Chapter 8
Improvement of Human Multilineage Hematopoietic Engraftment by Cytokine Knock-in Replacement in Human-Hemato-Lymphoid System Mice

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A primary aim of biomedical research is to gain a better understanding of human physiology and to use this knowledge to prevent or cure human diseases. Due to practical and ethical barriers to the experimentation on human subjects, many studies are conducted on small animal models such as the mouse. However, mice are not men and the knowledge gained from animal experimentation is not always applicable to humans [1, 2]. In this context, mice repopulated with a human hemato-lymphoid system (HHLS) represent a useful small animal model for the study of human hematopoiesis and immune function in vivo [2, 3].

HHLS mice are generated by transplantation of human hematopoietic stem and progenitor cells (HSPCs) and/or human fetal tissues into recipient mice deficient in the innate and adaptive arms of the immune response [2, 3]. The first models of HHLS mice were developed in the late 1980s [4–6] and have been undergoing successive improvements since then [7, 8]. The strains of mice currently used as recipients for human hematopoietic engraftment share three characteristics. First, they lack B and T cells due to the severe combined immune deficiency (*scid*) mutation in the gene encoding the DNA-activated protein kinase, DNA activated, catalytic polypeptide (PRKDC) protein [4, 5], or due to deletion of one of the two *Rag* genes [9–11]. Second, deletion of the *Il2rg* gene that encodes the common gamma chain (γ_c) of cytokine receptors abolishes interleukin (IL)-15 (and IL-2, -4, -7, -9, -21) signaling and results in the absence of natural killer (NK) cells [9, 11–13]. Third, the interaction between the signaling regulatory protein alpha (SIRPα) receptor

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expressed on mouse macrophages and the CD47 ligand on human cells provides an inhibitory signal to mouse macrophages and confers phagocytic tolerance for the human xenograft [14, 15].

Cross-species interaction between SIRPα expressed on mouse cells and CD47 on human cells can be achieved using several approaches. Mice of the nonobese diabetic (NOD) genetic background contain a strain-specific polymorphism in the *Sirpa* gene, which affects the glycosylation of the mouse SIRPα protein and renders it cross-reactive with human CD47 [14, 16]. This polymorphism in *Sirpa* is most likely the main determinant of the capacity of the NOD background to support high levels of human cell engraftment, because backcrossing of the NOD-*Sirpa* allele onto the Balb/c RAG2$^{-/-}$ $\gamma_c^{-/-}$ genetic background is sufficient to support engraftment levels comparable to those observed in NOD-based recipients [16]. An alternative approach to induce SIRPα/CD47 cross-reactivity consists in expressing, by lentiviral transduction, the mouse *Cd47* gene in human HSPCs prior to the transplantation into recipient mice [16]. Finally, our labs have generated a human *SIRPA* bacterial artificial chromosome (BAC)-transgenic mouse, in which the entire gene encoding human SIRPα is inserted in the mouse genome [17]. This transgene induces mouse-to-human phagocytic tolerance, as demonstrated by the delayed clearance of human red blood cells injected into those mice, compared to nontransgenic mice. Upon transplantation of human HSPCs, the human *SIRPA* transgene resulted in significantly increased engraftment and maintenance of human hematopoietic cells, as well as improved immune function such as the production of antigen-specific immunoglobulin M (IgM) and immunoglobin G (IgG) in response to immunization [17].

High levels of human hematopoietic cell engraftment, upon transplantation of human HSPCs, are achieved when using NOD *scid* $\gamma_c^{-/-}$ (NOG (12) or NSG (13)) or hSIRPα^{tg} RAG2$^{-/-}$ $\gamma_c^{-/-}$ (SRG (17)) mice as recipients. Furthermore, human multilineage hematopoietic development is observed, with the presence of most human cell types present including T and B lymphocytes, NK cells, monocytes/macrophages, dendritic cells and sometimes low levels of erythrocytes and platelets. However, the terminal differentiation, homeostasis, physiologic relative cellular composition, and/or effector function of most of these human cells is suboptimal. It has been hypothesized that this condition is due to reduced or absent cross-reactivity between cytokines secreted by mouse tissues and the human receptors expressed on hematopoietic cells as well as nonphysiologic education of human adaptive immune cells in the xenogeneic environment [2, 18, 19]. To circumvent this limitation, several strategies have been developed to deliver human cytokines in the mouse host.

The injection of recombinant cytokines into recipient mice is the most straightforward approach to deliver human factors, and it has been used since the earliest models of HHLS mice [20–23]. This method has the disadvantage of being costly and labor-intensive as cytokines have short half lives in vivo and commonly daily injections are needed for prolonged periods of time. More cost effective methods consist in using lentiviral delivery of cytokine-encoding cDNA that results in constitutive synthesis and secretion of the cytokine in vivo [24], or hydrodynamic injection of plasmid DNA that leads to transient high-level secretion in circulation of

cytokines, mostly by liver cells [25]. These methods can prove useful to boost the development of specific lineages of human cells, or for proof-of-concept experiments to test the efficacy of a candidate cytokine to support human hematopoietic development and function. However, the results of experiments performed using any of these protocols of cytokine delivery should be interpreted with caution, because they generally result in overexpression resulting in high concentrations of cytokines that may not be representative of physiological conditions and may induce artefactual effects on human cell development and function. Moreover expression is commonly systemic rather than physiologically normal local delivery which is the hallmark of cytokine biology.

To circumvent the need for exogenous administration of cytokines or cytokine-encoding vectors, genetically modified mice expressing human cytokines have been developed [26–28]. The transgenic (over)-expression of a cytokine-encoding cDNA under the control of a constitutive promoter, such as the cytomegalovirus (CMV) promoter, presents the same limitations as the previously described protocols of delivery (i.e., nonphysiological regulation of gene expression). In order to achieve more physiological expression of the human gene, therefore, BAC-transgenesis, in which the entire human gene (including the promoter and all regulatory sequences) is inserted in the mouse genome, should be favored over artificial ectopic expression in the wrong tissues by the use of constitutive promoters, cDNA constructs and the like.

Finally, our laboratories have been developing a method of gene humanization by knock-in replacement, mostly based on the velocigene technology of Regeneron Inc. [19, 29]. This method consists in replacing a portion of the mouse genome (from the initiation codon to the stop codon of a gene of interest) by its human counterpart. As most of the regulatory sequences (including promoter, 5' and 3' untranslated regions (UTR)) are of mouse origin, the transcription of the "humanized" gene is achieved in the most physiological conditions. The genes selected for such gene replacement have to meet at least two main criteria [18, 19]. First, the candidate genes have to encode cytokines that play the major roles in hematopoiesis; these cytokines should not be (fully) cross-reactive from mouse to human and mostly nonhematopoietic cells should produce them. Cytokines made by hematopoietic cells will be produced by the engrafted human cells and need not be provided endogenously. The second criteria relates to the cross-reactivity of the human cytokine on mouse cells. Indeed, in the case of homozygous replacement of a gene, defects in mouse hematopoietic cell development could ensue if the human cytokine is not fully cross-reactive on human cells as the mouse copies are eliminated in this way. On the one hand, this defect in mouse cells can be an advantage because it opens a niche that can be colonized by human cells upon engraftment of HSPCs. On the other hand, defects in these mouse cells could result in health defects or even lethality of the mouse. Therefore, only genes dispensable for health and survival of the mouse can be effectively humanized by knock-in replacement. We have recently reported the generation of three strains of mice with knock-in replacement of cytokine-encoding genes [30–32], all in the RAG2$^{-/-}$ $\gamma_c^{-/-}$ genetic background, and we describe these mice in the following sections.

8.1 Thrombopoietin

Hematopoietic stem cells (HSCs) are rare cells that have the unique properties of self-renewal capacity and the potential to generate all cell types of the hematopoietic system, for the entire life of the organism [33, 34]. Several lines of evidence suggest that functional human HSCs are not efficiently maintained in classical models of HHLS mice: Large numbers of $CD34^+$cells (which contain all HSCs) need to be injected in order to achieve successful engraftment; engraftment levels decline with time, and the serial transplantation of human $CD34^+$cells from a primary recipient mouse to a secondary recipient is highly inefficient, indicating a loss of human HSC (or *scid* repopulating cells, SRC) over time.

Thrombopoietin (TPO) is one of the critical factors secreted in the bone marrow niche and required for the maintenance of functional, quiescent, and self-renewing HSCs [35–38]. The homozygous humanization of the gene encoding TPO by gene replacement [30] ($TPO^{h/h}$) resulted in a reduction in the mouse $Lin^-Sca1^+cKit^+$ (LSK) cell population, which is known to contain mouse HSCs, leaving an open niche for human HSCs. After transplantation of human HSPCs ($CD34^+$cells) into $RAG2^{-/-}\gamma_c^{-/-}$ $TPO^{h/h}$, we observed increased numbers and frequencies of the human cell population known to be enriched in HSCs ($CD34^+CD38^{low}CD90^+CD45RA^+$cells) compared to control ($TPO^{m/m}$) recipient mice. Furthermore, human $CD34^+$cells isolated from the bone marrow of $TPO^{h/h}$ mice had a higher capacity to repopulate secondary recipient mice, showing that genetic humanization of TPO favors the maintenance of more functional and self-renewing human HSCs in the mouse. Phenotypically, this improved function of human HSCs resulted in increased engraftment levels of human hematopoietic cells in the bone marrow, that were maintained without decline for up to 6–7 months [30]. Despite this significant improvement through humanization of TPO, the maintenance and function of human HSCs remains suboptimal in HHLS mice. Additional factors or other creative approaches will be beneficial to reconstruct a more human bone marrow niche in the mouse [39].

8.2 Interleukin-3 and GM-CSF (CSF2)

The genes encoding IL-3 and granuocyte-macrophage colony stimulating factor (GM-CSF) are closely linked in the genome (less than 10 kb apart), and therefore we humanized both genes with a single knock-in replacement event, thus, generating $RAG2^{-/-}\gamma_c^{-/-}$ IL-3/$GM\text{-}CSF^{h/h}$ mice [31]. Both cytokines play important roles in the development and maturation of myeloid cells, but we have so far characterized in detail and reported only the role of GM-CSF in this model.

GM-CSF is critically required for the terminal differentiation and function of lung alveolar macrophages [40, 41]. As human GM-CSF is not cross-reactive on mouse cells, nonengrafted $GM\text{-}CSF^{h/h}$ mice phenocopied GM-CSF-deficient mice

and developed pulmonary alveolar proteinosis due to functional defects in mouse alveolar macrophages [31]. Upon transplantation of human $CD34^+$ cells, human alveolar macrophages developed in IL-3/GM-$CSF^{h/h}$ recipient mice, thereby replacing the defective mouse alveolar macrophages, but they were barely detectable in IL-3/GM-$CSF^{m/m}$ control recipients. Interestingly, in engrafted IL-3/GM-$CSF^{h/h}$ mice, human alveolar macrophages were able to partially rescue the pulmonary alveolar proteinosis phenotype observed in nonengrafted mice of the same strain. Besides the maintenance of lung homeostasis, alveolar macrophages play an important role in the immune response to mucosal infections by production of proinflammatory and antiviral cytokines, such as IL-6 or type I interferons. Accordingly, we observed robust expression of these human cytokines in the lung of engrafted IL-3/GM-$CSF^{h/h}$ mice in response to intranasal infection with influenza virus [31]. This model should be useful for studies of the response to lung mucosal infections or other lung pathologies.

8.3 M-CSF (CSF-1)

Macrophage colony stimulating factor (M-CSF) is another cytokine required for myelopoiesis, particularly for the development of monocytic/macrophage cells. The knock-in replacement of M-CSF in RAG2$^{-/-}$ $\gamma_c^{-/-}$ M-$CSF^{h/h}$ mice did not have any detectable phenotypic effect on mouse myeloid cell populations, suggesting that human M-CSF is at least partially cross-reactive on mouse cells [32]. After transplantation of human $CD34^+$ cells, the percentage of human myeloid cells ($CD33^+CD14^+$) in lymphoid tissues was increased from less than 5% in M-$CSF^{m/m}$ recipients to up to 20–30% in M-$CSF^{h/h}$ mice. These cells were also present in nonlymphoid tissues of M-$CSF^{h/h}$ mice, such as the liver, lungs and peritoneum. Finally, in response to lipopolysaccharide (LPS) stimulation in vivo, M-CSF gene humanization resulted in a 2–3-fold increase in the serum concentrations of human cytokines such as tumor necrosis factor alpha (TNFα) and IL-6 [32]. Major phenotypic and functional differences in monocyte subpopulations exist between the mouse and human species, and models to study human monocytes in vivo are currently lacking [42, 43]. M-CSF-humanized mice should prove a useful tool to study the development, activation, migration, and differentiation of human monocytes subsets in vivo, in homeostatic and diverse pathological conditions.

These three examples illustrate how the humanization by knock-in replacement of cytokine-encoding genes can impact favorably the development, homeostasis and function of human cells in HHLS mice. The same approach can be used to humanize additional cytokines and improve the development and function of other human hematopoietic cell types. Furthermore, we anticipate that the combination of multiple human cytokines in a single recipient mouse will have additive or synergistic effects on human hematopoiesis. This will result in HHLS mice with more complete and functional human innate and adaptive immune responses. Such an improved model will provide a much-needed tool for predictive preclinical human research in vivo.

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