REVIEW

Human lymphohematopoietic reconstitution and immune function in immunodeficient mice receiving cotransplantation of human thymic tissue and CD34$^+$ cells

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Small animal models with functional human lymphohematopoietic systems are highly valuable for the study of human immune function under physiological and pathological conditions. Over the last two decades, numerous efforts have been devoted towards the development of such humanized mouse models. This review is focused on human lymphohematopoietic reconstitution and immune function in humanized mice by cotransplantation of human fetal thymic tissue and CD34$^+$ cells. The potential use of these humanized mice in translational biomedical research is also discussed.

INTRODUCTION

The need for humanized mouse models is largely driven by the fact that many findings from conventional animal models do not apply to humans due to the intrinsic differences between species. Humanized mice with functional human hematopoietic and lymphoid systems are particularly useful in the study of pathogens and therapies that specifically target human lymphohematopoietic cells. Over the last two decades, a number of humanized mouse models have been created for the study of human immune responses *in vivo*.[1] Among these models, two are particularly worth mentioning. One is created by injecting human CD34$^+$ cord blood cells into newborn immunodeficient mice.[2,3] Although transplantation of hematopoietic stem/progenitor cells has been inefficient in achieving human T cell development in adult mice,[4,5] human thymopoiesis and T-cell development in the recipient mouse thymus can be achieved by transplantation of human CD34$^+$ cells into newborn immunodeficient mice.[2,3] However, the thymus in these mice remained small with a cellularity of approximately 3×10^5, which is less than 1% of an immunocompetent mouse thymus, indicating inefficient human thymopoiesis in the mouse thymus.[2] Furthermore, although such humanized mice can mount *in vivo* immune responses to viral infections (e.g., Epstein–Barr virus),[2] there is emerging evidence indicating the failure of these mice to develop efficient antigen-specific immune responses.[6–9] Furthermore, these mice were unable to produce T cell-dependent antibodies after immunization with antigens.[9,10] This is likely due to a lack of human leukocyte antigen (HLA)-restricted antigen recognition by human T

cells, as improved antigen-specific human T-cell and antibody responses were achieved by CD34$^+$ cell transplantation in transgenic mice expressing HLA molecules.[6–8,10] The thymus is also important for determining the major histocompatiblity complex restriction of human regulatory T cells (Tregs).[11] Moreover, thymic stromal lymphopoietin expressed by thymic epithelial cells within the Hassall corpuscles are essential for the development of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the human thymus.[12] Thus, although CD4$^+$Foxp3$^+$ cells are detectable in newborn mice receiving CD34$^+$ cells,[13] further studies are needed to assess the function of human Tregs in these mice, as hematopoietic cells do not express thymic stromal lymphopoietin[14] and human cells do not react to mouse thymic stromal lymphopoietin.[15] The other humanized mouse model is created by cotransplantation of human fetal thymic tissue and CD34$^+$ cells, which is discussed and illustrated in this review.

HUMAN LYMPHOHEMATOPOIETIC CELL RECONSTITUTION IN IMMUNODEFICIENT MICE RECEIVING COTRANSPLANTATION OF HUMAN FETAL THYMIC TISSUE AND CD34$^+$ CELLS

The first humanized mouse model with durable human thymopoiesis and T-cell development was established by implanting human fetal thymic (Thy)/liver (Liv) tissues under the renal capsule in CB17-scid mice by McCune and colleagues,[16] after they confirmed the potential of human fetal thymic tissue to grow in these mice.[17,18] The surviving Thy/Liv graft was found to be similar to that of normal human bone marrow, and to contain hematopoietic stem cells (HSCs)/progenitors

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that are capable of forming colonies of myelomonocytic and erythroid lineages *in vitro*.[16] Improved human thymopoiesis and T-cell development were later achieved by implantation of human fetal Thy/Liv tissues into NOD/SCID mice[19,20] that have significantly reduced natural killer (NK) cell activity and less mature macrophages.[21–23] However, in general, the human Thy/Liv-grafted mice showed poor reconstitution with human B and myeloid cells.[20] Furthermore, these humanized mice failed to develop efficient *in vivo* immune responses.[19,20] Since bone marrow-derived antigen presenting cells (APCs) play an important role in the induction of antigen-specific T-cell responses,[24–26] the lack of reconstitution with non-T cell human lineages (e.g., APCs)[20] may be responsible for the insufficient *in vivo* immune responses of human T cells in human Thy/Liv-grafted mice.

One strategy that was used to improve reconstitution with non-T-cell lineage human hematopoietic cells was intravenous injection of human hematopoietic stem cells into human Thy/Liv tissue-grafted mice. Since injection of ficolled human fetal liver mononuclear cells failed to achieve sustained human T-cell reconstitution,[17] purified human $CD34^+$ cells were used.[19,20] Cotransplantation of human Thy/Liv (under the renal capsule) and $CD34^+$ fetal liver cells (i.v.) significantly improved both reconstitution with human B cells and myeloid (including $Lin^-CD11c^+HLA-II^+$ myeloid and $Lin^-CD123^+HLA-II^+$ plasmacytoid dendritic cells) cells, and T-cell development in NOD/SCID mice.[19,20] Almost all major human T-cell subsets, including CD4, CD8, Treg and invariant natural killer T (NKT) cells, were detected in these mice. Human CD4 and CD8 T cells expressing HIV coreceptors, CXC chemokine receptor type 4 and CC chemokine receptor type 5, were also detected in the spleen and intestine of these humanized mice (Habiro K and Yang YG, unpubl. data, 2006), suggesting the suitability of these mice for the study of HIV pathogenesis. Furthermore, the humanized Thy/HSC mice showed significant repopulation with human $\gamma\delta TCR^+$ intraepithelial T lymphocytes, mostly $CD4^-CD8\alpha\alpha^+$ and $CD4^-CD8^-$, in the intestine (Habiro K and Yang YG, unpubl. data, 2007; Figure 1). The spleen from these humanized mice showed formation of white pulp, and a follicular structure was observed throughout the organ.[20,27] Similar results were also observed in subsequent studies from other groups, and such human Thy/Liv/$CD34^+$ cell-grafted mice were later called bone marrow–liver–thymus mice.[28,29] We recently demonstrated that implantation of human fetal liver tissue is unnecessary, and humanized mice with functional human lymphohematopoietic systems can be achieved by transplantation of fetal thymic tissue (under the renal capsule) and $CD34^+$ cells (i.v.) (Figure 2). Similar to Thy/Liv/$CD34^+$ cell-grafted mice, human thymic grafts in these mice underwent significant growth (with a cellularity of $>2\times10^8$) and

maintained a normal phenotypic distribution of human thymocyte subsets. Therefore, for the sake of simplicity, hereafter we refer to both Thy/Liv/$CD34^+$ cell- and Thy/$CD34^+$ cell-grafted mice as humanized Thy/HSC mice. Omission of liver tissue implantation makes intravenous injection of $CD34^+$ cells the only stem cell source for T-cell development, which thereby improves the ratio of transgene-expressing T cells (e.g., transgenic TCR) in humanized mice receiving genetically manipulated $CD34^+$ cells. The IL-2 receptor γ-chain (also known as common γ-chain) is an important component of the receptor complex for several interleukins including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, which are crucial for the development of T, B and NK cells.[30] Because of the complete NK cell deficiency in NOD/SCID/$\gamma c^{-/-}$ (NSG) mice, humanized Thy/HSC NSG mice showed significantly faster reconstitution with human hematopoietic cells than humanized Thy/HSC NOD/SCID mice.

IN VIVO ANTIGEN-SPECIFIC IMMUNE RESPONSES OF HUMANIZED THY/HSC MICE

Xenograft rejection

The immunocompetence and capability of mediating robust *in vivo* immune responses of humanized Thy/HSC mice was first shown by their ability to spontaneously reject porcine skin xenografts.[19,20] The fact that Thy/HSC mice, but not Thy/Liv mice, were able to spontaneously reject porcine skin grafts, indicates that intravenous injection of $CD34^+$ HSCs is critical for establishing a functional human immune system, presumably by improving reconstitution with human APCs and B cells.[20] Injection of human $CD34^+$ HSCs is also important for maintaining long-term immunocompetence because (i) engrafted $CD34^+$ cells are required for sustaining human thymopoiesis and T-cell development and (ii) $CD34^+$ HSC-derived APCs are essential for survival and homeostatic proliferation of human T cells in the periphery.[31] Further studies have demonstrated that Thy/HSC mice are also capable of rejecting porcine islet[32] and thymic xenografts.[33] Humanized Thy/HSC mice with an established human immune system provide a useful and thus far only model for assessing *in vivo* responses and tolerance of the human immune system to porcine xenoantigens.[20,33]

Anti-viral immunity

Humanized mice with a functional immune system are a valuable model for studying human-tropic viral infections, such as HIV and Epstein–Barr virus. Although humanized mice with viral-target human cells (e.g., human T cells for HIV) permit analysis of viral infections, the assessment of antiviral immunity and vaccine therapies requires humanized mice with a functional human immune system.
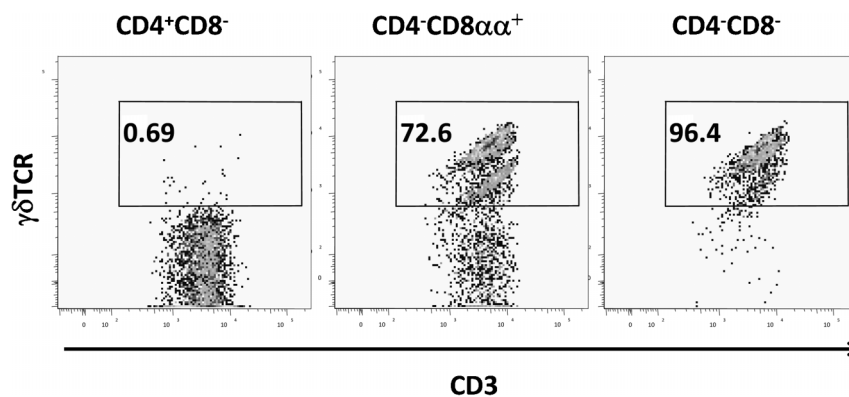

Figure 1 Repopulation with human $\gamma\delta TCR^+$ T cells in humanized Thy/HSC mice. Flow cytometric profiles of intestine intraepithelial T cells from a representative Thy/HSC mouse are shown. Human $\gamma\delta TCR^+$ T cells were also detected at lower levels in the spleen. HSC, hematopoietic stem cell; Thy, thymic.
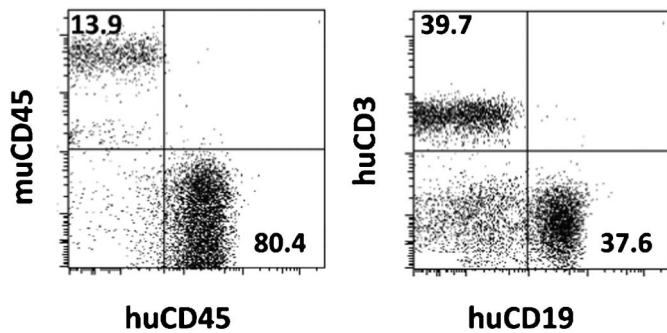
Figure 2 Human lymphohematopoietic reconstitution in humanized mice receiving human fetal thymic tissue (under the renal capsule) and CD34$^+$ cells (i.v.). Representative flow cytometric profiles depicting human chimerism in PBMCs at week 12 are shown. PBMC, peripheral blood mononuclear cell.

Studies by Melkus and colleagues demonstrated the ability of Thy/HSC mice to develop robust HLA class I- and class II-restricted adaptive immune responses to Epstein–Barr virus infection.[28] Humanized Thy/HSC mice were also found to develop robust HIV-specific CD4 and CD8 T-cell and antibody responses following HIV infection.[34] These studies demonstrate the utility of this humanized mouse model for the study of antiviral immunotherapies.

B-cell function and antibody production

The presence of high levels of human IgM and IgG antibodies in the sera of humanized Thy/HSC mice[19] indicates that human B cells developing in these mice are functional. A more detailed analysis of human B-cell function and T–B cell interaction was performed in humanized Thy/HSC mice after *in vivo* immunization with T-dependent antigen, 2,4-dinitrophenyl hapten-keyhole limpet hemocyanin (DNP23-KLH).[27] The immunized, but not control, humanized mice showed strong antigen-specific T-cell responses and T cell-dependent production of dinitrophenyl-specific human IgG (including IgG1, IgG2 and IgG3) antibodies. These results demonstrated functional human T–B cell interactions resulting in B-cell activation, antibody production and class switching in these humanized mice. The development of functional human B cells in these mice was further shown by the production of anti-pig xenoreactive antibodies following porcine xenotransplantation. Previous studies have shown that porcine islet xenograft rejection in humanized Thy/HSC mice was associated with significant intragraft deposition of human IgM and IgG antibodies.[32] Humanized Thy/HSC mice were also found to produce natural and induced cytolytic antibodies against pigs.[33]

Treg development and function

The development of phenotypically and functionally normal human Tregs was also demonstrated in Thy/HSC mice.[35,36] High frequencies of Foxp3$^+$ Tregs was detected in CD4$^+$CD8$^-$CD25$^+$CD127$^-$ thymocytes. Most of these cells were natural Tregs as shown by the expression of Helios,[35] a transcription factor that distinguishes natural Tregs from induced Tregs in humans and mice.[37,38] Most thymic natural Tregs in the Thy/HSC mice were CD45RO$^+$CD45RA$^-$, similar to those in the fetal thymus.[35,36] Human CD4$^+$CD25$^+$CD127$^-$Foxp3$^+$ Tregs were also detected in the blood, spleen and lymph nodes of Thy/HSC mice. Importantly, human CD4$^+$CD25$^+$CD127$^-$ cells from Thy/HSC mice were capable of suppressing the proliferative response of responder T cells to CD3/CD28 or allogeneic stimulation, and their suppressive ability was comparable to that of Tregs isolated from adult human peripheral blood mononuclear cells (PBMCs).[36,39] These

results demonstrate the utility of humanized Thy/HSC mice for the study of human Tregs, including their development and function under physiological and disease settings.

Invariant NKT (iNKT) cell development and function

iNKT cells are a rare cell population that develops in the thymus and recognizes glycolipid antigens presented by non-polymorphic CD1d molecules.[40] iNKT cells produce large amounts of interferon-γ and IL-4 upon activation,[40] and iNKT cell defects have been found to be associated with increased susceptibility to autoimmune diseases and cancers.[41,42] Studies from our group and others[43] have shown the development of functional human iNKT cells in humanized Thy/HSC mice. Flow cytometric analysis using anti-human Vα24-Jα18 and α-galactosylceramide-loaded CD1d tetramer revealed that human iNKT cells were present in PBMCs, spleen, liver, bone marrow and thymus from Thy/HSC mice (Figure 3a),[43] and that the majority of these cells were CD4$^+$ (Figure 3b). Human iNKT cells developing in these mice were functional as shown by production of interferon-γ and IL-4 after stimulation by α-galactosylceramide[43] (Figure 3c). Thus, these humanized mice provide a useful *in vivo* model for the study of human iNKT cell development and function.
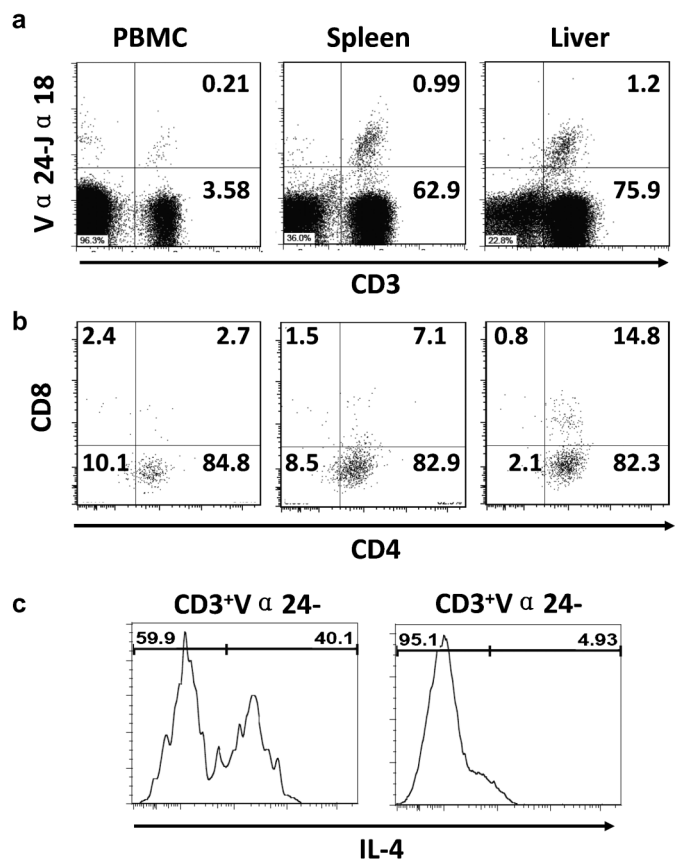

Figure 3 Human iNKT cell reconstitution in humanized Thy/HSC mice. (**a**) CD3$^+$Vα24-Jα18$^+$ iNKT cells in various tissues from a humanized Thy/HSC mouse. (**b**) CD4 and CD8 expression on iNKT cells. (**c**) IL-4 production by iNKT cells from humanized Thy/HSC mice immunized with α-galactosylceramide (i.v.). HSC, hematopoietic stem cell; iNKT, invariant natural killer T; PBMC, peripheral blood mononuclear cell; Thy, thymic.

THE LIMITATIONS IMPEDING HUMAN HEMATOPOIETIC RECONSTITUTION IN IMMUNODEFICIENT MICE

Although immunodeficient mice permit engraftment of human hematopoietic stem cells and blood cell differentiation, it has yet been able to achieve completely normal human hematopoiesis in any of the currently available immunodeficient mouse strains. Current strategies towards achieving this goal are broadly divided into the following two main categories.

Cytokine environment

One of the major strategies for improving human hematopoiesis in immunodeficient mice is to optimize the recipient hematopoietic cytokine environment. Previous endeavors were focused on cytokines, which are essential to hematopoiesis and blood cell development, but have no or poor cross-reactivity between human and mice. For example, IL-15 is important for NK cell development,[44] as are GM-colony-stimulating factor (CSF) and IL-4 for dendritic cells,[45] M-CSF for monocytes/macrophages[46] and IL-3 for myeloid and erythroid cells.[47,48] However, human cells do not respond to murine IL-15,[49] GM-CSF,[50] IL-4,[51] G-CSF[52] or IL-3.[53] Accordingly, treatment with human IL-15–IL-15Rα complexes (IL-15 coupled to IL-15Rα)[54] or enforced expression of human IL-15 by hydrodynamic injection of human IL-15 plasmids[55] were found to be effective in promoting human NK cell development and function in humanized mice. Similarly, enforced expression of human GM-CSF and IL-4 in humanized mice improved reconstitution with human dendritic cells and monocytes/macrophages.[55] Recent efforts have explored the use of human cytokine knock-in mice for improving human lymphohematopoietic reconstitution.[56] In these mice, human cytokine expression was achieved by knock-in replacement of the mouse counterpart cytokine gene to maintain its promoter and regulatory elements. Human IL-3/GM-CSF knock-in mice receiving human CD34^{+} cells showed improved human myeloid cell reconstitution in the lung, and functional mucosal immune responses to lung pathogens.[57] Improved human myeloid cell differentiation and function were also seen in human CSF-1[58] and thrombopoietin[59] knock-in mice receiving intrahepatic injection of human CD34^{+} cells at newborn age. The use of immunodeficient mice with knock-in expression of multiple human cytokines should allow for creating humanized mice with better human hematopoiesis and lymphohematopoietic reconstitution.

Macrophage-mediated xenorejection

It has been noted that human red blood cells (RBCs) and platelets are almost undetectable in human CD34^{+} cell-grafted immunodeficient mice, including NSG mice that have no T, B or NK cells.[29] Although enforced expression of human erythropoietin and IL-3 was found to improve human RBC reconstitution in NSG mice receiving human CD34^{+} cells, the level of human RBCs in these animals remained extremely low compared to the levels of human PBMCs,[55] suggesting that the lack of human RBCs in humanized mice likely resulted from poor survival or xenorejection. Recently, we investigated the possibility of xenoimmune rejection of human RBCs by macrophages in NOD/SCID and NSG mice. Both in vitro and in vivo analyses demonstrated the high susceptibility of human RBCs to rejection by mouse macrophages.[60] Human RBCs from healthy volunteers were rapidly rejected after transfusion into control, but not macrophage-depleted, mice, and macrophage depletion led to recovery of human RBCs in all humanized mice. Previous studies have shown that the inability of donor CD47 to functionally interact with recipient SIRPα poses a barrier to xenogeneic hematopoietic cell transplantation by triggering macrophage-mediated rejection.[61–63] However, the CD47–SIRPα pathway seems unlikely to induce rejection of human RBCs in NOD/SCID or NSG mice, as human CD47 has been shown to cross react with NOD SIRPα.[64] In support of this possibility, rejection of human RBCs in immunodeficient mice on the NOD background was found to be significantly more rapid than the rejection of CD47-deficient mouse RBCs.[60] Macrophage-mediated xenorejection was even more clearly evidenced in the study assessing human platelet reconstitution in humanized mice. Macrophage depletion resulted in full recovery of human platelet reconstitution (i.e., to a similar level as human PBMCs) that would be otherwise extremely low (~1%) in blood circulation in humanized Thy/HSC mice or those receiving CD34^{+} cells along (Hu Z and Yang YG, manuscript submitted). This may explain why human platelet reconstitution was not improved in human CD34^{+} cell-grafted mice with knock-in expression of human thrombopoietin.[59]

CONCLUDING REMARKS

Humanized Thy/HSC mice provide a mouse model that allows human T-cell development in a human thymus and effective human T cell–APC interaction in the periphery. In this model, cotransplantation of human fetal thymic tissue and CD34^{+} HSCs results in a highly functional human immune system with multilineage repopulation of human lymphohematopoietic cells, effective class-switched antibody responses, T-cell responses to viral infections, and spontaneous allogeneic and xenogeneic graft rejection. The humanized Thy/HSC mouse model is considered a valuable model system for the study of human immune function in vivo.

ACKNOWLEDGEMENTS

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