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Humanized mouse models for preclinical evaluation of HIV cure strategies

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Abstract

Although the world is currently focused on the COVID-19 pandemic, HIV/AIDS remains a significant threat to public health. To date, the HIV/AIDS pandemic has claimed the lives of over 36 million people, while nearly 38 million people are currently living with the virus. Despite the undeniable success of antiretroviral therapy (ART) in controlling HIV, the medications are not curative. Soon after initial infection, HIV integrates into the genome of infected cells as a provirus, primarily, within CD4$^+$ T lymphocytes and tissue macrophages. When not actively transcribed, the provirus is referred to as a latent reservoir because it is hidden to the immune system and ART. Following ART discontinuation, HIV may emerge from the replication-competent proviruses and resumes the infection of healthy cells. Thus, these latent reservoirs are a major obstacle to an HIV cure, and their removal remains a priority. A vital aspect in the development of curative therapies is the demonstration of efficacy in an animal model, such as the humanized mouse model. Therefore, optimization, standardization, and validation of the humanized mouse model are a priority. The purpose of this review article is to provide an update on existing humanized mouse models, highlighting the advantages and disadvantages of each as they pertain to HIV cure studies and to review the approaches to curative therapies that are under investigation.

Keywords

Humanized mice. HIV cure. HIV latency. Functional cure. Sterilizing cure.

Introduction

Mice have made immeasurable contributions to the improvement of public health through their use in scientific research. Disease pathogenesis, therapeutic safety and efficacy, and biological characteristics of infectious agents are just a few areas of research in which mice have been deployed as a model system. In the development of therapeutics, murine models have been employed to evaluate a treatment's safety and efficacy. Since mice can provide researchers with complete access to all organ and tissue specimens, preclinical studies produce a holistic evaluation of a therapy that may be used to support clinical trials[1,2]. When compared to other animal models, mice boast significant benefits. Mice have small housing and nutrition requirements, as well as large litter sizes. In addition, numerous inbred strains of mice have been developed with genetic backgrounds that may support a variety of investigations into human diseases[1,2].

Despite their benefits, mice are not a perfect model for human systems. However, the recent development of humanized mouse models has begun to bridge the gap between murine and human systems. The insertion of human characteristics through cell engraftment or genetic manipulation, within mice, has allowed for the

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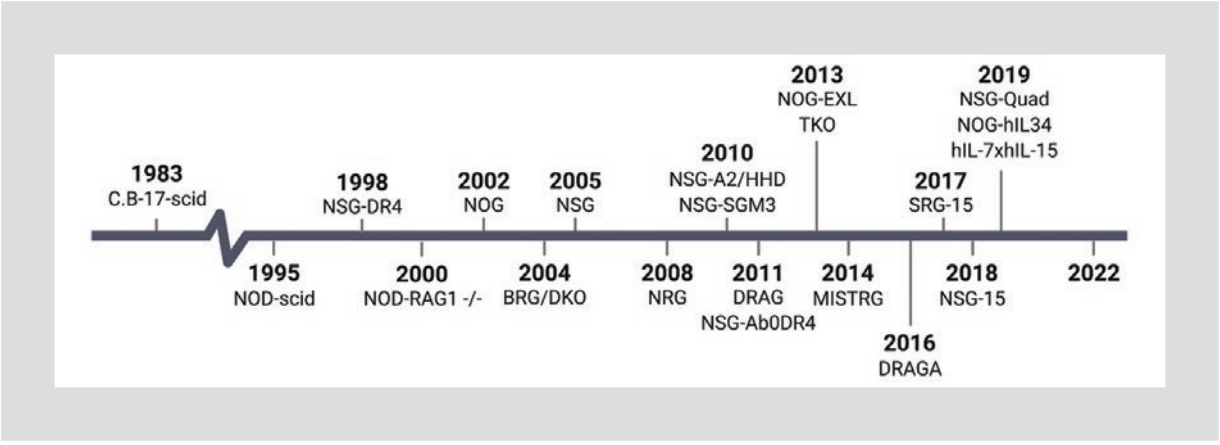

Figure 1. *Timeline for the identification and generation of immunodeficient mouse strains leading to murine hosts suitable for reconstitution with human immune cells.See Table 1 for a brief description of each strain. Created with BioRender.com.*

generation of human-like immune systems and accurate modeling of many human diseases (for the purposes of this review, a humanized mouse refers to a mouse reconstituted with human immune cells)[2]. Humanization requires an immunodeficient mouse strain, so the transplanted human cells are not rejected by the murine immune system. Since their inception, these models have evolved through transgenic crossing and knock-in/knock-out mutations to improve their capacity for a robust and sustainable engraftment of human immune cell populations (Fig. 1, Table 1).

HIV is a pathogen that only establishes infections in human-CD4$^+$ cells. Despite advances in antiretroviral therapy (ART), the main treatment for HIV, the virus continues to be an endemic threat to global public health. While ART effectively achieves viral suppression and promotes partial rebound of immune function, multiple morbidities associated with infection continue to impact patient health outcomes. These therapeutic regimens still do not address the latent viral reservoir, nor do they completely resolve immune abnormalities[3,4]. Furthermore, adherence to ART regimens is difficult for many people living with HIV (PLWH) because most formulations must be taken daily for the duration of their life. Obstacles to treatment adherence prevent many PLWH from maintaining viral suppression. Therefore, the development of a cure could potentially eliminate the burden of HIV and ART from PLWH. To date, several HIV cure approaches have been evaluated and involve a variety of pharmacological, genetic, and immunological techniques.

There are currently two preclinical animal models available to evaluate HIV cure strategies, humanized mice, and non-human primates (NHPs). Each animal model has its advantages and disadvantages. Unlike NHPs, humanized mice contain human-CD4$^+$ cells that can be infected with HIV. In contrast, NHPs may only be infected with simian immunodeficiency virus (SIV) or chimeric simian-human immunodeficiency virus, whose sensitivities to ART may differ from that of HIV. Furthermore, SIV strains can use additional coreceptors, besides CCR5 and CXCR4, to potentially establish viral reservoirs in unconventional cell types. Compared to NHPs, mice can be obtained and housed in larger numbers, allowing for the testing of multiple conditions while ensuring statistical power. However, despite these differences, HIV cure studies in humanized mice and NHPs have demonstrated similar outcomes between the models[5]. Therefore, it is reasonable to conduct initial animal studies in humanized mice, due to their convenience, then confirm the data in NHPs.

Methods of humanization in mice

There are several commonly used methods for the generation of humanized mouse models (Fig. 2). The first method involves injection of human PBMCs. This model (Hu-PBMC or Hu-PBL) supports rapid CD3$^+$ T cell expansion and is useful for the observation of the T cell response[2]. Human lymphocytes found in this model maintain the donor's immunological memory and become highly activated due to exposure to mouse antigens. B cells are present, but only at very low levels. While there are many cases where this simple model is preferred, as for the evaluation of new HIV drugs, it is not suitable to study chronic infection

Table 1. Description of the immunodeficient strains discussed in this review

Strain/Reference	Description
C.B-17-scid[60-63]	Severe combined immunodeficiency
NOD-scid[64]	Non-obese-diabetic-scid
NSG[65]	IL-2rg KO on NOD-scid background
NOG[66]	Il-2rg mutation on NOD-SCID background
NRG[67]	Rag1 KO on NOD-scid background
BRG/DKO[68]	Rag 2 and IL2rg KO on BALB/c background
TKO[8,69]	Rag 2, CD47, and IL2rg KO on C57BL/6 background
NSG-A2[16]	binding domain of HLA class I-A2 Tg on NSG background
NSG-A2/HHD[70]	binding domain of HLA class I-A2 and human β2-microglobulin Tg on NSG background
NSG-DR4[71]	HLA-DR4 Tg on NSG background
NSG-Ab0DR4[72]	HLA-DR4 Tg and mouse MHC II KO on NSG background
DRAG[73]	HLA-DR4 Tg on NRG background
DRAGA[21]	HLA-A2 Tg on DRAG background
NSG-SGM3[74]	SCF, GM-CSF, IL3, and Tg on NSG background
NOG-EXL[66,75,76]	GM-CSF, IL3, Tg on NOG background
NSG-Quad[32]	M-CSF Tg on NSG-SGM3 background
MISTRG[33]	M-CSF, SCF, GM-CSF, IL3, thrombopoietin, and Sirp-α KI on DKO background
NSG-15[22]	IL15 Tg on NSG background
SRG-15[77]	IL15 and Sirp-α KI on DKO background
NSG hIL-7×hIL-15[78]	IL15 and IL7 KI on NSG background
NOG-hIL34[34]	IL34 Tg on NOG background

Tg: transgenic; KO: knock out; KI: knock in. *Adapted and updated from Terehara et al.*[9]

because the longevity of this model is severely limited by the development of graft versus host disease (GVHD)[2,3,6]. To address this substantial shortcoming, McCann et al. postulated that administration of purified CD4+ memory T cells would reduce GVHD development by decreasing the diversity of the T cell receptor repertoire. A study in immunodeficient mice showed that transplantation with total T cells led to the development of GVHD within 7 weeks, while GVHD did not occur in mice injected with CD4+ memory T cells[7]. Another method of preventing GVHD in this model is to perform experiments in the Triple Knockout (TKO) mouse strain. The TKO strain is immunodeficient and, importantly, does not express murine CD47. In the absence of CD47, TKO mice do not signal through the CD47-SIRPα cascade, which mediates the recognition and elimination of the mouse cells by the human cells. Holguin et al. showed that the Hu-PBMC TKO model delays GVHD development by 28 days compared to Hu-PBMC models in other mouse strains[8].

The engraftment of CD34+ human hematopoietic stem cells (HSCs) derived from bone marrow, cord blood, or fetal liver has been shown to be an effective method of generating a diverse and durable immune

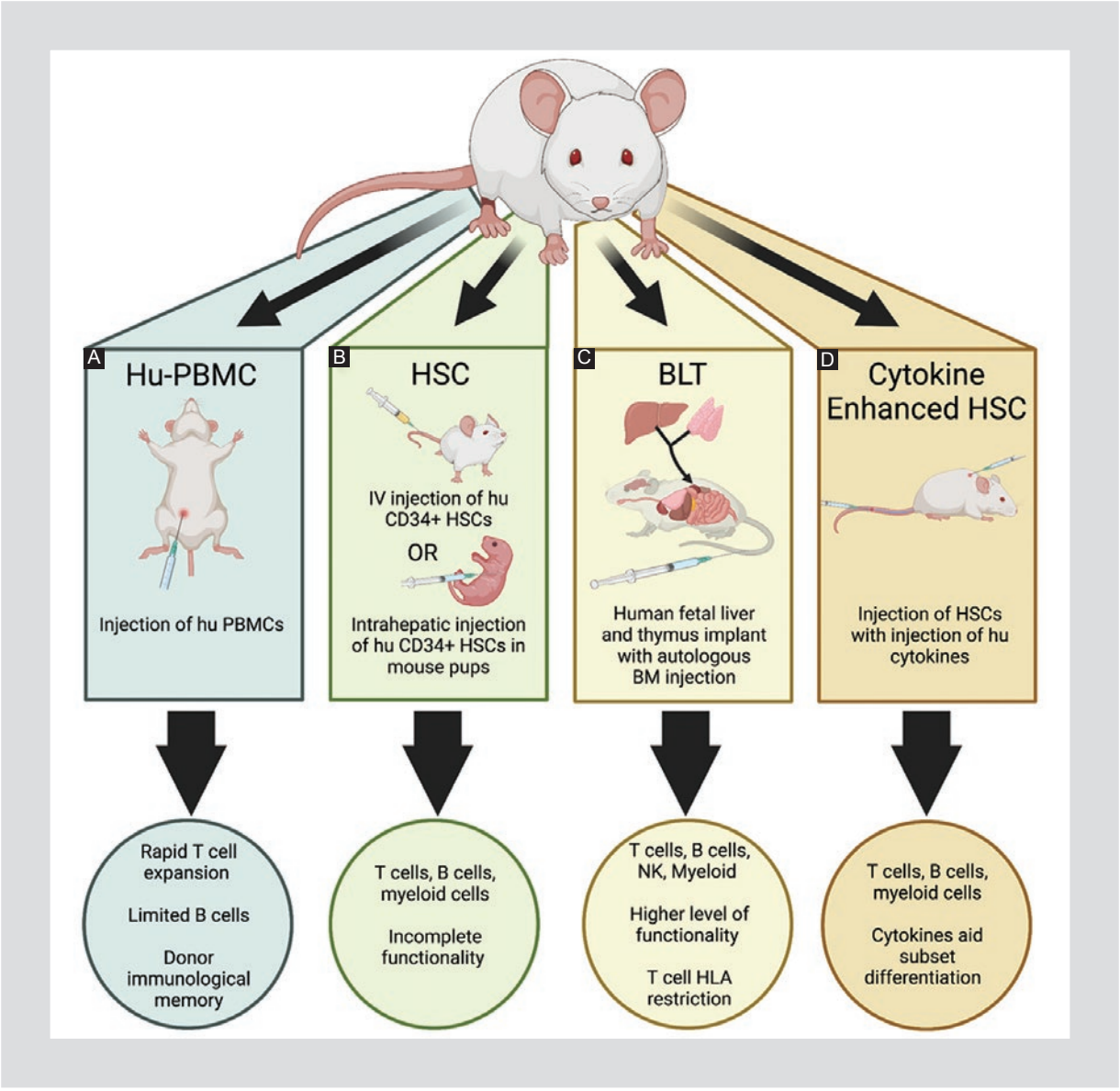

Figure 2. *Depiction of methods commonly used for generation of humanized mice and a general description of the resulting reconstitution for each.* **A:** *generation of a Hu-PBMC-NSG mouse by intraperitoneal injection of human PBMCs.* **B:** *generation of HSC-NSG mouse by intravenous injection of human HSCs in an adult mouse or intrahepatic injection of human HSCs in newborn mouse pups.* **C:** *generation of BLT mouse by surgical implantation of human fetal liver and thymus followed by intravenous injection of bone marrow or fetal liver derived HSCs from the same donor.* **D:** *generation of HSC-NSG mouse by intravenous injection of human HSCs enhanced with subsequent injection of human cytokine. Created with BioRender.com.*

system reconstitution[2,9]. HSCs are often introduced by intrahepatic injection in newborn pups or by intravenous injection in adult mice[10,11]. This model (Hu-HSC) requires sublethal irradiation to preferentially kill rapidly dividing cells, including bone marrow cells, before CD34$^+$ cell injection. This depletion of bone marrow cells allows for human CD34$^+$ HSCs to seed and proliferate in their place. A derivative of the Hu-HSC model, the CD34T$^+$ model, follows the injection of umbilical cord blood CD34$^+$ cells with IL-7 injections. This procedure improves the human immune presence in gut associated lymphoid tissue. Another model expands on Hu-HSC by implanting human fetal liver and thymus under the kidney capsule before the injection of human CD34$^+$ cells autologously derived from the fetal liver or bone marrow. This model, which also requires irradiation preconditioning, is referred to as bone marrow/liver/thymus (BLT) and is considered one

of the most effective engraftment methods[12]. BLT mice have robust, highly functional immune reconstitutions. The implantation of the human thymus allows for the education of T cells in human tissues, rather than in the mouse thymus, resulting in human leukocyte antigen (HLA) restriction[6]. Another benefit of this model, compared to its exclusively HSC-engrafted counterpart, is that the human immune cells are more widespread in tissues, including reproductive and gastrointestinal tissues[12,13]. While there are many advantages, this model is limited by the difficulty in obtaining fetal tissues, as well as the development of GVHD[2,7,8]. An alternative to fetal thymus tissue is thymus tissue obtained from neonatal cardiac surgeries. Brown et al. have shown that transplantation of neonatal thymic tissue fragments in conjunction with autologous-cord-blood- derived HSCs into mice gives comparable results to those in BLT mice[14].

Recent efforts to improve on humanized mouse models have sought to enhance the method of engraftment and promote complete immune reconstitution. A major area of development is the improvement of myeloid cell differentiation. Honeycutt et al. developed a humanized mouse model, myeloid-only mice (MoM) using the NOD/SCID strain. This strain can support engraftment with myeloid and B cell populations, but not human T cells[15]. MoM are generated by transplanting human $CD34^+$ HSCs into NOD/SCID mice. This model supports macrophage development throughout many tissues, including the brain, and is useful for evaluating HIV cure interventions within macrophage reservoirs. However, the utility of this model in HIV cure studies is limited because there are no interactions between macrophages and T cells. In another approach, the use of exogenous cytokine injection and hydrodynamic gene delivery to supplement HSC engraftment has proved beneficial. Introduction of exogenous cytokines though injection of recombinant human FMS-like tyrosine kinase 3 ligand (FLT3L) induced functional dendritic cell (DC) development in HSC-NSG-A2 mice[9,16]. Using hydrodynamic gene delivery, Chen et al. showed that the transfection of HSC-NSG mice with human cytokine genes resulted in cytokine expression for 2 - 3 weeks and larger populations of some myeloid cell types[17]. However, hydrodynamic gene delivery has significant limitations. For long-term experiments, repeated hydrodynamic injections are necessary to maintain cytokine expression and cell differentiation. Therefore, transgenic mouse strains that constitutively express human cytokines may provide a preferable alternative model.

Current generation immunodeficient strains for the creation of humanized mice

The first immunodeficient strain for successful humanization was CB.*17-Prkdcscid* (commonly referred to as the *scid* mouse), which carries the severe combined immunodeficiency (*scid*) mutation. This mutation impairs development of mature T and B lymphocytes. After the *scid* mouse, the NOD/Lt-scid (NOD-scid) strain was developed, followed by NOD.Cg-*PrkdcscidIL2rgtm1WjN/Sz* (NSG) and NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Sug}*/JicTac (NOG). The non-obese-diabetic (NOD) background results in innate immune deficiencies. Compared to NOD-scid, NSG and NOG carry a deletion (in NSG mice) or alteration (in NOG mice) of the IL-2 common gamma chain (IL2rg), which disrupts the production or function, respectively, of several cytokine receptors, namely, IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21[18]. In both cases, the mutations inhibit the cytokine communication that is crucial for the development of lymphoid cell populations including natural killer (NK) cells[2,3]. Mutations in the Rag1 locus result in similar B and T deficiencies to the *scid* models. When the Rag 1 mutation is coupled with an NOD background and the IL2rg mutation, this strain, referred to as NRG, produces comparable reconstitution to NSG mice, but has increased radiation tolerance[19]. Similar to the NRG strain, the BRG/DKO mouse contains Rag2 and IL2rg mutations, but uses a BALB/c background rather than NOD.

Together, these mice make up the current generation of strains for humanized mouse models. The most commonly used of which are the NSG, NOG, and BRG/DKO[2]. Reconstituted cell populations vary depending on method of engraftment, but, generally, these models support the development of the major subsets of a human immune system. Notably, for HIV research, these models produce large quantities of $CD4^+$ T cells in multiple tissues, such as the peripheral blood and the primary and secondary lymphoid organs[9,12]. In addition, these models support B cell populations, mostly the immature phenotype, and some innate immune cells[9]. While these strains are widely used and extremely versatile, their translation to a human system is limited by their imperfect and incomplete reconstitution of cell subsets[1,6]. Furthermore, humoral immunity is impaired by limited class switching, low levels of immunoglobulin (Ig) production, and poorly developed germinal centers and lymphoid tissues. Additional shortcomings of these models stem from the species-specific nature of the cytokines neces-

sary for the differentiation of immune cells. To overcome these shortcomings, the next generation strains, which express human cytokines, have been developed[20].

Next generation strains for the creation of humanized mice with improved lymphoid development

Newer strains incorporate knock-in mutations to improve the capacity for immune system reconstitution. A popular method for improving existing models has been the incorporation of HLA genes, so engrafted T cells are educated with matched HLA, rather than mouse MHCs, in the mouse thymus. Examples of this include the NSG-A2 and NSG-A2/HHD strains, both of which express the binding domain of HLA Class I-A2, while the NSG-A2/HHD mouse also expresses human β2-microglobulin. In addition, HLA Class II-expressing strains have been developed on both an NSG (NSG-DR4 and NSG-Ab0DR4) and NRG (NRG-DR4 (DRAG)) background. Genetic manipulation of the DRAG strain to include HLA-A2 expression has resulted in the DRAGA mouse[21]. Each of these strains listed above have increased functionality of $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells. In some models, the $CD4^+$ T cells support Ig isotope class switching, which has not been previously observed in the current generation of mouse strains[6,9].

To address the lack of NK cells in the current generation of humanized mice, NK development has been stimulated by the introduction of IL7 and/or IL15 genes. These cytokines are produced in the transgenic NSG-15 and the NSG hIL-7xhIL-15 mice[22]. In addition, the SRG-15 strain contains IL15 and Sirp-α knock-in mutations on a DKO background[9]. The increased availability of NK cells, within these strains, provides an opportunity to study the role of antibody-dependent cellular cytotoxicity (ADCC) in an HIV cure[23].

Next generation strains for the creation of humanized mice with improved myeloid development

Although the role of resting memory $CD4^+$ T cells as a reservoir of HIV infection has been clearly established, there is evidence that macrophages also represent a durable HIV reservoir. Tissue-resident macrophages, including microglia in the central nervous system (CNS), have a lifespan of months to years and are resistant to the cytopathic effects of HIV[24,25]. Studies in both SIV-infected macaques and HIV-infected humanized mice demonstrate that tissue macrophages are productively infected and represent a source of rebound viremia upon cessation of ART[15,26,27]. Thus, the development and evaluation of HIV cure strategies should investigate the impact on macrophages.

In most cases, the current generation mouse strains used for humanization (e.g., NOG, NSG, NRG, BRG) do not support sufficient development of human myeloid cells (monocytes, macrophages, or DCs). One reason for this is that mouse cytokines do not stimulate the human cytokine receptors that are important for myeloid lineage differentiation. This hurdle to myeloid cell differentiation is being addressed by the development of transgenic mice expressing human cytokines. The specific cytokines controlling hematopoiesis in humans are shown in figure 3. Transgenic NSG mice expressing the human cytokines stem cell factor (SCF), granulocyte macrophage colony stimulating factor (GM-CSF), and IL-3, called NSG-SGM3 mice, have displayed an increased production of myeloid cells and regulatory T cells (Tregs) following the injection of $CD34^+$ human HSCs[28,29]. Similarly, transgenic expression of human GM-CSF and IL-3 on the NOG background (NOG-EXL model) produces comparable reconstitution[30]. However, these HSC-NSG-SGM3 and HSC-NOG-EXL mice still exhibit poor macrophage development.

An improvement in macrophage development within humanized mice may be achieved through the incorporation of human macrophage colony stimulating factor (M-CSF) genes[31]. To this end, a new mouse strain, termed NSG-Quad, has been created by the crossing of NSG-SGM3 mice and M-CSF-expressing transgenic mice[32]. Studies are currently underway to assess macrophage reconstitution in NSG-Quad. Another approach to achieve reconstitution with human macrophages is the MISTRG strain. The MISTRG is a human M-CSF, SCF, GM-CSF, IL3, thrombopoietin, and SIRPA knock-in mouse on a DKO background[33]. This model supports robust development of highly functional T, B, NK, and myeloid cells, without the need for irradiation pretreatment[9]. Despite these advantages, the MISTRG is severely limited by its shortened lifespan (about 10-16 weeks after engraftment) compared to other strains[12]. These models, supporting differentiation of human myeloid lineages, provide important platforms for targeting HIV reservoirs in macrophages.

In addition, an IL-34 transgenic strain (NOG-hIL34) has recently emerged[34]. This model uniquely supports differentiation of microglia, which allows for the study of

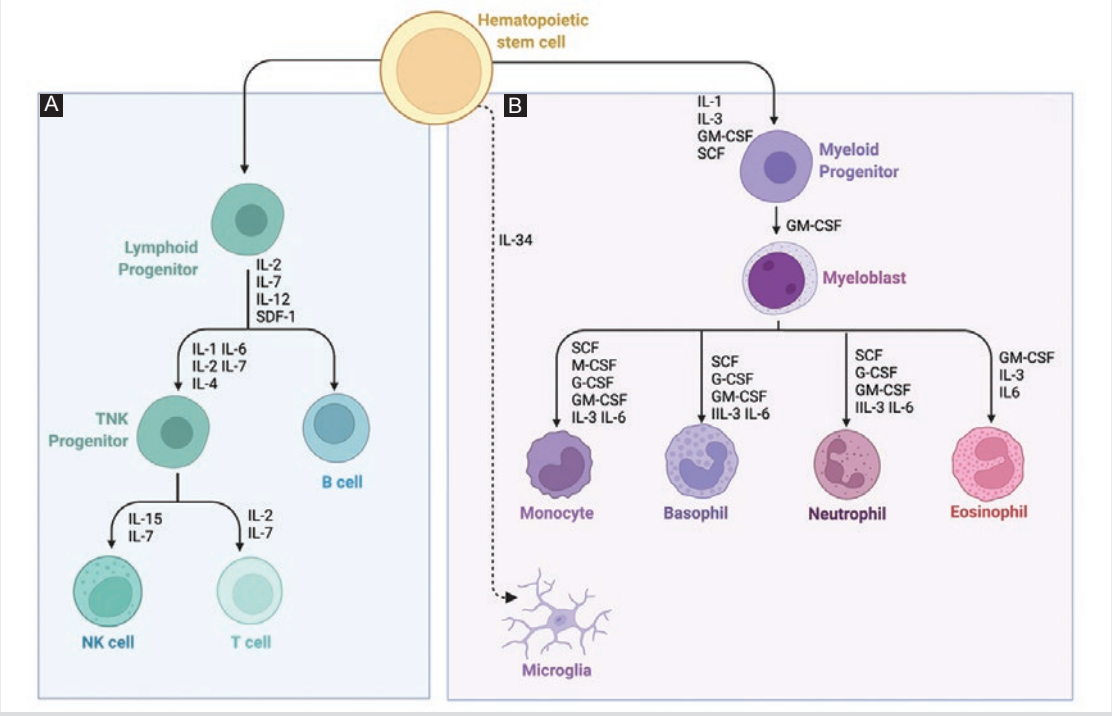

Figure 3. *Flow chart of human immune cell differentiation.* **A:** *differentiation of lymphoid lineage cell types that can be found in some humanized mouse models, including the important cytokines necessary for their development.* **B***: differentiation of myeloid lineage cell types that can be found in some humanized mouse models, including the important cytokines necessary for their development. A dotted line represents the pathway for the differentiation of microglia from a unique stem cell type in the yolk sac (not depicted in figure). Created with BioRender.com.*

HIV infection in the brain and CNS. This area has been particularly elusive in the pursuit of a HIV cure because many therapies struggle to cross the blood-brain barrier and target reservoirs within the CNS[2,34]. Therefore, these mice provide an unprecedented opportunity to study the effectiveness of novel therapies on viral suppression or viral clearance in the brain and the CNS[2].

HIV cure strategies and their preclinical evaluation in humanized mouse models

While many cure strategies for HIV have found success *in vitro*, the need for preclinical testing is crucial to ensure that a therapy can be safely utilized in clinical trials. Through constant development, the humanized mouse model has become an excellent resource in preclinical studies of HIV cure strategies. The generation of a human-like immune system within the mouse allows for the evaluation of immunological therapies. In addition, the HIV-infected CD4$^+$ cells in humanized mice can be targets of novel pharmaceutical and genetic therapies.

As shown previously, it is apparent that humanized mouse models have experienced rapid developments. Similarly, HIV cure strategies have experienced their own advances. From improvements of existing procedures to the incorporation of new technologies, cure strategies have drawn closer toward their goal of eradicating the global burden of HIV.

A curative therapy for HIV falls into two categories: a functional cure and a sterilizing cure. Functional cures can control viremia in PLWH for an extended period without the support of ARTs. In this situation, the virus persists, but the individual will not progress to AIDS and cannot transmit the virus to others. In contrast, sterilizing cures eradicate all functional proviruses within the host. The current

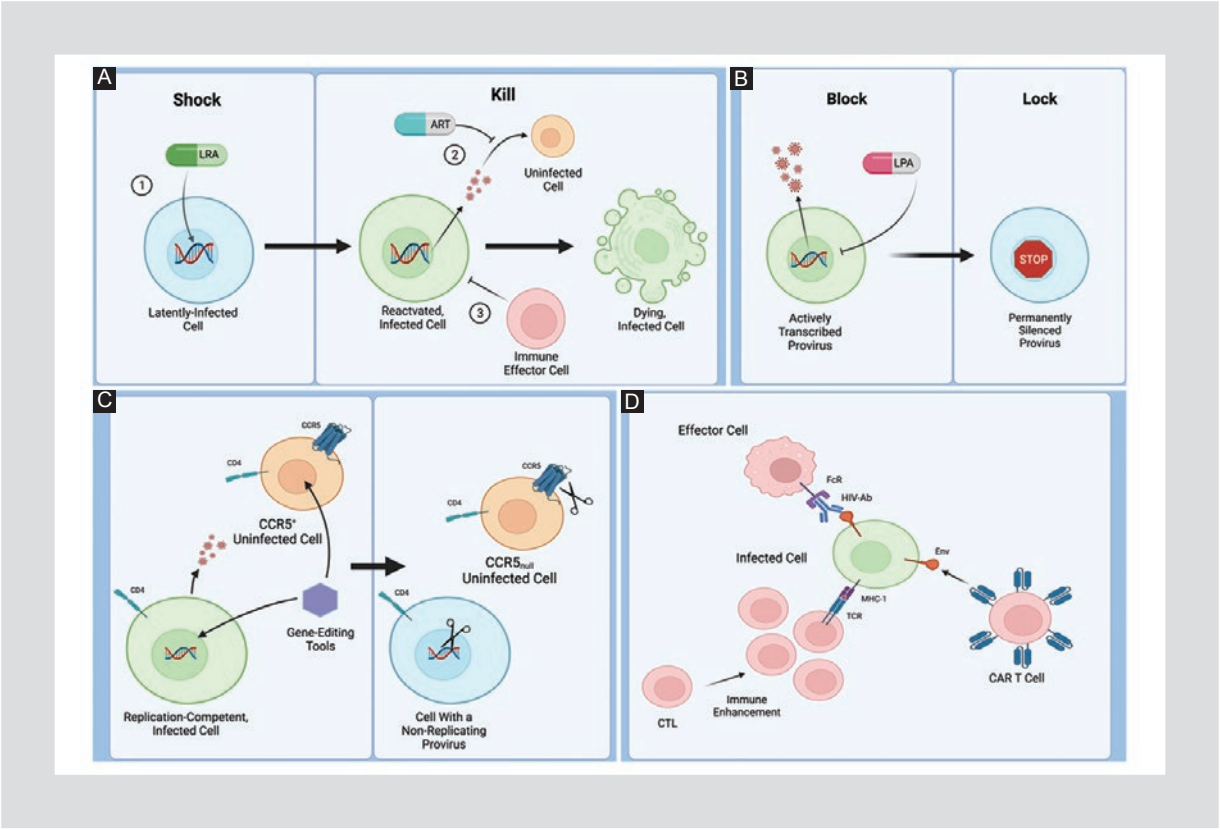

Figure 4. *Review of the cure strategies described in this paper.* **A:** *shock and kill cure strategy. (1) A LRA is administered and enters the cell to induce transcription of the latently-infected cell's provirus. (2) The reactivated and infected cell begins transcribing the provirus and producing virions. However, ART treatment prevents the spread of the virus to uninfected cells. (3) Reactivated and infected cells produce markers of viral infection during production of virions, which triggers a response from immune effector cells that lead to the death of the infected cell.* **B***: block and lock cure strategy. An LPA enters an infected cell with an actively-transcribed provirus and induces changes that permanently cease transcription of the provirus.* **C:** *genetic cure strategies. Gene editing tools can act on replication-competent, infected cells to excise or disrupt provirus genes. This prevents the provirus from producing functional viruses. Gene editing tools may also act on uninfected cells to prevent CCR5 production or to interfere with CCR5 genes. Interference allows CCR5 proteins to be produced, but these proteins cannot interact with HIV glycoproteins.* **D:** *immunological cure strategies. Immune enhancements can promote the proliferation and activity of anti-HIV CTLs. Antibodies with Fc-mediated effector functions can facilitate the eradication of infected cells through interactions with HIV proteins on infected cells and the Fc receptors of effector cells. CAR T cells can recognize HIV proteins, such as the envelope protein (Env), on an infected cell and produce a cytotoxic response. Created with BioRender.com.*

research into curative therapies involves three main approaches: an immunological approach, a genetic approach, and a pharmacological approach (Fig. 4)[35-37].

A cure for HIV is an elusive goal because the virus employs multiple mechanisms to preserve its genome within the host. In the usual disease course, the natural host immune response alone is usually insufficient to eradicate or control an established HIV infection. This mainly occurs because HIV can persist in a latent state within some infected cells. These latently infected cells have integrated the HIV provirus into the host genome but are not transcriptionally active. While most proviruses are dysfunctional, a portion remains replication competent. Latent cells avoid triggering the host immune response because they do not produce markers of viral infection, such as double stranded RNA or antigens. Furthermore, while ART can inhibit the spread of the virus to uninfected cells, these medications cannot remove or destroy the provirus within the host genome. Therefore, if ART treatment is stopped or becomes ineffective, HIV virions from latent cells can infect new cells, leading to a rebound of viremia and progression of the disease[35]. Therefore, the production of latent reservoirs stands as the greatest obstacle to a cure for HIV.

Immunological techniques

The immunological approaches to HIV cures have two aims: enhance the host immune response or introduce artificially modified immune cells and effectors.

These include immune enhancement therapies, anti-HIV antibodies, and chimeric antigen receptor (CAR) T cells. Most immunological HIV cures, alone, may produce functional cures, but in combination with other approaches, they could produce sterilizing cures.

Immune enhancement therapies

HIV vaccines that enhance the $CD8^+$ T cell response are a strong candidate for a functional cure. HIV-specific $CD8^+$ T cells can naturally control HIV infection by destroying infected cells and suppressing infection. However, their function is limited by T cell depletion and exhaustion, as well as the presence of latent reservoirs. Therapeutic vaccines can provide a functional cure by amplifying the HIV-specific $CD8^+$ T cell population and inducing other antiviral mechanisms. The current research into therapeutic vaccines utilizes DCs to stimulate HIV-specific immunity. In these vaccines, DCs are transfected to produce HIV antigens and other immunomodulatory proteins. These vaccines have shown promise in humanized mice and clinical trials. A study in BLT-humanized mice by Norton et al. found that a DC vaccine transfected with a lentiviral vector containing a plasmid that encodes HIV-1 antigens, CD40 ligands, and a soluble programmed cell death 1 dimer can enhance the antiviral response to HIV. While the enhancements from therapeutic vaccinations are significant, there is a concern that the effects may not be durable[38]. Therefore, routine vaccinations may be required. However, in combination with other therapies, the immune enhancements from a therapeutic vaccine could lead to a sterilizing cure by supporting the complete eradication of infected cells.

An alternative form of immune enhancement was demonstrated by the Goldstein laboratory. The laboratory designed synthetic proteins known as synapse for T cell activation (synTac) that selectively stimulates HIV-specific $CD8^+$ T cells to expand and attack HIV infected cells. In NSG mice injected with PBMC in their spleens and infected with HIV, treatment with synTacs increased HIV-specific $CD8^+$ T cells by 32-fold, which potently triggered the killing of HIV infected cells and suppression of viral replication[39]. Thus, multiple methods of enhancing the anti-HIV response are emerging.

HIV antibodies with Fc-mediated effector functions

Antibodies are an important component of the host's immune response to viral infections. They may suppress HIV by neutralizing virions and/or mediating the killing of infected cells through Fc receptor-mediated effector functions. The Fc-mediated effector functions include: ADCC, antibody-dependent cellular phagocytosis, and complement-dependent cytotoxicity[40]. ADCC is mainly mediated by NK cells, macrophages, and neutrophils. Studies of broadly neutralizing antibodies (bNAbs) in humanized mouse models have demonstrated the importance of antibody effector functions. The treatment of HIV-infected HSC-NRG humanized mice with a combination of three bNAbs and three provirus inducers, followed by removal of all treatments, led to an absence of HIV rebound in 57% of the mice. The delay in virus rebound, in the treated mice that did rebound, suggests a reduction of the HIV reservoir by bNAb-mediated killing[41]. A reduction of the HIV reservoir has also been demonstrated by non-neutralizing HIV antibodies with potent Fc-mediated effector functions through interactions with NK cells in the HSC-SRG-IL15 humanized mouse model[23]. Together, humanized mouse models have demonstrated the potential for combinations of latency reversing agents (LRAs) and HIV antibodies (both bNAbs and non-neutralizing) in HIV cure studies.

CAR T cells

CAR T cells are a popular therapy for blood malignancies, such as B-cell leukemias. However, these cells have potential as a curative therapy for HIV because they can be engineered to display broad and efficient antiviral activity. A CAR is a modified receptor that consists of an extracellular domain, which is formed through a fusion of the heavy and light variable chains of an Ig. The extracellular domain is connected to the transmembrane domain through a hinge region. Inside the T cell, the transmembrane region connects to a signaling domain that activates specific T cell functions. In HIV-specific CARs, the most common extracellular domains contain Ig-like CD4 domains (CD4ζ-based CARs) or a fused bNAb[42]. Previously, CAR T cells were generated by obtaining a host's PBMCs, then transducing and amplifying the cells *in vitro*. At present, delivery systems for CAR-encoding genes can transduce T cells to express CARs *in vivo*. A paper by Weidner et al. describes a protocol for the delivery of a CAR-encoding plasmid to engrafted T cells within a humanized mouse[43]. The *in vivo* generation of CAR T cells would improve the therapy's scalability by producing the cells inside of the patient instead of a laboratory.

T cells can be transduced to express one (mono-CAR), two (duoCAR), or more unique CAR molecules. The incorporation of additional CAR molecules can increase specificity. A study by Anthony-Gonda et al. involved the development of bispecific and trispecific duoCAR T cell models. In humanized mice, these duo-CAR T cells suppressed HIV infection in the spleen more effectively than natural T cells[44]. Therefore, CAR T cells in combination with other therapies have great potential as sterilizing cures because these cells efficiently eradicate viral reservoirs.

Genetic techniques

The two successful HIV cures achieved in the Berlin and London patients involved allogeneic hematopoietic stem-cell transplantation from donors with the CCR5 Δ32 mutation, which confers resistance to CCR5-tropic HIV infection[45,46]. These successes have provided the rationale for targeting CCR5 in gene editing approaches. In addition, the disruption of the HIV provirus has been an elusive goal that may soon be possible due to the advent of novel gene editing therapies. The current genetic techniques for HIV cures include endonuclease therapy and recombinase therapy.

Endonuclease therapy

Endonucleases, such as zinc-finger nucleases (ZFNs) and clustered regularly interspaced short palindromic repeat-Cas9 (CRISPR-Cas9), are enzymes that selectively cleave double stranded DNA (dsDNA) sequences. To repair the double-stranded breaks, both endonucleases usually utilize non-homologous end joining (NHEJ), which is a system that regularly deletes or adds nucleotides at the cleavage site. This is a concern for cure therapies because this system is prone to mutations, which may lead the HIV target to generate resistance to the therapy[36]. ZFNs recognize DNA sequences according to their zinc finger array, while Cas9 binding requires the target sequence to interact with a single guide RNA (sgRNA) and to be adjacent to a protospacer adjacent motif (PAM). Xu et al. developed a CRISPR/Cas9-mediated CCR5 ablation method for long-term transplantation of HSCs in HSC-NSG mice[47]. In these mice, challenge with a CCR5-tropic HIV strain produced significantly lower viral loads and spared $CD4^+$ T cells. In another study, in BLT mice, the HIV provirus was excised by SaCas9 with multiple sgRNA, delivered through adeno-associated virus 9 infection[48]. Furthermore, in a seminal study by Dash et al., HIV-infected HSC-NSG humanized mice were treated with long-acting slow-effective release ART (LASER ART) and CRISPR/Cas9 therapy. The results of this study showed that a third of the mice were cured of HIV[49].

Recombinase therapy

Engineered recombinase enzymes control gene expression by integrating, excising, or inverting specific sections of DNA to alter a sequence's activity. Unlike endonucleases, recombinase enzymes directly repair cleavage sites, which prevents damage to the genome[50]. Therefore, recombinases have lower rates of viral escape when altering HIV sequences. The engineering of recombinases has produced a number of enzymes that target different sequences of the HIV provirus. Brec1 is an engineered recombinase that recognizes sequences within the HIV provirus' long terminal repeats (LTR). *In vitro* studies have found that HIV-infected cells that are transfected with Brec1 genes can effectively excise the HIV provirus. The *in vivo* validity of Brec1 recombinase was evaluated in NRG mice engrafted with PBMCs from an HIV-infected patient. In these mice, the treatment with Brec1 successfully eliminated the HIV provirus in the blood, spleen, lung, and liver[51].

Pharmacological techniques

Pharmacological techniques to generate an HIV cure are difficult to establish because few therapies can act on the provirus. The two categories of pharmaceutical cure strategies that have shown promise are shock and kill and block and lock. Both of these therapies interact with the latent reservoir by activating or silencing it.

Shock and kill

Shock and kill is an approach that target the latent reservoirs of HIV. First, a pharmacological agent forces transcription of the latent provirus (the "shock" step). Then, reactivated, infected cells can be eliminated by the immune system (the "kill" step), while ART prevents the spread of HIV to uninfected cells. Agents that induce provirus expression are called LRAs and include disulfiram, histone deacetylase inhibitors (HDACi), DNA methyltransferase inhibitors, histone methylation inhibitors, toll-like receptor agonists, protein kinase C agonists, bromodomain inhibitors and, more recently, selective activators of noncanonical NF-κB signaling pathways (e.g., AZD5582)[37,52]. Some of these LRAs

have shown potent activation of HIV expression *in vitro*, which has been confirmed in humanized mouse models. The protein kinase C agonist, SUW133, demonstrated the reactivation, and ultimately, the death of latently infected cells in ART-treated BLT mice[53]. However, the dose of SUW133 needed to produce therapeutic effects was close to the lethal dose. In a study by Nixon et al., another LRA, AZD5582, reversed HIV latency in ART-suppressed, infected BLT mice, as shown by an increase of viral RNA in both plasma and tissues. These results were then confirmed in SIV-infected rhesus macaques. Importantly, no inflammatory cytokines or cellular markers of activation were induced by AZD5582 in either animal model, suggesting the drug is safe[5]. Clinical trials with AZD5582 are currently being planned.

The available clinical data on LRAs suggest that HIV reactivation could be effective in PLWH, but the elimination of infected cells may not be sufficient. Ineffective killing of reactivated, infected cells may be due to inadequate $CD8^+$ T cell and NK cell responses. Thus, shock and kill needs to be combined with therapies that promote the clearance of infected cells to achieve a cure.

Block and lock

The aim of block and lock therapy is to permanently block the transcription of the HIV provirus. This may be achieved by inhibiting the HIV proteins required for virus transcription, such as Tat, or by inhibiting cellular transcription factors, such as CDK9[54]. Several latency-promoting agents (LPAs) have been explored, including the Tat inhibitor didehydro-corticostatin A (dCA), triptolide, curaxin CBL0100, and heat shock protein 90 inhibitors. *In vitro* studies have found that dCA potently and persistently induces transcriptional suppression of the provirus, which cannot be reversed by LRAs. In HIV-infected BLT mice, a combination of ART and dCA treatment for 1-week delayed HIV rebound for 19 days after the treatment was discontinued[55]. In another study, a zinc finger protein (ZFP-362) targeting the HIV promoter region was fused to the active domains of DNA methyltransferase 3A to create a ZPAMt HIV protein repressor[56]. The protein repressor was packaged into exosome nanoparticles to facilitate delivery to tissues. Testing in Hu-PBMC-NSG and HSC-NSG humanized mouse models showed that the protein was capable of suppressing HIV expression in the bone marrow, spleen, and brain. This exosome-based therapy is important for the development of cure strategies because it has shown, for the 1st time, the delivery of block and lock to HIV reservoirs in the brain. Therefore, this therapy has the potential to be adopted for clinical trials along with ART for PLWH, decreasing the stringency of drug regimen and enhancing their quality of life. A novel block and lock strategy could involve exogenous expression of the naturally occurring HIV antisense transcript (Ast). Zapata et al. have reported that Ast RNA naturally impairs HIV expression by interacting through base pairing with homologous DNA sequences in the 5'LTR and by recruiting the Polycomb Repressor Complex 2. This repressor complex introduces the inhibitory epigenetic mark, H3K27me3 into the surrounding chromatin, reducing HIV transcription[57,58]. Although not yet evaluated *in vivo*, exogenous expression of Ast may provide a potential block and lock cure.

Conclusion

While individual HIV cure therapies are still in early development, a growing body of evidence from cure studies in humanized mouse models and clinical trials suggests that a collaborative approach between therapies is needed to facilitate the control or eradication of the virus. A study by the Zack lab in BLT mice demonstrated synergistic combinations of latency-reversing agents and immunological therapies. The treatment with SUW133, a LRA, followed by administration of NK cells cured HIV (no virus detectable in the examined tissues, including spleen) in 40% of the mice, which was a higher percentage than either treatment alone[59]. Further studies involving a combination of cure therapies could utilize a series of LRA cycles followed by the administration of LPAs or gene-editing tools. Since LRAs, LPAs, and gene-editing tools have unique target sequences, utilizing different combinations of these therapies could enhance treatment outcomes and prevent viral escape. As studies of these cure approaches and their combinations progress *in vitro*, humanized mouse models continue to develop and the gap between the bench and the bedside continues to shrink for an HIV cure.

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