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# Human Hemato-Lymphoid System Mice: Current Use and Future Potential for Medicine

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## Abstract

To directly study complex human hemato-lymphoid system physiology and respective system-associated diseases in vivo, human-to-mouse xenotransplantation models for human blood and blood-forming cells and organs have been developed over the past three decades. We here review the fundamental requirements and the remarkable progress made over the past few years in improving these systems, the current major achievements reached by use of these models, and the future challenges to more closely model and study human health and disease and to achieve predictive preclinical testing of both prevention measures and potential new therapies.

### Keywords

humanized mice; stem cells; hematopoiesis; infectious disease; cancer

## INTRODUCTION

Knowledge of human physiology and patho-physiology is largely gained by observation, by surrogate in vitro assays, and by cautious, primarily safety-driven clinical trials. However, although clinical trials are ultimately essential for progress in medicine, many do not have a major impact, some are surpassed by new knowledge in basic research that cannot be implemented by trial adaptation, and finally, clinical trials have high financial costs. Conversely, rigorous proof-of-principle forward experimentation is, for ethical risk-benefit evaluations, tightly restricted.

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E.E.E., R.A.F., and M.G.M. filed patent applications on genetically modified mice and the use thereof. M.G.M. is on the scientific advisory committee of TransCure Biosciences, a company devoted to provide humanized mouse models for HIV research.

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Progress in medicine is therefore mostly slow, with many false paths taken and likely even more opportunities lost. This seems particularly true for complex pathophysiological processes in which the hemato-lymphoid system is involved, such as infectious diseases, autoimmunity syndromes, and multigenetic lesion-driven cancers as well as aging. In fact, the evolving HIV pandemic over the past few decades, with now over 30 million people infected and about 2 million dying each year, as well as the rising cancer incidence in aging societies are prime examples of human diseases with relative species specificity that have been cured in surrogate in vitro or animal experiments but that continue to be major challenges in clinical settings.

Most societies agree on the use of small animals such as worms, flies, and small vertebrates for biomedical research, whereas the use of models more closely related to humans, such as nonhuman primate models, is restricted for ethical considerations as well as economic reasons. Thus, small mammals such as mice have become key surrogate model systems in biomedical research. However, as outlined below, mice are not men, and findings in mice often are not supported by the results obtained with human studies.

There is therefore a need to develop better, broadly available, and easy-to-handle human species–specific, highly predictive, preclinical, in vivo model systems that have the potential to solve some of these problems. One promising approach is the generation of human-to-mouse xenotransplantation models in which functional elements of the human hemato-lymphoid system are implemented in an in vivo setting that allows consecutive experimentation. Generating these models has the potential both to accelerate fundamental knowledge acquisition and to improve clinical approaches (**Figure 1**). It has been 22 years since this journal last reviewed mice that carried human lymphoid cells, which then focused on studying HIV (1). We provide here a resource review on the efforts over the past three decades to improve human hemato-lymphoid system (HHLS) mice, with a focus on the principal requirements for such systems, the remarkable progress made over the past few years, and the current major achievements reached by use of these models. Also, we highlight the limitations and the future challenges that remain to closely model the human hemato-lymphoid system in health and disease.

## OF MICE AND MAN

The most recent common ancestor of mice and men lived approximately 91 Mya (2). Thus, the two species have been evolving independently for a cumulative time of more than 180 million years. During this long evolutionary period, the respective ancestors lived in increasingly different environments and under distinct pressures of natural selection. As a consequence of this genetic adaptation, they accumulated genetic differences that led to the species that we know today, with major differences in their morphology, size, life span, cellular dynamics, diet, and ecosystem.

The availability of genome-wide sequences of mammalian species has allowed the identification of loci that evolved under positive natural selection. Interestingly, in humans some of the strongest evidence of positive selection was identified among genes associated with functions in immunity and host defense (3, 4). This observation suggests that

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microorganisms, through their interaction with host immune defense, have been a major factor in shaping the present-day human genome and the mechanisms of host defense (3, 5–7).

Although we often think of microorganisms as pathogens that cause disease, most microorganisms that populate human mucosal surfaces are actually harmless or symbiotic commensals, and the immune system has to maintain a delicate balance between eliminating pathogenic organisms and preserving beneficial microbiota (8). Since their divergence from other lineages of nonhuman primates, approximately 6.5 Mya, humans underwent several fundamental environmental and cultural changes. For example, the control of fire, which allowed humans to cook their food and, later, the invention of agriculture and domestication induced major shifts in the diet, with consequences for metabolism, digestion, and the associated microbiome (9– 11). Relatedly, the proximity to domesticated animals, the increasing densities of human populations, and the resulting alterations inflicted on the environment created conditions in which new pathogens could spread and cause devastating diseases such as plague and malaria (12, 13). Furthermore, accumulating evidence suggests that dietary changes and their effect on gut microflora are contributing to the rise of autoimmune and metabolic disorders in Western societies (14). Finally, with the increased life expectancy in modern societies, defects in immune function associated with aging are becoming more prevalent (15, 16). This immunosenescence is characterized by a reduced capacity to mount an effective immune response, higher susceptibility to infection, and reduced immunosurveillance that, along with the accumulation of deleterious mutations, might also contribute to the increased incidence of tumors with age (17).

In light of the successive events that have marked the evolutionary history of humankind, it is hardly surprising that humans and mice present major differences in their immune systems and microbiota, as well as in their susceptibility to infectious agents and respective diseases (18, 19) (**Figure 2**, **Table 1**). Therefore, immunologists interested in understanding human immunology cannot rely solely on the use of animal models such as the mouse or nonhuman primates. Although clinical observation, in vitro experimentation, and genetic analyses at the level of individuals or of populations can be informative in delineating the genetic basis of the human immune response (20), the possibilities for forward experimental approaches are limited. Balancing halfway between experimentation on animal models and studies on human subjects, humanized mice represent a complementary approach that combines some of the species specificities of humans with the experimental possibilities and convenience of small animal models.

## REQUIREMENTS FOR HUMAN HEMATO-LYMPHOID SYSTEM DEVELOPMENT, MAINTENANCE, AND FUNCTION IN MICE

For success of any xenogeneic transplantation system, some fundamental requirements need to be met: (*a*) Donor tissue or cells need to be available in sufficient amounts and of sufficient quality; (*b*) the tissue needs to be accepted by the host immune system, and the potential cotransplanted immune components of the graft need to be inactive against the recipient, i.e., bidirectional host-donor tolerance is required; (*c*) the grafted tissue needs appropriate physical space, and the recipient must require and make use of the graft to

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achieve physiologic homeostasis; (*d*) the graft is ideally in the orthotopic site to be embedded in its most appropriate environment; (*e*) essential graft-supporting and maintenance factors that are not produced by the graft itself need to be cross-reactive from host to graft; and (*f*) xenograft effector functions need to be cross-reactive from graft to host (**Table 2**).

These principal requirements can be applied to the specific context of xenotransplantation of a human hematopoietic system into a mouse host. The development of HHLS mice during the past few decades has been a continuing quest to meet more of these requirements. To be useful, an ideal model of HHLS mice would meet at least four criteria: (*a*) All lineages and subsets of human hematopoietic cells should develop in the mouse host, and they should be maintained in proportions and localizations similar to those observed in healthy humans; (*b*) these cells should be functional, i.e., capable of mounting innate and adaptive immune responses in vivo and to clear pathogens; (*c*) the model should replicate human diseases and pathology such as infection and malignancies; and (*d*) it should allow the testing of therapeutic interventions and faithfully predict the outcome in clinical settings. Although some of the necessary conditions have already been fulfilled in early or current models, others remain a challenge and will require the development of improved strains of recipient mice.

## Donor Cells/Tissue

Different sources of human hematopoietic cells can be used for transplantation into mice. The most readily available samples are cells from human peripheral blood. Lymphocytes can expand by homeostatic proliferation and thus repopulate the mouse host for extended periods of time. In contrast, other cell types such as myeloid cells lack proliferative capacity, and their engraftment is low and transient.

Engraftment of human hematopoietic stem and progenitor cells (HSPCs) with long-term blood production potential represents a better approach for the reconstitution and maintenance of a full hematopoietic system. Human HSPCs are contained in the $CD34^+$ fraction of hematopoietic cells. They can be isolated from human fetal liver, cord blood, adult bone marrow, or peripheral blood after chemotherapy or cytokine- or CXCR4 antagonist–mediated mobilization. While frequency and efficacy of engrafting cells within the $CD34^+$ population are higher when cells are isolated from fetal or neonatal origin, adult-derived cells are also used successfully. The latter enables the investigator to select donors with specific genetic polymorphisms or patients affected by given diseases of interest and thus to generate a humanized mouse model of a particular genetic or pathologic condition.

Human fetal tissues including liver, bone, and thymus can be cotransplanted into the recipient hosts, such as in the bone-liver-thymus (BLT) model (see sidebar, below). These tissues not only contain hematopoietic progenitors that can repopulate the entire mouse, but also provide a human microenvironment for proper development and education of human cells in the mouse host.

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## Bidirectional Tolerance of Host and Donor

Injection of hematopoietic cells from one species into another species generally results in immediate rejection. To avoid the xenoreactive response of the mouse immune system against the human graft, mice deficient in both adaptive and innate immune responses must be used as recipients. T and B lymphocytes are the mediators of the adaptive immune response, whereas natural killer (NK) cells and phagocytic cells are major innate cell types responsible of xenograft rejection.

Genetic deficiencies in enzymes involved in the mechanism of V(D)J recombination of T and B cell receptors result in the absence of both cell types. One of two mutations is commonly used to achieve this: the severe combined immunodeficiency (*Scid* ) mutation, which affects the protein kinase, DNA-activated, catalytic polypeptide (PRKDC) involved in DNA repair (21–23), or deficiency in one of the recombination-activating genes (*Rag1* or *Rag2*) (24–28).

NK cells are innate lymphocytes specialized in the recognition and elimination of cells that lack expression of MHC class I molecules (29). Given that mouse NK cell receptors do not cross-react with human HLA molecules, human cells are recognized as MHC-negative and eliminated by NK cells. The interleukin (IL)-2Rγ (also known as the common γ-chain or γc) is a cytokine receptor chain shared by multiple cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). IL-15 is a critical cytokine required for NK cell development and maintenance. Antibody-mediated depletion of NK cells or mutation or deletion of the *Il2rg* gene results in the absence of NK cells owing to the absence of IL-15 signaling (28, 30–32).

Phagocytic tolerance is achieved in part by engagement of the SIRPα receptor on macrophages by the ubiquitously expressed receptor CD47, leading to the delivery of a don't-eat-me signal. Thus, for phagocytic tolerance, this receptor-ligand pair needs to interact successfully. Cross-reactivity and phagocytic tolerance of macrophages is achieved either by a polymorphism in the mouse *Sirpa* gene found in the nonobese diabetic (NOD) genetic background, by the transgenic expression of the human *SIRPA* gene on the mouse background, or by the transgenic expression of mouse *Cd47* on human hematopoietic cells (33–36). Alternatively, phagocytic tolerance can be achieved, at least temporarily, by eliminating recipient phagocytic cells by application of clodronate-containing liposomes (37).

While tolerance of the mouse host for the human cells is necessary for efficient engraftment and survival of the graft, tolerance of human immune cells to the mouse is also required to maintain health and survival of the host. This problem is particularly evident when human differentiated cells such as T cells are transplanted that can be activated in the xenogeneic environment by mouse MHC, causing graft-versus-host disease (GvHD) (22, 38). This is likely to become an even more prevalent issue in future models with a more complete and functional human immune system. Under such conditions, the fully competent human immune system would have the potential to destroy the murine organ systems, which must of course be obviated. Strategies will need to be developed to maintain graft-versus-host tolerance, for both T cell–mediated and phagocytic responses.

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### Space for Cell/Tissue Replacement of Host by Donor

The ablation of mouse cell populations (such as T, B, and NK cells) required to induce tolerance to the graft also results in unoccupied cellular space for given lineages that can be populated with cells from their human counterparts. However, these deficiencies in mature cellular compartments are generally insufficient to enable human HSPC engraftment; thus, additional preconditioning of the recipient mice is required to facilitate reduction of mouse HSPCs and generation of respective niche space. This preconditioning is commonly performed by sublethal X-ray or γ-irradiation, as ongoing mouse hematopoiesis is still required until effective production of human platelets and red blood cells (RBCs) can be achieved to substitute for respective mouse cell production (discussed below).

An alternative preconditioning protocol consists of treating the mice with chemotherapeutic agents, such as the drug busulfan (39). Also, one can envision that the same outcome can be achieved by selective antibody-mediated deletion of mouse progenitor cells such that residual antibody should not recognize transplanted human hematopoietic counterpart cells (40). Moreover, with additional genetic modifications that further reduce mouse hematopoietic cells and better support human hematopoiesis, the need for preconditioning to achieve significant hematopoietic xenogeneic chimerism will likely become less important (discussed below).

The delivery of human hematopoietic cells in synchrony with the natural time-course of the establishment and expansion of the endogenous hemato-lymphoid system in the mouse also favors engraftment. Thus, administration of human HSPCs into newborn recipients—i.e., mice that naturally expand their hematolymphoid system for several weeks—which is a situation that resembles HSPC transplantation in human infants, results in particularly high hemato-lymphoid engraftment levels compared with that in adult recipients (28).

### Orthotopic Localization of Donor Cells/Tissues

Orthotopic localization of hematopoietic cells is critical to enable their support by growth, differentiation, and survival factors supplied by the natural environment. HSPCs have a high tropism to postnatal bone marrow, and their systemic application results in highly efficient homing that allows intravenous transplantation of respective cells in clinical medicine (41, 42). This likely reflects naturally occurring occasional hematopoietic stem cells (HSCs) that travel through peripheral blood and return to the marrow (43). As the appropriate human and mouse homing and retention signals are cross-reactive (discussed below) (44), human cells efficiently home to mouse bone marrow upon intravenous injection. Nevertheless, we and others had the impression that limited numbers of human HSPCs show better and more reliable engraftment when injected directly into the liver of newborn mice or into the bone marrow of adult mice, i.e., primary hematopoietic sites, although this is not formally proven for all cell populations (28, 45–48).

Regarding a hemato-lymphopoietic graft, not only do primary HSPCs need to locate to appropriate niche sites, but also offspring hemato-lymphoid cells need subsequently to migrate to primary and secondary lymphoid organs and tissues. The respective adhesion and

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migration molecules therefore need to cross-react appropriately. This is discussed in context below.

### Differentiation and Maintenance of Donor Cells/Tissues

Hematopoiesis is a complex and highly regulated process during which HSCs proliferate and differentiate into all blood cell types (49, 50). This vital process ensures the maintenance of a fully functional hemato-lymphoid system for the entire life of the organism. Multiple factors are involved in the tight regulation of hematopoiesis, from the maintenance of self-renewing HSCs in the bone marrow niche to their differentiation into multiple cell lineages, their localization and maintenance in peripheral tissues, and their activation under conditions of infection or other challenges (**Figures 3** and **4**).

The extrinsic factors involved in the regulation of this process include cytokines and attractant molecules such as chemokines. Although some of these soluble factors are secreted by hematopoietic cells, many of them are produced by nonhematopoietic tissues and are thus of mouse origin in HHLS mice. Cytokines and their receptors have coevolved so that divergence in one of the binding partners is compensated by a change in the other interacting molecule. However, with accumulating divergence, interspecies cross-reactivity between cytokine and receptor can be lost. The level of conservation between species is highly variable for different cytokines (**Supplemental Table 1**; follow the **Supplemental Material link** from the Annual Reviews home page at **http://www.annualreviews.org**): For example, human and mouse TGF-β family members share more than 90% identical amino acids, whereas less than 30% of the amino acid sequence is conserved for IL-3. Unfortunately, although sequence identity is the best objective value of conservation, there is no direct correlation between the percentages of identical amino acids and the interspecies functional cross-reactivity of proteins. Furthermore, in vitro assays of cross-reactivity, in which recombinant proteins are generally used at supraphysiological concentrations, are not always predictive of bioactivity in the in vivo microenvironment. Therefore, the identification of mouse factors that are not fully cross-reactive on human receptors in vivo is currently one of the major challenges for the development of models of HHLS mice (**Figures 3** and **4**).

Interspecies cross-reactivity of adhesion molecules—such as integrins and selectins—and chemokines is also required for the correct migration and retention of cells in tissues. Most integrins are highly conserved between species (80–90% amino acid identity), but amino acid identity of selectins and chemokines is lower (50–80%) and could be a cause of inadequate homing of human cells into mouse tissues (**Supplemental Table 1**).

Besides cytokine support, a process of selection and education is required for the generation of functional lymphocytes such as T and NK cells. T cells are an important part of the adaptive immune response. They derive from lymphoid progenitors that leave the bone marrow to seed the thymus, where they undergo a complex process of positive and negative selection that generates a functional T cell repertoire able to respond to foreign pathogens while avoiding autoimmunity. A theoretical representation of human T cell repertoire selection in the context of a mouse thymic microenvironment is depicted in **Figure 5**. Undoubtedly, the suboptimal education of human T cells can be accounted for by the

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selection of a human T cell repertoire on mouse MHC molecules and by the suboptimal interaction between human T cell coreceptors (CD4 and CD8) and mouse MHC. Priming of naive T cells occurs in lymph nodes and is mediated by dendritic cells (DCs) (**Figure 6**). Both mouse and human DCs are present, but none of them is likely to efficiently activate human T cells. Also, the repertoire of human T cells capable of binding human MHC (on human DCs) is likely limited, given that the T cell receptor (TCR) repertoire was positively selected on mouse MHC. Although in the xenogeneic context, and possibly also in special situations in humans, an alternative positive selection pathway with direct human thymocyte-thymocyte interaction might be active (51, 52), this is likely not efficient (45). Furthermore, optimal priming of human T cells by mouse DCs would require functional cross-reactivity of costimulatory molecules (40–70% amino acid identity) and cytokines produced by the DCs (**Supplemental Table 1**).

Moreover, NK cells undergo an educational process called licensing by the interaction with MHC class I–expressing cells. Binding of MHC class I to inhibitory receptors on NK cells, such as killer cell Ig-like receptors (KIRs), inhibits target cell lysis and allows NK cells to distinguish between self and nonself/altered-self (missing self) such as tumor cells. From the descriptions of these mechanisms of lymphocyte education, it is evident that the expression of human MHC molecules in the mouse host is essential for the optimal function of T and NK cells and, consequently, for appropriate innate and adaptive immune responses.

### Functionality of Donor Cells/Tissues

A final requirement for the successful xenotransplantation of a human hemato-lymphoid system is the functionality of human cells in the mouse host. As for their development and maintenance, cross-reactivity of cytokines, chemokines, and adhesion molecules is necessary for the optimal activation of human cells (**Figure 4**).

Additionally, effector cytokines produced by human cells must have the capability to act on mouse tissues. In this case, cross-reactivity of human cytokines on the mouse receptor is needed. Examples of such effector cytokines include the proinflammatory molecules IL-6 and TNF-α, the antiviral type I interferons (IFNs), and molecules involved in tissue repair such as IL-22 (**Figure 4**).

## HISTORY OF HHLS MICE

Because the development of humanized mice has been reviewed in detail before (e.g., Reference 53), we here give only a brief summary (**Table 3**). The first models of HHLS mice were reported in 1988 by three independent groups who transplanted human hematopoietic cells into immunodeficient mice (22, 23, 54). Two of these studies used CB17-*Scid* mice as recipients for human peripheral blood (22) or human fetal tissues (23). Subsequently, CB17-*Scid* mice were also transplanted with human bone marrow hematopoietic progenitor cells (55). Those early models initiated a new era for the in vivo study of human hematolymphoid cells and provided a much needed small animal model of HIV infection. However, engraftment levels were low and transient in CB17-*Scid* recipients.

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In 1995, backcrossing of the *Scid* mutation onto the NOD genetic background resulted in an immunodeficient recipient in which increased levels of human engraftment were achieved (56, 57). The major genetic factor that contributes to the capacity of NOD to support higher levels of human engraftment was identified only 12 years later. A strain-specific polymorphism in the NOD *Sirpa* gene encodes a variant of the SIRPα protein that cross-reacts with the human form of its ligand, CD47 (33, 34). The NOD *Sirpa* polymorphism thus confers phagocytic tolerance for the human xenograft. Although other polymorphisms in the NOD genetic background may be involved, such as deficiency in lytic complement activity or reduced NK cell function (56), expression of NOD *S*irpa in the Balb/c background is sufficient to confer phagocytic tolerance and to support enhanced human cell engraftment (36).

Several strategies have been tested to abolish the function of mouse NK cells in recipient mice. While deficiency in the genes encoding β2-microglobulin (β2m) or perforin resulted in partial inactivation of host NK cells function (58, 59), complete elimination of NK cells was achieved in the early 2000s by crossing mutant forms of the Il2rg gene onto the Balb/c Rag2−/− (BRG) and NOD-Scid (NOG and NSG) backgrounds (28, 31, 32, 60). Concomitantly, the optimization of the protocol of human cell transplantation by intrahepatic injection into newborns resulted in better engraftment levels, multilineage development, thymic lymphopoiesis, and some functional adaptive immunity (28, 32, 46).

In 2006, two groups independently reported a model (now commonly termed the bone-liver-thymus or BLT model; see sidebar) in which they cotransplanted human fetal tissues and hematopoietic progenitor cells into NODScid recipients (61, 62). This model, in which human T cells develop and are educated in a human thymic microenvironment, has become a frequently used model for HIV studies (63).

Overall, these successive improvements to the HHLS mouse models resulted in increasing levels of human engraftment and lineage differentiation (**Tables 3** and **4**). Indeed, in the well-established models most commonly used today (NOG/NSG, SRG, and BLT), peripheral engraftment levels are in the range of 30–60% human $CD45^{+}$ cells and are maintained for periods exceeding 6–9 months. Furthermore, all lineages of human hematopoietic cells develop at least to some degree in the mouse, including T and B lymphocytes, NK cells, DCs, monocytes, macrophages, erythrocytes, and platelets (28, 31, 32, 35, 36). The established models of HHLS mice thus represent useful small animal models for the study of human hematopoietic and immune function in physiological conditions and in diverse disease models. These models are listed and discussed in the next section.

## ACHIEVEMENTS WITH ESTABLISHED MODELS

One purpose of humanized mouse models is to provide a preclinical platform where human-specific physiology and disease are recapitulated, which allows deeper understanding and the development and testing of potential new preventive and therapeutic approaches. In this section, we summarize the current achievements in dissecting human hemato-lymphoid cell physiology and pathology, including infectious diseases, as well as efforts to test new

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preventive or therapeutic measures. Naturally, these achievements also revealed limitations of the models on which we focus later in this review.

### Physiology of Early Human Hematopoietic Development

The detailed characterization of early human hematopoiesis is probably the discipline of biomedical research that has benefited the most from the development of strains of mice that can be transplanted with human hematopoietic cells. In analogy to mouse hematopoiesis, human cells have been identified by prospective isolation of candidate populations followed by xenotransplantation into recipient mice and the reading out of respective hematopoietic lineages. Using this approach, researchers have identified human self-renewing HSCs and lineage-committed progenitors (64–66). These studies revealed a model of human hematopoietic development that slightly differs from the mouse system (reviewed in 50) (see also **Supplemental Table 2**).

Recently, two groups have shown that limiting numbers or even a single cell of defined human cell populations containing HSC(s) could successfully engraft NOG or NSG mice and sustain long-term hematopoiesis over serial transplantations (65, 67). Furthermore, analogous to mouse aged HSCs, bone marrow from elderly human individuals showed a significantly increased number of phenotypic HSCs, and their differentiation potential skewed toward myelopoiesis over lymphopoiesis when transplanted into mice (68).

The timely and sufficient availability of human HSCs with defined MHC for the patient in need poses a problem for clinical hematopoietic transplantation; thus, the expansion of human HSPCs would represent a major advance for application of these therapies (69), and HHLS mice represent the best possible preclinical tool for a meaningful readout. A recent and prominent example of this is the identification of StemRegenin1, a small compound that expands the number of HSCs with NSG-repopulating capacity ex vivo (70).

Thus, although limitations in HSC maintenance remain (discussed later in this review), HHLS mice currently represent the best possible standard to identify in vivo long-term human HSCs, as reflected by several months' engraftment in mice.

### Precursor Hematopoietic Neoplasms

Compared with cancers of other organ systems, hematopoietic neoplasia samples can relatively easily be obtained from patients. Indeed, after the establishment of the first HHLS models, *Scid* and later NOD-*Scid* recipients have been used to successfully isolate acute myeloid leukemia (AML)-initiating cells from other leukemia cells that do not have in vivo tumor repopulating ability (71) and to further identify the cells that share cellular characteristics with normal HSCs but are capable of reconstituting the heterogeneous leukemia phenotype (72). These cells were termed cancer stem cells (CSCs; also known as leukemia-initiating cells, LICs), and collectively these findings fueled a whole new field of research in tumor biology aimed at defining tumor heterogeneity and the respective cells with tumor-initiating capacity (73, 74). CSCs supposedly exist mostly in a dormant cell cycle phase, similar to normal HSCs, and thus are more resistant to conventional chemotherapy, often leading to failure of eradication and relapse of disease. While the CSC

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concept likely holds true for many hierarchically organized tumors, CSCs are, owing to continuous subsequent genetic alterations, a moving target. This research also emphasizes that the selection pressure exerted by the environment, i.e., the xenogeneic assay itself, impacts the results obtained, with the potential to compromise the validity of any conclusions: With the increasing immunodeficiency of the host, higher and broader CSC readout was observed (75).

Notably, engraftment and propagation of human hematopoietic cancer cells in mice seem to depend on the aggressiveness of disease, which is typically characterized by dysregulated cell growth in an increasingly environment-independent and cell-autonomous fashion. Thus, highly aggressive behavior of a human hematopoietic neoplasm in the mouse correlates with poor clinical outcome (76). In contrast to aggressive forms of acute myeloid and lymphoid leukemia, some less aggressive acute leukemias and other less aggressive early hematopoietic neoplasms do not engraft in currently available mice. In fact, there has been little progress toward establishing in vivo xenogeneic models for more chronic hematopoietic neoplasms such as myelodysplastic syndromes, myeloproliferative neoplasms, or multiple myeloma that are also difficult to maintain in vitro. A likely reason for the difficulty to engraft these neoplasias is that they depend on environmental factors that are not provided or are not cross-reactive in currently available recipient mice. Indeed, it has been demonstrated that bone marrow stromal cells in some instances play an important role in both initiation and progression of myelodysplastic syndromes (77). Consequently, the humanization of the mouse bone marrow microenvironment, by either genetic modification to express human factors or by implantation of human biomaterials containing niche cells derived from patients, would likely supply survival or maintenance factors for some neoplasias. These in turn might serve as targets for therapy in the future.

Beyond aiding the understanding of pathologic features of disease, HHLS models have proven useful as platforms for the development of novel, molecularly targeted therapies and their preclinical evaluation. In fact, some extracellular receptors have been found to be up-regulated in AML cells compared with normal HSCs, and specific targeting of these molecules by antibody or cytokine injection led to efficient eradication of AML-CSCs in vivo (78– 82). Some of the key studies on precursor hematopoietic neoplasms are listed in **Supplemental Table 3**.

## Infectious Diseases with Human Tropism

Another discipline that has greatly benefited from the availability of HHLS mice is the study of pathogens that have exclusive human tropism (83). In particular, viral and bacterial pathogens such as HIV, hepatitis C virus (HCV), Epstein-Barr virus (EBV), and tuberculosis affect millions of individuals and cause a significant societal and financial burden around the globe. A better understanding of the pathology and immune responses induced by these infectious agents as well as the development of new vaccines and therapies are critically needed. The use of HHLS mouse models simplifies and enables in vivo studies (see also **Supplemental Table 4**) that were previously not feasible owing to the prohibitive costs and ethical restrictions associated with other animal models such as nonhuman primates.

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### HIV

The first HHLS mouse models used to study HIV infection were the Hu-PBL-SCID and the SCID-hu models (see **Table 3**) in which HIV-mediated $CD4^+$ T cell depletion could be demonstrated (1, 84, 85). These models have also allowed the testing of experimental drugs in vivo, but the utility of these early models was restricted by the lack of significant adaptive immune responses, the activation and decline of transferred human peripheral blood leukocytes (PBLs) (hu-PBL-SCID), and the absence of human cells in circulation (hu-SCID).

BRG, NOG, and NSG mice overcome some of these limitations. After HIV infection, sustained viremia and $CD4^+$ T cell depletion is observed in the blood, but virus-specific adaptive immune responses are weak and are detected in only a fraction of mice, thus complicating the use of these models for preclinical vaccine research (86–90). In contrast, BLT mice feature more robust adaptive immune responses that are detectable in most infected mice, with both humoral and T cell responses being present (91). Moreover, as BLT mice are repopulated with $CD4^+$ T cells in the mucosal epithelium, HIV infection in these mice can be achieved via routes resembling human infection, such as intrarectal and intravaginal infections (92, 93). BLT mice are currently the preferred model for HIV research, and this model has enabled proof-of-concept studies using microbicides to prevent mucosal HIV infection in vivo (63).

### Epstein-Barr Virus

EBV is a γ-herpesvirus that is restricted to humans and infects hematopoietic cells. Studies have demonstrated that EBV can infect humanized mice, albeit via unnatural routes such as intraperitoneal or intravenous infection (28, 94). Human T cell responses in NSG/NOG conferred protection against virus-induced tumor development in a CD4- and CD8-dependent manner (94, 95). Additionally, these models have for the first time allowed the characterization of the in vivo contribution to EBV-associated diseases of individual viral genes regulating the lytic function or the latency of the virus (96, 97). Regarding the testing of novel anti-EBV immunization strategies, one study demonstrated that an experimental vaccine candidate was able to induce T cell responses at low levels, but it remained untested whether these responses were protective (98).

### Dengue Virus

Dengue virus (DENV) is a mosquito-borne virus belonging to the Flaviviruses that in some infected individuals causes dengue hemorrhagic fever (99). This complication is thought to be mediated by preexisting immunity to a different serotype of DENV, but causal studies have been impossible due to its restriction to humans. Several studies have demonstrated that adaptive immune responses are detectable, although relatively inefficient, in HHLS mice after DENV infection (100–102). As in studies with HIV and EBV, HLA-restricted responses are detected in both BLT and HLA-transgenic mice. A side-by-side comparison of BLT mice and HLA-A2-transgenic mice demonstrated similarly increased levels of antiviral IgM compared with non-HLA-transgenic mice, but all strains lack significant DENV-specific IgG responses (102).

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### *Salmonella enterica* Typhi

The bacterium *Salmonella enterica* Typhi (*S*. Typhi) causes typhoid fever in humans. Because of its human tropism, no animal model was available to study its pathogenesis in vivo or to test vaccines. Recently, the first mouse models for acute and chronic *S*. Typhi infection have been generated using humanized mice based on BRG or NSG mice (103, 104). In both studies, intraperitoneal infection with *S*. Typhi was used. An important future challenge is to develop a mouse model that allows infection by the oral, i.e., natural, route. This will likely depend on achieving better human hematopoietic cell reconstitution in the intestine.

### Other Infectious Diseases (HBV, HCV, HSV, HTLV, Malaria)

HHLS mice have also been used to study other viruses including HCV, herpes simplex virus (HSV)-2, and human T-lymphotropic virus (HTLV)-1 (see **Supplemental Table 4**). In the case of HSV-2, infection of BRG mice induced even specific IgG responses, which has been reported only rarely for other viruses (105). In the case of hepatotropic diseases such as HCV, a human hematopoietic system is insufficient to model the pathology and immunity in humanized mouse models, as HCV infects only human hepatocytes. Cotransplantation of human fetal hepatocytes and HSPCs has allowed the generation of an autologous in vivo system following HCV infection, but HCV-specific immune responses were barely detectable (106, 107).

The generation of model systems featuring both human hepatocytes and hematopoietic cells would also allow the study of other hepatotropic pathogens such as HBV or the parasites *Plasmodium falciparum* and *P. vivax* responsible for human malaria (106). In particular, for *P. falciparum* and *P. vivax*, it would allow studies of the whole life cycle of these parasites as they first infect hepatocytes and subsequently erythroid cells.

### Species-Specific Immune Responses to Pathogens

Certain pathogens have a broad species-tropism but elicit distinct immune response in different host species. A prime example of a human-specific immune response to a pathogen is the granuloma, a typical lesion found in humans infected with tuberculosis (108, 109). The granuloma consists of an accumulation of macrophages and T cells. In human tuberculosis, granulomas have a well-defined structure that typically contains multinucleated giant (Langhans giant) cells that arise from the macrophage fusion. In contrast, infection of mice with *Mycobacterium tuberculosis* only leads to the formation of loosely organized structures that resemble granulomas but that lack Langhans giant cells. Because it is controversial whether the granuloma plays a protective role during tuberculosis in humans or favors the dissemination of mycobacteria, the generation of humanized mouse models that develop human granulomas will prove interesting. The improved development and function of human macrophages as well as of human $CD4^+$ T cells in HHLS mice are likely required to achieve this goal.

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## Solid Tumors and Human Immunity

Most solid tumor research has relied on the use of murine tumor models and revealed important basic principles (17). However, primary human tumors are mostly more complex in their nature and evolve more slowly compared with those tested in mouse models, and human immune responses vary from those observed in mice. Thus, better in vivo models of human antitumor immune responses are needed. This is particularly necessary with the current development of immunomodulating therapies, some of which are becoming available for clinical use (110). Humanized models require transplantation of both primary human tumors and, in the best case, hemato-lymphoid cells or HSPCs from the same individual (see also **Supplemental Table 5**). Moreover, this would ideally occur in an environmental context where the same HLAs, or at least some of them, would be expressed. This is difficult to achieve for both practical and ethical reasons.

An important step in this direction was taken by transplanting human HSPCs and same-donor peripheral T cells as well as breast cancer cell lines into NOD-*Scid* β2m$^{-/-}$ mice. The authors demonstrated that CD4$^{+}$ T cells promote early tumor development in a DC-dependent fashion that could be partially prevented by IL-13 antagonists (111). More recently, they also showed the role of TSLP-OX40L in promoting tumor growth in vivo (112). These in vivo observations were paralleled by respective in vitro studies on human primary breast cancer samples, demonstrating the principal value of respective combined in vitro and in vivo analysis for solid tumor immune interaction research.

## Autoimmune Disease

As with tumor immunology, studies of autoimmunity require a functional human immune system and the respective target tissues. The latter could be of mouse origin carrying HLA components or, likely better, of human origin matching the HLA of human immune system cells. In theory, all human autoimmune diseases should be able to be modeled. A first step in this direction was taken with the generation of NOD-*Rag1*$^{-/-}$*Il2rg*$^{-/-}$*Ins2*$^{Akita}$ (NRGAkita) mice that spontaneously develop diabetes owing to the Akita mutation (113). Upon transplantation with both human islets and non-matched human HSPCs, these islets were infiltrated and rejected in some cases. Therefore, while potentially suitable to model autoimmune diabetes, this model thus far has been used only to measure an adaptive immune rejection of an allograft.

Another study demonstrated that transferred human PBLs were prevented from chemotaxis upon treatment with an agonist of CXCR3 in an air-pouch model of arthritic inflammation (114). While this is a good example of a transient engraftment to test a therapeutic, again it clearly does not reflect the complexity of the human disease (**Supplemental Table 6**).

To this end, HHLS mouse models will need to incorporate endogenous expression of human MHC for T cell selection as well as on the target tissue. Some of these limitations have been overcome with the use of the BLT model and the modified BLT (115). In this model, when the target tissue is the immune system itself, it is possible to generate fully antigen-specific immune responses. However, for autoimmune targets outside the immune system, the target cells must also express the appropriate human MHC molecules. Additionally, because

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different human MHC alleles are the most closely linked genes associated with autoimmunity, having the appropriate human MHC molecules may be critical for our understanding of these diseases.

### Human Hemato-Lymphoid Cell–Directed Gene Therapy

HHLS mouse models appear to be suitable for preclinical testing of genetic modification of both HSPCs and mature hematopoietic cells in order to correct inborn or acquired disorders, including metabolic disorders, immune dysfunction, and hematopoietic malignancies and infections (**Supplemental Table 7**). HIV gene therapy is particularly well suited to be tested in HHLS mice, as both an in vivo readout and an infection can be combined (116–119). Whether HHLS mice with their overall short readout times are also suitable to allow safety testing to elucidate mechanisms of gene therapy–mediated insertional mutagenesis still needs to be determined (120–122).

In summary, HHLS models that have been established thus far are capable of recapitulating some aspects of human pathology and immune responses. These models already have the potential to accelerate the development of novel drugs as well as the identification of prognostic and therapeutic molecular targets. However, several limitations remain, and improvements of the recipient mice are necessary, as discussed in the next section.

## REMAINING LIMITATIONS AND the NEXT GENERATION OF HHLS MICE

The list of applications for which established HHLS mice have been successfully used highlights the potential of these models for biomedical research. However, it also reveals fundamental limitations that are likely a result of insufficient mouse-to-human cross-reactivity of development, education, maintenance, activation, and migration factors for human hemato-lymphoid cells in the xenogeneic environment (45) (see also **Figures 3** and **4** and **Supplemental Table 1**). Naturally, the field is trying to address these deficiencies in a sequential manner by focusing first on ensuring cell development, then on cell maintenance, and finally on appropriate cell function. With the increasing feasibility of complex genetic engineering, recent years have witnessed a renaissance in the development of HHLS mice, with ambitious programs worldwide that are currently leading to remarkable progress (123).

Different protocols have been used to deliver a variety of human cytokines in HHLS mice (124). **Table 5** lists cytokines that have been delivered exogenously into HHLS mice by injection of recombinant cytokines, hydrodynamic injection of plasmid DNA, or lentiviral delivery in vivo. Another effective approach employs genetically expressing human cytokines in the genome of recipient mice (**Table 6**). This can be achieved by transgenic overexpression, by BAC transgenesis, or best by knockin replacement of a mouse gene by its human counterpart. The overexpression of cytokines under the control of a constitutive active promoter has to be used with caution, as high level or aberrant expression of a cytokine can induce nonphysiological effects (125). BAC transgenesis and knockin replacement, where the human gene directly replaces the mouse gene and is driven by the mouse promotor, result in more physiological expression of the encoded protein (35, 126–128). In the case of knockin replacement, which might indeed represent the most physiological way of expression, several general and specific issues need to be considered:

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(*a*) As with other technologies, factors that are not cross-reactive mouse to human and are not produced by the transplanted cells themselves should be chosen; (*b*) knockout of the respective mouse gene should not substantially influence viability and breeding efficacy of the mouse strain or the human knockin should compensate for this by rescuing the deficiency via sufficient human-to-mouse cross-reactivity; and (*c*) if the mouse knockout induces a defect in mouse cell populations, it might confer an additional competitive advantage to the corresponding human cell population.

Below, we focus our discussion on the remaining limitations and improvements implemented in the most recent models of HHLS mice (BRG, NSG/NOG, and BLT) based on the specific hematopoietic cellular lineages.

## Hematopoietic Stem and Progenitor Cells

HSCs, like all adult stem cells, are characterized by two defining properties: self-renewal and the potential to differentiate into all lineages of blood cells (49, 50). All HSCs are found within the human $CD34^+$ cell population. However, while $CD34^+$ cells account for up to 5% of human fetal liver, cord blood, and bone marrow, less than 1% of the $CD34^+$ fraction represent HSCs (49). Transplantation of 100,000 human $CD34^+$ cells, a number frequently used in HHLS transplantation assays, should contain a significant number, i.e., more than one human HSC. However, even in mice that receive several-fold these numbers of human $CD34^+$ cells, the levels of human cell engraftment declines, starting within five months after transplantation (see, for example, Reference 126). Furthermore, the efficiency of transplantation into secondary recipients is low. Thus, the number of so-called *Scid*-repopulating cells declines over time in current HHLS models. This is in stark contrast to the respective mouse-to-mouse transplantation experiments in which limited numbers of HSCs expand to steady-state levels over 3–5 months and can even be used for subsequent serial transplantations (49). These observations suggest that bona fide human HSCs either do not engraft or are not maintained or expanded (or any combination) in the mouse microenvironment.

Thrombopoietin (TPO) and stem cell factor (SCF) are two cytokines that are indispensable for the maintenance of quiescent self-renewing adult HSCs in the bone marrow niche (129–133) (**Figure 3**). The humanization of the gene encoding TPO by knockin replacement in the *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ background led to a significant increase in the number and the maintenance of functional long-term HSCs, as demonstrated by immunophenotypic characterization and enhanced capacity to repopu-late secondary recipients, respectively. As a result, TPO humanization led to increased levels and prolonged maintenance of human cell engraftment in the bone marrow (126). Also, the transgenic expression of membrane-bound human SCF under a cytomegalovirus promoter in NSG mice resulted in higher engraftment levels after human HSPC transplantation, even in the absence of preconditioning of the recipients (134, 135). However, a direct effect of human SCF on the maintenance and self-renewal capacity of human HSCs in the mouse host has not yet been demonstrated in this model.

Despite the improvements conferred by humanization or overexpression of the genes encoding TPO and SCF, the maintenance of truly self-renewing human HSCs still remains largely defective in the mouse host. Thus, further analytical investigation is required to

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identify the critical factors available and produced in the complex three-dimensional microenvironment termed the HSC niche (136–138), which are not sufficiently cross-reactive and would need to be humanized in order to reconstruct a fully functional niche for human HSCs.

### Erythropoiesis and Thrombopoiesis

Mature RBCs are produced through the highly regulated process called erythropoiesis in which progenitor cells of the megakaryocytic-erythroid lineage differentiate into reticulocytes in the bone marrow. Reticulocytes then emigrate and differentiate into erythrocytes in the circulation (139). Important steps in this process, such as the differentiation of erythroid progenitors, the accumulation of hemoglobin, and the subsequent enucleation of normoblasts, are regulated in part by cytokines. Erythropoietin (EPO) is the most important cytokine involved in the terminal differentiation of RBCs, and mouse EPO is not, or is insufficiently, cross-reactive to the respective human receptor (45) (**Figure 3** and **Supplemental Table 1**). In all models of HHLS mice, erythroid lineage cells develop in the bone marrow, but mature erythrocytes are strikingly absent from the blood, indicating either a block in terminal differentiation or destruction in the periphery. Provision of human EPO and IL-3 boosts human erythropoiesis (140, 141). Nonetheless, strong evidence suggests that it is the destruction of human erythrocytes that contributes to their absence in the periphery. Phagocytosis is one of the major effectors of this process, as clodronate-mediated depletion of mouse phagocytic cells results in increased frequencies of human erythrocytes in the periphery (141). Also, transgenic expression of human SIRPα on the mouse background improves survival of transferred human RBCs (35).

Platelets, or thrombocytes, are produced by fragmentation of megakaryocytes that also derive from megakaryocytic-erythroid progenitors in the bone marrow (142). TPO is the major cytokine involved in this process. Similar to RBC development observed in HHLS mice, human megakaryocytes are also present in the bone marrow, but unlike in humans, human platelets are almost completely absent from peripheral blood in these mice. Cytokine support, through TPO humanization, does not significantly enhance the number of platelets in circulation, but clodronate-mediated elimination of phagocytic cells induces a boost in human platelets in the blood (126, 143).

Overall, these observations show that novel strains of recipient mice are needed for the efficient repopulation of HHLS mice with human RBCs and platelets. These strains need to combine cytokine support for the development and maturation of human erythrocytes and platelets with genetic deficiencies in the mechanisms that contribute to the elimination of these cells.

### Myelo-Monocytic Cell Lineages

Major differences in myelo-monocytic lineages exist between human and mouse. One of the most notable is the ratio of myeloid to lymphoid cells among white blood cells: Human blood is rich in myeloid cells, whereas mouse blood is dominated by lymphocytes (**Figure 2*a***) (19). However in HHLS mice, human myeloid cells rarely exceed 5–10% of human

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white blood cells (**Table 4**). Despite their relative paucity, human myeloid cells are largely functional in HHLS mice (144), although some impairments have also been reported (145).

Given the major defects that exist in the development of human myeloid populations in HHLS mice, this lineage has been the first to be targeted for improvement by transgenic expression of cytokines known to support myelopoiesis. Transgenic mice expressing human SCF, GM-CSF, and IL-3 (SGM3) under the control of a ubiquitous cytomegalovirus promoter were generated in *Scid*, NOD-*Scid*, and NSG backgrounds (125, 146–148). These transgenes induced an increase in myeloid differentiation and improved the engraftment of AML samples. However, continuous high expression of these cytokines came at the cost of terminal differentiation and subsequent further reduction of the human HSPC compartment in the mouse bone marrow (125). Furthermore, the most noticeable effect of SGM3 transgenesis in NSG mice was an increase in human T cells, particularly regulatory T cells, through a thus far not determined indirect mechanism (148). The transgenic expression of membrane-bound human SCF (described above) also had a positive effect on myelopoiesis, particularly on the development of mast cells (135).

Knockin humanization of the two adjacent genes that encode IL-3 and GM-CSF had a major effect on the populations of lung alveolar macrophages (127). Owing to the lack of the mouse cytokines and the non-cross-reactivity of human cytokines, mouse alveolar macrophages exhibited a defect in terminal differentiation and function, leading to development of pulmonary alveolar proteinosis similar to the phenotype observed in GM-CSF-deficient mice (149, 150). After engraftment with human $CD34^+$ cells, the lungs of these recipient mice were repopulated with human alveolar macrophages that were able to partially rescue the proteinosis phenotype. Furthermore, those cells were able to mount a pulmonary innate immune response, with the robust production of human type I IFNs and proinflammatory cytokines in response to intranasal influenza infection (127). This effect on alveolar macrophage population replacement is mostly due to the humanization of GM-CSF. The effect of IL-3 humanization in these mice has not yet been fully characterized.

M-CSF is another cytokine critically required for myelopoiesis. Hydrodynamic injection of plasmid DNA encoding human M-CSF increased the population of $CD14^+$ monocytes in multiple tissues (140). Similarly, the knockin replacement of the gene encoding mouse MCSF by its human counterpart led to a signifi-cant improvement in the engraftment of functional $CD33^+CD14^+$ monocytes in tissues of recipient mice (128).

Finally, besides its effect on the maintenance and function of human HSCs, the humanization of TPO resulted in enhanced frequencies of bone marrow myeloid cells, particularly $CD66^+$ granulocytes (126).

Although these newly developed recipient strains represent major steps toward a fully functional human myelo-monocytic system in the mouse host, several challenges remain. For example, humanization of other factors might be necessary for efficient development and maturation of granulocytes in peripheral blood. Furthermore, the combination of multiple humanized alleles will most likely result in synergistic effects and will be needed to generate a complete human myeloid compartment. Human and mouse monocyte

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subpopulations differ in many respects (**Table 1**) (151, 152). The surface markers used to identify these populations are not shared between species. Furthermore, the populations that look most similar based on gene expression profiles have distinct functional properties. Thus, mouse monocyte biology can be only partially translated to the understanding of human monocyte function. And given that migration, homing, and in vivo differentiation are important aspects of monocyte biology that cannot be replicated in vitro (151–153), improving human myeloid function in HHLS mice will undoubtedly help advance our understanding of the function of human myelo-monocytic cells in vivo (154). The cross-reactivity of mouse chemokines required for the correct migration of human monocytes will then need to be evaluated in vivo. Conversely, humanization of cytokine receptors might be necessary for the complete function of effector molecules, such as proinflammatory cytokines, secreted by human monocytes.

Finally, improved HSC maintenance combined with efficient myeloid development should favor better engraftment and development of myeloid malignancies. It might also become possible to transplant myeloid leukemias/neoplasias of lower aggressiveness that have been difficult to engraft in currently available models.

### Dendritic Cells

DCs represent a specific lineage of cells that link the innate and the adaptive immune responses. Flt3L is the most important cytokine required for DC development. DCs have the uniquely efficient capacities to capture and process antigens, to present them on MHC molecules, and to activate naive T cells (155). In the mouse, several DC subsets have been identified (including classical DCs, inflammatory DCs, and plasmacytoid DCs; **Table 1**), and each contains subpopulations that are in part functionally characterized (156–158). DC populations are also heterogeneous in humans, and although the markers used to identify the different subsets are distinct between human and mouse, the functional counterparts of the different subsets can be found in each species (**Table 1**) (159, 160).

In HHLS mice, all subpopulations of conventional (including BDCA1$^{+}$ and BDCA3$^{+}$ subsets) and plasmacytoid DCs can be found in percentages similar to those in human physiological conditions relative to human engraftment levels (28, 144, 161). Increased frequencies of human DCs can be obtained by the hydrodynamic injection of plasmids encoding human GM-CSF, IL-4, and Flt3L, but these frequencies are probably supraphysiological (140). These observations suggest that mouse Flt3L is sufficiently cross-reactive on the human Flt3 receptor and that no additional cytokine support is critically required for human DC development. However, as in other myeloid compartment cells, it might be beneficial to reduce the mouse DC compartment in order to get more niche space for human counterparts. Functionally, human DCs isolated from HHLS mice recapitulate the functions of cells isolated from human donors (28). Interestingly, HHLS mice represent a suitable platform to test in vivo DC-specific targeting. One group (98) demonstrated that an antigenic peptide fused to an anti-DEC205 antibody induced antigen-specific T cell and antibody responses against EBV infection. Furthermore, antigen-specific human T cell tolerance can be induced in HHLS mice by targeting DCs (162).

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Although all reports so far suggest that human DCs are functional in the mouse host, further characterization is needed. It will be a particularly important task in the coming years to determine if human DCs also sufficiently populate nonlymphoid tissues and if they subsequently localize in appropriate lymphoid tissue microarchitecture. Furthermore, as both mouse and human DCs are present in HHLS mice, cells from the two species are competing for the capture of antigens and presentation to human T cells. Mouse costimulatory molecules are not highly conserved between species (**Supplemental Table 1**), and therefore mouse DCs may not prime human T cells optimally or could even induce T cell anergy. This will, in addition, depend on the positive and negative selection status of human T cells in the mouse background (**Figure 5**). Thus, genetic elimination of mouse DCs would result in a situation in which presentation of antigens to human T cells is accomplished exclusively by human DCs.

## Natural Killer Cells

NK cells represent 10–15% of lymphocytes in human blood. They play important roles in the host defense against viruses (specifically herpesviruses in humans) and probably also in immunosurveillance against tumors (29, 163–166). Two subsets of NK cells can be distinguished based on the expression of cell surface markers: an immature subset that mainly produces cytokines and a mature subset that expresses inhibitory receptors and has cytolytic activity. Major differences exist between human and mouse NK cells, including the cell surface markers used to distinguish the different subsets, the ligands of their activating receptors, and the effector mechanisms of cytotoxicity (**Table 1**). Interestingly, human and mouse NK cells rely on different families of inhibitory receptors (KIR and Ly49, respectively). Although these families of receptors have similar functions, they are not structurally or evolutionarily related and may represent an example of convergent evolution (167).

The development and maintenance of NK cells is critically dependent on the cytokine IL-15. In contrast to other cytokines, direct binding of IL-15 to the target cell (and its receptor IL-2Rβ) is not sufficient for biological activity. Instead, IL-15 has to be *trans*-presented in complex with IL-15Rα by a different cell than the target cell (168). For mouse NK cells, the cellular source of *trans*-presented IL-15/IL-15Rα is mainly hematopoetic, i.e., myeloid cells and DCs. However, it is unclear which cell type is required for human NK cell homeostasis in vivo. In addition to IL-15, IL-7 is required for the homeostasis of thymic NK cells in the mouse. In contrast to IL-15, IL-7 is exclusively expressed by nonhematopoetic cells.

All current HHLS mouse models exhibit considerable limitations regarding human NK cell development, maintenance, and function. Human NK cells seem to be particularly susceptible to phagocytosis by mouse macrophages, as the interaction between CD47 and SIRPα increases human NK cell proportions (35, 36). However, even in these conditions, the number of human NK cells in HHLS mice is low compared with that in humans, and their terminal differentiation is impaired (169, 170). For example, the reduced frequency of cells expressing KIRs may reflect an absence of NK cell licensing due to missing KIR–MHC class I interactions. This correlates with functional defects such as decreased levels of IFN-γ production and cytolytic activity after stimulation with cytokines and target cell lines.

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Consistent with this, few functional NK cell responses to pathogens or to tumors have been demonstrated in HHLS mice so far (140, 171).

Using various methods to provide human IL-15 and IL-15Rα, investigators have attempted to overcome these limitations (**Table 5**), resulting in increased numbers of human NK cells and their improved terminal differentiation and function (140, 172, 173). Future efforts might be directed at providing human IL-15/IL-15Rα without the need for exogenous application of IL-15/IL-15Rα complexes. In addition, to ensure proper licensing of human KIR-expressing NK cells, it may be necessary to have human MHC class I expression in the mouse host, i.e., on nonhematopoietic cells. After these improvements, functional human NK cell responses against human herpesviruses and tumors should be possible in HHLS mice.

## T Cells

In the established models of HHLS mice lacking the IL-2Rγ chain, human T cells develop de novo from transplanted human HSPCs in the mouse thymus, from where they exit and seed secondary lymphoid organs (28, 31, 32, 46, 174). This T cell production is transient, with an initial increase in thymus size and subsequent involution and, in particular, loss of CD4/CD8 double-positive cells (47, 175). This is accompanied by an increase of human T cells in the peripheral blood starting at about 6 weeks, leading to an inversion of the T and B cell ratio over time, and, with decline of overall human engraftment, to relative T cell dominance. This leaves a window of 2–6 months when, based on the presence of the relevant cells, immunity can potentially be studied. In peripheral tissues, the relative frequencies of $CD4^+$ and $CD8^+$ T cells with a broad Vβ repertoire as well as of γδ and regulatory T cells are close to human physiological values for a transient time of about 3 months (28, 32, 46), with a subsequent shift of T cell phenotype toward effector and central memory, while naive T cells are underrepresented (36, 176, 177).

Altogether, these observations suggest several limitations in the development, maintenance, and function of T cells in HHLS mice. With the concommitant decline of HSPCs in the bone marrow, the mouse thymus may not be appropriately supplied with progenitors; the mouse thymus might not appropriately sustain respective human early thymocyte progenitors, and the survival and proliferation of human thymocytes may be inadequate. As a consequence, the output of mature T cells from the thymus is low, which is likely compensated by homeostatic proliferation of T cells that therefore acquire an effector/memory phenotype. Furthermore, the inefficient or altered selection of a human T cell repertoire on mouse and possibly also human MHC molecules in the thymus also contributes to the impaired maintenance of T cells and subsequent function in the periphery (see also **Figure 5** and discussion in Reference 45).

Several factors contribute to adequate T cell homeostasis in the periphery, including survival of human T cells in the presence of mouse macrophages, cytokine support (e.g., IL-7 and IL-15), and the continued interaction of TCRs with MHC molecules (178). Strategies for enhancing human T cell development, homeostasis, and function have focused on those three parameters.

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Like NK cells, human T cells seem to be particularly susceptible to phagocytosis by mouse macrophages, which can be inhibited through successful CD47-SIRPα signaling. Indeed, cross-reactivity between these two molecules resulted in proportionally increased T cell development, higher frequencies of naive T cells, and as a result, significantly enhanced humoral responses, likely also due to better T cell help for antibody-producing cells (35, 36).

The provision of IL-7 and IL-15 has had different effects depending of the protocol of administration used (**Table 6**). Injection of recombinant IL-7 had a (transient) effect on thymic lymphopoiesis, but did not improve peripheral T cell homeostasis (174, 179). When delivered at a supraphysiological concentration through in vivo lentiviral delivery, however, IL-7 induced a striking increase in T cell numbers without affecting the naive versus effector/central memory ratio (180). Repeated injection of the human IL-15/IL-15Rα complex resulted in increased numbers of both $CD4^+$ and $CD8^+$ T cells and in antigen-specific IgG production after tetanus toxoid immunization (181).

Several groups have reported the generation of recipient mice that transgenically express human MHC class I or class II (**Table 6**). Human MHC increased peripheral T cell numbers in some models but did not significantly affect the naive versus effector/central memory phenotype. As expected, human MHC expression induced better cytotoxic and humoral adaptive immune responses. For example, transgenic expression of HLA-A2 resulted in an antigen-specific, HLA-restricted response to EBV or DENV immunodominant epitopes similar to those observed in $HLA\text{-}A2^+$ human individuals (94, 102, 176). In the case of MHC class II, expression of HLA-DR4 resulted in the production of antigen-specific human IgG after immunization with tetanus toxoid or ovalbumin (175, 177).

Overall, most of the described strategies had a positive effect on human T cell development, homeostasis, and/or function. However, adaptive immune responses in HHLS mice are still not comparable to respective responses in humans (in the range of one order of magnitude lower), and it is likely that multiple improvements will need to be combined to produce robust T cell–dependent responses. First, better continuous population of the thymus with progenitors should result in larger size and longer maintenance of the thymus and thus a more diverse repertoire. Second, complete humanization of the MHC class I and class II loci should enhance the development, selection, and function as well as the homeostasis of human T cells. Third, genetic replacement of additional genes encoding cytokines could have a positive effect without requiring injection of nonphysiologic levels of cytokines. Fourth, genetic ablation of mouse DCs will result in the priming of naive T cells exclusively by human DCs, as discussed above. Finally, more robust development of lymph nodes and better lymphoid organ structuring are key requirements to achieve better adaptive immune responses.

In the case of the BLT model, in which HSPCs and fetal tissues are cotransplanted (see sidebar), T cell selection occurs efficiently in a human microenvironment in the thymic organoid, resulting in better adaptive responses (61, 62). However, humanization of MHC molecules and provision of human cytokines remain necessary for peripheral T cell homeostasis and effector function as well as for ensuring tolerance of the human T cells to the mouse host.

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### B Cells

B cells are essential for the humoral immune response, as they produce antigen-specific antibodies in response to infection and vaccination. B cells develop in the bone marrow from a common lymphoid progenitor in distinct steps via pro- and pre-B cells to immature B cells that leave the bone marrow and home to secondary lymphoid organs, where they differentiate into mature B cells (182). Early during B cell development, B cell survival is dependent on signaling from the pre-BCR, but at later stages, B cell survival factors such as BAFF and APRIL are central. Similar to T cells, B cells are subjected to a selection process during the differentiation to avoid the development of auto/polyreactive mature B cells that may harm the host. The main selection is thought to occur by elimination of developing B cells with high affinity for self-antigens, through mechanisms of clonal deletion, receptor editing, or induction of anergy. This process results in a pool of B cells with limited auto/polyreactivity. Upon antigenic stimulation, antigen-specific B cells undergo affinity maturation, class-switching, and differentiation into memory B cells or antibody-secreting plasma cells. In contrast to these classical B cells, also referred to as B2 cells, a subset of B cells, present in mouse and human and referred to as B1 cells, is characterized by a higher degree of auto/polyreactivity and almost complete absence of memory formation. These cells are thought to produce antibodies with low affinity for different antigens, called polyspecificity, but this may occur with faster kinetics compared with B2 cells. Notably, although class switching occurs in these cells, somatic hypermutation is absent.

In all HHLS models, human B cells are detectable in high frequencies. However, several observations indicate that unusual subsets of B cells dominate and that the function of the B cell compartment is impaired. First, HHLS mice are characterized by relatively low levels of human IgG, around 100-fold lower than humans, in the steady state, whereas IgM concentrations are closer to levels observed in humans (28, 32). Second, several studies using different recipient strains as well as different sources of $CD34^+$ cells have reported accumulation of $CD5^+$ B cells that resemble B1 cells, which are normally a small subset in humans (183). Third, detailed analysis of the B cell repertoire has revealed a broad repertoire in both NSG and BLT mice, but without significant somatic hypermutation even in mature B cells (184–187). Fourth, one study reported an unusually large frequency of auto/polyreactive cells even within the mature B cells, suggesting that B cell selection is not efficient in the mouse host (186). Finally, all studies show that, upon immunization of HHLS mice, the antigen-specific humoral immune responses are predominantly of the IgM serotype, and antibody titers only increase marginally with repeated boosting. Many of these characteristics are typical of immunity mediated by B1 cells. Hence, the development of classical B2 cells and their responses may be impaired in HHLS mice.

Compared with these basic models, improved human B cells responses, including antigen-specific IgG responses, have been observed in BLT mice and in mice that express human MHC class II molecules, which likely improves the cross talk between human T cells and B cells (61, 62, 175, 177). Interestingly, the accumulation of $CD5^+$ cells and the relative absence of B2 cells may reflect the early developmental stage of the hematopoietic system in HHLS mice, as similar observations were made in human neonates as well as after bone marrow transplantation (188). Hence, to further improve B cell responses in HHLS mice,

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additional genetic modifications will be needed to improve B cell development, maturation, and differentiation or to support memory cell development, maturation, and activation. Evidence suggests that providing IL-7 and BAFF may improve B cell development and survival in HHLS models, but these increases are likely incremental (179, 189). Another major limitation for B cell functions is thought to derive from defects in the organization of secondary lymphoid organs (see below) and the impairments in antigen-specific T cell function that are required for T-dependent B cell responses.

## Follicular Dendritic Cells

Follicular dendritic cells (FDCs) are of nonhematopoietic, perivascular precursor origin (190). FDCs can be induced by lymphotoxin-expressing B cells in lymphoid organs and even in nonlymphoid organs during inflammatory lymphoneogenesis (191, 192). FDCs represent important structural and immunological components for immune complex presentation and B cell maturation in B cell follicles (193). FDCs are absent from SCID mice, but they can be induced upon lymphocyte transfer (194). Similarly, when BRG or other IL-2Rγ-deficient recipient mice are constituted with human cells and develop B and T cells, mouse FDCs are induced in the follicles (28, 45). Follicle and FDC formation are particularly enhanced once mice are vaccinated in combination with adjuvants or are infected with live pathogens (M.G. Manz, unpublished results). The extent to which species' differences in FDC-lymphocyte interaction impinge on appropriate immune responses remains to be determined.

## Lymph Nodes

Secondary lymphoid organ development represents a complex organogenetic process involving both hematopoietic and nonhematopoietic lymph node–inducer and –organizer cells (195). Whereas common IL-2Rγ–/– mice have small lymph nodes, nonengrafted BRG mice lack these, apart from small mesenteric ones (195, 196). Interestingly, it was demonstrated that the lymph node anlagen can be rescued by transfer of T and NK cells in an IL-7/IL-7R-dependent fashion for up to one week after birth (196). Neonatal BRG and NSG/NOG mice transplanted with human CD34+ cord blood cells reliably develop mesenteric lymph nodes, although of varying size, whereas, for example, inguinal, axillary, mediastinal, and cervical lymph nodes are only rarely observed in highly engrafted animals (28, 47). Interestingly, in BRG mice transplanted with human peripheral blood mononuclear cells in a way that leads to xenogeneic GvHD or with genetically modified CD4+ T cells, mesenteric, inguinal, and axillary lymph nodes populated with human cells are occasionally observed (37, 118; M.G. Manz, unpublished observations). Thus, lymph node development upon induction of human cells is possible, and it will be important to determine if more robust T cell development obtained by better HSC maintenance, cytokine availability, and correct graft and tissue MHC interaction will lead to better peripheral lymph node formation and functional immune responses.

## Human Hemato-Lymphoid Cell Populations in Mouse Peripheral Tissues

To achieve appropriate immune responses, cells need to be able to reach peripheral tissues. Human myeloid and lymphoid cells can be detected in human HSPC–transplanted HHLS mouse organs such as liver, lung, brain, skin, reproductive organs, and intestine (28, 92, 127,

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197–199). Also, in the setting of xenogeneic GvHD, human lymphocytes can home to mouse nonlymphoid organs such as liver, lung, gut, kidney, and skin in human PBL-transplanted BRG mice (37). Thus, the principal requirement of cross-reactivity of necessary tissue homing interactions seems to be fulfilled.

However, the extent to which the steady-state distribution, organization, and functionality of human cells in nonlymphoid tissues reflect physiology is thus far not well studied. For example, investigators have calculated that T cells in the small intestine represent almost 50% of all T cells in mice, whereas the human T cell population in HHLS mouse intestine seems to vary widely (200, 201). It was recently suggested, based on comparative studies using NOD-Scid-BLT, NSG-BLT, NSG, and BRG mice and the identification of CD8αα-expressing human cells, that the mouse IL-2Rγ chain is critical for the intestinal human T cell population (197, 202). However, this study does not exclude the possibility that the more efficient intestinal population is due to some form of moderate GvHD, caused by human T cells selected on human HLA in the cotransplanted human thymus that might recognize typical mouse peripheral GvHD target tissues.

Thus, as with lymph node development, it will be important to determine if more robust T (and other) cell development by better HSC maintenance, cytokine availability, and correct graft and tissue MHC interaction will lead to overall increased peripheral nonlymphoid tissue and mucosal lymphoid tissue formation.

## MOVING FORWARD

Using novel genetic engineering technology, research on HHLS mice has taken great strides during the past decade, leading to incremental, stepwise improvements toward the generation of easy-to-handle, economically reasonable, predictive preclinical model systems for human health and disease. It also is clear that every improvement step reveals limitations at subsequent levels that need to be addressed in the future (**Table 7**). In fact, it is unlikely that a unified model for all purposes will be available any time soon.

However, given the progress made, one can hope that combining genetically engineered HHLS mice will lead to model systems that are shaped toward and can be applied to study specific disease. For example, human primary hemato-lymphoid malignancy studies may require better hemato-lymphoid development support, whereas, for instance, HIV research will require long-term engrafted, immuno-competent mice with the respective growth factors and HLA expression required for faithful immune responses; and malaria research may need to rely on human RBC and liver presence.

The breadth of possible uses of HHLS mice is tremendous, given the potential combination of coemerging, complementary technologies such as the generation of disease-specific embryonic stem cells, induced pluripotent stem cells, and their offspring tissue-specific somatic stem cells, including HSCs, with genetic, antibody, or small molecule–mediated in vivo targeting of cells, tissues, and microbes. Thus, within the next decade, HHLS mice research will likely gain further momentum and shift from its technical developmental phase toward direct, highly specific, and efficient preclinical use, as already demonstrated for human hematopoietic development and, partially, for primary hematopoietic disease.

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## Supplementary Material

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## THE BONE-LIVER-THYMUS (BLT) MODEL

BLT mice are generated by transplantation of human-derived $CD34^+$ cells (mostly from fetal liver) into a NOD-*Scid* or NSG recipient mouse also implanted with autologous fetal liver and thymus tissues (61–63, 203). These implanted tissues provide an entirely human microenvironment in which human hematopoietic cells can develop. As a result, high levels of multilineage engraftment are observed in all tissues of BLT mice, including mucosal surfaces such as the gut and the female reproductive tract (62, 92, 197). Importantly, because T cells develop and undergo selection in a human thymus, BLT mice contain a highly diverse, HLA-restricted T cell repertoire able to mount effective adaptive immune responses (204). Because of the efficient repopulation of mucosal surfaces and T cell function, BLT mice become the model of choice for the study of HIV (91, 93).

Limitations to the use of the BLT model are the necessity to have access to fetal tissues and the impossibility of using samples from adult donors with genetic conditions or diseases of interest. Furthermore, positive selection in the thymic organoid occurs exclusively on human cells, and T cells with affinity for mouse MHC are not eliminated (see also **Figure 5**). As a result, BLT mice are more prone to the development of GvHD than other models.

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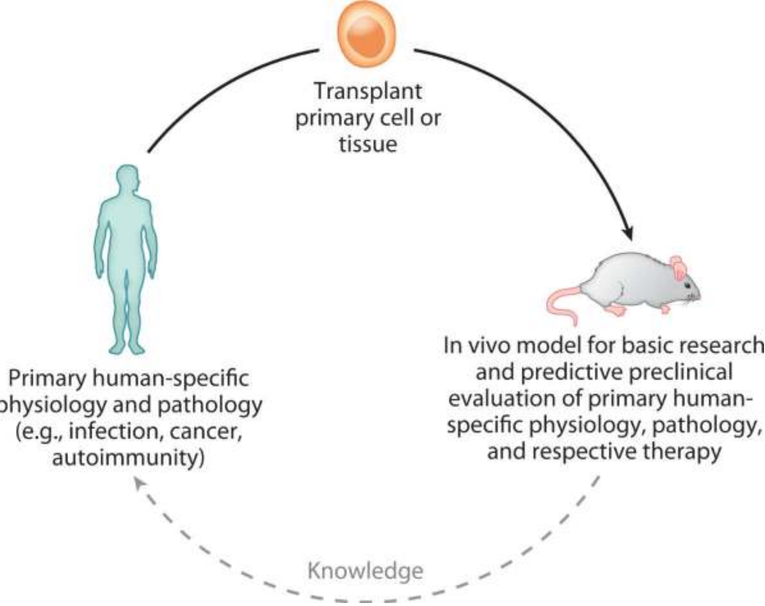


**Figure 1.**

Utility of humanized mouse models for predictive in vivo preclinical testing of physiology, pathology, and therapy. With the generation of predictive humanized mouse model systems, knowledge of primary human-specific physiology and pathology can be accelerated and utilized for medical improvements.

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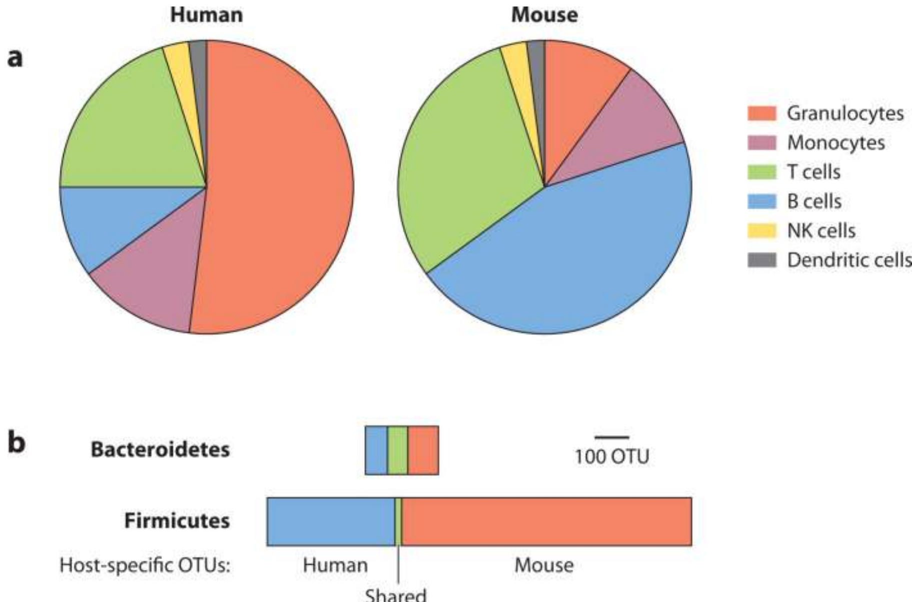


**Figure 2.**

Differences in the white blood cell composition and in the gut microbiota of human and mouse. (*a*) White blood cell differential of human and mouse. Whereas human blood is rich in myeloid cells (granulocytes and monocytes), the composition of mouse blood is dominated by lymphocytes (B and T cells). The functional and evolutionary significance of this difference is currently unknown. (*b*) Bacteroidetes and Firmicutes are the two most abundant phyla present in the gut microbiota of vertebrates. However, significant differences in bacterial species representation exist, depending on the species of the host. Operational taxonomic units (OTUs) shared between human and mouse ( *green*), specific for human (*blue*), and specific for mouse (*red* ) hosts are represented for each bacterial phylum. The host-specific microbiota is required for adequate maturation and function of the immune system. Data modified from Reference 207.

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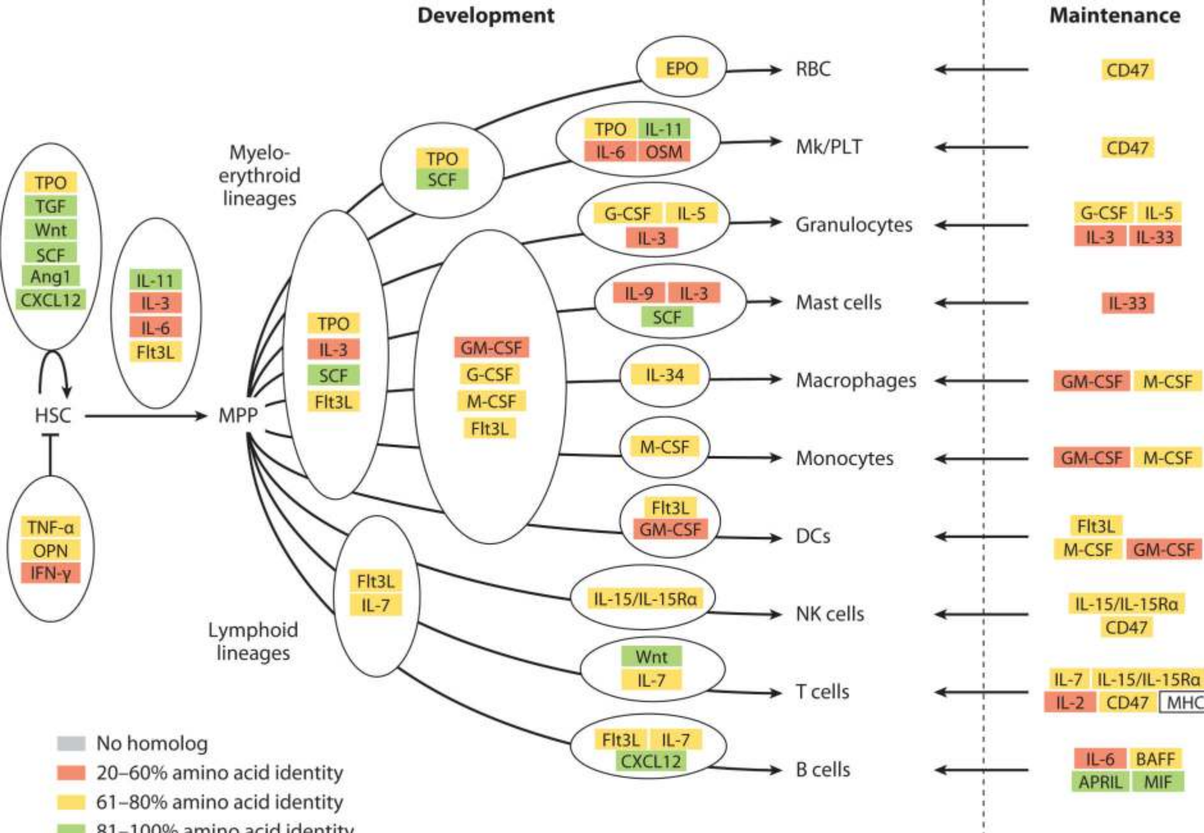


**Figure 3.**

A nonexhaustive list of key factors required for hemato-lymphoid system development and maintenance. Only factors that are produced mostly by nonhematopoietic cells are depicted. The color code indicates the percentage of amino acid identity between the human and mouse proteins (see also **Supplemental Table 1**).

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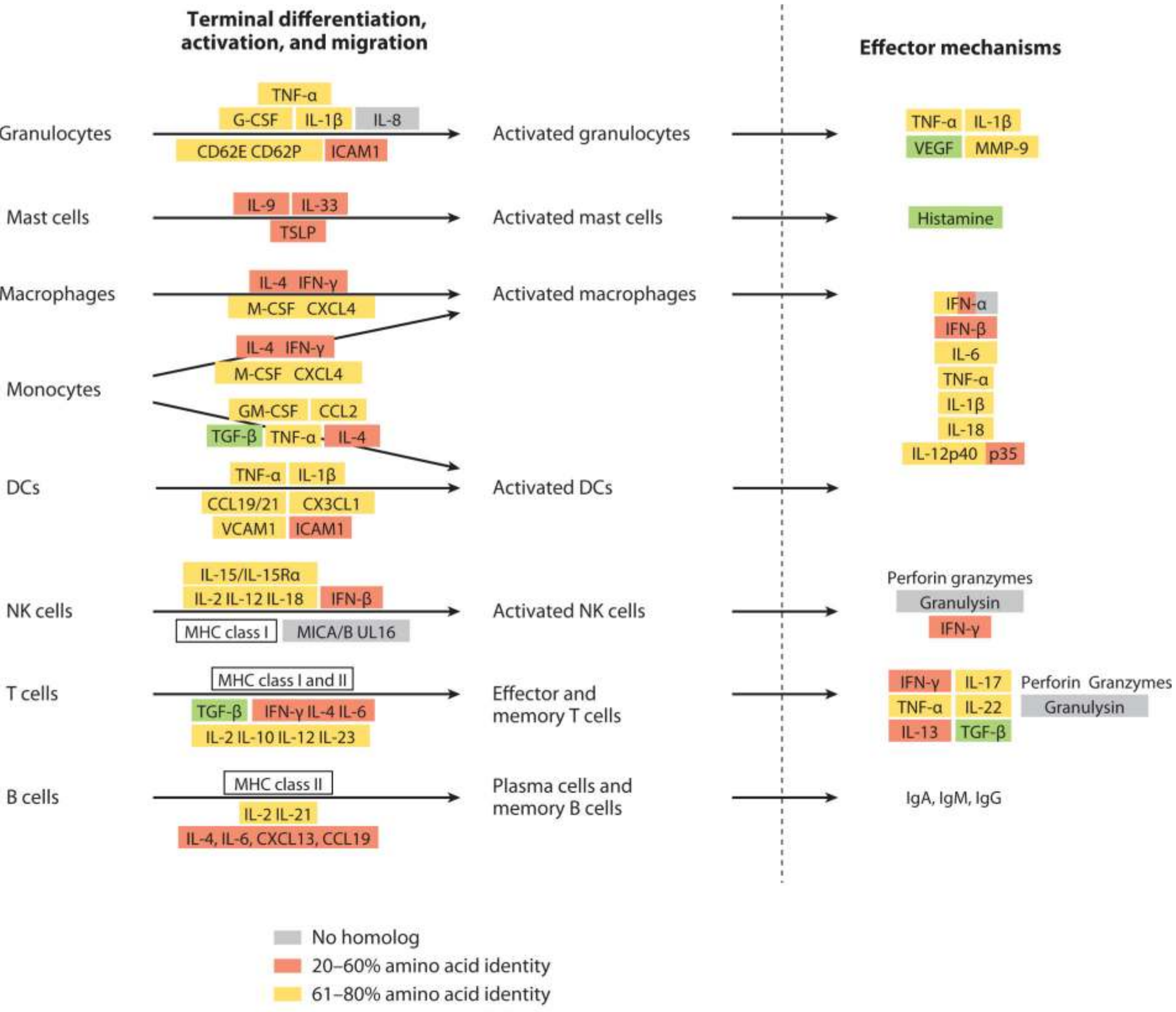


**Figure 4.**
A nonexhaustive list of key factors required for the terminal differentiation, activation, and migration of hematopoietic cell lineages. Also shown are factors that mediate the effector functions of hematopoietic cells and act on nonhematopoietic tissues. The same color code as in **Figure 3** is used (see also **Supplemental Table 1**).

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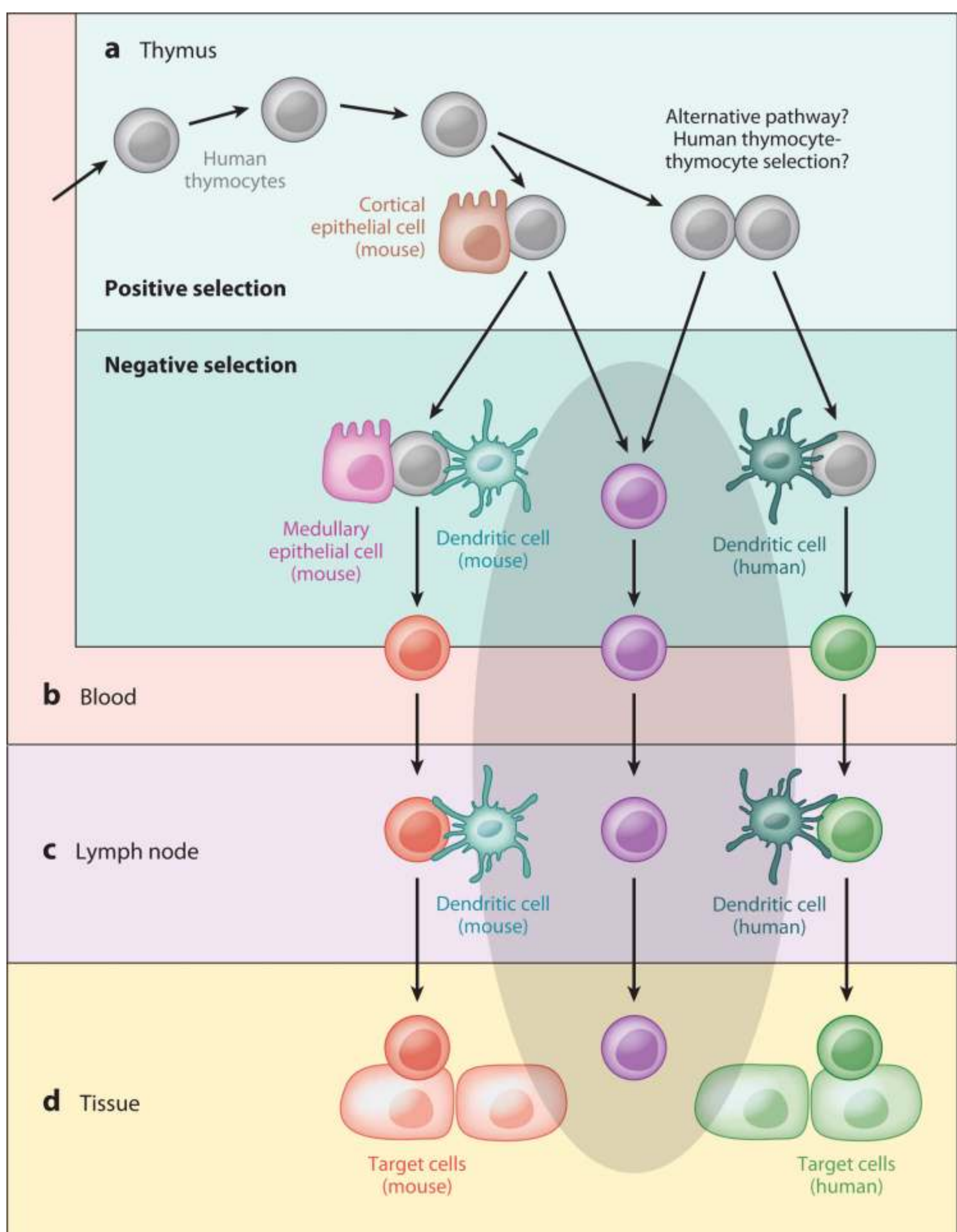


**Figure 5.**

Theoretical representation of human T cell selection and function in a mouse environment. (*a*) A functional T cell repertoire is selected in the thymus. Thymocytes first undergo positive selection (i.e., the survival of thymocytes expressing a T cell receptor that interacts with MHC, and death by neglect of those that do not interact) in the thymus cortex. This process is in physiology mediated by MHC-expressing epithelial cells of mouse origin. However, in HHLS mice, an alternative thymocyte-thymocyte selection pathway might be active for some human thymocytes (see text). Next, positively selected thymocytes undergo negative selection in the thymus medulla. This process consists of the elimination of T cells that bind to MHC/self-peptide complexes with high affinity, and it results in the establishment of central tolerance. Negative selection is mediated by mouse medullary

epithelial cells and by dendritic cells that can be of mouse or human origin. (*b*) Once T cells are released from the thymus, their homeostatic maintenance in the periphery requires continued interaction with MHC molecules and cytokine support. (*c*) Upon antigen recognition in the lymph node, naive T cells are primed by dendritic cells. This process requires interaction between MHC and T cell receptor and coreceptor as well as between costimulatory molecules and cytokines provided by the dendritic cell. Both mouse and human dendritic cells are present and can in theory activate naive T cells, provided that costimulatory molecules and cytokines are sufficiently cross-reactive. (*d* ) Finally, cytotoxic T cells eliminate their target cells after recognition of MHC/peptide complexes through their T cell receptor. Two extremes of human T cell selection and function in HHLS mice that do not express human MHC on nonhematopoietic cells are depicted on the right and left side of the figure, where human T cells consistently interact with either mouse or human MHC. However, in the HHLS xenogeneic setting, some interaction with respective mouse or human MHC likely occurs at every step, leading to inefficient and possibly inappropriate T cell reactivity (symbolically depicted in the gray area in the middle).

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**Table 1**

Comparison of the phenotypic and functional characteristics of human and mouse subpopulations of immune cells

| **Monocytes** | **Human** | **Shared** | **Mouse** |
|---|---|---|---|
| | **Classical (CD14$^+$ CD16$^-$)**<br>IL-10 production<br>**Intermediate (CD14$^+$ CD16$^+$)**<br>Pro-inflammatory cytokines | CD11b, CCR2<br>Gene expression profile<br>Phagocytic capacity | **Inflammatory (Ly-6C$^{hi}$, CX3CR1$^{lo}$)**<br>Proinflammatory cytokines |
| | **Nonclassical (CD14$^{dim}$ CD16$^+$)**<br>Low phagocytic capacity<br>Response to viral infection | CD11b, CX3CR1<br>Gene expression profile<br>Blood vessel patrolling | **Resident (Ly-6C$^{lo}$, CX3CR1$^{hi}$)** |
| **Dendritic cells** | | | |
| **Conventional DCs** | BDCA1 | CD11c, MHC-II<br>Presentation of exogenous antigens on MHC-II<br>Activation of naive CD4$^+$ T cells | CD11b, DCIR2 |
| **Conventional, cross-presenting DCs** | BDCA3 | CD11c, MHC-II, Clec9A<br>Cross-presentation of exogenous antigens on MHC-I<br>Activation of naive CD8$^+$ T cells | CD8α, DEC205 |
| **Plasmacytoid DCs** | BDCA2, BDCA4 | MHC-II<br>Type I IFN secretion | CD11c$^{lo}$, mPDCA1, CD45R/B220 |
| **Inflammatory DCs** | Poorly described in vivo | | Mo-DCs (DC-SIGN)<br>TiP-DCs |
| **Natural killer cells** | | | |
| **Immature** | CD56$^{hi}$ CD16$^-$ | Cytokine secretion | CD11b$^+$ CD27$^+$ |
| **Mature** | CD56$^{lo}$ CD16$^+$ | Cytolytic activity | CD11b$^+$ CD27$^-$ |
| *NKG2D ligands* | *MICA, MICB, ULBP1-6* | | *H-60, Rae-1* |
| *Inhibitory receptors* | *KIRs* | | *Ly-49* |
| *Lytic factors* | *Granulysin* | *Perforin, granzymes* | |

Abbreviations: DC, dendritic cell; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; IFN, interferon; iNOS, inducible NO synthase; KIR, killer cell Ig-like receptor; MHC-I/II, major histocompatibility complex class I/class II; Mo-DC, monocyte-derived DC; TipDC, tumor necrosis factor/iNOS-producing DC.

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## Table 2

Requirements for xenogeneic transplantation

| General requirements for xenotransplantation | Specific requirements for HHLS mice |
|---|---|
| Availability of donor cells or tissue | For long-term experiments:<br>■Human HSCs with capacity to generate and maintain all hemato-lymphoid cells from fetal liver, cord blood, bone marrow, peripheral blood (direct or preexpanded) hematopoietic predifferentiated ESC or iPSC<br>■Human fetal tissues (liver, thymus)<br>For short-term experiments:<br>■Human hematopoietic effector cells from peripheral blood or any other tissue |
| Bidirectional host-donor tolerance | Innate immunity:<br>■ NK cell and phagocytic system deficiency or tolerance of mouse to human<br>■newly generated NK cell and phagocytic system tolerance of human to mouse<br>Adaptive immunity:<br>■ T and B cell deficiency of or tolerance of mouse to human<br>■newly generated T and B cell tolerance of human to mouse |
| Physical space and/or biological need for cell or tissue replacement | Reduction or elimination of mouse hemato-lymphoid tissue by<br>■ X-ray or $\gamma$-irradiation<br>■pharmacologic means<br>■ genetic engineering<br>Introduction of human cells in synchrony with hemato-lymphoid tissue expansion in mice<br>■upon mouse cell-depletion procedure<br>■intrauterine or newborn transplantation |
| Orthotopic localization of donor cells or tissue | Appropriate cross-reactivity of migration and tissue-retention factors:<br>■ homing of human HSCs to mouse bone marrow<br>■migration of human T cell precursors to mouse thymus<br>■migration of human effector cells to blood, secondary lymphoid organs, and tissues |
| Differentiation and/or maintenance of donor cells or tissues | Provision and cross-reactivity of differentiation and maintenance factors that are not provided by human hemato-lymphoid cells themselves (see **Figures 3** and **4** and **Supplemental Table 1)** |
| Functionality of donor cells or tissues | Effector function of human hemato-lymphoid cells in mouse environment (see **Figure 4** and **Supplemental Table 1**) |

Abbreviations: ESC, embryonic stem cell; HSC, hematopoietic stem cell; iPSC, induced pluripotent stem cell; NK, natural killer.

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**Table 3**

The history of HHLS mice

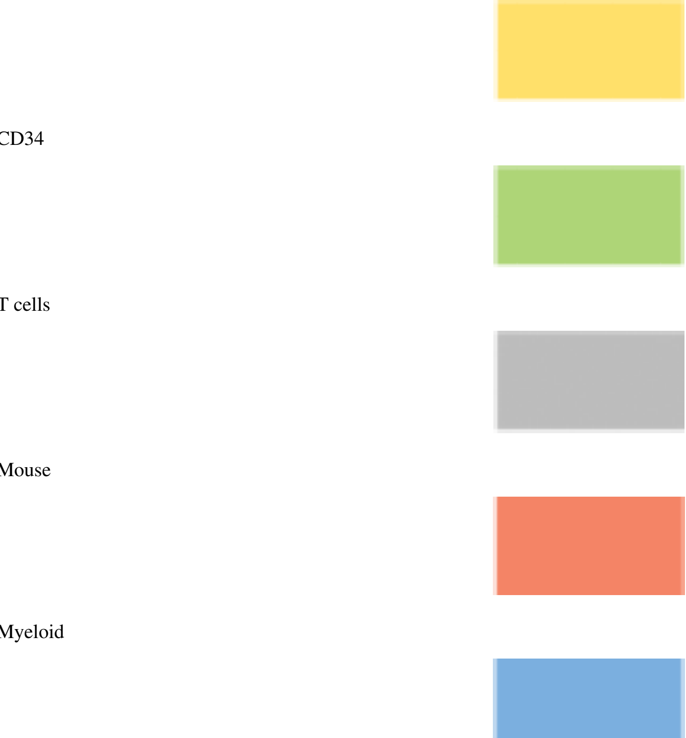
| | Strain | Acronym | T/B cell deficiency | NK cell deficiency | Phagocytosis tolerance | Features | Reference(s) |
|---|---|---|---|---|---|---|---|
| 1988 | CB17-*Scid* | Hu-PBL-SCID | *Scid* | – | – | Transplantation of PBL | 22 |
| 1988 | CB17-*Scid* | SCID-hu | *Scid* | – | – | Transplantation of fetal tissues | 23 |
| 1992 | CB17-*Scid* | Hu-SRC-SCID | *Scid* | – | – | Transplantation of HSCs | 55 |
| 1995 | NOD-*Scid* | NOD-*Scid* | *Scid* | Reduced function | NOD *Sirpa* | Increased phagocytic tolerance | 57, 205, 206 |
| 2002 | NOD/Shi-*scid Il2rg–/–* | NOG | *Scid* | Truncated IL-2Rγ | NOD *Sirpa* | No NK cells and phagocytic tolerance | 31 |
| 2004 | BALB/c-Rag2–/– *Il2rg–/–* | BRG | *Rag2–/–* | *Il2rg–/–* | – | No NK cells transplantation in newborn | 28 |
| 2005 | NOD/LtSz-*scid Il2rg–/–* | NSG | *Scid* | *Il2rg–/–* | NOD *Sirpa* | No NK cells and phagocytic tolerance | 32, 174 |
| 2006 | NOD-*Scid* | BLT | *Scid* | – | NOD *Sirpa* | T cell development in human thymic tissue | 61, 62 |
| 2011 | *hSIRPA*$^{tg}$ *Rag2–/– Il2rg–/–* | SRG | *Rag2–/–* | *Il2rg–/–* | Human *SIRPA* | Similar to NOG/NSG | 35 |

Abbreviations: BLT, bone-liver-thymus; HSC, hematopoietic stem cell; NK, natural killer; NOD, nonobese diabetic; PBL, peripheral blood leukocytes; *Scid,* severe combined immunodeficiency; SRC, *Scid*-repopulating cell; – indicates no deficiency or no tolerance.

## Table 4

Characteristics of established models of HHLS mice[a], [b], [c]

| Strain | Bone marrow | Blood | Human HSC homeostasis | Thymus stroma | Thymus tumors | LN | Functional human adaptive and innate immunity |
|---|---|---|---|---|---|---|---|
| Human | | | + | Human | – | + | + |
| NOD/SCID | | | – | Mouse | + | – | – |
| BRG | | | – | Mouse | – | – (+) | – (+) |
| NOG/NSG/SRG | | | – | Mouse | – | – (+) | – (+) |
| BLT | | | – | Human organoid | ? | + (–) | + (–) |



CD34

T cells

Mouse

Myeloid

B cells

[a]The data on erythroid lineage for each model are incomplete in the literature and therefore are not shown.

[b]Symbols indicate the following: – (+) absent or defective compared with human; + (-) consistently present and functional, but at lower levels than in humans; ? status unknown.

[c]Data from References 28, 31, 32, 35, 59, 60.



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**Table 5**

Improvement of HHLS mice by exogenous cytokine administration

| Lineages | Cytokine(s) | Strain | Delivery | Targeted cell type(s) | Reference(s) |
|---|---|---|---|---|---|
| **Erythropoiesis** | IL-3+EPO | NSG | Hydrodynamic DNA or recombinant | Presence of human erythrocytes | 140, 141 |
| **Myeloid/DC** | Flt3l+GM-CSF+IL-4 | NSG | Hydrodynamic DNA | Increased number of DCs | 140 |
| | M-CSF | NSG | Hydrodynamic DNA | Increased number of monocytes/ macrophages | 140 |
| **Lymphoid** | IL-7 | NSG | Fc-fusion protein | Enhanced thymopoiesis in mice engrafted as adults | 174 |
| | IL-7 | BRG | Recombinant | Enhanced thymopoiesis and pDC numbers | 179 |
| | IL-7 | BRG | Lentiviral in vivo (high concentrations of IL-7) | Increased peripheral T cell numbers | 180 |
| | IL-15/IL-15Rα | BRG | Recombinant | Increased number and maturation of NK cells<br>Increased T cell number and humoral response | 172, 181 |
| | IL-15 | BRG | Recombinant or adenovector | Improved development and maturation of NK cells | 173 |
| | IL-15+/–Flt3l | NSG | Hydrodynamic DNA | Increased number of NK cells | 140 |

Abbreviations: BRG, BALB/c-Rag2$^{-/-}$ Il2rg$^{-/-}$; DC, dendritic cell; EPO, erythropoietin; GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; M-CSF, macrophage colony-stimulating factor; NK, natural killer; NSG, *NOD/LtSz-scid Il2rg*$^{-/-}$.

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## Table 6

Improvement of HHLS mice by genetic expression of cytokines and HLA molecules

| | Protein(s) expressed | Strain | Expression method | Effect | Reference(s) |
|---|---|---|---|---|---|
| **HSC cytokines** | TPO | BRG | Knockin replacement | Better HSC maintenance and myeloid differentiation | 126 |
| | Membrane SCF | NSG | CMV transgenic | High engraftment without irradiation, development of human mast cells | 134, 135 |
| **Myeloid cytokines** | IL-3/GM-CSF | BRG | Knockin replacement | Replacement of alveolar macrophages | 127 |
| | M-CSF | BRG | Knockin replacement | Increased numbers of monocyte/ macrophage | 128 |
| | SCF/GM-CSF/IL-3 | NSG | CMV transgenic | Terminal myeloid differentiation<br>Increased regulatory T cells<br>Loss of functional HSCs | 148 |
| **MHC-I** | HLA-A2 | NSG | Transgenic expression | HLA-restricted response to EBV and Dengue<br>Protection against EBV-induced tumors | 94, 102, 176 |
| **MHC-II** | HLA-DR4 | NRG | Transgenic expression | Increased number and function of T cells<br>Tetanus toxoid-specific IgG | 175 |
| | HLA-DR4 | NOG I-A-/- | Transgenic expression | Slightly increased $CD4^{+}$ T cell numbers<br>Ovalbumin-specific IgG | 177 |

Abbreviations: BRG, BALB/c-Rag2$^{-/-}$ *Il2rg*$^{-/-}$; CMV; cytomegalovirus; DC, dendritic cell; EBV, Epstein-Barr virus; GM-CSF, granulocyte/ macrophage colony-stimulating factor; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; IL, interleukin; M-CSF, macrophage colony-stimulating factor; MHC-I/II, major histocompatibility complex class I/class II; NK, natural killer; NOG, NOD/Shi-*scid Il2rg*$^{-/-}$;NRG, *NOD-Rag-I-Il2rg-I-;* NSG, NOD/LtSz-*scid Il2rg*$^{-/-}$; SCF, stem cell factor; TPO, thrombopoietin.

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## Table 7

Limitations of established HHLS models and possible solutions

| Limitation | Main reason for limitation | Possible solutions |
|---|---|---|
| Insufficient standardized graft to generate appropriate numbers of mice for well-controlled experimentation | Low HSC numbers in available donor samples | Primary HSC expansion<br>HSC generation from ESCs or iPSCs |
| Inadequate maintenance of human engraftment | Lack of human HSCs maintenance | Construction of faithful human HSC niche by<br>■genetic modification of mouse niche<br>■implementation of human niche cells in mouse bone marrow<br>■implementation of human niche by transplantation of human ossicles |
| Low numbers of human RBCs and platelets | Low RBC and platelet production and survival | Provision of human growth factors, protection from mouse innate immune response |
| Inefficient human innate and adaptive immune responses | Suboptimal immune cell differentiation, selection, maintenance, activation, and migration | Faithful expression of human growth factors and adhesion molecules<br>Deletion of nonlethal mouse growth factors to create respective space for human cells<br>Protection from mouse innate immune response<br>Deletion of mouse and substitution with human MHC class I and II (preferentially haplotypes) |
| Inefficient support and reaction to human-specific infectious diseases | HIV: human immune system<br>Malaria: human RBCs, human immune system, human liver<br>HBV/HCV: human immune system and liver | Same as above and implementation of human liver |
| Inefficient models for human autoimmune diseases | Inefficient immune response<br>Lack of MHC-matched immune system and target tissue | Human hematopoietic-directed autoimmunity (e.g., aplastic anemia, ITP, AIHA): provision of functional immunity<br>Human nonhematopoietic-directed autoimmunity (e.g., IDDM): provision of functional human immunity and implementation of MHC-matched target tissue |
| Inefficient human primary neoplasia and primary neoplasia and immune cell interaction | Hemato-lymphoid system neoplasia: lack of disease-initiating cell maintenance (particularly in clinically less-aggressive diseases)<br>Solid organ neoplasia: lack of immune system and primary tumor-MHC match | Same as for HSC and T/B cell maintenance<br>Human solid organ neoplasia: provision of functional human immunity and implementation of MHC-matched target tissue |

Abbreviations: AIHA, autoimmune hemolytic anemia; ESC, embryonic stem cell; HBV, hepatitis B virus; HCV, hepatitis C virus; HSC, hematopoietic stem cell; IDDM, insulin-dependent diabetes mellitus; iPSC, induced pluripotent stem cell; ITP, immune thrombocytopenia; MHC, major histocompatibility complex; RBC, red blood cell.