Humanized hemato-lymphoid system mice

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ABSTRACT

Over the last decades, incrementally improved xenograft mouse models, supporting the engraftment and development of a human hemato-lymphoid system, have been developed and now represent an important research tool in the field. The most significant contributions made by means of humanized mice are the identification of normal and leukemic hematopoietic stem cells, the characterization of the human hematopoietic hierarchy, and their use as preclinical therapy models for malignant hematopoietic disorders. Successful xenotransplantation depends on three major factors: tolerance by the mouse host, correct spatial location, and appropriately cross-reactive support and interaction factors such as cytokines and major histocompatibility complex molecules. Each of these can be modified. Experimental approaches include the genetic modification of mice to faithfully express human support factors as non-cross-reactive cytokines, to create free niche space, the co-transplantation of human mesenchymal stem cells, the implantation of humanized ossicles or other stroma, and the implantation of human thymic tissue. Besides the source of hematopoietic cells, the conditioning regimen and the route of transplantation also significantly affect human hematopoietic development *in vivo*. We review here the achievements, most recent developments, and the remaining challenges in the generation of pre-clinically-predictive systems for human hematology and immunology, closely resembling the human situation in a xenogeneic mouse environment.

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Introduction

Over the last decades humanized mouse models have become an important tool in human hematopoiesis research. The information extracted from experimentation on primary human cells that have been maintained or generated *in vivo* in a mouse host can be used to understand the physiology and pathophysiology of human hematopoiesis (Figure 1). While the characterization of hematopoietic stem cells (HSC) by multicolor flow cytometry and in depth genetic analysis improves steadily, the xenograft model remains the only broadly accessible assay to functionally define HSC and their malignant counterparts, leukemic stem cells (LSC). The significant differences in cell surface marker expression between mouse and human stem and progenitor cells (HSPC) highlights the importance of assessing the development of human hematopoietic cells *in vivo* and shows that both murine and humanized models are complementary. It is, therefore, essential to assess the advantages and limitations of each experimental system in the light of the scientific and clinical question being addressed.

Although humanized mice have contributed extensively to the characterization of the physiology of human hematopoiesis, one of the biggest contributions of the model lies in the understanding of malignant hematopoiesis and the leukemic hierarchy. Today, primary human leukemia research relies heavily on the leukemia

xenograft model because of several key advantages over murine models. Humanized mouse models, unlike genetically modified mouse models of leukemia development, can reflect the disease heterogeneity observed in patients. Disease heterogeneity can be assessed at the clonal level and has led to the identification of the sub-clonal architecture of leukemia. Humanized mouse models are also used extensively as preclinical models to test novel therapeutic approaches. This allows assessment of therapeutic response in a heterogeneous disease such as leukemia.

However, several limitations remain; the composition of the hematopoietic system does not fully recapitulate human hematopoiesis, and the long-term maintenance of human cells is limited. In addition, the functionality of human hematopoietic cells (both healthy and diseased) may be altered in a mouse environment, possibly because of the lack of cross-reactivity of specific factors between the mouse host and the human graft. Finally, less aggressive HSC/HSPC neoplasms do not develop efficiently in mouse xenograft models.

This review focuses on the most recent developments in humanized mouse models for healthy hematopoiesis and primary hematopoietic malignancies. We highlight the differences and advantages of next-generation recipient mouse strains over previous models and their potential application in future research.

Key requirements for successful xenotransplantation models

Humanized mice are generated by transplantation of human hematopoietic cells into recipient mice.[1,2] An ideal model would be a humanized mouse in which the human graft fully replaces the endogenous mouse hematopoietic and immune systems, in both space and function. To achieve, this goal, three basic requirements need to be met (Figure 2). First, the recipient strain of mouse needs to be deficient in both its adaptive and innate immune compartments to prevent the xeno-rejection of the human graft by the endogenous mouse immune system. Second, a niche

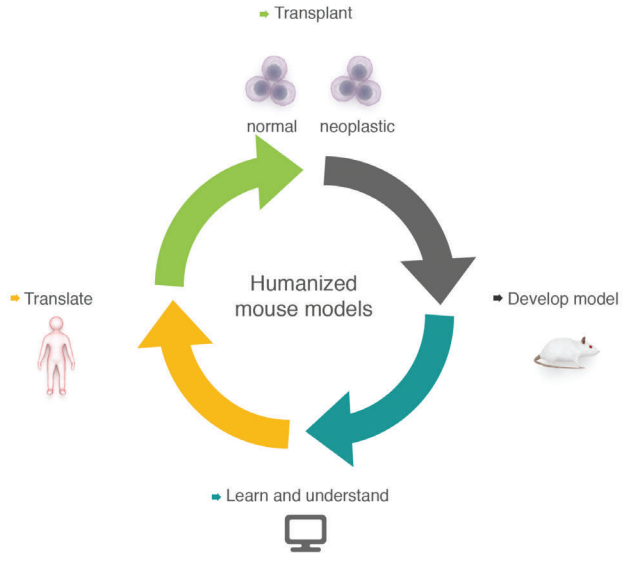

Figure 1. The principle of humanized mouse models. Normal (healthy) or neoplastic hematopoietic cells are transplanted into immuno-compromised mice to develop humanized mouse models. The models help to learn and understand the physiology and pathophysiology of human hematopoiesis. The knowledge gained with these models can then be translated to humans.

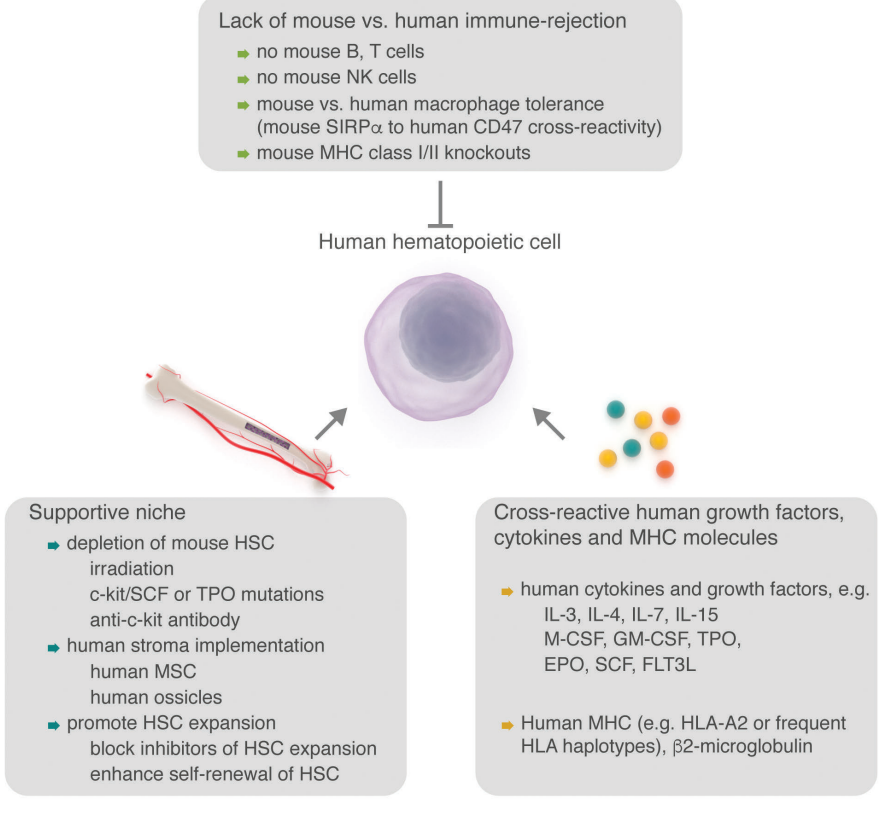

Figure 2. Prerequisites for successful xenotransplantion of human hematopoiesis. The (mouse) host must be tolerant for the transplanted human hematopoietic cells and provide a supportive niche, including cross-reactive growth factors, cytokines and MHC molecules. Novel models carry multiple genetic modifications in the mouse host to meet these three criteria and support successful human hematopoietic engraftment.

Table 1. History of the development of humanized mouse models.

Year	Strain	Acronym	Characteristics	References
1988	Nude-beige-xid	bg/nu/xid	T and NK cell deficiency	3
1988-1992	CB17-*Scid*	SCID	T and B cell deficiency	4, 5, 6
1995-1996	NOD-*Scid*		T and B cell deficiency Phagocytic tolerance	7, 8, 9
2002-2005	NOD-*Scid Il2rγ*$^{-/-}$	NOG/NSG	T, B and NK cell deficiency Phagocytic tolerance	16, 17, 18
2004	BALB/c-*Rag2*$^{-/-}$*Il2rγ*$^{-/-}$	BRG	T, B and NK cell deficiency	13
2011	Tg(hSIRPA)*Rag2*$^{-/-}$*Il2rγ*$^{-/-}$	SRG	T, B and NK cell deficiency Phagocytic tolerance	22

needs to be generated to accommodate the human cells. Third, the human cells need to obtain supportive cross-reactive signals from the created niche.

Mouse immune-deficiency

The development of mouse strains that support human hematopoiesis *in vivo* started in the late 1980s with *bg/nu/xid* (*beige-nude-xid*) mice, which are athymic and have a reduced number of natural killer (NK) and so-called lymphokine activated killer cells (Table 1).[3] This was followed by the generation of CB17-SCID mice, which are deficient in mouse T and B cell development.[4-6] Today, immunodeficient mice lacking T lymphocytes, B lymphocytes and NK cells are used as recipients to prevent the immune rejection of the human graft. Defective V(D)J recombination results in the absence of mature B and T cells and is found in mice harboring the severe combined immunodeficiency (SCID) mutation in the gene encoding the DNA-PKcs enzyme or in mice deficient for either of the two recombination activating genes (*RAG1* or *RAG2*).[5-14] Deficiency of NK cells is achieved by disrupting the signaling downstream of interleukin (IL)-15, a critical cytokine required for NK-cell development and maturation. The common gamma chain of cytokine receptors (IL-2Rγ, CD122) is shared by multiple members of the IL-2 family of cytokines, including IL-15.[15] Hence, mutation or deficiency in the *Il2rγ* gene results in the complete absence of NK cells in the mouse host.[12,13,16-18]

Macrophage tolerance

The development of mice with phagocytic tolerance against human cells was a major improvement and was accomplished by the generation of SCID mice on a non-obese diabetic (NOD)-background.[7-9] The CD47-SIRPα axis is a signaling pathway by which endogenous CD47-expressing cells, i.e. most, if not all, healthy cells of the body, prevent their phagocytosis by providing an inhibitory signal (the so-called "don't eat me" signal) to macrophages.[19] Human CD47 and mouse SIRPα do not cross-react in most mouse strains and consequently mouse macrophages engulf the transplanted human cells.[20] The *Sirpa* gene is highly polymorphic between different mouse strains. In particular, the *Sirpa* allele of NOD mice encodes for a protein with a 20 amino acid difference and a distinct glycosylation pattern in the extracellular domain of SIRPα compared to the C57Bl/6 allele. In fact, the NOD allele of *Sirpa* is very similar to the human *SIRPA* allele and NOD-SIRPα binds to human CD47, whereas C57Bl/6-SIRPα does not.[20] As a consequence, immunodeficient mice on the NOD background are significantly more supportive of human hematopoietic development than any other mouse strain in which SIRPα does not cross-react with human CD47.[20] A method used to induce mouse-to-human phagocytic tolerance consists in introducing the human *SIRPA* gene into the mouse genome of "non-NOD" mice, either by BAC-transgenesis or by knock-in humanization of the mouse gene.[21,22] Alternatively, murine CD47 expressed in transplanted human cells can induce tolerance.[23,24]

NOD/ShiLtJ-*Prkdc*^{scid} (NOD-SCID), NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1wjl}/SzJ (NSG), and NOD.Cg-*Prkdcscid Il2rgtm1Sug*/Jic (NOG) mice, the three most commonly used recipient mouse strains for humanized mouse models, combine murine T-, B-lymphocyte and NK-cell deficiency (NOD-SCID mice can be depleted of NK cells by anti-CD122 antibody treatment) with macrophage tolerance towards human cells (Table 1)[7-9,16-18] Furthermore the lack of the IL-2Rγ limits the spontaneous development of murine thymoma, a phenomenon commonly observed in NOD-SCID mice.[25]

Niche space

In addition to immunodeficiency, the ablation of mouse cells can create open niches in which transplanted human cells can home and develop. This was recently demonstrated in mice harboring mutations in the gene encoding c-kit (CD117), which is important for HSC maintenance and function.[26,27] In c-kit mutant mice, mouse HSC are reduced in number and function (see below). The reduction in mouse HSC confers a competitive advantage to human HSC homing, maintenance and differentiation, and eliminates the need for irradiation pre-conditioning of the host mice prior to transplantation. A similar observation was made in mice with knock-in replacement of mouse cytokine-encoding genes by the human ortholog.[28-30] In particular, the knock-in humanization of *THPO*, encoding thrombopoietin (TPO) and *CSF2* [encoding granulocyte-macrophage colony-stimulating factor (GM-CSF)] results in deficiency of the respective mouse cytokines and functional impairment of mouse HSC and alveolar macrophages, respectively.

Cytokine support for human hematopoietic development and immune function

While human HSPC development and differentiation are well recapitulated in NOD-SCID and NSG/NOG mice, several developmental and functional defects remain.[31-33] For example, the differentiation of human HSC into func-

tional myeloid cells and NK cells is limited,[34,35] B cells do not undergo sufficient maturation to become memory and antibody-producing cells,[36,37] and, unless a fetal thymus is co-transplanted, the selection and maturation of T cells in adult animals is disturbed due to the lack of thymic support, and the ratio of B- to T-lymphocytes is strongly biased towards B-cell differentiation and does not mimic the human system.[38,39] It has been hypothesized that, despite the issue of MHC restriction, the limitations are due to a lack of cross-reactivity of mouse cytokines, and possibly chemotactic factors.[1,32] To circumvent some of these limitations, several methods have been developed to deliver human cytokines *in vivo* (Figure 2). Exogenous recombinant cytokines were first injected into SCID mice transplanted with human bone marrow in the early 1990s.[4] However although this approach is simple, it is expensive and not suited for long-term experiments. The hydrodynamic injection of cytokine-encoding plasmids, which results in transient, systemic expression of candidate proteins, is more convenient and results in significant support of human NK cells and monocytes when human IL-15 or macrophage colony-stimulating factor (M-CSF) is expressed, respectively.[40] Similarly, the transgenic expression of cytokine-encoding genes under the control of a strong constitutive promoter provides support for human hematopoietic cell development.[41-44] However, these approaches all result in the expression of systemic supra-physiological concentrations of human cytokines. In the case of transgenic mice expressing human IL-3, GM-CSF and stem cell factor (SCF) this induces the mobilization of HSPC and limits long-term engraftment.[44] This example highlights the importance of achieving physiological expression of human cytokines and other factors, which can be achieved either by BAC-transgenesis or by knock-in replacement of the mouse gene.[29] We have reported individual gene knock-in humanization for four cytokines (Table 2): TPO, IL-3/GM-CSF (the two contiguous genes were replaced simultaneously) and M-CSF, conferring sup-

Table 2. Comparison of next-generation humanized mouse models in their support of normal hematopoiesis.

Strain	Background	Genetic modification	Conditioning regimen	Cells injected	Compared to	Key features	Functional assays	Reference(s)
MISTRG	BRG	Human knockin: M-CSF, IL-3, GM-CSF, TPO BAC transgene: human SIRPα	Sublethal irradiation or no irradiation	FL, intrahepatic	BRG NSG	- Median human BM CD45$^+$ (85%) similar to NSG (80%) at 10-12 weeks - Increased human myeloid (CD33$^+$) and monocytic (CD14$^+$/CD16$^+$) differentiation in the PB and BM - Supports human macrophage and NK-cell development	- Monocytes (cytokines, phagocytosis) - Human response to Listeria, Influenza infection - Human NK-cell-mediated lysis - Human tumor-associated macrophages	46
TPO	BRG	Human TPO knockin	Sublethal irradiation	FL and CB	BRG	- Median human BM CD45$^+$ (80%) significantly higher compared to BRG (45%) at 3-4 months - Increased myeloid (CD33$^+$CD66$^+$) differentiation - Increase in human CD34$^+$CD38$^-$, increase in human CD34$^+$CD38$^-$CD90$^+$CD45RA$^-$	- CD34$^+$ cell secondary transplantation	28
BRgWv (also some neonates)	BALBc	Mouse c-kit mutation Kit Wv	No irradiation	CB	NSG (irradiated)	- Median human BM CD45$^+$ in non-irradiated BRgWV: 80%, similar to irradiated NSG at 17-24 weeks - Increased myelo-monocytic differentiation - Increased neutrophils and neutrophil precursors - Significant increase in human CD34$^+$CD38$^-$ and CD90$^+$CD45RA$^-$ (frequency equivalent to human BM) - Similar results in neonates and adults - Supports human lymphopoiesis, block in B-cell differentiation	- Secondary transplantation	26
NBSGW	NSG	Mouse c-kit mutations Kit W41	+/- irradiation	CB	NSG irradiated NSG non-irradiated	- Median human PB CD45$^+$ similar in non-irradiated NBSGW and irradiated NSG at 4 (30-40%), 8 (50-60%), and 12 (50-60%) weeks - Median human BM CD45$^+$ significantly higher (97%) in non-irradiated NBSGW compared to non-irradiated NSG (30%) at 12 weeks - Signifcantly more human CD34$^+$, CD33$^+$, CD45 GlyA$^+$	- Secondary transplantation	27

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port for human HSC, alveolar macrophages and monocytes/macrophages, respectively.[29,30,45]

Finally, hematopoiesis is a complex process in which successive developmental steps are regulated by multiple cytokines.[1,31] To optimize multi-lineage human hematopoiesis, it is, therefore, necessary to provide multiple cytokines that support the successive differentiation steps, from the HSC to terminally differentiated mature cells, and their subsequent maintenance and function.[40,46] This principle is best illustrated in the MISTRG mouse, in which four cytokines (M-CSF, IL-3, GM-CSF and TPO) are genetically "humanized" in one mouse strain.[46] As a consequence, MISTRG mice strongly support the development of human myelo-monocytic cells and show a strong human innate immune response in response to viral and bacterial infections. An additional and indirect consequence of improved myelopoiesis in MISTRG recipients is the expression of the IL15/IL15Rα complex on human monocytes, which in turn supports the development and function of human NK cells.[46] The MISTRG model thus demonstrates the necessity of combining multiple factors that synergize, directly and indirectly, to support the development of a functional human immune system.

Generation of a human microenvironment in humanized mice

To create a microenvironment that is more similar to the human bone marrow (BM) and thymic niche, additional human tissue can be co-transplanted along with the human hematopoietic cells.

Bone marrow microenvironment

Human hematopoiesis is regulated by a specialized microenvironment, the BM niche. Besides hematopoietic cells, the BM niche is composed of adipocytes, osteoblasts/osteoclasts, megakaryocytes, endothelial cells, macrophages and Schwann cells, as well as specialized stromal cells including Nestin-positive mesenchymal stromal cells (MSC), CXCL12-abundant reticular (CAR) cells and leptin receptor-positive cells.[47] The BM niche cells provide survival and maintenance signals to HSPC and, in hematologic cancers, to leukemia-initiating cells and malignant plasma cells, for example.

Friedenstein and co-workers showed that MSC can be separated from other cells in the BM by their tendency to adhere to tissue culture plastic.[48] Furthermore they found that MSC in culture can differentiate into several lineages such as osteoblasts, chondroblasts and adipocytes.[49-51] As

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Strain	Background	Genetic modification	Conditioning regimen	Cells injected	Compared to	Key features	Functional assays	Reference(s)
hSCFTgNSG	NSG	Human transgene: Membrane-bound SCF	Sublethal irradiation	CB	NSG	- Median human BM CD45$^+$ significantly higher (97%) compared to NSG (63%) at 8-35 weeks - Increased myeloid (CD33$^+$) differentiation - Supports human mast cell development	None	43
hu-mSCF, neonates (also some adults)	NSG	Human transgene: Membrane-bound SCF	+/- irradiation	CB	NSG irradiated NSG non-irradiated	- Non-irradiated: trend towards higher human BM CD45$^+$ (57%) compared to NSG (37%) at 12 weeks - Irradiated: trend towards higher human BM CD45$^+$ (72%) compared to NSG (51%) at 12 weeks - Increased support of CD71$^+$/CD235a$^+$ development	- Rejection of human skin graft	41
IL-3/GM-CSF	BRG	Human IL3, GM-CSF knockin	Sublethal irradiation	FL and CB	BRG	- Deficiency in mouse lung alveolar macrophages - Development and function of human lung alveolar macrophages	- Rescue of pulmonary alveolar proteinosis Lung influenza infection	30
IL-3/GM-CSF	NOG	Human transgene: IL3, GM-CSF	No irradiation	CB	NOG	- BM: 3-4x more human CD34$^+$CD38$^-$ - Increased myeloid (CD33$^+$) differentiation - Increased support of dendritic cell, basophil and mast cell differentiation.	- Inducible cutaneous anaphylaxis	42
CSF1 neonates	BRG	Human CSF1 knockin	Sublethal irradiation	FL	BRG	- Increased myelo-monocytic differentiation	- Human cytokine response to LPS - Human phagocytosis - Enhanced chemotaxis	45
hSIRPαTg (neonates)	BRG	BAC transgene: human SIRPα	Sublethal irradiation	FL	BRG NSG	- Median human BM CD45$^+$ significantly higher in hSIRPαTg (60%) compared to BRG (35%) at 12-14 weeks - Median human BM CD45$^+$ similar in hSIRPαTg and NSG - Increased human CD34$^+$, CD34$^+$CD38$^-$ engraftment - Similar human CD45$^+$ engraftment - Similar human CD34$^+$, CD34$^+$CD38$^-$ engraftment	- Improved humoral immune response to ovalbumin	22

FL: fetal liver; CB: umbilical cord blood; BM: bone marrow; PB: peripheral blood; GMP: granulocyte macrophage progenitor; CMP: common myeloid progenitor; LPS: lipopolysaccharide.

MSC are able to differentiate into chondrocytes through a process that is called endochondral ossification, they can be used as a source to form a BM microenvironment *in vivo* (Figure 3).[52] Additionally, *in vitro* maturation (chondrogenic differentiation) of human BM-derived MSC results in accelerated formation of larger, hematopoiesis-supporting bone tissue.[53] Furthermore, several studies have shown that upon subcutaneous implantation of human MSC into immune-deficient mice a supportive BM cavity can be formed through a vascularized cartilage intermediate which is replaced by hematopoietic tissue and bone and can attract and support murine hematopoiesis.[54-57] Reinisch *et al.* showed that only BM-derived MSC are able to form a BM cavity through a vascularized cartilage intermediate.[58] Holzapfel *et al.* developed an engineered humanized bone construct by seeding MSC on a tubular medical-grade polycaprolactone scaffold followed by culture in a rotating bioreactor before implantation into NSG mice.[59] By inducing differentiation of MSC *in vitro* they were able to recapitulate morphological features and biological functions of the human HSC niche.

Several studies now suggest that the BM niche can actively participate in the initiation of leukemia and consequently novel therapeutic approaches aim at targeting not only the leukemia clone, but also the supportive microenvironment.[60,61] Indeed, Chen *et al.* developed an ectopic human BM niche that supports human normal and leukemic engraftment (Table 3).[62] In this model, knockdown of hypoxia-inducible factor 1α in human MSC leads to a significant reduction of human leukemic engraftment and demonstrates that the human BM niche can be genetically manipulated. In a recent myelodysplastic syndrome (MDS) model, patient-derived MSC were co-injected along with CD34$^+$ cells into NSG mice and NSG mice expressing human SCF, GM-CSF and IL-3 (NSG-SGM3).[63] Interestingly, myelodysplasia was only observed in mice co-injected with human MSC in this study. Moreover, Groen *et al.* demonstrated the engraftment of primary human multiple myeloma cells into a scaffold-based ectopic BM niche.[64] By using luciferase gene marking of multiple myeloma cells they were able to visualize myeloma growth and therapeutic response. From these interesting observations it can be inferred that stromal cells, through their interaction with diseased hematopoietic cells, contribute actively to disease development and that a functional human BM niche may be necessary to fully recapitulate human disease *in vivo*.

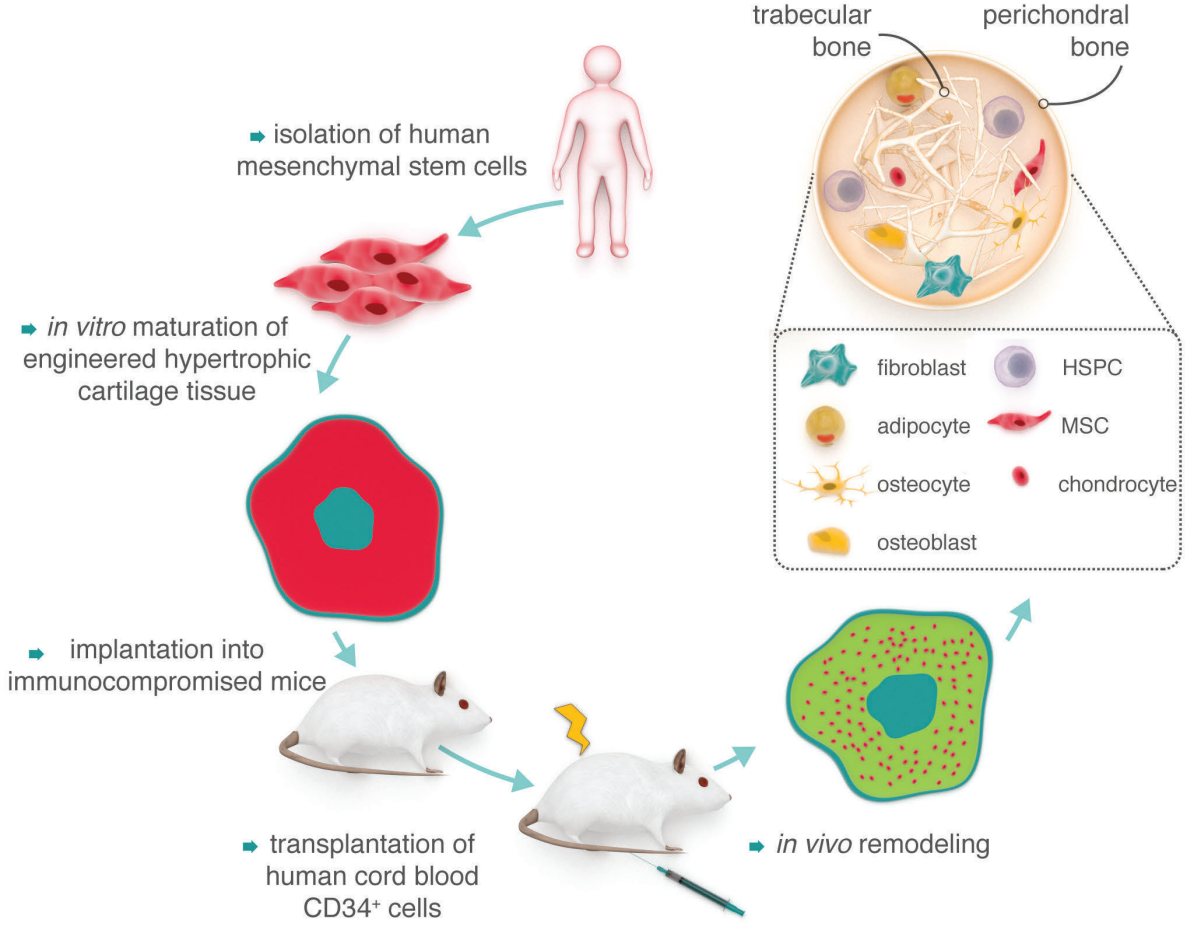

Figure 3. Engineering of heterotopic human niches in a xenograft mouse model. Human adult BM-derived mesenchymal stromal cells (MSC) are *ex vivo*-differentiated into hypertrophic cartilage, and implanted into immune-compromised mice. CD34$^+$ human umbilical cord blood cells are transplanted into sublethally irradiated mice with implanted ossicles. Upon *in vivo* implantation the hypertrophic cartilage develops into a fully mature bone organ through endochondral ossification.

Thymic microenvironment

The idea behind co-transplanting human thymus is to provide a human microenvironment that supports the development of human T cells, as well as their selection on human MHC molecules.[5] In the so-called "BLT" (bone marrow-liver-thymus) model, small fragments of human fetal liver and thymus are co-transplanted under the mouse kidney capsule.[65,66] Mice transplanted according to the BLT protocol sustain efficient human thymic lymphopoiesis, and T cells are the main component of the human graft.[39] Although the BLT model was initially reported as a system in which human adaptive immune responses would be improved compared to those of other models, the evidence for improved adaptive immunity in BLT mice, in comparison to transplantation of human CD34$^+$ cells without co-transplantation of thymic tissue, is still scant.[67,68] Recently, it was demonstrated that the transplantation of human embryonic stem cell-derived thymic epithelial progenitors into thymus-deficient nude mice supports the development of functional human T cells.[69] Neither approach, however, is accessible to most investigators due to the limited availability of embryonic and fetal tissues. Moreover, the biology of human thymus educated T cells and their reactivity against the xenogeneic environment needs to be further characterized. In addition, serum immunoglobulin levels and antigen-specific human antibody responses (particularly IgG responses) in BLT mice remain several orders of magnitude lower than in humans.[37,39,68] Nevertheless, despite the remaining functional deficiencies, BLT mice are particularly useful for studies of human immunodeficiency virus infection because of the high frequency of human T cells in lymphoid and mucosal tissues.[70-72]

Sources of human hematopoietic cells, routes of transplantation and mouse age at transplantation

Human HSPC are the standard source of cells for transplantation into humanized mice (Figure 4). HSC can differentiate into all hematopoietic lineages, self-renew and sustain hematopoiesis for the entire life of the organism. The frequency of HSC is highest in the CD34$^+$ cell population, although a lower number of HSC might be found in the CD34$^-$ fraction as well.[31,73-75] CD34$^+$ cells can be isolated from different sources: fetal liver, cord blood, adult BM or G-CSF-mobilized peripheral blood cells. The frequency of HSC among CD34$^+$ cells declines from fetal life to adulthood.[46] The number of cells that needs to be injected and the engraftment that can be expected are, therefore, highly variable depending on the source of CD34$^+$ cells used.

CD34$^+$ cells are transplanted into adult mice by either intravenous or intrafemoral injection (Figure 4). Transplantation into newborn mice (up to 3 days after birth) results in efficient engraftment and multilineage human hematopoietic differentiation, including T-cell development.[13] Human CD34$^+$ cells can be engrafted into newborn mice by intravenous, intracardiac or intrahepatic injection, with the liver being a natural site of hematopoiesis during fetal development and the first few days of the life. It has been demonstrated that female NSG mice are more supportive of human engraftment than male NSG mice when HSC are transplanted at a limiting dose.[76]

It is, therefore, crucial that the appropriate transplantation protocol is chosen in order to enable the experimental question to be answered.

Table 3. Comparison of ectopic human BM niches in xenograft models.

Source	Matrix	Number of cells seeded	Culture condition	Additions	Mouse model	Irradiation	Human cells transplanted	Number of transplanted cells	Timepoint of transplantation	Functional readout	Reference(s)
Human MSC from BM	Matrigel	1.5x10^6 MSC	MSC expanded, mixed with Matrigel, injected subcutaneously	Human BM-derived MSC mixed with 1.5x10^6 ECFC	NSG	Sublethal	MNC from CB, human acute myeloid leukemia cell line MOLM13	2x10^6 MNC or 2x10^6 MOLM13	After ossicle implantation	No	62
Human MSC from BM	4 Hybrid scaffold consisting of biphasic calcium phosphate particles	2x10^5 MSC	MSC expanded, loaded on scaffold, 7 days *in vitro* culture, implanted subcutaneously	No	BRG	-	CD34$^+$ from CB	1-5x10^5 CD34$^+$ or 1-5x10^6 MNC	8 weeks after ossicle implantation	No	64
Human MSC from BM, WAT, UCB, Skin	Matrigel-equivalent matrix	2x10^6 MSC	MSCs expanded, mixed with Matrigel, injected subcutaneously	No	NSG	Sublethal	CD34$^+$ from CB	1x10^4 to 2x10^5	8-10 weeks before ossicle transplantation	Serial transplantation	58
Human MSC from BM	Tubular medical-grade polycaprolactone scaffold	3x10^5 MSC	4 weeks static culture followed by 4 weeks dynamic culture in a bi-axial rotating bioreactor	No	NSG	Sublethal	CD34$^+$ from BM	1.3x10^5 CD34$^+$ and 1x10^6 CD34$^-$	10 weeks after ossicle implantation	No	59

BM: bone marrow; ECFC: endothelial colony-forming cells; MNC: mononuclear cells; MSC: mesenchymal stroma cells; CB: umbilical cord blood; WAT: white adipocyte tissue.

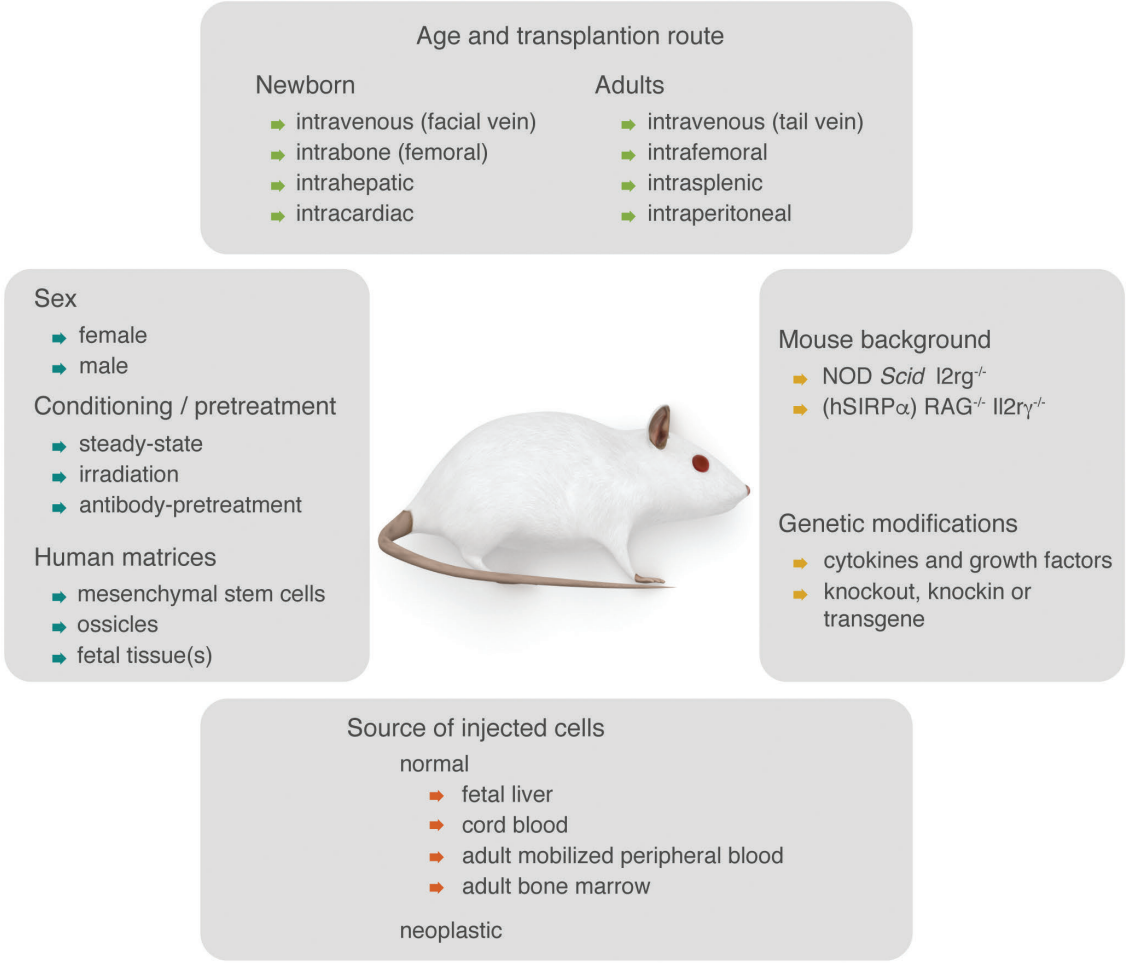

Figure 4. Parameters that influence the engraftment of human hematopoietic cells *in vivo*. Depending on the experimental conditions, different strains of mice, methods of pre-conditioning and routes of injection can be used to transplant human hematopoietic cells obtained from various sources.

Normal human hematopoiesis in humanized mice

The development of human hematopoiesis in mice was pioneered and recently reviewed by John Dick's group.[31] HSC are very rare with an estimated frequency of 1 in 10^6 human BM cells.[77] To be defined a HSC, the cell must have the potential to self-renew and differentiate into all hematopoietic lineages. Most investigators assess these properties 12-16 weeks after transplantation in mouse xenografts. The time-point of lineage engraftment assessment is particularly critical since the engraftment of certain lineages is transient and limited to a specific time window. In this regard engraftment of myelo-eythroid cells is observed early after transplantation, while the engraftment of T cells can increase significantly beyond 12 weeks.[78]

The $CD34^+$ population remains a heterogeneous mixture of cells with a minority of cells being *bona fide* HSC. Considerable effort has, therefore, been taken to identify markers that may reliably help to isolate HSC. Most publications define the human HSC population as $CD34^+CD38^-Thy1^+CD45RA^-$.[31,79] The closest progenitors to HSC are multipotent progenitors (MPP) and it was suggested that these intermediates may be distinguished from HSC by the lack of Thy1 expression.[80] However some MPP retain serial repopulation capacity. Additional markers are, therefore, needed to discriminate MPP from HSC. One such marker is CD49f, as demonstrated by the lack of long-term engraftment of $CD49f^-$ cells.[81] Interestingly, single-cell transplants of $CD49f^+$ cells generated long-term engraftment independently of Thy1 expression. Thus, while HSC express CD49f, MPP should be defined as $CD49f^-$ cells. Importantly, gene expression analysis of HSC and MPP based on CD49f expression revealed distinct profiles of these two populations.

Hematopoietic stem and progenitor cell engraftment in next-generation humanized mice

The engraftment of HSC is a prerequisite to ensure multilineage engraftment and one approach to increase human HSC engraftment is to reduce the mouse HSC compartment. This has been successfully achieved in NSG and BALB/cJ mice (NBSGW and BRgWv mice, respectively) that carry mutations in c-Kit (Table 2).[26,27] Strikingly, NBSGW and BRgWv mice are highly supportive of human hematopoiesis even without irradiation. The level of human engraftment at non-limiting doses in non-irradiated NBSGW and BRgWv mice is comparable to that in irradiated NSG mice and dramatically higher in NBSGW than

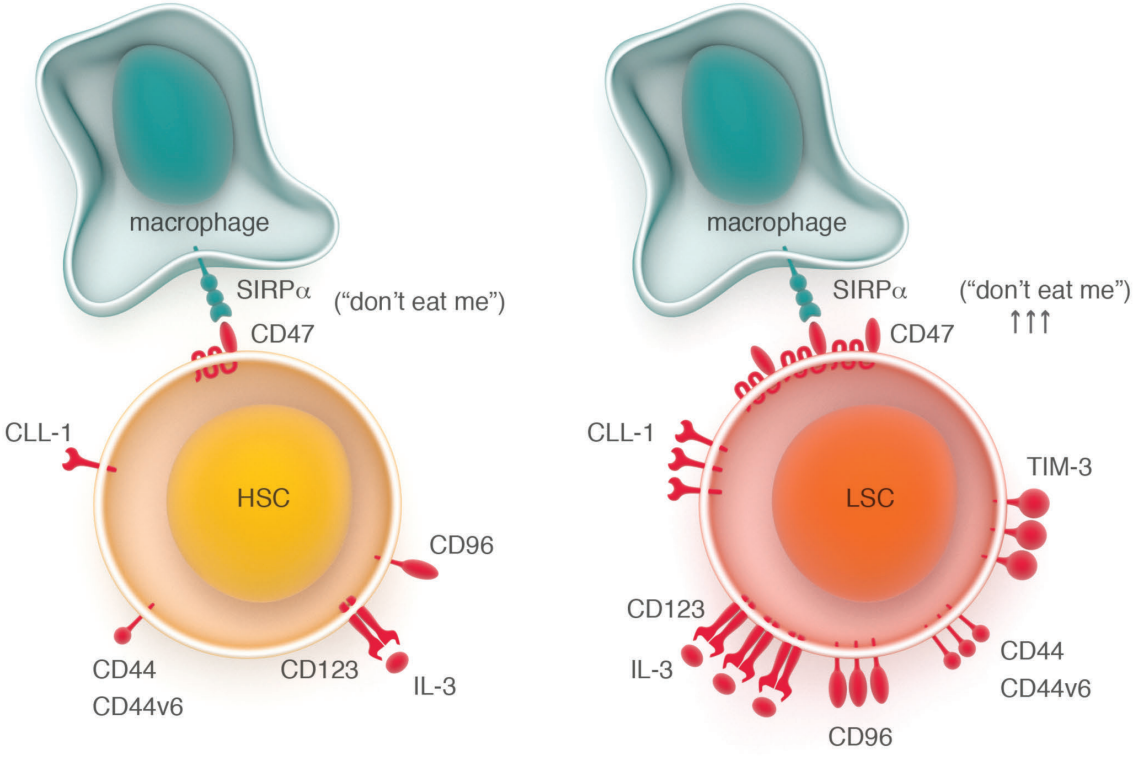

Figure 5. The expression of certain surface markers is significantly increased on LSC and allows these cells to be distinguished from their normal counterparts. LSC express high levels of CD47 and evade innate immune attacks by macrophages. The expression of TIM-3 has only been reported on LSC, but not on HSC. The expression of CD44 and the v6 isoform of CD44 are increased on LSC. These and other markers such as CLL-1, CD96 and CD123 allow LSC to be distinguished and targeted by monoclonal antibodies.

in non-irradiated NSG mice. In BRgWv mice, the frequency of human *bona fida* HSC ($CD45RA^{-}CD90^{+}$) 17-24 weeks after transplantation is similar to that in human BM. As the HSPC pool in next-generation humanized mice has not so far been characterized extensively, it will be interesting to see how these compare to BRgWv mice in this respect. Clearly, TPO knock-in mice demonstrate enhanced HSC maintenance and promote granulocyte-macrophage progenitor (GMP) differentiation, and the increased support of myelopoiesis in TPO and MISTRG mice most likely results from an increased GMP pool (see below).

Myeloid development in humanized mice

Human peripheral blood contains a significant proportion of myeloid cells, a situation not reflected in xenografted NOD-SCID mice and NSG mice.[1,82] Several approaches have, therefore, been taken to develop models that more faithfully mimic human hematopoiesis with a focus on myeloid lineage development *in vivo*.[1] Very early in the development of humanized mouse models sublethally irradiated SCID mice were injected with the "myeloid" cytokines IL-3, GM-CSF and SCF to assess myeloid development *in vivo*.[3,4] After 4 months myeloid cells were still detectable in the host, demonstrating the multilineage potential of human HSC. Several approaches have been taken to develop mouse models that promote myeloid differentiation of human HSC (Table 2). Mice that express the classic human "myeloid" cytokines IL-3, GM-CSF or human M-CSF (CSF1) show increased support of myeloid differentiation.[42,45] This confirms that the introduction of human genes into mice leads to the production of functional proteins. Interestingly, however, the support of myeloid differentiation is not confined to the presence of classical myeloid cytokines. In fact, most next-generation mouse models have been reported to promote improved human myeloid differentiation.[26-28,46] As described above, increased support of myelopoiesis is observed in mice that express human TPO.[28,46] Furthermore, the number of murine platelets is significantly reduced in TPO mice compared to control mice. Nevertheless, no significant increase in human megakaryocytes or platelets is observed, suggesting that human TPO is insufficient to promote full human megakaryopoiesis *in vivo*. Finally, NBSGW mice seem to be more supportive of human erythropoiesis. In summary, next-generation humanized mice promote myeloid differentiation, in part likely because they favor the development of myeloid progenitors.

Development of functional hematopoietic cells in mice

One particular focus in xenograft models has been the assessment of functional properties of engrafted human cells. MISTRG mice support development of functional monocytes characterized by the production of human tumor necrosis factor-α and IL-6 as a response to stimulation with lipopolysaccharides or pathogens, and by phagocytic activity against *E. coli*.[46] MISTRG mice also

support the development of functional NK cells and the development of CD163$^+$ tumor-infiltrating macrophages in a melanoma model. Strikingly, tumor growth was significantly higher in MISTRG mice than in NSG mice, which were both engrafted with human hematopoiesis, probably as a result of increased numbers of human tumor-associated macrophages, a process reversible by anti-vascular endothelial growth factor antibody therapy. A human cytokine response was also observed in CSF1 mice injected with lipopolysaccharide, and human monocytic cells purified from mice showed increased phagocytic activity against bacteria.[45] Furthermore the cells showed enhanced chemotaxis to MIP3β. In addition to models that develop a human immune response against pathogens, humanized models of human allergic reactions and immune-rejection have been established; mature human basophils and mast cells were detected in IL-3/GM-CSF transgenic mice and when exposed to serum from patients with cedar pollinosis mice developed passive cutaneous anaphylactic reactions.[42] Moreover, mice that express membrane-bound human SCF rejected HLA-mismatched human skin grafts.[41] These observations demonstrate that next-generation humanized mouse models support the development of functional human immune cells *in vivo*. For more detailed information on human immune responses in humanized mice, we refer to the respective review articles.

Malignant human hematopoiesis in humanized mice

Murine, surrogate genetically modified models of HSC malignancies and cell lines might not reflect the complexity of human disease. In contrast, humanized mouse models can reproduce disease characteristics of individual patients and allow assessment of disease heterogeneity, which is essential for the genetic characterization of neoplastic clones and to test therapeutic responses.

Humanized mouse models for acute myeloid (AML) and lymphoid leukemia are the most widely used xenograft models. The engraftment of less aggressive, chronic diseases such as MDS, myeloproliferative neoplasms and mature B-cell (including multiple myeloma) and T-cell lymphomas remains a challenge and, as for healthy hematopoiesis, novel approaches aim at generating robust and more consistent models.

The identification of LSC in 1994 was the foundation for the cancer stem cell hypothesis.[82,83] Human LSC are functionally defined as cells that can be engrafted into mice and propagated into secondary recipients. While it is well accepted that the frequency of LSC is highest in the CD34$^+$CD38$^-$ fraction in most AML, LSC are also found outside this population.[84,85] The search for additional markers that may allow more stringent identification of LSC is, therefore, ongoing. In contrast to healthy hematopoiesis, leukemia shows a very heterogeneous behavior and the surface phenotype of LSC varies between leukemia subtypes. Additional markers that have been used to characterize AML stem cells include CD123, CD44, CD47, CLL1 (in AML with *fms-related tyrosine kinase 3* mutations), CD96 and TIM3 (Figure 5).[82,86-90] These markers are expressed at a significantly higher level on the surface of AML stem cells compared to normal HSC. This allows the distinction of

Table 4. Comparison of next-generation humanized mouse models in their support of malignant hematopoiesis.

Disease	Injected population	Route of injection	Number of samples compared	Mouse strain	Conditioning	Additional treatment/ scaffold	Timepoint of analysis	Features	Reference(s)
Primary AML	Bulk AML cells	Tail vein	8	NOD.SCID N.S-β2m$^{-/-}$ N/S-S/GM/3	Sublethal irradiation	None	8-12 weeks	- BM engraftment: N.S-β2m-/- and N/S-S/GM/3 > NOD.SCID - only 1/8 with high and stable BM engraftment in N/S-S/GM/3 mice	107
CB-MA9-Nras	CB-derived line (leukemic)	Tail vein		NOD.SCID NSS (NOD.SCID-SGM3) NSG NSG-SGM3	None	None	When sick (d+40 or later)	- Increased homing in NSG and NSG-SGM3 - BM engraftment: NSG-SGM3 > NSS > NSG > NS	108
CB-AML1-ETO CB-CBFβ-MYH11	CB-derived line (pre-leukemic)	Intrafemoral		NSG NSG-SGM3	Sublethal irradiation	None	16 weeks	- BM engraftment: NSG-SGM3 > NSG	108
Primary AML	Bulk AML cells	Tail vein	5	NSG NSG-SGM3	Sublethal irradiation	None	12-16 weeks	- 3/5 samples with higher BM engraftment level in NSG-SGM3 (2 NSG non-engrafters)	108
Primary AML	Bulk AML cells	Tail vein	6	NSG NSG-SGM3	None	None	12-16 weeks	- 4/6 samples with higher BM engraftment level in NSG-SGM3 - Higher CD34$^+$ expression in NSG-SGM3 - Distinct clonal architecture in NSG and NSG-SGM3	106
Primary MDS	MDS CD34$^+$	Intrafemoral	24 (low risk, n=5; intermediate-1 risk, n=19	NSG NSG-SGM3	Sublethal irradiation	5E+5 patient-derived MSC co-transplanted	16-28 weeks	- 1/7 samples engrafted in NSG without MSC - 14/20 samples engrafted in NSG-MSC - 4 samples compared side-by-side - increased engraftment in NSG-GM3 mice - Dysplasia only observed in mice transplanted with MSC	63

AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; CB: umbilical cord blood; MSC: mesenchymal stem cells.

LSC from HSC, and has been used as a rationale to develop targeted therapies.[91] Differential expression of "LSC-specific" antigens provides a therapeutic window for novel therapeutic approaches. Indeed, antibodies directed against these antigens have been assessed in the leukemia xenograft model and have shown promising results, which are now translated into clinical trials.

LSC have also been detected in the $CD34^-$ fraction, particularly in AML with mutated *Nucleophosmin 1*.[92] An interesting phenomenon is that the leukemia-initiating potential may also reside in more mature progenitors that have acquired LSC properties. This has been demonstrated in an AML xenograft model in which lymphoid multipotent progenitor (LMPP)-like ($CD34^+CD38^-CD45RA^+$) and GMP-like ($CD34^+CD38^+CD45RA^+$) populations function as putative LSC.[93] Furthermore, recent studies have demonstrated pre-leukemic stem cells upstream of LSC.[94,95]

The characterization of LSC is most advanced in AML, while the identification of disease-initiating cells in other HSC neoplasms remains a challenge. Although disease heterogeneity may explain the lack of suitable markers to some extent, one of the main reasons for the difficulties in characterization is related to the low engraftment potential of the less aggressive disease in humanized mouse models. A recent study, however, identified MDS 5q- stem cells in the $Thy1^+CD45RA^-$ fraction of $CD34^+CD38^-$ cells.[96]

The identification of LSC in B- and T-lymphoblastic leukemia has been more difficult since there does not seem to be a consistent phenotype among disease subtypes. Although some studies found that $CD34^+CD38^-$ LSC lack CD19 expression in B-lymphoblastic leukemia other studies have shown that putative LSC express CD19.[97-100] Similarly the expression of T cell markers and CD34 may vary in T-lymphoblastic leukemia initiating cells.[100-102] Finally, a seminal study identified chronic lymphocytic leukemia initiating cells in the $CD34^+CD38^-CD90^+$ fraction.[103]

These studies demonstrate that humanized mouse models can help to identify premalignant and disease-initiating cells in a wide variety of HSC neoplasms and next-generation humanized mouse models may further increase the spectrum of hematopoietic malignancies that can be investigated in such models.

Leukemia engraftment and clonal heterogeneity in humanized mouse models

Gene expression profiling of a functionally validated LSC enriched population identified a LSC signature that correlates with clinical outcome.[104] This demonstrates that LSC and the AML xenograft are not artifacts, but reproduce human disease. However in the AML xenograft model, robust and reproducible engraftment is limited to 40-50% of patients' samples. A recent correlative study

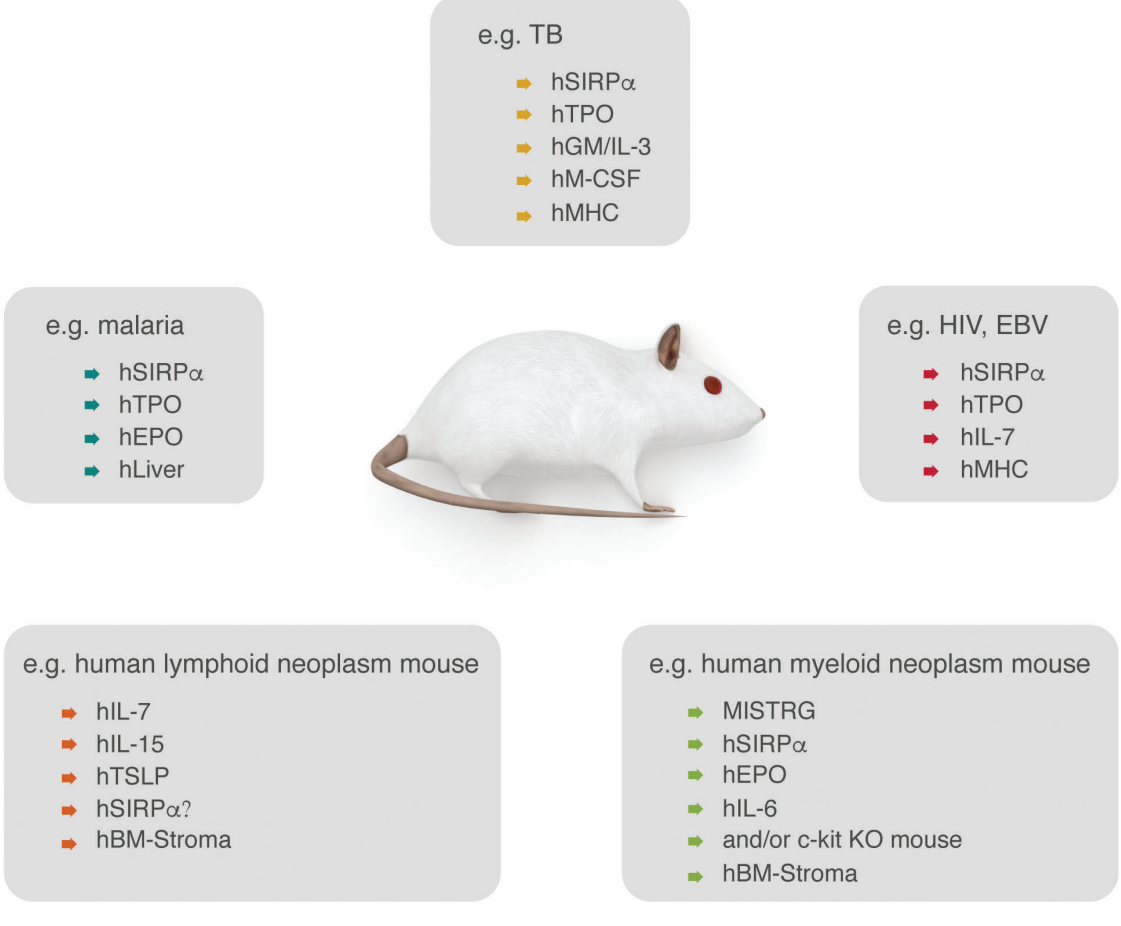

Figure 6. Possible disease-specific, tailored mouse models in future research. Different humanized mouse models that might serve specific research questions.

with 307 AML samples showed that only 44% generated a graft that reproduced the human correlate, while 28% did not engraft and 29% generated a T-cell or multi-lineage graft.[105] The same study demonstrated that a higher proportion of relapse samples compared to diagnostic samples (66% *versus* 44%) engrafted the mice and the engraftment potential of AML correlated with the aggressiveness of disease. Caution is, therefore, needed when interpreting data and conclusions may only be drawn for a specific disease subtype. Indeed, most published AML xenograft models do not mention the efficacy (engrafting *versus* non-engrafting samples) of AML engraftment. Consequently, there is an unmet need for mouse strains that support engraftment not only of high-risk, but also of lower-risk disease.

Development of malignant hematopoiesis in next-generation humanized mice.

Four studies demonstrated that engraftment of AML and MDS was significantly improved in NSG-SGM3 mice (Table 4).[63,106-108] Larger comparative studies are required to determine whether next-generation humanized mice can replace the standard mouse strains in some AML and other less aggressive myeloid neoplasms. However, the AML study by Klco *et al.* shows that NSG-SGM3 mice preferentially support certain AML sub-clones that are probably more sensitive to regulation and support by human cytokines produced *in vivo* from the transgene.[106] Interestingly, in that study, the most abundant clone found in NSG-SGM3 mice was not the most abundant clone found in the AML sample injected. This suggests that minor sub-clones present in the injected sample can become dominant depending on the different driving or selecting milieu present in the mouse. Moreover NSG-SGM3 mice also seem to affect the differentiation process since greater CD34 expression was found in AML cells in NSG-SGM3 mice than in NSG mice. Importantly, in this model none of the xenografts had the same sub-clonal architecture as the one of the injected AML. Preclinical AML therapy models have also revealed heterogeneity in response to treatment between mice transplanted with the same AML sample, suggesting that certain AML sub-clones may be more or less susceptible to a specific therapeutic intervention – an observation that has also been made in humans. In line with the data outlined above, AML sub-clones with strong dependence on myeloid cytokines may be more susceptible to therapies that target these pathways. Thus, xenograft models for AML not only define LSC and are preclinical therapy models, but also represent a tool allowing dissection of the clonal architecture of AML and the functional properties of AML sub-clones that could become targets for therapy.

Conclusions

Humanized mouse models have contributed significantly to the understanding of healthy and diseased human hematopoiesis and have led to the translation of promising therapeutic compounds into clinical applications. However there is room for improvement as several limitations remain.

Human adaptive immune responses remain limited in humanized mice, even when the human T-cell repertoire is selected on human fetal thymic tissue. Therefore, further improvement is needed to strengthen the development of human B and T cells, their education and maturation, the homeostasis of those cells in the periphery, the appropriate formation of secondary lymphoid structures (i.e. lymph nodes with functional germinal centers), and the functional interaction between human B cells and mouse follicular dendritic cells. Several strains of mice that express human HLA molecules have been reported in the past few years, conferring some improvement in the amplitude and/or in the repertoire of the adaptive response.[109-112] However, a combination of multiple factors (human cytokines, human HLA molecules, human lymphoid tissues) will be required to sustain an adaptive immune response comparable to that observed in humans, a critical step to make these models suitable for e.g. vaccine testing.

Furthermore, with the increasing functionality of human immune cells in the new models of humanized mice developed in the past few years, a new challenge emerges: the human immune system is not entirely tolerant for the mouse host. This was first observed in the BLT model in which human T cells selected on human HLA in the human fetal thymus mediate xenogeneic graft-versus-host disease, as peripheral tissues exclusively express mouse MHC molecules.[39,113] Moreover human phagocytic cells in mice that support increased myelopoiesis can engulf mouse red blood cells and platelets.[43,46] Thus, while previous efforts in the field of humanized mice consisted in generating strains in which the mouse immune system would tolerate the human graft, the development of newer models with a better functional human immune system will also need to focus on inducing tolerance of the human immune system for the mouse host.

While equally substantial improvements have been made in the development of humanized mice that support diseased hematopoiesis, significant limitations remain. Future models will need to support the engraftment of the majority of patient samples for a specific disease to allow solid conclusions regarding disease biology and response to therapy. Although mice that produce "myeloid" cytokines seem to move the field in the right direction, new challenges such as the selective and preferential support of hematopoietic sub-clones need to be taken into consideration. Finally, while humanized mouse models for HSC malignancies have led the field of xenograft models in the past, models for human solid organ malignancies and for autoimmune disorders will contribute significantly to disease understanding and development of novel therapeutic approaches in the future. Thus, one can envision that tailored humanized mouse models might be used for specific disease questions in the future (Figure 6).

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