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Review

CAR-NK cell therapy: a potential antiviral platform

Ming-Hao Lin [a,1], Li-Juan Hu [a,1], Jeffrey S. Miller [b,*], Xiao-Jun Huang [a,c,*], Xiang-Yu Zhao [a,*]

[a] Peking University People's Hospital, Peking University Institute of Hematology, National Clinical Research Center for Hematologic Disease, Beijing Key Laboratory of Cell and Gene Therapy for Hematologic Malignancies, Peking University, Beijing 100044, China
[b] Department of Medicine, University of Minnesota, Minneapolis, 55455, USA
[c] Peking-Tsinghua Center for Life Sciences, Beijing 100871, China

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ABSTRACT

Viral infections persist as a significant cause of morbidity and mortality worldwide. Conventional therapeutic approaches often fall short in fully eliminating viral infections, primarily due to the emergence of drug resistance. Natural killer (NK) cells, one of the important members of the innate immune system, possess potent immunosurveillance and cytotoxic functions, thereby playing a crucial role in the host's defense against viral infections. Chimeric antigen receptor (CAR)-NK cell therapy has been developed to redirect the cytotoxic function of NK cells specifically towards virus-infected cells, further enhancing their cytotoxic efficacy. In this manuscript, we review the role of NK cells in antiviral infections and explore the mechanisms by which viruses evade immune detection. Subsequently, we focus on the optimization strategies for CAR-NK cell therapy to address existing limitations. Furthermore, we discuss significant advancements in CAR-NK cell therapy targeting viral infections, including those caused by severe acute respiratory syndrome coronavirus 2, human immunodeficiency virus, hepatitis B virus, human cytomegalovirus, and Epstein-Barr virus.

1. Introduction

Viral infections are capable of initiating various chronic diseases, such as acquired immune deficiency syndrome (AIDS), hepatitis B and hepatitis C, which pose profound threats to both human health and societal stability [1]. Moreover, although coronavirus disease 2019 (COVID-19) was originally identified as an acute infectious disease, emerging research suggests that its pathogen may persist in the human body for extended periods, potentially evolving into a chronic infection as well [2]. Unfortunately, existing antiviral treatments have not yet achieved complete eradication of these infections. This is primarily due to the ability of infected cells to establish a latent reservoir, where the viruses can remain dormant for extended periods and potentially resume production of infectious viruses at any point, even developing resistance to antiviral drugs [3–7]. As a result, there is an imperative necessity to devise therapeutic strategies that can effectively manage and eradicate this viral reservoir over the long term. Natural killer (NK) cells are integral components of the innate immune system, and are able to control viral infections through several killing mechanisms. Chimeric antigen receptor (CAR)-NK cell therapy possesses the capability to redirect the cytotoxic function of NK cells towards virus-infected cells both via the CAR and their innate receptors, further enhancing their cytotoxic activity.

This review explores the biological study of NK cells in the context of controlling viral infections, with a focus on the mechanisms by which viruses escape from NK cell-mediated cytotoxicity. Additionally, it provides a comprehensive overview of clinical trials investigating NK cell-based antiviral therapy, highlighting the potential benefits and limitations of CAR-NK cell therapy in the treatment of viral infections. The review further discusses the optimization strategies of CAR-NK cells and their application across various viruses, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), human immunodeficiency virus (HIV), hepatitis B virus (HBV), human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV).

2. NK cell in antiviral immunotherapy

2.1. Biological study of antiviral effect of NK cells

NK cells, a crucial subset of innate lymphoid cells, constitute approximately 10%–15% of the total peripheral blood (PB) lymphocytes, and also present in the liver, lungs, uterus, intestine, lymph nodes, and skin, forming a first line of defense against viral

* Corresponding authors.
E-mail addresses: mille011@umn.edu (J.S. Miller), xjhrm@medmail.com.cn (X.-J. Huang), zhao_xy@bjmu.edu.cn (X.-Y. Zhao).
[1] These authors contributed equally to this work.

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infections within the human immune system [8,9]. NK cells originate from $CD34^+$ hematopoietic progenitors, progressing into common lymphoid progenitors, and eventually maturing into NK cells. This maturation process is marked by the progressive upregulation of CD56 and the downregulation of CD34 [10]. NK subsets are predominantly divided into two main subgroups: $CD56^{dim}CD16^+$ and $CD56^{bright}CDl6^-$. The $CD56^{dim}CD16^+$ subset represents the predominant NK cell population in PB, and is recognized as the most inherently cytotoxic subset. Conversely, the $CD56^{bright}CD16^-$ subset primarily functions in cytokine secretion and plays a pivotal immunoregulatory role. In contrast to adaptive immune cells, such as T and B lymphocytes, NK cells possess the distinctive capability to rapidly respond to threats without the need for antigen stimulation or being constrained by the major histocompatibility complex (MHC). NK cells are characterized by the expression of a diverse array of activating receptors on their surface, including activating killer Ig-like receptors (KIRs), natural cytotoxicity receptors (NCRs), NKG2D, CD16, and DNAX accessory molecule-1 (DNAM-1), as well as inhibitory receptors, such as inhibitory KIRs, TIM-3, TIGIT, and NKG2A [11]. Through intricately balancing these activating and inhibitory signals, NK cells maintain tolerance to normal host tissue cells and kill abnormal cells, including malignant cells and virus-infected cells. Several models for NK cell activation have been proposed [12]. Virus-infected cells possess the capacity to downregulate MHC-I molecule expression to evade detection by virus-specific $CD8^+$ cytotoxic T cells. This downregulation reduces inhibitory signals, thereby leading to NK cell activation, a process referred to as NK cell 'missing-self' recognition [13]. In addition, viruses can enhance the expression of activating receptor ligands on the surface of target cells, thereby amplifying activation signals and triggering the response of NK cells through the 'induced-self' recognition mechanism [14]. Upon identification of virus-infected cells, NK cells swiftly secrete perforin and granzyme or engage antibody-dependent cell-mediated cytotoxicity (ADCC) via the surface IgG Fc receptor (CD16), resulting in the direct or indirect elimination of the infected cells, respectively. NK cells are capable of expressing apoptosis-inducing ligands, such as Fas ligand and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which induce programmed apoptosis in target cells. Additionally, NK cells can secrete a variety of proinflammatory cytokines, such as interleukin (IL)-2, IL-15, interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α), in order to activate a stronger immune response to effectively control viral infections [15,16].

What's more, it has been observed that NK cells can develop a long-lasting, antigen-specific memory after being infected by viruses and vaccination in animal models and even in human experiments, named adaptive NK cells [17–21]. The precise phenotypes of memory NK cells are not yet fully understood, and different viral infection animal models have identified distinct characteristics. Among them, NK cell memory formation during HCMV infections is the most well understood and characterized. In individuals infected with HCMV, the $NKG2C^+CD57^+$ NK cell subset is the most well-defined population of memory-like cells. These cells are characterized by low or no expression of NKG2A, FcRγ, NKp30 and NKp46, and high expression of KIR for self-human leukocyte antigen (HLA)-C [22]. Transcriptomic studies have revealed shared epigenetic and transcriptional regulations between memory NK cells and memory $CD8^+$ T cells. A notable difference from conventional NK cells is that memory NK cells exhibit significantly reduced expression levels of signaling molecules, including SYK, FcRγ, and EAT-2, as well as transcription factors, like PLZF, attributed to elevated DNA methylation levels [18]. It has been proven that memory NK cells exhibit an enhanced cytotoxic response against virally infected cells; however, the underlying mechanisms remain incompletely understood [19,23–25]. One potential mechanism for this enhancement in the context of HCMV and HIV, is that CD16 may amplify downstream signaling pathways through its alternative adapter molecule CD3ζ to enhance ADCC capacity in the absence of FcRγ expression in memory NK cells [18,23]. Our research group has determined that the donor NKG2C genotype further influenced HCMV reactivation and refractory HCMV infections by imparting the reconstruction of adaptive $NKG2C^+$ NK cells following transplantation. Investigations on humanized mice infected with HCMV have directly demonstrated that adaptive $NKG2C^+$ NK cells contribute to HCMV clearance [26]. Additionally, inflammatory cytokines, such as IL-12, IL-15, and IL-18, are capable of imparting memory-like properties to both murine and human NK cells in the absence of antigen, which are referred to as cytokine-induced memory-like (CIML) NK cells with enhanced IFN-γ production [27].

2.2. NK cell dysfunction and viral immune evasion

Despite the pivotal role that NK cells play in antiviral immunity, viruses have developed various mechanisms to evade immune surveillance mediated by NK cells. One such mechanism involves the expression of inhibitory ligands for NK cell receptors by the virus-infected cells. In the case of HCMV, the virus can express peptides derived from the viral glycoprotein UL40 on the surface of infected cells, which then bind to HLA-E molecules. The resulting complexes of HLA-E and UL40 peptides serve as ligands for the inhibitory receptor NKG2A expressed on NK cells. This interaction leads NK cells expressing NKG2A to recognize the infected cells as self and subsequently restrict their cytotoxic activity [28]. It has shown that during SARS-CoV-2 infections, especially in severe patients, the NK cell counts significantly decreased [29]. The precise cause of this reduction remains uncertain, and it may be attributed to the redistribution of NK cells from the bloodstream to infected tissues, such as the lungs, or to an increase in NK cell death. In this regard, Zheng et al. [30] identified a potential mechanism underlying NK cell exhaustion. They observed that, in COVID-19 patients, the inhibitory receptor NKG2A was overexpressed in comparison to activating receptors, in contrast to healthy controls, resulting in the functional exhaustion of NK cells. Secondly, viruses possess the capability to downregulate ligands of NK cell activating receptors on the surface of infected cells. An unbiased proteomic analysis revealed alterations in the protein expressions on SARS-CoV-2-infected lung epithelial cells. The analysis showed a downregulation of NK cell activating ligands, including B7-H6, MHC class I chain-related (MIC)-A, UL16-binding proteins (ULBPs), and Nectin1, which were correlated with reduced NK cell activation [31]. In addition, some certain viruses have been identified to directly evade the cytotoxic activity of NK cells by manipulating host cell signaling pathways, including signaling lymphocyte activation molecule (SLAM)-family receptors and related molecules, FcγR-family receptors, and TRAIL death receptor signaling [32–34]. The soluble protein A43 encoded by monkey cytomegaloviruse exhibits excellent binding kinetics with 2B4, and has the ability to disrupt the interaction between the receptor 2B4 and its ligand CD48, which interferes with the formation of the immune synapse between human NK cells and target cells, resulting in inhibiting cytotoxic activity [35]. Furthermore, transforming growth factor-beta (TGF-β), an immunosuppressive cytokine that can be induced during various viral infections, inhibits NK cell function through multiple mechanisms, and has become an attractive target for viral immune evasion strategies [36,37].

2.3. Antiviral products based on NK cells

Given the effective antiviral effects of NK cells and the potential viral immune evasion mechanisms, NK cells can be designed and modified into various products to enhance their antiviral efficacy.

As of now, a significant number of NK cell products have advanced to the clinical stage for antiviral therapy. There are three principal strategies for NK cell-based antiviral therapies: therapy enhancing NK cell activity, NK cell adoptive transfer, and CAR-NK cell therapy. The enhancement of NK cell's antiviral functions can be achieved through several fundamental methods, including cytokine stimulation (such as IL-2, IL-15, IL-12, and anti-TGF-β) via exogenous administration or gene editing, the use of monoclonal antibodies targeting NK cell surface receptors, and the application of NK cell engagers [38–42]. For example, there are some ongoing clinical trials evaluating the safety, tolerability, and efficacy of IL-15 superagonist in individuals with HIV infections (NCT04505501, NCT04340596). In addition, NK cell infusion can enhance the body's ability to clear the viruses, and this approach is sometimes integrated with therapy enhancing NK cell activity to maximize efficacy. Published clinical trials of antiviral therapy based on NK cells infusion are summarized in Table 1 [43–47]. Our research team has achieved substantial clinical-grade expansion of NK cells *in vitro* through feeder cells loaded with membrane-bound IL-21 and 4-1BB ligand [48]. We demonstrated that compared to primary NK cells, expanded NK cells exhibited enhanced ability to inhibit HCMV proliferation and transmission *in vitro* studies [46]. Furthermore, we prospectively enrolled 20 patients diagnosed with hematological malignancies undergoing hematopoietic stem cell transplantation (HSCT), and adoptively infused expanded NK cells derived from the same donors after transplantation. By comparing with a control group of patients who did not receive NK cell infusion, we confirmed that adoptive NK cell infusions were able to prevent HCMV infections, promote HCMV clearance, and enhance NK cell reconstitution after HSCT, and these clinical efficacies would be optimized when used in conjunction with IL-2 (NCT04320303). It provides a new idea for the prevention and treatment of clinical HCMV infections. Furthermore, CAR-NK cell therapy represents a promising strategy for managing viral infections. This approach involves the genetic modification of NK cells to artificially construct activation signaling pathways, thereby specifically enhancing their cytotoxic capabilities [49].

As previously discussed, viruses have developed various strategies to suppress the function of NK cells during pathogenesis. Although CAR-based immunotherapy presents certain drawbacks, CAR-NK cells demonstrate improved targeting abilities and decreased vulnerability to viral immune evasion through both CAR-independent and CAR-dependent pathways. As a result, the use of CAR-NK cell therapy in antiviral treatments is garnering heightened interest in the scientific community.

3. Advantages, limitations, and optimization strategies for CAR-NK cell therapy

3.1. Potential advantages and limitations of CAR-NK cell therapy

In comparison to CAR-T cell therapy, CAR-NK cell therapy has the advantages of abundant cell sources, enhanced safety, and a range of killing mechanisms [50]. Additionally, the development of clinical-grade, off-the-shelf CAR-NK cell products have greatly reduced production costs. The graft-versus-host disease (GvHD), cytokine release syndrome (CRS), and immune effector cell-associated neurotoxicity syndrome (ICANS) are the primary adverse effects associated with current cell therapies. The disparity in MHC between donors and recipients is the primary factor contributing to acute GvHD [51]. Unlike T cells, NK cells lack the T cell receptor (TCR) to directly or indirectly recognize MHC. On the contrary, some studies have identified an inverse correlation between the quantity of NK cells in the graft and the occurrence of severe acute GvHD [52]. The proposed mechanism suggests that donor-derived NK cells may mitigate acute GvHD by killing recipient-derived antigen-presenting cells. Additionally, NK cells may attenuate GvHD by eradicating over-activated T cells through the secretion of cytokines. Upon activation, CAR-T cells can release a substantial quantity of inflammatory cytokines, including IL-2, IL-6, and TNF-α, which are recognized as triggers of CRS and ICANS. Conversely, NK cells typically secrete IFN-γ and granulocyte–macrophage colony stimulating factor, which have not yet been implicated in the pathogenesis of CRS and ICANS [53]. The safety of CAR-NK cell therapy has been validated in several clinical studies in hematological malignancies [54]. In the context of antiviral therapy, research on CAR-NK cell therapy remains nascent, necessitating further investigation and clinical trials to ascertain the safety of CAR-NK cells.

Nonetheless, CAR-NK cell therapy is hindered by several challenges as follows: the limited persistence of NK cells *in vivo*, inadequate homing capabilities, and suppressive effects of the microenvironment. Research indicates that unengineered NK cells activated *in vitro* exhibit a lifespan of approximately two weeks *in vivo*, which is shorter than that of T cells [55,56]. Tang et al. [57] employ CAR-NK cells targeting CD33 with CD28 and 4-1BB costimulatory domains for the treatment of acute myeloid leukemia. While this therapeutic strategy did not lead to any significant adverse events, it was unable to achieve durable remission, likely due to the limited persistence of CAR-NK cells *in vivo*. In contrast, in a Phase I/II clinical trial, Liu et al. [58] genetically modified NK cells using a retroviral vector to express an anti-CD19 CAR alongside IL-15 and inducible caspase-9 as safety mechanisms. The study assessed the cellular kinetics of CAR-NK cells in the context of treating CD19-positive lymphoid tumors. The findings demonstrated that CAR-NK cells underwent *in vivo* expansion within three days post-infusion and maintained their presence for a duration of at least 12 months. The authors propose that IL-15 may significantly contribute to the sustained persistence of CAR-NK cells. Factors influencing the homing of NK cells encompass integrins, selectins, and chemokine receptors, along with their respective receptor or ligand profiles [59]. A diminished capacity of NK cells to migrate to sites of infection correlates with inadequate containment of systemic viral dissemination [60]. Enhancing the ability of NK cells to localize to infection sites may be achieved through the expression of chemokine receptors in CAR-NK cells or by altering the delivery method of infected cells, such as direct application to the infection site. Furthermore, the majority of currently available CARs have been specifically designed and engineered for CAR-T cells, which may not be optimally suited for application in NK cells. Consequently, it is imperative to refine CAR-NK cell therapy to enhance its cytotoxicity, leveraging insights primarily derived from cancer treatment research.

3.2. CAR design optimization

Functional CAR structures expressed in NK cells are primarily composed of four key components: an extracellular binding domain, a hinge region, a transmembrane region, and an intracellular domain [61]. The successful design of CARs is accomplished through a meticulous design process coupled with comprehensive functional testing. Leveraging the traditional CAR design, substituting T cell components with NK cell's activating receptors and adaptor proteins to form NK-specific CARs can notably boost signal transduction and cellular activation (Fig. 1).

The extracellular binding domain consists of a single-chain variable fragment (scFv), formed by fusing the variable heavy and light chains of an antibody through a short peptide linker. The scFv grants CAR-NK cells the ability to more specifically recognize and target antigens. It is necessary to design an appropriate scFv with

Table 1
Published clinical trials of anti-viral infection based on adoptive NK cell therapy.

NCT number	Study title	Virus	Intervention	Phase	Published results
NCT04900454 [43]	Allogeneic natural killer (NK) cell therapy in subjects hospitalized for COVID-19	SARS-CoV-2	Allogeneic NK cells (DVX201)	I	A total of nine hospitalized COVID-19 patients successfully received a single infusion of DVX201 across three dosing cohorts, with the treatment being well-tolerated at all dosage levels.
NCT04365101 [44]	Natural killer cell (CYNK-001) infusions in adults with COVID-19	SARS-CoV-2	Allogeneic NK cells (CYNK-001)	I/II	Of the six treated patients, four were evaluable. The infusions were generally well-tolerated, with one Grade 5 event of hypoxic respiratory failure. Early efficacy was observed in three of the four evaluable patients, showing improvements in oxygenation, inflammatory markers, and radiographic findings.
NCT04578210 [45]	Safety infusion of natural killer cells or memory T cells as adoptive therapy in COVID-19 pneumonia or lymphopenia	SARS-CoV-2	T memory cells or NK cells	I/II	Eighty-one patients evaluated were randomized into the infusion of $CD45RA^-$ memory T cells or the standard of care. The experimental group met the primary outcome for recovery. A faster lymphocyte recovery was observed in the experimental group, and there was no any treatment-related adverse.
NCT04320303 [46]	CMV infection and immune intervention after transplantation	HCMV	Allogeneic NK cells	I	Twenty post-transplantation patients who received adoptive NK cell infusions had a significantly lower cumulative incidence of HCMV infection and refractory HCMV infection compared to controls. Additionally, they exhibited better NK cell reconstitution by day 30 post-infusion.
NCT03899480 NCT03346499 [47]	Adoptive transfer of haploidentical NK cells and N-803/IL-2	HIV	NK cells plus either an IL-15 superagonist (N-803) or IL-2	I	Six individuals with HIV received an infusion of haploidentical related donor NK cells, along with either IL-2 or N-803. The approach was well tolerated with no unexpected adverse events. There was a moderate decrease in the frequency of viral RNA-positive cells in lymph nodes.

HCMV: human cytomegalovirus; HIV: human immunodeficiency virus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

ideal affinity and specificity, so that the CAR can be specific for virus-associated antigens without inducing serious side effects on self-antigens [62]. Previous studies have confirmed that the order of scFv heavy and light chains does not affect the level of CAR expression on T cells, but most of the current CAR-NK products follow the VH-VL direction [63,64]. Besides, some other types of antibodies or antibody alternatives have also been used as antigen-binding domains for CARs, including single variable domain on a heavy chain, shark variable domain of new antigen receptor (VNAR) and lamprey-derived variable lymphocyte receptor [65–67]. It has revealed that VNAR-based CAR-T cell designs may offer clinical advantages, while not be reported in the setting of CAR-NK cells [68].

The hinge region, connecting the scFv and transmembrane domains, is typically derived from CD8α, CD28, IgG-based hinges (such as IgG1, IgG4 and CH2/CH3 domains of IgG Fc) or other structures [64]. One study revealed that CAR-NK cells with CD28 hinge region exhibited superior cytotoxicity than those with IgG1 hinge region, while the data of CAR-NK is still insufficient [65]. This region enhances the flexibility of contacting the target antigen. By modifying the length and structural characteristics of the hinge region, it is possible to achieve optimal spacing between engineered cells and target cells, thereby minimizing off-target effects [69,70]. The transmembrane domains, composed of hydrophobic α-helices, serve to connect the extracellular domain of the CAR with its intracellular signaling domain, effectively anchoring the receptor to the cell membrane. A variety of transmembrane domains have been developed for CAR construction, including those derived from CD3ζ, CD4, CD8, CD28, NKp44, NKp46, NKG2D, DNAM-1, and so on [64].

The CAR architecture of CAR-NK cells has experienced numerous modifications and is evolving towards greater diversification in alignment with advancements in intracellular signaling domains. The first-generation CAR is characterized by the presence of a single intracellular signaling domain. In contrast, the second and third generations incorporate one or more co-stimulatory signaling domains into the intracellular component [71,72]. Although the signal transduction domain of CARs typically involves the TCR/CD3ζ chain or theFcεRIγ chain, due to shared downstream signaling pathways between T cells and NK cell, NK cells possess the capability to initiate signaling through distinct proteins, including DNAX-activating proteins (such as DAP10 and DAP12). Several studies have evaluated the stimulatory potential of CD3ζ, DAP10, and DAP12 in enhancing NK cell activity, revealing that the integration of these domains within the CAR-NK structure yields a more potent cytotoxic response compared to the use of any single domain alone [71,73,74]. Other costimulatory molecules are frequently derived from the CD28 family (including CD28 and ICOS), the tumor necrosis factor receptor family (such as 4-1BB, OX40, and CD27), and the SLAM-related receptor family (notably 2B4), which is predominantly expressed in NK cells [75,76]. Researches have indicated that CAR-NK cells incorporating DNAM-1 and 2B4 exhibit stronger proliferation ability, cytokine secretion ability and anti-tumor activity than those utilizing CD28 or 4-1BB, which are commonly employed in the design of CAR-T cells [77–79]. The fourth-generation CAR, also referred to as armored CAR, represents a distinct conceptual approach in its design, which increases the molecular payload through genetic modification, thereby giving CAR-NK cells additional characteristics and functions to solve the inherent limitations of immune cell therapy [80]. For instance, some scholars have successfully overcome the inhibitory effects of the tumor microenvironment by co-expressing cytokines or chemokines based on CAR. Moreover, they are able to integrate suicide genes to achieve precise regulation of the treatment process [81]. Furthermore, current research also attempts to overcome functional exhaustion by knocking out genes that negatively regu-

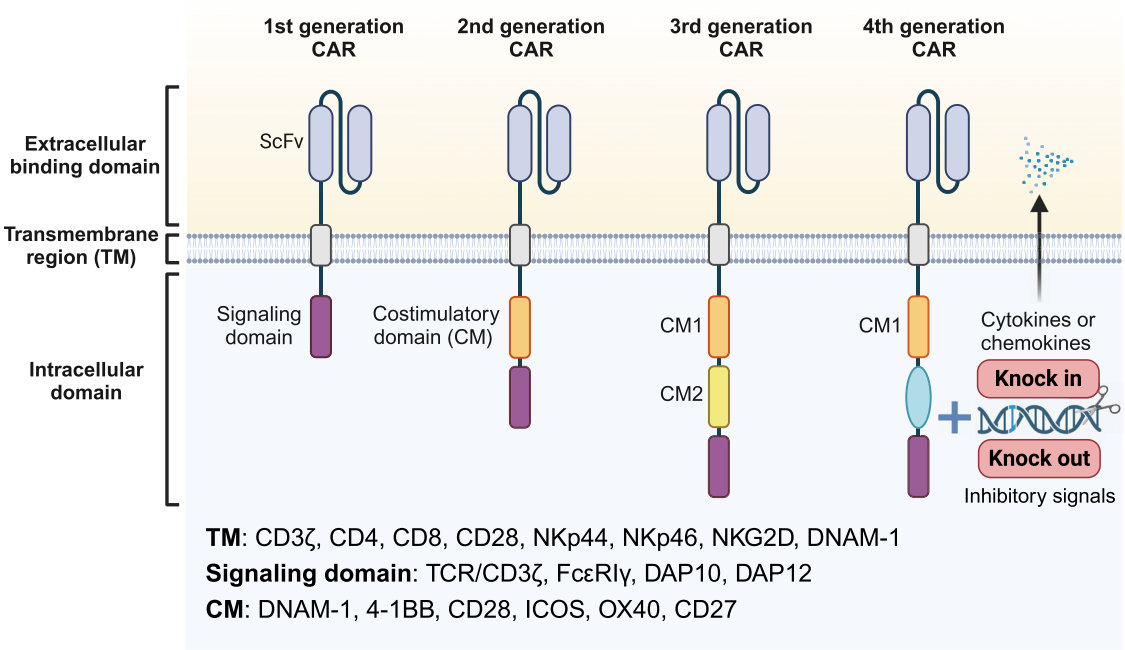

Fig. 1. The overview of CAR design optimization based on biological characteristics of NK cells. With advancements in intracellular domain technology, CAR structures have evolved to the fourth generation. Building on traditional CAR designs, NK cell-activating receptors and their downstream adaptor proteins are now used to replace T cell components, creating NK-specific CARs. This enhances signal transduction and cell activation capabilities, including applications in the transmembrane domain, signaling domain, and costimulatory domain. CAR: chimeric antigen receptor (Created with Biorender.com).

late NK cell function, mainly including various inhibitory immune checkpoint receptors [82]. As mentioned previously, TGF-β signaling has been proven to play a crucial role in the progression of tumors and infections, and it can inhibit NK cell function [83]. Prior studies have found that knocking out the TGF-β receptor 2 or expressing a dominant-negative form of TGF-β receptor 2 to disrupt TGF-β signaling helps maintain the persistence and cytotoxicity of CAR-NK cells [84].

It is worth mentioning that one research team has developed an artificial intelligence tool called CAR-Toner [68]. This platform enables users to input the protein sequence of CARs in order to obtain a score that quantifies positively charged patches, which are indicative of CAR tonic signaling associated with CAR-T cell activity. Additionally, it can provide strategies for optimizing CAR-T cell design, thereby providing substantial support for the development and application of CAR-T cell therapy. The platform holds potential for extending its utility to CAR-NK cell therapy. Overall, more detailed studies are necessary to design and optimize CAR constructs specific to NK cells for NK cell-mediated therapy.

3.3. NK cell selection, engineering and transduction process optimization

CAR-NK cells can be derived from a wide range of sources, including PB, cord blood (CB), NK cell lines, and stem cell, such as human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) [64]. Each source of NK cells has its own unique advantages and limitations, as summarized in Table 2. Overall, the expansion capacity of NK cells derived from PB and CB is constrained by significant donor variability, resulting in an inconsistent yield of NK cells, which poses challenges for long-term storage [85]. The NK-92 cell line, originating from the blood of lymphoma patients, requires irradiation for *in vivo* application; however, this process significantly diminishes the efficacy of CAR-NK cells [86]. Meanwhile, NK cells derived from PB, CB or the NK-92 cell line were hard to be genetic modification. Notably, hESCs and iPSCs exhibit the advantageous characteristics of both primary NK cells and NK cell lines, making them a focal point of current research. Additionally, they are amenable to genetic modification, with various strategies available to enhance their cellular functions [77]. Nonetheless, there remains a paucity of direct comparative studies evaluating the clinical efficacy of CAR-NK cells derived from different NK cell sources.

Given the wide range of sources for NK cells and the superior safety of allogeneic NK cells, CAR-NK cells hold the potential to

Table 2
Advantages and limitations of NK cell sources.

NK cell source	Advantages	Limitations
PB	• Mature phenotype • High cytotoxicity • High safety	• Need for cell isolation and expansion *in vitro* • Potentially immunosuppression (auto-PB NK cells) • Relatively low transduction efficiency
CB	• Easy to collect and store • High proliferation and persistence potential	• Immature phenotype, low expression of activating receptors • Heterogeneity
NK cell lines	• Easy to obtain (NK92, KHYG-1, NKL, YT) • High cytotoxicity due to low expression of inhibitory receptors • Homogeneous cell composition	• Requires irradiation owing to possible tumorigenicity • Short lifespan • Impaired ADCC activity due to low or no expression of CD16
Stem cell (hESC, iPSC)	• Integrated the advantages of primary NK cells and NK cell lines	• High technical difficulty • Potential immunogenic • Potential malignant transformation

ADCC: antibody-dependent cell-mediated cytotoxicity; CB: cord blood; hESC: human embryonic stem cell; iPSC: induced pluripotent stem cell; PB: peripheral blood.

Table 3
Advantages and limitations of CAR delivery.

Methods	Advantages	Disadvantages
Retrovirus	• Mature process technology • Large transgenic load	• Risk of insertional oncogenesis • Uncontrolled immune response • Requiring expanded NK cells • Lower transgene capacity than lentivirus
Lentivirus	• Independent of cell cycle progression • High transduction efficiency • Safer than Retrovirus	• Risk of insertional mutations • Limited packaging capacity and litters
Electroporation	• Relatively easy to design • High transduction efficiency • Safe	• Requiring expanded NK cells
Transposon	• Relatively easy to produce • High transduction efficiency • Low immunogenicity • Cheap	• Risk of insertional mutations
CRISPR/Cas9	• Precise gene integration • Potential for further engineering of NK cells	• Limited standardization

become an off-the-self product. This is beneficial in shortening the production cycle and reducing treatment costs, thereby promoting clinical application. Furthermore, memory NK cells are a potential platform for the development of CAR-NK cells, based on their favorable safety profile, increased proliferation and extended persistence. The more potent anti-tumor ability CAR-NK cells derived from CIML-NK cells has been confirmed in preclinical animal model [87]. These achieved encouraging results have made it necessary to investigate whether this can be used to control viral infections. In addition, memory NK cells induced by viral infections are also promising sources of CAR-NK cells in the field of antiviral therapy.

Currently, there are various methods for CAR delivery to NK cells, including viral vectors such as retrovirus and lentivirus vectors (LVs), as well as non-viral vectors like electroporation, transposon and CRISPR/Cas9 gene editing technology, as seen in Table 3 [88,89]. The efficacy of LVs in generating CAR-NK cells is contingent upon the envelope proteins they express. Traditionally, vesicular stomatitis virus G glycoprotein (VSV-G) has been utilized as the primary glycoprotein for pseudotyped LVs. However, the limited expression of the VSV-G receptor, low-density lipoprotein receptor, on human NK cells hinders effective lentiviral transduction [90]. Consequently, it has been proposed to modify the viral pseudotype to enhance transduction efficiency. Notably, Baboon envelope pseudotyped lentiviral vectors, which utilize the amino acid transporters ASCT1 and ASCT2 as receptors, exhibit high expression levels on activated NK cells, have been proven to make a more efficient NK cell transduction [91]. More studies on NK cell-specific viral glycoprotein components may improve the transduction efficiency of lentivirus. In the future, we should focus on improving vector design and using new biological materials as nucleic acid vectors to improve gene transduction efficiency under the premise of ensuring safety.

4. The application of CAR-NK cell therapy in antiviral infections

Fig. 2 illustrates the prospective targets for CAR-NK cells in the context of antiviral infections. These targets span a range of viruses, encompassing SARS-CoV-2, HIV, HBV, HCMV, and EBV.

4.1. SARS-CoV-2

COVID-19 is a highly contagious respiratory disease caused by the SARS-CoV-2. Since its emergence at the end of 2019, it has swiftly and extensively spread across the globe, leading to catastrophic consequences for both healthcare systems and global economies [92]. SARS-CoV-2 is classified as a beta genus coronavirus, characterized by its enveloped structure and single-stranded positive-sense RNA genome. SARS-CoV-2 is undergoing constant changes account for the huge number of infections, giving rise to multiple variant strains, including the variants of concern renamed by the World Health Organization (WHO) as Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529). The surface of SARS-CoV-2 is adorned with glycosylated spike (S) protein, which has the capacity to bind to the host cell receptor angiotensin-converting enzyme 2 (ACE2), thereby enabling viral attachment and entry into the host cell [93]. Additionally, cell surface proteases, such as transmembrane protease serine 2, are involved in this process through cleaving the S protein, leading to its conformational changes that facilitate virus-cell fusion. Once entry into the cell, SARS-CoV-2 releases a large amount of nucleic acid for succeeding replication and transcription. Upon completing replication, the virus undergoes division into multiple particles and is subsequently released from the host cell, facilitating the invasion of neighboring cells and re-entering the cellular replication cycle.

Several medications have been developed for the treatment of COVID-19, primarily including antiviral agents and immunomodulatory drugs. However, the continuous mutations of SARS-CoV-2 have led to reduced efficacy of existing antiviral treatments, and increased susceptibility to drug resistance [94]. Additionally, although vaccines are considered crucial for controlling the pandemic, the rates of COVID-19 infections and hospitalization remain high among vaccinated individuals, considering vaccine development not keeping pace with the ongoing mutations of SARS-CoV-2 [95,96]. NK cells exhibit strong antiviral activity against SARS-CoV-2 and reduce lung fibrosis [97]. Early $NKG2C^{+}CD57^{-}$ adaptive NK cells during acute COVID-19 infections and $NKG2C^{+}$-$CD57^{+}$ mature adaptive NK cells in long-COVID patients were reported with enhanced target cell cytotoxicity [98,99]. The senescence and depletion of NK cells is an important factor affecting the severity of SARS-CoV-2 infections. For patients with COVID-19, those with severe symptoms exhibit a significantly higher viral load, accompanied by a reduction in the number of NK cells, as well as lower levels of IFN-γ and TNF-α production [29,97]. In addition, NK cells are reduced and less activated in COVID-19 patients with persistent SARS-CoV-2 infections [100]. Hence, increasing the number of functional NK cells, especially CAR-NK cells, can help to control COVID-19 at an early stage or severe COVID-19. Based on the unique characteristics of the virus, there are several potential targets available for designing CAR-NK cell therapy in the treatment of SARS-CoV-2 infections, and additional research and experimental studies are required to confirm their efficacy and safety.

In a preclinical study conducted by Ma et al. [101], they developed a novel method to generate CAR-NK cells that directly targeted SARS-CoV-2 by utilizing the scFv domain of S309 (S309-CAR-NK cell), which could accurately recognize highly conserved regions of the SARS-CoV-2 S protein, making it more likely to identify different variants of the SARS-CoV-2. The successfully engineered S309-CAR-NK cells demonstrated their capacity to bind to and be activated by the SARS-CoV-2 *in vitro*. To evaluate their functional activity, the researchers conducted experiments using cell lines expressing SARS-CoV-2 S protein. Notably, the S309-CAR-NK cells exhibited clear signs of activation after co-culture with these cells, indicating their ability to respond to viral presence.

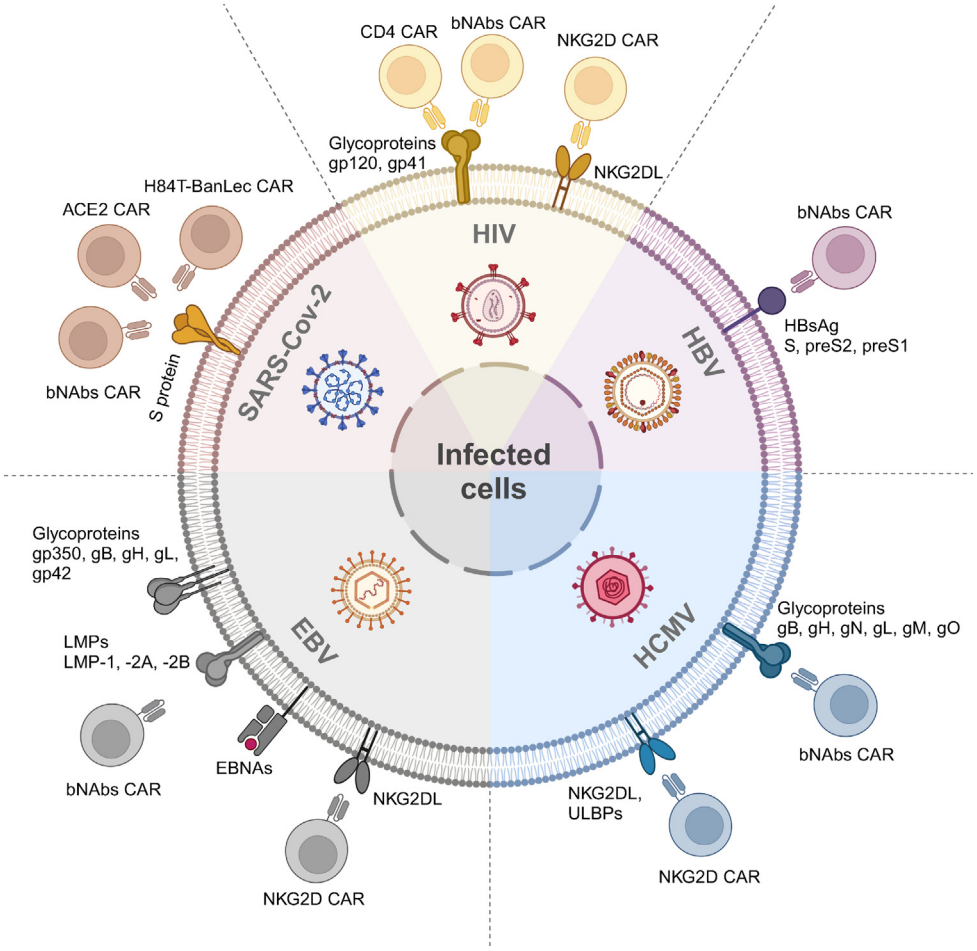

Fig. 2. Potential targets for CAR-NK cells in antiviral infections. A variety of attractive targets for CAR-NK cell therapy in antiviral infections are presented. ACE2: angiotensin-converting enzyme 2; bNAbs: broadly neutralizing antibodies; CAR: chimeric antigen receptor; EBNAs: EBV nuclear antigens; EBV: Epstein-Barr virus; HBsAg: HBV surface antigen; HBV: hepatitis B virus; HCMV: human cytomegalovirus; HIV: human immunodeficiency virus; LMPs: latent membrane proteins; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2. (Created with Biorender.com).

Extensive additional experimentation verified the potent antiviral capabilities of the S309-CAR-NK cells. They not only possessed the ability to kill target cells that were infected with SARS-CoV-2 S protein, but also surpassed the cytotoxic activity and cytokine production capacity of previously reported CR3022-CAR-NK cells, which targeted the S protein as well.

Unlike the sites targeted before, Christdoulou et al. [102] designed H84T-Banana Lectin (BanLec) CAR-NK cells with 4-1BB and TCRζ intracellular domains, which targeted high mannose glycosylation sites near the receptor binding domain of SARS-CoV-2 S protein. The H84T-BanLec CARs were stably expressed at high density on primary human NK cells after two weeks of *ex vivo* expansion. To examine the potential of H84T-BanLec CAR NK cells in reducing cellular pseudovirus infections, researchers engineered SARS-CoV-2 envelope-pseudotyped lentiviral particles containing firefly luciferase and utilized human ACE2 (hACE2)-expressing 293T cells. The findings indicated that the bioluminescence mediated by the SARS-CoV-2 pseudovirus in hACE2 293T cells was reduced in the presence of CAR-NK cells when co-cultured with hACE2 293T cells and free-circulating pseudovirus particles, compared to unmodified NK cells. Furthermore, H84T-BanLec CAR-NK cells were proven to show a stronger cytokine secretion ability, including IFN-γ and TNF-α. Given the widespread expression of high mannose polysaccharides in other viruses, the potential of H84T-BanLec CAR-NK cell therapy in the treatment of HIV, influenza and other viruses is worth further exploration [103].

Besides, Lu et al. [104] transduced NK cells isolated from CB with a mixture of two retroviral vectors carrying the extracellular domain of a mutant variant of ACE2 and human soluble IL-15 (sIL15) to generate mACE2-CAR_sIL15 NK cells. They expressed the S protein in the human lung cancer cell line A549 (A549-spike), and then co-cultured mACE2-CAR_sIL15 NK and control NK cells with A549 cells to evaluate the ability of effector cells to eradicate virus-infected cells. The results of long-term cytotoxicity assays and real-time cell analysis indicated that the cytotoxicity of mACE2-CAR_sIL15 NK cells against A549-spike cells was significantly higher than that of control NK cells. Additionally, to assess the potential clinical applicability of these CAR-NK cells, they also conducted freeze–thaw cycle tests, encompassing cryopreservation and subsequent recovery, on mACE2-CAR_sIL15 NK cells. The results indicated that over 80% of the cells demonstrated functional recovery, with cell viability surpassing 90%. Notably, even six hours post-thawing, the viability of mACE2-CAR_sIL15 NK cells remained above 80%. Subsequently, the researchers administered CAR-NK cells to transgenic mice that expressed hACE2 and infected by live SARS-CoV-2 *in vivo*. The findings reviewed that the transgenic mice treated with CAR-NK cells exhibited a reduced viral load of SARS-CoV-2, better maintenance of body weight, and extended survival, compared to those mice receiving the vehicle or control NK cells. Furthermore, the levels of several cytokines measured in the different treated groups of mice, such as IL-6 and TNF-α, did not show statistically significant differences. This

suggests that CAR-NK cell therapy may not induce toxic cytokine storm *in vivo*.

In a Phase I/II clinical trial (NCT04324996) targeting early-stage COVID-19 patients, researchers utilized CAR genetically modified NK cells derived from CB. These cells were engineered to express NKG2D-ACE2 and were augmented with an IL-15 agonist. The primary objective was to inhibit the infection of ACE2-expressing cells by the novel coronavirus and to enhance the cytotoxic efficacy of the NK cells. As of now, the results of this clinical trial have not been reported. Further clinical trials are warranted to validate these findings.

4.2. HIV

HIV/AIDS poses a major threat to global public health, and it was estimated that there were 40 million people globally living with HIV/AIDS, with 1.65 million new HIV infections and 0.78 million HIV-related deaths in 2021 [105]. HIV belongs to the *Retroviridae* family of lentiviruses, and is primarily divided into two types, HIV-1 and HIV-2, with HIV-1 being the most common and highly transmissible strain for AIDS. HIV consists of two components: the viral core and the envelope. The core is composed of capsid protein and contains two identical single-stranded RNA, nucleocapsid protein, and essential enzymes for viral replication, including reverse transcriptase, integrase, and protease. HIV-1 envelop is a trimer gp160 formed by the monomers of a heterodimer, with each monomer containing the receptor binding protein gp120 and the transmembrane fusion protein gp41, connected by non-covalent interactions. Below the envelope structure is the matrix protein, forming the viral inner shell. In the process of infection, gp120 first binds to the CD4 molecule on the surface of target cells, then binds to co-receptors, mediating the fusion of the viral envelope with the cell membrane. This allows the viral core to enter the cytoplasm of the infected cell and integrate its genetic material into the host chromosome, leading to subsequent assembly and release of new viral particles [106].

Currently, the primary approach for treating HIV infection is through the use of combination therapy with multiple antiretroviral drugs, known as antiretroviral therapy (ART) [107]. However, despite the ability of ART to effectively suppress HIV replication and improve clinical outcomes, the presence of a stable reservoir of latent proviruses within memory $CD4^+$ T cells hinder the complete cure of viral infections [108]. Researchers are actively exploring strategies for achieving a functional cure of HIV. These strategies including shocking or blocking the viral reservoir, immunotherapies, such as broadly neutralizing antibodies (bNAbs), targeted therapies, and vaccines, stem cell transplantation, and gene editing [47,109]. NK cells are potentially important for the treatment of HIV infections and clearance of the viral reservoir. Patients with high NK cell counts during primary HIV infections had lower viral loads, which can be used to predict HIV disease progression and immune recovery after ART [110]. Pohlmeyer et al. [111] identified a subset of $CD11b^+CD57^-CD161^+$-Siglec-$7^+CD56^{dim}CD16^+$ NK cells which distinguished HIV controllers from non-controllers. It was confirmed that this subset secreted more IFN-γ and CD107a *in vitro*. Jost et al. [112] recognized biomarkers of memory NK cells for HIV infections by flow cytometry, which showed elevated expression of KLRG1, $\alpha4\beta7$, and NKG2C. In brief, these cells can be isolated for future functional studies and clinical application. However, HIV can cause NK cell dysfunction through inflammatory environment, surface receptor imbalance, and viral proteins impairment [113]. Therefore, anti-HIV CAR-NK cell therapy stands out as a potential approach.

Initially, the design of CAR-NK cell therapy to target HIV-1 infections was based on modifying hhESCs and iPSCs with the extracellular portion of CD4 and CD3ζ intracellular signaling domain, since CD4 protein is the gateway for HIV entry into T cells [114]. Compared with unmodified NK cells, these CAR-NK cells showed stronger inhibition of HIV replication *in vitro*, but no difference was observed in the *in vivo* mouse model. This may be due to the lack of costimulatory domains leading to insufficient persistence and proliferative capacity of the cells, and CD4 receptors are not the best target choice for anti-HIV therapy in general. Given that HIV-infected cells express gp160 on their surface, anti-gp160 CAR can be used to target and eliminate these infected cells [115]. HIV is highly diverse and prone to mutation, resulting in a variety of gp160 variants being expressed. HIV gp120 encompasses five conserved regions (C1-C5) and five variable regions (V1-V5). HIV gp41 consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular domain is composed of three important functional regions. Several major bNAbs epitopes of HIV-1 envelope glycoprotein have been revealed, including the conserved CD4 binding site on gp120, the glycan region of V1-V3, the gp120-gp41 interface, the membrane-proximal external region (MPER) on gp41, and fusing peptides [116]. Previously developed anti-HIV CARs that recognize a single epitope of gp160 are unable to cover multiple HIV strains. Hence, Lim et al. [117] have developed a chemically guided universal CAR-NK cell, which utilizes a CAR with surface antibody that can recognize the small molecule ligand 2,4-dinitrophenyl (DNP). They subsequently endowed the CAR-NK cells with the ability to target multiple epitopes of gp160 by combining it with various HIV-1 gp160 antibodies conjugated with DNP, allowing the killing of different HIV-1 subtype-infected cells. The researchers proved that anti-DNP CAR-NK cells were able to target and kill HIV-1-infected $CD4^+$ T cells. After further testing the differential killing ability of the CAR-NK cells against multiple gp160 antibodies targeting different antigenic sites, the results revealed that CAR-NK cells showed a stronger targeting ability towards the distal membrane epitopes. Relying on numerous off-the-shelf anti-gp160 antibodies, this universal CAR-NK cell can overcome the diversity of HIV and has the potential to eradicate infected cells. It should be noted that some human natural antibodies can also recognize DNP, and attention should be paid to designing anti-DNP CARs with higher affinity to enhance therapeutic efficacy.

Nowadays, there are more studies focused on the application of CAR-T cell therapy for combating HIV infections than CAR-NK cell therapy. Liu et al. [118] conducted a single infusion of bNAbs-derived CAR-T cells in fourteen HIV-1-infected patients who had received ART. The results showed that all patients tolerated the CAR-T cell therapy well, and the infused CAR-T cells persisted in the PB of recipients for over six months. And the researchers found that CAR-T cell therapy significantly reduced the size and diversity of HIV reservoir. Building upon these findings, ART was temporarily discontinued in six patients who received CAR-T cell therapy, and a comparison with historical controls demonstrated further evidence for the effective suppression of HIV-1 in plasma, with the longest viral rebound time extended to 10 weeks. Within the envelope protein sequence of rebound strains, multiple characteristic escape mutations that confer resistance to VRC01 antibody were identified, further underscoring the selective pressure exerted by CAR-T cell infusion on the viral reservoir. Unfortunately, it did not achieve the goal of cure in the end. In order to further enhance the antiviral effect, the design of CAR-T cells can target multiple independent recognition sites on HIV-1 envelope proteins. Anthony-Gonda et al. [119] conducted a study in which they developed over 40 LVs based on HIV-1, encoding single, dual, and trispecific anti-HIV CARs. These CARs were designed to target three potential sites on the trimeric envelope glycoprotein: the gp120 CD4-binding site (mD1.22), the gp120 coreceptor-binding site (m36.4), and the gp41 near the MPER (C46 peptide). Their findings indicated that the most promising candidates were comprised of

two CAR molecules, including mD1.22-CAR and m36.4-CAR. Through duoCAR-T cell therapy, over 99% immune cells infected with different HIV strains, including drug-resistant ones, were effectively eliminated. The research team performed simultaneous injections of CAR-T cells and HIV-infected human cells into the spleens of humanized mice. After one week of duoCAR-T cell therapy, HIV DNA was not detected in the spleens of 5 out of 6 mice, with an average decrease of 97.5% in viral levels. In contrast, the inhibitory rates of HIV DNA were 42% and 61% in the two mono-CAR-T cell products, respectively. It is worth mentioning that the Phase I/IIa clinical trial (NCT04648046) for the duoCAR-T cell therapy targeting HIV has received FDA approval [120]. Furthermore, future research should explore the potential of combination therapy. For instance, previous study has demonstrated that, in comparison to ART alone, the integration of CAR-T cells with ART can expedite the suppression of viral reactivation in HIV-infected murine models [121]. It may be advantageous to ensure that ART reduces the viral load to low level prior to the administration of CAR-T cells, akin to the role of conditioning in diminishing susceptibility to viral infection. The question of whether ART prophylaxis should be maintained following CAR-T cells infusion in high-risk patients warrants further investigation. Additionally, the application of latency reversing agents to activate latent HIV-1 facilitates the recognition and elimination of HIV-infected cells by CAR-T cells, thereby accelerating the eradication of viral reservoirs. Mao et al. [122] developed anti-HIV-1 CAR-T cells, termed M10 cells, which incorporates endogenous bNAbs and the follicular homing receptor CXCR5. In a Phase I clinical trial, participants received two infusions of M10 cells, followed by two challenges with chidamide to activate the HIV-1 reservoir within the patient. The findings demonstrated that M10 cells infusions significantly inhibited HIV-1 viral rebound. In conclusion, the experience obtained from CAR-T cell therapy for HIV infections is worthy of further validation and optimization in CAR-NK cell therapy.

4.3. HBV

According to the latest data from the WHO, the global positivity rate for HBV surface antigen (HBsAg) stands at 3.8%. In 2019, there were 296 million people suffering from chronic hepatitis B (CHB), and it is estimated that 331,000 people died from HBV-related cirrhosis and chronic liver disease [123]. HBV, a member of the *Hepadnaviridae* family, comprises a complete particle that includes an envelope and a nucleocapsid. The HBV envelope is characterized by the presence of three distinct proteins: the small protein (HBsAg), the middle protein (HBsAg+PreS2), and the large protein (HBsAg+PreS2+PreS1), while the nucleocapsid contains the core protein (HBcAg), a relaxed circular DNA molecule, and the HBV DNA polymerase [124]. During the replication process, the viral DNA is transported into the host cell nucleus, where it is converted into a supercoiled covalently closed circular DNA (cccDNA) molecule, which is also the key indicator in determining whether CHB has been cured [4]. The antiviral treatments for CHB mainly include interferons and nucleoside analogues, which inhibit viral replication by competitively suppressing the reverse transcriptase activity of the HBV DNA polymerase. However, they struggle to completely eradicate the virus [125].

NK cells constitute a principal category of immune cells within the liver, possessing the capability to manage CHB through direct antiviral mechanisms and by orchestrating adaptive immune responses [126]. However, CHB induces functional exhaustion in NK cells by upregulating inhibitory receptors and downregulating activation receptors [127]. In this context, a CAR capable of recognizing HBV envelope proteins is needed to develop, presenting novel therapeutic opportunities for CHB. To date, there is a lack of direct studies on CAR-NK cells targeting HBV infections, but insights can be drawn from the results of existing CAR-T cell therapy. Bohne et al. [128] have engineered CAR-T cells that specifically target HBV small and large proteins, with a CD3ζ signaling domain and a CD28 costimulatory domain. These CAR-T cells have been previously shown to effectively eliminate HBV-infected and thus cccDNA-positive primary hepatocytes *in vitro*. To evaluate the efficacy *in vivo*, a prior study demonstrated that HBsAg-targeted CAR-T cells significantly reduced plasma levels of HBsAg and HBV DNA in comparison to the control group in human liver chimeric mouse models [129]. Similarly, another study developed novel HBV-specific CAR-T cells targeting the preS1 region, which successfully induced production of pro-inflammatory and antiviral cytokines, and decreased HBV cccDNA to an extremely low level after CAR-T cells infusion *in vitro* [130]. Furthermore, Festag et al. [131] generated a fully human CAR targeting HBsAg, designated as S-CAR, with CD3 and CD28 signaling domain. They evaluated the efficacy of S-CAR-T cells in an immunocompetent preclinical mouse model. The findings indicated that the infused S-CAR-T cells exhibited sustained antiviral effects without inducing significant treatment-limiting side effects, but they were ultimately unable to cure HBV infections. This limitation may be attributed to the insufficient affinity of the employed to detect low concentrations of HBsAg on the infected cell membrane. Schreiber et al. [132] employed single B cell sorting technology to identify novel recombinant human monoclonal antibodies targeting HBV. Their research led to the discovery of two high-affinity antibodies, designated 4D06 and 4D08, which exhibited broad neutralizing capabilities and reactivity across various HBV genotypes. The scFvs of these antibodies were subsequently cloned into a second-generation CAR configuration, incorporating CD28 and CD3ζ intracellular signaling domains. This configuration demonstrated enhanced functional affinity when re-expression in T cells. Considering the inherent antiviral potential of NK cells and the superiority of targeted therapy, CAR-NK cells hold promising prospects for the eradication of HBV, and more data is forthcoming.

4.4. HCMV

HCMV is a double-stranded DNA virus classified within the beta-subgroup of the *Herpesvirus* family, and it is prevalent globally with an estimated seroprevalence rate of approximately 83% [133]. Following primary infection, HCMV establishes latency within the host and can subsequently reactivate, particularly when the host's immune system is compromised. This reactivation can result in recurrent episodes of infection, potentially causing multi-organ damage. Such complications are notably severe in organ transplant recipients and individuals with immunodeficiencies. Data indicate that the incidence of HCMV disease, defined as HCMV infections accompanied by clinical symptoms in haploidentical HSCT recipients is approximately 3%, with HCMV pneumonia and gastroenteritis being the most prevalent manifestations, and the overall mortality rate associated with HCMV disease is around 40% [134]. In clinical practice, preemptive therapy is frequently employed to manage HCMV infections after HSCT. This approach involves initiating antiviral treatment upon detection of HCMV viremia, prior to the onset of clinical disease. The primary therapeutic agents for HCMV infections and disease are antiviral drugs, such as ganciclovir, valganciclovir, foscarnet sodium and cidofovir, which inhibit HCMV DNA polymerase (UL54). In addition, some novel drugs, like maribavir (inhibition of viral UL97 kinase) and letermovir (inhibition of viral terminase complex), have improved the outcomes of resistant/refractory HCMV [135]. Despite the emergence of new drugs targeting novel mechanisms, drug-resistant mutations continue to arise under selective pressure [136,137]. Study revealed that the proportion of refractory CMV was approximately 15.0% after letermovir treatment [138].

Adoptive cell therapy specifically targeting HCMV has been demonstrated to be an effective method to counteract drug resistance. As previously discussed, NK cells play a crucial role in combating HCMV infections. Despite the demonstrated efficacy of NK cell infusion, HCMV poses a significant challenge by employing various mechanisms to evade NK cell-mediated cytotoxicity. HCMV-specific cytotoxic T lymphocytes are frequently utilized, achieving a cumulative response rate exceeding 80% at six weeks in patients with refractory HCMV [139,140]. Nonetheless, their application is constrained by the necessity for compatible donors who are both HCMV-seropositive and HLA-matched. To address this limitation, the use of CAR targeting HCMV proteins emerges as a promising alternative.

Research into the biological functions of proteins encoded by HCMV can aid in the design of CAR structures targeting HCMV. The structural proteins of HCMV primarily include capsid proteins, tegument proteins, and envelope glycoproteins. Envelope glycoproteins are integral to the viral entry into host cells and facilitate intercellular transmission. Among these, several glycoproteins, including gB, gH, gN, gL, gM, and gO, have been characterized [141]. These glycoproteins, expressed on the surface of cells infected with HCMV, represent promising targets for CAR-based cell immunotherapy. Olbrich et al. [142] engineered CARs specific to HCMV gB with 4-1BB or CD28 costimulatory domains, in $CD4^+$ and $CD8^+$ T cells derived from HCMV-seronegative adult PB or CB. *In vitro* assays demonstrated revealed that gB CAR-T cells effectively recognized and eliminated HCMV-infected cells. Furthermore, CAR-T cells incorporating the 4-1BB signaling domain exhibited superior antitarget cytotoxic performance. In a murine model of HCMV infections, gB CAR-T cells also demonstrated resistance to HCMV infections without inducing GvHD. Ali et al. [143] engineered eight novel CAR constructs, excluding gB, and incorporated the 4-1BB signaling domain. There constructs were transduced into primary $CD8^+$ T cells using a lentiviral vector. In the assessment of cytokine release, upregulation of surface CD107a, cell proliferation, cytolysis of infected cells, and suppression of viral replication *in vitro*, only the CAR derived from the 21E9 antibody exhibited superiority across all these parameters, which has significant implications for the subsequent selection of scFvs. Moreover, NKG2D, an essential NK cell activating receptor, interacts with stress-induced ligands, such as MIC-A, MIC-B and HCMV ULBPs, which represent ideal targets for immunotherapy [144]. Overall, more targeting elements should be evaluated in CAR-T cells, and ultimately guides the development of CAR-NK cells.

4.5. EBV

EBV, also known as human herpesvirus 4, is a linear and double-stranded DNA virus as a member of the *Gammaherpesvirus* family, and more than 90% of the human population worldwide showing serological positivity [7]. EBV infections can be categorized into two distinct phases: latent and lytic infection. Initially, EBV traverses the oropharyngeal epithelium to infect B lymphocytes, facilitated by the interaction between the viral surface membrane glycoprotein (gp350/220) and the B cell surface receptor CD21. Additionally, EBV is capable of sporadically infecting T lymphocytes and NK cells through alternative pathways. Under conditions of balanced virus-host interactions, EBV can establish a stable latent infection state, replicating in synchrony with the host's nuclear genes. When host immunity function is compromised, EBV may transition from a latent to a lytic infection phase, leading to extensive replication of EBV and release of viral particles. At this stage, capsid proteins serve as the primary structural components of EBV, including gp350, gB, gH, gL, and gp42. During the latent infection, the expression of EBV viral proteins is strictly limited to evade host immune surveillance. These encoded proteins include three latent membrane proteins (including LMP-1, -2A, and-2B) and six EBV nuclear antigens (including EBNA-1, -2, -3A, -3B, -3C, and -LP) [145]. The expression products of latent infection may induce malignant transformation of cells, resulting in various malignant tumors originating from lymphocytes or epithelial cells, such as lymphoma, nasopharyngeal carcinoma (NPC), gastric carcinoma and EBV-related post-transplant lymphoproliferative disorder (PTLD). Various EBV-associated tumor cells display distinct patterns of latent gene expression, categorized as latency III, II, I, and 0 [146]. In malignancies exhibiting latency III, such as PTLD, EBV-infected cells express a comprehensive array of latent proteins. In latency II tumors, such as NPC, the expression is predominantly limited to EBNA-1, LMP-1, and LMP-2. In contrast, tumor cells associated with latency I, exemplified by Burkitt's lymphoma, express only EBNA-1. Latency 0 is characterized by a complete absence of antigen expression and is typically observed in memory B cells, which act as a reservoir for the virus.

The main treatments for EBV-associated malignancies include antiviral drugs, chemotherapy, radiotherapy, and operative treatment. These approaches often come with significant side effects and are not always effective in eliminating tumor cells, leading to risks of recurrence and distant metastasis. NK cells are crucial in controlling EBV infections. A deficiency in the cytotoxic function of NK cells against EBV is a contributing factor to the persistence of EBV infections and is associated with an increased risk of EBV-related malignancies [147]. EBV has developed several immune evasion strategies to circumvent NK cell-mediated responses [148]. Similar to anti-CMV treatment, there has been development in the adoptive transfer of EBV-specific T cells (EBV-CTLs), which are obtained by stimulating T cells *ex vivo* with EBV antigen peptides and various cytokines [149,150]. However, there are several issues limiting their widespread application. EBV-CTLs usually require donors who are serologically positive for the virus. Additionally, EBV infections can induce a decrease in the expression of HLA molecules in infected cells, allowing the pathogen to escape the cytotoxic effects of EBV-CTLs [151].

In light of these challenges, CAR gene therapy targeting EBV-encoded proteins may be a superior solution, and the research field is primarily focused on CAR-T cell therapy. Slabik et al. [152] generated an EBV-specific gp350-targeted CAR containing CD28/CD3ζ signaling domains. These gp350 CAR-T cells demonstrated gp350-specific activation and cytotoxic effects in several EBV strains, including 293T, B95-8, and B-LCL, *in vitro*. They constructed a humanized model in Nod.Rag.Gamma mice transplanted with CD34 cells from CB and infected with the EBV/M81/fLuc lytic strain. The results reviewed that protectively infusions of gp350 CAR-T cells showed a significantly slower pace of EBV progression and lower virus-induced inflammation, while purified $CD4^+$ CAR-T cells promoted xeno-GvHD. And CAR-T cells therapeutically infusions played an important role in slowing the spread of EBV infections, lymphoproliferation, and inflammation, without tumor development. Researchers have advanced towards the clinical development of gp350 CAR-T cells under good-manufacturing practices [153]. In addition, targeting latent membrane proteins is essential for the eradication of EBV during its latent stage. In a preclinical study, Tang et al. [154] demonstrated that CAR-T cells specific to LMP-1 can effectively recognize and eliminate LMP-1 positive NPC cells in both *in vivo* and *in vitro* experiments. Their findings indicated that the inclusion of 4-1BB alongside CD28 as a costimulatory signaling domain enhances the efficacy of CAR-T cells compared to the use of CD28 alone. An early Phase I clinical trial is currently underway to assess the application of anti-LMP1 CAR T cells in treating LMP1 positive infectious and malignant hematological diseases (NCT04657965). In order to specifically target malignant EBV-positive PTLD, Dragon et al. [155] developed a CAR utilizing the scFv of the TCR-like monoclonal antibody

TÜ165, with the expression of inducible IL-12 to enhance T cells redirected for universal cytokine-mediated killing. *In vitro* studies demonstrated that TÜ165 CAR-T cells successfully recognized the intracellular antigen EBNA-3C in EBV-infected cells, and effectively eliminated EBV-positive cells. The presence of IL-12 augmented the cytotoxic function of the CAR-T cells and facilitated the recruitment of monocytes and NK cells. Moreover, CAR-T cells targeting B cell markers, such as CD19 and CD20, has been reported in the treatment of EBV-associated diseases [156,157]. The findings from the aforementioned studies compellingly illustrate the critical role of CAR-targeted therapy in combating EBV infections. In the design of CAR-NK cells, it is essential to assess a variety of target combinations and CAR structures to develop more effective and safer CAR-NK products for EBV-related diseases.

5. Conclusions

In this review, we examine the advancements in research concerning CAR-NK cell therapy for viral infections. Despite the fact that the majority of studies remain in the preliminary preclinical phase, the promising outcomes suggest that CAR-NK cell therapy holds potential as an effective strategy for the eradication of various viruses. Future developments in the design of CAR-NK cells should concentrate on leveraging the autologous components of NK cells to augment their responsiveness to activation signals. This indicates a continued need for extensive optimization efforts to assess and enhance the safety and efficacy of newly constructed CAR-NK cell products. To mitigate the risk of viral resurgence due to mutation, CAR-NK cells can be engineered to target multiple, distinct viral proteins, thereby enhancing their antiviral efficacy. Furthermore, the integration of CAR-NK cell therapy with other antiviral treatments presents a promising avenue for future clinical trials. Through comprehensive research into the interaction mechanisms between the virus and the host, coupled with advancements in genetic engineering technologies, it is anticipated that CAR-NK cells infusions are able to sustainably and effectively regulate viral replication, potentially achieving long-term or even lifelong viral control.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Xiang-Yu Zhao and Xiao-Jun Huang designed and supervised the project. Ming-Hao Lin and Li-Juan Hu collected the literatures and drafted the original manuscript. Xiang-Yu Zhao, Xiao-Jun Huang, and Jeffrey S. Miller revised the draft. All authors discussed and commented on the manuscript.

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