# Chapter 4
# Mouse Genetic Background and Human Hematopoietic Stem Cells Biology; Tips for Humanization

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## 4.1 Genetic Backgrounds and Hematopoietic Cytokines for Better Function of Human HSC and Immune System

Xenotransplantation is defined as transplantation of living cells, tissues, or organs from one to another species. Major barrier for successful xenotransplant engraftment is bidirectional immune response between host and donor, triggered by recognition of "non-self" antigens and eventually resulting in deconstruction of host tissues or donor xenograft. Degree of this xenogenic immune reactions depends on what host immuno-components are activated by donor xenografts [1]: (i) humoral adoptive immune response mediated by presence of host natural antibodies that reacts to xenograft-derived molecules, and subsequent activation of complement system causing graft rejection; (ii) T cell-mediated cellular immunity as blockage of T-cell response significantly suppressed xenograft rejection; (iii) innate immune cells such as natural killer (NK) cells, macrophages, dendritic cells (DCs), neutrophils that detect exogenous molecules with species-specific modification or polymorphism via cell surface receptors, e.g., pattern recognition receptors. Ligation of the receptors on innate immune cells leads to cell activation and immediate rejection of xenografts via engulfment, complement system- or antibody-mediated mechanism.

Minimum requirement for xenotransplantation model to avoid the abovementioned xenogeneic immune reactions is the use of immunodeficient animals that lack acquired and ideally innate immunity, and thus permit sustained engraftment of xenotransplant. Among laboratory animals, small rodents, such as mice, have been utilized for the last several decades to develop immunodeficient strain by

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spontaneous gene mutation or artificial gene targeting, and engraft with human cells or tissues to reconstitute human hemato-lymphoid system (HHLS) for understanding human physiology and pathophysiology [2, 3]. Since history of development of immunodeficient mice has been well described in many other reviews [4, 5], major breakthroughs are discussed here briefly: (1) Severe combined immunodeficiency (SCID) mice was found on C.B17 background, which lack functional B and T cells because of impaired V(D)J recombination [6–8]; (2) Non-obese diabetic (NOD) mice crossed with SCID mice (NOD/SCID) have additional functional defects in innate immunity (macrophages, NK cells, and complement system), allowing better engraftment of xenotransplant than C.B17-SCID [9]; (3) Mice with homozygous deletion of interleukin-2 receptor gamma chain gene (*Il2rg*$^{-/-}$), a common cytokine-receptor gamma chain shared by other cytokine receptors (IL2-, IL4-, IL7-, IL9-, IL15- and IL21-R), was genetically engineered to have NK cell deficiency [10, 11], and subsequently crossed with Balb/c mice deficient for *Rag2* gene encoding a V(D)J recombination enzyme [12] (Balb/c Rag2$^{-/-}$IL2rγ$^{-/-}$, termed as BRG hereafter), or with NOD/SCID (termed as NSG or NOG) [13, 14]. Not only gene mutation but also mouse genetic background *per se* seems to be a determinant for high human cell engraftment: SCID or Rag2$^{-/-}$Il2rγ$^{-/-}$ mice on Balb/c genetic background is more permissive for human cell engraftment compared to equivalent mutants on C57BL/6 background [15, 16]. NOD background is superior to other strains, e.g., C.B-17, Balb/c or C57BL/6 [17] because NOD mice have lower levels of NK cell activity [9, 14] and a polymorphism in signal regulatory protein alpha (*Sirpa*) gene that cross-reacts with human ligands and suppresses phagocytosis of human cells by mouse macrophages ([18, 19] and in detail, discussed below).

Hematopoiesis is one of the somatic organ systems with high cell turnover and highly-organized developmental hierarchy, in which self-renewing hematopoietic stem cells (HSCs) in the bone marrow (BM) produce all hematopoietic and immune cells to maintain functional hemato-immune system throughout lifetime of an individual. Differentiation, maturation, and cell death of blood and immune cells are tightly controlled by many growth factors, chemokines, cytokines, and adhesion molecules through receptor-ligand interaction, and the subsequent intracellular signal activation. Some of these soluble factors are secreted by hematopoietic cells, while others are produced by non-hematopoietic cells in the BM, e.g., mesenchymal stroma, osteoblasts, endothelial cells [20]. In HHLS models, the factors that are produced only by non-hematopoietic mouse cells, need to be cross-reactive to respective human receptors to support human cell engraftment and reconstitution [21]. Therefore, non-cross-reactive human factors have been supplemented in HHLS mice by in vivo administration of recombinant protein, lentiviral or plasmid vector coding human proteins, or genetic replacement of mouse factors with human counterparts, and shown to enhance human hematopoiesis and immune function [22–26].

## 4.2 Human HSC Biology in Mouse Environment

Human HSC has been immunophenotyped by prospective isolation of living subpopulation with antibodies against cell surface markers, and subsequent functional test of the isolated cells. In vitro culture systems have been developed to presumably test multilineage and self-renewal potential of the isolated HSC candidate, including methylcellulose-based colony forming-unit (CFU) assay or long-term culture-initiating cells (LTC-IC) on stromal cells. However, as these *in vitro* assays often use hyper/hypo-physiologic concentration of cytokines and limited to maintain HSC self-renewal, they do not reflect *in vivo* situations and distinguish self-renewing HSCs from other non-self-renewing progenitors. Thus, transplantation of human HSC candidates into HHLS mice has been employed as so far "a gold standard" surrogate assay to test in vivo function of authentic HSCs. Human HSCs capable of repopulating HHLS mice in vivo, so-called SCID repopulating cells (SRCs), have been enriched in lineage marker negative ($Lin^-$), $CD34^+$, $CD90^+$ [27, 28] in human fetal liver (FL)/ bone marrow (FBM), umbilical cord blood (UCB), and adult BM (ABM) [29], and further subdivided as $CD38^-$ [30, 31], $CD45RA^-$ [32], Rhadamin-$123^-$ [33], and $CD49f^+$ [34]. However, serial transplanting ability of the human HSCs defined above are limited in a mouse, unlike mouse HSCs that are transplantable over a couple of rounds of transplantation [35], suggesting that mouse environment *per se* does not support human HSC self-renewal.

HSCs are localized in a specialized BM microenvironment, referred to as "niche," which provides necessary and sufficient factors to maintain HSC quiescence, self-renewal and multilineage differentiation capacity [20]. Cellular niche component consists of various cell types including osteoblasts/osteoclasts [36–38], endothelial cells [39], adipocytes [40], Schwann cells [41], macrophages [42, 43], CXCL12 abundant reticular (CAR) cells [44], and leptin $receptor^+$ [45], $nestin^+$ [46], or platelet-derived growth factor receptor (PDGFR)-$\alpha^+$ [47] mesenchymal stromal cells (MSC) (Fig. 4.1). These niche cells in different location of BM produce a variety of molecular niche factors to control steady state HSC homeostasis through ligand/cell surface receptor interactions: Angiopoietin-1 (ANG-1)/Tie2 [48], CXCL12/CXCR4 [39, 49, 50], Stem cell factor (SCF)/c-Kit [45], Thrombopoietin (THPO)/c-Mpl [51, 52], Tumor growth factor (TGF)-β/TGF receptor-2 [41], Wnt/Frizzle [53, 54], N-cadherin [36, 54, 55], E-selectin ([56]; Fig. 4.1).

The knowledge on cellular and molecular BM niche components as mentioned above has been obtained from the studies with genetically modified mice, whereas human counterpart niche components remain largely unknown. Given that LTC-IC represents human HSC potential *in vitro*, stromal cells that maintain LTC-ICs presumably recapitulate human BM microenvironment and serve as HSC niche cells. Similarly, it has been demonstrated that $CD146^+$ MSCs that are found in human BM can self-renew and form hematopoietic microenvironment *in vivo* in a heterotopic site with expression of ANG-1 [57]. Since little is known about other niche cells and factors for human HSC maintenance, and cross-reactivity of mouse factors to human HSCs, establishment of *in vivo* system to engineer human BM microenvironment might help to better support human HSCs in a mouse environment.

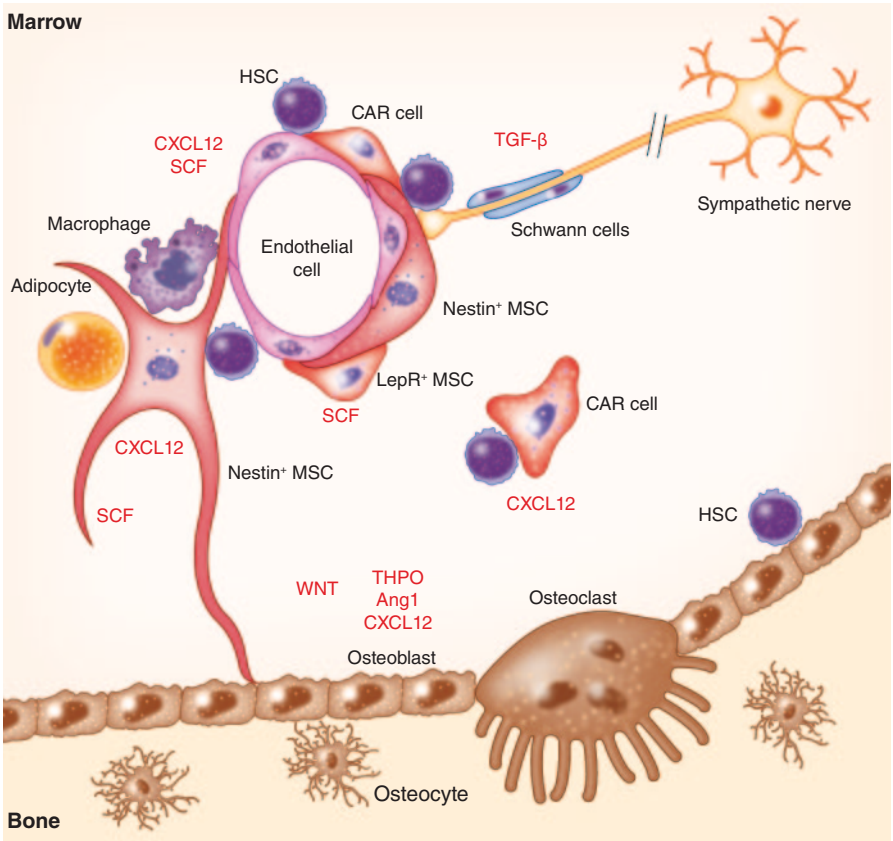


**Fig. 4.1** Possible cellular and molecular HSC niche components in mouse BM. Niche cells (*black* instead of black) and factors (*red*) previously reported are depicted. HSC hematopoietic stem cell, CAR cell, CXCL12 abundant reticular cell, MSC, mesenchymal stromal cell, LepR, leptin receptor, ANG-1, angiopoietin-1, SCF, stem cell factor, THPO, thrombopoietin, TGF-β, tumor growth factor-β

## 4.3 Mouse Hematopoietic Stem Cell or Niche Defect in SCID BM

First, HHLS model was developed by two groups, by engrafting CB17-SCID mice with human tissues [58] or peripheral blood leukocytes [59], and observed human B/T differentiation as well as human antibody secretion in the peripheral blood. C.B17-SCID mice have a spontaneous point mutation in *Prkdc* gene (protein kinase, DNA activated, catalytic polypeptide) involving in V(D)J recombination [7, 8], and therefore, no functional B and T cells but intact innate immunity and NK function, leading to low level of human cell engraftment.

Prkdc also regulates non-homologous end-joining (NHEJ) pathway, a process to repair double stranded breaks upon DNA damage [60]. It was found that DNA-damaging agents restore T cell receptor rearrangement, thereby resulting in normal

T cell development, but also induce lymphoma with high frequency [61]. Furthermore, C.B17-SCID show "leaky" phenotype: with age, normal functional B and T cell are spontaneously generated and serum immunogloblin level is increased accordingly [62–64].

It was recently demonstrated that CB17-scid mice have impaired self-renewal capacity of HSC in lethal irradiation transplantation, and upon non-conditioned transplantation, are more permissive for exogenous mouse HSC engraftment than control recipient, allowing 8–10% of donor hematopoietic stem/progenitor cell (HSPC) in BM [65]. As $Rag1^{-/-}$ mice, another immunodeficient strain with defective V(D)J recombination, did not show similar result, this defect is not due to immunodeficiency but impaired competitive repopulating ability of SCID mutant HSCs leading to niche occupancy defect. In addition, genetically modified *Prkdc* mutants show HSPC loss caused by DNA damage accumulation during embryonic development and start to die of pancytopenia shortly after the birth [66]. Since similar phenotype was observed in HSCs lacking NHEJ-related gene [67, 68], functional impairment of SCID HSCs is dependent on NHEJ machinery rather than V(D)J recombination, as in part supported by the recent finding that HSCs have active NHEJ machinery to be resistance to DNA damage [69].

## 4.4 Role of Mouse Innate Immunity in the Engraftment of Human Cells: NOG versus NSG

Innate immune cells such as NK cells, DCs, or macrophages play an important role for xenograft rejection. Human cell engraftment in HHLS mice was significantly improved when C.B17-SCID mice were crossed to NOD strain that lacks complement system, NK cells, and macrophages [70, 71]. NK activity in NOD/SCID was significantly reduced compared to C.B17-SCID but not completely abrogated [64]. Further elimination of NK cells has been achieved by anti-CD122 antibody that targets both NK and myeloid cells or genetically engineered mutation in *Il2rg* gene [13, 14], and lead to facilitate engraftment of human SRCs [72, 73]. Two types of mutations in IL2Rγ have been introduced into NOD/SCID independently: one mutant with complete null mutation resulting in no cell surface expression of IL2Rγ, and no binding to ligands at all (NSG, NOD/LtSz-scid $Il2r\gamma^{-/-}$ [14]), and another with a truncated intracellular domain of IL2Rγ that still binds ligands but does not transduce downstream signals (NOG, NOD/Shi-scid$Il2r\gamma^{-/-}$ [13]). Recent comparative analysis, where limited number of human HSCs were transplanted into NSG, NOG, and anti-CD122 treated NOD/SCID, demonstrated that NOG and NSG mice show significant improvement in human SRC detection over NOD/SCID model [74]. In addition, the higher sensitivity to detect SRCs in NSG than NOG suggests that the remaining IL2Rγ chain extracellular domain in NOG might leave HSCs undifferentiated in the BM and thus result in less repopulation and ignorance of SRCs.

Besides NK cell-mediated innate immune barrier, another key genetic determinant for xenograftment has been identified in *Sirpa* gene responsible for high human

cell engraftment in NOD strain [18]. SIRPα is mainly expressed on macrophages, granulocytes, and DCs, and upon binding CD47 transduces a negative "don't eat me" signal in macrophages preventing phagocytic activity [75, 76]. It was shown that NOD-type SIRPα with polymorphism in immunoglobulin like domain V (IgV) can cross-react with human CD47 unlike SIRPα from other strains, and therefore, block mouse macrophage engulfment against engrafted human cells, leading to efficient homing and engraftment [18]. This finding was further proved by significant improvement of human cell engraftment in HHLS mice with overexpression of mouse CD47 in transplanted-human $CD34^+$ cells, genetic introduction of human SIRPα in BRG strain, or crossing of C57/BL6.$Rag2^{-/-}$Il2r$\gamma^{-/-}$ with C57/BL6.NOD harboring NOD-type SIRPα [19, 77, 78]. Consistently, disruption of SIRPα–CD47 signal axis in macrophages by neutralizing antibody or chemical-mediated depletion of macrophages enhances human HSC engraftment [79, 80].

## 4.5 Lymphoid Tissue Development: NOD/SCID versus NOG versus NSG Mice

Introduction of SCID mutation into NOD background reduces activity of mouse innate immunity against engrafted human cells, and also rescues NOD mice from development of T cell-mediated autoimmune insulin-dependent diabetes mellitus [81]. Despite improved human cell engraftment, NOD/SCID mice develop a spontaneous thymic lymphoma with high incidence, in 70% of mice by the age of 40 weeks [64, 82]. A mean lifespan of NOD/SCID mice is only 8.5 months [64] and thus severely impedes long-term study of human immunity and hematopoiesis. Genetic crosses of NOD/SCID onto IL2R$\gamma^{-/-}$ mice extend their lifespan up to on average 22 months with free of thymoma [14].

Engraftment of human $CD45^+$ cells in thymus was significantly higher in NOG or NSG than NOD/SCID [12, 13, 64, 71, 74] (Table 4.1). The reconstituted thymi of NOG or NSG contain human $CD45^+CD3^+$ cells, majority of which are CD4 and CD8 double positive T cells [74], and these human T cells display polyclonal T cell receptor (TCR)-αβ [83] and functional antigen response [84, 85]. Similarly, spleen has been better reconstituted with $CD19^+IgM^+$ B, $CD3^+$ T, and $CD56^+$ NK cells in NOD or NSG than other strains. Functional analysis revealed that human B cells developed in mouse spleen resemble the characteristics of B-1 B cells that represent a minor population in humans and express auto/polyreactive antibody [86]. Although the human antibody repertoire in HHLS mice is similar to that observed in humans [87, 88], class switching from IgM to IgG and affinity maturation of human antibody occur poorly in current HHLS models [2, 64]. Development of peripheral lymph nodes (pLNs), e.g., axillary, inguinal LNs is also limited in any of IL2Rγ-deficient immunocompromised mice including BRG, NOD, NSG, while mesenteric LN is usually formed [5, 89]. Given the fact that transfer of relatively high number of T cells develops pLNs in BRG model [90, 91], it will be possible to overcome this limitation upon robust T cell reconstitution by long-term sustained HSC engraftment.

**Table 4.1** Representative humanization protocol in HHLS models

| Mouse strain abbreviation | Mouse age | Conditioning | Human cells | Cell dose | Injection route | Analysis (wkpt) | % hCD45$^+$ | | | | % hCD34$^+$ in BM CD45$^+$ | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | PB | Thy | SPL | BM | | |
| BRG | newborn | 400 cGy IR | CB CD34$^+$ | $3.8–12 \times 10^4$ | i.h. | 4–26 | n.d. | >70 | 10–80 | 10–70 | n.d. | [89] |
| | newborn | 350 cGy IR | FL CD34$^+$ | $2–20 \times 10^5$ | i.p. | 6–11 | >50 | 45–99 | >50 | >50 | n.d. | [5, 97] |
| BLT (NOD/SCID) | 6–10 week | Implantation of FL and FT and 200–300 cGy IR | FL CD34$^+$ | $1–5 \times 10^5$ | i.t.v. | 18–25 | 40 | n.d. | 50 | 50 | n.d. | [126] |
| | 6–8 week | Implantation of FL and FT and 325 cGy IR | FL CD34$^+$ | $2–25 \times 10^5$ | i.t.v. | 26 | 49 | 99 | 42 | 48 | n.d. | [116] |
| NOG | 8–13 week | 225 cGy IR | CB Lin$^-$ | $2.3–3.0 \times 10^5$ | i.f. | 11–13 | n.d. | 8 | 4 | 16–22 | 6 | [74] |
| | n.d. | 240 cGy IR | CB CD34$^+$ | $0.4 \times 10^5$ | i.t.v. | 20 | 30 | n.d. | 70 | 35 | 3 | [13] |
| NSG | newborn | 100 cGy IR | CB Lin$^-$CD34$^+$ | $10^5$ | i.f.v. | 12 | 69 | 78–96 | 55 | 73 | n.d. | [85] |
| | newborn | 100 cGy IR | CB CD34$^+$ | $3 \times 10^4$ | i.f.v. | 12 | 50 | n.d. | n.d. | n.d. | n.d. | [127] |
| | newborn | 150 cGy IR | CB CD34$^+$ | $1 \times 10^6$ | i.f.v. | 8–12 | >50 | >90 | 55 | 64 | n.d. | [96] |
| | 8–13 week | 225 cGy IR | CB Lin$^-$ | $2.3–3.0 \times 10^5$ | i.f. | 11–13 | n.d. | 6 | 4 | 43 | 6 | [74] |
| | n.d. | 325 cGy IR | mbPB CD34$^+$ | $7.0 \times 10^5$ | i.t.v. | 8–10 | 6 | 37 | 60 | 35 | n.d. | [14] |
| | 7–10 week | 2 × 25 mg/kg BSU i.p. | CB CD34$^+$ | $20 \times 10^5$ | i.t.v. | 24 | 60–65 | n.d. | n.d. | 5 | n.d. | [104] |
| | 3–4week | 2 × 25 mg/kg BSU i.p. | CB CD34$^+$ | $2 \times 10^5$ | i.t.v. | 22 | 41 | 99 | 74 | 83 | n.d. | [105] |
| BRG-hTHPO$^{KI}$ | newborn | 300 cGy IR | CB/FL CD34$^+$ | $1.0–2.0 \times 10^5$ | i.h. | 12–16 | 10 | n.d. | 27 | 80 | 12 | [22] |

**Table 4.1** (continued)

| Mouse strain abbreviation | Mouse age | Conditioning | Human cells | Cell dose | Injection route | Analysis (wkpt) | % hCD45$^+$ | | | | % hCD34$^+$ in BM CD45$^+$ | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | PB | Thy | SPL | BM | | |
| BRG-hIL3/GM-CSF$^{KI}$ | newborn | 400 cGy IR | CB/FL CD34$^+$ | 1.0–2.0 × 10$^5$ | i.h. | 8–12 | 18 | n.d. | 20 | 50 | 15 | [23] |
| BRG-hM-CSF$^{KI}$ | newborn | 300 cGy IR | FL CD34$^+$ | 1.0–2.0 × 10$^5$ | i.h. | 12 | 35 | n.d. | 25 | 42 | n.d. | [24] |
| BRG-hSirp$^{Tg}$ | newborn | 360 cGy IR | FL CD34$^+$ | 1.0–2.0 × 10$^5$ | i.h. | 10–12 | 23 | n.d. | 70 | 66 | n.d. | [78] |
| NSG-hIL-3/GM-SF/SCF$^{Tg}$ | newborn | 100 cGy IR | FL CD34$^+$ | 1.5–2.0 × 10$^5$ | i.h. | 8–12 | 50 | n.d. | n.d. | n.d. | 0.2 CD34$^+$ CD38$^-$ | [128] |
| NSG mbhSCF$^{Tg}$ | newborn | 150 cGy IR | CB Lin$^-$CD34$^+$ CD38$^-$ | 0.5–5.3 × 10$^4$ | i.f.v. | 8–35 | 83.1 | n.d. | 94.5 | 97.1 | n.d. | [125] |
| | newborn | No IR | CB CD34$^+$ | 3.0 × 10$^4$ | i.t.v. | 12 | 13 | 46 | 70 | 57 | 0.27CD34$^+$ CD38$^-$ | [121] |
| NSG-β2m$^{-/-}$ HLA-A2$^{Tg}$ | newborn | 150 cGy IR | CB Lin$^-$CD34$^+$ CD38$^-$ | 0.5–3.0 × 10$^4$ | i.f.v. | 16–32 | n.d. | n.d. | 87 | 71 | n.d. | [124] |
| NSG-HLA-A2$^{Tg}$ | newborn | 100 cGy IR | FL CD34$^+$ | 1.0–3.0 × 10$^5$ | i.h. | 12 | 40 | n.d. | >60 | 28 | n.d. | [123] |
| NSG-HLA-DR4$^{Tg}$ | n.d. | 350 cGy IR | CB CD34$^+$ | 8.0 × 10$^4$ | i.t.v. | 24 | n.d. | 84 | 82 | 69 | 7 | [122] |
| NOG-I-A$^{-/-}$HLA-DR4$^{Tg}$ | 6 week | 120 cGy IR | CB CD34$^+$ | 1.0 × 10$^5$ | n.d. | 12–20 | n.d. | n.d. | 20–80 | 70 | n.d. | [129] |

**Table 4.1** (continued)

| Mouse strain abbreviation | Mouse age | Conditioning | Human cells | Cell dose | Injection route | Analysis (wkpt) | % hCD45$^+$ | | | | % hCD34$^+$ in BM CD45$^+$ | Refs. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | PB | Thy | SPL | BM | | |

*BRG* Balb/c Rag2$^{-/-}$ IL2rγ$^{-/-}$, *NSG* NOD/SCID IL2rγ$^{-/-}$, *NOG* NOD/SCID IL2rγ$^{-/-}$, *NRG* NOD Rag2$^{-/-}$ IL2rγ$^{-/-}$, *BLT* bone marrow-liver-thymus, *IL* interleukin, *THPO* thrombopoietin, *GM-CSF* granulocyte macrophage colony-stimulating factor, *mbhSCF* membrane-bound human stem cell factor, *HLA* human leukocyte antigen, *KI* knock-in, *Tg* transgenic, *IR* irradiation, *BSU* busulfan, *CB* cord blood, *FL* fetal liver, *FT* fetal thymus, *i.f.v* intrafacial vein, *i.h.* intrahepatic, *i.p.* intraperitoneal, *i.f.* intrafemoral, *i.t.v.* intratail vein, *PB* peripheral blood, *Spl* spleen, *Thy* thymus, *BM* bone marrow, *wk* week old, *wkpt* weeks post transplantation, *n.d.* not discribed or determined

## 4.6 Parameters in Donor and Recipient that Influence Humanization Efficiency

Main source of human HSCs to engraft HHLS models is FL, UCB, ABM, and pharmacologically mobilized peripheral blood (mbPB). Frequency of $CD34^+$ cells containing human HSPCs is around 0.5–5 % in FL, UCB, and ABM, and 0.04–0.4 % in mobilized PB [29, 92]. Since $CD34^+$ cells are functionally heterogeneous, in vivo functional studies have been performed by transplanting limiting dose of cells into HHLS mice to determine the frequency of SRCs, and found the highest in FL and the lowest in mbPB (SRC frequency: FL≥CB>BM≥mbPB) [22, 23, 93, 94].

Choice of age and sex of recipient animals seems to matter: injection of CB-derived $CD34^+$ cells into 6–8 week BRG mice results in 20 ± 16 % engraftment in BM and partial thymic repopulation [95], whereas newborn of BRG shows 10–60 % human chimerism in BM and robust development of functional T cells [89, 96, 97] (Table. 4.1). This suggests more efficient de novo human cell repopulation in newborn than in young adult; with sex of recipient mouse, limiting dilution and serial transplantation of the defined human HSCs into NSG male or female demonstrated that NSG females support higher engraftment in BM and spleen, and more robust self-renewal of human HSC than do males, suggesting that sex-specific mechanism is crucial for human HSC engraftment and maintenance [98]. It is, however, of note that a range of $1–2 \times 10^5$ $CD34^+$ cells does not seem to make a significant difference in human cell engraftment [98].

A major hurdle for successful HSC engraftment during transplantation is BM homing, a highly coordinated multistep process in which injected HSCs circulate in blood, cross BM endothelium barrier, and finally settle down to an emptied BM niche to self-renewal and differentiate into multilineage blood cells [99]. In xeno-transplantation, this process is further complicated by other parameters as cross-reactivity of homing, migration and growth factors, availability of HSC niche space in host BM, growth competition between mouse and human HSCs. One strategy to avoid homing-associated complications is to inject cells directly into the hematopoietic site, either bone of adult mice (intra-femoral injection) to escape from host immune surveillance [100, 101], or liver of newborn (intra-hepatic injection) to recapitulate natural homing process [89]. Intraosseous injection allows detecting human SRCs with 15-fold higher sensitivity compared to intravenous injection [73, 100], although it might stress and disturb BM microstructure [102]. Other injection technique, intrafacial or intraperitoneal injection seems to work at least in newborn as comparable to or better than intrahepatic injection [85, 97].

To achieve high and sustained human HSC engraftment, niche space in host needs to be created by depletion of mouse HSCs and hematopoietic cells. To this end, most of the studies with HHLS models employ sublethal dose of irradiation. Since each immunodeficient strain has different irradiation sensitivity [74], the irradiation dose should be optimized considering both animal mortality and human engraftment. It will be interesting to test efficacy of human HSC engraftment with other myeloablative regimens than irradiation, such as cyclophosphamide [103],

busulfan [104, 105], or neutralizing antibody against mouse c-Kit [106]. Representative humanization protocols with HHLS models are shown in Table 4.1.

## 4.7 How to Improve "Humanization": Multilineage Cell Engraftment and Maintenance

For the past decades, a lot of efforts have been made to develop different HHLS models with genetic crossing and gene targeting, and to study physiology of human hematopoiesis and immunity, as well as pathogenesis of human diseases including autoimmunity, infection, and cancer (reviewed in [2, 3]). Despite remarkable achievements, there are some limitations that remain to be overcome to have robust HHLS models: (i) unstable human cell reconstitution and maintenance; (ii) limited functionality of human innate and acquired immunity; (iii) inefficient platelet and red blood cell production. This topic will not be discussed here, as it is not highly relevant to HIV infection and also covered in other review [2].

First, long-term stabilization of human cell engraftment over experiments and different donor cells requires more number of human HSCs to be transplanted and better maintenance of self-renewal capacity of transplanted HSCs *in vivo*. However, human samples available for transplantation are usually limited. One approach to improve this is to develop an *in vitro* culture system that allows expansion of functional HSCs. This has been already considered and up to 17-fold expansion has been achieved at the best as determined by in vivo NOD/SCID repopulating assay (reviewed in [107]). Another way to supply human HSCs is to generate human HSCs from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) that can be maintained and expanded *in vitro*. However, the process of HSC generation from pluripotent stem cells would be highly complicated by the evidences that many extrinsic and intrinsic factors are temporarily and spatially involved in the HSC emergence during hematopoietic ontogeny (reviewed in [108, 109]). Many studies have attempted to mimic *in vivo* early HSC development by in vitro culture of PSCs with supplementation of fluid factors or coculture with feeder cells, e.g., fibroblasts or stromal cells [110, 111]. Yet, *in vitro* HSC development from iPSCs is largely limited to production of *in vivo* transplantable HSCs. To bypass exogenous factor-mediated HSC specification, a recent work has demonstrated that human iPSC-derived teratoma formed in NSG mice contain human HSCs that are transplantable into another recipient [112]. Introduction of a certain sets of transcription factors in mouse or human fibroblasts, or in human iPSCs have shown to activate hemogenic program that generates in vitro hematopoietic colony forming cells or *in vivo* blood repopulating cells with limited self-renewal [113–115]. Although these studies have significantly advanced our knowledge to expand or develop HSC *ex vivo*, unlimited supply of transplantable human HSCs for preclinical and clinical application remains challenging.

To improve *in vivo* engraftment or maintenance of human HSCs, human *SIRPA* and *THPO* gene have been introduced into BRG mice by genetic engineering

(SIRPα$^{Tg}$ BRG and THPO$^{KI}$ BRG, respectively), and these models showed high engraftment level and enhanced self-renewal activity of human HSC in BM with no obvious impact on quality of mouse life [22, 78] (Table 4.1). An alternative strategy to support human HSC function is providing mice with human microenvironments by cotransplantation of human tissues or human niche cells. Some groups have developed a model where human FL-derived CD34$^+$ cells are transplanted into sublethally irradiated NOD/SCID or NSG previously implanted with autologous human fetal thymus and liver, so-called BM-liver-thymus (BLT) model, and achieved high human engraftment and robust immune response [116, 117]. Yet, a broad application of BLT model is limited by necessity of easy access to human fetal tissues. In contrast, implantation of human MSCs is relatively easy, as they can grow from their self-renewing progenitors in *in vitro* culture of human BM efficiently [57]. *In vivo* implantation of MSC progenitor or MSC-derived ossicle develop heterotopic bone organ that recruits functional HSC to the site [118, 119]. This system might serve as "stem cell trap" to provide more optimal microenvironment for robust human HSC maintenance.

Second, because of low or little cross-reactivity of some of the mouse-soluble factors to human receptor [21], mouse environment provides suboptimal conditions for human HSPCs to fully differentiate to the mature cells that in turn migrate to an appropriate tissue and exert their function upon activation. Various strategies have been taken to improve differentiation and maturation of human immune cells as follows: *in vivo* injection of recombinant human cytokines, human cytokine-coding plasmid or virus to enhance erythropoiesis, myelopoiesis, thymopoiesis, and NK cell development (reviewed in [120]); genetic replacement of myeloid-supporting mouse cytokines with human counterparts to promote myeloid cell differentiation [22–25]; transgene expression of human SIRPα, membrane-bound SCF, MHC I and II to augment human cell engraftment, mast cell development, and functional T cell development [78, 121–125]. More new animals or novel methodology are expected to be developed and revolutionize HHLS models.

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