adeno-associated viruses (AAVs) rely on coinfecting viruses, such as adenoviruses (AdVs), for efficient viral replication. The replication process, coordinated by DNA-PK and ATM pathways, involves the phosphorylation of multiple downstream targets, including CHK1/2 kinases, RPA2, NBS1 and histone H2AX [70]. In the case of human papillomaviruses (HPVs), DDR signalling is activated in differentiated cells infected with HPV, and various DNA damage repair components involved in the ATM-related DDR pathway localise to the HPV replication site. Studies indicate that proteins, including γ H2AX, CHK2, 53BP1 and members of the MRN complex (MRE11, RAD50, and NBS1), contribute to viral DNA replication [71, 72].

The complexity of DDR association increases with the advanced viruses. For example, Herpes Simplex Virus 1 (HSV-1) infection activates ATM kinases, providing advantages for viral genomic replication. Conversely, the ATR pathway, which is usually involved in canonical pathways in DNA damage signalling seems to be dysfunctional [73]. While the ATR-CHK1 pathway remains inactive, certain components of this pathway are recruited by virus replication machinery, aiding the infection [73, 74]. Similarly, the Epstein-Barr Virus (EBV) recruits ATM but may encounter challenges from the ATR-CHK1 pathways, which regulate nucleotide pools and can impede virus transformation [75]. In viruses, such as Kaposi Sarcoma-Associated Herpesvirus (KSHV), the interaction with PI3KKs is complex and can vary depending on the infected cell type [76, 77].

9. Drivers and therapeutic targets

Replication stress is a well-established driver of genomic instability [78], underscoring the importance of understanding its underlying factors. Replication stress can arise from both extrinsic and intrinsic causes, with certain intrinsic factors being well-known oncogenes (**Figure 4**). Uncontrolled expression of multiple oncogenes has been reported to trigger replication stress [79]. Notably, H-RAS, c-Myc and cyclin E have emerged as significant contributors to replication stress in numerous studies across different species [80–83]. These oncoproteins have been found to increase the frequency of unscheduled origin firing, depleting nucleotide pools and leading to collisions between the transcriptional machinery and replication origins, thus inducing replication stress. RNA binding protein ZFP36L1 depleted cellular models demonstrate differences in cell growth, clonogenicity, migration, and replication stress as compared to wild-type cells (unpublished data of genome engineering laboratory, University of Westminster). In yeast, overexpression of *c-myc* promotes unscheduled origin firing by targeting *cdt1* and *cdc45*, resulting in the activation of replication origins [80, 81]. Similarly, elevated levels of cyclin E hinder the loading of MCM proteins, impeding efficient pre-replication complex formation and affecting replication initiation in human cells [82]. Furthermore, mutated RAS oncogenes enhance transcriptional activity, leading to collisions between transcriptional components and the replication machinery, thereby inducing replication stress [83]. In addition to oncogenes, cellular processes that affect DNA accessibility, such as chromatin condensation, can also pose challenges to the replication fork. Recent studies have suggested that the enrichment of the DSB marker γ H2A (H2AX) in the

heterochromatic region of yeast is replication-dependent. Fragile sites are prevalent in repressive chromatin environments, and chromatin relaxation reduces the breakage of fragile sites [84]. These findings imply a potentially higher occurrence of DNA DSBs in heterochromatic regions. However, the exact causes of these breaks remain unknown with several potential factors, including chromatin structure inhibition, warranting further investigation.

Replication stress has long been a prime target for anticancer drugs currently in use [85–87]. Moreover, several novel drugs targeting DDR pathways are undergoing clinical trials (**Table 1**). These drugs enhance the replication stress levels in cells, leading to cell death, particularly cancerous cells, which heavily rely on their DDR pathways to escape cell death. Recent trends in anticancer strategies involve targeting checkpoints, primarily ATM-CBK2, ATR-CBK1 and WEE-1 as viable options [87]. Effective inhibition of protective pathways, such as ATR-CBK1, which aid the survival of cancer cells, could be detrimental, particularly in cases where the Myc oncogene is over-expressed [113, 114]. In parallel with CBK1, the WEE1 protein plays a crucial role in regulating the G2/M checkpoint. Inhibition of WEE1 drives the cell into the mitotic phase with impaired DNA, which enhances replication stress, leading to a mitotic catastrophe [115]. Given the significance of DDR pathways, it is crucial to evaluate the impact of DDR inhibitors on normal cells concurrently. Encouragingly, human cells harbouring mutant endogenous CBK1 with disrupted phosphorylation sites S317 and S345 demonstrated that CBK1 functions are controlled by different phosphorylation events, and the baseline function of CBK1 is independent of the stress response pathway [116]. In contrast, cancer cells heavily rely on ATR kinase activity for survival under stressed conditions [116]. Our expanding knowledge of DNA replication and replication-stress-associated pathways opens avenues for identifying new potential targets for cancer therapy. Furthermore, exploring replication stress markers as early molecular diagnostic tools holds promise for the development of novel approaches for improved treatment.

10. Conclusion

To advance our understanding of replication stress, it is crucial to research further into the underlying pathways and molecular factors that either promote or restrict its occurrence. By gaining a more comprehensive understanding of the intricate molecular events associated with replication stress, we can uncover the diverse types of replication stress that manifest in distinct genomic locations. Additionally, exploring the fate of cells that accumulate DNA damage and resulting replication stress that are formed due to loss of newly identified cancer driver genes will provide valuable insights into the consequences and potential therapeutic implications. Recent advances in sequencing, proteomic and metabolomic methods offer unprecedented opportunities to investigate, identify and dissect the molecular signatures with replication stress.

Targeted DDR molecule	Drug name	Targeted cancer	Clinical trial number	Clinical Trial.gov URL
ATR inhibitors	AZD4547	Non-small cell lung carcinoma (NSCLC), squamous cell carcinoma, adenocarcinoma	NCT02664935	https://clinicaltrials.gov/search?term=NCT02664935 [88]
	AZD6738	Breast cancers	NCT03740893	https://clinicaltrials.gov/search?term=NCT03740893 [89]
		Prostate cancer	NCT03787680	https://clinicaltrials.gov/search?term=NCT03787680 [90]
		Advanced solid tumours, advanced pancreatic adenocarcinoma	NCT03669601	https://clinicaltrials.gov/search?term=NCT03669601 [91]
		Clear cell renal cell carcinoma (CCRCC), locally advanced pancreatic cancer, metastatic renal cell carcinoma, metastatic urothelial carcinoma, metastatic pancreatic cancer	NCT03682289	https://clinicaltrials.gov/search?term=NCT03682289 [92]
	Ceralasertib	Advanced solid tumours, head and neck squamous cell carcinoma, NSCLC, gastric cancer, breast cancer and ovarian cancer	NCT02264678	https://clinicaltrials.gov/search?term=NCT02264678 [93]
		Head and neck squamous cell carcinoma	NCT03022409	https://clinicaltrials.gov/search?term=NCT03022409 [94]
		Metastatic triple-negative breast cancer	NCT03330847	https://clinicaltrials.gov/search?term=NCT03330847 [95]
	ART0380	Advanced cancer or metastatic cancer, ovarian cancer, primary peritoneal cancer, fallopian tube cancer	NCT04657068	https://clinicaltrials.gov/search?term=NCT04657068 [96]
ATM inhibitors	AZD0156	Advanced solid tumours	NCT02588105	https://clinicaltrials.gov/search?term=NCT02588105 [97]
	AZD1390	Glioblastoma, brain cancers and leptomeningeal disease	NCT03423628	https://clinicaltrials.gov/search?term=NCT03423628 [98]
CHK1 inhibitors	LY2603618	NSCLC	NCT01139775	https://clinicaltrials.gov/search?term=NCT01139775 [99]
	LY2880070	Ewing sarcoma and Ewing-like sarcoma	NCT05275426	https://clinicaltrials.gov/search?term=NCT05275426 [100]
	SCH 900776	Adult leukaemia	NCT01870596	https://clinicaltrials.gov/search?term=NCT01870596 [101]
	SRA737	Advanced solid tumours	NCT02797977	https://clinicaltrials.gov/search?term=NCT02797977 [102]
CHK2 inhibitors	PHI-101	Platinum-refractory or resistant ovarian carcinoma, platinum-resistant fallopian tube carcinoma and platinum-resistant primary peritoneal carcinoma	NCT04678102	https://clinicaltrials.gov/search?term=NCT04678102 [103]

Targeted DDR molecule	Drug name	Targeted cancer	Clinical trial number	Clinical Trial.gov URL
DNA-PK inhibitors	AZD7648	Advanced cancers	NCT03907969	https://clinicaltrials.gov/search?term=NCT03907969 [104]
	Peposertib (M3814)	Advanced solid tumours	NCT02516813	https://clinicaltrials.gov/search?term=NCT02516813 [105]
		Solid tumours	NCT03724890	https://clinicaltrials.gov/search?term=NCT03724890 [106]
WEE1 inhibitors	AZD1775	Hypopharynx squamous cell carcinoma, oral cavity squamous cell carcinoma, larynx cancer	NCT03028766	https://clinicaltrials.gov/search?term=NCT03028766 [107]
		Chronic myelomonocytic leukaemia, myelodysplastic syndrome with isolated del(5q), myelodysplastic/myeloproliferative neoplasm, recurrent adult acute myeloid leukaemia, untreated adult acute myeloid leukaemia	NCT02666950	https://clinicaltrials.gov/search?term=NCT02666950 [108]
	Metastatic colorectal cancer		NCT02906059	https://clinicaltrials.gov/search?term=NCT02906059 [109]
	Solid tumours		NCT01748825	https://clinicaltrials.gov/search?term=NCT01748825 [110]
	Metastatic pancreatic adenocarcinoma, unresectable pancreatic carcinoma		NCT02194829	https://clinicaltrials.gov/search?term=NCT02194829 [111]
	Adavosertib	Solid tumours	NCT00648648	https://clinicaltrials.gov/search?term=NCT00648648 [112]

Targeting DDR pathways has emerged as a promising strategy for cancer therapy as it exploits the vulnerabilities of cancer cells while sparing normal cells. This table offers a comprehensive overview of novel anticancer drugs targeting DDR pathways alongside details of cancer types and associated clinical trial numbers. The data shown below has been curated from registered, ongoing clinical trials with the U.S. National Library of Medicine (NIH).

Table 1.
DDR pathways are defining features of genomic stability and in turn cell health and cancers.

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CAR-NK Cell Therapies in Tumor Microenvironment: Dilemma and Controversies?

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Abstract

Chimeric antigen receptor-natural killer (CAR-NK) cell therapy holds significant promise for cancer immunotherapy due to its efficient recognition and lysis of malignant cells. Furthermore, despite the potential of CAR-NK therapy as a safer and more effective immunotherapeutic strategy, researchers are actively focused on addressing its limitations. These include enhancing persistence, optimizing genetic engineering methods, and standardizing the production process for broader clinical applicability. Developing novel generations of CAR-NK cells, combined with a deeper understanding of their behavior in solid tumors, might potentially revolutionize cancer cell therapy and improve patient outcomes. However, to improve clinical outcomes and facilitate the broader application of CAR-NK cell therapies, we must address challenges related to the optimization of CAR constructs, in vivo persistence, tumor penetration, safety, and regulatory considerations. Overall, the article presents an extensive review of the challenges and potential strategies for improving the long-term anti-tumor efficacy of CAR NK cell therapy, emphasizing the importance of combination therapies, drug delivery methods, and immune checkpoint blockade in enhancing the effectiveness of NK cell-based immunotherapy. The paper provides valuable insights into these strategies' intricate mechanisms and potential future applications in cancer immunotherapy.

Keywords: tumor microenvironment (TME), adaptive immune cells, natural killer (NK) cells, cancer-associated fibroblasts (CAFs), chimeric antigen receptor (CAR), cancer immunotherapy

1. Introduction

Natural killer (NK) cells are large granular lymphocytes (LGLs) that emerge from hematopoietic stem cells (HSCs) in the bone marrow. A subset of innate lymphoid cells (ILCs) with miscellaneous killing mechanisms, they have recently become important in the application of immunotherapy. NK cells are a kind of innate

immune cells that can distinguish and demolish aberrant cells, transformed cells, virus-infected cells, and cancer cells by using a variety of surface receptors that may determine diverse molecules on target cells. These cells can directly lyse tumor cells in a non-MHC-restricted way [1–3]. NK cells represent a mixture of activations and inhibitory receptors on their surface. The equivalence between activating and inhibitory receptor signaling determines whether an NK cell will eradicate a target cell. Furthermore, NK cells play a significant role in boosting anti-tumor immunity [1]. After all, NK cells in the TME are numerous and interact directly with tumor cells, limiting tumor growth. These cells improve outcomes in patients with diverse categories of cancer, like hepatocellular carcinoma (HCC), melanoma, breast cancer, non-small cell lung cancer (NSCLC), squamous cell lung cancer, pulmonary adenocarcinoma, renal cell carcinoma (RCC), hematological tumors, solid tumors, glioblastoma, ovarian cancer, and gastric cancer [4–8].

Genetic modification techniques, including chimeric antigen receptor (CAR) engineering and the inhibition of inhibitory genes, have demonstrated the potential to enhance the function of natural killer (NK) cells. Patients with blood cancers can effectively eradicate their tumor cells using these techniques. However, previously, when treated with the same natural killer cells lacking CARs, their tumors were unsusceptible to eradication [2]. CAR-NK cells have exhibited comparable *in vivo* efficiency to CAR-T cells in xenograft mouse models, leading to enhanced survival rates [9, 10]. Through the productive transfer of allogeneic NK cells to patients, there has been a hopeful platform for CAR engineering and “off-the-shelf” products used in various fields [7].

In this review, we present CAR-NK cell therapy for solid tumors as a potential platform to revolutionize cancer treatment and improve patient outcomes in comparison to CAR-T cell therapy due to its advantages over CAR-T cell therapy, such as reduced risk of graft versus host disease (GVHD) and Cytokine Release Syndrome (CRS). Additionally, we discuss the challenges and effects of immunosuppressive TME mechanisms for successful progression in clinical trials. Moreover, we provide data on recently published preclinical and clinical studies of CAR-NK therapy. Finally, we evaluate the progress of CAR-NK clinical trials, highlighting current challenges. We then describe existing strategies aimed at optimizing the persistence, functionality, safety, and efficiency of CAR-NK cells, as well as enhancing their penetration into solid tumors. These strategies can contribute to the development of effective CAR-NK cell therapies.

2. CAR-NK cell therapy for solid tumors

Natural killer (NK) cells are innate immune cells that naturally target and eliminate non-self cells, such as infected cells and various tumor cells, without prior sensitization. They also play a crucial role in enhancing the overall immune response [11, 12]. These cells have features like MHC-unrestricted identification, cell cytotoxic activity, and tumor-infiltrating capability. In addition, they have the least side effects, like graft versus host disease (GVHD), cytokine release syndrome (CRS), and immune effector cell-associated neurotoxicity syndrome (ICANS) [13, 14]. Consequently, researchers are utilizing NK cells to generate CAR-NK cells that target various tumor antigens in numerous clinical trials (**Table 1**). CAR-NK therapies are the most efficient therapies for eradicating solid tumors in a group of CAR cell therapies [15].

Targeted specific antigen	CAR-NK product or name	Source of NK cells	Treated cancer types	Stage of trials or phase	Study objectives	Year	ClinicalTrials.gov ID/REF
MUC1	Anti-MUC1 CAR-NK cells	NK92 cell line	Hepatocellular carcinoma Non-small cell lung cancer Pancreatic carcinoma Triple-negative invasive breast carcinoma Malignant glioma of the brain Colorectal carcinoma Gastric carcinoma	Phase 1/2	Evaluation of safety and effectiveness of CAR-pNK cell immunotherapy in patients with MUC1-positive relapsed or refractory solid tumor.	2016	NCT02839954
HER2	(NK-92/5.28.z) anti-HER2 CAR-NK cells + Ezabenlimab	NK92 cell line	Glioblastoma	Phase 1	Evaluation of safety and tolerability of NK-92/5.28.z in patients with recurrent HER2-positive glioblastoma.	2017	NCT03383978
NKG2D	CAR-NK cells targeting NKG2D ligands	Autologous or allogeneic NK cells	Solid tumors	Phase 1	Evaluation of safety and feasibility of CAR-NK cell therapy in subjects with metastatic solid tumors.	2018	NCT03415100
PSMA	Anti-PSMA CAR-NK Cells	Human primary NK cells	Metastatic castration-resistant prostate cancer	Early Phase 1	Evaluation of safety and efficacy of anti-PSMA CAR-NK cells in metastatic castration-resistant prostate cancer.	2018	NCT03692663
ROBO1	BiCAR-NK/T cells (ROBO1 CAR-NK/T cells)	N/A	Malignant tumor	Phase 1/2	Evaluation of safety and efficacy of ROBO1 CAR-NK/T cell immunotherapy for malignant tumors.	2019	NCT03931720

Targeted specific antigen	CAR-NK product or name	Source of NK cells	Treated cancer types	Stage of trials or phase	Study objectives	Year	ClinicalTrials.gov ID/REF
ROBO1	ROBO1 CAR-NK cells	Human primary NK cells	Solid tumor	Phase 1/2	Evaluation of safety and efficacy of cell therapy using ROBO1 CAR-NK cells to treat solid tumors.	2019	NCT03940820
ROBO1	BiCAR-NK cells (ROBO1 CAR-NK cells)	N/A	Pancreatic cancer	Phase 1/2	Evaluation of safety and efficacy of ROBO1 CAR-NK cell immunotherapy for pancreatic cancer.	2019	NCT03941457
Mesothelin	Anti-Mesothelin Car-NK Cells (Mesothelin Car-NK-HNRM-01)	Human primary NK cells	Epithelial ovarian cancer	Early Phase 1	Evaluation of safety, tolerability and efficacy of anti-Mesothelin Car-NK Cells with Epithelial ovarian cancer	2019	NCT03692637
NKG2D	NKG2D CAR-NK Cell	N/A	Refractory metastatic colorectal cancer	Phase 1	Investigation of NKG2D CAR-NK cell therapy in patients with refractory metastatic colorectal cancer.	2021	NCT05213195
PD-L1	PD-L1-targeted CAR-NK cells (PD-L1 t-haNK), with IL-15 fusion protein (N803) + pembrolizumab	Human, allogeneic, NK cell line	Gastroesophageal junction cancers, advanced head and neck squamous cell carcinoma	Phase 2	To test the effectiveness of irradiated PD-L1 CAR-NK cells combined with pembrolizumab and N-803 in people with advanced forms of gastric or head and neck cancer.	2021	NCT04847466
5 T4	Anti-5 T4 CAR-NK	N/A	Advanced solid tumors	Early Phase 1	Evaluation of safety, tolerability, initial efficacy, and pharmacokinetics (PK) of anti-5 T4 CAR-NK cells in patients with advanced solid tumors.	2022	NCT05194709

Targeted specific antigen	CAR-NK product or name	Source of NK cells	Treated cancer types	Stage of trials or phase	Study objectives	Year	ClinicalTrials.gov ID/REF
DLL3	DLL3-CAR-NK cells	N/A	Extensive stage small cell lung cancer	Phase 1	Evaluation of safety and efficacy of DLL3-CAR-NK cell infusion in treatment of relapsed/refractory extensive stage small cell lung cancer.	2022	NCT05507593
Claudin6, GPC3, Mesothelin, or AXL	Claudin6, GPC3, Mesothelin, or AXL-targeting CAR-NK cells	Human primary NK cells	Stage IV ovarian cancer Testis cancer Refractory endometrial cancer	Phase 1	Evaluation of safety and preliminary efficacy of Claudin6, GPC3, Mesothelin, or AXL-targeting CAR-NK cells in patients with Claudin6, GPC3, Mesothelin, or AXL-positive advanced solid tumors.	2022	NCT05410717
NKG2D	NKG2D CAR-NK cells	N/A	Ovarian cancer	Not Applicable	Evaluation of safety and efficacy of NKG2D CAR-NK cells in treatment of platinum-resistant, relapsed epithelial ovarian cancer.	2023	NCT05776355
	SZ011 CAR-NK	N/A	Advanced triple-negative breast cancer	Early Phase 1	Evaluation of clinical safety and efficacy of SZ011 in treatment of advanced triple-negative breast cancer.	2023	NCT05686720
	SZ011 CAR-NK	N/A	Ovarian epithelial carcinoma	Early Phase 1	Evaluation of clinical safety and efficacy of SZ011 in treatment of ovarian epithelial carcinoma.	2023	NCT05856643
	SZ003 CAR-NK	N/A	Advanced hepatocellular carcinoma	Not Applicable	Evaluation of clinical safety and efficacy of SZ003 in treatment of advanced hepatocellular carcinoma.	2023	NCT05845502

Targeted specific antigen	CAR-NK product or name	Source of NK cells	Treated cancer types	Stage of trials or phase	Study objectives	Year	ClinicalTrials.gov ID/REF
TROP2	TROP2-CAR-NK cells	Human primary NK cells	Advanced solid tumors	Phase 1	Determination of safety, tolerability, optimal cell dose, maximum tolerated dose, and recommended Phase 2 dose of TROP2-CAR-NK cells in participants with solid tumors with high TROP2 expression.	2023	NCT06066424
TROP2	TROP2-CAR-NK cells	N/A	Pancreatic cancer, ovarian cancer, adenocarcinoma	Phase 1/2	Determination of safety and optimal cell dose of TROP2-CAR/IL15-transduced CB-NK cells delivered intraperitoneally to patients that are resistant to treatment.	2023	NCT05922930
CD70	CAR.70/IL-15-transduced cord blood-derived NK cells + fludarabine + cyclophosphamide	Cord blood-derived NK cells	Advanced renal cell carcinoma, advanced mesothelioma, advanced osteosarcoma	Phase 1/2	Determination of safety, tolerability, and optimal cell dose of CAR.70/IL15-transduced CB-derived NK cells in patients with lymphodepleting chemotherapy to patients with advanced renal cell carcinoma, mesothelioma, or osteosarcoma.	2023	NCT05703854
MICA/B	FT536	Allogeneic NK cell-produced iPSC	Gynecologic cancer, ovarian cancer, fallopian tube cancer, primary peritoneal, cavity cancer	Phase 1	Treatment of recurrent gynecologic cancers.	2024	NCT06342986

Table 1.
Clinical trials of CAR-NK therapy.

2.1 Activation signal for CAR-NK

2.1.1 CAR-independent mechanisms

NK cells use CAR-independent mechanisms, and CAR-NK cells activate against tumor cells [16]. In a CAR-independent manner, CAR-NK cells can recognize and eliminate tumor cells that do not express, overexpress, or abnormally express the target antigen. This feature allows CAR-NK cells to lyse tumor cells through various mechanisms (**Figure 1**) [8, 17, 18].

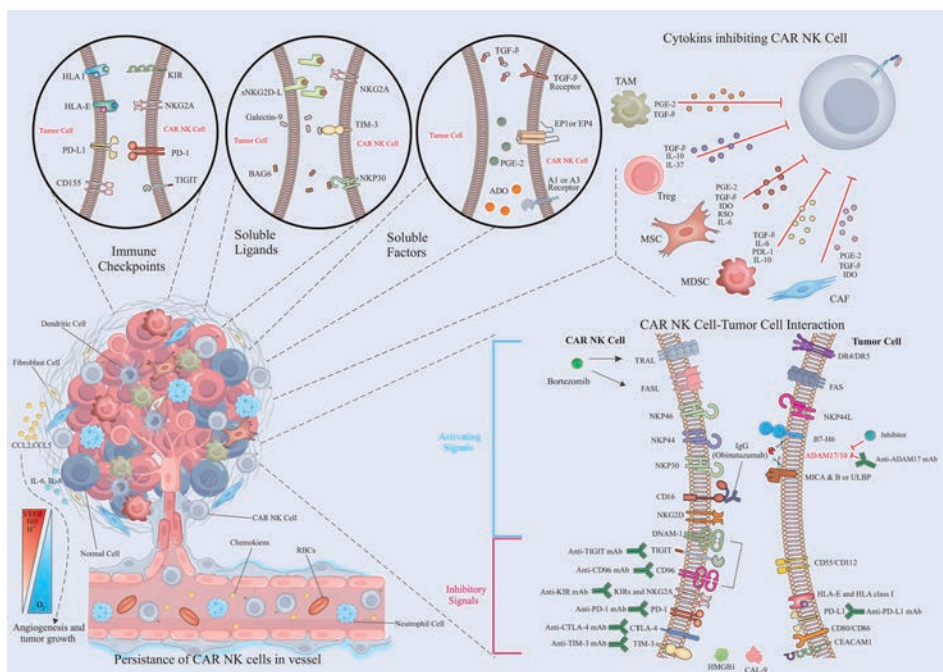


Figure 1.

The possible involvement of immunosuppressive variables in the tumor microenvironment (TME) that could lead to the ineffectiveness of CAR-NK cells. It also describes treatment strategies aimed at enhancing the detection and activation of natural killer (NK) cells in the solid tumor microenvironment. TME inhibits identifying tumor cells by natural killer (NK) cells through multiple mechanisms. In response to cytokine signaling, the expression of internal checkpoints such as CLBL, SOCS3, and CIS regulates the activation of natural killer (NK) cells and the development of immunological synapses with tumor cells. The inhibitory tumor microenvironment also contributes to suppressing CAR-NK cells by releasing suppressive molecules such as TGF- β . Within this particular structure, the potency of CAR-NK is diminished, resulting in reduced efficacy against tumor cells. The homing of CAR-NK cells is also controlled by chemokine receptors, such as CCR5, which directs the cells to the liver in response to CCL3. This reduces the effectiveness of CAR-NK cells against malignancies located in the bone marrow. The function and cytotoxicity of CAR-NK cells are regulated by intrinsic mechanisms within natural killer (NK) cells. For instance, when tumor ligands interact with NK immunomodulatory checkpoint receptors, including TIGIT, PD-1, NKG2A, TIM-3, and Siglec-7, it hinders the response of CAR-NK cells to target cells. ADAM17 inhibits the NK cell antibody-dependent cellular cytotoxicity (ADCC) response by releasing the CD16 receptor from the surface of the NK cell. The primary agents involved in these processes are potential targets for therapy to reinstate NK cell recognition and activation. (1) The proteasome inhibitor bortezomib enhances the ability of natural killer (NK) cells to kill cancer cells by increasing the cytotoxicity of TRAIL and FasL. (2) The ability of natural killer (NK) cells to identify tumor antigens is greatly hindered by the action of proteases ADAM10 and ADAM17, which cause the shedding of CD16, B7-H6, and NKG2DL (MICA, MICB, and ULBP). Therefore, administering anti-ADAM17 monoclonal antibodies and ADAM inhibitors can enhance the identification and activation of natural killer (NK) cells through the engagement of CD16, NKP30, and NKG2D receptors. (3) Furthermore, the genetically modified "off-the-shelf" NK cells, known as FT516, have a

high affinity for non-cleavable CD16 and are resistant to protease-mediated cleavage. Currently, clinical trials are underway to evaluate the therapeutic efficacy of FT516. (4) The activating receptor DNAM-1 competes with the inhibitory receptors TIGIT and CD96 for ligands CD155 and CD112. Monoclonal antibody treatments that specifically target TIGIT and CD96 have been shown to significantly hinder the advancement of cancer and the spread of cancer cells to other parts of the body by inhibiting the depletion of natural killer (NK) cells generated by TIGIT and CD96. (5) Killer cell immunoglobulin-like receptors (KIRs) and NKG2A have essential functions in the "missing self" process of natural killer (NK) cells. Nevertheless, tumor cells exploit this process to elude the immune monitoring carried out by natural killer (NK) cells. By using monoclonal antibodies, the inhibitory signals can be blocked, which in turn restores the ability of natural killer cells to destroy tumor cells. (6) The immune checkpoint molecules PD-1, PD-L1, CTLA-4, and TIM-3 cause NK cell fatigue by binding to specific ligands on tumor cells. ICIs primarily increase the identification and cytotoxicity of natural killer (NK) cells against tumor cells by targeting these molecules. This illustration was generated using biorender.com. Cancer cells express immunological checkpoint ligands on their cell surface, which provide a suppressive interaction with NK cells. In addition, tumor cells can inhibit the activity of NK cells by releasing soluble ligands into the surrounding environment, such as BAG-6, galectin-9, and soluble NKG2D-L (sNKG2D-L), as well as other soluble substances, including cytokines like transforming growth factor- β (TGF- β), enzymes, and metabolites. Several soluble factors are also generated by immune cells found in the tumor microenvironment (TME), including Tregs, tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC). Platelets release the metalloproteinases ADAM-10 and ADAM-17, which cause the shedding of NKG2D-L. Additional non-immune cells, such as derived-mesenchymal stromal cells (MSC) and cancer-associated fibroblasts (CAF), also generate indoleamine 2, 3 dioxygenase (IDO) or reactive oxygen species (ROS) that diminish the function of NK cells. In addition, hypoxia, elevated levels of fatty acids, food deprivation, acidity, and other metabolic variables collectively create a complex immunosuppressive tumor microenvironment that impairs the efficiency of natural killer (NK) cells against hematologic malignancies. Various techniques can counteract the immunosuppressive process of the tumor microenvironment (TME). (A) Antibodies that block immunological checkpoints prevent the suppression of NK cell cytotoxicity. When the adenosine A2A receptor (A2AR) binds to extracellular adenosine (ADO), it also inhibits the function of NK cells. Inhibiting the CD73 ectoenzyme, responsible for producing ADO, decreases the concentration of this metabolite in the tumor microenvironment (TME), consequently enhancing the cytotoxic activity of NK cells. Moreover, the inhibitory impact of NK cells is hindered by anti-TGF- β neutralizing antibodies, which disrupt the connection between this cytokine and its receptor (TGF- β R). (B) The expression of the dominant-negative receptor (DNR) obstructs the inhibitory signaling activated by PD-1 and TGF- β R when PD-L1/L2 or TGF- β is present. (C) Small molecule inhibitors targeting GSK-3 β affect the metabolism of NK cells and enhance their ability to kill target cells. Additional inhibitors are specifically designed to impede the kinase activity of TGF- β R: PD-1, Programmed Death 1; TIGIT, T cell immunoglobulin and ITIM domain; TIM-3, T cell immunoglobulin and mucin-domain containing-3; NKG2A, natural killer group 2A; PD-L1/2, Programmed Death ligand-1/2; HLA-E, HLA class I histocompatibility antigen, alpha chain E; ADAM-17, A disintegrin and metalloprotease 17; TGF- β , Transforming growth factor beta; TGF β R-2, Transforming growth factor beta receptor type 2; CCL2 Chemokine (C-C motif) ligand 2; CAR, chimeric antigen receptor; CIS, cytokine-inducible SH2-containing protein; SOCS3, suppressors of cytokine signaling; LAT, linker for activation of T cell; CBLB, Casitas B-lineage lymphoma protooncogene B; ADCC, Antibody-dependent cellular cytotoxicity; HLA-I, HLA class I histocompatibility antigen; KIR, Killer-cell immunoglobulin-like receptor; HLA-E, HLA class I histocompatibility antigen, alpha chain E; PD-L1, Programmed Death ligand-1; PD-1, Programmed Death 1; TIGIT, T cell immunoglobulin and ITIM domain; BCL2-associated Athanogene 6 (BAG-6); sNKG2D-L, soluble natural killer group 2D ligands; TIM-3, T cell immunoglobulin and mucin-domain containing-3; PGE-2, prostaglandin E2; NO, nitric oxide; A Disintegrin And Metalloproteinase (ADAM).

2.1.2 CAR-dependent mechanisms

If certain tumors express a particular antigen, the CAR distinguishes it, and it is used as a target for tumor treatment [19]. Next-generation CAR-NK cell designs often incorporate intracellular co-stimulatory domains like DAP10, DAP12, and 2B4 to enhance signal transduction [20–22]. Research suggests that CAR-NK cells incorporating DAP10 and CD3 ζ in their signaling domains can effectively recognize and eliminate tumor cells expressing specific antigens. These engineered cells exhibit enhanced cytotoxic activity against both solid tumors and leukemias in preclinical studies [23]. Based on some studies, the NK cells' cytotoxicity against unsusceptible PSCA-positive tumor cells improves with DAP12 into anti-PSCA (prostate stem cell antigen) CAR, as opposed to CD3. As per some research, the cytotoxic function of NK cells against mesothelin-expressing ovarian tumor cells, both in vitro and in vivo, intensified with anti-mesothelin CAR containing 2B4 and CD3 ζ . Contrary to the

capability of therapeutic antibodies (provoking antibody-dependent cellular cytotoxicity (ADCC)) or CAR-NKs against tumor cells, some tumors are unsusceptible to NK cell attack [24–26].

“Bispecific CARs” incorporate two distinct single-chain variable fragments (scFvs) within a single CAR structure, enabling the recognition of two specific target antigens. Bispecific CARs are activated when both antigens are recognized simultaneously by their respective receptors. This dual recognition enhances the precision of tumor identification and improves the cytotoxic activity of these cells [27, 28]. Li et al. researched bispecific CAR engineering in CAR-NK cells that boost the cytotoxic function of NK cells against gastric cancer by transducing a retroviral bispecific CAR (PD1-DAP10/NKG2D), effectiveness of CAR-NK cells against antigen heterogeneity, and tumor escape from solid tumors boosted by these double-targeting receptors.

NK cells can recognize MHC-I molecules through engineered receptors like TCR-CARs (T-cell Receptor-like Chimeric Antigen Receptors) or Proteome Detector CARs. Engineered NK-92 cells equipped with TCR-CARs can specifically target MHC-I-expressing tumor cells. By integrating TCR-based recognition with CAR-NK cell technology, we can enhance tumor identification capabilities and potentially prevent tumor escape mechanisms that involve downregulating MHC-I expression [29–31].

2.2 CAR-NK cell activation and expansion *ex vivo*

If there is no cellular contact, the expansion of NK cells in *ex vivo* is insufficient. As a result, the count of NK cells is not enough for the clinical approach, so investigators have characterized CAR-transduced lymphocytes and feeder cell-based NK expansion strategies that require cytokines and artificial antigen-presenting feeder cells (aAPC), including PBMCs, EBV-transformed lymphoblastoid cell lines (LCLs), and the K562 cell line [32–35]. The proficiency of NK cell stimulation and proliferation is enhanced when K562 cells are transduced with mbIL-15, IL-18, mbIL-21, or 4-1BB ligand (4-1BBL). MbIL15 in K562 can raise telomere length, magnify log-phase NK cell expansion, and significantly decrease senescence during their *ex vivo* propagation [36–43]. Given the specificity of CAR-NK cells, further research is needed to determine whether Inverted Cytokine Receptors (ICRs) such as IL-15 and IL-21 are suitable for CAR-NK cells. This can be achieved by merging IL-4 and IL-7 receptor domains with the overturned cytokine receptor (4/7 ICR) and combining them into CAR constructs. Eventually, the study highlights the importance of comprehending and utilizing metabolic processes for enhanced therapeutic effects and offers insightful information on potential approaches for improving CAR-NK cell therapy. To enhance the effectiveness of CAR-NK cell therapy, researchers have recently looked into silencing NK inhibitory receptors during the CAR-NK cell creation process [3]. CAR-NK cells, when combined with cytokine-induced memory-like NK cells, enhance responses to cytokines and stimulate activating receptors for weeks to months following pre-activation. This combination results in a more robust response to tumor cells. These cells enhance *in vivo* persistence and responses to unsusceptible tumors [44–47]. CD19-CAR-NK and CD70-CAR-NK cells significantly release IL-15 independently of antigen stimulation. This autonomous IL-15 production enhances CAR NK cell proliferation [47]. CAR-NK cells that target CD19 produced an overall response of 73% and a complete response of 64% in 11 patients with lymphoma and chronic lymphocytic leukemia, respectively, in one published phase I/II trial. These patients showed no signs of cytokine release syndrome [48].

2.3 CAR-NK cell activation and expansion *in vivo*

After cell therapy, IL-2 and IL-15 are injected to improve the *in vivo* expansion and activation of NK cells. However, side effects such as capillary leak syndrome and excessive activation of NK cells occur, which causes cell exhaustion. If CAR-NK cells include intracellular signaling domains (i.e., 2B4, DAP10, and DAP12), NK cell activity is enhanced, and their persistence *in vivo* is elevated [49–52].

“Armored CARs are engineered with stimulatory cytokine transgenes, such as IL-2, IL-7, IL-15, and IL-21. These CARs are designed to release cytokines and other molecules. These CAR-engineered NK cells can enhance NK cell expansion, persistence, and anti-tumor activity [13, 53]. TRUCK NK cells are generated to release the immunostimulatory cytokine. For example, scientists have produced iCas9/IL15/CD19-CAR-NK cells. These cells generate IL-15 upon identification of the CD19 antigen. Furthermore, CAR-NK cells can be eliminated after pharmacologic activation of the inducible caspase-9 (iC9) suicide gene [54].

In Liu et al.’s study, “Universal antigen-presenting cell (uAPC)-expanded CAR-NK” cells were used as feeder cells. To exploit the “missing self” phenomenon as a “signal 1,” K562 cells, which are MHC class I negative, were used in conjunction with CAR-NK cells engineered to express IL-15. Furthermore, the anti-tumor activity of uAPC-expanded CAR-NK cells has been enhanced due to their high metabolic fitness [55]. CAR-NK cells engineered with “Synthetic Notch” (SynNotch) receptors or “Inverted Cytokine Receptors” (ICRs) exhibit enhanced activation when exposed to tissue-specific ligands that interact with these engineered receptors [56, 57].

3. Understanding the tumor microenvironment

We have a lot of inhibitory ingredients in the tumor microenvironment, like myeloid-derived suppressor cells (MDSCs), hypoxic microenvironments and nutrient deprivation during tumor growth, concentrated immunosuppressive cytokines (TGF- β , IL-10, IL-37, IDO, adenosine, and prostaglandin E2 (PGE2)), the atypical vasculature of solid tumors, and reduced adhesion molecules on the endothelium [58–63]. This immunosuppressive environment in solid tumors can deteriorate the anti-tumor function of NK cells by the interaction of the immune checkpoint receptor PD-1 on NK cells and PD-1 ligands (PD-L1) on tumor cells and tumor-associated neutrophils (TANs) [64–67]. NK cell cytotoxicity can be suppressed by components of the tumor microenvironment, particularly by tumor-associated macrophages (TAMs), specifically M2-like TAMs, and cancer-associated fibroblasts (CAFs). These cells release anti-inflammatory cytokines and immunosuppressive mediators within the tumor microenvironment (**Figure 1**) [68, 69]. Tumor spheres exhibit uncontrolled proliferation and a dense extracellular matrix (ECM), which creates a physical barrier that hinders the movement of immune cells toward them [70].

By employing CRISPR/Cas9 technology to knock out related genes of NK cells, researchers attempt to generate CAR-NK cells that counteract some of these immunosuppressive effects. Using genome editing to remove checkpoint components and enhance their function is another approach to combating NK cell exhaustion in the tumor microenvironment (TME) [1]. Oncolytic adenoviruses (OAd) that express TNF- α and IL-2 can change the immunosuppressive TME in pancreatic cancer and

cause both CAR-dependent and CAR-independent host immunity [71]. The killing potency has increased following the knockout of CISH in NK cells, activating the mTOR pathway and enhancing both tumor metabolism and the tumor microenvironment [72].

4. Immune checkpoint receptors

Immune checkpoint receptors expressed on tumor cells, such as the inhibitory receptor PD-1 and IL-1R8, can significantly impair the effectiveness of CAR-NK cell therapy. Lu et al. developed “Chimeric Co-stimulatory Converting Receptors” (CCCRs) for NK cells. These engineered receptors suppress PD-1 signaling and its immunosuppressive effects within the tumor microenvironment (TME) while simultaneously enhancing activating signals. This results in improved anti-tumor cytotoxicity against human lung cancer in preclinical models [73, 74]. Scientists are looking for a way to switch or cease immune checkpoints in CAR-NK cell therapy to improve the anti-tumor performance of CAR-NK cells [43, 75]. Immune checkpoints are essential components of the immunosuppressive TME, aiding in tumor immune evasion and NK cell exhaustion. These checkpoints may potentially cause CAR-NK cells to become exhausted, making them less effective against tumors [71].

4.1 ICIs and combinational NK therapy

NK-92 cells can combine a high-affinity PD-L1-specific CAR and CD16 with endoplasmic reticulum-retained IL-2. These cells release increased amounts of granzyme and perforin, leading to the lysis of MDSCs and M2-like TAMs. By eliminating these inhibitory cells, CAR-NK cells can effectively reshape the immunosuppressive tumor microenvironment [76, 77]. Some studies recommend using cytokines like IL-2 and IL-15 in CAR-NK cells to enhance the durability and efficiency of NK cells. Enhancing NK cell activity in ADCC and the activity of therapeutic antibodies is another crucial aspect of clinical trials and scientific advancement. Researchers have been exploring ways to prevent CD16 shedding, which is an essential component of antibody-dependent cellular cytotoxicity (ADCC). For example, altering CD16's cleavage domain or focusing on the metalloproteinase ADAM17 can prevent CD16 from detaching from NK cells [78, 79]. Using CAR-NK cells with cytokines like IL-18 and IL-21 can improve in vivo persistence of various NK cells [80].

4.2 Infiltration capabilities of CAR-NK cells

Based on the research, if the chemokine receptor CXCR3 on human NK cells during ex vivo expansion is overexpressed, they can interact with CXCL10 on solid melanoma tumors, so their migration into solid tumors can be improved [4, 81]. Also, if CXCL16 is increased in the pancreatic tumor environment, it can enhance the tumor tissue infiltration of NK cells, the anti-tumor activity, and decrease the tumor burden in mice [82]. The interaction between chemokines and their receptors on CAR-NK cells represents a promising strategy to enhance tumor infiltration. For example, combining CAR-NK cells that express the chemokine receptor CXCR4 with the chemokine CXCL12/SDF-1 α , which is often overexpressed in glioblastoma cells, can significantly enhance tumor infiltration [83, 84]. Additionally, the combination of CAR-NK cells and the mRNA of the chemokine receptor CXCR1 (an IL-8 receptor) can enhance migration into the tumor microenvironment in vitro and strengthen

infiltration into human ovarian tumors in vivo in xenograft models [85]. CXC chemokine ligand 9 (CXCL9) and CXC chemokine ligand 10 (CXCL10) are chemokines that enhance the migration of CAR-NK cells [86]. Various engineering techniques have been investigated to enhance the migration of NK cells to tumor regions. For example, to improve migration to lymph nodes that express the chemokine CCL19, NK cells have been electroporated with mRNA encoding the chemokine receptor CCR7. Nevertheless, additional clinical trials are needed to confirm the efficacy of these strategies [1].

4.3 CAR-NK cell-based cytotherapies: Challenges and new adventures

A significant drawback that restricts the efficacy of NK cell immunotherapy is the limited in vivo survival of infused cells in the absence of cytokine support [1]. To obtain therapeutic results by utilizing the NK cell-induced ADCC effects, therapeutic antibodies that precisely target tumor-associated antigens—such as cetuximab, which targets the Epidermal Growth Factor Receptor (EGFR), and rituximab, which targets CD20—are used. The conclusion of three Phase I/II clinical trials indicates that Robo-1 is a promising candidate for CAR-NK cell treatment. The purpose of these studies was to evaluate the safety and efficacy of CAR-NK cell therapy, which specifically targets ROBO-1, in patients with pancreatic cancer and other solid tumors [87].

Some strategies, including the engineering of induced pluripotent stem cell (iPSC), differentiated CAR-NK (iPSC-NK) cells, and the enhancement of cryopreservation techniques, are used to overcome these barriers and increase the effectiveness of NK cell-based therapeutics. iPSC-NK therapy provides an opportunity to overcome many of the difficulties associated with NK cell therapy by utilizing a new source of NK cells. A homogeneously differentiated population of NK cells is derived from iPSCs. The limitations of the NK-92 cell line could be addressed if it is scaled up to clinical scales. Specific research teams have effectively demonstrated that iPSCs can produce functional NK cells in vitro. However, under long-term in vitro culture conditions, iPSC-NK cells may be carcinogenic and likely result in genetic instability. To address safety concerns and enable large-scale production, further research is required [88]. Heterogeneity with hematopoietic progenitor cells and shared antigen expression are significant obstacles in building CARs against acute myeloid leukemia (AML) [1]. Cryopreservation has been identified by researchers as a significant technological challenge in the study of NK cells. Furthermore, the survival, recovery, and cytotoxic function of CAR-NK cells after thawing—especially following long-term storage—were markedly enhanced by IF-M, a Me2SO-free cryopreservation medium [88]. It is critical to use CAR structure and genetic engineering to optimize NK cell reconstitution, increase cell proliferation, and optimize cytotoxic activation. Therefore, breakthroughs in cryopreservation procedures, large-scale preparation methods, and general efficacy are urgently needed. Unresolved issues in the field include how to address the challenges of relatively limited in vivo persistence and the potential for exhaustion [71].

5. Clinical trials and research progress

5.1 Clinical trials for CAR NK cell therapy

There is promotion in CAR-NK cell therapy, for example, combined CAR-NK cells and NKG2D ligands in multiple myeloma, CD138-targeting CAR-NK cells for

AML, BCMA-CAR-NK cells for multiple myeloma, and combined BCMA-CAR-NK cells and CXCR4 for decreasing tumor burden and boosting the survival of tumor-bearing mice [89–91]. A novel CAR-NK cell line designed by Murakami, this type of CAR-NK cell named CARKHYG-1 specially represses the growth of GBM cells and EGFR-CAR-NK cells developed by Liu et al., which prevented breast cancer cell line-derived xenograft (CLDX) and patient-derived xenograft (PDX) tumors in mice [92, 93]. CD73 mCAR pNK cells were developed by Wang et al. and can act as an anti-glioblastoma (GBM) treatment. Additionally, CAR-NK-92/5.28.z was created by Zhang, targeting the growth factor receptor tyrosine kinase ErbB2 (HER-2) to provide anticancer effects [94, 95]. Preclinical studies have demonstrated the potential of CAR-NK cells in treating glioblastoma and EpCAM-positive colorectal cancer in xenograft models. However, clinical data supporting their effectiveness against solid tumors remains limited. Three phase I/II clinical research in China showed that CAR-NK cells can be a helpful treatment option for non-hematological tumors, pancreatic ductal adenocarcinoma, and relative solid tumors with ROBO-1 expression [96–99]. According to the study, the combination of regorafenib and CAR-NK-92 cells showed greater efficacy in inhibiting the growth of established EpCAM-positive tumor xenografts than either CAR-NK-92 cells or regorafenib used alone. Previous studies have shown the safety and feasibility of cord blood-derived anti-CD19 CAR NK cell therapy in patients with B-cell lineage malignancies [37, 42, 43]. Another study showed promising treatment against solid tumors using FT596, an iPSC-derived off-the-shelf CAR-NK product, with NKG2D, 2B4, and IL-15 signaling. The efficiency of FT596 monotherapy and its mixture with CD20-targeting monoclonal antibodies like rituximab or obinutuzumab for the treatment of chronic lymphocytic leukemia and B-cell lymphoma was investigated in a phase I trial (NCT04245722) [100, 101]. In a previous study, anti-FITC CAR-NK cells were examined for treating heterogeneous solid tumors. A bi-specific FITC-DUPA adapter was synthesized to target prostate-specific membrane antigen (PSMA) expressed on prostate tumor cells. This approach demonstrated the potential of anti-FITC CAR-NK cells to accurately target tumors by redirecting their cytotoxicity [80].

5.2 Safety and feasibility of CAR-NK therapy

The purpose of the ongoing clinical trials is to assess the safety and efficacy of CAR-NK therapy, as well as its ability to improve patient outcomes and induce tumor regression. These studies are essential for demonstrating the best dosage administration, assessing the expansion and survival of CAR-NK cells in vivo, and finding any possible side effects related to this innovative immunotherapy strategy. No notable toxicities were found in the study, which underlined the significance of donor selection for allogeneic cell treatments and the influence of cord blood unit characteristics on therapeutic outcomes. The amount of nucleated red blood cells present and the time between collection and cryopreservation are the two factors that demonstrate the ideal cord blood units. These elements significantly affect the efficacy of CAR-NK cell therapy [102]. The CAR-NK cell matching procedure enhanced the cells' reactivity to chemokines released by tumors, particularly the IL-8 receptor CXCR1. The CAR-NK cell matching procedure prevents tumor migration and invasion [85]. The efficacy of CAR treatment that targets NKG2DL in treating solid tumors is now being evaluated in a clinical trial (NCT03415000). Additionally, to improve the therapeutic outcome against solid tumors, the combination of CAR-NK therapy with additional treatments like chemotherapy, radiotherapy, and oncolytic viruses is being investigated [43, 103].

6. Advancements in CAR NK cell therapy for cancer

6.1 Limitations of NK cells as a CAR platform

A significant limitation of CAR-NK cell therapy is their relatively short lifespan, typically around 2 weeks. This limited persistence can significantly impact their therapeutic efficacy and sustained anti-tumor activity. To enhance the therapeutic efficacy and persistence of CAR-NK cells, significant efforts are being made to extend their longevity [104]. The durability and immobility of NK cells in living organisms are significantly influenced by the interleukins IL-2 and IL-15. However, excessive dosages of this cytokine have been associated with organ damage, specifically in the kidney, heart, lung, and central nervous system. Unlike, low levels of IL-2 cause suppressive Treg cells to mobilize, which impacts NK cell function. Furthermore, cytokine transgenes and NK cell genetic alteration can prolong the effects of this treatment and increase the lifespan potency of CAR-NK [105–107]. Trials have been conducted to use an IL-15-armed CAR to prolong the lifespan of CAR-NK cells in vivo, based on clinical findings. In a phase I/II investigation, researchers studied NK cells modified with a vector that included an anti-CD19 CAR, IL-15, and an inducible suicide gene. The study revealed a low level of toxicity and a high rate of positive responses [108]. In this regard, CAR-NK cell therapy exhibits promising results in cancer immunotherapy yet, challenges remain [109]. Lentiviral transfections may be ineffective, whereas the bioengineering of CARs may improve insertion mutations [109].

7. Challenges of the clinical application of CAR-NK therapy

One of the principal challenges in advancing CAR NK cell therapy is the efficient expansion of NK cells for clinical application. Although it is possible to obtain NK cells from peripheral blood mononuclear cells (PBMCs), the relatively low abundance of NK cells in PBMCs poses a significant challenge to their isolation and subsequent expansion for therapeutic applications. Furthermore, the expansion procedure can be made more difficult by the NK cell population's heterogeneity from many resources, such as peripheral blood or cord blood [109]. While iPSC-derived NK cells may carry the risk of teratoma formation, UCB-derived NK cells may have limitations regarding cell number and purity. To overcome the limitations of CAR-NK cells, such as restricted proliferative capacity and inconsistent CAR expression across different cell sources, researchers have explored the use of CAR-NK cell-derived induced pluripotent stem cells (iPSCs) [86]. To achieve consistent and reproducible outcomes in clinical applications, it is essential to optimize methods for producing and expanding CAR-NK cells from various sources, as each source differs in potency and persistence. [104, 110]. The susceptibility of NK cells to the freeze-thaw process, which significantly decreases their viability and capability to eliminate target cells, is another challenge. Nevertheless, several studies have shown that adding IL-2 can help cryopreserved NK cells operate again to some extent. Therefore, optimizing cryopreservation methods is crucial for maintaining the purity, viability, functionality, and potency of CAR-T and CAR-NK cells after thawing. This is particularly important for the future development of off-the-shelf products that require robust and reliable cryopreservation strategies [3, 111]. With little information on their safety and effectiveness in curing solid tumors, the use of CAR-NK cells in clinical trials for cancer treatment is still in the early stages. To fully assess their potential

across different cancer types, further comprehensive clinical research is necessary [109]. Furthermore, NK cells can target cancer stem cells that are resistant to standard treatment [112, 113].

7.1 Fratricide and trogocytosis in NK cells

Trogocytosis is another new problem with CAR treatments and should be speculated since it may change the CAR cell's function. In CAR therapy, fratricide occurs when the tumor's CAR target antigen is transmitted to CAR-T or CAR-NK cells, which can then be killed by other NK cells [114, 115]. This tendency could be countered by creating a dual-CAR system. This system would consist of an inhibitory CAR that detects its own presence and emits a "do not kill me" signal to NK cells when they interact with their TROG+ siblings, as well as an activating CAR targeting the unique tumor antigen. This technique preserved the activation of CAR signaling against the tumor antigen while successfully inhibiting fratricide generated by the Trogocytic antigen. Consequently, it caused CAR-NK cells to become more active [114].

Additionally, prior research has shown that specific structural changes to the various components of the CAR manufacture may be able to diminish fratricide and/or the loss of antigens through trogocytosis. To neutralize the impact of trogocytosis-mediated signaling, the researchers proposed using blocking antibodies against the target antigen [114, 115]. Additionally, specialized gene knockouts (KOs) have been developed to stop fratricide. CAR-NK cells lacking CD38 and CD7 genes have been engineered to treat hematologic malignancies [116–118]. These findings underscore the importance of considering antigen loss and trogocytosis-mediated signaling when developing novel cell-based immunotherapies [115]. Liao's team made CD38-specific CARs into the CD38 gene locus, which showed strong anti-tumor function and improved accuracy while minimizing off-target effects. A "two-in-one" CRISPR/Cas9 strategy was employed to enhance the anti-fratricide capabilities of CAR-NK cells. CD7-specific CAR-NK cells demonstrate improved anti-tumor function while potentially reducing the risk of off-target effects and increasing tumor specificity [119].

7.2 Challenges that affect cytotoxicity and persistence in solid tumors

To date, the effective targeting of solid tumors by CAR-NK cells depends on a combination of factors, including their cytotoxicity, durability, and ability to migrate and infiltrate the tumor microenvironment. However, while treating solid tumors, these aspects are affected by many obstacles. The dense stroma that prevents CAR-NK cells from penetrating the tumor location is one major obstacle that acts as a barrier to CAR cell capability. Additionally, the immunosuppressive tumor microenvironment (TME) and continuous exposure to target antigens can lead to exhaustion and dysfunction of intratumoral CAR-NK cells [111, 120, 121]. In this regard, various engineering techniques have been explored to enhance the migration of NK cells to the tumor site. For example, to improve migration to lymph nodes expressing the chemokine CCL19, NK cells can be electroporated with mRNA encoding the chemokine receptor CCR7 or CXCR2. However, more clinical trials are needed to confirm these strategies' efficacy [83, 122, 123]. The limited persistence of infused NK cells without cytokine support is another issue that limits the efficacy of NK cell immunotherapy. Exogenous cytokines have been used to increase the durability and replication of adoptive NK cells to extend the lifespan of NK cells. Nevertheless, this strategy

may have unfavorable side effects, such as the growth of regulatory T cells and other suppressive immune subsets. Further research is necessary to fully elucidate the mechanisms by which transmembrane-bound IL-15 enhances the long-term persistence of NK cells [124, 125]. The cytotoxicity and durability of CAR-T and CAR-NK cells in solid tumors are also impacted by tumor escape [111, 126]. TME shows an immunosuppressive state that impedes anti-tumor immunity. Accordingly, in CAR cell therapy, we face significant challenges, such as overcoming the immunosuppressive tumor microenvironment (TME) in solid tumors (**Figure 1**).

Recent studies have explored strategies to engineer NK cells resistant to the suppressive effects of TGF- β . These approaches include: Utilizing CRISPR-Cas9 technology to disrupt the expression of the TGF- β receptor 2 (TGF- β R2) gene in NK cells and introducing a dominant-negative form of TGF- β R2 into NK cells using retroviral vectors [127, 128]. In some research, “Converter CARs” are used to make a beneficial environment for the immune effector cells, and these types of CARs enable the development of a new generation of CAR-NK cells to exchange the immunosuppressive TME and enhance outcomes in the therapy of solid tumors. NK-92 cells engineered to express a chimeric receptor incorporating the extracellular domain of TGF- β R2 and the intracellular domain of NKG2D exhibit enhanced IFN- γ production and cytotoxic activity while becoming resistant to the suppressive effects of TGF- β [129, 130]. Chemical inhibitors such as galunisertib, LY2109716, and P6 peptide can enhance the anti-tumor function of NK cells and promote their infiltration into the tumor region by blocking TGF- β signaling pathways. CAR-NK-cP6 showed significant cytotoxic potency [131].

Another key immunosuppressive metabolite within the TME is adenosine. This metabolite interacts with the A2A adenosine receptor (A2AR) in NK cells, inhibiting their function. Some studies have explored strategies to enhance NK cell function by generating NK cells with conditional deletion of the A2AR gene [132].

7.3 Transduction of CAR-NK cells and delivery

The most frequently used vectors in recent gene therapy experiments are lentivirus, retrovirus, and adeno-associated virus vectors. Because of their innate antiviral ability, increased sensitivity to apoptosis, and constrained capacity for expansion, NK cells are more difficult to engineer than T cells [133, 134]. Substitute nonviral techniques, like electroporation or lipofection, produce temporary expression of the CAR construct, which may not offer long-term efficacy [104]. However, the innate resistance of NK cells to lentivirus impedes effective transduction [1].

Additionally, studies have demonstrated the high efficacy of these methods, such as using CRISPR/Cas9 technology for the precise integration of the CAR construct into the primary NK cell genome and utilizing AAV vectors for efficient and targeted delivery of the CAR construct [104, 135]. However, researchers are examining nonviral delivery routes as cost-effective substitutes due to the disadvantages associated with elevated immunogenicity, potential insertional mutagenesis, limited insert size, high costs of good manufacturing practice-grade viral production, and related regulatory complications. However, they are less effective than viral systems [111, 133, 136]. CD33-CAR-NK cell models generated through lentiviral transduction have demonstrated cytotoxic potency both in vitro and in vivo against AML cells, leading to a significant reduction in tumor burden. Furthermore, CD19/CD20 dual-CAR-NK cells have been developed to enhance cytotoxicity against ALL cells [137].

8. Comparative analysis of CAR-NK therapies

8.1 Advantages of NK cells as a CAR platform

Human leukocyte antigen (HLA)-matching restriction, frequently a drawback of T cell-based treatments, is absent from NK cells. Research indicates that CAR-NK therapy has a safer profile than CAR-T cell therapies, with lower toxicity levels. CAR-NK cells have demonstrated lower rates of cytokine release syndrome (CRS), encephalopathy syndrome (CRES), neurotoxicity, and hemophagocytic lymphohistiocytosis [138] as well as less graft-versus-host disease (GVHD) than CAR-T therapy. As an “off-the-shelf” remedial product, CAR-NK cells can also be generated from allogeneic resources, such as healthy donor blood. This characteristic enables more affordable, accessible, and cost-effective production, increasing the scalability and patient accessibility of CAR-NK therapy [104]. Patients undergoing CAR-T treatment often have low T cell counts due to prior intensive treatment, which may pose a risk of insufficient collections [88].

Peripheral blood is the most readily available source. However, it has drawbacks such as high expense, low cell replication ability, and limited survival time. However, because of its benefits in gene expression, ease of collection, and decreased risk of GVHD, UCB is recognized as a superior source of NK cells. NK cell lines, particularly the NK-92 and KHYG-1 cell lines, are emphasized as potential resources for the rapid and abundant production of NK cells for immunotherapy [90, 139]. Furthermore, CAR-NK cells’ short lifespan decreases the danger of CRS, making cell-based immunotherapy safer and more adjusted [109]. The ability of CAR-NK cell treatment to decrease antigen loss relapse is another advantage, unlike CAR-T cell therapy. On the other hand, CAR-NK cells can identify cancer cells through both CAR-dependent and CAR-independent mechanisms. This characteristic of CAR-NK cells underscores their ability to address challenges related to immunological escape and antigen loss following immunotherapy [109, 140, 141]. In addition to the CAR-dependent pathway, NK cells are naturally cytotoxic. In contrast to T lymphocytes, which primarily utilize CAR-specific targeting, NK cells possess inherent cytotoxicity receptors that enable TAA-unrestricted lysis of target cells. Furthermore, IgG-Fc-Receptor III family expression on NK cells promotes antibody-dependent cell-mediated cytotoxicity. CAR-NK cells may be more effective than CAR-T cells as they offer both CAR-dependent and CAR-independent killing abilities [88].

NK cells have a wider range of developmental origins than T cells. CAR-NK cell therapy is a topic of great interest because it can target various antigens and may reduce immunosuppression induced by the tumor microenvironment. Compared to T cell immunotherapy, NK cells may have a wider range of applications in treating pancreatic cancer because of their capacity to penetrate the inner sites of solid tumors [87]. Additionally, NK cells’ innate ability to target tumors may lessen antigen escape, which is considered a resistance mechanism in CAR-T clinical trials [48]. These cells demonstrate safety in clinical applications. For example, several Phase I/II clinical trials have indicated that cancer patients without graft-versus-host disease (GVHD) or severe toxicities show favorable tolerance and outcomes. Using HLA-mismatched anti-CD19 CAR-CB-NK cell infusion, 63.6% (7/11) of the enrolled patients with relapsed or refractory leukemia (non-Hodgkin’s lymphoma or CLL) experienced a complete remission without cytokine release syndrome, hemophagocytic lymphohistiocytosis, GVHD, or neurotoxicity. Natural killer cells are suitable for cell immunosurveillance because they boost T cell and antibody responses and quickly

eliminate cancer cells with oncogenic transformation-associated surface biomarker expression, distinguishing them from the previously described checkpoint inhibitors and adoptive CAR-T treatment. As a result, NK cells are commercially available items for cancer immunotherapy that work through paracrine effects, antibody-dependent cell-mediated cytotoxicity (ADCC), and direct cytolytic action [142]. Autologous NK cells are not necessary for the production of CAR-NK cells. As a result, CAR-NK therapy avoids the difficulties related to autologous cell utilization [88]. An increasing number of clinical trials indicate that adoptive T cell and NK cell therapies are effective in treating hematologic cancers; however, their effectiveness in patients with solid tumors has been limited [70].

By using NK cell receptor-dependent processes to target tumor cells that do not express the CAR-targeted antigen, CAR-NK cells can effectively eliminate a heterogeneous tumor. In general, CAR-NK cell immunotherapy shows promise as a more adaptable and secure substitute for CAR-T cell therapy [19]. In contrast to T cells, NK cells can mediate cytotoxicity without prior antigen-specific sensitization. Furthermore, they can recognize and eliminate target cells in an MHC-independent manner [143]. NK-92 cell lines engineered to express chimeric antigen receptors (CARs) targeting EGFRvIII, HER2, and B7-H3 have demonstrated effectiveness in preclinical glioblastoma (GBM) models. CAR-NK cells derived from human peripheral blood, targeting EGFRvIII or CD73 and GD2, have demonstrated capability in GBM xenograft models. When utilizing B7-H3 CAR-NK cells, tumor burden prominently decreases [144].

9. Deployment of CAR-NK cells in tumor combination therapy

To alter the tumor microenvironment or enhance the diversity of the NK cell receptor/ligand group, CAR-NK cell treatment is used in combination therapy with radiation, chemotherapy, and other immunotherapy techniques. By increasing the sensitivity of tumor cells and the anti-tumor activity of CAR-NK cells, this method aims to produce a more successful therapeutic result (**Figure 2**) [145].

9.1 Combination of CAR-NK immunotherapy with chemotherapy

Based on current research, combining lymphodepletion chemotherapy with CAR-NK immunotherapy is a strategy that has been shown to lower the refusal rates of injected CAR-NK cells and reduce tumor size by decreasing the levels of MDSCs and T-regs in the tumor microenvironment (TME) [103, 146]. In the immunodeficient mouse model of Burkitt lymphoma, using Romidepsin in combination with chemotherapy and anti-CD20-CAR-NK cells has shown promising therapeutic outcomes. Utilizing CAR-NK cells as biological carriers to carry biological or cytotoxic medications to the tumor site has also been considered a viable option. Implementing this approach would increase the intracellular levels of the chemotherapeutic drug while reducing off-target effects. To successfully deliver the chemotherapeutic medication to the tumor, a study created modified CAR-NK-92 cells with Paclitaxel (PTX)-coated nanoparticles, which showed promising anti-tumor results [147, 148].

9.2 Immune checkpoint inhibitors and CAR-NK cell activity

Immune checkpoint inhibitors can be used therapeutically to increase NK cells' ability to destroy PD-L1+ malignancies [145]. Specific investigations have also shown

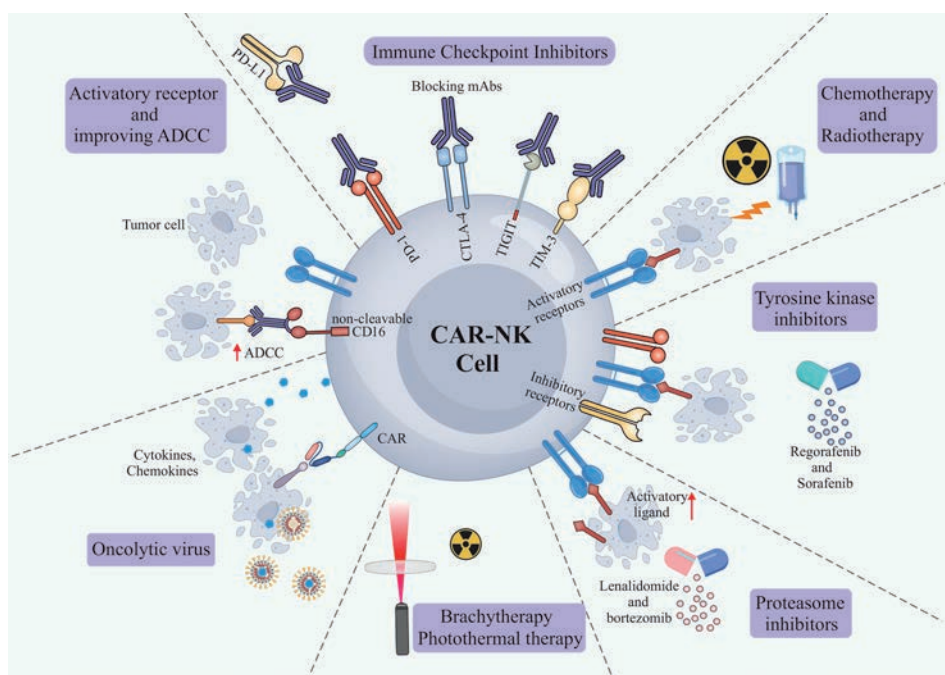


Figure 2.
 Utilization of CAR-NK cells in the context of tumor combination therapy. The tumor microenvironment (TME) represents a significant obstacle to CAR-NK therapy, primarily because of many variables, such as the secretion of immunosuppressive components by inhibitory cells inside the TME. The primary focus of CAR-NK therapy is to address the immunosuppressive role of the tumor microenvironment (TME). This is crucial when considering combination therapies, including anti-PD-1/PD-L1, chemotherapy, kinase inhibitors, proteasome inhibitors, oncolytic virus, and photothermal therapy. These techniques can induce the release of tumor antigens and DAMPs, facilitating the activation, proliferation, and elimination of CAR-NK cells. Many molecules can be targeted and classified as TSA (tumor-specific antigens), TAA (tumor-associated antigens).

that PD-1 antibodies can inverse the beneficial depletion of particular NK cell subsets. The inhibitory effect of macrophages on NK cell proliferation and ADCC can be effectively reduced by combining NK cells with an anti-PD-L1 antibody [149–151]. PD-L1 expression on tumor cells is associated with a decrease in the cytotoxic response of NK cells and promotes the development of more aggressive tumors in vivo. Furthermore, studies have demonstrated that inhibiting the PD-1/PD-L1 axis enhances the anti-tumor activity of NK cells and increases their sensitivity to tumor cells [145, 152–154]. Additionally, the elevation of PD-L1 expression is limited by the inhibition of JAK pathway activation. As a result, this makes tumor cells more susceptible to NK cell activity [145, 155]. It is hypothesized that anti-PD-L1 may stimulate AMP-activated protein kinase (AMPK) activation, increasing tumor susceptibility to NK cells, and that AMPK regulates the immunological response to immune checkpoint blockade [145, 156, 157].

9.3 Enhancing anti-tumor activity of CAR-NK cells with chemoradiotherapy

According to recent research, CAR-NK cells' anticancer activity can be increased by combining them with chemotherapy and radiation therapy. Additionally, research has shown that cisplatin can enhance CAR-T/NK cell-mediated cytotoxicity, resulting in intense anti-tumor activity. The CAR-T/NK cells retain their cytotoxicity

when exposed to cisplatin [158, 159]. Notably, recent studies have demonstrated that CAR-NK cells that target CD44 continue to be cytotoxic when cisplatin is present; the combination of cisplatin and CD44-CAR-NK cells has more anti-tumor effect than sequential therapy [160]. Similarly, researchers have discovered that combining CD133-targeting CAR-NK92 cells with cisplatin achieves the most potent anti-tumor impact on ovarian cancer while preserving the cytotoxicity and viability of the CAR-NK cells [159].

9.4 Utilizing NK cells as drug delivery carriers

Employing immune cells as carriers to deliver anti-tumor medications straight to the tumor site has drawn interest in this context [161, 162]. In particular, the study clarifies NK cells have significant concentrations of free sulfhydryl groups on their surface, which can be used to target medication delivery [148]. Furthermore, after encapsulating paclitaxel in synthetic cross-linked multilayer liposome vesicles (cMLV) nanoparticles, prior work showed that maleimide stably attached the drug to the sulfhydryl groups on the surface of NK cells. By using the targeting ability of CAR-NK cells, this novel approach enables paclitaxel-loaded cMLV to be delivered to the area of tumor cells, combining the effects of chemotherapy and tumor cell immunotherapy [145, 162].

9.5 Combination of CAR-NK immunotherapy with tyrosine kinase inhibitors (TKIs)

Furthermore, researchers have observed that another TKI, cabozantinib, can enhance the cytotoxic potential of CAR-NK-92 cells against renal cancer cells in vitro. It also decreases the expression of PD-L1 on the cell membrane and increases the expression of EGFR in renal cancer cells [163]. It has been noted that lenalidomide's inhibitory impact on tumors is enhanced by encouraging peripheral T cells and dendritic cells to secrete interleukin-2 (IL-2) and interferon-gamma (IFN- γ), which in turn increases NK cell killing and proliferation [164]. It has been reported that bortezomib and CAR-NK-92 cells have a more significant inhibitory effect on carbonic anhydrase IX (CAIX)-positive xenografts than either CAR-NK-92 cells or bortezomib alone. This suggests that their combined use may increase tumor cell sensitivity to NK cells, indicating a potential synergistic impact [165].

9.6 Enhancing anti-tumor activity of CAR-NK cells in combination with oncolytic virus

The synergistic suppression of tumor growth by OV-IL15C and EGFR-CAR-NK cells was associated with increased intracranial infiltration and activation of NK and CD8⁺ T cells, and the durability of CAR-NK cells was prolonged. Similarly, the combination of EGFR-CAR-NK cells and oncolytic herpes simplex virus-1 therapy for brain metastases of breast cancer demonstrated enhanced tumor cell elimination. They markedly increased the survival duration of tumor-bearing mice [166, 167]. Recent research suggests that oncolytic viruses can enhance CAR-NK cell therapy by modifying the tumor microenvironment and improving anti-tumor activity. For instance, NK cell aggregation in tumor foci was increased by vaccinia virus expressing CCL5, and oncolytic vaccinia virus expressing particular chemokines demonstrated capability in augmenting the effectiveness of NK cell therapy [168]. A combination of CAR-NK cells and *E. coli* K-12 DH5 α , a non-pathogenic *E. coli*, used to transfer critical

immune-activating cytokines to tumors, like decoy-resistant IL18 mutein (DR18) and suppress tumor development in immune-competent colorectal carcinoma and melanoma mouse models, boost migration of CAR-NK cells into the tumor site and survival of NK cell therapy [169].

9.7 Potential benefits of combined photothermal therapy therapies in CAR-NK therapy

Adoptive transfer of CAR cells or CAR-NK cells in cancer immunotherapy combined with thermal therapies like mild hyperthermia, photothermal therapy (PTT), and MWA increased therapeutic efficiency, potentially addressing residual tumor cells and improving the treatment of solid tumors, lung cancer, and pancreatic cancer [145, 170].

9.7.1 Synergistic effects of immunotherapy and brachytherapy radiation

The application of Robo1-specific CAR-NK immunotherapy in conjunction with 125I seed exertion for brachytherapy in an orthotopic pancreatic cancer mouse model suggests a potential synergistic impact between immunotherapy and brachytherapy in treating pancreatic cancer. The results show that the Robo1-specific CAR-NK cells improve the effectiveness of 125I seed brachytherapy [171].

10. Future and perspective

Future improvements in patient outcomes and a revolution in cancer cell treatment may result from the development of new generations of CAR-NK cells and a better understanding of how these cells behave in solid tumors. Clinical-grade Universal Antigen Presenting Cells (uAPC) are currently used in MD Anderson clinical trials to produce NK and CAR-NK cells. This novel technique facilitates the production of pure GMP-grade CAR-NK cells in the precise amounts required for adoptive CAR NK cell-based cancer immunotherapy [55]. It might be possible to use SNAP-CAR-NK cells as a powerful adapter technique in subsequent research to precisely target modified cells to different antigens using covalent chemistry [43]. Combining engineered targets with NK cell receptor modification can potentially increase the effectiveness of NK cell treatment by reprogramming immune responses to cancer cells. By forecasting patient reactions, AI can assist in developing combination treatments with traditional medicines or alternative immunotherapies in addition to various engineering techniques. Crucially, safety concerns, including off-target activation, remain a significant challenge for modified NK cell therapies. Ensuring the specificity of NK cell treatment is essential to minimizing potential side effects and preventing detrimental effects on healthy tissue [118]. Insertional mutagenesis, which can result in NK cell lymphomas, is another concern associated with the frequent use of integrative vectors to alter NK cells. The FDA advises a vector copy number (VCN) of less than five for gene therapy products [118, 172]. To assess the NK cells' engineering efficiency, purity, phenotype, and tumorigenicity, robust quality control standards must be established [173]. Since high dosages and several injections are needed for these latter points, innovative methods to grow, alter, and cryopreserve primary NK cells *ex vivo* would be very helpful for scaling up production for clinical applications [102, 118].

11. Conclusion

Over the past ten years, there has been a notable advancement in cellular immunotherapy. The therapeutic promise of CAR-NK therapy is further supported by the lack of documented side effects such as GVHD and CRS, which are frequently linked to CAR-T therapy. Furthermore, CAR-NK cells were proven to be as effective at killing cancer cells as CAR-T cells. The creation and application of CAR-NK treatment are not without difficulties, though. These challenges include the transduction of NK cells, the need for effective in vitro proliferation, a shorter in vivo durability compared to T cells, and the potential biohazard associated with the use of animal products. To overcome these obstacles, researchers are working to create safe, efficient, and expandable protocols for acquiring CAR-NK cells. Considering the biological characteristics of CAR-NK cells, improving CAR compositions, and developing procedures for CAR-NK cell preparation are the main goals of current research and clinical studies. Notably, several clinical trials are currently underway to evaluate the effectiveness of CAR-NK therapy in the treatment of solid tumors, hematological malignancies, and other types of cancer. To further enhance their therapeutic potential, CAR-NK cells are being investigated in combination with other immunotherapy approaches. Additionally, researchers are working to enhance the effectiveness of creating “off-the-shelf” products by advancing the manufacturing process for CAR-NK cells. This includes attempts to improve the potency and insistence of CAR-NK cells, clarify the production process, and maximize recovery of CAR-NK cells during freezing. The development of recombinant CAR-NK cells with memory capabilities and methods to reprogram CAR-NK cells to resist tumor suppression and escape are also being investigated [174]. The efficacy of iPSC-derived quadruple-engineered NK cells is now being evaluated in clinical trials [175, 176]. In conclusion, the significant developments in cellular immunotherapy reflect scientific advances in clinical trials and innovative approaches for reprogramming NK cells [176].

Authors’ contributions

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Abbreviations

AML	acute myeloid leukemia
AMPK	AMP-activated protein kinase
ADCC	antibody-dependent cellular cytotoxicity
DAP10	DNAX-activating protein 10
IDO	indoleamine 2,3-dioxygenase
iPSCs	induced pluripotent stem cells
MDSCs	myeloid-derived suppressor cells
MSLN	mesothelin
NKG2DL	natural killer group 2D ligands
PD-1	programmed cell death 1
TGF- β	transforming growth factor beta
Treg	regulatory T

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Characteristics of Natural Killers and their Functional Activity in Patients with Chronic Epstein-Barr Virus Infection

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Abstract

The Epstein-Barr virus (EBV) is a type 4 lymphotropic herpes virus. It is the cause of infectious mononucleosis, which results in significant alterations in the immune system. It is known from the literature that the role of natural killers (NK) cells in chronic EBV infection has not been thoroughly investigated. We conducted a study of 200 patients suffering from chronic Epstein-Barr virus infection (140 women and 60 men). The average age of patients was 37.87 ± 0.78 years. The duration of the patient's chronic EBV infection from the first symptoms to the laboratory examination and confirmation of the diagnosis was 3.58 ± 0.19 years. The aim of the work is to study the content of natural killers (NK cells (CD3-CD16+ CD56+), and TNK cells (CD3+ CD16+ CD56+), T cells (CD3+ CD16 + CD56-)) in peripheral blood; cytotoxic activity of NK, which was assessed by spontaneous and induced expression of CD107a-LAMP on the cell membrane of lymphocytes; production of interferon gamma in the culture of blood lymphocytes by ELISA; and the production of the proinflammatory cytokine TNF-alpha in the culture of blood lymphocytes. At the time of the study, patients did not receive any antiviral therapy.

Keywords: Epstein-Barr virus, natural killer cells, interferon gamma, tumor necrosis factor-alpha, cytotoxic activity of killer cells

1. Introduction

1.1 Epstein-Barr virus

Epstein-Barr virus (EBV) belongs to the family of human γ -herpesviruses, which affects about 90% of the adult population in the world and is the etiological factor of various lymphoproliferative, malignant neoplasms, and autoimmune diseases [1–6]. EBV was first isolated and identified using electron microscopy by Anthony Epstein and Yvonne Bar in a culture of tumor cells brought back from an expedition

to Equatorial Africa (Uganda). The cells were obtained by biopsy from a patient with Burkitt's lymphoma (BL) in 1964 [7]. EBV was registered as the first human onco-genic virus with a high infection rate of the population worldwide [8]. In 1968, it was discovered that the Epstein-Barr virus (EBV) is the cause of infectious mononucleosis (IM) [9]. Later, the World Health Organization recognized EBV as a class I oncogenic virus [10–12].

Primary human infection in infancy and early childhood is often asymptomatic. In childhood, it is not uncommon for children to exchange toys and utensils. The second stage of EBV infection usually happens in adolescence and early adulthood. This is because people come into contact with the virus through kissing and the exchange of saliva [13, 14]. Moreover, the risk of infection is highest in girls. This is because they go through puberty earlier than boys [15]. Thus, EBV spreads rapidly through contact between children who are not infected with EBV and EBV-positive adults or children of the same age.

Primary infection may occur later if the patient has not been in contact with an EBV-infected person in early childhood. About 50% of adolescents and adults may develop acute infectious mononucleosis (IM) [16, 17]. Worldwide, 90% of adults and 50% of children are seropositive for EBV [18]. In low-income countries, most children become infected with EBV in early adolescence [19]. In high-income countries, peak EBV seroconversion occurs in children aged 2–4 years and 14–18 years, with the peak infection rate occurring in late adolescence and declining after age 35 [20]. Thus, almost all children are seropositive by age 6 [21].

After primary infection, the following factors are important:

1. The probability of infection depends on the amount of viral DNA that the patient receives during the first contact (e.g., through saliva when kissing) [22];
2. Proliferation of early differentiated CD56^{dim} NKG2A⁺ immunoglobulin-like receptor (–) NK cells, which carry out immune control during primary infection [23];
3. An increase in the content of cross-reactive memory CD8⁺ T cells in the blood, activated by EBV, which induces the development of lymphoproliferation [24].

After primary infection, EBV persists in the host body throughout its life [25]. During the period of latent persistence, the virus expresses a minimal number of genes, which allows the virus to evade the host's immune response. Acute infection and reactivation of the virus allow viruses to spread and infect the cells of a new host, while the virus has minimal impact on its host. In this struggle between the virus and the host, if the host wins, the virus is eliminated; if the virus wins, the host may die [26]. Hadinoto Vey et al. showed in their work that in the Waldeyer's ring of EBV carriers, the frequency of infected B cells varies from $1/10^7$ to $1/10^4$ [27]. The authors of the work showed that during the day, the virus replicates in $\sim 5 \times 10^3$ cells; that is, on average, 0.51 cells are replicated in each palatine tonsil or in each Waldeyer's ring at any given time. Viral replication is induced by the differentiation of memory B cells into plasma cells [28]. It has been shown that there is no correlation between virus shedding and the level of infected cells. However, EBV carriers continuously shed the virus every 2 minutes, thus renewing the saliva reservoir before the act of swallowing [27].

After primary infection, EBV is characterized by two phases of the life cycle: latent and lytic replication. To enter the cell, the EBV genome produces nine distinct

glycoproteins (GPs). To date, 13 GPs have been discovered, with 12 of them being expressed exclusively during the productive phase of lytic replication [29]. The tropism of each new EBV virion is determined by the GPs of the target cell envelope, which is regulated by interactions with cell receptors that ensure attachment, initiate signaling cascades, or internalize the virus [30].

Epithelial cells are the first to be infected, and after EBV is isolated from oropharyngeal epithelial cells, B cells are infected [29]. B cell infection occurs through the binding of the gp42 glycoprotein to the Major Histocompatibility Complex (MHC) class II Human Leukocyte Antigen (HLA) molecule expressed on B cells [31]. Virions in B cells contain a lot of gH/gL, with the help of which the virus can infect epithelial cells through direct binding to integrins $\alpha V\beta 5$, $\alpha V\beta 6$, or $\alpha V\beta 8$ [32]. Virions formed in epithelial cells contain a lot of gp42/gH/gL and are capable of infecting B cells. It has been suggested that the tropism-switching mechanism ensures the persistence of infection in host cells [33]. In epithelial cells, the virus enters lytic replication with subsequent lifelong latency in circulating memory B lymphocytes, periodically reactivating. Lytic replication increases the pool of latently infected cells [34].

In the latent phase, the virus resides in the cell nucleus as a circular episome. It is attached to the host genome's chromatin through the viral protein EBNA-1. During this phase, the virus produces a limited number of viral proteins and non-coding RNA molecules. The EBV latent phase depends on normal cell division processes and on the host cell DNA replication mechanism, which is important for the passive replication of its viral genome and transmission to daughter cells. During the latent phase, the virus remains latent in the human body throughout life, only occasionally manifesting itself in the form of reactivation and lytic replication. During the reactivation period, the virus enters the lytic replicative cycle, expresses all genes, and produces daughter virions. Then the cell dies and releases viral particles that infect the cells of another host [35].

1.2 Natural killer cells

NK cells are the first defenders of innate immunity, capable of destroying infected cells in the absence of antigen presentation and recognition. Many works have been published worldwide on the regulation of human NK cell maturation, but the exact localization of these cells and the mechanisms of production have not been fully elucidated [36]. Two major populations of peripheral blood NK cells were first identified and described by Lanier, L.L. et al. in 1983 and 1986 based on the differential expression of CD56 and CD16, namely $CD56^{bright}CD16^{lo/-}$ and $CD56^{dim}CD16^{+}$ [37, 38].

NK cells belong to the family of innate lymphoid cells (ILC) [39]. These cells perform the following tasks:

- Secrete perforin and granzyme to eliminate the goal cells;
- Produce cytokines to control immune reactions;
- Bind death-inducing receptors to the goal cells and trigger apoptosis [40].

The functions of NK cells are controlled by the ratio of activating and inhibitory receptors, cytokines and chemokines, and interactions with other immune cells [41].

- Inhibitory receptors include receptors of the Major Histocompatibility Complex (MHC) class I with varying molecular structures and specificities for varied

alleles of the Human Leukocyte Antigen (HLA) class I molecules. They are playing the role of negative regulators of NK cytotoxicity [40, 42]. This group includes killer immunoglobulin-like receptors (KIRs), which recognize polymorphic determinants of HLA-A, -B, or -C molecules, and heterodimeric receptors CD94-NKG2A/B, which identify peptides of various HLA class I molecules [43].

- Activating receptors include the C-lectin family member NKG2D, which includes polypeptide sequences associated with MHC-I, and members of the immunoglobulin superfamily, such as the natural cytotoxicity receptors (NCRs) and the leukocyte adhesion molecule DNAX accessory molecule-1 (DNAM-1, also known as CD226) [43, 44].

The NCR family includes heterogeneous molecules of the Ig superfamily that associate with various polypeptides that signal to immunoreceptors tyrosine-based (ITAM). The NCR group consists of the NKp30, NKp44, and NKp46 receptors. Today it is known that NKp46 [45], NKp30 [46], and NKp44 [47] induce the process of disintegration of cancer cells; NKp30 and NKp46 appear on the membrane of resting and activated NK cells. It is also known that NKp44 expression is limited to activated NK cells [44].

The early stage of NK cell development in humans occurs in the bone marrow, and the later stage of differentiation occurs in secondary lymphoid organs—lymph nodes and tonsils [48, 49]. NK cell receptors are germline encoded. It has been shown that NK cell precursors in adults can be present in the bone marrow [50], spleen [50], thymus [51], intestine [52], secondary lymphoid tissues [53], uterus [54], and liver [55]. However, none of these organs, except bone marrow, are critical for the generation of mature circulating NK cells. That is, NK cell development depends on the microenvironment, which leads to the generation of diverse tissue-specific cell subtypes with unique functions [56].

Currently, 7 (1, 2, 3, 4a, 4b, 5, and 6) stages of NK cell development are considered. They can be identified by the expression of CD16 (killer immunoglobulin receptors - KIR), CD34, CD94, and CD117 (c-Kit) [57]. NK cells are produced by CD34⁺ hematopoietic stem cells (HSCs), which are found in the human bone marrow, thymus, spleen, and liver. The progenitor, which belongs to stage 1, expresses CD34⁺CD45RA⁺CD10⁺. These cells are capable of ex vivo differentiation into NK cells, T cells, and dendritic cells (DC) [48]. Bipotent precursors of T and NK cells have also been described in the thymus [58, 59].

At stage 2, CD34 expression is maintained, and CD117 is expressed in minimal amounts; these NK cells are characterized by the phenotype CD34⁺CD45RA⁺CD38⁺CD10⁺CD7⁺CD123⁻CD127⁻ [60, 61]. At stage 3, cells lose the CD34 marker, but CD117 expression is retained. The stage 3 population is heterogeneous and expresses surface antigens CD7, CD56, CD121A (IL-1R1), CD127, CD336 (NKp44, NCR2), and leukocyte function associated antigen-1 (LFA-1) [62, 63]. During the transition to the 4th stage, CD94 expression on the cell membrane is characteristic, and NK cells are characterized by the CD34^{neg}/CD117^{low}/CD94^{pos}/CD16^{neg} phenotype. In recent years, two separate 4th stages of NK cells have been described: 4a and 4b, which differ in the induced expression of NKp80 [57]. The stage 4a NK cell subpopulation NKp80^{neg}/CD56^{br} is characterized by high expression of the markers NKG2D, NKp30, NKp46, CD94/NKG2A, and CD161 and does not have effector functions. Stage 4b NK cells are characterized by the production of IFN- γ and perforin-dependent cytotoxicity in vitro [64]. The precursors of terminally differentiated and cytotoxic CD56^{dim} NK cells

are stage 4a and 4b NK cells (stage 5). This is followed by terminal maturation, which is characterized by the expression of CD16 and/or KIR and cytotoxic granules, forming a transient population of CD56^{br}/CD16^{pos} NK cells (stages 5 and 6) [65]. During stage 4, NK cells lose expression of CD117, CD127, and CD94/NKG2A receptors and express CD94/NKG2C, inhibiting expression of CD56 [66, 67]. It has been shown that at stage 5, CD56dim/KIRpos NK cells differ from stage 6 NK cells in the expression of the surface marker of replicative senescence CD57, decreased expression of CD56, and NKG2A expression ceases [60, 68]. At stage 6, CD56dim NK cells are highly cytotoxic and proliferate in response to infection, resulting in the expansion of both NK cell subtypes [69]. In the later stages of NK cell development, inhibition or loss of some surface receptors may occur, such as CD56, CD117, NKp46, CD94/NKG2A, TNF family receptor, and CD27. Expression of CD94 on the cell surface is involved in the formation of heterodimeric C-type lectin receptors. Mature NK cells acquire the CD34^{neg}/CD117^{pos/neg}/CD94^{pos}/HLADR^{neg}/CD10^{neg}/CD122^{pos}/CD94^{pos}/NKp44^{low}/NKG2D^{pos}/CD161^{pos} phenotype, which is divided into two final stages of differentiation depending on the expression of CD56 and CD16 [49, 70, 71].

NK cells (CD56 + CD3-) constitute 1–20% of peripheral blood mononuclear cells in humans and are divided into two large subpopulations depending on the level of CD56 and CD16 expression: CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻. NK cells CD56^{dim}CD16⁺ are innate cytotoxic killer cells, and CD56^{bright}CD16⁻ produce cytokines, in particular interferon- γ (IFN- γ), tumor necrosis factor α (TNF- α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [28, 56, 72]. About 90% of NK cells are CD56^{dim}CD16⁺ (“mature” cytotoxic NK cells), present in the bloodstream, bone marrow, spleen, and lungs. This NK cell subpopulation expresses CD16, KIR, and CD94/NKG2C to induce antibody-dependent and natural cytolytic function. Thus, CD56^{bright}CD16⁻ and CD56^{dim}CD16⁺ NK cells perform different functions: CD56^{bright} NK cells perform an immunomodulatory role, and CD56^{dim} NK cells perform a cytotoxic role [73–75].

It has also been shown that CD56^{bright} NK cells specifically respond to the Epstein-Barr virus (EBV) and protect host cells from infection [76, 77]. 10% of NK cells with the CD56^{bright}CD16⁻ phenotype (“immature” immunomodulatory NK cells) can produce IFN- γ under the influence of IL-12 and IL-18 but contain few cytotoxic granules and have low expression of the low-affinity Fc receptor CD16 and KIR. That is, they have weak cytotoxic properties [78] and are present in lymphoid tissues, tonsils, and intestines. IFN- γ production and cytotoxicity are the defining functions of mature NK cells.

NK cells play a crucial role in fighting EBV. They do this by destroying cells that have been transformed by EBV during the acute phase. Additionally, they help to limit the amount (viral load) of EBV in the body. When activated, NK cells employ three primary mechanisms to eliminate virus-infected cells:

- a. Production of cytokines [79, 80];
- b. Secretion of cytolytic granules;
- c. Use of cytotoxicity triggered by death receptors that induce the extrinsic pathway of apoptosis [64, 81].

Death receptors include TNF-associated apoptosis-inducing ligand receptor (TRAIL-R) and Fas (CD95), Fas ligand (CD95L), and TNF-related

apoptosis-inducing ligand (TRAIL), present in NK cells [82, 83]. Death receptors are characterized by the presence of a cytoplasmic death domain, which helps these receptors activate the apoptotic mechanism, including caspases 8 and 10 [83]. At the moment of activation of NK cells, the contents of lytic granules are released, which consist of secretory lysosomes, perforin, and granzymes involved in the cytotoxic function and are delivered to the target cell through membrane fusion in the region of the immunological synapse (IS) [64, 84]. As a result, there is a reorganization of the cytoskeleton [85, 86], and the microtubule organizing center is polarized toward the target cell [87]. The polarized lytic granules, guided by microtubules within the IS, approach the target cell's membrane, merge with it, and release enzymes that initiate the target cell's intrinsic apoptotic process [88]. The inner surface of the granules is coated with lysosomal-associated membrane protein-1 (LAMP-1 or CD107a), a highly glycosylated protein that is expressed on the cell surface following the fusion of lysosomes with the plasma membrane. Degranulation results in cell surface expression of CD107a and depletion of intracellular perforin [89, 90]. All resting NK cells, when receiving signals for degranulation, are capable of expressing surface CD107a and mediating cytotoxicity. CD107a expression is a marker for determining cytotoxic activity. Lytic granules contain glycoprotein, perforin, granzymes (a class of serine proteases), FasL (CD178), TRAIL (CD253), and granulysin [90]. Granzyme B induces apoptosis by caspase-dependent and caspase-independent mechanisms through the release of cytochrome C from mitochondria by proteolytic cleavage of the proapoptotic protein Bid (Pinkoski MJ, (2001)). However, polarization and degranulation cannot completely ensure effective destruction of target cells. Destruction of virus-infected cells by NK cells occurs until the cytolytic granules are depleted [91].

Th1 cytokines (Granulocyte-Monocyte Colony-Stimulating Factor (GM-CSF), TNF, IFN- γ) are produced by NK cells in response to tumor ligands and intracellular pathogens. They participate in the activation of T cells and other mediators of innate immunity [92]. NK cell function is regulated by positive and negative signals provided by various receptors on the cell surface. Cytokines (IL-12, IL-15, IL-18, IL-21, and IFN- $\alpha\beta$) induce NK cell proliferation, enhance NK cell cytotoxicity, and/or IFN- γ production [93]. Classical inducers of IFN- γ production by NK cells and T cells are IL-12 and IL-18 [94]. IL-12 has a pronounced immunomodulatory effect on cells of innate and adaptive immunity. Expression of IL-12 genes coordinates expression of IFN- γ , and expression of IFN- γ genes induces IL-12 production. The effects of IL-12 can be manifested in the increased production of IFN- γ and the transformation of CD4⁺ T cells toward a Th1-type phenotype. Synergy of IL-12 with IL-18 induces IFN- γ production in B cells [95]. IL-15 is involved in the differentiation of NK cells and increases the expression of NKp30 on NK cells, which results in increased NK cell cytotoxicity. IL-15 is the most potent regulator of IFN- γ production in human NK and T cells compared to IL-21. IL-15 also promotes the development of adaptive immunity and is involved in stimulating the maturation of dendritic cells under the influence of NK cells [41]. The synergistic effect of IL-21, IL-18, and IL-15 enhances the production of IFN- γ . When NK cells are stimulated *in vitro* in the presence of IL-21 together with FLT3 and IL-15, differentiation and proliferation of cytotoxic CD56^{dim} NK cells from bone marrow precursors is stimulated [96, 97]. IL-7 plays an important role in the early differentiation of NK cells [98]. By increasing the number of subunits and enhancing proteasome activity, IFN- γ induces the expression of HLA (major histocompatibility complex) class I and II antigens. As a result, the host's sensitivity to an infectious pathogen increases, and the ability to identify and respond to this pathogen increases [99]. Thus, IFN- γ has many important immunostimulatory and immunomodulatory effects.

1.3 The role of NK cells in EBV infection

The role of NK cells in EBV infection was demonstrated in studies of patients with primary immunodeficiency with selective deficiency in NK cell production, who suffer from increased susceptibility to this virus [100, 101].

In 2005, Williams H. et al. published the results of their study on the content of NK cells during primary EBV infection and showed that in the first month after infection, the number of CD56^{bright} cells with increased cytotoxic activity increases. The authors showed that patients with low peripheral blood viral load had a more pronounced expansion of NK cells and concluded that NK cell content controls the clinical outcome of EBV infection. Thus, NK cells play an important role in primary EBV infection, destroying infected B cells, boosting the antigen-specific T cell response, and producing cytokines [102]. Balfour H.H.J. et al. in 2013 conducted a study on a large group of students and showed a correlation between the expansion of NK cells and viral load during acute EBV infection. The authors showed a correlation between clinical complaints of patients and viral load [103].

Interesting data were obtained in the work of Azzi T. and Lünemann A. in 2014. It was shown that in patients with IM, NK cell expansion occurs due to the proliferation of early differentiated CD56^{dim} NKG2A⁺ immunoglobulin-like receptor⁻ NK cells (CD56^{dim} NKG2A⁺ KIR⁻ cells) with migration to secondary lymphoid organs. This population of early differentiated NK cells increases during primary EBV infection and persists in elevated numbers for 6 months. The authors showed that these NK cells specifically identify B cells in the lytic stage of EBV replication, correlate with the number of EBV DNA copies, exhibit degranulation activity, and persist for a long time compared to healthy or EBV-negative children with IM-like diseases. The study showed no difference in the NK cell content in the peripheral blood of EBV seropositive and seronegative control groups. The authors suggested that asymptomatic and acute EBV infections affect NK cells differently [104]. Thus, early differentiated NK cells may play a key role in the immune control of primary infection caused by viral persistence.

Healthy children have a high content of CD56^{dim} NKG2A⁺ KIR⁻ CD57⁻ cells at birth, which gradually decreases during the first 10 years. This process is due to the progression of differentiation of these cells into more mature NK cell subpopulations (due to a decrease in NKG2A expression, an increase in the expression of KIRs, and the maturation marker CD57) [105, 106]. This may be explained by infection with other viruses. Ultimately, the loss of EBV-reactive early differentiated NK cells with age increases the risk of IM in infected individuals. CD56^{bright} NKG2A⁺ and mostly KIR⁻ NK cells were detected in higher numbers in the tonsils of EBV seropositive patients, indicating long-term lytic replication of EBV in the tonsils of asymptomatic EBV carriers [107]. A constant increase in the absolute content of CD56^{dim} NKG2A⁺ KIR⁻ NK in peripheral blood is observed only in the acute period of IM. This is probably due to either a longer survival of these NK cells in the blood or continuous proliferation under the influence of EBV, which occurs in tissues with further expansion in the peripheral blood. After the acute period of IM, the number of EBV-infected B cells in the peripheral blood rapidly decreases [108].

The main innate barrier against EBV infection is tonsil NK cells, which produce higher levels of IFN γ than peripheral blood NK cells, inhibit the proliferation of EBV-infected B cells, and inhibit the expression of latent EBV antigens [109–111]. CD56^{bright} NKG2A⁺ NK cells of the tonsils perform the main function of effector NK cells, which limit the growth of EBV-susceptible B cells in the tonsils 18 hours after

infection. It has been shown that the content of CD56^{bright} NK cells with an immature phenotype (NKG2A + CD94 + CD54 + CD62L⁻) is increased in the tonsil area of EBV-infected patients. These cells produce IFN- γ and inhibit viruses [109–111]. Thus, tonsillar CD56^{bright}NKG2A⁺IFN- γ ^{high} NK cells play an important role at the site of viral entry.

The aim of our study was to investigate the content of NK cells in peripheral blood, the relationship of cells with viral load, clinical complaints of patients, and the production of TNF- α , IFN- γ , and cytotoxic activity in patients with chronic EBV infection.

2. Material and methods

2.1 Patients

The study included 200 patients suffering from chronic Epstein-Barr virus infection (cEBVI). The group consisted of 140 women and 60 men. The average age in the overall group was 37.87 ± 0.78 years (95% CI: 36.35–39.42). The duration of the disease was 3.58 ± 0.19 years (95% CI: 3.21–3.97). From the medical history, it is known that all patients were frequently ill children in childhood, suffered from chronic tonsillitis, and 132 patients (66%) suffered from acute infectious mononucleosis, after which there was a subfebrile temperature, severe weakness, and physical fatigue for a long time. The diagnosis of chronic EBV infection was diagnosed and confirmed by laboratory tests by the relevant specialists. After that, patients were referred for treatment by an immunologist. All patients underwent differential diagnostics of EBV with other viral infections (human immunodeficiency virus, viral hepatitis, cytomegalovirus infection, toxoplasmosis), helminthic invasions, and autoimmune diseases associated with EBV infection. The study did not include patients who received antiviral or immunomodulatory therapy in the last 6 months.

2.2 Examination methods

The clinical examination methods consisted of collecting anamnesis, data on previous therapy (immunotherapy or antiviral), and the presence of concomitant diseases. The clinical condition of patients was assessed using a generally accepted method, consisting of recording complaints and objective data of the patient at the time of contacting an immunologist. To record the severity of patient complaints, a 3-point subjective assessment scale was used (0 – no symptoms, 1 – mild symptoms, 2 – moderate symptoms, 3 – significant symptoms).

2.2.1 Determination of viral DNA by PCR in saliva samples

Viral DNA was determined in saliva samples using polymerase chain reaction (PCR) with real-time hybridization-fluorescence detection. The AmpliSens EBV/CMV/HHV6-screen-FL test systems of the Central Research Institute of Epidemiology (Russia) were used. The unit of measurement of viral load during DNA isolation from saliva was defined as the number of EBV DNA copies in 1 ml of sample (cDNA). According to the instructions, this indicator is calculated using the formula: $\text{cDNA} = \text{cDNA} \times 100$, where cDNA is the number of copies of viral DNA in the sample. The analytical sensitivity of the test system is 400 copies/ml.

2.2.2 Identification of NK cell number

The relative number of NK cells was determined by multicolor flow cytometry by studying the subpopulation composition of peripheral blood lymphocytes. Blood was taken from the cubital vein and transferred to vacutainers with the addition of ethylenediaminetetraacetate. Sample preparation was performed according to the manufacturer's instructions. The following monoclonal antibodies were used: anti-HLADR-FITC, anti-CD4-PE, anti-CD3-ECD, anti-CD56-PC5.5, anti-CD25-PC7, anti-CD8-APC, anti-CD19-APC-AF700, anti-CD45-APC-AF750. VersaLyse was used for erythrocyte lysis. Cell samples were analyzed on a Navios flow cytometer (Beckman Coulter, USA). 5000 events were accumulated in the lymphocytic region CD45 + brightSSdim. NK lymphocytes were identified as CD3-CD56+ CD45 + ^{bright}SSdim. Absolute NK cell counts were calculated based on complete blood count.

2.2.3 Production of tumor necrosis factor-alpha (TNF- α) in the culture environment (spontaneous, induced production) and in blood serum

Heparinized peripheral blood was diluted 5 times with RPMI-1640 nutrient medium with L-glutamine (Biolog, Russia) and then cultured in Petri dishes with or without a cytokine synthesis inducer. The inducer was pyrogenal (*Salmonella typhi* lipopolysaccharide 100 μ g/ml, N.F. Gamaleya Research Center for Epidemiology and Microbiology, Russia). Blood cells were cultured for 24 hours at 37°C in a CO₂ incubator. After incubation, the supernatant was transferred to Eppendorf tubes and frozen for further work. Spontaneous cytokine production was assessed in the supernatant of the blood cell culture without the addition of the inducer, and induced production was assessed with the addition of pyrogenal. The cytokine content in the blood serum and supernatant of the peripheral blood cell culture was determined by enzyme immunoassay using ELISA reagent kits (Vector Best, international certificate ISO 13485) according to the manufacturer's instructions. The sensitivity of the test system to TNF- α did not exceed 1.0 pg./ml.

2.2.4 Identification of production rate (spontaneous, serous, and induced) of IFN γ in the lymphocyte culture

Identification of production rate (spontaneous, serous, and induced) of IFN γ in the lymphocyte culture was conducted by means of solid-phase enzyme multiplied immunoassay (EMI) by the use of test systems gamma-interferon-IFA-BEST (Vector Best, Russia). As an inductor of the production of IFN- γ , we used phytohemagglutinin (FGA-P) ("PanEco," Russia) with a dose of 10 mcg/ml. The quantitative content of cytokines was identified in the serum and supernatant liquid of a 24-hour culture of whole blood via a method of solid-phase enzyme multiplied immunoassay with the use of test systems gamma-interferon-IFA-BEST (Vector Best, Russia). The test systems producer provided reference values of spontaneous, serous, and induced production of IFN- γ .

2.2.5 Cytotoxic activity of killer cells

The cytotoxic activity of killer cells is assessed by spontaneous and induced expression of the degranulation marker CD107a-LAMP (Lysosomal-associated membrane protein). Expression of the marker on the cell membrane of lymphocytes is an

indicator of lysosome degranulation. Expression of the CD107a marker was assessed after co-cultivation of peripheral blood mononuclear cells (PBMCs) with target cells. The K562 cell line (human chronic erythromyelosis) is used as target cells. K562 cells express several ligands (MICA, MICB, ULBP2, and ULBP4) to the NKG2D receptor of cytotoxic lymphocytes. Interaction of NKG2D with ligands results in the degranulation of lysosomes of NK cells, NK T cells, and lymphokine-activated CD8+ T cells with the expression of CD107a marker on their membranes. That is, the test allows us to evaluate the ability of killer cells to participate in NKG2D-dependent cytolysis of killer cells. Peripheral blood taken for the study is transferred to vacutainers with lithium heparin as an anticoagulant. During sample processing, suspension of peripheral blood mononuclear cells is separated on a density gradient with subsequent washing. Then PBMC and K562 are co-cultivated in a ratio of 10:1 under CO₂ incubator conditions for 20 hours in the presence of monoclonal antibodies and anti-CD3-FITC/CD(CD16 + 56)-PE and anti-CD45PC5 (Beckman Coulter). To evaluate spontaneous cytotoxic activity, RPMI nutrient medium (Biolot) was added to the peripheral blood mononuclear cell suspension in the appropriate volume instead of K562. The samples were analyzed using a Navios flow cytometer (Beckman Coulter). Accumulation was performed up to 1000 events in the minimal cell population (NK- or NK-T). The lymphocyte population was identified as CD45 + ^{bright}SSdim cells. The relative number of cells expressing the degranulation marker CD107a (CD107a+) on the membrane of the NK, NK-T, and T-lymphocyte subpopulations was estimated. The stimulation index was calculated as the ratio of the induced expression of the CD107a marker to the spontaneous expression.

2.3 Statistics

For statistical processing of the obtained data, the IBM SPSS Statistics software package, version 26 (Armonk, NY: IBM Corp.), was used. Group results are presented as the arithmetic mean $M \pm$ standard error. Parametric (Pearson's method) and non-parametric (Spearman's method, Kendall's tau (τ)) methods were used in the work. Compliance with the condition of observation independence was also checked. For this purpose, linear regression analysis (with calculation of the determination coefficient (R-square) and the Durban-Watson criterion) and variance analysis (ANOVA Analysis of Variance) with calculation of the Fisher criterion (F) for checking the significance of the model were used. The standardized β indicator was calculated with 95% confidence intervals. The critical level of significance of the difference in indicators was equal to 0.05.

3. Results

A total of 200 patients were examined. All of them underwent a study of the number of EBV DNA copies using the PCR method in saliva samples, which was 253390.73 ± 36821.68 copies/ml (95% CI: 192030.45–332918.08). When conducting a correlation analysis of the relationship between the number of copies and the patient's age and the duration of the disease, statistically significant correlations were not obtained. Therefore, in order to assess the dependence of the number of EBV DNA copies on the patient's age, an analysis was performed using the linear regression method, which showed that the level of DNA copies depends on the patient's age - $R^2 = 0.271$, $F = 71.162$, $\beta = 0.521$, $p = 0.000$ (**Figure 1**).

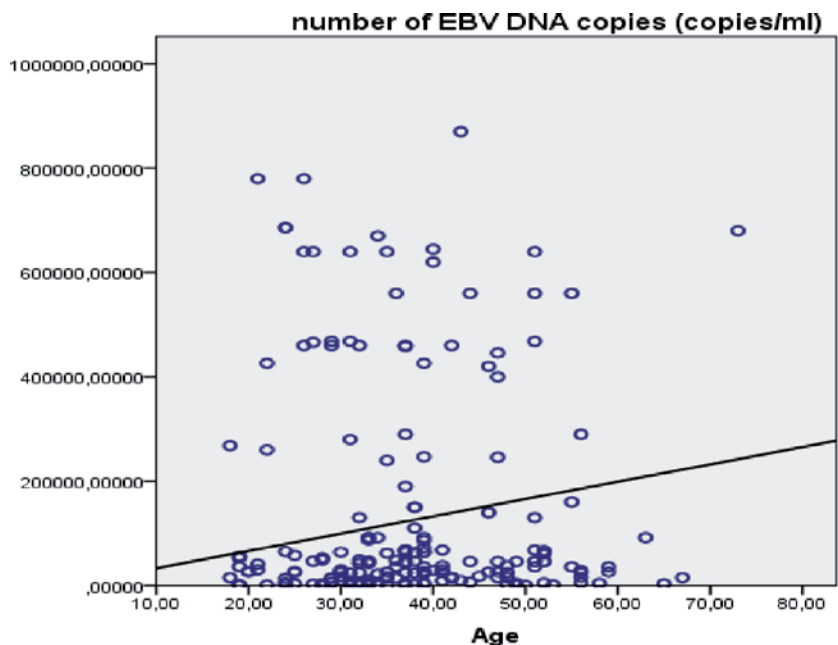


Figure 1.
Regression model of the relationship between patient age and the number of EBV DNA copies in a saliva sample (—linear regression).

When analyzing the number of EBV DNA copies with the duration of the disease, a weak but significant linear regression was obtained ($R^2 = 0.219$, $F = 30.313$, $\beta = 0.468$, $p = 0.000$).

3.1 Relationship between the number of EBV DNA copies and clinical complaints

The next step was to analyze the relationship between the number of EBV DNA copies in saliva samples and the severity of clinical complaints in patients. For this purpose, a correlation analysis was performed. The prognostic significance of the amount of EBV DNA in saliva samples was assessed using linear regression analysis

Clinical complaints	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
Fever	0.310	42.253	0.557	0.0001
Chills	0.367	54.559	0.606	0.0001
Headaches	0.196	22.895	0.443	0.0001
Weakness	0.327	45.726	0.572	0.0001
Postnasal drip	0.233	28.537	0.483	0.0001
Sore throat	0.290	38.310	0.538	0.0001
Lymphadenitis	0.220	26.563	0.469	0.0001

Table 1.
Regression models of the significance of clinical complaints from the number of EBV DNA copies in patients with chronic EBV infection.

with the calculation of the coefficients of determination R^2 (R-square) and the Durbin-Watson criterion and ANOVA (analysis of variance) using the F criterion, as well as with the calculation of the standardized beta coefficient (β) with a 95% confidence interval. The results are presented in **Table 1**.

3.2 Clinical complaints

Long-term with frequent relapses, not only clinical but also laboratory signs of viral activity—this is the chronic form of EBV infection, and this is described in detail in the literature [112–117]. Patients complain of prolonged subfebrile temperature (37.1–37.3°C), weakness, and unmotivated fatigue. They are also bothered by increased sweating (especially at night), constant discomfort and/or sore throat, lymphadenitis, swelling of the nasal mucosa with abundant mucous discharge, stomatitis, and possibly conjunctivitis. Some patients experience cough, skin rash, and pain in the joints, muscles of the trunk, and limbs. Neurological disorders develop headaches, memory and sleep disorders, decreased concentration, irritability, tearfulness, and a tendency to depression. Patients also complain of frequent colds and other herpesvirus infections. Such patients often have long-term stressful situations, and psycho-emotional and physical overloads in their medical history, which worsens the condition of the patients. The patients were examined by a neurologist, consulted psychiatrists, and attended psychotherapy sessions. As prescribed by psychiatrists, the patients received antidepressant therapy. It is characteristic that the occurrence of the described complaints was aggravated by long-term stressful situations, and psycho-emotional, and physical overloads. We analyzed the frequency of occurrence of the described complaints in the general group of patients (**Table 2**).

Clinical complaints	Frequency of clinical complaints, % (n = 200)
Subfebrile temperature	80.00
Lymphadenitis	55.00
Sore throat	80.00
Weakness	74.00
Physical fatigue	74.00
Chills	60.00
Sweating	80.00
Postnasal drip	55.00
Stomatitis	54.00
Arthralgia	45.00
Irritability and tearfulness	60.00
Headaches	40.00
Decreased concentration	47.50
Decreased memory	45.00
Sleep disturbances	54.00
Daytime sleepiness	40.00

Table 2.
Frequency of clinical complaints (%) in patients with chronic EBV infection in the general group.

3.3 Content of NK cells in peripheral blood

Next, a study was conducted on the content of NK cells in peripheral blood (Table 3 and graphically in Figure 2).

NK cell subpopulations in blood	NK cell content in blood (%)	Reference values
NK cells (CD3-CD16 + CD56+)	11.10 ± 0.43 (95% CI: 10.31–12.00)	8–16
TNK-cells (CD3 + CD16 + CD56+)	6.92 ± 0.31 (95% CI: 6.34–7.59)	1.5–8.0
T cells (CD3 + CD16 + CD56-)	1.60 ± 0.07 (95% CI: 1.45–1.75)	0.1–0.8

Table 3.
NK cell content (%) in the blood of patients with chronic EBV infection.

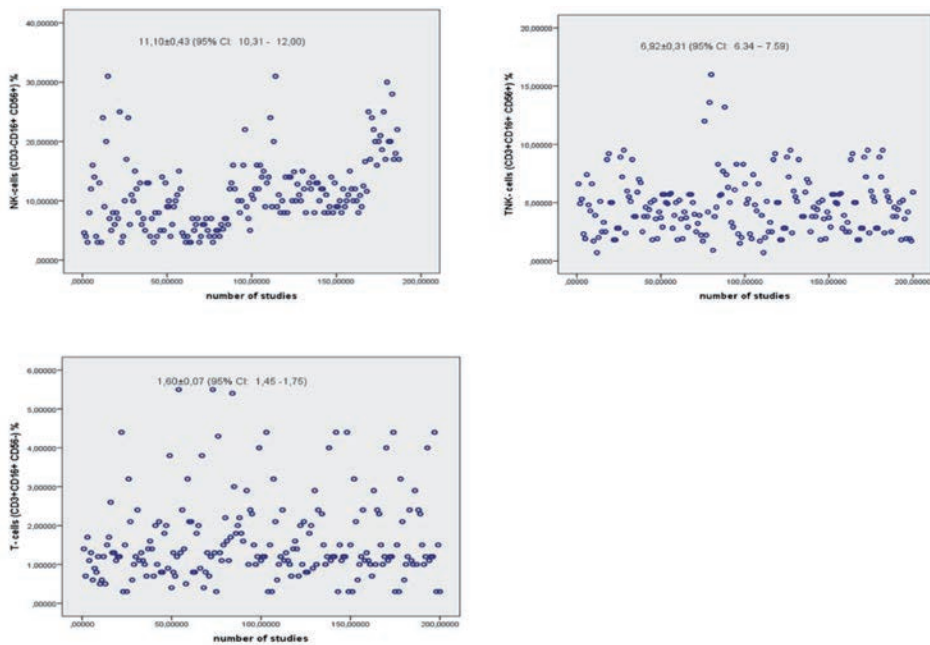


Figure 2.
Content of NK cells (%) in the blood of patients with chronic EBV infection.

3.4 Prognostic significance of NK cell content in peripheral blood

Next, a regression analysis was performed using the linear and exponential regression method of the dependence of the NK cell content in the blood on the duration of the disease. The results obtained are presented in Table 4 and Figure 3.

Next, a regression analysis was performed on the relationship between the content of NK cells in the blood and the age of patients (Table 5, Figure 4).

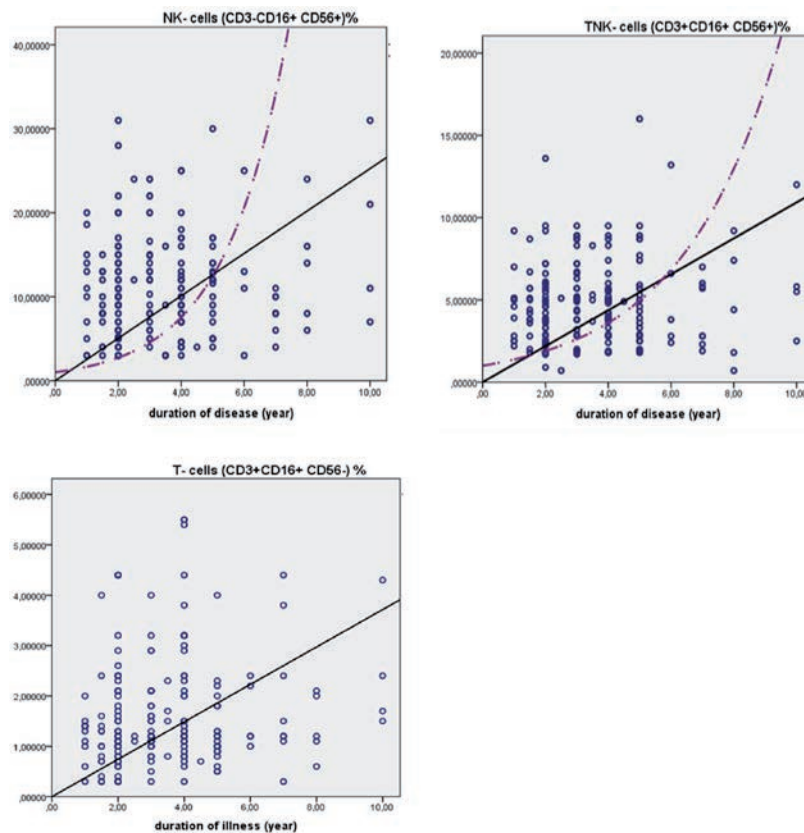
The obtained results of the regression analysis of the relationship between the content of NK cells in the blood and the number of EBV DNA copies in saliva samples are presented in Table 6.

From the table data, it is clear that the resulting regression models are quite weak and not very informative, despite the statistical reliability of these models. The next

NK cell subpopulations in blood	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
NK cells (CD3-D16 + CD56+)	Linear	0.647	186.649	0.804	0.0001
	Exponential	0.692	229.343	0.832	0.0001
TNK cells (CD3 + CD16 + D56+)	Linear	0.648	193.046	0.805	0.0001
	Exponential	0.744	305.138	0.863	0.0001
T cells (CD3 + CD16 + CD56-)	Linear	0.586	134.337	0.765	0.0001
	Exponential	0.112	13.164	0.349	0.0001

Table 4.

Regression models of significance between the content of NK cells in the blood and the duration of the disease in patients with chronic EBVI.

**Figure 3.**

Graphical representation of regression models of the dependence of NK cell content on the duration of the disease in patients with chronic EBV infection (—, exponential regression; —, linear regression).

step was to perform a regression analysis of the relationship between the content of each subpopulation of NK cells and the clinical complaints of patients. The results are presented in **Tables 7–9**.

From the data presented in the table, it follows that the content of NK cells (CD3-CD16+ CD56+) in the blood plays a role in the development of clinical complaints in patients with chronic EBV infection.

NK cell subpopulations in blood	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
NK cells (CD3-D16 + CD56+)	Linear	0.726	488.652	0.852	0.0001
	Exponential	0.804	752.555	0.896	0.0001
TNK cells (CD3 + CD16 + D56+)	Linear	0.682	402,740	0.826	0.0001
	Exponential	0.864	1197.679	0.930	0.0001
T cells (CD3 + CD16 + CD56-)	Linear	0.671	365.545	0.819	0.0001

Table 5.
Regression models of significance between the content of NK cells in the blood and age in patients with chronic EBV infection.

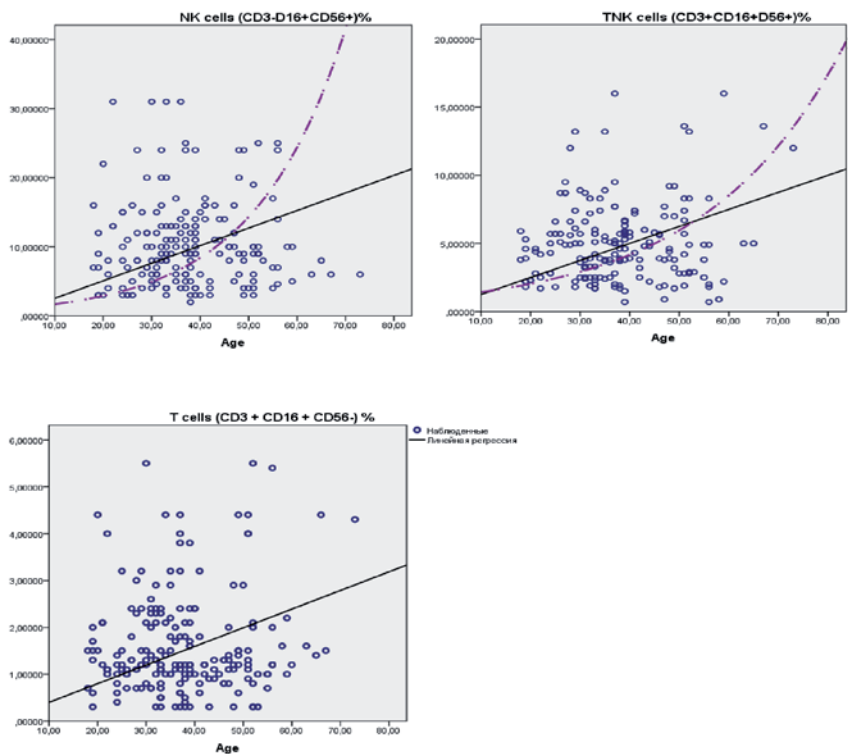


Figure 4.
Graphical representation of regression models of the dependence of NK cell content on the age of patients with chronic EBV infection (— exponential regression; — linear regression).

The data presented in **Table 9** show that only linear regression models of the influence of T cells (CD3 + CD16 + CD56-) content in the blood on the development of clinical complaints in patients were obtained. No reliable exponential regressions were found.

3.5 TNF- α production in the culture medium

Next, a study was conducted on the spontaneous and induced production of the proinflammatory cytokine TNF- α in the culture medium and in the blood serum (**Table 10, Figure 5**).

NK cell subpopulations in blood	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
NK cells (CD3-D16 + CD56+)	Linear	0.189	35.353	0.435	0.0001
	Exponential	0.256	54.962	0.506	0.0001
TNK cells (CD3 + CD16 + D56+)	Linear	0.176	33.551	0.402	0.0001
	Exponential	0.206	40.786	0.452	0.0001
T cells (CD3 + CD16 + CD56-)	Linear	0.228	25.463	0.478	0.0001
	Exponential	0.061	5.583	0.248	0.020

Table 6.

Regression models of significance between the content of NK cells in the blood and the number of EBV DNA copies in saliva samples in patients with chronic EBV infection.

Clinical complaints	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	P
Sore throat	Linear	0.581	245.309	0.762	0.0001
	Exponential	0.719	453.573	0.848	0.0001
Fever	Linear	0.536	213.527	0.732	0.0001
	Exponential	0.829	406.212	0.829	0.0001
Postnasal drip	Linear	0.576	240.613	0.759	0.0001
	Exponential	0.695	403.791	0.834	0.0001
Lymphadenitis	Linear	0.497	175.686	0.705	0.0001
	Exponential	0.601	268.431	0.775	0.0001
Irritability	Linear	0.585	249.489	0.765	0.0001
	Exponential	0.653	333.554	0.808	0.0001
Chills	Linear	0.501	177.658	0.708	0.0001
	Exponential	0.585	249.977	0.765	0.0001
Sleep disturbances	Linear	0.537	205.000	0.733	0.0001
	Exponential	0.620	288.731	0.787	0.0001
Weakness	Linear	0.714	441.001	0.845	0.0001
	Exponential	0.858	1072.305	0.926	0.0001

Table 7.

Regression models of significance between the content of NK cells (CD3 + CD16 + CD56+) and clinical complaints in patients with chronic EBV infection.

As can be seen from the data in the table, the level of TNF- α production in patients is within the reference values.

3.6 Prognostic significance of the level of induced TNF- α

To determine the prognostic significance of the level of induced TNF- α in patients, an analysis was performed using the linear and exponential regression methods. A reliable regression model of the dependence of the level of induced TNF- α on the duration of the disease was obtained (**Figure 6**):

linear $R^2 = 0.451$, $F = 155.147$, $\beta = 0.671$, $p = 0.0001$.

exponential $R^2 = 0.750$, $F = 567.927$, $\beta = 0.866$, $p = 0.0001$.

Clinical complaints	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
Sore throat	Linear	0.558	239.489	0.747	0.0001
	Exponential	0.628	321.350	0.793	0.0001
Fever	Linear	0.596	293.956	0.772	0.0001
	Exponential	0.661	388.564	0.813	0.0001
Postnasal drip	Linear	0.550	230.896	0.742	0.0001
	Exponential	0.609	294.643	0.781	0.0001
Lymphadenitis	Linear	0.474	171.888	0.688	0.0001
	Exponential	0.532	217.333	0.730	0.0001
Irritability	Linear	0.522	206.451	0.723	0.0001
	Exponential	0.569	249.137	0.754	0.0001
Chills	Linear	0.424	139.088	0.651	0.0001
	Exponential	0.487	179.094	0.698	0.0001
Sleep disturbances	Linear	0.537	217.799	0.732	0.0001
	Exponential	0.585	263.063	0.763	0.0001
Weakness	Linear	0.690	420.146	0.830	0.0001
	Exponential	0.769	630.283	0.877	0.0001

Table 8.

Regression models of significance between the content of TNK cells ($CD3 + CD16 + CD56+$) and clinical complaints in patients with chronic EBV infection.

Clinical complaints	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
Sore throat	Linear	0.524	209.210	0.724	0.0001
Fever	Linear	0.484	185.391	0.695	0.0001
Postnasal drip	Linear	0.511	197.589	0.715	0.0001
Irritability	Linear	0.427	140.686	0.653	0.0001
Weakness	Linear	0.592	274.425	0.770	0.0001

Table 9.

Regression models of significance between the content of T cells ($CD3 + CD16 + CD56-$) in the blood and clinical complaints in patients with chronic EBV infection.

Conducting an analysis using the linear and exponential regression method showed the presence of the following regression models of the dependence of the level of induced TNF- α and clinical complaints (**Table 11**).

The data in the table show that the level of induced TNF- α production affects the severity of the clinical picture of the disease in patients.

The regression analysis of the dependence of the level of induced TNF- α and the number of EBV DNA copies in saliva samples are presented below:

- linear $R^2 = 0.268$, $F = 39.905$, $\beta = 0.518$, $p = 0.0001$
- exponential $R^2 = 0.296$, $F = 45.914$, $\beta = 0.544$, $p = 0.0001$

Next, a regression analysis of the relationship between the level of induced TNF- α production and the content of NK cells was performed (**Table 12**, **Figure 7**):

Parameter	TNF- α level (pg/ml)	Reference values (pg/ml)
Serum level	4.75 $\pm$ 0.86 95% CI: 3.31–6.58	0–6
Spontaneous level	7.37 $\pm$ 0.71 95% CI: 6.09–8.89	7–30
Induced level	2932.46 $\pm$ 140.60 95% CI: 2671.53–3212.71	2810–5700

Table 10.
TNF- α production in the culture medium in patients with chronic EBV infection.

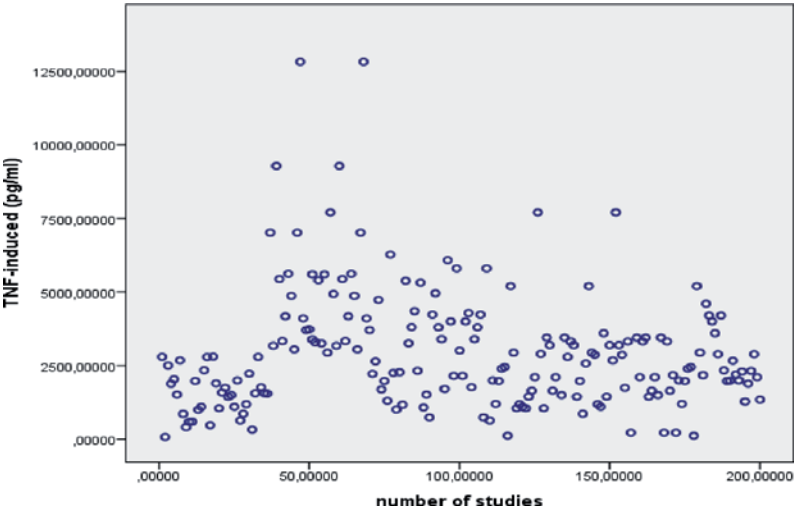


Figure 5.
Production of induced TNF- α in patients with chronic EBV infection.

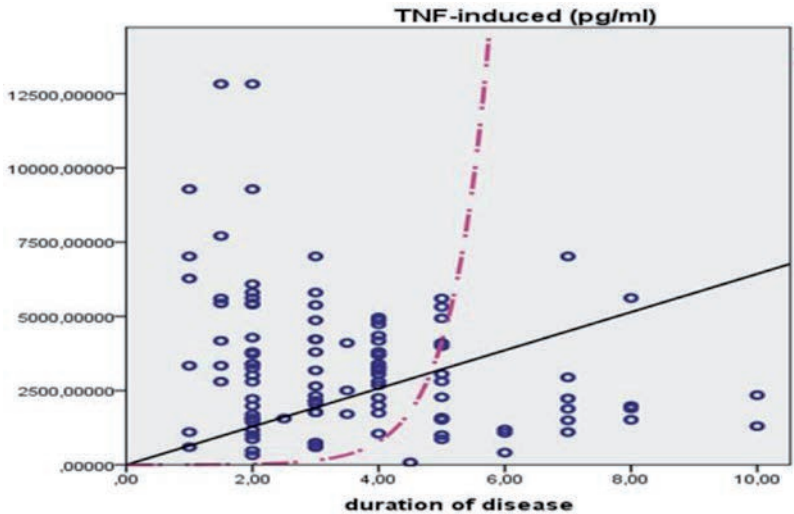


Figure 6.
Dependence of the level of induced TNF- α production on the duration of chronic EBV infection (— exponential regression; — linear regression).

Clinical complaints	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
Sore throat	Linear	0.500	188.699	0.707	0.0001
	Exponential	0.732	517.537	0.856	0.0001
Fever	Linear	0.541	233.326	0.735	0.0001
	Exponential	0.750	593.870	0.866	0.0001
Lymphadenitis	Linear	0.413	133.418	0.642	0.0001
	Exponential	0.614	301.927	0.783	0.0001
Postnasal drip	Linear	0.428	140.385	0.654	0.0001
	Exponential	0.709	457.268	0.842	0.0001
Weakness	Linear	0.594	274.983	0.771	0.0001
	Exponential	0.886	1466.910	0.941	0.0001
Irritability	Linear	0.454	156.285	0.674	0.0001
	Exponential	0.664	371.270	0.815	0.0001
Chills	Linear	0.389	119.876	0.624	0.0001
	Exponential	0.602	284.733	0.776	0.0001
Sleep disturbances	Linear	0.389	119.876	0.624	0.0001
	Exponential	0.602	284.733	0.776	0.0001

Table 11.
Regression models of the dependence of the level of production of induced TNF- α on clinical complaints in patients with chronic EBV infection.

Lymphocyte subpopulation	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
NK cells (CD3-CD16+ D56+)	Linear	0.308	72.980	0.555	0.0001
	Exponential	0.419	118.140	0.647	0.0001
TNK cells (CD3 + CD16 + CD56+)	Linear	0.398	116.181	0.631	0.0001
	Exponential	0.436	135.923	0.660	0.0001
T cells (CD3 + CD16 + CD56-)	Linear	0.500	197.032	0.707	0.0001
	Exponential	0.123	27.554	0.350	0.0001

Table 12.
Regression models of the dependence of the level of induced TNF- α on the content of NK cells in patients with chronic EBV infection.

3.7 INF γ production

The results of the interferon- γ level (serum, spontaneous, induced) are presented in **Table 13**, **Figure 8**.

Parameter	INF γ level (pg/ml)	Reference values (pg/ml)
Serum level	5.66 $\pm$ 1.24 95% CI: 3.62–8.51	0–6
Spontaneous level	8.53 $\pm$ 1.30 95% CI: 6.32–11.37	0–10
Induced level	1976.35 $\pm$ 161.34 95% CI: 1675.99–2307.15	281–4335

Table 13.
Content of induced INF γ in the culture medium in patients with chronic EBV infection.

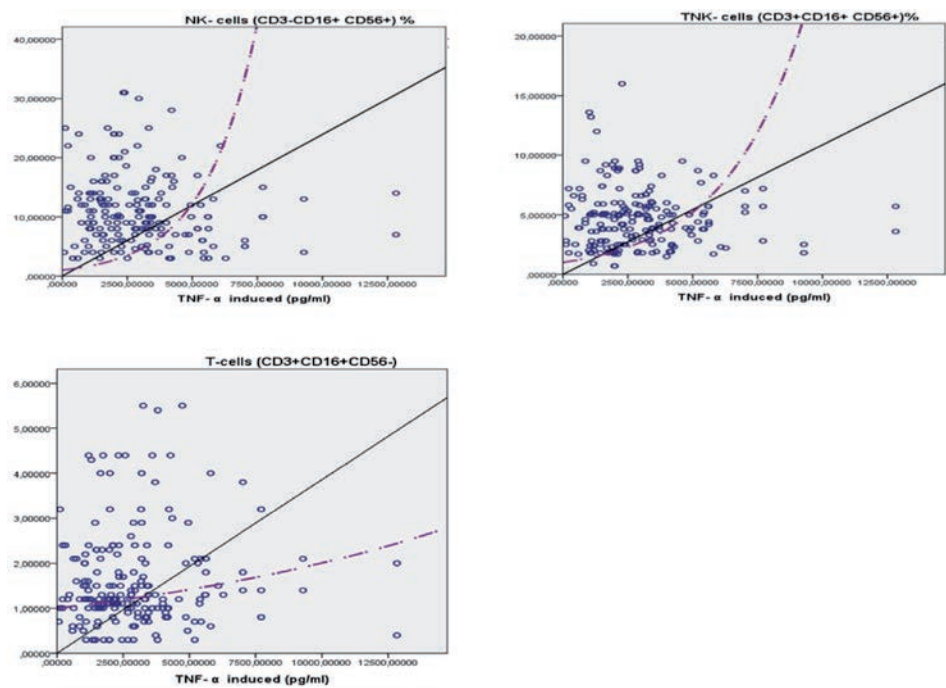


Figure 7. Regression model of the relationship between TNF- α production and the content of NK cells in the blood (—, exponential regression; - - -, linear regression).

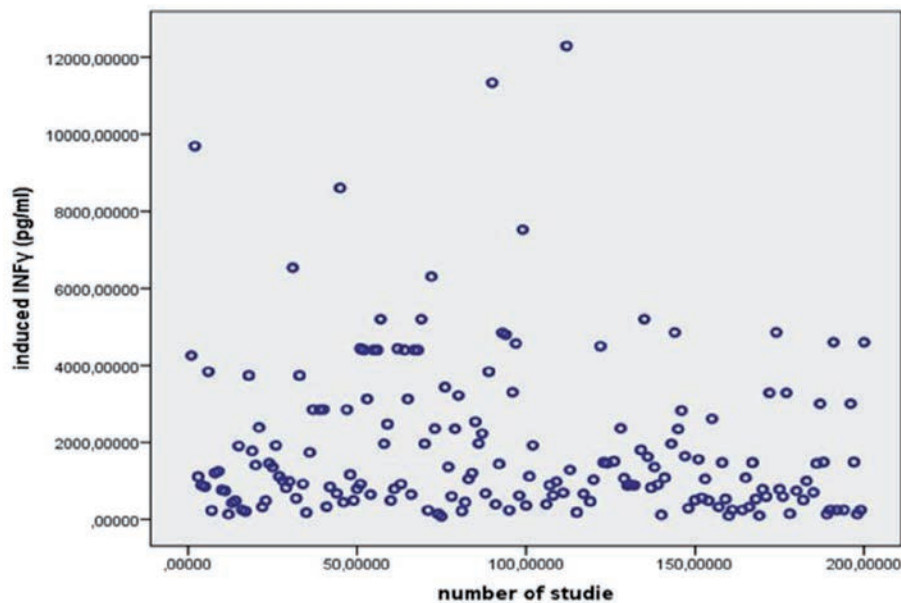


Figure 8. Level of induced INF γ production in culture medium in patients with chronic EBV infection.

3.8 Prognostic significance of the level of induced $\text{INF}\gamma$

To determine the prognostic significance of the level of induced $\text{INF}\gamma$ in patients, an analysis was performed using the linear and exponential regression methods. A reliable regression model of the dependence of the level of induced $\text{TNF-}\alpha$ on the duration of the disease was obtained (**Figure 9**):

- linear $R^2 = 0.451$, $F = 155.147$, $\beta = 0.671$, $p = 0.0001$
- exponential $R^2 = 0.750$, $F = 567.927$, $\beta = 0.866$, $p = 0.0001$

Regression analysis of the dependence of the level of induced $\text{INF}\gamma$ and the number of EBV DNA copies in saliva samples are presented below:

- linear $R^2 = 0.277$, $F = 37.472$, $\beta = 0.526$, $p = 0.0001$
- exponential $R^2 = 0.338$, $F = 49.995$, $\beta = 0.581$, $p = 0.0001$

Regression models of the dependence of the level of induced $\text{INF}\gamma$ on clinical complaints in patients with EBV were also identified. The results are presented below (**Table 14**).

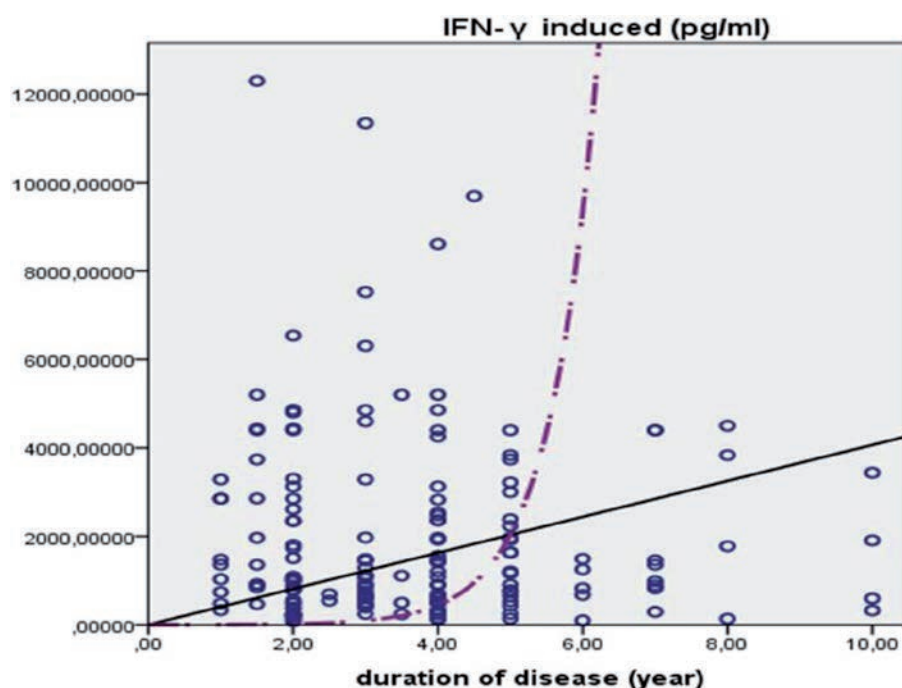


Figure 9.
Dependence of induced $\text{INF}\gamma$ production on the duration of the disease (--- exponential regression; — linear regression).

Clinical complaints	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
Sore throat	Linear	0.404	109.076	0.636	0.0001
	Exponential	0.752	505.557	0.867	0.0001
Lymphadenitis	Linear	0.285	63.064	0.529	0.0001
	Exponential	0.607	260.800	0.779	0.0001
Fever	Linear	0.327	82.76	0.577	0.0001
	Exponential	0.752	511.538	0.867	0.0001
Weakness	Linear	0.420	120.203	0.648	0.0001
	Exponential	0.827	1130.005	0.934	0.0001
Irritability	Linear	0.306	73.230	0.553	0.0001
	Exponential	0.641	296.202	0.801	0.0001
Sleep disturbances	Linear	0.302	71.981	0.550	0.0001
	Exponential	0.618	268.276	0.786	0.0001

Table 14.

Regression models of the significance of the level of induced $INF\gamma$ on clinical complaints in patients with chronic EBV infection.

Lymphocyte subpopulation	Equation	Determination coefficient (R^2)	Fisher criterion (F)	Standardized coefficient (β)	p
NK cells (CD3-CD16+ D56+)	Linear	0.328	40.942	0.572	0.0001
	Exponential	0.719	214.581	0.848	0.0001
TNK cells (CD3 + CD16 + CD56+)	Linear	0.448	67.669	0.668	0.0001
	Exponential	0.767	276.671	0.876	0.0001
T cells (CD3 + CD16 + CD56-)	Linear	0.347	44.706	0.589	0.0001
	Exponential	0.676	175.250	0.822	0.0001

Table 15.

Regression models of the dependence of the level of induced $INF\gamma$ on the content of NK cells in patients with chronic EBV infection.

Next, a regression analysis of the relationship between the level of induced $INF\gamma$ production and the content of NK cells was performed (**Table 15, Figure 10**).

3.9 Dynamics of cytotoxic activity of NK cells

Next, the dynamics of cytotoxic activity of NK cells were assessed based on the expression of the degranulation marker CD107a on the cell membrane in patients (**Table 16**).

The data in the table show that the values of cytotoxic activity in patients with chronic EBV infection are within the reference values. Correlation analysis of the relationship between the number of EBV DNA copies, the content of NK cells, and the stimulated expression of the degranulation marker of cytotoxic granules on the membrane of CD107a cells did not reveal any positively significant relationships. Regression analysis also did not provide significant linear and exponential models of the relationship between the number of EBV DNA copies, the content of NK cell subpopulations in the peripheral blood, and stimulated expression of the degranulation marker CD107a.

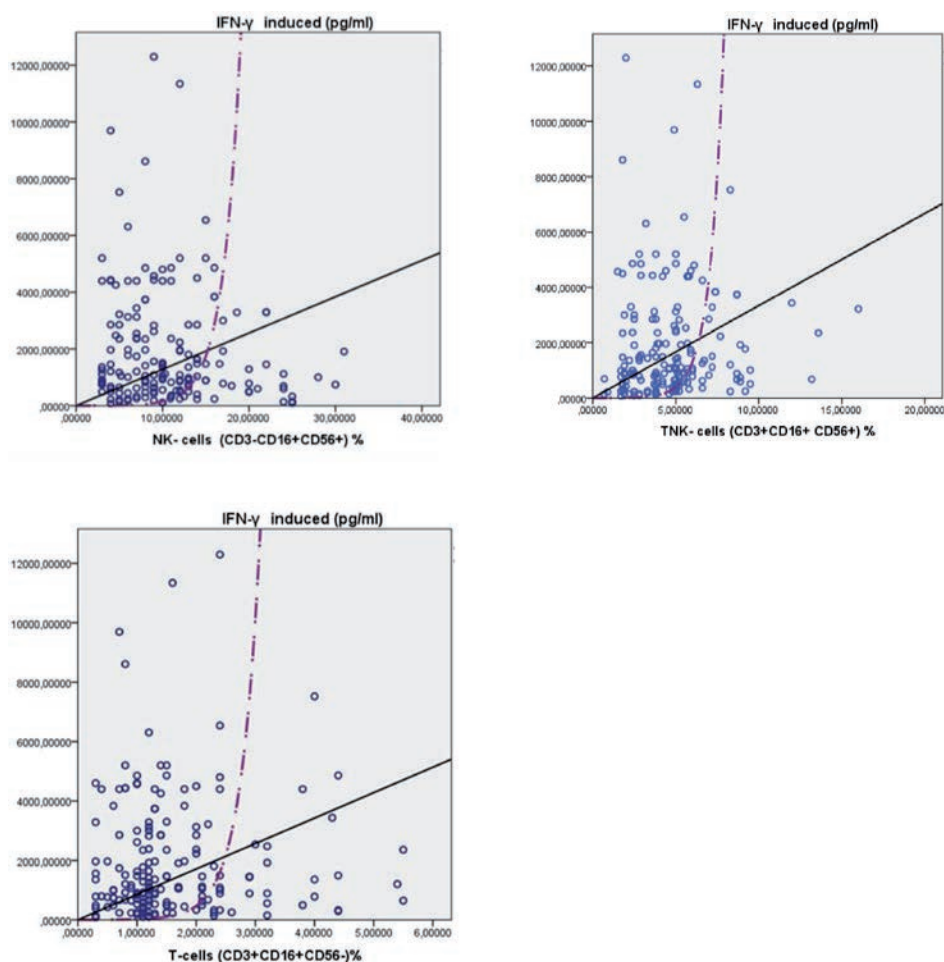


Figure 10.
 Regression model of the dependence of the level of induced $\text{INF}\gamma$ in the blood on NK cells in patients with chronic EBV infection (— exponential regression; — linear regression).

4. Discussion

In our work, we tried to analyze subpopulations of NK cells in peripheral blood, their ability to produce $\text{INF}\gamma$ and $\text{TNF-}\alpha$, and cytotoxic activity in patients suffering from long-term chronic Epstein-Barr viral infection. Analysis of the content of NK cell subpopulations (NK cells (CD3-CD16+ CD56+); TNK cells (CD3 + CD16+ CD56+); T cells (CD3 + CD16 + CD56-)) in the peripheral blood of patients with EBV infection did not reveal changes in the number of these cells. However, the content of each individual NK cell population reliably depended on the patient's age and the duration of the disease, which was shown by the regression analysis method. This coincides with and confirms previously published data on the correlation dependence of the NK cell content on the patient's age, which is explained by the age-related depletion of EBV-reactive early differentiated NK cells.

At birth, the content of CD56dimNKG2A + KIR-CD57- cells in humans is maximal; as humans grow older, during the first 10 years, the content of this population in

Expression of degranulation marker CD107a		Reference values
Expression of cytotoxic granule degranulation marker CD107a on NK cells (CD3-CD16 + CD56+)		
Spontaneous	2.91 ± 0.29 95% CI: 2.39–3.49	0.9–3.3
Stimulated	19.01 ± 0.91 95% CI: 17.15–20.91	11.0–24.0
Stimulation index	11.95 ± 0.79 95% CI: 10.45–13.44	5.5–17.0
Expression of cytotoxic granule degranulation marker CD107a on TNK cells (CD3 + CD16 + CD56+)		
Spontaneous	1.47 ± 0.13 95% CI: 1.20–1.74	0.4–1.6
Stimulated	2.60 ± 0.18 95% CI: 2.24–2.99	0.5–3.0
Stimulation index	2.69 ± 0.21 95% CI: 2.27–3.13	1.0–2.5
Expression of cytotoxic granule degranulation marker CD107a on T cells (CD3 + CD16 + CD56-)		
Spontaneous	0.31 ± 0.02 95% CI: 0.26–0.35	0.1–0.4
Stimulated	0.38 ± 0.02 95% CI: 0.31–0.40	0.1–0.4
Stimulation index	1.09 ± 0.04 95% CI: 1.01–1.17	≤ 1.0

Table 16.
Cytotoxic activity of NK cells in patients with chronic EBV infection.

the blood constantly decreases due to the constant differentiation of cells into mature NK cell subpopulations [104]. The process of maturation is accompanied by constant interaction of humans with various infections, which results in a decrease in NKG2A expression, the acquisition of KIRs, and the maturation marker CD57 on the cell membrane [105, 106, 118].

It is known from the literature that when a person is infected, EBV induces the expansion of certain NK cell subpopulations, which persist and circulate in the peripheral blood for several months after the primary infection. A decrease in the NK cell population leads to uncontrolled replicative activity of the virus [117]. NK cells control EBV infection by killing infected cells and by suppressing T cell hyperactivation. In vitro cocultures were used to study the phenotype and function of NK cells that identify EBV [119]. The authors showed that the compartment of NK cells that identify and eliminate EBV-infected cells has the phenotype CD56^{dim}CD16⁻NKG2A⁺2B4⁺NKG2D⁺NKG2C⁻CD57⁻ cells [104]. That is, this NK cell phenotype plays a major role in the immune control of primary EBV infection. A decrease in the content of immature CD56bright NK cells in the blood has been shown in the acute and latent stages of EBV infection. This indicates the differentiation of this NK cell phenotype into early differentiated NK cells [120].

In our work, when conducting a correlation analysis of the content of each subpopulation of NK cells in peripheral blood and the number of EBV DNA copies in saliva samples, no statistically significant relationships were obtained. Regression analysis showed

the presence of weak linear and exponential regressions indicating the relationship of each NK cell subpopulation with the number of EBV DNA copies in the saliva sample. However, the values of the regression models, despite statistical reliability, are uninformative. This is not consistent with the results published by other researchers on the relationship between NK cells in the blood and the number of EBV DNA copies in peripheral blood mononuclear cells [121, 122]. Probably, the data we obtained can be explained by the long period of illness in patients (more than 3 years), as well as the absence of reactivation of viral particles directly in the mononuclear cells of the peripheral blood.

Interesting data were obtained by analyzing the content of the NK cell compartment with the severity of clinical complaints of patients. The study found the highest linear and exponential regression values between the content of subpopulations of NK cells (CD3 + CD16 + CD56+) and TNK cells (CD3 + CD16 + CD56+) and clinical complaints in patients. The content of T cells (CD3 + CD16 + CD56-) showed only the presence of linear regression with clinical complaints of patients. That is, NK cells (CD3 + CD16 + CD56+) and TNK cells (CD3 + CD16 + CD56+) have the greatest significance in the development of clinical complaints. Based on this, we can suppose that NK cells play an important role in controlling not only primary infection but also the reactivation of the virus by destroying EBV-infected cells. Our results confirm the earlier suggestion made by Williams Hilary et al. in their work published in 2005 [121]. The study authors found a significant inverse correlation (Pearson correlation coefficient (r) was -0.87 ($p < 0.001$)) between elevated viral load and the percentage of NK cells in peripheral blood. The high EBV DNA copy number reflects the large number of latently infected cells present in the circulating B cell subpopulation.

In the work of Abbott Rachel J et al., no correlation was found between the number of EBV DNA copies in a saliva sample and clinical complaints [123]. We also did not find any correlations between these parameters. However, regression analysis demonstrated the presence of linear and exponential regression between the number of EBV DNA copies in the saliva sample and the following clinical complaints: fever, chills, headache, postnasal drip, weakness, sore throat, and lymphadenitis. Later, Balfour, H.H.J., and co-authors in their work showed the presence of a correlation between the expansion of NK cells in the blood and the viral load; that is, the severity of clinical complaints is due to the activation and expansion of NK cells [122].

In our study, the level of TNF- α production in patients was found to depend on the duration of the disease and the number of EBV DNA copies in saliva samples. It is known that the effect of TNF- α inhibition is due to the influence of EBV at the transcriptional level [124, 125]. Selective regulation of cytokine production may promote viral reactivation in the host organism [125]. TNF- α is the main proinflammatory cytokine that is involved in the development of clinical complaints in the patient. In experimental conditions on rats, it has been shown that local or systemic administration of TNF- α leads to the appearance of a whole spectrum of behavioral signs of the disease in animals, depending on the dose and time of administration [126]. Robert Dantzer et al. in their work observed that rats lose interest in their physical and social environment. They also reported a decrease in motor activity, social isolation, reduced food and water intake, an increase in the duration of slow-wave sleep, and changes in cognitive functions. Additionally, their study suggested that the rats may become more sensitive to pain, which could lead to the development of hypoalgesia [127]. In another experimental study, the authors showed that in mice, TNF- α stimulates serotonin uptake in synaptosomes of the midbrain and striatum, which is due to the activation of mitogen-activated protein (MAP) kinase p38 [128]. In experimental conditions in mice, it was found that the amount of TNF α produced in the blood in response

to infection was related to the severity of the disease in patients. The introduction of a drug that blocks TNF α production leads to a decrease in the severity of clinical complaints and depression [129, 130]. It has been shown that increased levels of TNF α production in patients cause the development of sick behavior and weight loss [131]. TNF α crosses the blood-brain barrier (BBB) and affects areas of the brain that are responsible for motor coordination and fatigue [132]. Krzyszton C.P., et al. published research data indicating that the development of fever, fatigue, sleep disturbances, depressive, and cognitive impairments can be induced by increased production of proinflammatory cytokines, in particular TNF- α [133]. Our results on the influence of the level of TNF α production on the development of such clinical complaints as fever, weakness, fatigue, sleep disturbance, irritability, depression, and tearfulness fully confirm the data of other authors. Moreover, the level of TNF α production is associated with the NK cell compartment in the peripheral blood, the content of which is related to the number of EBV DNA copies in saliva samples and affects the development of clinical complaints in patients, which is also shown in our work.

In 1984, a study was done that demonstrated that mononuclear cells from the blood of adults with no immune response to EBV or from newborns cannot be activated by EBV to further induce IFN- γ production [134]. Hilary Williams et al. showed in their work that EBV infection of peripheral blood mononuclear cells from people with an immune response to the virus in vitro induces IFN- γ production 6 hours after contact with the virus and reaches a peak 12–24 hours after induction [135]. It has been shown that NK cells localized in the tonsils produce higher amounts of IFN- γ compared to NK cells from peripheral blood. These NK cells, which are derived from the tonsils, can reduce the transformation of B cells caused by EBV in vitro. This finding supports the idea that IFN- γ plays a role in the cytotoxicity of NK cells against EBV infection [28, 136, 137]. Our work demonstrated the relationship between the level of IFN- γ production, the duration of the disease, and the number of EBV DNA copies. A dependence of IFN- γ production on the content of NK cells in the blood was also revealed. We showed that the level of IFN- γ production affects the development of clinical complaints of patients. This is inconsistent with the data published by Barbara Cameron et al. on the lack of correlation between IFN- γ production and clinical complaints [138]. This is probably due to the fact that our study assessed the effect of induced IFN- γ production, while the study by Barbara Cameron et al. assessed only the serum level of IFN- γ . In infected patients, an increase in the concentration of neopterin in the blood serum ($p < 0.01$) and a decrease in the concentration of tryptophan ($p = 0.01$) were detected, which indicates the development of Th-1 immune activation using IFN- γ [139].

In patients with long-term chronic EBV infection, expression of the degranulation marker CD107a on the NK cell membrane was shown to be within reference values. No reliable significant correlations and regression models were found between the expression of the degranulation marker CD107a on the NK cell membrane, the content of NK cells in the blood, and the number of EBV DNA copies.

5. Conclusion

Our study of the role of NK cells in patients with long-term chronic EBV infection has shown that these cells play an important role in the host's response to the infection. They are involved in the formation of an immune response, the production of proinflammatory cytokines, and the expression of the degranulation marker CD107a, which activates the cytotoxic function of NK cells that induce apoptosis of infected cells.

Authors' contribution

conception and research design—Rakityanskaya I. A.;
material gathering and processing—Rakityanskaya I. A., Ryabova T. S.;
data analysis and interpretation—Rakityanskaya I. A., Ryabova T. S.;
statistical processing of data—Rakityanskaya I. A.;
script composition—Rakityanskaya I. A., Ryabova T. S.;
editing—Ryabova T. S.;
research supervision—Rakityanskaya I. A.;
text writing and editing—Rakityanskaya I. A., Ryabova T. S.;
responsibility for integrity of all article's parts—Rakityanskaya I. A.;
Script further revision for important intellectual content—Rakityanskaya I. A.,
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The authors declare no conflict of interest.

Data sharing policy

The statistical code and dataset used in support of the findings of this study are included within the article.

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Vimentin IFs as a Shield of Cells against Doxorubicin

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Abstract

Vimentin is a cytoskeleton protein of type III intermediate filaments (IFs) that is expressed in mesenchymal cells. Unlike other tissue-specific IF proteins vimentin may be found in different cell types. Thus, the expression of vimentin is a hallmark of an epithelial-to-mesenchymal transition (EMT), which is observed during early embryogenesis, wound healing, and the malignant transformation of various tumor cells. Besides the well-established function of vimentin IFs to provide the mechanical strength of the cells, we discovered that their interaction with mitochondria causes the decrease of their motility and the elevation of the mitochondrial membrane potential. These observations suggested an idea that vimentin could play a role in stabilization of mitochondria in the harsh conditions of chemotherapy and thus protect cancer cells against oxidative stress. Doxorubicin is used for treating a wide range of human tumors, including gastric carcinoma, breast carcinoma, leukemia, lung cancer, malignant lymphoma and others. Though it has been used clinically for over five decades, precise molecular pathways leading to the cancer cell death remain unexplored. However, various mechanisms have been proposed including topoisomerase inhibition, oxidative stress, and redox cycling of doxorubicin in mitochondria. Here, we analyze the recent data demonstrating vimentin's role in cell protection against doxorubicin.

Keywords: vimentin, mitochondria, doxorubicin, verapamil, ROS

1. Introduction

One of the major obstacles in an antitumor therapy is the elevated resistance of tumor cells to cytotoxic drugs. Tumor cells have the capacity to avoid the apoptosis by different mechanisms. First, some of tumor cells are able to form a propitious micro-environment [1]; second, the disruption of tumor suppression regulatory pathways is often observed in malignant cells [2]; and third, they usually develop a multidrug resistance by hyper expression of drug efflux transporters such as P-glycoprotein [3, 4]. Enhanced expression of vimentin is characteristic to many types of tumors, and it is generally believed that this IF protein is responsible for the malignant properties of tumor cells [5]. Several attempts were made to find a link between vimentin expression and the properties of tumor cells that enhance their survival [5, 6]; however, the molecular mechanisms of vimentin involvement are still to be elucidated.

Doxorubicin is among the most commonly used drugs for treating a wide range of human cancers, including gastric carcinoma, breast carcinoma, leukemia, lung

cancer, malignant lymphoma, and others. Though it has been used clinically for over five decades, precise molecular pathways leading to the cancer cell death remain unexplored. However, various mechanisms have been proposed including topoisomerase inhibition, oxidative stress, and redox cycling of doxorubicin in mitochondria [7–9].

DNA topoisomerases are enzymes that play essential roles in the DNA replication, transcription, recombination, and repair. They reduce supercoiling in DNA by breaking and rejoining one or both strands of the DNA molecule. DNA topoisomerases bind to either single-stranded or double-stranded DNA and cut the phosphate backbone of the DNA. This intermediate break allows the DNA to be unwound, and, at the end of these processes, the DNA backbone is resealed again [10]. Doxorubicin binds to DNA by intercalation between base pairs, and it inhibits DNA topoisomerase activity by stabilizing the DNA-topoisomerase complex. The result of this interaction includes DNA strand breaks and inhibition of DNA religation. Inhibition of topoisomerase is widely considered as a central mechanism underlying doxorubicin's deleterious effect on rapidly proliferative cancer cells [11].

Oxidative stress is another widely accepted mechanism of doxorubicin-induced cytotoxicity. The first argument is the elevated intracellular levels of reactive oxygen species (ROS) following doxorubicin treatment. The second is the protection of the cells treated with doxorubicin by the administration of antioxidant compounds. Increased ROS levels after doxorubicin treatment in cells may result from different mechanisms. First, increased ROS level may be related to disturbance of mitochondrial electron transport chain due to disruption of mitochondrial genome. Second, a surplus ROS may be produced by redox cycling of doxorubicin between its quinone and semi-quinone forms [8]. Thus, the main target of doxorubicin is DNA [12] and topoisomerase involved in DNA reparation [9], while mitochondria are also the targets of doxorubicin-induced cytotoxicity.

Although increased tumor invasiveness correlates with vimentin hyper expression in many tumors [5], the role of this IF protein in cancer progression remains obscure. In this chapter, we summarize the data demonstrating vimentin's effects on mitochondria functions that could provide an explanation of elevated resistance of some tumor cells against cytostatics. Targeting vimentin simultaneously with doxorubicin may suggest a new approach in antitumor therapy.

2. Vimentin affects properties of mitochondria

Disturbances of mitochondrial distribution, morphology, and functions in different cells and tissues with damaged IFs were documented by many authors [13–15]. Impairments in mitochondrial function were also observed in fibroblasts obtained from vimentin gene knockout mice [16]. Tolstonog and co-authors suggested that vimentin provides a protection from oxidative damage of mitochondria that was observed in cells from mice with knockout of vimentin [17]. As soon as mitochondria play a paramount role in the cellular physiology, as the source of energy [18], being involved in the induction of apoptosis [19], and regulating the intracellular Ca^{2+} concentration [20], it seems important to consider vimentin IFs as one of the determinants of cellular homeostasis.

According to our earlier data deletion of vimentin IFs in mice fibroblasts causes the increase of mitochondrial motility [21]. It was found that N-terminal part of vimentin molecule contains the sequence responsible for binding to mitochondria [21, 22] with features of mitochondria localization signal found in other proteins [23]. We have

found that the interaction of vimentin with mitochondria leads to elevation of their membrane potential [24]. Interestingly, the increase of mitochondrial potential was observed even in the presence of small fragment of vimentin molecule containing this binding site [24]. It was also shown that this binding is controlled by GTPase Rac1 through protein kinase PAK1 that phosphorylates Ser-55 of vimentin [25]. Activation of this cascade in fibroblasts results in disruption of the interaction and causes a decrease of mitochondrial potential and increase of their motility [25].

In earlier studies of impact of vimentin gene knockout in murine fibroblasts analysis of mitochondria showed a decline of respiratory function and the increased level of ROS, which by itself caused a further mitochondrial damage in a vicious circle [17]. We have found later that fragmentation of mitochondria is observed in fibroblasts devoid of vimentin at a very low concentration of H_2O_2 , while restoration of vimentin IFs make them more stable [26]. This effect of vimentin IFs on mitochondrial resistance against oxidative stress was dependent on its ability to bind mitochondria since the Vim(P57R), the mutated form without such ability had no effect [26]. Thus, the interaction of mitochondria with vimentin IFs not only provides their correct distribution in cells and ensures an appropriate level of the membrane potential but also guarantees their protection against oxidative damage. Such protection of mitochondria by vimentin implies the possibility that hyper-expression of this IF protein in tumor cells can cause at least partially their increased resistance against anticancer drugs.

3. Vimentin IFs provide doxorubicin resistance when restored in vimentin-null cells

To address the possible role of vimentin's interaction with mitochondria in protection of cells against doxorubicin, we used the murine cell line MFT-16 isolated from vimentin knockout mouse [27]. Several derivative cell lines were prepared that expressed wild-type variant and some mutant forms of human vimentin that differed in the ability to bind mitochondria. These cell lines contained well-developed IF networks that were concentrated around nuclei and radially distributed toward the cellular periphery (**Figure 1**, [25]). However, the effects on mitochondria of these IFs were different [24, 25]. While in cells containing wild-type vimentin and mutant Vim(S55A) mitochondria were less motile and possessed an increased membrane potential, those in cells expressing Vim(P56R) and Vim(S55E) had an

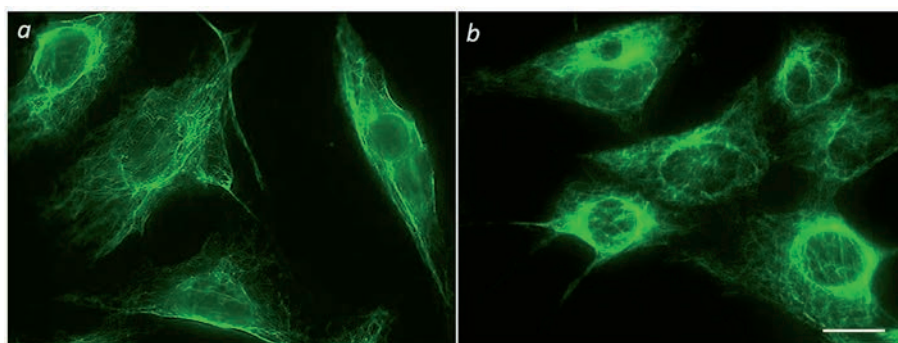


Figure 1.
(adopted from ref. [28]) Immunofluorescent staining of vimentin IFs in cells MFT-16 stably expressing vim(WT) (a) and vim(P56R) (b). Scale bar, 10 μ m.

enhanced motility and decreased potential similar to mitochondria in vimentin-null cells. Importantly, the viability of these cell lines in the presence of doxorubicin was essentially different [28]. Vimentin-null cells were five times more sensitive to the drug than those expressing human vimentin when compared using MTT test. The survival curve for vimentin-containing cells in doxorubicin showed a considerable shift toward the higher concentrations (**Figure 2a**). Viability in doxorubicin of cells with mutated forms of vimentin was different depending on the ability to bind mitochondria. Cells expressing Vim(P56R) and Vim(S55E) were as sensitive to the drug as those without vimentin. On the contrary, the cells expressing Vim(S55A) showed the drug resistance similar to cells with wild-type protein (**Figure 2b**). In order to exclude the possible artefactual effect of different vimentin mutants on the mitochondrial (NADPH)-dependent oxidoreductase activity that is involved in the reduction of tetrazolium dye in the MTT test, we confirmed our data by using another test based on the accumulation of the neutral red (NR) dye in the lysosomes of live cells [29]. This approach gave very similar results (**Tables 1 and 2**) [28]. Thus, the protective effect on cells of vimentin IFs against doxorubicin depends on their ability to interact with mitochondria. Interestingly, these results are in a good agreement with the earlier data from other laboratories that demonstrated correlation between increased level of vimentin expression and elevated resistance to doxorubicin of the human colocal carcinoma cell line LoVo [6], MCF-7 cell line of breast adenocarcinoma [30],

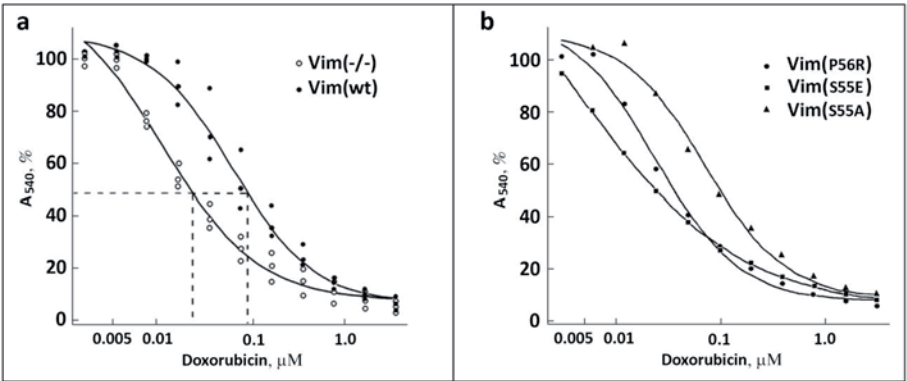


Figure 2. (adopted from ref. [28]) viability curves of MFT-16 cells and MFT-16 stably expressing vim(WT) (a) and cells stably expressing vim(P56R), vim(S55E), or vim(S55A) in the presence of doxorubicin plotted according to the data obtained using MTT-test. Symbols indicated for each cell line represent mean values of optical density expressed in percentage of drug-free control.

Cell line	IC ₅₀ , nM	IC ₉₀ , nM
Vim-/-	17 ± 5	181 ± 56
Vim WT	83 ± 11	643 ± 222
Vim PR	24 ± 4	183 ± 52
Vim SE	4.7 ± 7.4	217 ± 113
Vim SA	71 ± 9	506 ± 166

Values of IC₅₀ and IC₉₀ are presented as mean ± SEM.

Table 1.
Cell viability in the presence of doxorubicin estimated with MTT-test.

Cell line	IC ₅₀ , nM	IC ₉₀ , nM
Vim-/-	24.4 ± 4.8	251 ± 73
Vim WT	65.9 ± 9.5	699 ± 255
Vim PR	27.4 ± 4s.4	234 ± 64
Vim SE	20.2 ± 5.5	260 ± 84
Vim SA	79.4 ± 9.3	543 ± 152

Values of IC₅₀ and IC₉₀ are presented as mean ± SEM.

Table 2.
Cell viability in the presence of doxorubicin estimated with NR-test.

and gastric cancer cells SGC7901 [31]. Noteworthy, the reversal of EMT in these cells with concomitant decrease of vimentin expression caused a reduction of doxorubicin resistance [6, 30, 31].

4. Conclusions

Considering the discussed above vimentin’s ability to provide a protection of mitochondria in the conditions of oxidative stress some practical implications could be suggested. First, over-expression of vimentin in different tumor cells may cause their additional drug resistance and should be regarded as an obstacle in chemotherapy using doxorubicin. Second, targeting vimentin in cancer cells may be advantageous in order to increase their sensitivity to anticancer drug such as doxorubicin. The promising approach in this direction could be the application of Withaferin-A [32], a compound from *Withania Somnifera* that was shown to disrupt vimentin IFs and itself possesses anticancer activity.

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Tissue Engineering Scaffolds: The Importance of Collagen

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and Ingrid Silva-Cote*

Abstract

Tissue engineering focuses on developing replacement tissues and organs to maintain, restore, or improve their function. To achieve this goal, an optimal scaffold is required to promote cell growth and biomolecules release involved in the repair process. In tissues, the extracellular matrix (ECM) provides spatial and mechanical cues to cells and physical support. Therefore, creating a scaffold that mimics the ECM of a tissue or organ of interest to facilitate its repair represents an urgent need. Collagen is the most abundant protein in the ECM and is essential for maintaining the biological and structural integrity of the tissue as well as providing physical support. Collagen-based scaffolds can be obtained from a decellularized collagen matrix, preserving the original tissue shape and ECM structure, or by extracting, purifying, and polymerizing collagen alone or with other natural or biosynthetic polymers and ceramics, which can be chemically or physically cross-linked, modified with natural/synthetic polymers or inorganic materials, or supplemented with biochemical factors. The properties of collagen for obtaining tissue engineering products and the intellectual property of collagen-based scaffolds in clinical trials and patents are discussed. Here, we described the importance of collagen for tissue and organ repair.

Keywords: tissue engineering, collagen, biological scaffold, decellularized, biosynthetic scaffold, repair

1. Introduction

Collagen represents the primary structural protein in the body, comprising approximately 30 wt% of the body's total protein content [1]. Its different types sustain mechanical and bodily stresses, support various cell types, and anchor many growth factors [1, 2]. Besides, this protein effectively modulates the biological behavior of cells to stimulate native tissue repair [3]. To date, about 28 distinct types of collagens have been identified in vertebrates [4]. Of which, the most abundant fibrillar protein is Type I collagen.

As well known, collagen is a key component of the extracellular matrix (ECM), which strongly regulates the extracellular scaffolding, maintains the biological and structural integrity of ECM, and plays critical roles in the regulation of the phases of wound healing [5]. This protein induces platelet activation and aggregation during

the early phase to induce homeostasis. Besides, collagen degradation releases fragments that promote fibroblast proliferation and synthesis of growth factors that regulate angiogenesis and reepithelialization [5].

Tissue engineering is an emergent strategy that combines cells, biomaterial scaffolds, and biologically active molecules to repair a specific damaged tissue. When these products are implanted at the lesion, the scaffold will be replaced by the cells' own matrices during the regeneration. Therefore, the scaffolds should be biocompatible and biodegradable and provide appropriate signals for the seeded cells that regulate the process [6].

Biological scaffolds are structures used in tissue engineering to support cells and bioactive molecules required to repair or replace injured tissues. These scaffolds are fabricated from natural materials by removing the cellular content from source tissues while preserving the structural and molecular composition of the remaining ECM [7]. This complex is crucial in tissue engineering because the ECM provides physical support to tissues, maintains their architecture, and can regulate cellular functions, providing spatial and mechanical signals to cells [6, 8]. The microstructure of the ECM varies depending on the type of tissue. However, their proteins, such as collagen, fibronectin, laminin, and glycosaminoglycans (GAGs), are responsible for maintaining the 3D structure, mechanical support and favorably influence the mitogenesis, chemotaxis, and cell differentiation fate [9, 10]. Each of these processes is key to promoting the formation of new tissues and organs [6].

The decellularization process is highly relevant in tissue engineering due to its efficient removal of the cellular and nucleic components while preserving the structural and functional proteins of the extracellular matrix (ECM) [11, 12], especially the collagen fibers, which are the main component of ECM, therefore of the acellular matrices [3, 6]. During a procedure to obtain an acellular matrix, the spatial arrangement of collagen fibers within the ECM represents an important structural aspect due to the mechanical properties associated with fiber direction. In addition, the presence and integrity of ECM proteins and their three-dimensional organization strongly affect the quality and downstream clinical outcome when the scaffolds are used for tissue repair and reconstruction (10). The ECM regulates cell behavior and phenotype while cells, in turn, continuously produce, degrade, and remodel the ECM. This reciprocal process is crucial to tissue development, homeostasis, and wound healing [9].

The decellularized matrices have been widely used as biological scaffolds to repair various tissues and organs such as cartilage, skin, bone, bladder, blood vessels, heart, liver, and lung, among others [6, 13, 14]. It has been reported that collagen strongly regulates the microstructure of decellularized matrices for repairing tissue. To date, a decellularized matrix from porcine cornea was used for *in vivo* repair by transplantation into rabbit cornea for corneal tissue engineering. Based on the results, no immune reaction occurred, and the turbid corneas became clear, which is suggested by the structure of the aligned collagen fibrils after decellularization, producing transparency after transplantation [15].

This chapter focuses on the importance of collagen for tissue engineering products because the protein has garnered broad interest as a biomedical material. In particular, collagen is a highly biocompatible macromolecule with potent biological functions for tissue repair and regeneration [1]. One of the objectives of this chapter is to offer a complete overview of the use of acellular matrices as biological scaffolds because they are mainly used for tissue repair, considering key factors such as biocompatibility and biodegradability attributable to ECM composition, especially collagen content.

2. Structure and function of collagen

Collagen consists of a triple helix chain formed by α chains. Each alpha (α) chain contains about 1000 amino acids with a molecular weight of approximately 100 kDa. It is composed of a specific set of amino acids repeating sequence (Gly-Xaa-Yaa)_n [3]. In this case, glycine residues are located in every third position, and the amino acids in the Xaa and Yaa positions are often proline and hydroxyproline. It should be noted that collagen is cleaved from a precursor molecule known as procollagen by the C-terminal proteolytic processing of soluble procollagen precursors [3].

Collagen is a crucial regulator of the structural integrity of tissues and organs. In particular, regulates the structure of connective tissues, such as bone and cartilage [8]. Moreover, collagen interacts with diverse types of cells through its specific peptide repeat unit and triple helix structure, promoting their adhesion, proliferation, and differentiation by interactions with specific receptors such as integrins, glycoproteins, and proteoglycan receptors [3, 16]. Another feature that ratifies the importance of collagen is its capacity to store and deliver endogenous growth factors and cytokines, providing a suitable microenvironment for cells that participate in tissue and organ development and wound repair [3].

Using acellular matrices naturally provides collagen, an essential tool for producing tissue engineering technologies (**Figure 1**). Collagen offers low immunogenicity, a porous structure, permeability, good biocompatibility, and biodegradability [8, 17, 18].

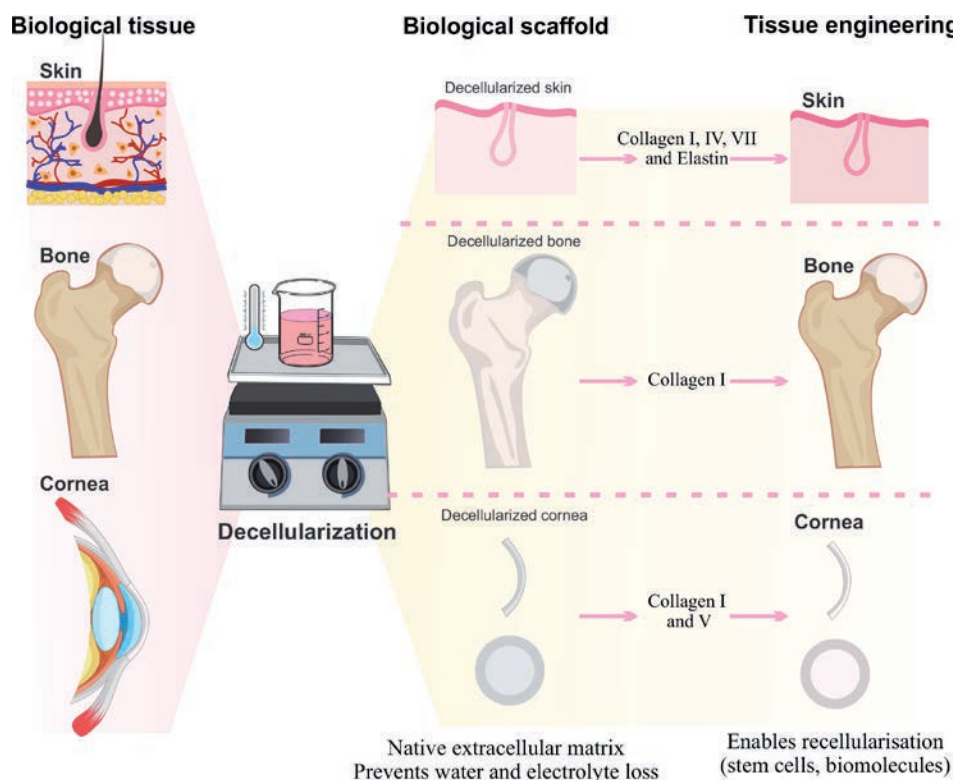


Figure 1. Decellularized scaffolds with application in tissue engineering. Acellular scaffolds from different tissues can be obtained by physicochemical and enzymatic process that preserve the native collagen content.

Each property of collagen represents an opportunity to explore the diverse processes in which this protein participates, especially in tissue maintenance and repair. Here, we mentioned the collagen properties in decellularized matrices widely used in tissue engineering.

3. Decellularized scaffolds

3.1 Corneal decellularized matrix

Decellularized corneas have been reported as a viable alternative that acts as a scaffold for corneal engineering. Several strategies have been used to lyse cells and remove cellular material from the cornea [12]. Acellular corneal matrices (ACM) have been proposed as an alternative to mimic the native structure and composition of the human cornea. These matrices contain collagen I and V [19] and can be recellularized with corneal cells due to their ability to provide a structure similar to the native cornea [20].

Preclinical studies and human clinical applications of decellularized corneas have been successfully described. In animal lamellar keratoplasty experiments, porcine ACM was shown to integrate into the corneal wound bed and improve the reconstruction of the lamellar integrity of the corneal stroma, suggesting that acellular bioengineered cornea may be a good alternative for the treatment of feline corneal sequestrum [21]. Another *in vivo* study demonstrated corneal regeneration using ACM. Briefly, porcine corneas were decellularized and transplanted into a 6.0 mm diameter keratectomy wound in rabbits. Histological analysis showed that the implanted ACM was completely integrated into the rabbit cornea, and infiltrated keratocytes were detected in the decellularized corneal matrix six months after transplantation. In addition, ACM improved corneal transparency [22].

Regarding the use of ACM as a scaffold for tissue engineering constructs, one study reported the *in vivo* biocompatibility of grafts composed of decellularized human corneal stroma with or without human adipose-derived adult stem cells in the rabbit cornea. The grafts were implanted in a 50% stromal pocket. Complete and stable graft transparency was reported during the study, with excellent biocompatibility and integration and no clinical signs of rejection [23].

A comprehensive evaluation of decellularized porcine corneas after clinical transplantation has been reported. In this study, porcine ACM was transplanted in the center of the left cornea of a 40-year-old male patient with a 1.5-mm-diameter corneal ulcer caused by bacterial keratitis, whose vision had rapidly deteriorated. Porcine ACM (Acornea, Product, 350 μ m thick) and a lamellar graft were customized to the patient's corneal defect size. Within the 2-month follow-up period, the corneal ulcer graft became transparent and his visual acuity improved from 0.01 to 0.1. However, the patient's visual improvement with porcine ACM was insufficient. Therefore, to improve his vision, the patient underwent a second deep lamellar keratoplasty using a human donor cornea. It is important to note that the porcine ACM showed good integration with the host cornea, keratocytes had grown within the ACM, and no inflammatory cells were detected [24].

3.2 Bone repair/regeneration

The natural matrix of bone is highly composed of collagen fibrils, specifically type I collagen. Therefore, this type of collagen is widely used in bone repair [3]. However, the poor mechanical properties offered by collagen in bone tissue require

the preservation of the inherent collagen and inorganic components within the bone. This condition is one of the more relevant parameters during the bone decellularization process [10].

In vivo evaluations have been reported to determine the potential of acellular bone matrix (ABM). To date, acellular bovine bone matrix sections were implanted into the left mandible of two-month-old male Sprague-Dawley rats (5 mm diameter round defect). The ABM was dispersed in the bone defect area after six and 12 weeks, and the new bone volume ratio was significantly increased in the ABM-treated group compared to the untreated group [25]. A similar study reported the effect of ABM enriched with hydroxyapatite and extracellular vesicles on the repair of the rabbit mandibular bone defect model, increasing the number of bone-specific cells, vascularization, and bone matrix formation [26].

3.3 Acellular dermal matrix

Acellular dermal matrix (ADM) is a biological scaffold that provides skin-native tissue biochemical properties and ultrastructural architecture to support tissue repair [27]. These matrices have high collagen types I, IV, and VII and elastin content, promoting the graft's favorable elastic properties [14]. In a preclinical context, ADM has been used to treat chronic skin wounds because it provides molecules that improve intercellular communication and neovascularization in wound surface repair [28].

Numerous preclinical evidence has demonstrated the repair effect of ADM, especially on skin wounds. Construct based on ADM recellularized with mesenchymal

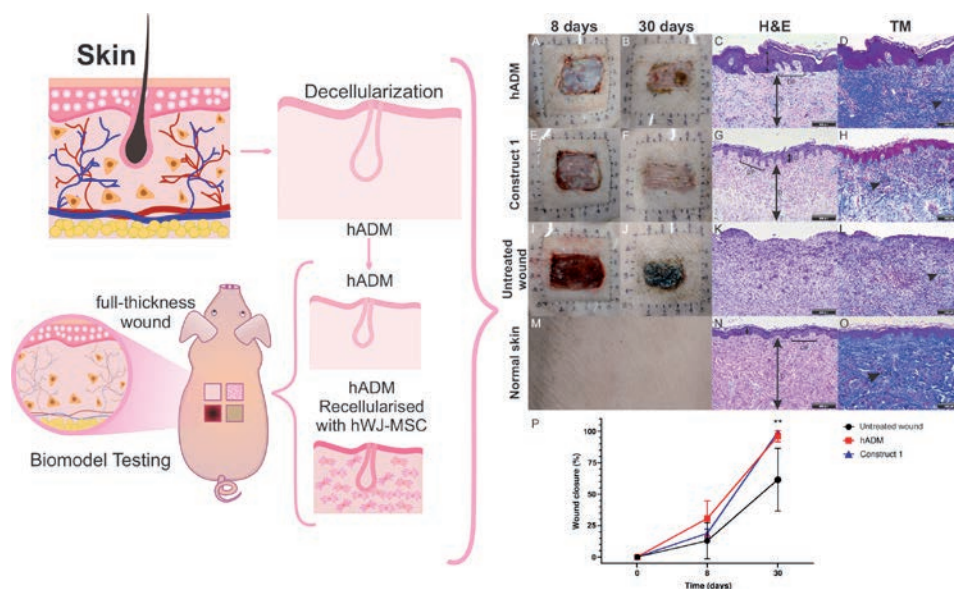


Figure 2. *In vivo* skin wound repair potential of hADM and construct based on hWJ-MSC seeded on hADM. Macroscopic appearance of the wounds at 8 and 30 days after treatment: hADM (A and B), construct (E and F), untreated wound (I and J), normal skin (M). Representative images of histological sections of repaired wounds at day 30 using hADM (C and D), construct (G and H), untreated wound (K and L), and normal skin (N and O). Hematoxylin & eosin (C, G, K, N) and Masson trichrome stain (D, H, I, O). Wound closure rate (P). hADM: Human acellular dermal matrix, SE: stratified epithelium, BM: basement membrane, DP: dermal papillae, D: Dermis, COL: collagen fibers. Scale bar = 200 μ m; n = 6; p < 0.01 (**). Image adapted from Correa-Araujo et al. [29].

stromal cells from Wharton jelly (hWJ-MSC) promoted the close of porcine full-thickness excisional wounds through the formation of stratified epithelium, basement membrane, and dermal papillae, improving the appearance of the repaired tissue at 30 days posttreatment (**Figure 2**) [29]. In addition, an experimental study about human ADM seeded with adipose-derived stem cells was reported. The construct enhanced wound healing in a murine model demonstrated by increased reepithelialization in 12 days, synthesis of granulation tissue, and vascularization during early wound healing [30].

4. Collagen and natural biopolymers scaffolds

Natural polymers have biocompatibility and bioactivity but are challenging to engineer due to limited processability, high contamination risk, and poor mechanical properties. Examples include bioactive proteins such as silk, collagen, gelatin, fibrinogen, and polysaccharides such as cellulose, chitosan, and alginate, and glycosaminoglycans, such as hyaluronic acid.

4.1 Chitosan

Chitosan is a polysaccharide found in the exoskeleton of crustaceans, mollusks, and insects. Chitosan has low toxicity, is non-immunogenic, and has antimicrobial activity, making it an excellent choice for tissue engineering applications. Its use as a biomaterial is justified by its biodegradability and biocompatibility. In addition, it is inexpensive and can be obtained by partial deacetylation of chitin [31, 32]. Additionally, chitosan is the only positively charged biopolymer that can interact with the ECM's structural molecules [33]. This unique cationic biopolymer can be combined with another anionic biopolymer to form a two-component scaffold with optimal mechanical and biological properties [34]. The combination of collagen with chitosan is a promising alternative for tissue healing purposes, given the antimicrobial and hemostatic properties of this polysaccharide; in addition, chitosan improves the structure of collagen fibers, and it is used to try to control the degradation time of the scaffold and improve scaffold mechanical properties.

The main application forms of collagen/chitosan scaffolds in tissue engineering for skin and bone repair are membrane [35, 36], hydrogel [37], hydrogel 3D-bioprinter [38], and sponge [39]. *In vivo* studies have shown that collagen/chitosan sponges are suitable for stimulating osteogenesis in cranial defects, although this process is slow [32]. Also, the repair of mandibular defects filled with a collagen/chitosan scaffold, seeded or not with dental pulp-derived MSCs, was also evaluated. The scaffold showed osteoconductivity, no foreign body reaction, malleability, and ease of manipulation but did not show promising results for association with dental pulp cells as better bone repair was observed in the defect treated with dental pulp cells alone [40].

On the other hand, a freeze-dried chitosan-collagen sponge for wound dressing was found to be nontoxic and biocompatible in an animal model. In addition to excellent water retention properties, which are critical for reducing the risk of dehydration at the wound site, it also showed excellent neovascularization and fibroblast proliferation, which were shown to promote substantial full-thickness skin wound healing [39]. An *in vivo* study of full-thickness wounds in the rat model using curcumin-loaded chitosan/poly(ethylene oxide)/collagen nanofibers showed

significant improvement in the mean wound area closure [41]. These results showed that the electrospun nanofibers, sponge, and hydrogels collagen-chitosan could be a promising type of wound dressing in the wound healing process.

4.2 Alginate

Alginate is a naturally occurring anionic and hydrophilic polysaccharide. It is one of the most abundant biosynthesized materials, mainly derived from brown algae and bacteria [42]. Alginate is particularly interesting for a wide range of applications as a biomaterial, especially as a support matrix or delivery system for tissue repair and regeneration [43]. Due to its outstanding properties in biocompatibility, biodegradability, non-antigenicity, and chelating ability, alginate has been widely used in various biomedical applications, including tissue engineering and drug delivery [44, 45]. Chelation with divalent cations is the easiest way to prepare alginate hydrogels from an aqueous solution under mild conditions. As a result of the natural polysaccharide, alginate has a pH-dependent anionic nature and can interact with cationic polyelectrolytes and proteoglycans. Therefore, cationic drug and molecule delivery systems can be obtained through simple electrostatic interactions [44]. However, alginate lacks cell-binding sites, limiting long-term cell survival and viability. Collagen (Col) contains cell-binding motifs, facilitating cell attachment, interaction, and spreading, consequently maintaining cell viability and promoting cell proliferation. In particular, collagen–alginate hydrogel has attracted much attention due to its excellent biocompatibility, gelling under mild conditions, low cytotoxicity, controllable mechanical properties, wide availability, and easy incorporation of other biomaterials and bioactive agents [46].

It has been reported that collagen, in combination with alginate, improves the hydrogel's mechanical properties and tunes the stiffness simply by the concentration of Ca^{2+} [47]. Moreover, due to the collagen-containing cell-binding ligands and the inherent lack of cell-binding motifs in alginate, increasing the amount of collagen in the collagen/alginate hydrogel improves the cell-binding ligands, allowing more cell adhesion and attachment, thereby maintaining cell viability and promoting cell proliferation.

On the other hand, alginate is considered a nondegradable biomaterial because the human body lacks a specific enzyme called alginase to cleave alginate polymer chains. However, covalently crosslinked alginate can be gradually degraded by ion exchange, in which covalent ions, such as Ca^{2+} , are constantly released from alginate to replace monovalent cations, such as Na^+ , from the environment [46]. Currently, sodium alginate and collagen, combined with other biomaterials or alone, are widely used hydrogels in cartilage, skin, intervertebral disc, and bone tissue engineering.

A promising approach for bone tissue engineering is the *in situ* mineralization (ideally hydroxyapatite formation) of blue shark (*Prionace glauca*) collagen mixed with alginate to produce 3D printable cell-laden hydrogels [48]. As well as hydroxyapatite/collagen–alginate scaffolds as bone fillers and as carriers of growth factors, such as bone morphogenetic proteins (BMPs) [49]. In addition, highly porous layered collagen scaffolds coated with an alginate polymer, which exhibit improved mechanical properties and controllable drug release, have potential as biomedical scaffolds for clinical use in bone tissue regeneration.

On the other hand, 3D bioprinting is an innovative technology for cartilage tissue engineering. Collagen type I (COL) mixed with sodium alginate (SA) to serve as 3D bioprinting bioinks and incorporated chondrocytes to construct *in vitro* 3D

printed cartilage tissue [50]. Likewise, collagen can be incorporated into alginate-based shape-memory scaffolds offering advantages as mechanical stability, injectability, and biological activity, mainly in annulus fibrosus of the intervertebral disc repair [51, 52].

Collagen and alginate wound dressings already on the market include DermaCol/Ag (advanced wound dressing containing collagen, sodium alginate, carboxymethylcellulose, ethylenediaminetetraacetic acid (EDTA), and silver chloride); ColActive® Plus (collagen, sodium alginate, carboxymethylcellulose and ethylenediaminetetraacetic acid dressing); and FIBRACOL™ plus (a soft, absorbent and conformable 90% collagen, 10% calcium alginate dressing).

4.3 Hyaluronic acid

Hyaluronic acid (HA) is a natural glycosaminoglycan found in the extracellular matrix of most connective tissues and is composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid. There are several ways in which HA can mimic the ECM: (1) Like other ECM components, it can provide a physical scaffold for cells to attach and migrate. (2) It can regulate cell behavior and signaling pathways by interacting with cell surface receptors. (3) It can serve as a reservoir for growth factors and other signaling molecules that can be released in a controlled manner to regulate cell behavior and tissue repair processes. Other known capabilities of this material include immune regulation and regeneration induction [53].

HA, due to its chemical composition, is a polymer that has a strong affinity for water and breaks down quickly. HA-based scaffolds have been extensively studied in the field of tissue engineering because they are highly biocompatible, biodegradable, and can be easily modified chemically. Hydrogels, sponges, injectable hydrogels, and electrospun scaffolds are all possible forms of HA-collagen scaffolds [54, 55]. The collagen-HA substrate is cell-compatible *in vitro* and enhances collagen synthesis and new blood vessel formation *in vivo*. Also, may offer robust, freely permeable 3-D matrices that enhance mammary stromal tissue development *in vitro* [56, 57]. Collagen/chondroitin sulfate/hyaluronic acid (Co/CS/HA) scaffolds and the cross-linked-HA/collagen/poloxamer sheets can be used for dermal tissue engineering [58]. Chondrocytes were encapsulated in the thiolated HA/type I collagen hydrogels, where they maintained their phenotype and secreted a considerable amount of matrix [59]. Niu et al. designed hyaluronic acid/collagen core-shell nanofibers, showing a reduction in the inflammatory level, as well as an increase in cell proliferation [60]. Tsai et al. generated a biocompatible 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)-cross-linked HA-collagen composite as a candidate mechanical barrier for preventing postoperative intraperitoneal adhesion [61].

Otherwise, the layer-by-layer (LBL) self-assembly technique is a highly effective method for immobilizing the major components of the extracellular matrix, such as collagen and hyaluronic acid, on titanium-based implants and forming a polyelectrolyte multilayer (PEM) film by electrostatic interaction, and this covalently immobilized film may be beneficial for early osseointegration of implants [62]. Likewise, multilayer coatings on Ti64 substrates based on jellyfish collagen and HA polyelectrolytes with incorporation of methylglyoxal as antimicrobial agent are surface coatings that are promising candidates for future bone tissue engineering applications to provide antimicrobial activity with bone-inducing functions [63].

5. Collagen and synthetic biopolymers scaffolds

Synthetic biopolymers are preferred scaffolding materials for their defined chemistries, cost-effectiveness, ability to tailor physicochemical properties, and longer shelf life. However, they are not bioactive and can induce inflammatory responses. Examples include polylactic acid (PLA), polyglycolic acid (PGA), poly-L-lactide (PLLA), poly ϵ -caprolactone (PCL), polylactic-glycolic acid (PLGA) copolymers, and polyhydroxyalkanoates (PHA).

5.1 Polycaprolactone

Polycaprolactone (PCL) is a biodegradable polyester composed of a sequence of methylene units between which ester groups are formed. This semicrystalline polymer has a melting point of 58–60°C, low viscosity, and easy processability. At room temperature, short-chain polycaprolactone is amorphous, soft, and rubbery. Due to the uniform structure, however, it crystallizes easily, resulting in material reinforcement. PCL is degraded by hydrolysis of its ester bonds under physiological conditions (such as in the human body) and has, therefore, received much attention for its use as an implantable biomaterial. In particular, it is especially interesting to prepare long-term implantable devices due to their degradation, which is even slower than that of polylactide. PCL has been approved by the U.S. FDA in specific applications used in the human body, such as a drug delivery device, suture (sold under the brand name Monocryl), or adhesion barrier.

PCL is a candidate material for bone defect repair because of its favorable properties, such as biocompatibility, biodegradability, non-toxicity, low degradation rate, and good mechanical properties. However, PCL's surface is hydrophobic and does not promote cell adhesion and proliferation [64]. Therefore, combining PCL and natural polymer can improve the hydrophilicity, substrate softness, and biological properties of cell adhesion and proliferation. Gun Woo et al. used a novel method that combines PCL 3D scaffolds with fish collagen (Col) and the osteogenic abalone intestine gastrointestinal digests (AIGIDs) from *Haliotis discus hannai* and demonstrated strong osteoinduction capability in the rabbit tibia defect model [65]. Kim et al. designed a 3D-printed polycaprolactone (PCL) scaffold reinforced with different percentages of carbonated HA (CHA) and coated with marine atelocollagen (MC). The results obtained by culturing MC3T3-E1 cells on the 10% CHA/MC/PCL scaffold showed a 1044% higher osteogenic differentiation than the pure PCL, and bone regeneration was confirmed in a mouse calvaria defect model. These results suggest that materials derived from marine byproducts may be valuable alternatives to materials derived from terrestrial animals for bone tissue engineering [66]. Similarly, the injectable nanofibrous PCL-COL prepared by interspersing PCL nanofibers within pre-osteoblast cell-embedded collagen type I promotes osteoblast proliferation, phenotype expression, and formation of mineralized matrix for bone regeneration [67].

Otherwise, the development and fabrication of biocompatible synthetic vascular grafts with hybrid structures using biopolymers are highly needed. The poly(ϵ -caprolactone)/collagen/heparin composite vascular graft is the main advantage of this composite: it not only has mechanical properties similar to autologous blood vessels and good biocompatibility. A synergistic effect between collagen and heparin released upon degradation promoted proper tissue regeneration when used as a vascular graft [68]. Also, Park et al. reported the fabrication of PCL-based scaffolds (D = 3 mm) with collagen (lined inner layer) and silica (random outer layer) to enhance vascular

cell responses. Human umbilical vein endothelial cells (HUVEC) and mouse fibroblasts were seeded into the inner and outer layers, respectively. Collagen in the lined inner layer is essential for the antithrombogenic design as it prevents platelet adhesion and activation. The seeding of mouse fibroblast cells allows the maintenance of vascular tone by improving the surface hydrophilicity of the PCL stimulating the secretion of angiogenic and epithelial growth factors involved in wound healing [69].

In skin scaffolds using polycaprolactone and collagen, Lizarazo et al. reported that PCL/Col stimulates the secretion of angiogenic and epithelial growth factors involved in wound healing in human Wharton's jelly mesenchymal stromal cells [70]. Jouibary et al. demonstrated that electrospun polycaprolactone/collagen/graphene oxide scaffolds had mechanical properties similar to those of normal skin [71]. Li et al. integrated antibacterial ZnO quantum dots into the biocompatible PCL/Col fibrous scaffolds by electrospinning method to achieve synergistic wound-healing effect and the PCL-Col/ZnO fibrous scaffolds containing vascular endothelial growth factor (VEGF) also promoted wound-healing effect through promoting expression of transforming growth factor- β (TGF- β) and the vascular factor (CD31) in tissues in the early stages of wound healing [72].

5.2 Polylactic acid

Poly(lactic acid) (PLA) can be divided into three different sub-families, namely: PDLLA (poly DL-lactic acid), PLLA (poly(L-lactic acid), and PDLA (poly(D-lactic acid)). These three subgroups of PLA have the same chemical makeup but differ in their three-dimensional molecular structure. PLA is a biodegradable polymer produced from renewable resources, including corn and potato starch, sugar beet sugar, and sugar cane. Poly(lactic acid) and its copolymers have attracted considerable attention in environmental, biomedical, and pharmaceutical applications [73]. Because it is a bioresorbable material, it can be used in medical applications to make hybrid scaffolds for mesenchymal cell cultures. Among the properties of PLA is its degradation process, which begins with hydrolysis of the polymer chain and ends with the production of CO₂ and H₂O, which are incorporated into the Krebs cycle [74]. PLA has been used and evaluated in fixation devices such as screws, pins, and washers to promote healing of thoracic, hand, leg, finger, and toe fractures; ligament reconstruction procedures; soft and hard tissue fixation; bone and osteochondral fragment alignment; meniscus repair; and hyaline cartilage fixation [75, 76].

Hybrid scaffolds are being used to improve bone repair therapies. Some examples of these are: tissue-engineered bone was constructed by combining bone marrow mesenchymal stem cells with nano-hydroxyapatite/collagen I/poly-L-lactic acid scaffolds and implanted into the bone tunnel of core decompression (CD) to treat early avascular necrosis of the femoral head. The construct showed promising results in enhancing the curative effect of CD and may provide a strategy for the treatment of this condition [77]. A nano-HAP/collagen/PLA composite scaffold was developed using biomimetic synthesis. Osteoblasts adhered, spread, and proliferated in the scaffold's pores within one week *in vitro*, and the scaffold was integrated 12 weeks after surgery *in vivo*. The composite shows promise for clinical significant bone defect repair [78]. Li et al. prepared Porous PLLA scaffolds with bioactive coatings using ice-based microporogens containing hydroxyapatite (HAP) and collagen, which served as both porogens to form the porous structure and vehicles to transfer the bioactive molecules to the inside of PLLA scaffolds in a single step. HAP/collagen coating improved the interactions between osteoblast cells and the polymeric scaffold [79].

Nanda et al. prepared dexamethasone (Dex)-releasing collagen microbead-functionalized PLA collagen hybrid scaffolds as an osteoinductive platform for human bone marrow-derived mesenchymal stem cells. The scaffolds were prepared by a combined method of emulsion freeze-drying and porogen-leaching using pre-prepared ice collagen particulates as a porogen material. The released Dex was useful for osteogenic differentiation of MSCs, and the hybrid scaffolds can be useful for regeneration of a functional bone tissue [80].

Researchers used 3D printing to create PLA scaffolds that were treated with collagen, minocycline, and citrate-hydroxyapatite nanoparticles. The resulting scaffolds have enhanced osteogenic activity and antibiofilm properties, making them suitable for bone repair [81]. Likewise, porcine skin collagen (PSColl) or tilapia fish scale collagen (TFColl) was immobilized onto PLA. PSColl-PLA or TFColl-PLA film showed significantly enhanced mineralization of osteoblast-like cells [82]. Similarly, electrospun scaffolds composed of PLA/Col/ amorphous calcium phosphate show significant production of bone-repair-related growth factors [83] and PLA 3D printed loaded with SDF-1–collagen support cell growth of endothelial cells and induce neo-vessel formation [84].

In cartilage tissue engineering, significant innovation is the fabrication of chitosan/collagen hydrogel scaffolds from 3D-printed PLA struts and cellulose nanofibers, which showed no cytotoxic effect on mesenchymal stem cells and allowed cell growth, attachment, proliferation, and migration through the scaffolds [85]. PLLA-collagen hybrid sponge cell seeding prevented scaffold collapse and promoted cartilage tissue formation *in vivo* [86].

For tendon and ligament regeneration, PLA-Pluronic® (PLA-P) and PLA-Tetronic® (PLA-T) copolymers formed into knitted patches and associated with collagen I/chondroitin sulfate (Coll CS) 3-dimensional matrices. PLA-based copolymers associated with collagen and CS sponge showed perfect tissue integration and allowed neotissue synthesis after 12 weeks *in vivo*. [87].

6. Acellular matrix and polymer scaffold technology search

Acellular matrices and polymeric scaffolds are two approaches in regenerative medicine that provide structural support for cell growth, differentiation, and adhesion. They both aim to regenerate damaged tissues. Patent searches are a valuable tool for research related to tissue engineering, acellular matrices, and biosynthetic scaffolds as they contain detailed information about research, processes, and technological advances not published in scientific articles. Patents help us understand the current state of the art, including existing technologies, research, and active areas of work. They also provide ideas for new techniques and process improvements to enhance acellular matrices and biosynthetic scaffolds. Considering this information, a technological search has been carried out in relation to these two types of technologies.

Similarly, patent documents often provide details about the composition of a product by describing the materials used, proportions, and processing techniques, which allows one to focus and customize the matrix or scaffold in specific tissue engineering research. On the other hand, continuous monitoring of emerging technologies, trends, inventors, companies, or institutions can be performed, which allows the identification of behaviors that influence research decision-making.

A search for patents related to acellular matrices and biosynthetic scaffolds was carried out using a strategy that involved selecting keywords, search fields, and

relevant technologies. The goal was to obtain information on existing patents [88]. For the selection of keywords and search fields, a previous bibliographic review was carried out, from which the following keywords were selected:

Acellula*, decellulariz*, extracellula*, graft*, matrix, bone*, dermal*, corneal, collagen*, repa*, regenerativ*, and biosyntheti* scaffold*.

A search equation was designed with limited keywords related to tissue engineering, including bone, dermis, and cornea. Then, a robust search equation was designed that included the International Patent Classification (IPC) codes and the cooperative patent classification (CPC) codes as a filter, excluding words that did not contribute to the terminology, such as “cosmeti*” and other types of acellular membranes, in order to obtain precise information.

There were 140 search results, including 67 relevant results related to acellular matrices and 42 patent documents related to synthetic polymers and synthetic matrices, including collagen. Of the 67 biological acellular matrices, 43% of the relevant results are related to bone acellular matrices, 31% are related to skin acellular membranes, 18% do not specify the type of acellular matrix, and 7% are related to acellular cornea. Of the 42 results for biosynthetic matrices, 50% are related to scaffolds for bone regeneration, 21% are related to skin, 19% do not specify, and 10% are related to cornea. Similarly, 31 of the 42 technologies include collagen. It should be noted that in cases where the type of repair is not specified, the acellular matrix or biosynthetic scaffold may be used in several types of tissues. No related technologies have an FDA application number. On the other hand, it was found that the publication dates of the 140 technologies found ranged from 1995 to 2023 (**Figure 3**).

In conclusion, it was observed that most of the technologies of both acellular matrices and biosynthetic scaffolds (unlike clinical trials) are related to the engineering of bone tissues, followed by dermal matrices; taking into account this, it was deduced that it is necessary to increase the technological production in corneal tissues. It is noteworthy that from 2015 to 2019 more patent applications were registered related to acellular matrix technologies and biosynthetic scaffolds for bone, skin, and cornea. Their production decreased for 2020, possibly due to the global contingency of COVID-19. However, the year in which more patents were conceived was 2021 (**Figure 3**). Finally, the information contained in these documents can be used as inspiration to create new technologies or improve existing methods.

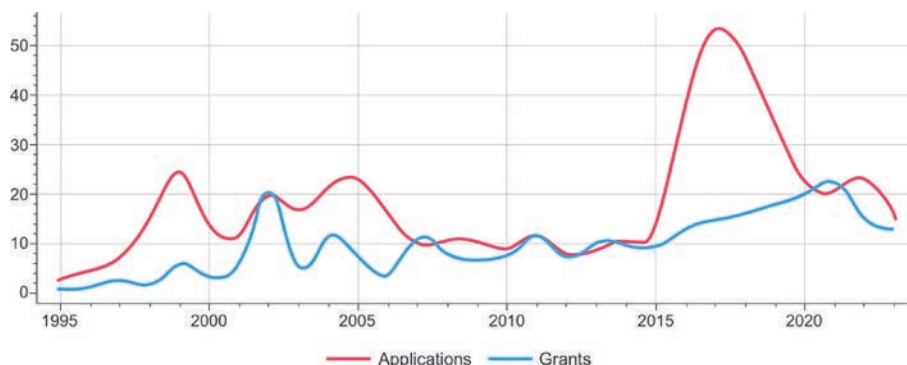


Figure 3.

Advances on the development of related technologies on decellularized matrices and biosynthetic scaffolds used in tissue engineering. Patent applications (red line) and granted technologies (blue line) from 1995 to 2023.

7. Clinical trials

Several clinical trials have been conducted with favorable results using collagen matrices, acellular membranes of the skin, cornea, and acellular bone matrices for their used in tissue repair or regeneration.

Strategies based on biopolymers combined with collagen for tissue treatment have been described. Since collagen is an abundant protein in connective tissue and an essential component of acellular matrices, collagen-based materials are indispensable in regenerative medicine [89], and collagen membranes have been studied in the regeneration of various tissues. For example, the use of a collagen membrane in bone repair has been reported with results comparable to endoprostheses, in this case in guided bone regeneration surgery in dentistry [89–91]. Results related to using a hydroxyapatite-collagen sponge biomaterial enriched with stem cells have been reported. These technologies were implanted in patients with alveolar cleft defects. The results showed reduced morbidity and decreased intensity and frequency of donor site pain in the group treated with the resorbable collagen sponge and stem cells at six months [92, 93]. Furthermore, in the case of articular cartilage regeneration, collagen membranes have been used in conjunction with autologous chondrocytes to form a construct capable of repairing articular defects [94].

On the other hand, corneal tissue engineering uses corneal tissue with cells; these corneal substitutes have been evaluated for viability and compatibility in patients and have been shown to promote healing and regeneration in corneal wounds. However, it has also been shown to have the possibility of improving corneal wound healing and regeneration [95, 96]. Similarly, clinical trials using acellular dermal matrices obtained from human skin [97–99] have demonstrated the efficacy of acellular dermal matrix in treating chronic wounds, diabetic foot ulcers, and other complex skin defects [100, 101]. In one clinical trial, 86 patients with diabetic foot ulcers were randomized into the study, with 47 patients receiving acellular matrix (study group) and 39 patients receiving standard-of-care therapy (control group). The proportion of healed ulcers between the groups was statistically significant ($P = 0.0289$), with the odds of healing being 2.7 times higher in the study group than in the control group, which indicated the potential of ADM by single application as an effective treatment of chronic diabetic ulcers [102].

On the other hand, a clinical study described using a human dermal matrix in combination with a skin graft as an alternative reconstructive solution for treating three different clinical cases related to full-thickness skin wounds. According to histological and ultrastructural analysis from repaired skin biopsies one year after the treatment with ADM, regenerative healing of the wound area with well-organized/oriented connective tissue, as well as cellular infiltration and blood vessel formation was observed in all clinical cases [103]. Several commercially available ADMs have shown significant repair effects for treating these diseases [104]. In addition, the extracellular matrix obtained from the dermis provides a support structure and growth factors that allow the expansion and proliferation of cells. This has great potential in tissue engineering and regenerative medicine [98, 105]. Human application of different acellular scaffolds is the ultimate and most important goal of tissue engineering and regenerative medicine [106]. Due to their biological composition and natural origin, these matrices promote biocompatibility, integrity, and cell and molecular movements crucial during tissue repair.

Finally, collagen-elastin matrices have been used as skin substitutes in treating burns hand and skin reconstruction; grafting with the matrix and unmeshed skin was

carried out in a one-stage procedure. After three months, the pliability of the grafted area was excellent. A full range of motion was achieved in all hands, and no blisters or unstable or hypertrophic scars occurred [107]. we found more studies related to the use of mesenchymal stem cells [108], adipose-derived cells, and xenogeneic grafts based on ultrapure collagen [109]; all this for the repair of different tissues; however, these trials do not yet have specific results. Clinical studies that do not present results may be because most of the clinical study still needs to be completed, or it was not possible to complete it due to failures in its planning [110].

8. Conclusion

Numerous techniques for obtaining collagen-based scaffolds, their preclinical and clinical application, and technology watch related to technologies based on this protein have been reported in this manuscript, highlighting the property of collagen as the main component of ECM. In tissue and organ repair, each process undoubtedly requires the participation of collagen because it predominantly forms the structure of the tissue and contains cellular and protein binding sites. These features confirm the importance of collagen in all sceneries of applications in tissue engineering, from obtaining acellular biological matrices to generating biosynthetic scaffolds for treating damaged organs and tissues.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 6

Collagen-Based Therapies for Accelerated Wound Healing

Alireza Ghofrani and Zahra Hassannejad

Abstract

Wound healing is a complex and dynamic process essential for maintaining tissue integrity and functionality. As a key component of the extracellular matrix (ECM), Collagen plays a crucial role in orchestrating this regenerative process. Acting as a vital fibrous protein, collagen serves as a dynamic conductor, coordinating tissue regeneration and repair. This chapter explores the application of collagen in accelerating the wound healing process, starting with the fundamental role of collagen in ECM remodeling. It discusses how collagen promotes wound healing through different types of scaffolds, micro/nanoparticles, synthetic peptides, and interactions with extracellular vesicles (EVs). The chapter also delves into the regulatory function of collagen in cellular processes and evaluates strategies to stimulate collagen synthesis. In conclusion, it provides an overview of upcoming advancements in the dynamic field of collagen-based therapies for wound treatment.

Keywords: wound healing, collagen synthesis, extracellular matrix remodeling, tissue regeneration, collagen-based wound therapies

1. Introduction

The largest organ of the human body, having an average surface area of 1.85 m^2 , is the skin [1]. Skin, apart from its critical function of homeostasis maintenance, serves as a barrier against the external environment, inhibiting the spread of infections and fluid losses [2]. Wounds are conditions characterized by skin defects or ruptures that result from physical or chemical traumas [3]. Bacterial maladies present a significant peril to human health, as stated by the World Health Organization [4]. Unhealed infected wounds represent a significant health concern, imposing substantial financial burdens on both patients and the healthcare system [5].

Wound healing involves critical stages including hemostasis, inflammation, proliferation, and remodeling, all influenced by extracellular matrix (ECM), collagen, and its derivatives [6–9]. Collagen induces platelet activation and aggregation, leading to fibrin clot formation at the injury site. In the inflammatory phase, proinflammatory cytokines, triggered by immune cell activation, influence fibroblast, epithelial, and endothelial cell migration. Fibroblasts deposit collagen and its degradation releases fragments that promote fibroblast proliferation and growth factor synthesis, driving angiogenesis and re-epithelialization. The acquisition of tensile strength relies

on ECM remodeling, balancing new matrix synthesis, and matrix metalloproteinase (MMP) degradation activities [10–13].

The intricate interplay among cells, soluble factors, and ECM expedites inflammation resolution, fostering fibroblast and keratinocyte proliferation [14]. Platelet activation, secretion of inflammatory cytokines, migration of macrophages, fibroblasts, and keratinocytes, as well as the expression of matrix MMPs and growth factors, are all crucial for wound closure. These processes ultimately lead to the formation of mature ECM and new tissue. Collagen plays a pivotal role in ECM remodeling during wound healing, offering structural support and facilitating adhesion ligand binding to cell surface receptors. Tissue stiffness and matrix composition influence specific signaling pathways, profoundly impacting cell fate [15, 16].

This chapter explores the crucial role of collagen in wound healing and investigates diverse collagen-based therapeutic approaches, including scaffolds, drug delivery systems, synthetic peptides, and extracellular vesicle (EV) interactions. By analyzing collagen's impact on cellular processes and its role in signaling pathways, the chapter provides valuable insights for enhancing therapeutic outcomes. It also examines factors influencing collagen synthesis, highlighting both chemical and physical inducers. Despite acknowledging constraints, the chapter concludes by outlining prospective developments, emphasizing the potential of collagen-based methods to advance wound care and regenerative medicine.

2. Collagen's role in ECM remodeling during wound healing

The dermis relies on fibrillar collagens, mainly types I and III, with subsequent involvement of types XII, XIV, XVI, and VI [17]. Aging increases collagen types I and III concentration in the epidermis [18]. In healthy skin, ECM is 80% type I and 20% type III, crucial for skin elasticity. Imbalances in their ratio affect wound healing outcomes [19]. Fetal healing has higher type III collagen, promoting scar-free recovery [20].

During remodeling, myofibroblasts exit *via* apoptosis or clumping, leaving ECM proteins and collagen. Early repair favors type III collagen, shifting to type I as healing progresses [21]. Molecules precisely regulate collagen I and III synthesis, culminating in tissue closure. Remodeling, concluding in months, involves substituting healing collagen III with normal collagen I, enhancing wound strength [22–24].

Wound repair requires angiogenesis, driven by vascular endothelial growth factor (VEGF), transforming growth factor- β (TGF- β), and type I collagen, mediated by integrin receptors [25–27]. Fibroblast migration forms granulation tissue, rich in connective tissue and blood vessels, driven by TGF- β synthesis during the proliferative phase [28, 29].

Several studies have investigated the effectiveness of collagen in cutaneous wound healing, emphasizing its biocompatibility. Research conducted by Wiegand et al. [30] demonstrated that native collagen promotes the migration of keratinocytes and fibroblasts, fostering a three-dimensional microenvironment that stimulates cell proliferation. Additionally, Jridi et al. [31] identified a positive interaction between collagen and human cells, leading to an increased hydroxyproline concentration at the injury site. In vivo experiments by Helary et al. [32] and Chen et al. [33], further supported collagen's ability to enhance granulation tissue, collagenization, neovascularization, re-epithelization, and the expressions of epidermal growth factor (EGF),

fibroblast growth factor (FGF), and a cluster of differentiation 31 (CD31), expediting the recovery process without eliciting an immune response.

However, abnormalities in ECM reconstitution during wound healing contribute to hypertrophic and keloid scars, characterized by elevated concentrations of collagen I and III, fibronectin, and laminin [34]. The collagen fiber orientation in these scars is perpendicular to the epithelial surface, contrasting with the three-dimensional network in normal skin [35]. Keloid scars exhibit abnormally thick, weakly organized collagen bundles with fewer cross-links in the deep dermis compared to the superficial dermis, while normotrophic and hypertrophic scars show variations in collagen bundle thickness [36, 37]. Keloids also have a higher proportion of collagen I to collagen III than normotrophic lesions, with heterogeneity in the collagen I/III ratio within keloid scars [11].

3. Collagen-based scaffolds

Collagen-based biomaterials can expedite the healing process of diverse tissue types by acting as a matrix [38]. These collagen scaffolds not only strengthen wounds but also facilitate vascularization and closure [39]. To enhance their therapeutic efficacy, bioengineered scaffolds can incorporate bioactive molecules, stem cells, and immunomodulation moieties. The effectiveness of these scaffolds depends on their architectures and material properties, emphasizing the importance of advances in biomaterial science and bioengineering [40]. Numerous collagen wound dressing formulations, designed to address challenging skin lesions, have been developed, showcasing the versatility of collagen in promoting wound healing [41–45]. Additionally, oral collagen-based supplements have been studied as a treatment to enhance the healing process of burn wounds. Miyab et al. [46] conducted a pilot clinical trial involving 31 men (aged 18–60) with 20–30% whole body burns. Their study revealed that an oral collagen supplement can effectively improve wound healing, elevate pre-albumin levels, and lead to reduced hospital stays. a pilot trial on 31 men (18–60 years) with 20–30% burns. Their study found that an oral collagen supplement significantly improved wound healing, increased pre-albumin levels, and clinically reduced hospital stays.

Even though collagen-based wound dressings have many benefits, there are inherent differences between the types, therefore a thorough evaluation of each kind is necessary to determine which is best for a specific case (**Figure 1**). This section aims to provide an insightful exploration of the distinct forms of collagen-based wound dressings.

3.1 Hydrogels

Hydrogels are highly sought-after substances for promoting the regenerative healing of injured tissues on account of their biomimetic porosity and effective water retention, which enable them to establish a moist environment locally [47]. Collagen hydrogels have the potential to serve as effective vehicles for functional nanoparticles and drug delivery, specifically as novel platform materials in the field of biomedical surgical dressings [47, 48]. A study by Ying et al. [49] has yielded a collagen-hyaluronic acid hydrogel that functions as a biomimetic dressing to facilitate uncomplicated wound recovery. According to the findings of the investigation, the hydrogel actively stimulated the development of vasculature. It has been demonstrated by

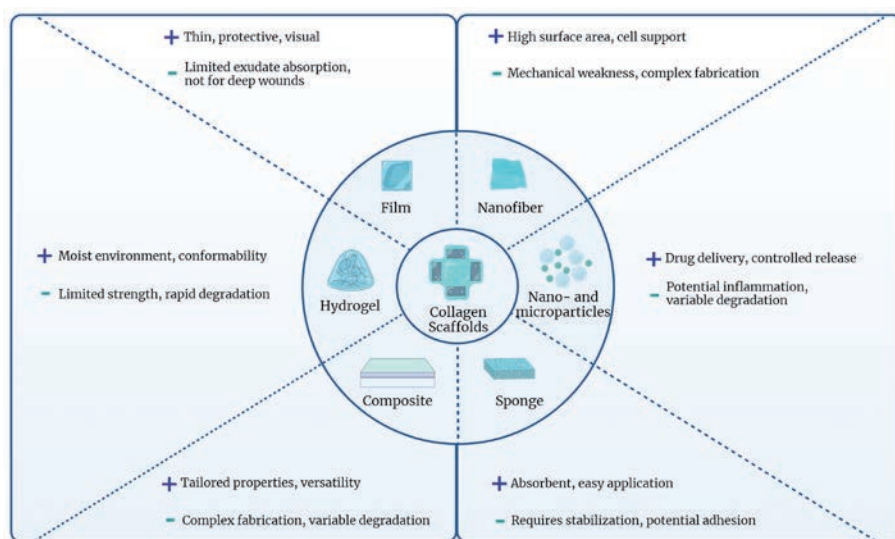


Figure 1.
Collagen wound dressings: Pros and cons (created with Biorender.com).

Chen et al. [50] that a pullulan-collagen hydrogel wound dressing promotes dermal remodeling and wound healing more effectively than commercially available collagen dressings. This hydrogel dressing promoted the formation of healed tissue characterized by collagen fibers that were shorter, less dense, and more irregularly aligned. For cutaneous wound repair, a tough and tissue-adhesive polyacrylamide/collagen hydrogel incorporating dopamine-grafted oxidized sodium alginate as a crosslinker has been synthesized by Bai et al. [51]. The hydrogel exhibited favorable mechanical characteristics, adhesion to cutaneous tissue, water absorption, and sustained biological activity. Experiments on wound healing in vivo demonstrated that the hydrogel can hasten the healing process and may find utility as a wound dressing.

It has been discovered that hydrogels containing collagen induce neovascularization, alleviate pain, and expedite the healing of burn lesions, rendering them more effective than conventional methods like paraffin dressings [52]. A collagen hydrogel containing curcumin has also been found to promote burn wound healing through its antioxidant and anti-inflammatory properties [53]. Moreover, Chakrabarti et al. [54] suggested that their bFGF-collagen-silver sulfadiazine hydrogel accelerates burn healing by promoting fibroblast proliferation. In an in vivo study, using hydrogels led to a faster wound closure, with nearly complete epithelialization in 16 days, compared to standard and control groups. The accelerated healing was attributed to increased re-epithelialization and granulation tissue formation facilitated by collagen and growth factors. The results of numerous investigations on collagen-based hydrogels and their uses in wound healing are compiled in **Table 1**.

3.2 Nanofibers

The integration of nanofibers and collagen, which possess advantageous characteristics including high porosity, exceptional bioactive agent delivery capacity, superior surface-area-to-volume ratio, and enhanced mechanical properties,

Materials	Experimental method	Outcome	Study
Collagen, Cysteine-modified ϵ -poly(L-lysine), <i>In situ</i> -polymerized polypyrrole	<i>In vitro</i>	Enhanced tissue adhesion, low cytotoxicity, improved cell migration, blood coagulation, and accelerated full-thickness wound healing.	[55]
Human placental collagen/adipose-derived stem cells (ADSCs)	<i>In vivo</i>	Significant wound healing and contraction with enhanced fibroblast proliferation, angiogenesis, and collagen deposition.	[56]
Curcumin-loaded liposomes, Lysine–collagen	<i>In vitro</i> and <i>in vivo</i>	Improved wound healing efficacy with sustained drug release and enhanced tissue regeneration.	[57]
Collagen, Laccase-protocatechuic aldehyde, Fe^{3+}	<i>In vitro</i> and <i>in vivo</i>	Enhanced mechanical properties, antioxidant activity, cell proliferation, and accelerated wound healing.	[58]
Recombinant human collagen type III, Multifunctional antibacterial nanoparticles	<i>In vitro</i> and <i>in vivo</i>	Programmed release of antibacterial nanoparticles and collagen type III for accelerated wound healing.	[59]
Collagen and polyethylene glycol, umbilical cord stem cell factor	<i>In vitro</i> and <i>in vivo</i>	Injectable, self-healing hydrogel promoting cell response, collagen deposition, and angiogenesis for diabetic wound repair.	[60]
Methacrylated collagen, 2-hydroxypropyl-beta-cyclodextrin/triclosan complex	<i>In vitro</i> and <i>in vivo</i>	Improved wound healing with controlled release of triclosan and enhanced antibacterial activity.	[61]
Collagen	<i>In vivo</i>	Super-ductile, injectable, self-healing hydrogels with accelerated wound-repair properties.	[62]
Human amnion, Rabbit collagen, Carboxymethyl cellulose sodium salt, Citric acid, Prawn shell chitosan	<i>In vitro</i> and <i>in vivo</i>	Rapid burn wound healing, improved re-epithelialization, and wound contraction when covered with a membrane.	[63]

Table 1.
Collagen-based hydrogels for accelerating wound healing.

has the potential to facilitate wound healing and stimulate skin regeneration [64]. Regenerative medicine and wound healing benefit from nanofibers due to their ability to mimic ECM and promote cellular proliferation and migration [65, 66]. In a rat model, electrospun tilapia collagen nanofibers were found to promote rapid and effective cutaneous wound healing, according to a study by Zhou et al. [67]. Collagen nanofibers accelerated wound healing in vivo, thereby substantially stimulating re-epithelialization [68]. It has been reported that appropriately dense nanofiber scaffolds hasten the healing of skin wounds, promote re-epithelialization, and generate cutaneous skin appendages in the skin [69]. Xie et al. [69] have shown that the utilization of nanofiber scaffolds significantly expedites the process of wound healing through the reduction of collagen I/collagen III and TGF- β 1 ratios, which ultimately leads to a decrease in collagen deposition. Fibrosis was inhibited by polycaprolactone (PCL)/gelatin nanofiber scaffolds *via* the TGF-1/tumor necrosis factor (TNF)-stimulated gene-6 (TSG-6) pathway and in a manner dependent on TGF-1. By facilitating granulation tissue formation, sustaining re-epithelialization, and stimulating angiogenesis, nanofiber systems may hasten the process of wound healing [69]. Nevertheless, further research is needed to determine the specific impacts of nanofiber scaffolds on collagen synthesis and scar formation.

The potential of biomimetic nanofibrous scaffolds in wound healing applications is further highlighted by their recent achievements. Using graphene oxide quantum dot nanocomposites and collagen crosslinked modified chitosan, Dutta et al. [70] created asymmetric scaffolds by electrospinning. These scaffolds demonstrated superior cytocompatibility, anti-inflammatory properties, and antibacterial activity. Rapid re-epithelialization and collagen deposition were shown in an in vivo assessment using a rat model, highlighting its applicability for quick wound healing. Similarly, nanofibrous membranes based on recombinant human collagen type III were developed by Dong et al. [71] and showed superior mechanical qualities, flexibility, and water absorption. These membranes' capacity to accelerate wound closure and improve collagen deposition was demonstrated in vivo experiments on a mouse full-thickness wound model, underscoring their effectiveness in skin regeneration. For skin regeneration, Amini et al. [72] introduced a unique biomimetic hybrid scaffold made of decellularized dermis and collagen fibers that included stromal cell-derived factor-1 alpha (SDF-1 α). According to this study, the prolonged release of SDF-1 α from the scaffold demonstrated a strong capacity to quicken wound healing in diabetic rat models, pointing to its potential for skin tissue engineering.

3.3 Sponges

Collagen sponges possess exceptionally interconnected and porous architectures, allowing them to efficiently absorb blood and wound exudate as efficient wound dressings [73]. Collagen sponges are particularly advantageous in the context of wound healing due to their ability to suture delicate tissue and serve as a template for new tissue due to their wet strength [43]. It is possible to modify the porosity of collagen sponges through the manipulation of collagen content and the freeze-drying process [43, 74]. It is necessary to replace sponge dressings once they become completely saturated with absorbed exudate, with replacement occurring on a weekly or biweekly basis [75].

The efficacy of a collagen-based sponge as an active wound material has been demonstrated in a study by Aravinthan et al. [76] through its ability to promote

Materials	Vascular growth	Anti-bacterial	Moist microenvironment	Hemostasis	Study
Tilapia collagen	✓			✓	[80]
Aminated β -Glucan (A-Glu), Type I collagen				✓	[81]
Gentamicin collagen (Genta-Coll)	✓	✓		✓	[82]
Tilapia skin collagen		✓		✓	[83]
Zwitterionic betaine, Collagen			✓	✓	[84]
Hydrolyzed collagen, S-nitrosoglutathione			✓		[85]

Table 2.
Summary of collagen-based sponges in wound repair studies.

speedier re-epithelialization. A dry sponge dressing can retain a moist environment for skin repair while absorbing a greater quantity of exudate from the lesion [77]. Wu et al. [77] have produced a Tau@Col sponge that, by incorporating taurine, exhibits anti-inflammatory and antioxidant properties. This is due to the sustained release of taurine, which promotes enhanced wound healing *via* inhibition of inflammation and stimulation of proliferation. When the Tau@Col sponge was utilized to treat a full-thickness wound model in vivo on rodents, it demonstrated the most rapid closure of the wound, as well as the most effective granulation formation and collagen deposition. Another work was conducted by Yao et al. [78] to assess the safety and effectiveness of employing an absorbable collagen sponge loaded with recombinant basic FGF topically to promote wound repair in cases of traumatic ulcers. Recombinant basic FGF-containing absorbable collagen sponge substantially accelerated the wound healing process, according to the study. Shi et al. [79] developed non-toxic sponge scaffolds using porcine skin-derived collagen (PSC) and fish scale-derived collagen (FSC). In rabbit studies, PSC and FSC scaffolds showed better burn wound healing with no scars compared to gauze and vaseline gauze groups. **Table 2** summarizes the current research on collagen sponges.

3.4 Films

Primarily as a barrier, collagen films have been utilized in tissue engineering and wound healing. Collagen solutions can be used to cast and air-dry films with a thickness of approximately 0.1–0.5 mm, as with ophthalmological shields [43]. Collagen films produced *via* wind drying or freeze drying exhibit a supple and flexible texture, thereby enhancing their convenience of use and improving their ability to adhere to the wound. Furthermore, the thinness and transparency of these materials facilitate easy observation of the lesion, thereby enabling more accurate monitoring of the healing process [86]. They offer a more comfortable fit, are flexible and soft, and promote better wound adhesion [87]. Collagen possesses the ability to exert direct control over the wound microenvironment, facilitate cellular attachment and function by acting as a scaffold, and transport biologically active agents or antimicrobials to promote wound healing [21]. Rathod et al. [88] have demonstrated that collagen

film infused with calendula flower extract has an enhanced capacity for wound healing. Incorporating collagen into wound healing can help speed up the recovery process as an additional measure [89].

3.5 Composites

In contrast to alternative types of wound dressings, collagen-based composites present a multitude of benefits. They promote tissue regeneration by modulating the wound microenvironment and providing an environment conducive to cell growth [90]. More importantly, it is imperative to comprehend the distinct physiological characteristics of each patient and the characteristics of the wound to determine the most suitable wound dressing for specific instances [91]. A study by Grigore et al. [92] has yielded a collagen-nanoparticle composite scaffold that exhibits promise in facilitating tissue regeneration and wound healing while also controlling infections. Another work by Sun et al. [93] has produced a bi-layered composite dressing composed of non-crosslinked collagen with the potential to expedite the inflammatory response during the initial phases of wound healing. For wound healing, a research study by Lakra et al. [42] has developed a collagen scaffold that is reinforced with furfural. This scaffold offers both mechanical support and protection against the external environment. Information on several collagen-based composites for accelerating wound healing is compiled in **Table 3**.

3.6 Collagen nano- and micro-particles for drug/protein/peptide delivery

For accelerating wound healing, collagen nanoparticles and microparticles have demonstrated promise in the delivery of proteins, drugs, and peptides. These particles have the potential to be utilized in a multitude of medicinal domains, encompassing cosmetic purposes and the regeneration of nerve tissue, articular cartilage, skin, and bone [101]. Collagen nanoparticles possess the ability to penetrate skin lesions with relative ease, rendering them optimal candidates for therapeutic delivery under control [102]. It has been demonstrated that nanoparticles, including collagen nanoparticles, enhance the quality of wound healing and promote its progression as a result of their high ratio of surface area to volume, which enables more effective drug encapsulation and controlled release [103]. In addition, these particles possess benefits for wound healing applications [104] because their complex release profiles, which are unattainable with alternative delivery systems, can be modified *via* a multitude of parameters including material type, particle size, surface charge, encapsulation method, etc.

In the study by Li et al., the injectable granular hydrogel composed of collagen microparticles and hydroxyapatite nanoparticles connected with tannic acid demonstrated dynamic reversibility, making it effective in promoting wound healing and suppressing inflammatory responses [105]. Conversely, despite being evaluated as prospective grafting materials, collagen-type I scaffolds incorporating Aloe vera and gelatin-collagen microparticles failed to demonstrate a substantial enhancement in wound healing in the study by Gil-Cifuentes et al. [106]. This lack of improvement may be attributed to the scaffolds' low biodegradability. For the treatment of full-thickness burn infections, the curcumin-loaded microspheres encased in collagen-cellulose nanocrystal scaffolds by Guo et al. [107], exhibited encouraging outcomes in terms of preventing local inflammation and accelerating dermal regeneration. The aforementioned results collectively emphasize the adaptability and potential of collagen microparticles and nanoparticles in promoting strategies for wound healing.

Materials	Form	Outcome	Study
Type I collagen, silver nanoparticles	Nanocomposite	Antimicrobial, non-toxic, and biocompatible with skin cells and fibroblasts	[94]
Collagen, zinc oxide nanoparticles, polydopamine	Nanocomposite	Broad-spectrum antimicrobial, anti-inflammatory, promotes blood vessel generation and collagen deposition	[95]
Curcumin, PCL, collagen	Electrospun fibers	Increased mechanical properties, effective against <i>S. aureus</i> , suitable for wound management	[96]
Biogenic nano-zinc oxide, collagen	Membrane	Enhanced wound healing activity, reduced MMP-9, TNF- α , MPO, NAG, increased IL-10, comparable to standard group	[97]
Mussel-inspired scaffold with collagen and hyaluronic acid	Porous scaffold	Reduced inflammation, sustained growth factor release, excellent swelling ability, potential for diabetic wound treatment	[98]
Fish collagen, hyaluronate, sodium alginate	Lyophilized scaffolds	Multifunctional wound dressing, enhanced water absorption, controlled drug release, biocompatible	[99]
Collagen, carboxymethylcellulose, silver nanoparticles	Freeze-dried composites	Prevented infection, prospective wound healing, biocompatible, no cytotoxic effects	[100]

Table 3.
Collagen-based composites for accelerating wound healing.

4. Synthetic collagen-mimetic peptides

As wound healing is a complex biological process, it is crucial to develop novel approaches to facilitate this process. Synthetic peptides known as collagen mimetic peptides (CMPs) are engineered to simulate the characteristics and operations of endogenous collagen. Their capacity to expedite the process of wound healing has attracted considerable interest. The purpose of collagen CMPs is to replicate the appearance and functionality of collagen, a significant ECM protein that is indispensable for wound healing and physiological processes [108, 109]. The capacity of CMPs to aggregate into triple helices resembling collagen allows them to bind to partially denatured collagen, potentially playing a role in their efficacy as wound healing agents [110].

Hwang et al. [111] devised a method to deliver controlled vancomycin *via* elastin-like peptide and collagen-like peptide nanovesicles (ECnVs) affixed to collagen-containing matrices, to advance antibiotic therapeutics. The research conducted by the authors revealed that vancomycin exhibited improved entrapment efficacy, resulting in prolonged antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Furthermore, Chattopadhyay et al. [112] brought attention to the effectiveness of a bifunctional peptide that promotes wound closure in mice and facilitates collagen deposition by clustering TGF- β receptors on damaged collagen. Moreover, Baratta et al. found that CMPs can accelerate corneal lesion healing and promote regeneration of corneal epithelial cells [109].

Deng et al. [113] investigated advancements in hydrogel formulations intended for wound repair. They created a thermosensitive hydrogel composed of chitosan conjugated with recombinant collagen-peptide. This hydrogel demonstrated exceptional mechanical strength and facilitated both cell infiltration and wound healing. Mistry et al. [114] investigated the potential of nutraceutical collagen peptides for cutaneous wound healing. Their findings revealed that significant wound closure was achieved in both young and aged fibroblasts *via* increased cellular proliferation and migration. Thapa et al. [115] developed vancomycin-containing liposomes tethered to collagen-based hydrogels as an advancement in wound infection control *via* CMP-tethered liposomes. By its sustained vancomycin release and enhanced antibacterial effects against MRSA, this method offers the potential for a topical formulation that can be utilized to prevent MRSA wound infections. The wound-healing potential of collagen peptides derived from the epidermis of *Tilapia nilotica* and *Salmo salar* was investigated by Mei et al. [116]. They found that altered wound microflora colonization was associated with accelerated healing rates.

5. Collagen and extracellular vesicles

Extracellular vesicles (EVs) are vital intermediaries of intercellular communication; they are secreted by a variety of cells and perform indispensable functions in processes that promote regeneration [117]. EVs serve as mediators that enable communication between parent and recipient cells; they actively participate in the complex processes of wound healing, which encompass remodeling, hemostasis, inflammation, and proliferation [117]. The potential for harnessing the intrinsic capabilities of EV-based signaling during each of these stages of wound healing indicates a viable approach to stimulating compromised healing processes.

Extracellular vesicles (EVs) originating from a wide range of sources, including blood, skin cells, stem cells, immune cells, and epithelial cells, have exhibited the

capacity to stimulate the deposition of ECM, remodel tissue, and contract wounds [118]. For example, EVs derived from mesenchymal stem cells (MSCs) and fibroblasts have been shown to promote wound healing by optimizing the functions of endothelial and fibroblast cells [119, 120]. Furthermore, EVs derived from MSCs exhibit considerable promise in facilitating the process of wound healing due to their favorable characteristics, including minimal immunogenicity, exceptional stability, and diverse therapeutic impacts on skin regeneration [120]. An unprecedented approach to managing chronic wounds involves the integration of bioactive molecules, including miRNAs and pharmaceuticals, with transformed EVs [121]. MSCs-derived nanovesicles imitate EVs secreted by cells in nature; these nanovesicles promote wound repair in vitro and in vivo [122].

Efforts to optimize the efficiency of wound healing encompass novel approaches, including the integration of collagen and EVs, as well as hydrogel dressings that provide sustained EV release [123]. The potential of this method is illustrated by the application of collagen III protein hydrogels that release EVs for an extended period, thereby facilitating angiogenesis-dependent cutaneous wound healing [124]. The collaboration between EV release and collagen synthesis to expedite wound healing is illustrated in **Figure 2**. This process involves an inflammatory response, cellular ingestion of vesicles, and improved cell functions that ultimately result in tissue repair.

6. Collagen's regulation

Collagen, a crucial protein, actively regulates various regenerative processes such as proliferation, differentiation, inflammation, and angiogenesis. In terms of cell proliferation and cycle progression, monomeric collagen is implicated in promoting cell cycle advancement by suppressing endogenous p21 levels, thereby releasing the “regulatory brake” on the cell cycle [125]. Regarding angiogenesis, collagen fibers offer guidance for endothelial cell migration, with collagen I supporting migration and collagen IV ensuring proper lumen formation and vascular integrity [126]. Notably, collagen and fibrin collaboratively influence sprout angiogenesis, while the collagen-derived inhibitor Arresten suppresses squamous cell carcinoma invasion [127, 128]. In the realm of cell differentiation, fibroblast-derived collagens, particularly type VI collagen, play a pivotal role in modulating key steps in cancer development, including apoptosis, proliferation, angiogenesis, invasion, and metastasis [129].

The induction of collagen synthesis involves both physical and chemical factors. Physical factors like mechanical stress, electrical stimulation, and ultrasound, along with chemical factors such as ascorbic acid, copper, and retinoic acid, contribute to this process. Various studies reveal that physical and biological factors can either stimulate or hinder collagen biosynthesis at different gene expression levels. For instance, during muscle wound healing, an increase in collagen synthesis rate is observed, indicating that collagen deposition is primarily achieved through heightened synthesis during this process [130, 131]. Neocollagenesis and neoelastinogenesis are recognized for their significant roles in addressing esthetic conditions like cutaneous aging and scarring [132].

6.1 Physical factors

The process of collagen synthesis is subject to strict regulation at the transcriptional level by various cytokines [133]. A multitude of trans-acting protein factors and

cis-acting regulatory elements participate in the control of collagen gene expression [133, 134]. As an illustration, the synthesis of type I collagen fibers is controlled by a variety of positive or negative transcription factors in reaction to distinct signaling pathways [133].

Although the available evidence does not explicitly discuss the precise impacts of ultrasound, mechanical stress, and electrical stimulation on collagen synthesis and regulation, it is established that such stimuli can alter cellular behavior and gene expression, including collagen gene expression. However, additional research may be necessary to determine the precise mechanisms and effects of these physical factors on collagen synthesis and regulation. It has been shown that electrical stimulation can direct the formation of new collagen, even in the absence of neural influences [135]. The results of this study indicate that electrical stimulation has a substantial impact on the regulation of collagen synthesis in the course of wound healing. Additionally, the studies emphasize the significance of mechanical stress in regulating collagen synthesis. There is a suggestion that when using electrical stimulation at levels that affect both motor and sensory functions, the resulting mechanical environment might not completely mimic the natural stress and strain that collagen fibers typically experience during regular daily activities. In other words, the effects of electrical stimulation may not precisely replicate the usual conditions that contribute to the maturation and cross-linking of collagen fibers in the body [136].

Similarly, recent studies investigate the impact of ultrasound on the synthesis and regulation of collagen. It has been demonstrated that ultrasound can induce fibroblast proliferation and migration, thereby stimulating collagen secretion and augmenting the tensile strength of connective tissues [133]. Research on porcine lesions has

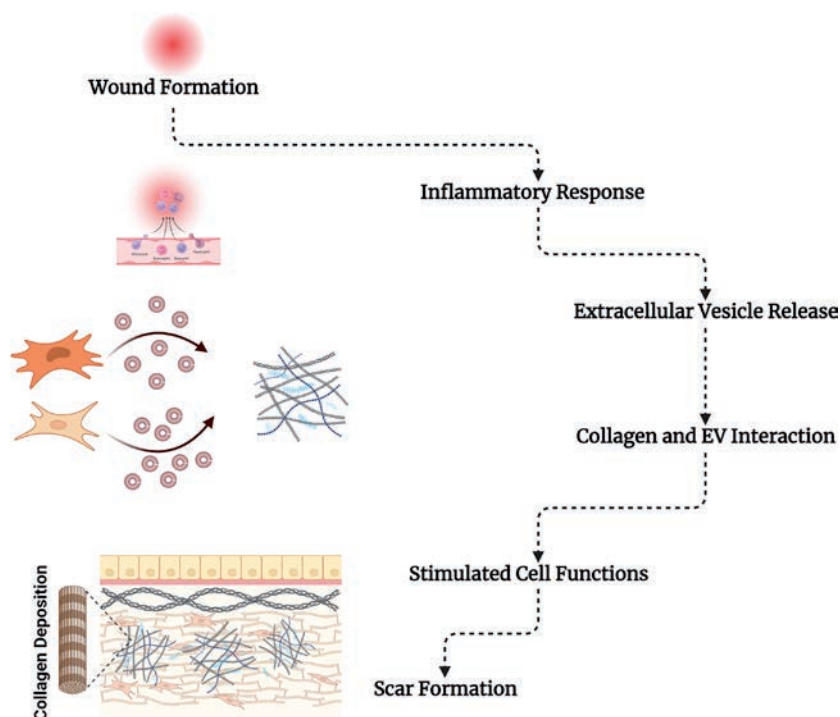


Figure 2.
Accelerating wound healing with collagen and EVs.

documented that the application of low-amplitude ultrasound promotes increased collagen deposition and density, suggesting that it has a beneficial effect on the healing process [137]. Furthermore, it has been discovered that therapeutic ultrasound can alter the biological and mechanical characteristics of fibroblasts, thereby impacting the phases of wound healing involved in regeneration and remodeling. Research has shown that the application of high-intensity ultrasound therapy can substantially enhance the synthesis of ECM constituents, such as collagen type I and fibronectin, which are indispensable for the processes of tissue regeneration and wound repair [138]. In addition, a clinical trial revealed that an ultrasonic surgery system stimulated collagen synthesis, demonstrating its potential to improve wound healing [139].

6.2 Chemical factors

A multitude of chemical factors, such as cytokines, ascorbic acid, glucocorticoids, and signaling pathways, regulate collagen synthesis. These factors are of paramount importance in the control of collagen gene synthesis and expression, which has serious implications for tissue morphogenesis, fibrosis, and even cancer.

The intricate regulation of type I collagen gene expression encompasses a multitude of cytokines operating at the level of transcription. Skin lesions, including scleroderma, may result from an overproduction and accumulation of collagen in different tissues [133]. It has been shown that ascorbic acid significantly enhances collagen synthesis while leaving other protein synthesis unaffected; furthermore, this effect seems to be unrelated to its function as a cofactor in the hydroxylation of lysine and proline [140]. Glucocorticoids inhibit collagen synthesis specifically at micro-molar concentrations, whereas cancer cells utilize mutated genes, transcription factors, signaling pathways, and receptors to modulate collagen biosynthesis [141]. Furthermore, the regulation of collagen can be achieved through the use of various inhibitory agents that target biosynthesized processes and distribution arrangements. Furthermore, indirect methods of collagen modification are also possible and are partially ascribed to other complex signaling pathways and interrelated molecular mechanisms [141].

7. Limitations and future trends

Collagen-based therapeutics, such as wound dressings containing diverse polymers, are indispensable for the process of wound healing. These scaffolds enable effective drug delivery, tissue engineering, and hemostatics through their ability to modulate the wound microenvironment and support cellular processes. Despite these advantages, collagen-based therapies have significant drawbacks, such as a dearth of high-quality research and randomized control trials substantiating their clinical utility, an absence of agreement regarding the most effective treatment approaches, and financial considerations that might impede patient access [142, 143].

Future developments in collagen-based therapies aim to overcome current limitations and enhance effectiveness, thus addressing present challenges. An example of this is the investigation of combination therapies, which entail the integration of collagen-based products with additional components for wound healing, including stem cells, EVs, and growth factors [144, 145]. Another promising approach involves using electrospun nanofibrous membranes made of recombinant human collagen type III. These membranes have demonstrated effectiveness in promoting the healing

of skin wounds [145]. Furthermore, current investigations are concentrated on the advancement of innovative collagen-based products, such as a collagen-like protein formed *via* elastin-like polypeptide fusion, which seeks to enhance the efficacy of wound healing [145]. In brief, while collagen-based therapies show promise in accelerating wound healing, the ongoing pursuit of advancements and novel methodologies, such as the development of combination therapies and new products, highlights the constantly evolving nature of this field.

8. Conclusion

Collagen is crucial to tissue preservation and regeneration, and this chapter covered wound healing in detail. Collagen coordinates tissue regeneration and repair as a dynamic conductor during ECM remodeling. The chapter covers collagen's basic activities in matrix remodeling and its many wound-healing applications, including scaffolds, micro/nanoparticles, synthetic peptides, and EV interactions. The chapter also examines collagen's regulatory role in cellular processes and collagen production stimulation methods. It looks ahead to collagen-based wound healing medicines and their potential. This chapter aims to contribute to the ongoing wound healing dialog by providing a thorough overview of current advancements and potential directions, inspiring further research and innovation in collagen-based therapeutic interventions for patients and healthcare practices.

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Collagen in Orthopedics: From Molecules to Therapies

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Abstract

Collagen, the primary constituent of the extracellular matrix (ECM) in most living organisms, is a structurally unique protein that has been classified into seven categories based on its supramolecular structure. The abundance of collagen in the human musculoskeletal system implicates it in the pathogenesis of several orthopedic conditions. Consequently, its metabolic products are useful biomarkers for the prognosis, diagnosis, and monitoring of orthopedic ailments. Collagen also finds therapeutic applications in orthopedics because of its biocompatibility, biodegradability, and mechanical stability. Several collagen-based biomaterials (CBBs) including sponges and nanofibers are currently used in orthopedic therapy. This chapter begins with a concise description of the biosynthesis of collagen as well as its classification and distribution in the human body. Subsequently, the chapter discusses the potential of collagen in orthopedic diagnostics and therapeutics while also delineating the challenges posed by collagen-based biomarkers, the risks associated with collagen from different sources, and the drawbacks of the conventional methods used to fabricate CBBs. Finally, the chapter explores the use of modern techniques like 3D bioprinting for the synthesis of highly structured collagen matrices and emphasizes the need for future research into collagen-based diagnostics and therapeutics in orthopedic surgery.

Keywords: arthritis, biomaterial, biomarker, bone tissue regeneration, cartilage, collagen, orthopedics

1. Introduction

Collagen is a morphologically diverse biopolymer found extensively in the extracellular matrix (ECM), a structural network found in most eukaryotic cells that consists of non-fibrillar amorphous substances like glycoproteins and fibronectin as well as fibrillary components such as elastin and reticular fibers [1, 2]. Collagen is the most abundant human protein and is largely present in the ECM of multiple tissues including the skin, cartilage, bones, and blood vessels. Up to 35% of the total protein mass in mammals corresponds to collagen [3, 4]. Based on their structure, function, and composition, 29 different types of collagens have been elucidated, each designated by a Roman numeral. Type I collagen is the most common type found in most

tissues, especially skin, bones, and tendons, followed by type II collagen, which is restricted to the cornea and the hyaline cartilage in joints, and type III collagen, which is mainly seen in blood vessels [5]. As diagrammatically represented in **Figure 1**, collagens can be classified into seven categories based on their supramolecular assembly, namely, fibril-forming collagens, fibril-associated collagens with interrupted triple helices (FACITs), network-forming collagen, transmembrane collagens, endostatin-producing collagens, anchoring fibrils, and beaded-filament forming collagens [6].

As shown in **Figure 2**, the biosynthesis of collagen begins in the nucleus with the transcription of specific *COL* genes encoding the pro- α 1 and pro- α 2 polypeptide chains. These genes are particular to each collagen type—for instance, *COL1A1* and *COL1A2* encode type I collagen, while *COL2A1* encodes type II collagen [8]. The transcribed mRNA moves into the cytosol where the ribosomes translate it to produce pre-procollagen chains, which are then translocated into the lumen of the rough endoplasmic reticulum, where they are converted into procollagen chains by undergoing three major post-translational modifications—removal of the N-terminal signal peptide, hydroxylation of lysine and proline by hydroxylase with vitamin C as the co-factor, and glycosylation of lysine hydroxyl groups with glucose and galactose.

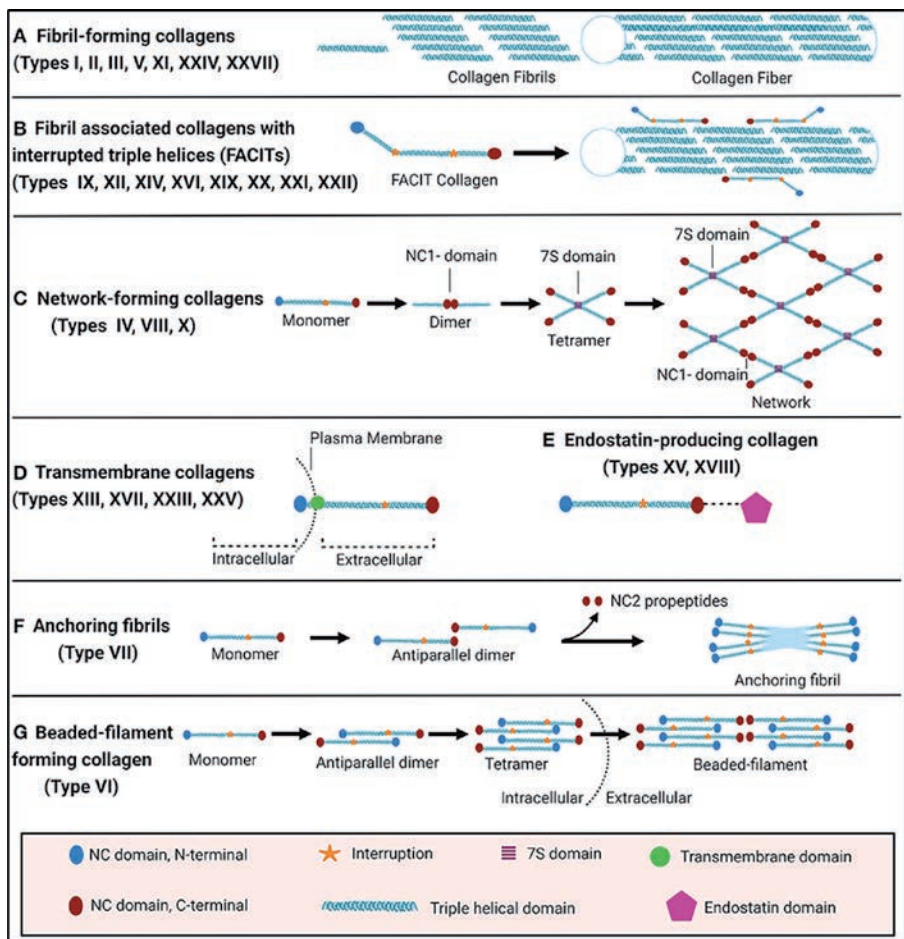


Figure 1.
Classification of collagens based on supramolecular assembly [6].

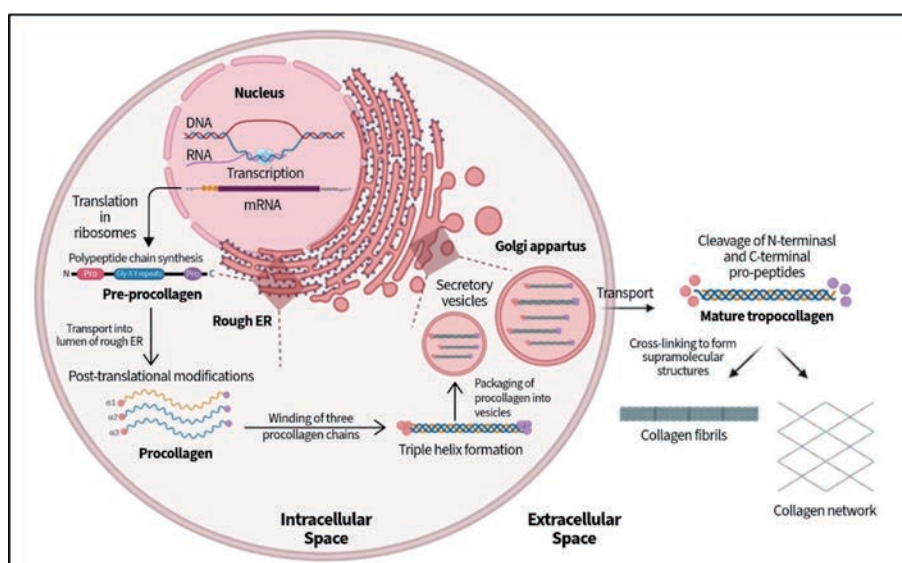


Figure 2.
 Schematic representation of collagen biosynthesis [7].

Three procollagen chains then wind around each other to form a coiled-coil triple helical structure, which moves to the Golgi apparatus, where it is packaged into secretory vesicles and transported to the extracellular space. Procollagen is converted to mature tropocollagen via cleavage of the C-terminal and N-terminal pro-peptides by collagen peptidases. Finally, in the case of type I collagen, five tropocollagen molecules assemble into a cross-linked network via covalent bonding by the action of lysyl oxidase on the lysine and hydroxylysine residues to form a supramolecule known as the collagen microfibril. These microfibrils are organized into collagen fibrils and fibers that provide structural integrity to the body tissues [6, 7]. All collagens are characterized by the presence of the Gly-X-Y motif, a repeating amino acid sequence that comprises glycine and, generally, proline and hydroxyproline as X and Y, respectively. Fibrillar collagens consist of a high percentage of uninterrupted Gly-X-Y motifs, which contributes to the stability of their fibrillar structure. The intramolecular hydrogen bonds between glycine residues impart tightness to the triple helix while proline and hydroxyproline confer resistance against proteases, thus limiting hydrolysis and maintaining structural integrity by virtue of their acyclic nature [9, 10]. Thus, they display structural, mechanical, and tissue-building properties through the regulation of critical cellular processes like proliferation, differentiation, and migration [5].

The bone is primarily a rigid, connective, osseous tissue that has two main parts—an inorganic component that constitutes 65% of the bone, comprising hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ crystals and mineral ions like sodium and potassium as well as an extracellular organic matrix known as the osteoid, 90% of which is made up of type I collagen while the non-collagenous organic molecules include proteoglycans, osteonectin, and osteocalcin. The four cell types in the osteoid are osteocytes, bone lining cells, osteoblasts, and osteoclasts. Osteoblasts are present on the bone surface and produce type I collagen. They also deposit hydroxyapatite (HA) crystals on collagen molecules to promote matrix calcification. HA provides rigidity, while collagen

provides tensile strength to the bone. Type I collagen accumulates along mechanical stress lines in the osteoid to create a structural framework for mineralization [11, 12]. Errors in collagen metabolism can lead to a variety of orthopedic disorders like osteogenesis imperfecta, Ehlers-Danlos syndrome (EDS), and certain types of skeletal dysplasia including spondyloepiphyseal dysplasia congenita (SEDC) and otospondyloepiphyseal dysplasia (OSMED) [4]. Osteogenesis imperfecta is a genetic disorder characterized by bone embrittlement due to a mutation in type I collagen while EDS leads to heritable connective tissue disorders (HCTD) caused by mutations in specific types of collagens [13, 14]. For instance, EDS type IV is an autosomal dominant disorder caused by the mutated *COL3A1* gene encoding type III procollagen [15]. Skeletal dysplasia, also known as osteochondrodysplasia, includes several genetic conditions that affect the bone and cartilage by different means. SEDC is a type II collagen disorder while OSMED is caused due to problems in type XI collagen [16].

The first reported collagen-based biomaterial (CBB) was the “catgut,” a collagen-rich suture material developed in 1881 by Joseph Lister, the father of modern surgery [17]. Given its biocompatibility, tensile strength, biodegradability, mechanical stability, and flexibility, collagen is an excellent therapeutic biomaterial. Currently, various CBBs like sponges, hydrogels, microspheres, nanofibers, and injectable solutions are commercially used for several applications including surgery, wound healing, oral therapy, tissue regeneration, and drug delivery [18]. Furthermore, collagen is a viable biomarker since it is abundantly present in most tissues and can be detected in blood plasma and tissue samples. The metabolic products of collagen are used to diagnose and monitor several conditions including cancer [19, 20].

This chapter focuses on the applications of collagen in orthopedic surgery. Orthopedics refers to the diagnosis, treatment, and prevention of injuries and disorders affecting different components of the musculoskeletal system. Being a predominant protein in the bone and cartilage, collagen and its metabolic products are widely used as diagnostic biomarkers for the detection of orthopedic conditions that require more efficient monitoring methods including ankylosing spondylitis, osteoarthritis, and rheumatoid arthritis. The orthopedic applications of collagen-based diagnostics have been delineated in the following section of the chapter. The next section discusses the use of collagen in orthopedic therapeutics such as sponges, hydrogels, and microspheres for the treatment of bone injuries, cartilage defects, athletic strain, and disorders like rheumatoid arthritis. The subsequent section concentrates on the challenges associated with the use of collagen in orthopedics including the drawbacks of different sources of collagen and the shortcomings of the techniques employed for its extraction, cross-linking, and sterilization. It also explores modern technologies such as plastic compression, electrospinning, and 3D bioprinting that have been introduced in recent years for fabricating highly structured CBBs. The chapter emphasizes the need for further research into the clinical applications of collagen-based diagnostics and therapeutics in the field of orthopedics.

2. Collagen-based orthopedic diagnostics

Due to its extensive presence throughout the ECM of the musculoskeletal system, the collagen network is affected during the pathogenesis of several orthopedic conditions. Thus, the levels of collagen degradation products serve as diagnostic indications of certain orthopedic disorders, especially those affecting the articular cartilage such as osteoarthritis (OA) and rheumatoid arthritis (RA). The hyaline articular cartilage

is a connective tissue lacking nerves, blood vessels, and lymphatics that is present between joints. It is made up of chondrocytes and has a dense collagenous network in the ECM that is rich in collagen type II followed by types IX and XI, while types III, IV, V, VI, X, XII, XIV, XVI, XXII, and XXVII are present in minor quantities. Based on collagen arrangement and chondrocyte proximity, the articular matrix is divided into three zones—the pericellular matrix (PCM) surrounding the chondrocytes, the territorial matrix surrounding the PCM, and the interterritorial matrix, which forms the largest zone. Type I and type III collagens are predominant in the peri-articular soft tissues, while the PCM consists of type III–VI collagens [7, 21]. During disease conditions, the articular cartilage gets damaged, leading to collagen degradation in the ECM by collagenases like A disintegrins and metalloproteinases with thrombospondin motifs (ADAMTS), cathepsins, and matrix metalloproteinases (MMPs), mediated by inflammatory molecules. Several fragments are released into the systemic circulation and the synovial fluid due to collagen degradation. Thus, these fragments act as highly specific and sensitive biomarkers indicative of the underlying clinical conditions. **Table 1** encapsulates the distribution of collagens in the articular cartilage and the proteinases that degrade them, especially during orthopedic conditions [22, 31].

2.1 Ankylosing spondylitis (AS)

Ankylosing spondylitis (AS), also known as axial spondyloarthritis (axSpA), is a systemic, chronic, inflammatory autoimmune condition brought about by genetic and environmental factors that affects the spine, sacroiliac joints (SIJs), and adjacent soft tissues. It is characterized by joint inflammation, fibrosis, calcification, and

Collagen type	Distribution in articular cartilage	Collagen-degrading proteinases	References
Type I	Fibrocartilage, elastic cartilage	MMP-2	[23]
Type II	ECM of all zones	MMP-1, 3, 13	[24]
Type III	PCM	MMP-3	[25]
Type IV	PCM	MMP-2, 9	[23]
Type V	PCM	MMP-2, 9	[26]
Type VI	PCM	MMP-2, 9	[27]
Type IX	Growth-plate cartilage	MMP-3, 13	[28]
Type X	Calcified zone and hypertrophic cartilage	MMP-1, 3, 13	[29]
Type XI	Articular cartilage	MMP-2	[30]
Type XII	Cartilage with more organized fibril orientation	Not available	
Type XIV	Uniformly throughout the articular cartilage	MMP-13	[22]
Type XVI	Territorial matrix of chondrocytes	Not available	
Type XXII	Articular surface of joint cartilage		
Type XXVII	Proliferative zone chondrocytes		

Table 1.
Distribution and susceptibility of collagens in the articular cartilage [22].

ultimately, a progressive increase in spinal rigidity resulting in loss of flexibility and movement due to complete fusion of the spine [32]. While the pathophysiology of AS is yet to be elucidated, it is associated with the HLA B27 gene polymorphism [33]. The onset of AS begins with immune dysregulation leading to chronic inflammation, brought about by the interleukin-17/23 (IL-17/23) pathway and tumor necrosis factor (TNF) as well as other pro-inflammatory cytokines such as IL-6, IL-10, IL-22, and interferon γ (IFN γ). This is followed by matrix erosion caused by MMP-1 and cathepsin K. Finally, bone fusion occurs during syndesmophyte formation by the action of the bone morphogenetic protein (BMP) and Wnt signaling pathway, the master regulator of bone remodeling [34]. Currently, C-reactive protein (CRP) is the only inflammatory marker that is clinically used to diagnose axSpA. There is an exigent need for sensitive biomarkers that facilitate effective diagnosis and monitoring of AS [35]. During AS, the cartilage intermediate layer protein 1 (CILP-1) produced by chondrocytes for the reduction of ECM proliferation is cleaved by MMP-1, MMP-8, and MMP-12 leading to increased levels of CILP-M, an MMP-generated neoepitope of CILP-1 [36]. Collagen fibril formation is disrupted in AS by the action of MMP-2 and MMP-9 which degrade type V collagen to produce C5M fragments. Thus, elevation of C5M levels also serves as an indicator of AS [26]. Further, due to extensive ECM remodeling, MMP-mediated fragments of collagen types I, III, IV, and VI, namely, C1M, C3M, C4M2, and C6M, respectively, are present in higher amounts in AS patients, thereby facilitating their use as blood-based biomarkers for tracking disease severity and progression [37].

2.2 Osteoarthritis (OA)

Osteoarthritis (OA) is a chronic, degenerative joint disease associated with aging. It is characterized by cartilage degradation, bone remodeling via BMPs, synovial membrane inflammation, subchondral osteosclerosis, synovial angiogenesis, and osteophyte formation by the action of vascular endothelial growth factors (VEGFs). As OA progresses, chondrocytes display a senescence-associated secretory phenotype (SASP) wherein they display mitochondrial dysfunction, leading to oxidative stress and ultimately undergo senescence. The cartilage breakdown products are phagocytosed by synovial cells which release pro-inflammatory and catabolic molecules including cytokines like TNF α and IL-6, chemokines, neuropeptides, bioactive lipids like prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), as well as adipokines like lipocalin, leptin, and adiponectin [22, 38, 39]. Though chondrocytes produce anti-inflammatory mediators like IL-3, IL-4, and IL-1Ra as well as tissue inhibitors of metalloproteinases (TIMPs), they are outnumbered by pro-inflammatory molecules, thus allowing synovial inflammation to increase [40]. CILP is overproduced in early OA and inhibits tumor growth factor β (TGF β), thereby hindering ECM proliferation [41]. The pathophysiology of OA has been diagrammatically elucidated in **Figure 3**. Oxidative stress and low-level inflammation lead to the breakdown of the collagen network in the ECM primarily via the degradation of type II collagen by MMP-13 (collagenase-3) along with MMP-1 (collagenase-1) and MMP-3, all three of which are upregulated by lipocalin and adiponectin while leptin upregulates MMP-1 and MMP-3 production [42]. Furthermore, collagen type II production is inhibited by IL-1 β , due to which reduction in its levels is suggestive of OA. The MMP-mediated cleavage of type II collagen also results in the accumulation of COL2-3/4C (short) neoepitopes and urinary C-terminal telopeptide (uCTX-II), which act as highly informative biomarkers as their levels increase with disease progression [43, 44].

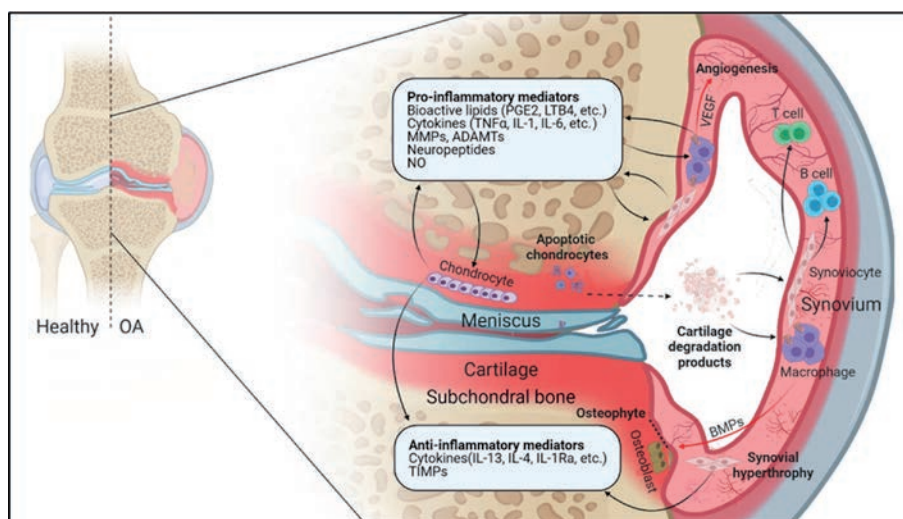


Figure 3.
 Pathophysiology of osteoarthritis [40].

Due to the increased deposition of type III collagen on the joints, the COL3/ADAMTS neoepitope, an ADAMTS-generated type III collagen fragment, serves as a serum biomarker for early OA [45, 46]. On the other hand, the presence of MMP-mediated type 3 collagen metabolites (C3M) indicates the degradation of soft tissues. Lipocalin and adiponectin increase the levels of inducible nitric oxide synthase, leading to the production of nitric oxide (NO), which causes protein nitration. Thus, increased serum concentrations of Coll 2-1 and Coll 2-1 NO₂ peptides are diagnostic OA markers [47, 48]. Leptin and lipocalin stimulate type X collagen (Col10) production, which contributes to the hypertrophy of chondrocytes, a characteristic of OA [49]. Thus, in addition to IL-1 β , osteocalcin, osteopontin, and VEGF, type X collagen is also a major biomarker for chondrocyte hypertrophy [50]. Moreover, the blood serum levels of Col10neo, a cathepsin K-generated neo-epitope of Col10, signify hypertrophic chondrocytes and can be used to detect OA in the knee joints [51]. The presence of type I collagen in the articular cartilage denotes the potential onset of OA since it is a component of the fibrocartilage repair tissue that fills cartilage defects [52].

2.3 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a systemic, chronic autoimmune disorder characterized by synovial joint damage, synovial inflammation, cartilage destruction, and matrix degradation [53]. As shown in **Figure 4**, there is an imbalance between the pro-inflammatory and anti-inflammatory cytokines in RA due to the overproduction of TNF α , IL-1 β , and IL-6 by synovial tissue macrophages (STMs), leading to chronic inflammation. This stimulates the release of matrix-degrading enzymes by the chondrocytes, leading to ECM degradation. Another major characteristic of RA is the tumor-like transformation of activated synovial cells into fibroblast-like synoviocytes (FLS), highly proliferative cells that produce MMP-1, MMP-3, MMP-13, ADAMTS4, ADAMTS5, and cathepsins. These collagenases cause tissue damage and promote the infiltration of immune cells like neutrophils and macrophages, which also release MMPs, ADAMTS, and pro-inflammatory cytokines, thereby exacerbating the

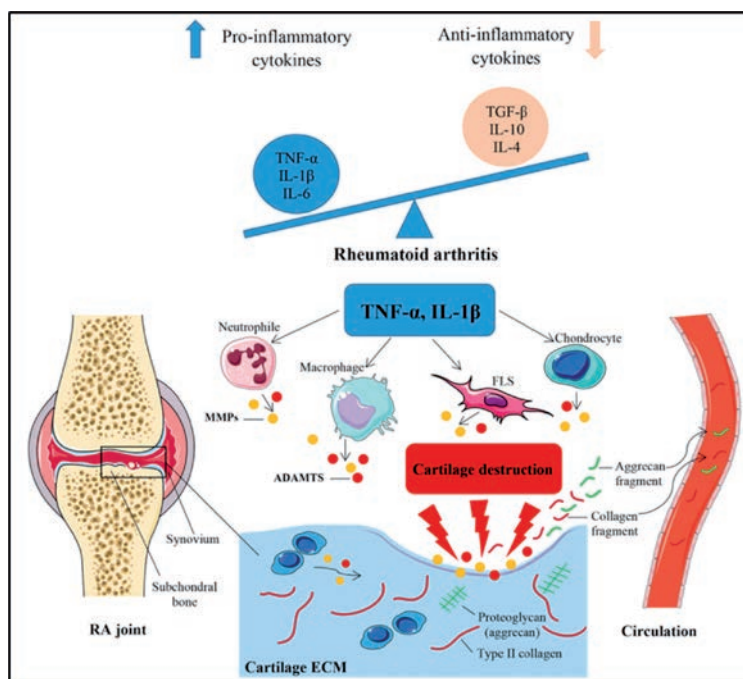


Figure 4.
Pathophysiology of rheumatoid arthritis [54].

inflammatory condition [55]. RA is characterized by several autoantigens including guanosine proteins, type II collagen, and circulating serum proteins. Autoantibodies like the rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) that are produced to counter these antigens serve as diagnostic markers of RA [22, 54, 56]. During disease progression, MMP-13 cleaves type II collagen, thus reducing its levels and leaving behind degradation products in the articular cartilage. The breakdown of soft tissues involves the MMP-mediated cleavage of type I and type III collagens, leading to the release of C1M and C3M, respectively—thus, their serum levels are used to track RA progression [57]. The metabolism of type IV collagen during destructive synovitis growth due to the tumor phenotype of FLS leads to elevated serum levels of C4M, another marker for disease prognosis [58].

3. Collagen-based orthopedic therapeutics

As a major structural protein in the human body, collagen plays a vital role in the repair and regeneration of tissues. Therefore, collagen-based therapies have been widely studied for clinical applications in bone and soft tissue regeneration. The role of collagen in maintaining joint strength and bone health, in combination with its breakdown during orthopedic conditions, makes collagen-based biomaterials (CBBs) a viable choice for managing musculoskeletal injuries and diseases, especially those involving cartilage damage, which cannot be reversed. Generally, naturally occurring type I and type II collagen proteins are extracted from human, bovine, murine, equine, and porcine sources such as bones, tendons, and skin as well as marine sources like fish skin, scales, and bones, via ECM decellularization. The undenatured

proteins are then subjected to protein hydrolysis to obtain hydrolyzed soluble bioactive peptides that have better digestibility, absorption, and distribution during oral administration. Collagen can also be produced synthetically as in the case of recombinant human collagen (rhCOL) obtained by transfecting COL genes into different prokaryotic and eukaryotic expression systems such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Zea mays*, and *Trichoplusia ni*, as well as transgenic animal models like mouse embryos and Chinese hamsters [59]. The composition, properties, and functionality of CBBs vary depending on the source of collagen and the hydrolysis process. While developing CBBs, the effect of the collagen source and extraction technique on properties such as biodegradability, immunogenicity, biocompatibility, and mechanical stability must be considered [60, 61]. CBBs are available in several forms including scaffolds, oral supplements, microspheres, and nanospheres, each of which lend themselves to specific therapeutic applications.

3.1 Collagen-based scaffolds

Collagen-based scaffolds are three-dimensional biomimetic matrices having porous, interconnected structures that facilitate the regeneration of damaged bone tissues. Two types of collagen scaffolds widely used in orthopedic therapy are sponges and hydrogels. Since the use of pure collagen does not facilitate control over scaffold properties like porosity and stability, most scaffolds use composites of collagen mixed with natural polymers like chitosan, silk fibroin, and hyaluronic acid, synthetic polymers such as polyethylene glycol (PEG), polyglycolide (PGA), and polyvinyl alcohol (PVA), as well as ceramics including hydroxyapatite, β -tricalcium phosphate (β -TCP), and silicate [62, 63]. Collagen sponges are generally developed by the process of freeze-drying, also known as ice-segregation-induced self-assembly or lyophilization. In this process, the collagen-based solution is frozen to entrap collagen molecules within hexagonal ice crystals, followed by sublimation of the water molecules, leading to the formation of intermolecular cross-links between the collagen aggregates, thus creating a highly porous, sponge-like structure, as shown in **Figure 5**. The shape and pore size of the sponge can be altered by controlling the fabrication parameters including duration, freezing temperature, and mold morphology. The optimal pore size must allow cell migration and nutrient diffusion while also facilitating cell attachment [64, 65]. Collagen sponges are generally loaded with growth factors like BMPs and fibroblast growth factors (FGFs) for use in bone tissue regeneration and cartilage defect repair to promote fibrin network formation in the ECM, chondrogenic differentiation of mesenchymal stem cells (MSCs), as well as osteoblast proliferation and migration [66, 67]. An atelocollagen sponge loaded with recombinant human BMP-2 (rhBMP-2) and implanted into mice models was shown to promote bone formation

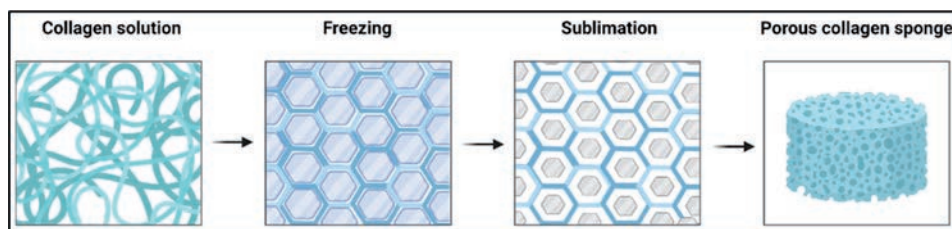


Figure 5.
Freeze-drying process of collagen sponge formation [64].

via osteoconduction [68]. Highly porous collagen implants were successful in treating injured rotator cuffs via connective tissue repair in human subjects [69]. Collagen sponges are also used to deliver antibiotics and biomolecules in orthopedic conditions like acute septic arthritis [70]. They are preferred due to their ease of processing, sterilization, loading, and preservation as well as their optimal functionality in inducing tissue regrowth [64].

Collagen hydrogels are hydrophilic, versatile scaffolds that can adopt a range of structures and physical properties based on factors like collagen concentration, pH, temperature, and ionic strength. Injectable hydrogels and hydrogel implants synthesized using collagen in combination with substances like hydroxyapatite, chitosan, and alginate are used for *in situ* bone tissue repair as they display physiological bone-like surface properties, minimal invasion, and high biocompatibility [71]. Given their highly absorbent, water-rich micro-environment and suitability for cell encapsulation, they provide an efficient biomimetic substrate for carrying adult human bone marrow-derived stem cells (hBMSCs), thus promoting ECM development and bone regeneration. They are also viable carriers of growth factors like rhBMP-2 and therapeutic drugs like doxycycline in orthopedic conditions since they form a continuous, direct delivery system and ultimately promote osteogenesis [72–74].

3.2 Collagen-based oral supplements

Oral supplementation is a popular form of collagen-based therapy, given its biocompatibility, digestibility, and bio-accessibility. Upon oral administration in the hydrolyzed form, collagen gets digested in the gastrointestinal tract and releases amino acids and peptides in the small intestine, which then enter the bloodstream. The high percentage of proline and hydroxyproline in collagen protects the peptides from intestinal proteolytic enzymes, thereby preserving their bioactivity. The two forms of collagen in oral supplements are undenatured type II collagen (UC-II), which retains its biological activity, and partially hydrolyzed collagen, which is degraded enzymatically or physically using heat/pH. Hydrolyzed collagen extracted from eggshell membranes reduces the levels of CTX-II and other inflammatory biomarkers [75]. Oral supplementation of collagen has been widely used to treat the symptoms of OA and RA including inflammation and joint pain. While both forms of collagen are effective, the analgesic benefits conferred by hydrolyzed collagen supplements were seen to be stronger in the case of OA, potentially due to better absorption [76]. The administration of an oral preparation comprising hydrolyzed collagen type II in combination with bioactive oligopeptides of natural hydrolyzed keratin, chondroitin sulfate, and hyaluronic acid was shown to reduce synovial fluid levels of the inflammatory cytokines, IL-6, IL-8, IL-10, and alleviate joint pain in patients with knee OA, thereby demonstrating its viability as an adjuvant orthopedic therapy [77]. Daily oral consumption of hydrolyzed type 1 collagen (hCol1) is another potential strategy to reduce pain and inflammation via chondrocyte protection during post-traumatic OA [78]. Collagen peptides are also viable analgesics in orthopedic treatments—they significantly reduce knee pain in OA patients through the chondroprotective effect brought about by reducing the expression of MMP-13, thereby inhibiting the degradation of type II collagen [79, 80]. The oral form of chicken type II collagen is effective in inducing oral tolerance and subsequently reducing inflammation, pain, joint swelling, and stiffness in RA patients [81, 82]. Type II collagen-mediated oral tolerance is also a viable treatment for collagen-induced arthritis (CIA) as it causes an anti-inflammatory effect by increasing the production

of anti-inflammatory IL-10, ultimately decreasing joint swelling and pain levels [83]. Bovine type II collagen induces oral tolerance in CIA, leading to reduced levels of pro-inflammatory IL-17 and subsequent downregulation of the IL-17 stimulated expression of receptor activator of nuclear factor κ B ligand (RANKL), an osteoclastogenic mediator, in CD4⁺ T-cells [82]. Bioactive collagen peptides (BCP) are also used as oral supplements for alleviating knee pain [84].

3.3 Collagen-based microspheres and nanospheres

Collagen-based microspheres are spherical microstructures ranging in diameter from 3 to 40 μ m. They are produced by the emulsification of native collagen in organic solvents like paraffin followed by glutaraldehyde-based cross-linking [85]. Mesenchymal stem cells encapsulated in collagen microspheres are used in articular cartilage repair since they promote chondrogenic differentiation and hyaline cartilage-specific gene expression. Further, the condensed collagen meshwork in microspheres is more stable with higher cell density as compared to collagen gels, and its superior chondroconductive capacity facilitates remodeling of the type I collagen template into a biomimetic, ECM-like matrix comprising type II collagen, glycosaminoglycan (GAG), and aggrecan [86]. Collagen microspheres can also efficiently maintain a cellular network of human osteoarthritic chondrocytes (hOACs). As shown in **Figure 6**, hOACs were enzymatically isolated from cartilage tissue samples of OA patients and encapsulated into collagen microspheres. The 3D hOAC-collagen microsphere model was shown to recapitulate the OA phenotypes more efficiently than the traditional 2D monolayer cultures and 3D pellet cultures in terms of physiologically relevant cell density, ECM interactions, MMP-13 levels, and extracellular deposition of type II and type X collagen. Thus, collagen microspheres are useful three-dimensional *in vitro* models for studying OA and screening potential therapies [87]. Fibrillar collagen microspheres of different sizes and morphologies can be prepared using water-in-oil emulsions by controlling the hydrophilic-lipophilic balance (HLB) and promoting the fibrillogenesis and gelling of collagen molecules, thus negating the need for cross-linking. These injectable microstructures are highly stable and biocompatible, and they mimic the ECM micro-environment, which is why they find application in the treatment of bone injuries and cartilage defects, especially in drug delivery systems [88]. Collagen microspheres are widely used in bone tissue regeneration as carriers of antibacterial drugs, glucocorticoids, and growth factors like BMP and VEGF since their degradation rate can be controlled via chemical

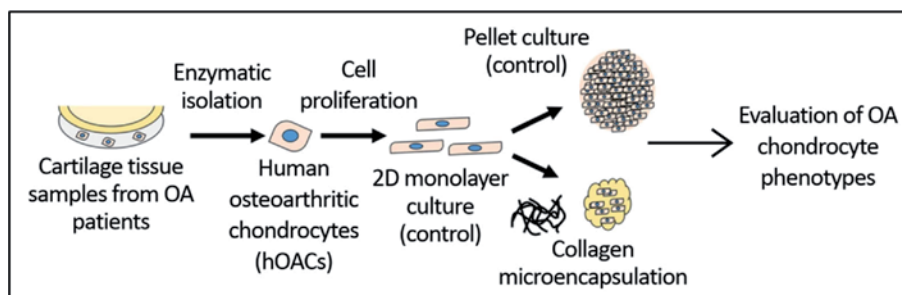


Figure 6.
 Synthesis and evaluation of a 3D hOAC-collagen microsphere model [87].

modifications like cross-linking thus facilitating the time-controlled, systematic release of the encapsulated molecules [89].

Collagen nanospheres are spherical structures with a diameter in the nano-range. They are generally synthesized using a high-voltage electrostatic field via self-assembly and fibrillogenesis. They can be used as drug delivery systems and as carriers of growth factors and other bioactive molecules. They also form a major component of scaffolds and implants used in bone tissue regeneration since they can effectively encapsulate stem cells and promote cell differentiation and proliferation [89, 90]. Factors like temperature and collagen type impact the size and structure of the nanospheres. Temperatures below 37°C are generally favored, and type II collagen is preferred for maintaining the spherical structure of microspheres. While type I collagen yields nanospheres of relatively smaller sizes, the sphericity of type II collagen nanospheres is better, due to which the latter is generally preferred for use in CBBs [91]. However, type I collagen nanospheres have been shown to promote the osteogenic differentiation and proliferation of bone marrow stromal cells *in vitro*, thus highlighting their application in orthopedic treatments for bone tissue formation [92].

4. Challenges and future perspectives

Despite the vast potential of collagen in orthopedic diagnostics and therapeutics, there are certain challenges that hinder its widespread use. Further research is necessary to elucidate reliable markers of collagen metabolism that are indicative of particular disease conditions in order to increase the accuracy of collagen-based diagnostics. Simple, non-invasive, rapid detection techniques must be developed for assessing the levels of collagen and its metabolic products with a high degree of sensitivity and specificity in order to facilitate the use of collagen-based biomarkers in detecting orthopedic disorders like OA and RA [22]. The source of collagen for use in CBBs poses a serious problem—while collagen is abundantly present in most living organisms, its extraction from animal sources presents several risks including allergy, disease transmission, degradability, and immunogenicity. Collagen derived from bovine and porcine sources was seen to cause allergic reactions such as anaphylaxis in up to 4% of the population [59]. Additionally, prion-contaminated collagen from terrestrial animals can transmit zoonotic diseases like foot-and-mouth disease (FMD), transmissible spongiform encephalopathy (TSE), and bovine spongiform encephalopathy (BSE) which leads to the variant Creutzfeldt-Jakob disease (vCJD) in humans [93]. While these issues may be negated by the use of marine-derived collagen, the lack of substantial clinical trials coupled with its comparatively low thermal stability, rapid degradation *in vivo*, and poor mechanical strength impedes its application in orthopedics [94, 95]. Furthermore, animal-derived collagen telopeptides are antigenic and may trigger immune responses, leading to hypersensitivity reactions and graft vs. host diseases [96]. The use of collagen derived from human placenta and skin, on the other hand, is hindered by the large batch-to-batch variation in the biophysical properties of collagen based on parameters such as age, environmental factors, genotype, and ethnicity, which cannot be standardized [97]. Moreover, human-derived collagen presents ethical concerns related to informed consent, religious beliefs, and data privacy. Despite ongoing research into artificially synthesized collagen-like peptides, they cannot substitute natural collagen due to high costs and absence of the natural tertiary structure, due to which they cannot reproduce

several biophysical properties necessary for biomedical applications [98]. Though it is considered a viable alternative, recombinant human collagen (rhCOL) also has certain shortcomings including the deficit of high protein throughput models, poor yield in mammalian cell cultures, high production costs, increased susceptibility to proteolytic degradation, higher frequency of misfolding in prokaryotic systems, and instability due to the lack of post-translational modifications in collagen obtained from expression systems that lack prolyl 4-hydroxyprolin (P4H), a heterotetramer enzyme necessary for the folding of collagen polypeptide chains to form triple helical structures [99, 100]. PH4-transduced plant systems like *Nicotiana tabacum* and *Zea mays* are currently preferred since they produce good yields of type I collagen [101]. Thus, despite the abundance of collagen, each of its sources has certain disadvantages that need to be addressed to optimize the cost-effective, large-scale production of collagen for orthopedic use.

Furthermore, the techniques employed for the extraction and cross-linking of collagen as well as the sterilization of CBBs, also cause certain issues. Fibrillar collagen, in its native form, is insoluble and resistant to proteolysis, which is why only type I collagen can be extracted in its original form from adult tissues. Thus, collagen is generally extracted in the form of hydrolyzed, soluble molecules through denaturing treatments using acids, alkalis, and proteolytic enzymes. However, the fibril-forming capacity of these molecules is lower than that of native collagen, especially due to the protease-mediated cleavage of terminal telopeptides [102, 103]. Further, the cross-linking of hydrolyzed collagen using chemical agents like glutaraldehyde and polyepoxy compounds may leave behind toxic residual compounds, thus rendering the CBB unfit for human use [104]. While physical cross-linking methods such as dehydrothermal treatment and UV irradiation do not pose the risk of toxicity, prolonged exposure could lead to the partial or even complete degradation of collagen [105]. Thus, non-conventional cross-linking methods using enzymes like transglutaminase and natural, biocompatible cross-linkers like genipin must be further developed [106]. The fragility and thermal sensitivity of CBBs prevent sterilization via several common methods like autoclaving and electron beam irradiation with gamma rays since these approaches cause molecular degradation and structural alteration of the collagen scaffolds. Even milder approaches like β -ray irradiation and ethylene oxide sterilization cause some level of damage, and their use is restricted to specific types of CBBs [103]. Currently, ethanol treatment is used for physically cross-linked collagen, while immersion in peracetic acid is employed in the case of acellular collagen ECM. However, no known method has left the molecular structure of collagen unaltered post-sterilization [107, 108].

Moreover, the inability of traditional CBBs to regenerate the fibrillar structure of natural collagen leads to issues such as poor tensile strength and loss of biophysical properties. In recent years, modern technologies such as plastic compression, electrospinning, and 3D bioprinting have been introduced for the synthesis of highly ordered collagen matrices [109]. Plastic compression involves the rapid expulsion of water from cell-seeded collagen gels in order to synthesize strong, tissue-like collagen matrices without the need for chemical cross-linking and cell activity. In order to improve the scalability and reproducibility of plastic compression, the technique has been modified using upward fluid flow, which facilitates the synthesis of multi-layered scaffolds [110, 111]. Electrospinning is widely used for CBB synthesis since it allows the fabrication of collagen nanofibers with different morphologies that mimic the structural properties of native collagen in the ECM. However, despite the architectural versatility and biocompatibility of electrospun collagen fibers, the solubilization

and processing of collagen prior to electrospinning leads to undesirable properties such as low elasticity, increased hydrophilicity, low mechanical strength, and high degradability [109, 112]. Near-field electrospinning, also known as melt electrowriting, is a modified electrospinning process that involves the extrusion of collagen fibers by the force of the electrical field followed by their immediate collection to avoid bending instabilities, which is achieved by reducing the air gap. The process allows precise control over individual collagen fibers, thereby facilitating the fabrication of tailored collagen microstructures and nanofibers with precise geometries. However, parameters like air gap distance, applied voltage, spinneret needle geometry, melt composition, and flow rate must be standardized prior to the widespread use of melt electrowriting [113]. 3D bioprinting is another highly advanced technique that produces hierarchical constructs via layer-by-layer deposition using collagen solutions as the bioink. The major disadvantage of this technique is the poor mechanical strength and stability of the CBBs, which can be overcome by using hybrid collagen-based bioinks containing substances like thrombin, hydroxyapatite, alginate, agarose, silk, and gelatin. Mechanical stability can also be improved by printing within a pre-formed mold, using thermo-reversible supports like gelatin slurries, increasing the density of the collagen bioink, and cross-linking the collagen fibers [106, 114, 115]. However, despite the advantages of modern fabrication techniques, they must be optimized through further research in order to promote the clinical use of CBBs in orthopedic therapy.

5. Conclusion

In conclusion, collagen is a unique component of the ECM with several desirable properties such as biocompatibility, biodegradability, and mechanical stability that can be harnessed for use in orthopedics. Given the involvement of collagen in the pathogenesis of orthopedic conditions, its metabolic fragments serve as biomarkers for the detection of diseases like ankylosing spondylitis and rheumatoid arthritis. With the development of efficient detection strategies and a deeper understanding of the mechanisms underlying disease pathogenesis, the potential of collagen-based diagnostics can be translated into widespread clinical applications. Since the invention of the catgut in 1881, several advanced CBBs including scaffolds and microstructures have been developed for orthopedic use. Innovative approaches are required to overcome the drawbacks associated with the different sources of collagen as well as the currently used extraction, processing, and sterilization methods. Further research into modern fabrication techniques like melt electrowriting and 3D bioprinting is of utmost importance for the integration of CBBs into orthopedic therapy. Despite its shortcomings, the unique characteristics of collagen place it at the forefront of biomedical research in the field of orthopedics.

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Conflict of interest

The authors declare no conflict of interest.

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Since the completion of the human genome sequencing, our understanding of cell and molecular biology has expanded at an extraordinary pace. Regions of DNA once dismissed as “junk DNA” are now recognized as critical elements that regulate gene expression and can influence the structure and activity of the cell. Investigators continue to discover new connections between well-characterized pathways and new modulators of the cell cycle. Advances in protein chemistry continue to elucidate the complexities of proteins involved in cell structure, extracellular matrix, and cell-to-cell communication between proteins expressed on the plasma membrane. This volume brings together exciting insights into the molecular mechanisms that define specialized cell types and explores how this knowledge can reveal insights into protecting healthy cells or triggering the death of transformed cells. We hope these chapters inspire readers as deeply as they have engaged the authors and editors of this edition.

Andrei Surguchov, Biochemistry Series Editor

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