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Natural killer cells for antiviral therapy

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Natural killer (NK) cell–based immunotherapy is being explored for treating infectious diseases, including viral infections. Here, we discuss evidence of NK cell responses to different viruses, ongoing clinical efforts to treat such infections with NK cell products, and review platforms to generate NK cell products.

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INTRODUCTION

Natural killer (NK) cells are immune cells capable of discriminating normal from abnormal cells, and NK cell deficiency generally results in lethal infections (*1*). NK cells respond to viral infections within hours, resulting in the killing of infected cells without prior activation, engagement of multiple targets simultaneously, secretion of antiviral cytokines, and interaction with the adaptive immune system. These capabilities make NK cells attractive for use as treatments, but NK cell responses to infection remain incompletely understood. Here, we review the current knowledge of NK cell roles in counteracting viral infections and speculate about NK cell products for treating HIV, cytomegalovirus (CMV), influenza, BK virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections.

NK cells originate in the bone marrow and thymus and share a common progenitor cell with T and B cells [reviewed in (*2*)]. NK cells lack T and B cell receptors (TCRs and BCRs, respectively) and expression of CD3 and instead express CD56 and CD16 [Fc gamma receptor III (FcγRIII)], the mediator of antibody-dependent cellular cytotoxicity (ADCC). Immature NK cells ($CD56^{bright}$/$CD16^{dim}$) develop into tissue-specific subpopulations (*3*), and mature NK cells ($CD56^{dim}$/$CD16^{bright}$) in these tissues and peripheral blood express killer cell immunoglobulin receptors (KIRs); secrete proinflammatory cytokines, perforins, granzymes, defensins, and cathelicidin; and mediate ADCC (*4*).

NK cells use various receptors to interact with other cells. Inhibitory receptors (e.g., NKG2A/CD94 and inhibitory KIRs) recognize human leukocyte antigen (HLA) surface molecules, protecting healthy cells from NK cell–mediated attack (*2*) and allowing NK cells to recognize a reduction in HLA class I (HLA-I) molecules on infected or malignant cells. Activating receptors, e.g., lectin-like receptors (NKp80 and NKG2 family), Fc receptors (CD16), and natural cytotoxicity receptors (NKp30, NKp44, and NKp46), allow NK cells to recognize increased expression of stress ligands, leading to NK cell–mediated killing. In addition, interleukin-2 (IL-2), IL-12, IL-15, IL-18, and type 1 interferons (IFNs) differentiate or activate NK cells. The chemokine receptors CCR1, CCR2, CCR5, CCR7, CXCR1, CXCR3, CXCR4, CXCR6, and CX3CR1 recruit NK cells to infected tissues (*2*).

Unlike T or B cells, NK cells do not require prior antigen exposure to mediate their effects. Although once believed to lack immune memory, recent evidence suggests that NK cells recall responses upon cytokine stimulation (*5*). In addition, NK cell subsets can show adaptive immune responses to viruses in mice, nonhuman primates, and humans (*6*–*9*). Furthermore, in a mouse model of CMV infection, primed NK cells exhibit enhanced IFN-γ secretion and degranulation and can protect against an otherwise lethal challenge with this virus (*10*).

Adoptive NK cell immunotherapy

NK cells are attractive for adoptive cellular therapy because they can identify and kill abnormal cells. Because of their non-HLA–restricted effector functions, NK cells are being developed as off-the-shelf therapies. NK cell lines, such as NK92 cells, which are IL-2–dependent (*11*), can be used to generate off-the-shelf products that express CD2, CD11a, CD28, and CD54 and are $CD56^{bright}$ but do not express TCRs or BCRs or lineage markers (Fig. 1). NK92-derived cellular products predominantly kill target cells through CD95 ligand rather than granzyme B and perforin-mediated mechanisms (*12*, *13*), and these cells can also be genetically modified to express desired receptors (e.g., CD16 for ADCC). Because NK92 cells were derived from a rare lymphoma (*12*), the cell product first must be irradiated, limiting its viability and thus requiring repeated infusions. However, even multiple high-dose infusions have been well tolerated and effective in persons with hematological malignancies; the product was immunogenic with anti-HLA antibodies detected in most recipients (*14*).

Peripheral blood is also a good source of NK cells for immunotherapy, especially mature NK cells. Distinct protocols have been used for peripheral blood–derived NK cell expansion and activation. When expanded on membrane-bound IL-15–expressing feeder cells, NK cell products display an activated phenotype, expressing NKG2D, NKp30, NKp44, NKp46, and DNAX accessory molecule 1 (DNAM-1), but produce IFN-γ at concentrations similar to those produced by nonexpanded NK cells. These expanded NK cells exhibit increased cytotoxicity compared with nonexpanded NK cells. They can also be genetically modified to overexpress NKG2D, a chimeric antigen receptor (CAR) targeted to *CD19* (CD19 CAR; a B lymphocyte antigen also expressed on cancers of the bone marrow), and membrane-bound IL-15 (which influences their in vivo persistence) (*15*). Membrane-bound IL-21–expressing feeder cells for NK cell expansion allow IL-21 signaling to activate telomerase, increasing telomere lengths and prolonging cellular proliferation. These expanded NK cells express high concentrations of IFN-γ, tumor necrosis factor–α (TNF-α), IL-22, and IL-23 (*15*), and NK activating receptors as well as the activation and terminal differentiation markers NKG2D, NKp30, NKp44, NKp46, NKp80, CD69, CD57, and DNAM-1 (*15*, *16*). CD16 is

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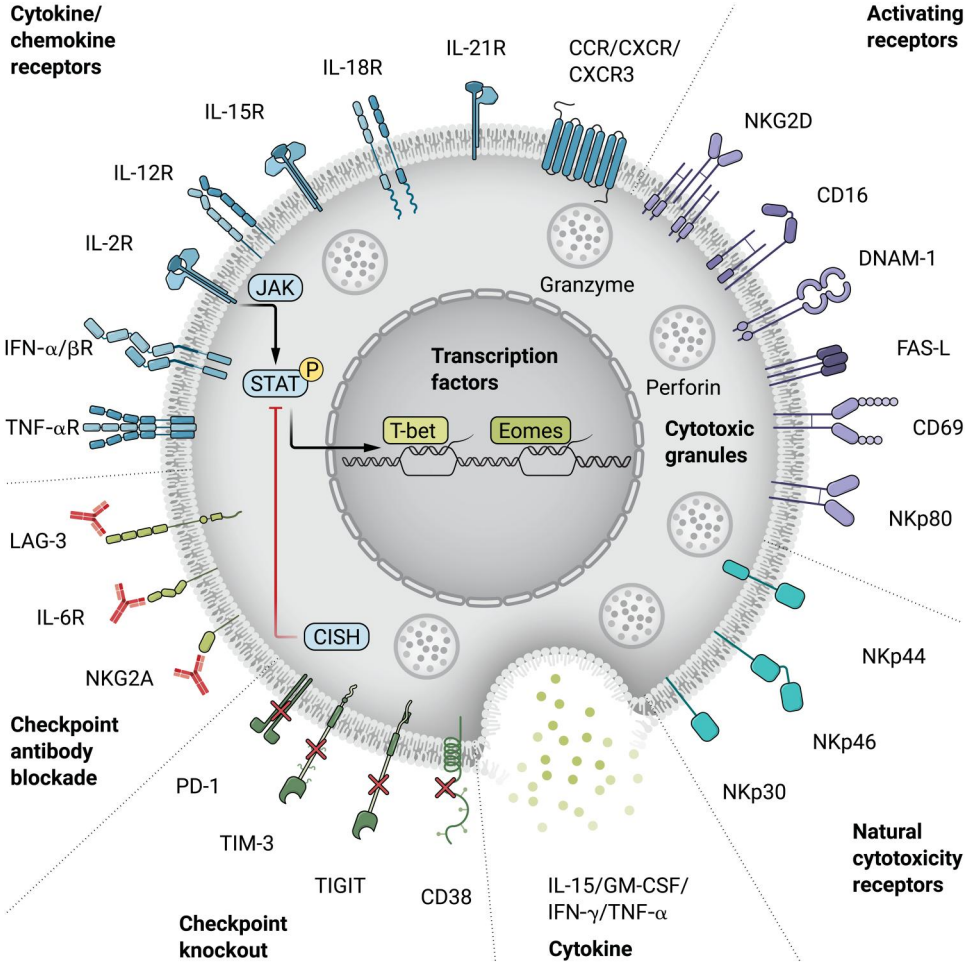

Fig. 1. Design of a generic antiviral NK cell infusion product. NK cells expanded in vitro undergo multiple changes, including up-regulation of receptors for chemokines and cytokines needed for these cells to traffic to and proliferate in regions of viral infection. Expanded NK cells also express activating receptors (e.g., NKG2D, CD16, DNAM-1, Fas-ligand, and CD69) and natural cytotoxicity receptors (e.g., NKp44, NKp46, and NKp30). The antiviral potential of NK cells can be enhanced by antibody blockade or genetic knockout of known NK cell immune checkpoint molecules. JAK, Janus kinase; GM-CSF, granulocyte-macrophage colony-stimulating factor; STAT, signal transducer and activator of transcription; LAG-3, lymphocyte activation gene-3; PD-1, programmed death 1; TIM-3, T cell immunoglobulin and mucin-containing domain-3; TIGIT, T cell immunoglobulin and ITIM domain.

also expressed, although it is susceptible to A disintegrin and metalloprotease 17 (ADAM17)–mediated cleavage, which could be modified genetically. Recent development of a feeder cell–free expansion platform using membrane particles produced from membrane-bound IL-21 feeder cells eliminates the risk of feeder cell contaminants (*16*). This cellular platform is being tested clinically to confirm the potential of membrane-bound IL-21–stimulated NK cells in hematological malignancies (*17*, *18*).

Umbilical cord blood–derived NK cells with genetic modification and CAR expression have also been expanded on membrane-bound IL-21 feeder cells. These NK cells express IL-15 and CD19-CAR, can treat B cell lymphoma, and may be long-lived (*19*). However, like peripheral blood–derived NK cells, their CD16 is cleavable, and they display an immature phenotype.

Induced pluripotent stem cell (iPSC)–derived NK cells can be generated (*20*) and expanded on membrane-bound IL-21 feeder cells (*15*, *16*) and express NKG2D, NKp44, NKp46, and CD94. These cells are also genetically modifiable (*21*). Examples include expressing CD19-CAR and a high-affinity noncleavable variant of CD16a (hnCD16) for enhanced ADCC (*22*). Currently, iPSC-derived NK cell infusion products are being used to target malignancies and SARS-CoV-2 infection (NCT04363346) (Table 1).

Umbilical cord blood–derived NK cells and iPSC-derived NK cells have an immature phenotype, lack KIR expression, and, consequentially, lack KIR-dependent NK cell "licensing." NK cell licensing is thought to be crucial for NK cell functionality and requires KIR–self–HLA-I interactions during NK cell development. However, about a third of all NK cells in mice and humans lack self-specific HLA-I receptor expression and are hence "unlicensed." Although naturally occurring, unlicensed NK cells are hyporesponsive, cytokine-induced activation (with IL-12, IL-15, and IL-18) can trigger potent antitumor effects, including CD16-mediated ADCC (*23*). Furthermore, there are no data that show that KIR expression affects efficacy of adoptively transferred peripheral blood–derived and cytokine-activated NK cells or iPSC-derived CAR-NK cells (*24*).

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Table 1. Clinical trials investigating adoptive NK cell therapies for treating viral infections. Clinical trials were identified on Clinicaltrials.gov (July 2021) using combinations of search terms. Of the 200 identified clinical trials, only open/active or completed interventional trials were included. Trials were excluded if they had unknown, withdrawn, or suspended status; if they were designed to treat malignancies or systemic lupus erythematosus; if they included other therapeutics that could affect infectious disease course or exert a direct effect on infused/endogenous NK cells; if they included immune checkpoint inhibitors, Toll-like receptor agonists, IL-2, peginterferon, direct-acting antivirals, corticosteroids, and dietary supplements; or if studies were performed solely for immune profiling or included vaccination. NK cell immunotherapy studies were included if they were performed during CMV reactivation after hematopoietic stem cell therapy.

Clinical trial status	Study title	Population	Intervention	Sponsor
NCT04634370 pending recruitment	Phase 1 clinical trial on NK cells	COVID-19	NK cell dose escalation	Hospital de Clinicas de Porto Alegre
NCT04578210 recruiting	Safety infusion of NK cells or memory T cells as adoptive cell therapy	COVID-19 patients with pneumonia requiring hospitalization	Arm 1: NK cells Arm 2: Memory T cells From allogeneic donors who have recovered from COVID-19	Instituto de Investigación Hospital Universitario La Paz
NCT04365101 pending recruitment	NK cell (CYNK-001) infusions in adults with COVID-19	COVID-19	CYNK-001 Genetically engineered placental–derived NK cells	Celularity
NCT04363346 pending recruitment	Study of FT516 for treating COVID-19 in hospitalized patients with hypoxia	COVID-19	FT-516 iPSC-derived NK cell product engineered to express noncleavable CD16	University of Minnesota
NCT04324996 recruiting	A phase 1/2 study of universal off-the-shelf NKG2D-ACE2 CAR-NK cells for treating COVID-19	COVID-19	Several arms, UB-CB–derived: NK cells, IL-15-NK cells, NKG2D CAR-NK cells, ACE2 CAR-NK cells, and NKG2D-ACE2 CAR-NK cells	Chongqing Public Health Medical Center
NCT04280224 recruiting	NK cells for treating COVID-19	COVID-19	NK cells (source or expansion not disclosed)	Xinxiang Medical University
NCT04320303 recruiting	CMV infection and immune intervention after transplantation	CMV viremia transplantation infection	membrane-bound IL-21/4-1bb-ligand NK cells	Peking University People's Hospital
NCT03899480 completed in 2021 (n = 9)	Adoptive transfer of haploidentical NK cells and N-803	HIV	Haplo NK cells activated and infused with N-803 (IL-15 superagonist)	University of Minnesota
NCT03346499 completed in 2018 (n = 4)	Adoptive transfer of haploidentical NK cells and IL-2	HIV	NK cells + IL-2	University of Minnesota

Placental stem cell–derived NK cell products are currently being tested in adults with coronavirus disease 2019 (COVID-19) (NCT04365101) (Table 1). As with iPSC-derived NK cells, the use of placental tissue allows for an almost unlimited source of cells, and these cells express NKG2D, CD94, DNAM-1, NKp30, NKp46, and NKp44 but require genetic modification for robust CD16 expression and ADCC functions (*25*).

Whereas NK cell products have garnered clinical interest, several limitations remain. First, upon infusion, NK cells do not proliferate as vigorously as T cells; however, larger numbers of NK cells ($>10^7$/kg recipient) can be infused, and the risk of graft-versus-host disease is low (*24*). Second, NK cell longevity is uncommon, although a recent report of IL-15–producing CD19-CAR NK cells in recipients a year after a single–NK cell infusion is encouraging (*19*). This longevity goal may be easier to achieve with iPSC-derived, hematopoietic stem cell–derived, or placental stem cell–derived NK cells rather than with terminally differentiated NK cells.

NK cell–based therapies for viral infections

NK cells fight both primary and chronic viral infections and are the first lymphocyte lineage to reconstitute after allogeneic hematopoietic stem cell transplant. On the basis of NK cell biology, an adoptive NK cell therapy could be designed to cure difficult-to-treat viral infections caused by key human pathogens.

Human immunodeficiency virus

Thirty-eight million people lived with HIV infection worldwide in 2019 and another 33 million people have died from HIV (*26*). Antiretroviral therapy has changed HIV from a universally lethal disease to a chronically managed disease, but major comorbidities of HIV infection remain.

NK cell effector functions play essential roles in host resistance to HIV disease. For example, NK cells that are expanded during early acute HIV infection inhibit HIV replication (*27*, *28*), and NK cell–mediated HIV-specific effector functions correlate with the emergence of certain viral escape mutants (*29*). In addition, vigorous NK cell activity correlated with protection from HIV infection in exposed persons (*30*–*32*), and increased NK cell–mediated ADCC

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inversely correlated with the risk of infection among participants in an HIV vaccine efficacy trial (*33*). In unvaccinated persons, specific combinations of NK cell KIR genes expressed in conjunction with their HLA ligands are associated with slower HIV disease progression and lower viral set point. Expression of HLA class I alleles of the Bw4 family with KIR3DL1 allotypes (*34*–*36*) and combinations of KIR3DS1 and HLABw4 or KIR2DL3 and HLA-C enhance NK cell activation (*37*). Moreover, HIV slow progressors and elite controllers with high expression of KIR3DL1*h and HLA-Bw4-80I have increased NK cell functionality, with their NK cells exhibiting enhanced K562 target cell–induced NK cell degranulation and IFN-α stimulation–induced IFN-γ secretion (*38*). In contrast, KIR3DS1 is associated with accelerated HIV disease progression, both in the presence and in the absence of coexpressed HLA-Bw4-80I (*39*), and ongoing HIV replication can induce the expansion of a dysfunctional $CD3^{-}CD56^{-}CD16^{+}$ NK cell subset (*40*).

To avoid the killing of HIV-infected T cells, HIV modulates NK cell receptor ligand expression. For example, the viral protein Vpr induces expression of the NKG2D ligands UL16 binding protein 1 (ULBP-1) and ULBP-2, triggering NK cell activation (*41*, *42*). In contrast, viral protein Nef prevents the expression of the NKG2D ligands MICA, ULBP-1, and ULBP-2 on HIV-infected cells (*43*) and induces the down-regulation of ligands to NKp44 (*41*, *44*). Similarly, Vpu reduces NK cell control of HIV-infected cells by down-modulating NK-T-B antigen (NTB-A) on infected cells, which engages the NTB-A coactivation receptor on NK cells in a homotypic fashion (*45*). Furthermore, Nef selectively down-regulates HLA-A and HLA-B, ligands for the NK cell–expressed KIR3DL1 inhibitory receptor, but not HLA-C and HLA-E. Vpu down-regulates HLA-C, but HLA-A and HLA-B are unaffected (*46*–*48*).

Whereas antiretroviral therapy for HIV improves IFN-γ production from NK cells and enhances ADCC function, it fails to completely restore subsets of NK cells. HIV infection reduces the frequency of $CD56^{+}$ NK cells; the expression of NKp30, NKp44, and NKp46; IFN-γ production; and ADCC function while increasing the number of $CD3^{-}CD56^{-}CD16^{+}$ NK cells. Ultimately, in HIV infection, $CD3^{-}CD56^{-}CD16^{+}$ NK cells dominate and exhibit poor NK cell effector functions (*40*).

NK cell products can be administered with cytokines or engineered to secrete growth or stimulating factors, e.g., IL-15. Theoretically, if NK cell products are engineered to recapitulate elite controller immunity, then a functional cure could be reached (Fig. 2). In vitro, IL-15 stimulation robustly activates NK cells from persons with HIV on antiretroviral therapy, restoring IFN-γ production to similar levels seen in HIV-negative persons and resulting in the killing of HIV-infected cells after treatment with the histone deacetylase inhibitor vorinostat, a viral latency-reversing drug (*49*). Furthermore, IL-15 stimulation can both reactivate HIV replication and activate endogenous NK cells to kill HIV-infected cells (*50*–*52*). Activation of NK cells by the IL-15 superagonist N803 inhibited acute HIV-1 infection in humanized mice (*50*). IL-15 treatment increased activated cytotoxic T lymphocytes and NK cells and reduced simian-human immunodeficiency virus (SHIV) in the lymph nodes of SHIV-infected nonhuman primates (*51*). A clinical study evaluating adoptively transferred haploidentical NK cells and N-803 (NCT03899480) for HIV is ongoing (Table 1). Along these lines, a recently reported study in macaques infected with simian immunodeficiency virus (SIV) found that IL-21 and IFN-α treatment during antiretroviral therapy promoted NK cell differentiation ($NKG2A/C^{low}CD16^{+}$) with potent HLA-E–restricted NK cell responses to SIV peptides that were associated with reduced SIV reservoirs (*53*).

CAR-modified hematopoietic stem cells differentiate into NK cells that are resistant to HIV infection and suppress viral replication in vitro (*54*). NK cell products can also be customized to avoid the harmful effects of certain HLA/KIR combinations, focusing instead on phenotypes and effector functions that are elevated in HIV elite controllers and viremic long-term HIV nonprogressors. For example, the $CD11b^{+}CD57^{-}CD161^{+}$Siglec-$7^{+}$ subpopulation of $CD56^{dim}$ $CD16^{+}$ NK cells is more abundant in elite controllers and HIV-negative persons than in viremic noncontrollers, and this NK cell subset's frequency is inversely correlated with HIV DNA levels, suggesting a lower viral reservoir (*55*).

NK cell infusion products could also be paired with additional immunotherapy and are being evaluated in persons with HIV ($n = 4$) in a phase 2 clinical trial studying the effects of haploidentical NK cell infusion after stimulation with IL-2 (Table 1). In addition, given that HIV escapes immune responses by inducing immune checkpoint molecules on cytotoxic T lymphocytes and NK cells, NK cell treatments could be combined with immune checkpoint blockade, as supported by studies of HIV-susceptible humanized mice (*56*–*58*). More studies are needed to assess the safety and efficacy of such NK cell–based therapies for HIV control, and NK cell longevity would need to be improved given that latent HIV is long lived.

BK virus

BK virus is a human polyomavirus that infects up to 90% of people (*59*). In general, BK virus establishes a latent, asymptomatic infection but can be reactivated in immunocompromised persons, including after hematopoietic stem cell transplant or renal transplant, leading to BK virus–associated nephropathy. This occurs in 5 to 10% of renal transplant recipients, of which 50 to 80% progress to graft loss (*60*). Hemorrhagic cystitis due to BK virus reactivation is also seen in 10 to 25% of bone marrow transplant recipients. Treatment options are limited to reducing or replacing immunosuppressive medications or treating with cidofovir (although in a systematic review of 40 studies, rejection of a transplanted kidney was not improved by cidofovir treatment) (*61*).

NK cell responses are relevant to BK virus control (Fig. 2). HLA-F, the ligand for the NK cell–expressed activating receptor KIR3DS1, is up-regulated on kidney tubular cells of BK virus–infected cultured renal biopsies, and enhanced HLA-F expression increases targeting of infected cells by NK cells expressing KIR3DS1 (*62*). Reduced expression of KIR3DS1 correlates with BK virus–associated nephropathy (*63*). The NK cell–expressed activating receptor NKG2D may also contribute to the recognition of BK virus–infected cells. However, the BK virus produces microRNAs that suppress the expression of the stress-induced NKG2D-ligand ULBP3, reducing NK cell activation (*64*). Evidence supporting NK cell infusion for BK virus–mediated renal failure comes from a clinical trial of NK cell products expanded ex vivo on membrane-bound IL-21–expressing feeder cells. NK cells were transplanted into haploidentical patients with high-risk malignancies during the peri-hematopoietic stem cell transplant period (days −2, +7, and +28 to 90); this was associated with improved NK cell numbers and function and a lower rate of disease relapse

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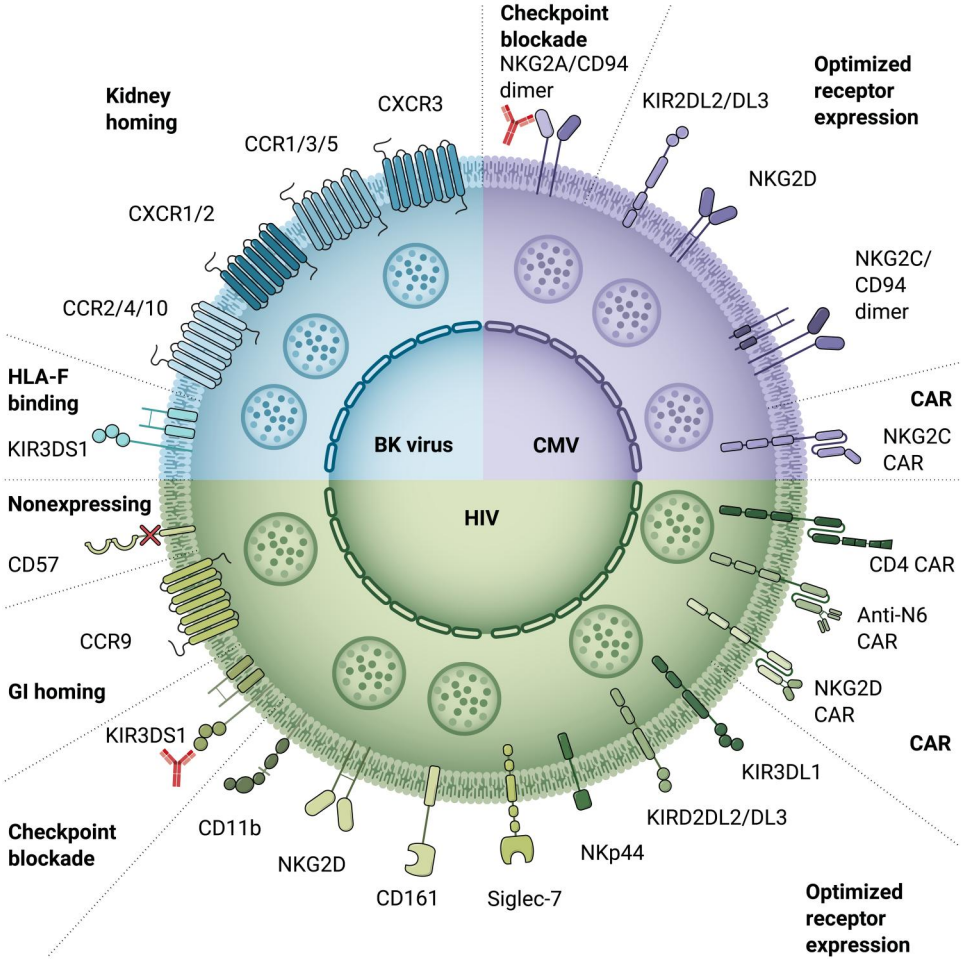

Fig. 2. Optimization of NK cell products for treating BK virus, CMV, and HIV infections. An optimal anti–BK virus NK cell product might include NK cells expressing chemokine receptors needed for trafficking to the kidneys and expression of KIR3DS1 for recognition of HLA-F on BK virus–infected renal tubular cells. For CMV infection, NK cell product optimization would involve manipulation of the NK cell response to HLA-E on infected cells through preferential expression of NKG2C, transfection of an NKG2C CAR, or blockade of NKG2A on mature ($CD57^+$) NK cells coexpressing KIR2DL2/DL3. An anti–HIV NK cell product might mimic features of HIV elite controllers, with optimization of the types of KIR expressed by NK cells relative to the HLA type at the site of infection to avoid NK cell inhibition by KIR ligation. This NK cell phenotype would be $CD11b^+CD57^-CD161^+$Siglec-7^+ with preferential expression of NKG2D and α4β7 integrin. Blockade of KIR3DL1 on NK cells or engineering them to express an HIV-targeting CAR may enable the NK cell product to prevent HIV from escaping the immune response. GI, gastrointestinal.

(*17*). There were no cases of BK virus cystitis in the treated group compared with 31.8% of a matched historical control group (*17*).

Human CMV

Human CMV (HCMV) is a double-stranded DNA herpesvirus type 5 that infects 60 to 90% of people worldwide. After acute infection, HCMV usually remains in various tissues for the lives of most infected persons without causing disease. However, immunocompromised individuals are at higher risk of HCMV reactivation and multiorgan disease (*65*). HCMV is usually treated by reducing immunosuppression where possible and with antiviral drugs, such as asganciclovir, valganciclovir, letermovir, cidofovir, and foscarnet.

HCMV infection and reactivation trigger expansion of $NKG2C^+$ NK cells (*66*), and $NKG2C^+$ NK cells have been identified in peripheral blood, liver, and lung (*3*). A higher percentage of these "adaptive" $NKG2C^+$ NK cells correlates with a lower risk of HCMV disease in transplant patients (*67*). NKG2C expression, $NKG2C^+$ NK cell frequency, HCMV reactivation, and disease risk are interlinked (*68*). NKG2C forms a heterodimer with CD94, and this complex recognizes HLA-E, which presents signal peptides derived from other HLA-I proteins (*69*). In HCMV-infected cells, HLA-E is also stabilized by peptides encoded by the polymorphic HCMV-UL40 region (*70*), resulting in peptide-specific recognition that modulates the differentiation and expansion of $NKG2C^+$ NK cells (*71*). In addition, HLA-E is the ligand for CD94/NKG2A, and signaling through the CD94/NKG2A receptor inhibits NK cell killing (*72*). NKG2A is predominantly expressed by immature NK cells; however, expanding $NKG2C^+$ NK cells generally have down-regulated NKG2A and exhibit a mature, terminally differentiated phenotype (*73*–*75*).

NKG2C allows NK cells to detect and eradicate HCMV-infected target cells, but HCMV has evolved strategies to evade NK cell killing. HCMV down-regulates major histocompatibility complex (MHC) class I molecule expression (*76*) and modulates the expression and posttranslational processing of several genes that encode

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ligands for NK cell–activating and inhibitory receptors (*77*, *78*). HCMV infection also up-regulates HLA homologs that bind to inhibitory KIRs, preventing NK cell–mediated killing of infected cells (*79*). In addition, the HCMV-encoded MHC class I molecular mimic, UL18, inhibits NK cells through ligation of the inhibitory receptor leukocyte immunoglobulin-like receptor subfamily B member 1 (*80*). The HCMV-encoded gene products UL16, UL112, and UL142 down-regulate ligands for the NK cell activating receptor NKG2D, and HCMV infection of NK cells may also lead to sequestering of the NKG2D ligand in the cytoplasm (*78*). NKG2C$^+$ NK cell infusion products can be engineered to overcome such immune evasion strategies and provide a robust therapy for HCMV disease, particularly in persons lacking NKG2C genes (*68*). Both NKG2C gene deletion and haploinsufficiency are associated with greater HCMV reactivation after transplant (*68*, *81*).

In contrast to chronic HIV infection, chronic HCMV and BK virus infections are generally asymptomatic in healthy persons, causing disease predominantly in immunocompromised persons. It is in immunocompromised individuals where NK cell infusions could make up for the lack of effective endogenous cytotoxic immune responses (Fig. 2). An infusion of NK cells, expanded using membrane-bound IL-21–expressing feeder cells, into haploidentical patients with high-risk malignancies could improve NK cell numbers and function and induce a lower rate of HCMV reactivation. These therapies are also being evaluated in posttransplant HCMV viremia (Table 1) (*17*). Future NK cell infusion products could incorporate features of HCMV-associated memory NK cell subsets, such as γ chain–deficient adaptive NK cells that exhibit enhanced ADCC (*82*–*84*).

Influenza virus

Influenza viruses are enveloped, single-stranded RNA orthomyxoviruses of three types (A, B, and C). Most infections are mild to moderate upper airway infections but can progress to severe illness (*85*). Influenza A can be treated with antiviral drugs that target the viral neuraminidase, but viral escape mutants often emerge during treatment, and the efficacy of these therapies is often limited when started late.

Influenza virus infects respiratory epithelial cells by the binding of virus hemagglutinin to sialic acid residues on the epithelial cell surface (*86*). The lung epithelium and tissue-resident innate immune cells sense viral RNA through pattern recognition receptors, producing proinflammatory cytokines and chemokines and promoting an antiviral state. This response activates and recruits immune cells, including NK cells, from the circulation. Activated NK cells secrete large amounts of IFN-γ and kill influenza virus–infected cells by perforin and granzyme release (*87*). B and T cells become activated and migrate to the lung, and the NK cell–expressed CD40 ligand augments influenza virus–specific humoral immunity. Influenza virus infection also elicits cross-reactive antibodies to hemagglutinin and neuraminidase, and internal epitopes of multiple viral strains promote CD16 (FCγRIII)–dependent NK cell–mediated ADCC against infected cells (*88*).

NK cell lymphopenia in the peripheral blood of persons with influenza correlates with severe disease. NK cells make up 10 to 20% of lymphocytes in healthy human lung tissue, and most have a CD56^{dim}CD16$^+$CD57$^+$NKG2A$^-$KIR^{++} phenotype (*89*). Initial reports described human lung NK cells as unresponsive to stimulation (*90*). However, this thinking has been challenged by reports of increased NK cell cytotoxicity in both the CD56$^+$ and CD56^{++} subsets from human peripheral blood and lung after influenza virus infection (*91*). Similarly, although it is thought that NK cells recruited from the circulation are responsible for IFN-γ production in the lung, a small but distinct subset of lung-resident CD56^{++}CD16$^-$CD49a$^+$CD103$^+$CD69$^+$ NK cells rapidly degranulate and up-regulate granzyme B and IFN-γ upon influenza virus infection (*92*).

Influenza virus–encoded proteins can directly activate NK cell antiviral activity. Virus hemagglutinin ligates the NK cell costimulatory receptors 2B4 and NTB-A, enhancing NK cell cytotoxicity (*93*). Early studies of lung NK cells suggested that the natural cytotoxicity receptors NKp44 and NKp46 bind to hemagglutinin on influenza virus–infected cells; however, the stimulatory potential of NKp46 and its binding to hemagglutinin have been challenged (*94*). Unsurprisingly, influenza virus has evolved NK cell–specific immune evasion mechanisms, such as virally encoded neuraminidase that removes sialic acid residues from NKp46 expressed on the surface of NK cells, thus disrupting recognition of hemagglutinin by NK cells (*95*). In addition, influenza virus infects NK cells directly through cell surface–expressed sialic acid residues, thus triggering apoptosis (*96*) and decreasing NK cell numbers (*97*). Furthermore, intact virus and free hemagglutinin protein inhibit NKp30- and NKp46-induced cytotoxicity by disrupting ζ chain signaling (*98*).

Emerging NK cell–based therapies may be useful for influenza virus infections (Fig. 3). First, ADCC is a cytokine-independent NK cell–killing mechanism relevant to influenza virus–specific immunity that does not rely on the development of neutralizing antibodies. Even nonneutralizing antibodies can elicit ADCC and may offer protection against emerging pandemic strains of influenza virus (*99*). Antibodies specific to the influenza virus nuclear protein or the ectodomain of the viral ion channel matrix protein 2 have protective effects in mice and activate human NK cell ADCC mechanisms (*100*, *101*). Treatment with NK cell ADCC-activating antibodies could complement more conventional influenza therapies by promoting NK cell killing of infected cells and potentially could offer protection against pandemic strains. Persons with severe influenza could be treated with nonneutralizing cross-reactive antibodies to elicit ADCC, such as antibodies specific to the N terminus of the matrix protein 2 proton channel, which is highly conserved across all influenza A virus serotypes and is required for viral entry, replication, and budding (*102*). A second potential treatment could be the use of bispecific antibody constructs, similar to those used in cancer immunotherapy (*103*), to target influenza virus–infected cells with NK cell cytotoxicity. Whereas T cells are necessary to clear influenza virus, excessive cytotoxic T lymphocyte responses can induce immunopathology (*87*), so the use of bispecific linkers targeting NK cell responses to influenza virus–infected cells may enable a robust but less immunopathogenic response that includes the effector functions of lung-resident CD56^{++}CD16$^-$CD49a$^+$CD103$^+$CD69$^+$ NK cells. NK cell products could be engineered to target infected cells using CARs. For both bispecific agents and CARs, influenza virus–encoded and universally conserved epitopes could be predeveloped to be deployed in the setting of an emerging pandemic. Whether modification of KIR expression could be used therapeutically remains to be evaluated, although NK cell responses to influenza virus may be serotype-specific (*104*), and both inhibitory and activating KIR receptors have been associated with severe disease during the 2009 H1N1 influenza pandemic (*105*). It remains unclear whether this correlation

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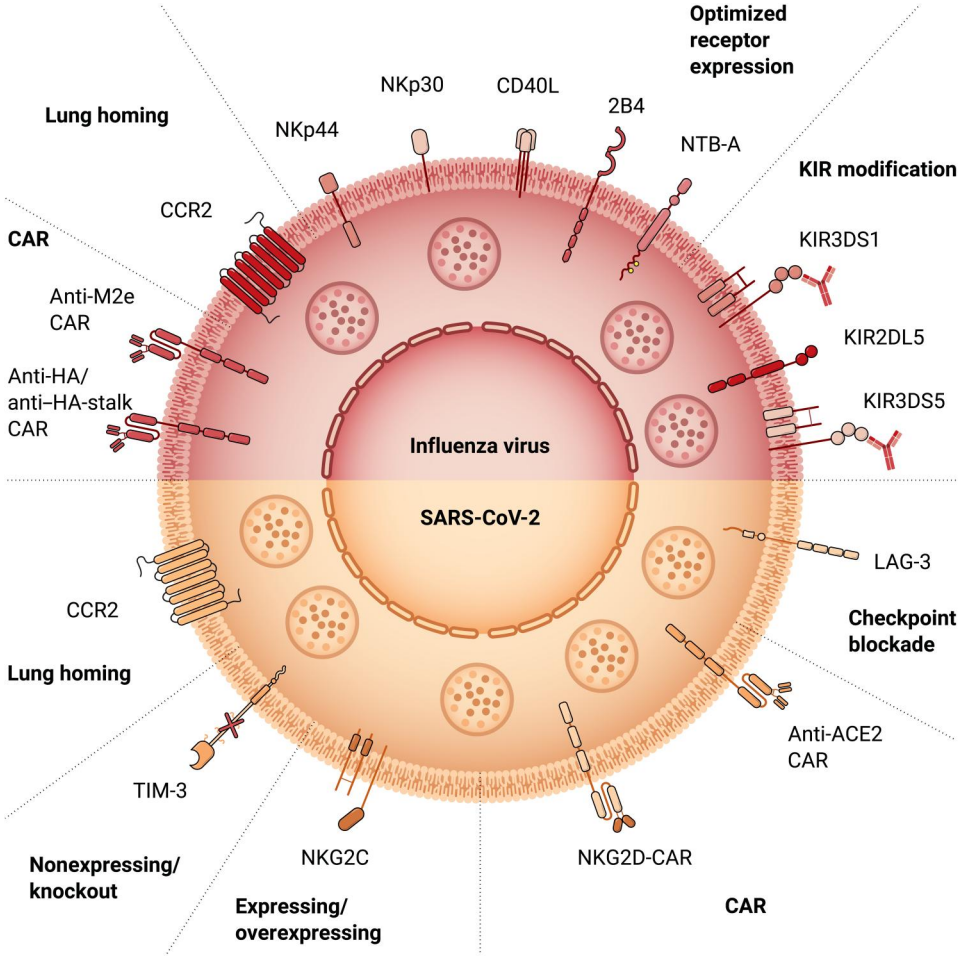

Fig. 3. NK cell products optimized for treating influenza virus or SARS-CoV-2 infections. NK cell products to treat pulmonary infections would express CCR2 for trafficking to the lungs. Preferential expression in the NK cell product of the natural cytotoxicity receptors NKp44 and NKp30, the costimulatory receptors 2B4 and NTB-A, and CD40 ligand is predicted to enhance the recognition of influenza virus–infected cells and augment ADCC and NK cell degranulation. Tailoring the types of KIRs expressed by NK cells or blocking specific KIRs locally to avoid known KIR-HLA combinations associated with severe disease may provide an influenza virus serotype–specific benefit. NK cell recognition and lysis of influenza virus–infected cells could be further enhanced by transduction of CARs that recognize conserved viral structures into the NK cell product. For an anti–SARS-CoV-2 NK cell treatment, studies suggest the potential benefit of knocking out or blocking putative NK cell exhaustion markers, such as TIM-3 or LAG-3. Also potentially beneficial may be overexpression of the activating receptor NKG2C, given that a decrease in NKG2C$^+$ NK cells and deficiency of the gene encoding NKG2C (*KLRC2*) are risk factors for severe COVID-19. HA, hemagglutinin.

results from enhanced NK cell inhibition or greater NK cell–mediated immunopathology, perhaps mediated by NK cells expressing both licensing and activating KIRs.

SARS-CoV-2

SARS-CoV-2 is the cause of COVID-19 and has become a global pandemic, with an estimated 645 million people infected and over 6.64 million deaths worldwide (as of November 2022). For people with severe COVID-19, widespread damage occurs in the lungs consistent with a hyperinflammatory state and macrophage activation syndrome (*106*). Current treatments for COVID-19 include direct-acting antivirals for early infection, like remdesivir, which is a nucleoside inhibitor of the viral RNA–dependent RNA polymerase. Other treatments include monoclonal antibodies, like casirivimab and imdevimab, and inflammatory modulators for later disease stages, like dexamethasone, tocilizumab, and baricitinib (*107*). NK cell products may also have a potential role (Fig. 3).

Multiple reports note a correlation between COVID-19 severity and phenotypic changes in peripheral blood NK cells (*108*–*111*). In persons with severe COVID-19 in the intensive care unit (ICU) compared with non-ICU patients, the absolute numbers of T cells and NK cells, but not B cells, are reduced (*112*, *113*). Furthermore, the frequency of NKG2A-expressing NK cells in peripheral blood is higher in persons with COVID-19 than in healthy controls. In contrast, the frequency of CD16-positive NK cells and NK cells expressing the effector function molecules CD107a, IFN-γ, IL-2, and TNF-α is reduced (*114*). In addition, a single-cell RNA sequencing analysis of peripheral blood mononuclear cells from persons with severe COVID-19 found an up-regulation of transcripts encoding LAG3 and HAVCR2 compared with healthy controls (*110*). Together, these changes may indicate an exhausted NK cell phenotype that corrects itself upon recovery (*115*, *116*). A role for SARS-CoV-2–specific adaptive NK cell–mediated immune responses has not yet been explored. However, persons with severe COVID-19 have fewer

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$CD56^{bright}$ and expanded mature $CD57^{+}$ $FcεRIγ^{neg}$ NK cells than those with moderate COVID-19 disease or COVID-19 survivors (*116*). In contrast, a decrease in a distinct adaptive NK cell subset, $NKG2C^{+}$ NK cells, correlates with more severe disease, suggesting that adaptive NK cell subsets are not equal in their ability to protect humans from severe COVID-19 (*117*). Similar to other viral infections, SARS-CoV-2 induces NK cell, monocyte, and dendritic cell apoptosis, thus contributing to immune suppression (*118*). In addition, the SARS-CoV-2 spike protein induces HLA-E up-regulation, which may inhibit NK cell killing by ligating the NK cell–expressed inhibitory NKG2A receptor (*119*). This inhibition can be abrogated in vitro with monalizumab, an antibody blocking NKG2A's inhibitory interaction with HLA-E (*120*). Whether changes observed in peripheral blood also occur in the lung remains to be fully explored. Single-cell RNA sequencing of bronchoalveolar lavage fluid–resident immune cells collected from persons with severe COVID-19 did not find increased NK cell–related transcripts compared with healthy controls, but NK cells appeared to be activated (*121*).

SARS-CoV-2 infection results in elevated serum concentrations of proinflammatory cytokines and chemokines (*122*), but how this increase modulates NK cell phenotypes and functions is currently unclear. Concentrations of the proinflammatory cytokine IL-6 inversely correlate with the frequency of granzyme A–expressing NK cells in patients with COVID-19 admitted to the ICU, and IL-6 blockade with tocilizumab restores NK cell cytotoxic activity and increases expression of granzyme A and perforin in NK cells (*112*). Other reports suggest that tocilizumab may prevent excessive inflammation and production of cytokines and chemokines and improve outcomes when given at an early stage of COVID-19 (*123*).

NK cell frequencies and their exhausted phenotypes suggest that NK cells may not contribute to severe inflammation in COVID-19; thus, NK cell immunotherapy may be beneficial (Table 1). One proposed off-the-shelf therapy is based on cryopreserved allogeneic NK cells generated from placental hematopoietic stem cells. This product, called CYNK-001, results in NK cells expressing the activating receptors NKG2D, DNAM-1, NKp30, NKp44, and NKp46 and the effector molecules perforin and granzyme B (clinical trial NCT04365101). Another strategy is to use neutralizing single-chain variable fragment (scFv)–secreting bispecific NKG2D-ACE2 (angiotensin-converting enzyme 2) CAR-NK cells derived from cord blood, combined with an IL-15 superagonist, to block SARS-CoV-2 infection of ACE2-expressing hostcells while up-regulating NK cell cytotoxicity; this approach is currently being tested in a clinical trial (NCT04324996). A different approach is the use of expanded NK cells on membrane-bound IL-21–expressing K562 feeder cells to generate highly activated $CD56^{bright}$ and $CD16^{bright}$ (i.e., "double-bright") NK cells. These double-bright NK cells lack the ACE2 receptor and are not targets for SARS-CoV2 (clinical trial NCT04634370). Another strategy is the use of allogeneic NK cell infusions generated from COVID-19 survivors as an adoptive therapy for lymphopenic persons with severe COVID-19 (clinical trial NCT04578210). A different phase 1/2 clinical study for severe COVID-19 is using allogeneic peripheral blood mononuclear cell–derived NK cell products collected from healthy donors and stimulated with IL-2/IL-15 (clinical trial NCT04344548). Another option is the use of CAR NK cells expressing the scFv domain of the virus spike protein receptor binding domain–specific neutralizing antibody, called S309. The use of an antibody specific to the highly conserved region of the SARS-CoV-2 spike protein increases the likelihood that S309-CAR-NK cells may recognize SARS-CoV-2 variants. In vitro, S309-CAR-NK cells kill target cells expressing SARS-CoV-2 spike protein and, compared with the recently published CR3022-CAR-NK cells, show superior killing activity and cytokine production (*124*). Along these lines, monoclonal antibodies are being used for early treatment of SARS-CoV-2 infection, and some have modifications in Fc domains—e.g., the YTE monoclonal antibody (carrying M252Y/S254T/T256E mutations) and the LS monoclonal antibody (carrying M428L/N434S mutations)—that increase their half-lives but decrease their ability to trigger ADCC (*125*). It is unknown whether such loss of ADCC will affect the effectiveness of these antibody-based therapies for COVID-19. In addition, antiviral medications are generally taken after symptom onset; therefore, early viral replication and associated mortality are not prevented. Thus, NK cell infusions are attractive potential treatments for acute viral infections, including lower respiratory virus infections, where antiviral therapy may be too slow to affect morbidity and mortality.

CONCLUSIONS

NK cells mount rapid, robust antiviral immune responses, combining the killing of infected cells with the secretion of large amounts of antiviral cytokines and chemokines and modulation of the adaptive immune system. Studies of NK cell responses in chronic and acute viral infections have shed light on resistance mechanisms and viral evasion strategies and offer therapeutic possibilities for some of our most intractable virus infections. Armed with this knowledge, advances in cell culture technology and genetic engineering may make it possible to propagate NK cells armed with new receptors and growth factors, redirecting their target specificity and ensuring their prolonged persistence in vivo. In addition, there are various opportunities for combination therapies such as combining NK cell products with antibodies to enhance ADCC. NK cell–based immunotherapy may have the potential to affect cancer treatments, but this approach has remained underexplored for antiviral treatments.

NK cell therapy may represent a new class of antiviral treatment, which is perhaps most promising for incurable viral infections, such as HIV and CMV, and persistent viral infections in immunocompromised persons, such as SARS-CoV-2 and influenza virus. Advantages of NK cell–based therapy include new options for persons with multidrug-resistant infections or persons who cannot tolerate other available therapies. It is also unlikely that viral drug resistance, as seen with HIV and influenza virus in response to antiviral drugs, will develop with NK cell–based therapies (*126*, *127*). Furthermore, off-the-shelf NK cell therapies (*128*) may offer immediate treatment for patients in extremis or those infected with new viral pathogens. However, there will be plenty of disadvantages to NK cell therapy. Although small-molecule antiviral therapy can be expensive, adoptive NK cell therapy will likely cost more for treating most infections and probably will be reserved for second- or third-line therapy in conjunction with small-molecule therapy. NK cell therapy may be less expensive than comparable CAR-T cell therapy (*129*). Storage of NK cells and infusion will likely be more complicated than for other cellular therapies. Several studies do suggest that cryopreserved NK cell infusion products can maintain their effector functions and therapeutic efficacy (*130*, *131*); however, cryopreservation requires further optimization. In

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addition, to successfully treat chronic virus infections, NK cell persistence will need to be improved, because infused NK cells are not thought to persist nearly as long in the recipient person as infused T cells do. To this end, key requirements, including costimulatory and cytokine signals essential for NK cell persistence, will need to be examined, and strategies to block the elimination of infused NK cells by the recipient allogeneic immune system will have to be developed. Of course, NK cell therapy remains unproven for any viral infection and side effects remain uncharacterized, which will require a number of robust clinical trials in the future. Much-needed research in this area could help to develop NK cell therapies for difficult-to-treat viral diseases.

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