With the recent advances in human-hemato-lymphoid-system mice, this commentary discusses the utility of these mice and further improvements required to generate an accessible system that allows predictive in vivo human hematology and immunology research.

Knowledge on human physiology and pathology, for good reasons, is predominantly gained by observation, cautious, safety-oriented in vivo experimentation, and in vitro surrogate assays. Thus, progress in clinical research is mostly slow, and rigorous scientific proof is often impossible. Given appropriate ethical consideration, most societies agree on research involving worms, flies, and small vertebrates. Because of easy assess and experimental feasibility, laboratory mice have become the main model for in vivo basic and applied biomedical science. However, approximately 65 million years of divergence in human and mouse evolution have shaped these two species that differ substantially in size, lifespan, reproductive activity, and exposure to environmental challenges, e.g., species-specific infectious agents that coevolved. Thus, with regards to hematology and immunology, mice are not men, and this is one of the reasons that achievements in mice are often lost in translation (Mestas and Hughes, 2004).

Increasing use of clinical hematopoietic-cell transplantation in malignant disease and immunodeficiencies, as well as the rise of the devastating HIV pandemic, led to the need of predictive testing assays, and thus experimentation with human-hematolymphoid system mice took off almost 20 years ago, resulting in major model improvements over the last few years. The detailed history and state of the art in this field has been reviewed (Legrand et al., 2006b; Macchiarini et al., 2005; Shultz et al., 2007). This commentary focuses on the current status and the necessary progress to be made in order to meet future expectations.

**Development of Human-Hematolymphoid-System-Permissive Mice**

For hematopoietic-cell engraftment, differentiation, and function, some basic requirements need to be met: Cells need to locate appropriately, must not be rejected, and must be supported by the host environment. For xenogeneic transplantation, this requires immune deficiency of the recipient as well as species cross reactivity of homing molecules and differentiation and survival factors, if not provided by transplanted cells themselves.

Mice with a mutation in the \( \text{Prkdc} \) gene, causing severe combined T and B cell immunodeficiency (SCID), led the way to human to mouse hematolymphoid cell and tissue transplantation. In pioneering work, Mosier et al. transferred human peripheral blood leukocytes (hu-PBL-SCID mice) (Mosier et al., 1988); McCune et al. transplanted human fetal liver hematopoietic cells, bone, thymus, and lymph nodes (SCID-hu mice) (McCune et al., 1988); and Dick and coworkers engrafted mice with human bone marrow cells (Lapidot et al., 1992). Both hu-PBL-SCID and SCID-hu mice are used for studying some aspects of hematolymphopoiesis and adaptive immune responses; however, several issues limited their broad use: hu-PBL-SCID mice produce recall, but rarely produce, if any, primary immune responses, and transplanted human T cells are activated in the xenogeneic environment; SCID-hu mice require multiple human fetal tissues and are thus labor and cost intensive, and again, primary immune responses were rarely detected (Legrand et al., 2006b; Macchiarini et al., 2005; Shultz et al., 2007).

A crucial step forward was achieved by crossing nonobese diabetic (NOD) and SCID strains in 1995: These NOD-SCID mice display additional defects in innate immunity as NK cell, macrophage, and complement deficiencies, leading to 5- to 10-fold greater human chimerism upon hematopoietic stem cell (HSC) and progenitor-cell transplantation compared to SCID mice (Shultz et al., 2007). Consequently, NOD-SCID mice became the gold standard in vivo model for human hematopoietic readout. However, because of lack of human T and B cell maturation, NOD-SCID mice did not satisfy the need for immunology research. Furthermore, most NOD-SCID mice develop lethal thymomas, limiting long-term follow up (Shultz et al., 2007).

Proof that cytokine-receptor targeting and further NK-cell depletion in NOD-SCID mice permit even higher engraftment rates and human T cell development in the mouse thymus came from addition of IL-2Rβ antibodies to pretransplantation conditioning (Kerre et al., 2002). Concurring with this work, the most recent improvement was achieved with the use of two new NOD-SCID mouse strains...
immunity

The upper panel shows spleen sections of a newborn intrahepatic CD34+-cord-blood-cell-transplanted Rag2−/−Il2rg−/− mouse. The lower panel shows control stains on human tonsils for comparison.

deficient for the common cytokine-receptor γ-chain (Il2rg−/−), common to the IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 receptors (Ishikawa et al., 2005; Ito et al., 2002; Shultz et al., 2005) and BALB/c, but not C57BL/6, Rag2−/−Il2rg−/− mice (Gimeno et al., 2004; Traggiai et al., 2004). All strains lack mouse T, B, and NK cells have no obviously high rate of tumor development and show normal life spans. Engraftment and differentiation of human HSC and progenitor cells are most efficient, when transplants are injected into newborn mice, i.e., in a setting where the recipient organism is naturally set for maximal hematolymphoid-system expansion, a situation reminiscent of hematopoietic-cell transplantation in human infants. Human CD34+ HSC and progenitor-cell transplantation into sublethally irradiated newborn NOD-SCID Il2rg−/− or BALB/c Rag2−/−Il2rg−/− mice leads to differentiation and more than half-year maintenance of all major cell populations of the human hematolymphoid system, including dendritic cells, natural-interferon-producing cells, T cells, B and immunoglobulin-producing cells, and, to somewhat lesser extent, NK cells, myelo-monocytic cells, platelets, and erythropoiesis, as well as formation and structuring of major hematolymphoid organs (Gimeno et al., 2004; Ishikawa et al., 2005; Traggiai et al., 2004). An example illustrating similarities of lymphoid-tissue structures in humans and newborn CD34+-cord-blood-cell-transplanted Rag2−/−Il2rg−/− mice is shown in Figure 1.

Human-Hematolymphoid-Cell Engraftment, Differentiation, and Function in Permissive Mice

Because up to now in vitro assays do not allow measurement of HSC maintenance or expansion, laboratory animals have been essential in HSC research. Single syngeneic mouse HSCs are able to generate greater than 50% hematopoietic chimerism for at least 10 months, and 100 syngeneic-HSC-enriched cells are capable of rescuing from hematopoietic lethal irradiation and of re-establishing HSC homeostasis, whereas at least ten times greater numbers are needed for radioprotection of fully MHC-mismatched recipients. HSC transplantation is currently the only clinical broadly used, long-term proven stem cell therapy. In four to six HLA-antigen-matched, unrelated human-cord-blood-cell transplantation, a minimum threshold number of 1.7 × 10^6 CD34⁺ HSC and progenitor cells/kg recipient weight has been suggested, and graft durability has been demonstrated for over 15 years (Grewal et al., 2003). In contrast, cell doses needed for robust (>10%) human to mouse xenogeneic engraftment are in a range of 5 to 50 × 10^6 CD34⁺ cells/kg recipient body weight (i.e., more than 10- to 100-fold greater); homeostatic expansion is not observed, and, although serial transplantation is possible, the frequency of SCID-repopulating cells (SRCs) and chimerism declines over time. Thus, human HSCs seem not to be maintained or expanded in currently used mice. Although some of this might be because of intrinsic differences in mouse versus human HSCs (e.g., different cycling times [McKenzie et al., 2006]), it is most likely to also reflect insufficient homing and cross reactivity of extrinsic, HSC-nurturing factors in respective niches. Thus, additional provision of human components might be beneficial.

Table 1 depicts cross reactivities of some hematolymphopoiesis supporting cytokines; it should be noted, though, that cross reactivity does not equal biologic activity. Furthermore, faithful expression will be critical because overexpression or injection of, for example, cytokines will not provide appropriate stimulation for steady-state hematolymphopoiesis. For human HSC maintenance, addition of human thrombopoietin and cotransplantation of human mesenchymal stroma cells to rebuild a human bone-marrow microenvironment might be useful (Muguruma et al., 2006) (Table 2). Although in vitro myeloid differentiation is readily achieved in colony-forming assays, human myeloid and erythro-thrombocytic readout is weak in currently used mice (Ishikawa et al., 2005; Legrand et al., 2006b; Shultz et al., 2007). As discussed above, in mice, few syngeneic HSCs are able to radioprotect recipients; radioprotection is a process dependent on erythro-megakaryocyte differentiation. In contrast, hematopoietic-cell transplantation in lethally conditioned humans regularly requires transient erythrocyte and thrombocyte transfusions, in part reflecting longer cellular differentiation times. In my laboratory, we did not observe radioprotection upon lethal irradiation of mice with high doses of human hematopoietic cells, nor were we able to detect robust numbers of human erythrocytes in mouse blood by using blood-group-testing systems (unpublished data).
Lymphoid-organ formation and structuring
Deletion of mouse IL-2R

Human myeloid-cell differentiation
Deletion of nonlethal, weak or not cross-reactive mouse cytokines;

Limitations
Possible Solutions

Table 2. Human-Hemato-Lymphoid-System Mice: Limitations and Some Potential Solutions

<table>
<thead>
<tr>
<th>Limitations</th>
<th>Possible Solutions</th>
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<tbody>
<tr>
<td>Human hematopoietic stem cell maintenance</td>
<td>Faithful expression of human cytokines (e.g., thrombopoietin); creation of humanized stem cell niche (e.g., human mesenchymal stem cell co-transplantation)</td>
</tr>
<tr>
<td>Human myeloid-cell differentiation</td>
<td>Deletion of nonlethal, weak or not cross-reactive mouse cytokines; faithful expression of preferentially weak or nonmouse cross-reactive human counterpart cytokines (e.g., GM-CSF, IL-3); deletion of mouse myeloid cell components (transient pharmacologic or genetic deletion)</td>
</tr>
<tr>
<td>Human T and B cell selection and maintenance</td>
<td>Deletion of mouse MHC class I and II; expression of human MHC class I and II (preferentially haplotypes)</td>
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<tr>
<td>Lymphoid-organ formation and structuring</td>
<td>Deletion of mouse IL-2R(-chain) instead of common γ-chain</td>
</tr>
<tr>
<td>Standardized human cell transplants</td>
<td>Generation of human HSCs from embryonic stem cells or multipotent adult progenitor cells; in vitro expansion of HSCs</td>
</tr>
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Cross-reactivity does not equal biologic activity.

Thus, besides species-intrinsic differences in cell size, differentiation, and survival dynamics, insufficient human myeloid-erythromegakaryocyte differentiation in mice possibly also results from inappropriate cytokine cross-reactivity. Furthermore, because recipient mice have grossly intact myeloid cellular compartments, there is no deficiency to be compensated, and human cells are likely to be outcompeted by mouse cells in recovery of irradiation-induced cytopenia. Thus, it is reasonable to expect that nonlethal genetic deletion of insufficient cross-reactive mouse myeloid growth factors, and replacement by respective human ones that ideally would not stimulate mouse receptors, will lead to improved human cell differentiation. In addition, transient deletion of mouse myeloid cells might be a reasonable approach.

In reconstituted mice, human NK cells are detected at low frequencies, but in vivo, functional properties have not been studied. Functional human dendritic cells (DCs) and natural type 1 interferon-producing cells (IPCs or plasmacytoid dendritic cells) are present in the bone marrow, spleen, lymph nodes, thymus, and liver, and Langerhans-cell differentiation was observed at least in one report (Palucka et al., 2003; Traggiai et al., 2004). Thus, in vivo antigen processing and presentation on human MHC should be possible.

The major improvement achieved by transplanting human HSC and progenitor cells in newborn NOD-SCID Il2rg<sup>-/-</sup> and Rag2<sup>-/-</sup>Il2rg<sup>-/-</sup> recipients is efficient intrathymic de novo human T cell development (Gimeno et al., 2004; Ishikawa et al., 2005; Traggiai et al., 2004), a process recapitulated weakly in adult transplanted NOD-SCID Il2rg<sup>-/-</sup> recipients upon addition of human IL-7 (Shultz et al., 2005). Thymic-generated cells include CD4<sup>+</sup> and CD8<sup>+</sup> T cells with a broad V<sub>β</sub> distribution, Foxp3<sup>+</sup> CD25<sup>+</sup> regulatory T cells, and γ<sub>δ</sub> T cells in fairly physiologic ratios. Upon maturation, T cells exit the thymus and seed secondary lymphoid organs. Isolated from these sites, the generated T cells are capable of proliferating and producing cytokines upon unspecific stimulation. What MHC restriction is to be expected from human T cells educated on a mouse background? Mostly mouse-generated data demonstrate that under normal developmental conditions, positive selection preferentially occurs on epithelial cells, whereas both epithelial cells and hematopoietic-derived DCs are involved in negative selection. As predicted by embryonic germ-layer derivation, we did not observe human thymic epithelial cells in CD34<sup>+</sup>-cell-transplanted mice, whereas both mouse and human cells constitute the thymic DC compartment (Traggiai et al., 2004). Human thymocytes should thus be positively selected on mouse, whereas negative selection could occur on both mouse and human MHC. However, it is reasonable to expect that thymocytes would continue to interact with mouse MHC during successive selection. Complicating the issue, several studies on mice point out that under certain conditions, hematopoietic offspring cells are involved in positive selection (e.g., Zinkernagel and Althage [1999]); moreover, species-specific differences might exist, allowing human thymocyte-thymocyte MHC class II interaction, and thus at least some CD4<sup>+</sup> T cell selection might occur on human MHC (e.g., Choi et al. [2005]). Once T cells exit the thymus, they depend on homeostatic factors, both MHC, and cytokines for survival. For the mice discussed here, human MHC is only present on hematopoietic but no other tissues. Presentation of both “self” and “nonself” antigens
will then depend on cellular tropism, as well as the efficacy of cells to take up extracellular antigens and present these in context of both MHC class I and II.

So what human T cell responses are observed in this complicated situation? First, in mixed lymphocyte reactions (MLRs), human T cells proliferate vigorously when stimulated with human allogeneic DCs but proliferate weakly or not at all when stimulated with human autologous DCs. Proliferative response to mouse DCs is overall low; however, T cell proliferation is stronger when stimulated with fully mismatched than with host-mouse-type DCs (Traggiai et al., 2004). Second, human T cells generated in mice display activity against human allogeneic target cells that could be blocked with human MHC class I or II antibodies, respectively (Ishikawa et al., 2005). Third, human T cells in mice mount some responses to in vivo infection with Epstein-Barr virus, mimicking to some extent infectious mononucleosis. Frequently, however, they were not capable of controlling EBV-driven B cell proliferation (Traggiai et al., 2004). Interestingly, by further testing of HLA-A2 and HLA-B8 cord blood CD34+ cell-transplanted mice, tetramer-staining positive CD8+ T cells were not detected with most commonly recognized EBV specific epitopes (unpublished data), whereas in acutely infected human beings, often up to half of CD8+ T cells show EBV specificity. Fourth, upon mouse infection with influenza virus, human T cells specific for viral epitopes are only observed in the context of mouse MHC (Legrand et al., 2006b); and, although based on limited data, robust specific T cell responses in HIV-infected mice have not been demonstrated so far (e.g., Baenziger et al., 2006). Last, no relevant homeostatic cell expansion was observed when mouse-derived human T cells were transferred to non-transplanted Rag2−/− Il2rg−/− mice. Along the same lines, others observed high peripheral T cell turnover rates and lack of long-term T cell maintenance (Legrand et al., 2006b).

In summary, current data allow no firm conclusion on the biology of T cell selection in this setting but suggest T cell tolerance, i.e., possibly negative selection, for both autologous human and mouse MHC. Reactions, at least against human allogeneic MHC, seem to be frequent, and rare specific responses in the context of autologous human and mouse MHC might be produced. These considerations do not account for insufficient cross-species co-stimulation and, overall, stirs some concern that any human T cell response observed in described settings might be the result of an “in vivo artifact.” Although this might be an opportunity to study some aspects of T cell biology, the obvious appropriate and urgently needed solution for generating robust human T cell responses and maintenance is replacement of mouse by human MHC components (Table 2); this replacement creates, at least for thymic selection, a similar situation as in mice cotransplanted with same human donor fetal thymic tissue. Along this way, it will be interesting to evaluate human T cell development in the absence of tissue MHC, a situation mimicking allogeneic hematopoietic cell transplantation in human MHC deficiencies.

Further indicating the impact of environment, the relative distribution of human cells reflects values observed in mice rather than in human blood. Indeed, B cells generated in the bone marrow and seeding secondary lymphoid organs are the major human cell population in transplanted animals (Legrand et al., 2006b; Macchiarini et al., 2005; Shultz et al., 2007). Maturation to human antibody-producing cells including class-switch recombination was only observed in mice with human T cell development, suggesting provision of some help. Human IgG concentrations increase over time and are on average approximately two logs and one log lower than in human adults and wild-type laboratory mice, respectively (Baenziger et al., 2006; Traggiai et al., 2004). Specific IgG antibody responses to T cell-dependent antigens such as tetanus toxoid, ovalbumin, and HIV epitopes are observed. However, absolute concentrations are low, and responses lacked consistency (Baenziger et al., 2006; Ishikawa et al., 2005; Traggiai et al., 2004), possibly in part because of inadequate T cell help. Although human cells (probably lymphotoxin-α-expressing B cells) induce formation of mouse follicular dendritic cells (FDCs), lack of human FDCs is likely to further impinge on B cell responses (Traggiai et al., 2004). Thus, providing adequate human MHC as well as supporting B cell maturation by addition of human stroma cells with FDC differentiation capacity (Munoz-Fernandez et al., 2006) should lead to improved humoral responses (Table 2).

Upon human CD34+ cell transplantation to newborn NOD-SCID il2rg−/− and Rag2−/− il2rg−/− recipients, newly formed human cells seed the thymus, spleen, and mesenteric lymph nodes, thus leading to an increase of these organs’ sizes. At least in thymus and spleen, 3D structures are formed that resemble physiologic organization, indicating that some cross-reactivity in organizational structures, e.g., chemokine networks, exist (Traggiai et al., 2004 and Figure 1). In contrast, mesenteric-lymph-node organization is less sufficient, and peripheral-lymph-node and intestinal-lymphoid-tissue formation does not compare well with wild-type mice (Legrand et al., 2006b; Macchiarini et al., 2005; Shultz et al., 2007). This might be because of the lack of IL-7R signaling and insufficient formation of lymphoid tissue anlage in il2rg−/− mice. Thus, the use of il2rb−/− instead of il2rg−/− mice might be a solution. However, because Rag2−/− il2rg−/− newborn transplantation with wild-type mouse bone-marrow cells or HSCs rescues peripheral-lymph-node development in recipients, additional defects in cross-species interactions of, for example, homing molecules are probable (Coles et al., 2006) and unpublished data.

Finally, with currently available human HSC and progenitors cells, engraftment levels vary, probably depending on intrinsic differences of donor cells as well as on pretransplant cell handling, and usually less than 10 animals can be transplanted from one graft. For broad use of these models as preclinical testing tools, it thus will be important that larger, standardized,
“off-the-shelf” hematopoietic transplants matched to human MHC components expressed on the mouse background will be available. This might be achieved by HSC expansion (use of genes with oncogenic potential will be less of a concern compared with clinical transplantation) and by human embryonic stem cell (ESC) or multipotent adult progenitor cell (MAPC) to HSC differentiation (e.g., Serafini et al. [2007] and Table 2).

Current and Future Applications

Given all the discussed complexities and limitations, what major scientific achievements have been made, and what might be reached given appropriate improvements of models that would justify the effort? No doubt, hematology research thus far profited most with characterization of human HSCs or SRCs, progenitor cells, and leukemia stem cells (LSCs), including identification of strategies to selectively target LSCs (Jin et al., 2006). Improved models are adding to this field as greater, more durable engraftment and lymphoid-lineage diversity is achieved, allowing, for example, genetic interference (e.g., Gimeno et al. [2004]). Furthermore, they might be permissive for engraftment of diseased cells, e.g., myeloproliferative syndrome cells. Reflective of above discussed limitations, possibilities in immunology research are just emerging, and similar impact on basic and clinical immunology still needs to be achieved. With recent improvements, current models are already useful to investigate in vivo issues such as pathology and intervention in human-specific lymphoptropic infections, effects of hematopoietic- and immune-system gene therapy, and lymphoid-system interactions with cancer (e.g., Aspord et al. [2007]) and cancer stem cells.

Given that ongoing efforts to achieve a fully functional human immune system in mice will work (Table 2), the breadth of possible use is going to be tremendous, including the following: in vivo manipulation of dendritic, T, and B cells, generation of T and B cell responses to autoantigens or tumors, preclinical vaccine testing, and evaluation of human-prion-disease immune-system interaction. Finally, powerful complementary technologies are coemerging as the capacity to replace large segments of the mouse genome with human sequences (e.g., T cell- and B cell-receptor components) or the ability to rapidly identify and reproduce human in vivo-selected high-affinity, neutralizing antibodies to infectious agents; all of these capabilites will enhance the field. Thus, it is anticipated that the use of improved, easy-to-generate, and broadly available human-hematolymphoid-system mice will gain momentum and impact on both basic and applied preclinical human immunology research over the coming years.

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