SUPPLEMENT ARTICLE

Overcoming Current Limitations in Humanized Mouse Research

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Immunodeficient mice engrafted with human cells and tissues have provided an exciting alternative to in vitro studies with human tissues and nonhuman primates for the study of human immunobiology. A major breakthrough in the early 2000s was the introduction of a targeted mutation in the interleukin 2 (IL-2) receptor common gamma chain (*IL2rg*^{null}) into mice that were already deficient in T and B cells. Among other immune defects, natural killer (NK) cells are disrupted in these mice, permitting efficient engraftment with human hematopoietic cells that generate a functional human immune system. These humanized mouse models are becoming increasingly important for preclinical studies of human immunity, hematopoiesis, tissue regeneration, cancer, and infectious diseases. In particular, humanized mice have enabled studies of the pathogenesis of human-specific pathogens, including human immunodeficiency virus type 1, Epstein Barr virus, and *Salmonella typhi*. However, there are a number of limitations in the currently available humanized mouse models. Investigators are continuing to identify molecular mechanisms underlying the remaining defects in the engrafted human immune system and are generating "next generation" models to overcome these final deficiencies. This article provides an overview of some of the emerging models of humanized mice, their use in the study of infectious diseases, and some of the remaining limitations that are currently being addressed.

Keywords. humanized; immunobiology; infectious disease.

Since the first description of the CB17-*Prkdc*^{scid} (CB17-*scid)* mouse in 1983 [1] and the observation in 1988 that these mice could be engrafted with human immune systems [2, 3], investigators have envisioned engrafting a functional human immune system in a small animal model that could be used for studies of human immunity, particularly human-specific infectious agents such as human immunodeficiency virus type 1 (HIV-1) [4–7]. To establish a functional human immune system in immunodeficient mice by engraftment of human hematopoietic or lymphoid cells, investigators have relied on 3 major model systems: (1) the Hu-PBL-severe combined immunodeficiency (SCID) (human peripheral blood lymphocyte severe combined immunodeficiency) model, established by injection of mature peripheral blood mononuclear cells, and 2 hematopoietic stem cell (HSC) engrafted models, (2) the Hu-SRC-SCID (human SCID repopulating cell) model and (3) the BLT (bone marrow, liver, thymus) model. The Hu-PBL-SCID model is established simply by injection of human peripheral blood mononuclear cells or spleen or lymph node cells into immunodeficient mice. The Hu-SRC-SCID model is established by injecting newborn or adult immunodeficient mice with HSCs from a variety of sources. The BLT model is established by subrenal capsule implantation of fetal liver and thymus fragments, and intravenous injection of autologous HSC derived from the same fetal liver donor. Each of these model systems have advantages and caveats, and the various models and their limitations have been completely discussed and reviewed recently [6–8].

A major technological breakthrough in the creation of humanized mice occurred in the early 2000s when investigators created immunodeficient stocks of *scid*, *Rag1*^{null} or *Rag2*^{null} mice that also harbored targeted mutations in the IL-2 receptor common gamma chain (*Il2rg*) gene, abbreviated as IL2rγ [9–11]. The IL2rγ chain is required for high-affinity ligand binding and signaling through

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multiple cytokine receptors, including IL-2 and interleukin 4, 7, 9, 15, and 21 [12]. Blocking signaling through these cytokine receptors leads to severe defects in both adaptive and innate immunity. Immunodeficient mice bearing a targeted mutation in the *IL2rγ* gene support significantly higher levels of human hematopoietic and lymphoid cell engraftment than all previous immunodeficient stocks of mice [6–8]. However, the lack of the *IL2rγ* gene leads to a decrease of lymphoid tissue inducer cells and consequently to underdeveloped lymph node anlagen and limited lymph node development [13].

Of the 3 models, the BLT model system is the most robust model of human immune system engraftment that has been achieved in humanized mice and is rapidly becoming the model of choice for the study of HIV-1 [6, 8]. Primary human T- and B-cell responses have been reported in BLT mice after immunization, particularly after virus infection, although the predominant antibody response remains immunoglobulin M (IgM) in both the Hu-SRC-SCID and BLT models [14–18]. However, some recent reports suggest that some class switching can be generated [19–21]. The BLT mice established using NOD-*scid* or NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl} (NSG) mice also develop robust human mucosal immune systems [22–29] that can be infected with HIV-1 via oral, rectal, or vaginal inoculation [23, 25, 26, 29–33]. Of particular interest, it has recently been reported that BLT mice infected with HIV-1 will induce human CD8 T-cell responses that closely resemble responses observed in humans infected with HIV-1 [34]. The humanized mouse immune response leads to a rapid virus sequence evolution that results in a reproducible escape from host immune responses, similar to that observed in HIV-1–infected individuals [34]. In some colonies BLT mice eventually develop a terminal wasting-like syndrome that can begin approximately 25–30 weeks after fetal liver and thymus engraftment [35]. However, this may depend on colony variables such as microbiota and/or prophylactic antibiotic administration, and it is an open question whether this variable is present in all colonies.

The availability of immunodeficient *IL2rγ*^{null} mouse models for studies of hematopoiesis, immunity, regenerative medicine, cancer and infectious diseases have been reviewed recently [6, 8]. However, there remain a number of limitations that prevent full use of these model systems. These limitations include difficulty in generating primary and recall human humoral immune responses that leads to class switching and immunoglobulin G (IgG) antibody production. The generation of memory T cells in these models can also be problematic. These observations exemplify a need for more mature, fully functional human peripheral and mucosal immune systems that can generate robust IgG responses and provide protective immunity. This impaired functionality may be due in part to the lack of formation of organized lymphoid structure in the peripheral lymph nodes and germinal centers. Moreover, an extensive listing of the species specificity of many factors required for human hematopoietic and immune system development and function has been developed [8], and these are candidates for enhancing human cell development in the immunodeficient mice. After human HSC engraftment, there is also a deficiency in the circulation in the blood of human platelets, red blood cells (RBCs), polymorphonuclear leukocytes (PMNs), and myeloid cells [36–39]. These limitations and approaches to overcome them can be summarized in 3 general areas: 1) Reduction of mouse innate immunity, 2)Enhancement of human innate immunity, and 3)Enhancement of human adaptive immunity.

APPROACHES TO FURTHER REDUCE MOUSE INNATE IMMUNITY

Some of the major limitations to engraftment of human cells and tissues in immunodeficient mice are host innate immune responses. In CB17-*scid* mice, major impediments to human cell engraftment include high NK cell activity; robust macrophage, neutrophil, and dendritic cell function; and high levels of hemolytic complement [40–42]. Generation of NOD-*scid* mice overcame many of these innate immune responses as NOD, and NOD-*scid* mice have reduced NK cell activity, although depletion of murine NK cells is not sufficient to increase human hematopoietic cell engraftment in C57BL/6 mice [43]. Additional defects are observed in myeloid and dendritic cell development and the lack of hemolytic complement due to a 2–base pair deletion in the coding region of the hemolytic complement *(Hc)* gene that encodes the C5 complement component. This mutation prevents the formation of the C5b-9 membrane attack complex [44]. Although NOD-*scid* mice engraft with human cells at higher levels than CB17-*scid* mice [45–47], they have a relatively short lifespan owing to the development of thymic lymphomas and die as early as 5 months of age, limiting long-term studies in these animals [44].

Engraftment of human peripheral blood lymphocytes (PBLs) into NOD-*scid* or NSG mice invariably leads to a lethal xenogeneic graft-vs-host disease (xeno-GVHD GVHD), with the onset of GVHD correlating directly with levels of human cell engraftment [48]. Surprisingly, NOD-*scid* mice are relatively resistant to the development of Epstein Barr virus–related lymphoproliferative disorders after injection of human PBLs, which are observed to develop in PBL-engrafted CB17-*scid* mice [41]. This resistance seems to be due to regulation of the lymphoproliferative disorder by the increased engraftment of human CD8 T cells in NOD-*scid* mice [49].

Engraftment of human HSC in the Hu-SRC-SCID NOD-*scid* model leads to the generation of all hematopoietic and immune cell lineages except for human T cells [7]. This was thought to be due to the residual NK cell activity in NOD-*scid* mice, but depletion of NK cells with anti-NK1.1 or anti-CD122 monoclonal antibodies in C57BL/6-*scid* or immunodeficient NOD mice does not lead to human T-cell development after

HSC engraftment [40]. This observation suggests that other factors in addition to NK cells are important in inhibiting human T-cell development from HSCs in immunodeficient mice.

The use of immunodeficient *IL2rγ*^{null} mice that totally lack NK cells for the first time permitted the generation of human T cells in the Hu-SRC-SCID model, but this generation is highly dependent on the age of the recipient at the time of HSC engraftment. Newborn NSG mice engrafted with HSCs, up to 3–4-weeks of age, support the development of human T cells [9, 36, 50], but older NSG mice develop an atrophied thymus with thymus cysts and fail to efficiently support human T-cell development after engraftment with mobilized peripheral blood HSCs [11] or cord blood HSCs [51]. In newborn or young NSG mice, the human T cells are restricted to mouse major histocompatibility complex (MHC) and fail to interact productively with human antigen presenting cell within the host [15]. To overcome this problem, investigators have developed HLA-transgenic NSG mice (see Approaches to Enhance Human Adaptive Human Immunity).

Another key component of the mouse innate immune system is the Sirpα-CD47 receptor-ligand axis. Sirpα (encoded by the *Sirpa* gene) is expressed on macrophages, and its interaction with CD47 expressed on most cells provides a "do not eat me" signal [52]. The NOD strain has a polymorphism in Sirpα that is very similar to human Sirpα but the Sirpα in BALB/c and C57BL6 have very low homology to human Sirpα [52]. The C;129S4-*Rag2*$^{tm1.1Flv}$ *Il2rg*$^{tm1.1Flv}$ (C;129RG *Rag2*^{null} *IL2rγ*^{null}) mice that transgenically express human Sirpα have increased levels of human HSC engraftment and immune development, attaining levels similar to those observed in NSG mice [53]. This observation documents that Sirpα is critical in regulating engraftment of human hematopoietic cells in immunodeficient mice. Based on these data, we have generated NSG mice transgenically expressing huSirpα, which are currently being characterized to determine whether this ability increases human cell engraftment and immune function in humanized NSG mice.

The use of NSG mice as the strain for establishing the HSC engraftment model systems leads to increased human immune cell engraftment and function relative to that obtained in NOD-*scid* mice according to a number of reports, probably owing to increased reduction of innate immunity as a result of the *IL2rγ*^{null} gene mutation [10, 26, 36, 54]. However, in some reports on the BLT model, similar engraftment levels are observed in NOD-*scid* and NSG mice [27, 55]. Interestingly, because the thymic lymphomas are highly dependent on IL-2, NSG mice—in contrast to NOD-*scid* mice—fail to develop thymic lymphomas and their lifespan approaches 2 years, well past the approximately 6 months that NOD-*scid* mice are expected to live [11].

To further decrease mouse innate immunity and enhance human hematopoietic and immune system engraftment and function, a number of genetic and technical approaches are being used. One potential approach to reduce development of GVHD is to use NSG mice deficient in mouse MHC class I and II [56]. With the NSG MHC class I or class II knockout mice in the Hu-PBL-SCID model system, it was observed that the development of GVHD was delayed, particularly in the MHC class I knockout mice [56]. This delayed GVHD suggests that the majority of the human xenoreactivity to mouse antigens may be to the murine MHC class I and II molecules in this model. It will be of particular interest to determine whether the GVHD that develops in HSC-engrafted mice is also directed predominantly against murine MHC class I and II. Development of NSG MHC class I and II double-knockout mice, and their use in the Hu-SRC-SCID and BLT models, will allow direct determination of whether the majority of the xenogeneic reactivity is to murine MHC antigens. These NSG MHC class I or class II knockout mice could also be crossed with NSG-HLA Tg mice to permit human T-cell restriction by a developing human immune system to be completely HLA restricted in the Hu-SRC-SCID model.

Additional approaches to further reduce host innate immunity include targeting Toll-like receptors (TLRs) in the host. A type of pattern recognition receptor, TLRs recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns. They are responsible for rapid innate immune responses to pathogens such as virus, bacteria, and fungi [57]. For example, we are developing NSG mice in which the genes encoding TLR4, type 1 interferon receptor, or MyD88 cytostolic adapter protein are knocked out. This should permit human innate immune responses to viral infections, such as HIV-1 infection, to be investigated in the absence of the molecules mediating the relevant murine host innate immune responses. Another approach to disarm innate immunity is the use of the neutrophil cytostolic factor 1(*Ncf1)* mutant mouse. This gene controls superoxide production by macrophages and neutrophils and is responsible for much of their cytotoxic activity [58]. A genetic mutation of *Ncf1*, which leads to a deficiency in reduced nicotinamide adenine dinucleotide phosphate oxidase activity, has been described for the NOD strain of mice [59], and we are currently generating NSG-*Ncf1*^{null} mice to further depress host innate immunity.

Finally, investigators have examined the modulation of human RBCs by host macrophages by intravenous injection of liposome-encapsulated CL2MDP (clodronate liposomes) [60]. In this study, NOD-*scid* BLT or NSG-BLT mice were injected with clodronate liposomes to deplete macrophages, and increased levels of circulating RBCs were observed. This study also ruled out the sole control of RBC circulation by the Sirpα-CD47 receptor-ligand axis [60]. However, long-term administration of clodronate is toxic, and we are currently developing NSG mice that express the diphtheria toxin receptor under control of

the CD11b or CD11c promoters to specifically delete murine myeloid and dendritic cell populations. Although human cells also express diphtheria toxin receptors, we have found by using rat insulin promoter-diphtheria toxin receptor transgenic mice that injection of low doses of highly purified diphtheria toxin ablates the murine beta cells that express high levels of receptor while not affecting human islets in the same recipient.

APPROACHES TO ENHANCE HUMAN INNATE IMMUNITY

To increase human innate (and adaptive) immunity in immunodeficient mice engrafted with hematopoietic cells, a number of approaches have been reported. Multiple human immune factors are species specific, and these have been described in 2 recent reviews [6, 8]. There are now many technological approaches for introducing human genes expressing human-specific molecules. These factors have been introduced into immunodeficient mice by transgenic expression using a promoter-driven construct, bacterial artificial chromosomes, adenovirus or adenovirus-associated vectors, or knock-in technology. These factors have also been provided through injection of recombinant proteins or hydrodynamic injection of plasmids containing complementary DNA for the specific human factor. Moreover, the recent development of NOD, NSG, and NOD-*Rag2*^{null} *IL2rγ*^{null} (NRG) embryonic stem cell lines for use in knockout or knock-in technology will facilitate rapid construction of new models of NSG and NRG humanized mice ([61] and http://research.jax.org/collaboration/escell). There are also new approaches for gene editing and creating new transgenic knockout, and knock-in mice, which include zinc finger nucleases [62–64], TALENs [65, 66] or the more recently described CRISPR-Cas9 system [67].

To further enhance human myeloid cell circulation in the blood of Hu-SRC-SCID and NSG-BLT mice, a number of approaches have been used. In 1 study, investigators injected recombinant granulocyte colony-stimulating factor and found increased levels of myeloid cells in the circulation, including PMNs [37]. C;129S4-*Rag1*^{null} *IL2rγ*^{null} mice transgenically expressing human interleukin 3 (IL-3)/granulocyte-macrophage colony-stimulating factor (GM-CSF) also exhibit increased levels of human myeloid cells in the circulation [39]. Hydrodynamic injection of plasmids containing interleukin 4 and GM-CSF has led to increased levels of human dendritic cells and improved human immune responses in the NSG Hu-SRC-SCID model system [21]. Triple transgenic NSG mice expressing IL-3/GM-CSF/SCF engrafted with human HSC display increased regulatory T cells and some increases in myeloid cells, specifically myeloid dendritic cells, in the circulation [68]. Because development of transgenic and knock-in mice is time consuming, the use of hydrodynamic administration of human cytokines should permit rapid screening of which cytokines and combinations of cytokines are likely to be most effective in enhancing human immune system development and function in humanized mice.

APPROACHES TO ENHANCE HUMAN ADAPTIVE IMMUNITY

Multiple approaches are being used to improve human immunity in humanized mice. These are focused on technologies designed to provide human-specific T- and B-cell molecules and cytokines required for hematopoietic and immune system development and function. These technologies are similar to those being used to enhance human innate immune development and function.

To improve human cellular immune responses, we and others are developing NSG mice that express a variety of HLA class I molecules that can be engrafted with HLA-matched HSC. For NSG-HLA class I Tg mice, this will provide appropriate human CD8 T-cell education and selection in the thymus of NSG mice. For example, NSG HLA–A2 mice engrafted with HLA-A2 umbilical cord blood-derived HSCs have been shown to develop HLA-A2–restricted human T-cell responses to virus infection [69, 70]. Cohorts of NSG HLA class II transgenic mice have also been developed to enhance human helper T-cell activity to improve humoral immune responses. NRG-HLA-DR4 and NOG-HLA-DR4 mice engrafted with HLA-DR4^{+} HSC display enhanced primary human immune responses [19, 20]. Another use of HLA Tg mice is to develop a model of human allogeneic-GVHD (allo-GVHD). NSG-HLA-DR4 Tg mice that are deficient in murine MHC class II have been engrafted with non-HLA-DR4 PBLs and develop a model of human allo-GVHD rather than xeno-GVHD [71].

Delivery of human species-specific cytokines has been used extensively for enhancing immune responses in humanized mice, and this has been recently reviewed by us and others [6, 8]. In the review by Rongvaux et al [8], a comparison of the homology sequence of murine and human proteins important in the development and function of a human immune has been summarized and classified into the following categories: (1) high homology (>80%), (2) significant homology (61%–80%), (3) low homology (<60%), or (4) absence of the murine equivalent protein. For the low homology and absence proteins, these are probably species specific and will need to be provided. This will aid greatly in identifying species-specific cytokines that may need to be provided in humanized mice for optimal human immune system function, but there are some caveats. For example, some of the human cytokines that have low homology might be expressed by the engrafted human hematopoietic cells, such as IL-2 secreted by engrafted T cells.

The technology used to provide the species-specific factor may also be important. For example, knock-in technology used to provide the human cytokine thrombopoietin led to increased engraftment and survival of human HSCs, but thrombocytopenia

in the mouse was also observed because of the observed decrease of mouse platelets and the relatively low level of circulating human platelets [72]. Similarly, the knock-in technology used to deliver the human cytokines IL-3 and GM-CSF led to increased human alveolar macrophage development and human immune responses in the lung but came with pulmonary alveolar proteinosis due to lower human physiological levels of human GM-CSF in the absence of murine GM-CSF [39]. Alternatively, it has been shown that a single amino acid difference in the B-cell cytokine BLyS (BAFF) between murine and human molecules leads to the binding of murine BLyS to its receptor on B cells, but this binding fails to lead to signaling through the receptor [73]. This lack of signaling would not be predicted based on the high protein homology. Moreover, immunodeficiency leads to higher levels of BLyS, probably owing to the lack of B cells to bind to the cytokine, resulting in a feedback loop for increased production [74].

Limitations in the current humanized mouse model systems include the development of wasting disease in highly engrafted humanized mice in all 3 model systems and poorly developed lymph nodes and germinal centers [13, 75]. Moreover, trafficking of lymphocytes has been observed to sites of HIV infection [76], inflammatory sites in arthritis models [77], and mucosal tissues [23, 27], but it is unknown whether the trafficking of human immune cells is optimal to all sites and in the migration to appropriate organizational sites within lymphoid tissues. Human-specific adhesion molecules may need to be expressed for optimal human immune cell migration and structural organization. The poor humoral immune responses and lack of appropriate class switching from IgM to IgG after immunization or infection may reflect the high levels of immature human B cells and low levels of mature B cells present in humanized mice [78]. Identification of these and other limitations that dampen the generation of robust immune systems in humanized mice is underway in multiple laboratories. Humanized mouse models permit insights into the pathogenesis of HIV-1 infection and evaluation of HIV-1 drugs, and in the future they may provide a platform for testing HIV-1 vaccines.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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