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Humanized Mouse Models of HIV Infection

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Abstract

Because of the limited tropism of HIV,* in vivo *modeling of this virus has been almost exclusively limited to other lentiviruses, such as simian immunodeficiency virus, that reproduce many important characteristics of HIV infection. However, there are significant genetic and biological differences among lentiviruses and some HIV-specific interventions are not effective against other lentiviruses in nonhuman hosts. For these reasons, much emphasis has recently been placed on developing alternative animal models that support HIV replication and recapitulate key aspects of HIV infection and pathogenesis in humans. Humanized mice, CD34$^+$ hematopoietic progenitor cell transplanted immunodeficient mice, and in particular mice also implanted with human thymus/liver tissue (bone marrow liver thymus mice) that develop a functional human immune system, have been the focus of a great deal of attention as possible models to study virtually all aspects of HIV biology and pathogenesis.

Humanized mice are systemically reconstituted with human lymphoid cells, offering rapid, reliable, and reproducible experimental systems for HIV research. Peripheral blood of humanized mice can be readily sampled longitudinally to assess reconstitution with human cells and to monitor HIV replication, permitting the evaluation of multiple parameters of HIV infection such as viral load levels, CD4$^+$ T-cell depletion, immune activation, as well as the effects of therapeutic interventions. Of high relevance to HIV transmission is the extensive characterization and validation of the reconstitution with human lymphoid cells of the female reproductive tract and of the gastrointestinal tract of humanized bone marrow liver thymus mice that renders them susceptible to both vaginal and rectal HIV infection. Other important attributes of all types of humanized mice include: (i) their small size and cost that make them widely accessible; (ii) multiple cohorts of humanized mice can be made from multiple human donors and each cohort has identical human cells, permitting control of intragenetic variables; (iii) continuous* de novo *production of human immune cells from the transplanted CD34$^+$ cells within each humanized mouse facilitates long-term experiments; (iv) both primary and laboratory HIV isolates can be used for experiments; and (v) in addition to therapeutic interventions, rectal and vaginal HIV prevention approaches can be studied. In summary, humanized mice can have an important role in virtually all aspects of HIV research, including the analysis of HIV replication, the evaluation of HIV restriction factors, the characterization of successful biomedical HIV prevention strategies, the evaluation of new treatment regimens, and the evaluation of novel HIV eradication strategies. **(AIDS Rev. 2011;13:135-48)**

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Introduction

According to WHO-AIDS there are 33 million HIV-infected people worldwide[1]. Treating these infected persons and preventing new transmissions are cornerstones of the biomedical response to HIV infection. A safe and efficacious vaccine would be the most cost-effective way to control the HIV pandemic, but such a vaccine is not yet available for clinical implementation. Therefore, there is an urgent need for improved, potentially curative HIV therapeutic regimens that can be universally accessed and a need for the rapid implementation of biomedical prevention approaches to stop the spread of HIV/AIDS. Patient-based research to develop these interventions requires extensive preclinical evaluations in robust, reproducible, and reliable models of HIV infection. Cell culture systems and *ex vivo* tissue explants have been used extensively to model HIV infection[2-5]. Explants in particular have been used by several groups because of their inherent increased complexity that includes both epithelium and HIV target cells. However, there are many aspects of HIV infection that can be best studied using *in vivo* models. For example, the entire dynamics of HIV transmission and disease progression in humans is extremely complex and cannot be replicated *in vitro*. Mucosal and peripheral organ systems all play important roles in transmission, replication, and pathogenesis. The ability to model the complexity of HIV disease makes animal models the most versatile experimental systems for the evaluation of HIV transmission, *in vivo* replication, pathogenesis mechanisms, novel therapies, and prevention approaches.

Replication of HIV is restricted to humans and chimpanzees. This strict species tropism has severely constrained *in vivo* experimentation[6]. As alternatives, multiple other lentiviruses have contributed to our understanding of HIV/AIDS when they infect their natural or related hosts as models of HIV infection. For example, wild chimpanzees and gorillas are naturally infected with the predecessor viruses to HIV, SIV_{cpz} and SIV_{gor}[7,8]. Additionally, increased mortality and AIDS-like immunopathology has been documented in wild chimpanzees infected with SIV_{cpz}[9]. These findings offer critical insights into the pathogenic potential of the viruses in these great ape populations; however, great apes are generally unavailable for HIV research. Therefore, alternative model systems have been sought such as lentiviruses capable of causing immunosuppression following infection of nonhuman primates (NHP), cats, and cattle. These viruses include simian immunodeficiency virus (SIV), SIV/HIV chimeric viruses (SHIV), feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV)[6,10-15]. Other lentiviruses that have also been evaluated as HIV models lead to immunoproliferation in horses, sheep, and goats. These are equine infectious anemia virus (EIAV), ovine lentivirus (OvLV) and caprine arthritis-encephalitis virus (CAEV)[15]. In particular, the NHP-SIV and NHP-SHIV models have contributed greatly to our understanding of HIV infection. However, the number of NHP available for research (especially females) is limited and the costs associated with NHP studies are high. It should also be noted that many biomedical interventions for HIV prevention and therapy are HIV-specific and that HIV has genetic and biological differences from SIV and SHIV. Those interventions that inhibit HIV specifically cannot be evaluated using NHP-SIV or NHP-SHIV models[6]. Therefore, *in vivo* infection models that utilize HIV itself are needed and humanized mice have been shown to help fulfill this need.

The term "humanized mice" has been applied to a variety of mouse models: (i) mice that have a single or multiple human transgene(s) in an otherwise wild-type animal; (ii) immunodeficient mice transplanted with human cells or implanted with normal or diseased human tissues; (iii) $CD34^+$ hematopoietic progenitor cell engrafted immunodeficient mice; and (iv) various combinations of (i), (ii), and (iii)[16-22]. The focus of this review is on humanized mouse models of HIV infection generated by $CD34^+$ hematopoietic progenitor cell transplantation. In these mice, $CD34^+$ cells generate *de novo* human immune cells capable of supporting *in vivo* HIV replication. One important point that needs to be emphasized is that whereas most lymphoid cells develop in bone marrow, T-cells do not. Thymic progenitors exit the bone marrow and migrate to the thymus because T-cells require the special microenvironment present in the thymus to develop. In $CD34^+$ transplanted mice this takes place in the mouse thymus[23-26]. However, bone marrow liver thymus (BLT) mice represent the only system where this takes place in the implanted human thymic organoid[27,28]. Thus, in BLT mice human T-cells are generated in the context of human thymic stroma where they are specifically educated in the context of human leukocyte antigen (HLA)[28]. Together, these relatively recently described humanized mouse models represent rapid, reliable, and reproducible experimental systems for HIV research, as evidenced by their extensive use since parenteral HIV infection of these mice was first reported five years ago (Table 1)[29,30].

Why humanized mice?

Their relative simplicity, small size and cost make humanized mice broadly accessible for *in vivo* HIV-infection

Table 1. Humanized mouse models and topical listing of HIV-related references

	CD34+ transplanted humanized mice			
Mouse strain/ humanized mouse	**DKO***	**NOG***	**NSG***	**BLT†**
In vivo HIV replication and pathogenesis	Baenziger, 2006 Berges, 2006 An, 2007 Gorantla, 2007 Zhang, 2007 Berges, 2008 Hofer, 2008 Jiang, 2008 Van Duyne, 2008 Berges, 2009 Choudhory, 2009 Ince, 2009 Hofer, 2010 Sango, 2010 Neff, 2010 Luo, 2010 Neff, 2011	Watanabe, 2007^ Watanabe, 2007^ Nie, 2009 Sato, 2010 Sato, 2010	Van Duyne, 2008 Kumar, 2008 Gorantla, 2010 Gorantla, 2010 Holt, 2010 Joseph, 2010^ Dash, 2011	Sun, 2007 Denton, 2008 Brainard, 2009 Denton, 2010 Kim, 2010 Garg, 2011
Human anti-HIV responses	Humoral – Baenziger, 2006§ – An, 2007 ‡ – Gorantla, 2007‡ – Ince, 2009‡ Cellular – An, 2007‡	Humoral – Sato, 2010§ – Watanabe,2007^§ APOBEC – Sato, 2010§	Cellular – Gorantla, 2010¶	Humoral – Sun, 2007¶ – Brainard, 2009¶ Cellular – Brainard, 2009¶
HIV therapy	ART – Choudhory, 2009 – Sango, 2010 *tat*-derived peptides – Van Duyne, 2008 Neutralizing Ab – Luo, 2010 aptamer delivered siRNA – Neff, 2011	Not evaluated	*tat*-derived peptides – Van Duyne, 2008 T-cell directed siRNA – Kumar, 2008 Zinc-finger Nuclease – Holt, 2010 Neutralizing Ab – Joseph, 2010^	T-cell directed siRNA – Kim, 2010
Mucosal HIV transmission	Vaginal – Berges, 2008 – Neff, 2010 Rectal – Berges, 2008 – Hofer, 2008	Not evaluated	Not evaluated	Vaginal – Denton, 2008 Rectal – Sun, 2007 – Denton, 2010
HIV prevention	Vaginal – Neff, 2010	Not evaluated	Not evaluated	Vaginal and Rectal – Denton, 2008 – Denton, 2010 Intravenous – Denton, 2010

Note that some papers covered multiple topics and therefore are repeated under the various appropriate headings on the last 4 lines of the table.
DKO: double knockout; NOG: *NOD/Shi-scid*γ*c*^{null}; NSG: *NOD/LtSz-scid/scid*γ*c*^{null}; BLT: bone marrow liver thymus.
*Unless indicated by the^, mice were generated by the typical protocol where the CD34+ cell transplant was given to newborns.
Those entries marked^ are studies in which the CD34+ cell transplant was given to adult mice.
†BLT mouse generation includes a bone marrow transplant of CD34+ hematopoietic progenitor cells into NOD/SCID or NSG mice previously implanted with autologous fetal liver and thymus tissue.
‡HIV-specific human immune responses were examined, but not detected.
§HIV-specific human immune responses were occasionally detected.
¶HIV-specific human immune responses were consistently detected.

studies. It is important to note that despite their overall similarities, there are significant differences between humanized mouse models, and that each model has its own advantages and limitations, both of which are addressed throughout this text. However, a key area where further improvements are needed is in the development of robust and sustained immune responses across different platforms (Table 1). Moreover, many organ pathologies caused by HIV are not perfectly recreated in humanized mice because *in vivo* human reconstitution is limited to the progeny of the transplanted human CD34$^+$ cells. One important exception is the BLT model where human thymic pathology can be studied in the context of human thymic epithelium. BLT mice exhibit robust and sustained levels of human cells in their gastrointestinal and female reproductive tracts, whereas differences in the levels and uniformity of reconstitution of these important tissues between humanized mice models using only CD34$^+$ transplants can result in different experimental outcomes. Other factors to consider when choosing humanized mice for HIV research is the relatively shorter life-span of mice (compared to NHP and humans), the heightened susceptibility of immunocompromised mouse stains (especially after irradiation) to opportunistic infections and death unrelated to HIV infection, and the differences in husbandry techniques used in different animal facilities. Nevertheless, specific advantages of humanized mice for HIV research include the ability to generate multiple cohorts of humanized mice from multiple human donors. Each cohort from a single human donor is composed of individual animals with genetically identical human cells, permitting control of intragenetic variables. Continuous *de novo* production of human immune cells from engrafted CD34$^+$ cells within each humanized mouse enables longitudinal experiments. Importantly, humanized mice do not require that a surrogate virus be used for infection studies. Humanized mice can be used for *in vivo* HIV infection studies because they support transmission and replication of laboratory adapted and primary HIV isolates. Finally, HIV prevention interventions can be studied in humanized mice because they are susceptible to multiple routes of HIV transmission.

An overview

Since the early stages of the AIDS epidemic it was realized that small animal models for HIV research would permit a wide range of studies of HIV pathogenesis, prevention, and therapy. The challenge noted was that rodent cells are fully refractory to HIV; HIV cannot bind and enter rodent cells as it does human cells, unless those cells express the primary HIV receptor and a coreceptor molecule (human CD4 and human CCR5 or CXCR4, respectively)[31,32]. However, even when these critical molecules are expressed on the surface of mouse cells and successful viral entry occurs, HIV encounters numerous additional blocks to its replication in rodent cells[33,34]. Since HIV will typically replicate robustly in human cells, several groups developed xenograft models by placing human cells in mice such that HIV could replicate *in vivo*[35,36]. Two of these mouse models, designated severe combined immunodeficient-human (SCID-hu) thy/liv and SCID-hu peripheral blood lymphocytes, have been extensively utilized to study viral cytopathic effects, for the evaluation of antiviral drugs and, to a lesser extent, to evaluate potential vaccine approaches[37-42]. These models are not optimal for the study of anti-HIV human immune responses, pathogenesis, or mucosal HIV transmission[18,43,44]. In contrast, some of the more recently described CD34$^+$ cell transplanted humanized mice have been shown to be well suited for these types of *in vivo* studies.

Common to all CD34$^+$ cell transplanted humanized mouse models is the use of one of several available immunodeficient mouse strains that are capable of engrafting and sustaining human hematopoietic cells[44]. The history and characteristics of mouse strains used for generating humanized mice have been recently described in great detail[44]. The three strains of immunodeficient mice that are most frequently used to generate humanized mice, as reviewed here, are: (i) *nonobese diabetic/SCID (NOD/SCID),* (ii) *Rag2$^{null}\gamma c^{null}$* and (iii) *NOD/SCIDγc^{null}* (Table 2).

The *NOD/SCID* mice (*NOD/Shi-scid*, Central Institute for Experimental Animals, Kanagawa, Japan; and *NOD/LtSz-scid/scid*, The Jackson Laboratory, Bar Harbor, Maine) have reduced mouse natural killer (NK) cell levels and are devoid of mouse T and B lymphocytes[45,46].

Rag2$^{null}\gamma c^{null}$ mice (*BALB/c-Rag2$^{null}\gamma c^{null}$*, Centre National de la Recherché Scientifique, Orleans, France) are frequently referred to as double knockout (DKO) mice, the term used herein. The DKO mice lack expression of the interleukin-2 (IL-2) receptor common gamma chain (γc^{null}) and are completely deficient in mouse T, B, and NK cells[47]. Humanized DKO mice are sometimes referred to as human immune system (HIS) mice or Rag-hu mice[26,30,48,49].

There are two *NOD/SCIDγc^{null}* strain variants originating from the NOD/SCID mice. The *NOD/Shi-scidγc^{null}* mice are referred to as NOG mice (Central Institute for Experimental Animals, Kanagawa, Japan), whereas *NOD/LtSz-scid/scidγc^{null}* mice are called NSG mice (The Jackson Laboratory, Bar Harbor, Maine). Both the NOG and NSG mice also lack expression of the IL-2 receptor common gamma chain (γc^{null}) and are completely deficient

Table 2. Key characteristics of immunodeficient mouse strains most frequently used for humanization

Parent strain	Additional mutation	Most commonly humanized strains	Strain characteristics[44]	Frequent labels for humanized mice of each strain
Rag2 knockout mice	IL-2 receptor gamma chain knockout	*BALB/c-Rag2$^{null}\gamma c^{null}$*	– Typical lifespan greater than 1 year – No mouse T, B, or NK cells – Radiation resistant	DKO-hu HSC RAG-hu mice HIS mice
Non obese diabetic/ severe combined immuno-deficient	IL-2 receptor gamma chain knockout	*NOD/SCIDγc^{null}* – *(NOG: NOD/ Shi-scidγc^{null})* – *(NSG: NOD/ LtSz-scid/scidγc^{null})*	– Typical lifespan greater than 1 year – No mouse T, B, or NK cells – Radiation sensitive – Fewer stem cells required for engraftment	NOG-hu HSC mice NSG-hu HSC mice NSG BLT mice
	no additional mutation	*NOD/SCID* – *(NOD/Shi-scid)* – *(NOD/LtSz-scid/ scid)*	– Lifespan of ~ 1 year due to thymic lymphomas that develop in some animals – No mouse T or B cells/reduced NK cell levels – Radiation sensitive – No human T-cells develop in absence of human thy/liv implant	BLT mice N/S BLT mice

BLT: bone marrow liver thymus; DKO: double knockout; hu: humanized; HIS: human immune system.

in mouse T, B, and NK cells. These two similar strains differ in their gamma chain mutations. In NOG mice, the gamma chain is truncated within the intracellular signaling domain while the targeted gamma chain mutation in NSG mice is a complete null phenotype[44,50,51].

While these are not the only immunodeficient strains utilized to generate CD34$^+$ cell transplanted mice[52-54], they account for the CD34$^+$ cell transplanted mice used in HIV research to date (Table 1).

There are significant differences observed in the levels of engraftment with human CD34$^+$ hematopoietic progenitor cells and in the levels of repopulation with CD34-derived human lymphoid cells between different immunodeficient mouse strains. The *NOD/SCID* mice engraft with human CD34$^+$ cells better than *SCID* mice due to their lower levels of mouse NK cells[45,46]. Better human engraftment in NOG and NSG mice compared to their two *NOD/SCID* parent strains has been reported[55]. Female NSG mice have also been reported to engraft somewhat better than NSG males[56,57]. This is a remarkable phenotype that could possibly be exploited to further increase cohort sizes by using reduced numbers of CD34$^+$ cells for the bone marrow transplants in NSG female mice. To generate CD34$^+$ cell transplanted humanized mice, individual mice are typically preconditioned (i.e. gamma radiation exposure or treatment with busulfan) to create a niche in the bone marrow for the transplanted human CD34$^+$ hematopoietic progenitor cells[25,58-62]. Human CD34$^+$ cells for transplantation are typically derived from umbilical cord blood, fetal liver, or granulocyte colony-stimulating factor mobilized peripheral blood[63]. One important consideration for the use of cord blood-derived CD34$^+$ cells is their relatively low abundance, limiting the number of mice that can be generated from a single sample. However, some investigators have overcome this limitation by combining cord blood CD34$^+$ cells from separate donors for transplantation[64,65]. It should be noted that in the case of humanized BLT mice, each animal receives both transplanted CD34$^+$ cells and implanted tissues from the same donor[27,28]. Regardless of the source of CD34$^+$ cells, engraftment with hematopoietic progenitor cells results in a lifelong (8-24 months, depending on the mouse strain and humanization strategy utilized; Table 2) supply of human immune cells in transplanted humanized mice[44,66].

The nature of humanized mice, which essentially harbor a complete human immune system, provides biomedical researchers with affordable, accessible, and flexible experimental platforms for *in vivo* modeling of human diseases and for the development and evaluation of methods to counter these diseases. In this regard, CD34$^+$ cell transplanted humanized mice have been primarily used to successfully model a variety of human-specific infectious diseases. However, the applications of humanized mice have not been limited solely to human infections. Other diseases and conditions that have been successfully modeled in humanized mice include

type 1 diabetes[67], inflammatory arthritis[68], tumor burden control[69], human breast tumor growth[54], and idiopathic nephrotic syndrome[70]. Besides HIV, human infectious diseases modeled in humanized mice include Epstein-Barr virus[71-74], hepatitis C virus[75], human cytomegalovirus[76], herpes simplex virus type 2 (HSV-2)[77], live attenuated influenza vaccines[52], *Salmonella Typhi*[78-80], sepsis[81], and dengue virus[82-87]. Additionally, autoregulatory lentiviral vectors that permit doxycycline-inducible gene expression in human hematopoietic cells have been successfully utilized in humanized mice as a model for inducible genetic therapeutic strategies being considered for humans[88].

Bone marrow liver thymus mice

As indicated previously, BLT mice are the only model in which human T-cells can develop in the context of a human thymic microenvironment. This significant difference sets this model apart and merits special consideration and description. In contrast to the other humanized mouse models discussed in the following section, in BLT mice implanted human tissue develops into a *bona fide* human thymus where human thymocytes are educated and mature into a diverse repertoire of functional HLA-restricted T-cells[28,89]. Furthermore, the fact that human T-cells were educated in a human thymus could be a contributing factor in the extensive and reproducible human reconstitution of the BLT mouse gastrointestinal tract[90,91].

Generation of BLT mice includes a bone marrow graft accomplished by the transplant of fetal liver-derived $CD34^+$ hematopoietic progenitor cells into preconditioned *NOD/SCID* or NSG mice previously implanted with autologous fetal liver and thymus tissue[27,28,92-95]. Human immune cells, including T, B, NK, myeloid, and dendritic cells, are found throughout BLT mice. Beyond peripheral blood reconstitution with human cells, BLT mouse tissues have been shown to reconstitute with high levels of human immune cells throughout the body, including in the human thymic organoid, bone marrow, spleen, lymph nodes, liver, lungs, small and large intestines, and the female reproductive tract[27,28,89-95]. In BLT mice both the intraepithelial and lamina propria compartment of the small and large intestines are populated with human T lymphocytes[90,91]. The exquisitely specific nature of the human reconstitution of the BLT mouse small intestine is highlighted by the fact that $CD8^+CD4^+$ T lymphocytes in NOD/SCID BLT mice were shown to exhibit a human gut-specific surface phenotype[91]. Specifically, the intestinal $CD8^+CD4^-$ T-cells express the typical alpha-beta heterodimer on their surface, but the $CD8^+CD4^+$ T-cells express a CD8 molecule composed of an alpha-alpha homodimer, which is a known human gut cell surface phenotype[96,97]. The BLT mouse female reproductive tract is also well reconstituted with human immune cells[90]. The vagina, ectocervix, endocervix, and uterus of BLT mice have been shown to harbor many human T-cells, including $CD4^+$ T-cells. In addition, each of these regions of the BLT female reproductive tract contains human monocyte/macrophages and human dendritic cells[90]. Thus, BLT mice are fully reconstituted with multiple human hematopoietic lineages throughout the body, including critical mucosal surfaces such as the intestines and female reproductive tract.

Human immune responses in BLT mice are relatively robust and exquisitely specific, which is in contrast to the limited immune responses described for $CD34^+$ cell transplanted DKO/NOG/NSG mice. For example, 2,4-dinitrophenyl hapten-keyhole limpet hemocyanin (DNP23-KLH) immunization in BLT mice results primarily in DNP-specific IgG1 and IgG2, with minimal DNP-specific IgG3 and no DNP-specific IgG4 detected[94]. This is quite similar to the IgG subclass distribution in humans after KLH immunization, in which IgG3 antibodies are less frequent and IgG4 antibodies are very slow to develop[94,98]. Other robust human immune responses in BLT mice include xenograft rejection, T-cell-dependent antibody development, antiviral responses, specific superantigen responses, and delayed-type hypersensitivity responses[27,28,93-95]. In addition, BLT mice generate the most extensive human immune responses against HIV of any humanized mouse model tested to date.

$CD34^+$ cell transplant mice

As indicated previously, in contrast to BLT mice, $CD34^+$ cell transplanted DKO/NOG/NSG mice do not contain a human thymic microenvironment where human T-cells can develop. Rather, in each of these models, human T-cells are generated in the mouse thymus[23-26]. The lack of HLA restriction could have major implications in the potential for developing robust human immune responses in $CD34^+$ cell transplanted DKO/NOG/NSG mice.

Despite this limitation, $CD34^+$ cell transplanted DKO/NOG/NSG mice have been extensively utilized in HIV research (Table 1). The primary method for generating these mice is to transplant $CD34^+$ cells by either an intrahepatic or intravenous injection into newborn DKO, NOG, or NSG mice (Table 1). This technique for the generation of humanized mice was first described by Traggiai, et al. and Gimeno, et al. and has been widely adopted[25,26,48,99-106]. In addition, $CD34^+$ cells have also been transplanted into adult NOG and NSG mice, resulting in similar levels of systemic human reconstitution[23,57,58,62,107-112].

In each of these instances, the transplanted immunodeficient mouse becomes systemically populated with human immune cells originating from the mouse's bone marrow. Human immune cells, including T, B, NK, myeloid, and dendritic cells, are found throughout CD34$^+$ cell transplanted DKO/NOG/NSG mice. Beyond peripheral blood reconstitution with human cells, tissues from these mice have been shown to harbor human immune cells in the thymus, bone marrow, spleen, lymph nodes, liver, and lungs[23,25,26,48,57,58,62,99,100,102-112]. Reconstitution of the intestines and female reproductive tracts of CD34$^+$ cell transplanted NOG and NSG mice has not been fully addressed in the literature[113]; however, immunofluorescence analysis of CD34$^+$ cell transplanted DKO mice showed the presence of human immune cells in the vagina, large intestine, and rectum of these mice[114]. A different research group also addressed the question of humanization of the female reproductive tract of CD34$^+$ cell transplanted DKO mice and reported a very different result[77]. Their report indicated that the vaginal tract of these mice did not harbor human immune cells unless stimulated by either HSV-2 vaccination or vaginal infection with replication-competent HSV-2. Interestingly, the human cells that were detected in the vaginal tissue of these mice following HSV-2 challenge were CD8$^+$ T-cells and CD3$^-$CD56$^+$ NK cells. Notably, no human CD4$^+$ T-cells were found[77]. Two additional research groups examined the human cell reconstitution of the gut from CD34$^+$ cell transplanted DKO mice. One group found that two of six mice were reconstituted with greater than 5% human lymphocytes when they examined the entire intestine using flow cytometry[115]. The other group reported that CD34$^+$ cell transplanted DKO mice harbor no to very few human immune cells in their small and large intestines after examining 14 mice by immunohistochemistry and three by flow cytometry[116]. Therefore, it is clear that human cells can migrate into the female reproductive tract and intestines of CD34$^+$ cell transplanted DKO mice. However, what is uncertain at this point is the extent to which the human immune cells are able to reconstitute these important mucosal sites of HIV transmission in a reproducible and robust manner. It should be noted that the differences in results obtained between these four research groups while characterizing CD34$^+$ cell transplanted DKO mice could be due to several factors including: use of different protocols for the handling and/or culturing of CD34$^+$ cells prior to injection, differences in the numbers of CD34$^+$ cells transplanted among research groups, age at which mice were tested for mucosal humanization, and perhaps other unknown factors. Overall, based on these reports, the humanization of the intestines and female reproductive tract of CD34$^+$ cell transplanted DKO mice appears sporadic and inconsistent[77,114-116]. These discordant results regarding the intestinal humanization in this model might help explain to a certain extent the differences reported in mucosal HIV transmission in humanized DKO mice (see below).

The functional capacity of the human immune systems in variants of CD34$^+$ cell transplanted DKO/NOG/NSG mice has been evaluated by multiple research groups. The results have indicated that both functional and dysfunctional human lymphocytes and NK cells can be identified in these systems, suggesting that further improvement will be needed before these models can be effectively used to evaluate human immune responses[25,26,58,99,104,111,112,117,118]. Therefore, different approaches have been used to improve the humanization and immune function in these mice. Exogenous human IL-15 and human Flt-3/Flk-2 ligand expression in CD34$^+$ cell transplanted DKO mice was reported to improve NK and myeloid cell levels and function[48,103,109]. Similarly, exogenous IL-7 was evaluated for its ability to enhance human thymocyte development and lymph node maturation in these mice[106]. While this work highlighted the importance of human IL-7 in human T-cell development within CD34$^+$ cell transplanted DKO mice, the IL-7 treatments did not improve homeostasis of peripheral T-cells in this system[106]. In addition to treatment with exogenous factors for improved immune reconstitution and immune function, there have been efforts to generate transgenically modified immunodeficient mice. The CD34$^+$ cell transplanted homozygous DKO mice that express human thrombopoietin (TPO), in place of mouse TPO, maintained peripheral human reconstitution of human lymphocytes for several months beyond what was observed to occur in DKO mice without human TPO. These human TPO-expressing mice exhibit higher levels of human granulocytes, but human T-cell levels were very low in these mice[119]. When human CD34$^+$ cells were transplanted into a modified NSG mouse strain that expresses human stem cell factor, granulocyte macrophage colony stimulating factor and IL-3 (called NSG-SGM3 mice), these mice generated robust human myeloid lineage reconstitution plus an abnormally high number of CD4$^+$ T-cells throughout multiple organs examined. The observed CD4$^+$ T-cell increase appeared to be due to higher numbers of functional CD4$^+$FoxP3$^+$ T regulatory cells, not T helper subsets[120]. In order to improve the human immune responses in humanized NSG mice, this strain has also been engineered to express HLA*A0201 covalently bound to human β2-microglobulin (NSG-HLA-A2/HHD mice)[105]. HLA-A2-restricted human T-cell responses against both Epstein-Barr and dengue viruses have been detected in CD34$^+$ cell transplanted NSG-HLA-A2/HHD mice[83,105].

This strain might offer an improved option for conducting human vaccine research in humanized mice. In short, the relatively poor human immune response observed in CD34+ cell transplanted DKO/NOG/NSG mice is leading to extensive and ongoing efforts to generate better human immune responses in these models.

HIV replication, pathogenesis, and antiviral responses

One observation is clear: virtually all types of HIV isolates tested to date replicate efficiently in humanized mice. They include: subtype B – R5-tropic (JRCSF, BaL, YU-2, ADA, NFN-SX[SL9], UG029A)[29,61,66,89,90,121-133], X4-tropic (NL4-3, LAI, MNp)[29,30,61,66,91,114,125,132-135], and dual-tropic (NL4-3RA, R3A)[132,136]; and subtype C (C1157)[61]. In addition, one SHIV (SHIV-C2/1) has also been shown to replicate in humanized mice[133]. HIV replication has been monitored in individual animals for over a year and mucosal HIV transmission typically results in the expected replication kinetics of peak viremia followed by viral set point levels in humanized mice[124,125]. Intravenous and intraperitoneal HIV exposures were used in most of these studies, while vaginal or rectal viral exposures were used in six reports[90,91,114,116,124,137]. Once infection occurs, HIV is systemically disseminated. Infection results in similar manifestations in all models, including CD4+ T-cell loss and CD8+ T-cell infiltration, regardless of the route of exposure[138]. Table 1 summarizes the humanized mouse model, the route of mucosal exposure, and the type of experiment performed for each of the studies cited above. In all these studies, peripheral blood plus primary and secondary lymphoid tissues were most commonly examined. The CXCR4-tropic HIV isolates in general showed the most dramatic pathogenic phenotypes and a single point mutation (V38E) in the gp41 region of a CXCR4-tropic HIV isolate was found to significantly alter the ability of the mutant virus to deplete CD4+ T-cells[134,138]. It should be noted that HIV pathology in humanized mouse intestines, including reduced gut effector memory CD4+ T-cells as seen in human HIV infections, have only been described in BLT mice[90,91,139].

HIV-specific immune responses have been examined in infected humanized mice (Table 1). However, HIV-specific humoral and cellular immune responses have only been consistently observed in BLT mice[89,91]. In particular, interferon-gamma producing T-cells specific for *gag*, *nef*, *pol* and *env*, plus HLA-restricted cytotoxic T lymphocytes specific for known *gag*- and *nef*-epitopes have been detected in BLT mice. All BLT mice evaluated (n = 9) developed human antibody responses to HIV by 12 weeks postexposure[89]. In addition to this work in BLT mice, CD34+ cell transplanted NOG, NSG, and DKO mice have also been reported to generate HIV-specific human immune responses, although human HIV-specific humoral responses are much less frequent in these models[138]. More recently, the role of CD8+ T-cells in HIV-infected CD34+ cell transplanted NSG mice was investigated by Gorantla, et al., who showed that viral RNA levels in plasma are increased and CD4+ T-cell loss is accelerated when human CD8+ T-cells are specifically depleted[126]. This is similar to what has been observed in NHP[140,141]. A pair of papers by Gorantla, et al. and Dash, et al. utilized CD34+ cell transplanted NSG mice to demonstrate multi-lineage human immune cells in the humanized mouse brain as well as significant neuropathology following HIV infection in these animals[122,123]. Jiang, et al. demonstrated that FoxP3+CD4+ regulatory T-cells are preferentially infected and depleted by HIV in CD34+ cell transplanted DKO mice, and that depletion of the FoxP3+CD4+ regulatory T-cells impaired HIV replication *in vivo*[136]. The role of immune activation in HIV infection in CD34+ cell transplanted DKO mice was examined by Hofer, et al., who found a positive association between chemically induced bacterial translocation, higher viral loads, and lower CD4+ T-cell levels[127]. Additionally, Ince, et al. examined the evolution of CCR5-tropic HIV and found evidence for CD4+ T-cell-induced selective pressure on HIV in CD34+ cell transplanted DKO mice[128]. In one animal, multiple clone sequences were obtained 44 weeks after infection. These sequences contained mutations that were functionally analyzed and determined to have an X4 phenotype, indicating that a tropism switch had occurred in this single humanized mouse[128]. Human anti-HIV innate immunity has also been examined in humanized mice. Specifically, Sato, et al. recently showed that human APOBEC3 proteins can induce mutations in the HIV genome in CD34+ cell transplanted NOG mice; however, there is a caveat to their interpretation in that these mutations occurred despite a functional *vif*[130]. Specifically, the data presented indicates that in three out of four amplicons, the *vif* sequence was found to be mutated, likely due to reverse transcriptase error, and this resulted in 3.5% of 86 *vif* amplicons being prematurely terminated. The five of 243 hypermutated (10+ G→A mutations) amplicons reported did not include *vif* sequence to confirm that *vif* was indeed intact in the hypermutated viral DNA[130]. However, there is convincing evidence that cell-associated DNA carried more G→A mutations at canonical APOBEC 3G, 3F, or 3B sites when compared to circulating virions[130]. Overall, both innate and adaptive human immune responses occur in humanized mice and these responses can result in viral evolution.

HIV therapy

Preclinical evaluation of novel treatments has been an important application of humanized mice in HIV research. *In vivo* efficacy reports have focused on antiretroviral (ART) and gene therapies. Both established and investigational antiretrovirals have been tested in CD34$^+$ cell transplanted humanized mice. Van Duyne, et al. described *tat*-derived peptides reducing plasma viral RNA at a single time point in CD34$^+$ cell transplanted DKO and NSG mice[142]. While this data is promising regarding the ability of the *tat*-derived peptides to reduce detectable plasma levels of HIV in these mice at two weeks post-intraperitoneal inoculation, longitudinal peripheral blood analysis during ongoing treatment would further clarify the potential of these peptides for future clinical applications and provide information regarding the likelihood of developing resistance mutations *in vivo*.

Antiretroviral therapy has brought many benefits to HIV patients, and demonstration of successful ART in humanized mice is critical to the future usefulness of these models. Choudhary, et al. determined peripheral blood and intestinal tissue levels of intraperitoneally delivered emtricitabine, tenofovir, and the integrase inhibitor L-870812. This comprehensive pharmacokinetic analysis was used to develop a triple-combination ART dosing regimen in CD34$^+$ cell transplanted DKO mice[115]. Longitudinal analysis of the effects of this antiretroviral combination showed reduced plasma viral RNA in HIV-infected humanized mice[115]. In two mice, failure of therapy occurred due to the development of resistance mutations to tenofovir and to the integrase inhibitor, suggesting that further optimization of this drug regimen is needed. Nevertheless, when ART was discontinued, the plasma viral RNA concentrations rebounded to pretreatment levels in all humanized mice tested[115]. Sango, et al. reported that ART consisting of zidovudine, lamivudine, and indinavir suppressed HIV in CD34$^+$ cell transplanted DKO mice[143]. This seven-week ART regimen, administered in sterile drinking water, was able to reduce recoverable replication-competent virus from spleen (which is the site of virus inoculation) 300-fold compared to untreated control mice. HIV suppression *in vivo* typically refers to a durable reduction in plasma viremia to below the limit of detection; unfortunately, in this study only a single data point was reported for pooled (n = 4) plasma RNA at the end of the seven-week ART regimen[143]. Analysis of pooled plasma can facilitate more sensitive viral RNA detection, and 69 copies/ml was reported for the treated group, indicating that a relatively sensitive assay was used. No breakthrough in plasma viremia during ART was observed, although longitudinal data was not shown[143]. This research group also evaluated the ability of the broadly neutralizing anti-HIV antibody 2G12 to reduce HIV infection when expressed in humanized mice[144]. Human CD34$^+$ cells were transduced with a lentiviral vector encoding 2G12 prior to being transplanted to generate humanized NSG mice. One week post-intraperitoneal HIV inoculation, mice were harvested and both the number of replication-competent viruses recovered from the spleen and the number of plasma viral RNA copies were quantitated. The results showed that 2G12 reduced recoverable virus 200-fold and the pooled plasma showed a 70-fold reduction in viral RNA copies versus control[144]. When a separate research group utilized dimeric 2G12 sustained at low levels in peripheral blood via "back pack" administration units on CD34$^+$ cell transplanted DKO mice, they found that this dimerized neutralizing antibody further reduced the detectable viral load and lessened the loss of CD4$^+$ T-cells associated with HIV infection when compared to treatment with monomeric 2G12[145]. The data from these manuscripts demonstrates successful therapy in humanized mice helping to fulfill the critical need for the validation of humanized mice for preclinical evaluation of novel therapeutic interventions.

HIV infection requires the presence of both a primary receptor (CD4) and a coreceptor (CCR5/CXCR4). Several therapeutic strategies targeting CD4 or CCR5 have been considered. There is great potential for the curative capacity of such approaches, as illustrated first by the effective clinical use of CCR5-targeted therapies and by the long-term control of HIV infection in an HIV-positive patient with acute myeloid leukemia[146]. This patient received two hematopoietic stem cell transplants from the same HLA-matched transplant donor who was homozygous for a 32 base pair deletion in CCR5 (delta 32 [Δ32]). The CCR5 containing this Δ32 deletion is no longer capable of acting as a coreceptor for HIV[147]. Therefore, the CCR5$^{\Delta 32/\Delta 32}$ cells derived from the donor stem cells are resistant to HIV infection and the transplant recipient's viral load has remained undetectable for more than 3.5 years in the absence of ART[146,148]. Holt, et al. tested an exciting application of therapeutically mutating CCR5 in the human cells of CD34$^+$ cell transplanted NSG mice. They evaluated the ability of CCR5-targeting zinc-finger nucleases to control HIV infection[113]. The CD34$^+$ cells were nucleofected with zinc-finger nuclease expression plasmids prior to transplantation. The human hematopoietic cells in the mice transplanted with nucleofected cells had disrupted CCR5 expression. An advantage of the zinc-finger nuclease approach is that this disruption of the CCR5 gene is permanent, and thus all progeny of altered CD34$^+$ cells are unable to express CCR5 with no

overt functional defects. The CCR5-deficient CD4+ T-cells (estimated at 5-7% of the original total CD4+ T-cell population) survived HIV-mediated cytopathology and expanded to pre-infection levels[113].

Novel strategies for targeted delivery of siRNA as HIV therapy have also been evaluated in humanized mice[149]. Kumar, et al. developed and tested a CD7-specific targeting construct that can deliver anti-CD4, -CCR5, -vif and -tat siRNA specifically to human T-cells. These constructs were shown to reduce surface CD4 expression on activated PBMC by 1 log[150]. The CD34+ cell transplanted NSG mice were treated intravenously with an anti-vif/tat construct and then infected intraperitoneally with HIV 18 hours later. Subsequent administrations of the construct followed at 4-5 day intervals for a total of 40 days. Despite the fact that this approach did not prevent infection, the peak plasma viral loads in the treated mice was 30-fold lower when compared to the control siRNA-construct treated mice. This reduction in viral levels was maintained for the duration of the experiment[150]. Kim, et al. utilized BLT mice to examine whether RNAi-mediated silencing of CCR5 expression could prevent infection after intraperitoneal injection of HIV[151]. Their novel immunoliposome siRNA delivery method specifically targeted lymphocytes for RNA interference based on lymphocyte function-associated antigen-1 expression. Targeted administration of the siRNA resulted in a reduction in plasma viral RNA levels and CD4+ T-cell loss, but did not prevent infection from occurring[151]. Neff, et al. have shown that aptamer delivered siRNA targeting HIV tat and rev suppressed plasma viral load levels by several orders of magnitude in CD34+ cell transplanted DKO mice[135]. In four treated animals, weekly treatment with the Ch A-1 aptamer was sufficient to suppress viral load to levels below detection for up to three weeks after treatment was ceased; however, viral breakthrough did occur in two mice after three weeks of treatment, suggestive of the development of resistance to Ch A-1[135]. None of these siRNA-based methods permanently alter the genomic DNA, as with the zinc-finger nuclease mentioned above. Still, they hold great promise as HIV therapeutics precisely because they do not require *ex vivo* manipulation of targeted cells.

Mucosal HIV transmission

Unprotected intercourse accounts for the majority of new HIV transmissions[1]. Reliable mucosal HIV transmission in humanized mouse models, like that observed for the BLT model, has opened new opportunities for *in vivo* evaluation of prevention strategies. Vaginal and rectal HIV infection in humanized mice is contingent on the presence of the human cells necessary for transmission in the female reproductive tract and the rectum. However, it is the chosen mouse strain and humanization protocol that determines the extent of human reconstitution in these important tissues. As described above, human CD4+ T-cells, monocyte/macrophages and dendritic cells extensively reconstitute all regions of the BLT mouse female reproductive tract[90,138,152]. The extensive reconstitution of these mucosal sites in BLT mice renders these animals susceptible to efficient vaginal HIV transmission (7/8 total [R5])[90,153]. Furthermore, the reconstitution of the intestines, including the rectum of BLT mice, with human cells required for HIV transmission renders them also susceptible to rectal HIV transmission (18/26 total [X4 and R5])[91,137].

The mucosal transmission of HIV in CD34+ cell transplanted NOG/NSG mice has not been addressed in the literature. However, mucosal transmission has been examined in CD34+ cell transplanted DKO mice. Particularly in the case of rectal exposure in this model, there is a discrepancy in the literature regarding their susceptibility to HIV transmission. Among the four research groups that have examined the human reconstitution of these mucosal tissues[77,114-116], only two groups have attempted either vaginal or rectal HIV transmission in these mice. Vaginal HIV transmission has been shown to be efficient in this model twice by the same group (20/21 total [X4 and R5])[114,124]. However, when both groups attempted rectal HIV transmission in CD34+ cell transplanted DKO mice, the results were entirely different[114,116]. In one case, transmission was efficient (11/14 total [X4 and R5]), while rectal transmission was rare in the other report regardless of whether inoculation occurred with cell-free or cell-associated HIV (1/23 total [R5])[114,116]. In this second report, the authors also unsuccessfully attempted to increase rectal HIV transmission in these mice by rectal administration of IL-1 beta 24 hours preceding viral exposure in the presence or in the absence of human seminal plasma (1/17 total [R5])[116]. Additional work must be done to address this dramatic discrepancy in the susceptibility of CD34+ cell transplanted DKO mice to rectal HIV transmission.

HIV prevention

HIV continues to spread at an alarming rate and successful biomedical HIV prevention tools are urgently needed. Mucosal HIV transmission accounts for the vast majority of new HIV infections. The fact that vaginal and rectal HIV transmission can be reproducibly performed in BLT mice makes this model, along with the CD34+ cell transplanted DKO mice, a critical component in our response to this pandemic in that they will

serve as outstanding models for the preclinical evaluation of HIV prevention strategies.

Clinical interventions designed to decrease HIV transmission currently being tested include topical pre-exposure prophylaxis (PrEP) and systemic PrEP with antiretrovirals. Recently, the CAPRISA 004 clinical trial reported that 1% tenofovir applied vaginally before and after intercourse resulted in a 39% reduction in incidence of HIV in the two high-risk populations of South African women enrolled[154]. Subsequently, the iPrEx clinical trial reported that daily oral emtricitabine (FTC) and tenofovir disoproxil fumarate (TDF), co-formulated as Truvada®, led to a 44% reduction in incidence in HIV in men who have sex with men at eleven sites in six countries[155]. When adjustments were made to account for adherence in the iPrEx trial participants as defined by detectable study drugs in plasma, the reduction in incidence was 92%. These very encouraging data show that antiretroviral PrEP has the potential to slow the spread of HIV in high-risk populations. These observations also bring us to a critical line of investigation in humanized mice: preclinical *in vivo* efficacy evaluations of biomedical interventions for HIV prevention such as topical and systemic PrEP.

To date, there are no reports published regarding the efficacy evaluation of topical PrEP (microbicides) for the prevention of HIV in humanized mice, but both BLT mice and CD34$^+$ cell transplanted DKO mice have been used to demonstrate *in vivo* the efficacy of systemic PrEP in preventing HIV transmission[90,124,137]. BLT mice were treated once daily for seven days with Truvada® in a manner that recapitulated the experimental design used for the iPrEx trial[155]. Three hours following the third FTC/TDF dose, mice were rectally exposed to HIV. Combined FTC and TDF were very effective at protecting BLT mice from rectal HIV transmission, with a protection level of 100%[137]. The iPrEx trial results indicated that the most adherent participants experienced a greater than 90% reduction in incidence and the BLT mouse result that 100% of mice were protected together show that daily systemic FTC/TDF can prevent rectal HIV transmission, and the similar protection levels observed in both studies serves to validate the BLT system for further preclinical efficacy evaluations of novel HIV prevention approaches. To this end, the same seven-day regimen of FTC/TDF used for the rectal study also prevented 100% of vaginal transmissions in BLT mice[90]. Therefore, given the success of systemic PrEP in humans and BLT mice for the prevention of rectal HIV transmission, as well as for the prevention of vaginal HIV transmission in BLT mice, there is reason to be optimistic regarding the outcomes of ongoing clinical trials evaluating systemic PrEP with FTC/TDF to prevent vaginal transmission in women. In the case of intravenous exposure, the seven-day PrEP regimen resulted in 88% protection from transmission in BLT mice[137]. However, when the FTC/TDF treatment was begun 24 hours following intravenous HIV inoculation, detection of the virus in plasma was only delayed. This postexposure prophylactic approach offered 0% protection from intravenous HIV transmission[137]. Consequently, having the drugs present systemically prior to exposure was important for the protection of BLT mice from intravenous HIV transmission. The drug investigation pipeline in humanized mice was expanded by Neff, et al. who tested closely related seven-day systemic PrEP regimens consisting of either oral raltegravir (an integrase inhibitor) or oral maraviroc (a CCR5 inhibitor)[124]. They found that each drug showed 100% efficacy at preventing vaginal HIV transmission in these CD34$^+$ cell transplanted DKO mice[124]. In conclusion, the iPrEx trial results validate the BLT model for the preclinical efficacy evaluation of biomedical interventions to prevent rectal HIV transmission, which highlights the usefulness of the model for the preclinical efficacy screening of other prevention interventions, including microbicides.

Conclusions and future directions

The versatility of humanized mice in HIV research is evident in the body of work presented here. The ability to study HIV biology and anti-HIV approaches *in vivo* using humanized mice is a major advance in the field of HIV research. These mouse models are already being utilized to perform preclinical efficacy evaluations of HIV prevention and HIV therapeutic interventions, but they can be used to a much greater extent in these efforts. To best harness the power of the humanized mouse models, we need to know more about the human immune responses occurring in these animals. It will be critical to discern whether anti-HIV NK responses are occurring as well as whether HIV-neutralizing antibodies (broad or narrow) are being generated within humanized mice, what is the magnitude of any detected responses, and whether secretory IgA or other antibody isotypes are involved in the response. An underexplored area of humanized mouse research is their potential usefulness for vaccines. Functional human antiviral immune responses in BLT mice combined with relevant routes of infection provide a unique opportunity to evaluate protective HIV-specific immune responses. Furthermore, the ability to reliably perform mucosal HIV transmissions, especially in BLT mice, presents numerous opportunities for examining the protective effects of antibodies at mucosal surfaces. Another critical experimental question to address is whether the development

and maintenance of a latent viral reservoir during ART occurs in humanized mice as it does in humans?

Focusing research in humanized mice on each of these critical aspects of the fight against HIV can accelerate the effort to help individuals infected with HIV or at risk of becoming infected with HIV. This effort will be further enhanced as humanized mouse researchers begin to diligently compare data from their humanized mouse research with data available from human and NHP studies whenever possible. In the future we expect significant refinements to these humanized mouse systems, which will enhance the already enormous potential of humanized mice to contribute to all aspects of HIV research.

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