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Review

The saga of the humanized mouse: a giant leap into the galaxy or a small step for humankind?

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Abstract

Laboratory animals have contributed greatly in understanding human physiology and pathology. However, differences in biology do not allow us to definitively interpret the results of experiments in animals. Transplanting human cells and tissues into mice has therefore been one of the milestones in humanizing laboratory animals. However, this necessitates complete replacement of the mouse system by the human system, the most explored of which is the immune system. But this has not been easy as each phenotypic deletion in mice has unraveled the complexity of host (mouse) defense mechanisms and at the same time shed more light on the development of the human immune system. Because humanized immune mice lack the armory to reject xenotransplants, they are being extensively used to implant and study other human systems, including infectious diseases. This review summarizes the history of humanizing the mouse, the technical advancements, the knowledge gained in the process, their use as well as the outlook in this fascinating and rapidly advancing field.

Keywords: humanized mice, transgenic, immunology

Overview

Social, ethical and economical considerations as well as technical limitations have constrained the study of physiology and pathology directly in humans and higher primates. Understanding biology from molecular to organismal level has included both expansionist and reductionist approaches. The problem has been in sythesizing them together because the reductionist methods do not appropriately reflect the complex interactions between molecules, tissues, organs, and organ systems *in vivo*. On the other hand, *in silico* modeling or *in vitro* reproduction of complex phenotypes is almost impossible. While gross pathology and exploratory studies have taught us a lot about normal and abnormal processes, microscopic and molecular studies have yielded the finer details of the underlying mechanisms as well as how to tackle diseases. Experimental animals have been used as surrogates to create human-like systems. Unlike humans, mice and rats can be manipulated by experimentally inducing disease, and the diseased tissue can be sampled at various stages when alive or *post mortem*. In addition, genetically modified or transgenic mice can be used for functional analysis of gene function(s) *in vivo*. Such animal models have provided important fundamental insights into biological processes. Indeed, the slogan "laboratory animals save more lives than the emergency telephone number" may not be an overstatement (Baenziger *et al.* 2008).

However, direct translation of the results from rodents to humans is intricate. Although similarities in genome, protein structure and function, and physiology are very high, humans and mice differ substantially. Thus one of the greatest reasons for major triumphs in mice often being lost in translational research is that mice are not men (Mestas and Hughes 2004, Steinman and Mellman 2004; Lin, 2008). Moreover, the phenotype of each inbred strain differs (Diwan *et al.* 1986; Zumbach *et al*. 2001), leading to potentially differing interpretations of results. Further, the mouse immune system rejects any transplant which is used study the biology of the implanted cell, tissue or organ. This has led to the discovery and engineering of several strains of mice which now serve as the basis to reconstitute human biological systems. However, the progress has been slow because ablation of a specific arm of the immune system leaves another arm at freedom to attack the transplant. Based on the knowledge accumulated over the years, humanized mice have been generated to investigate human hematopoiesis, innate and adaptive immunity, autoimmunity, infectious diseases, cancer biology, stem cell biology and regenerative medicine. However, further improvements are needed to achieve completely humanized mice.

A crash course in immunology

In order to understand the history of humanized mouse, it is essential to understand the biology of the immune system. The lymphoid organs can be divided into primary and peripheral. The primary lymphoid organs – bone marrow (BM) and thymus – generate and educate lymphocytes. While all lymphocytes originate in the BM, T and B cells mature in the thymus and the BM, respectively. The peripheral lymphoid organs – mainly spleen and lymph nodes (LN) – initiate and sustain specific responses. The cells of the immune system include granulocytes (neutrophils, basophils, eosinophils), lymphocytes, monocyte-macrophages, dendritic cells (DCs), natural killer (NK) cells and mast cells.

Immunity can be classified into innate and adaptive. Innate immunity is inborn and not specific to any pathogen. It is initiated by the activation of a group of receptors, including toll-like receptors (TLRs), by molecular patterns frequently encountered on pathogens. A variety of soluble factors, including lysozyme, interferons (IFNs), complements, collectins and acute phase proteins as well as phagocytes and NK cells contribute to innate immunity. Complements, which are activated either by certain pathogen molecules or via antibodies bound to pathogens, function by killing pathogens directly or by recruiting and/or aiding in the function of phagocytes that then clear the pathogens. IFN-α, β or γ block viral replication and also activate cells of both innate and adaptive immunity. NK cells act by recognizing aberrant cells and responding rapidly with the release of cytolytic granules and cytokines.

Adaptive immunity is initiated only upon exposure to antigen, and is characterized by specificity, diversity, memory, and self/non-self recognition. The specificity of each T and B cell is determined during maturation in the thymus or BM even before its contact with antigen. By a combination of multiple gene rearrangement and mutations, adaptive immunity generates an enormous diversity to recognize an infinite variety of antigens. This generation of diversity in B and T cell receptors (TCR) is mediated by recombination activating gene 1 and 2 (enciphered by *Rag1* and *Rag2* genes) as well as other proteins. Exposure to antigen activates T and B cells, and expands the population of cells with a given antigenic specificity, and produces effector (immediately functional) and memory (long-lasting) cells. Unlike B cells which recognize antigen directly, T cells can only recognize antigen bound to major histocompatibility complex (MHC) molecules (human leukocyte antigen or HLA in humans), and presented by antigen presenting cells (APC). There are two classical MHC molecules: class I, expressed by nearly all nucleated cells of vertebrate species, and class II, expressed only by specialized APC (B cells, monocytes, macrophages, DCs). Both are heterodimeric, class I containing a heavy chain and β_2-microglobulin (β_2m), class II made of α and β chains. T cells are educated to recognize non-self antigens presented by self MHC molecules.

Antibodies, or immunoglobulins (Ig's), of various types and subtypes, are the effector molecules of B cells. IgM is the first Ig produced upon exposure to an antigen (a primary response), as well as in neonates. IgG is the most abundant Ig in the serum, appears after IgM, is predominant in secondary or booster responses, and is produced by a phenomenon called class switching. Antibodies carry out their functions by inhibiting the invasion of pathogens, or by activating complements, or by aiding macrophages and NK cells to engulf or destroy pathogens.

There are two major subpopulations of T cells: helper (Th) and cytotoxic (Tc), which carry the surface molecules CD4 and CD8, and are hence typically referred to as CD4+ and CD8+ T cells, respectively. Stimulated Th cells secrete cytokines which activate various cells, including B cells, Tc cells, macrophages, and other immune cells. Th cells are further divided into Th1 and Th2, which originate from the common precursor Th0, depending on the initial signals and cytokine environment. Typically, Th1 and Th2 cells polarize immunity towards cell-mediated and antibody responses, respectively. The Tc cells exhibit cytotoxic activity mediated through the exocytosis of granules containing granzyme and *perforin*, and inhibit intracellular pathogens via the secretion of IFN-γ, thus playing a vital role in monitoring and eliminating aberrant cells, such as virus-infected cells, tumor cells, and cells of a foreign tissue graft. The other important T cell subpopulation is the regulatory T cell (Treg), which acts in controlling immune responses by regulating various pathways. NK cells function like Tc cells but are a part of innate immunity. NK cells express two distinct families of receptors: the activating receptors and the inhibitory receptors, belonging to one of the CD94/NKG2, Ly49, killer-cell Ig-like receptor (KIR), or leukocyte immunoglobulin-like receptor (LIR) families. In general, the most important inhibitory signal for NK cells is the presence of MHC class I molecules. Lack or absence of MHC class I molecules is the most important reason for NK cell activation, and mice lacking β2m have NK cell deficiency as they lack education during NK cell development.

All the cells of the immune system derive from the same

precursor cells, the hematopoietic stem cells (HSC), in the BM. HSCs are a heterogeneous population of cells with long-term and short-term regeneration capacities and differential repopulation kinetcs. *In vivo*, they are mostly found in the BM, but can be isolated from several fetal tissues (liver, spleen, aorta, and gonads), umbilical cord blood, placenta, peripheral blood as well as adult tissues like skin. The HSCs can be defined by the cell surface expression of specific markers, including CD34, and the absence of other markers, including CD38. Contact with stromal cells and soluble mediators secreted by them are necessary for HSCs to differentiate. Two major progenitor lines, myeloid and lymphoid, generate the hematopoietic cells. The myeloid progenitor undergoes thrombopoiesis (platelets), erythropoiesis (erythrocytes), granulopoiesis (granulocytes), monocytopoiesis (monocyte-macrophages, DCs) and differentiation into mast cells. Granulocytes are produced in increased numbers and migrate from blood to sites of infection or inflammation. Macrophages and DCs act as the major initiators of specific immunity.

The lymphoid progenitor produces T, B, and NK cells. T lymphopoiesis occurs in the thymus and absolutely requires contact with thymic stromal cells and the subsequent signal transduction events. The T cells then pass through a series of steps involving no, overlapping or exclusive expression of CD4 and CD8, during which, TCRs undergo gene rearrangement. The T cells, each being clonal but collectively bearing a vast array of specificities, come in contact with self MHC molecules and undergo selection and retention to react to non-self antigen (i.e., self-reactive T cells are deleted). The T cells can belong to one of the αβ (conventional) or γδ type based on the kind of TCR that they express. Other types include NKT and regulatory T cells.

B lymphopoiesis occurs in the BM, whose niche of stromal cells, extracellular matrix and cytokines is essential for the process. B cell development also goes through a series of well defined steps which can be recognized by the surface expression of particular markers. Further maturation of B cells occurs in spleen and lymph nodes. Similar to T cells, B cells rearrange their Ig genes to generate antibodies with a vast variety of specificities. These are then selected specifically on contacting the antigen in secondary lymphoid organs.

The identity of human NK cell precursors, their location, and factors involved in development are not as well defined. Fetal NK cell precursors arise in the liver, thymus, BM, and LN, and the BM is the major location of NK cell development in adults. Different HSC cell populations can generate mature NK cells in the presence of stromal cells and cytokines, and transcription factors play a critical role in the development of NK cells.

The generation, maturation, differentiation and effector functions of various immune cells are initiated or regulated by a variety of soluble mediators. The principle ones are the colony stimulating factors (CSF), interleukins (IL), IFNs, and tumor necrosis factor (TNF). CSFs stimulate HSCs to differentiate into various blood cell lineages, and include macrophage, granulocyte and granulocyte-macrophage CSFs as well as erythropoietin and thrombopoietin. Among the IFNs, only IFN-γ is secreted exclusively by cells of the immune system. It protects cells by initiating molecular antiviral pathways, and along with TNF-α, an inflammatory cytokine, it is involved in activation and regulation of innate and adaptive immune cells.

Interleukins are a group of >30 cytokines which regulate the generation, differentiation and function of immune cells. IL-1, produced by many cells, particularly macrophages and epithelial cells, performs multiple immune and non-immune functions, including the initiation and regulation of acute inflammation. IL-2 is a T cell growth factor, and in synergy with IL-15, it induces and regulates T and NK cell activation, proliferation and function. IL-3 and IL-7 are mostly produced by stromal cells and promote commitment and differentiation of myeloid and lymphoid lineages, respespectively. IL-4 and IL-13 induce Th0 cells to differentiate into Th2 cells, and their action is further supported by IL-10, a pleiotrophic immune regulator. IL-6 has multiple effects, the principal of which is end stage B cell differentiation. IL-8 is a neutrophil chemotactic factor. IL-9 supports IL-2- and IL-4-independent growth of Th cells. IL-11 supports megakaryopoiesis and osteoclast activation. IL-12, produced by APCs upon antigen stimulation, polarizes Th0 cells to differentiate into Th1 cells, which in turn secrete IFN-γ. IL-17, along with IL-1 and TNF-α, mediates delayed type and inflammatory reactions, is produced by Th type 17 (Th17) cells. IL-17, IL-21, IL-22 and IL-23 control the differentiation and function of Th17 cells. IL-10 and TNF-α are the major effectors of immune regulation mediated by Treg cells.

The interleukins function by binding mostly unique, and sometimes shared, cell surface receptors, many of which have oligomeric components. The IL-2 receptor (IL2R) common γ-chain (IL2Rγc), is a common receptor subunit used by IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, and is indispensable for high-affinity ligand binding and signaling of all these cytokines. Knocking out *IL2Rγc* therefore cause profound immunodeficiency. The deficiencies in the immune system can be generated by knocking out genes encoding immune effector molecules like granzyme and *perforin* B or specific cytokines or CSFs. These immunodeficient mice have played an important role in the study of in vivo function of human cells and tissues.

Humanization of mice: the genesis (or transgenesis) and refinement

The "humanized mouse" is one in which human proteins are transgenically expressed or human tissue is transplanted (Nomura *et al.* 2008; Pearson *et al.* 2008). Transgenesis can be used to (a) study immune responses, e.g., identification of antigenic epitopes presented by HLA molecules, (b) study infectious diseases specific to humans through the expression of virus-specific receptors, or by knocking out genes encoding IFNs or their receptors, or TLRs, and (c) validate and further evaluate autoimmune and inflammatory diseases. Study of several diseases, including those caused by hepatitis C, human corona, dengue and other viruses, as well as rheumatoid

arthritis, spondylitis, diabetes, encephalomyelitis etc. has been facilitated by these mice. Alternatively, engraftment of immunodeficient mice with human cells and tissues permits investigation of the development and function of these tissues *in vivo*, and the study of diseases.

Implantation of human cells and tissues (humanization) started with athymic *nude* mice, which lack both T and B cell functions (Segre *et al.* 1995), and other immunodeficient mice, either individually or in combination. However, initial attempts to engraft human hematopoietic cells were not successful. The breakthrough was the discovery of CB17-*scid* mice (Bosma *et al.* 1983), which contain a spontaneous mutation in the DNA-dependent protein kinase gene *Prkdc* (protein kinase, DNA activated, catalytic polypeptide), which is involved in the DNA double-strand repair and in the rearrangements of TCR and Ig gene segments (Blunt *et al.* 1995, Kirchgessner *et al.* 1995; Miller *et al.* 1995). Consequently, the mice lack T and B cell progenitors, and are unable to reject grafted cells or tissues. *Rag1 and Rag2* mutations also result in a similar phenotype (Mombaerts *et al.* 1992; Shinkai *et al.* 1992). Later, it was demonstrated that human fetal tissues and peripheral blood mononuclear cells (PBMC) could engraft to produce human hematopoietic cells in these mice (McCune *et al.* 1988; Mosier *et al.* 1988).

The *Scid*, *Rag1* or *Rag2* mice, however, retain innate immunity, posing a significant barrier to acceptance of the graft, especially of hematopoietic origin (Christianson *et al.* 1996). The major reason is the high level of host NK cell activity, to deplete which, antibodies to asialoGM1 were used unsuccessfully as this also targets human NK and activated $CD8^+$ T cells and macrophages present in the graft inoculum (Habu *et al.* 1981; Yoshino *et al.* 2000). On the other hand, IL-2 receptor β-specific antibodies, which target murine, and not human, NK cells, are a better option (Tanaka *et al.* 1993). Other approaches have targeted neutrophils with anti-Gr1 antibodies, and macrophages with liposome-encapsulated clodronate (Pearson *et al.* 2008). Genetic attempts to reduce innate immunity have included combining *scid* mutation with macrophage abnormalities, reduction in complement activity or defects in NK cell function, e.g., generation of NOD (non-obese diabetic)-*scid* mice (Lowry *et al*. 1996; Pflumio *et al.* 1996; Shultz *et al.* 1995; van der Loo *et al.* 1998). Efforts to further decrease innate immunity have included targeted mutation in β_2m (Christianson *et al.* 1997; Zijlstra *et al.* 1989), *perforin* (Kagi *et al.* 1994; Shultz *et al.* 2003) or granzyme B (Shresta *et al*. 1995) genes, leading to deficiency or reduced toxicity of NK cells.

An obstacle to the development of human hematopoietic cell functions is the lack of cross-reactivity of many mouse hormones, growth factors, and cytokines for the development, survival, and function of these cells (Auffray *et al.* 1994; Uze *et al.* 1990). In addition, many required human-specific factors are produced by nonhematopoietic stromal cells not present in the inoculum. Exogenous factors important for the engraftment, homing, and differentiation of human HSC as well as the function or regulation of the differentiated cells have been used in an attempt to overcome these obstacles (Pearson *et al.* 2008; Chen *et al.* 2009). Alternate approaches have included transgenic expression (Bock *et al.* 1995; Nicolini *et al.* 2004) or manipulating the human HSC inoculum ex vivo, especially with cocktails containing multiple human cytokines (Pearson *et al.* 2008). The latter approach has been hampered by the difficulty in achieving ex vivo expansion of human CD34+ HSC without loss of their capacity to repopulate stem and progenitor cells in vivo (Sorrentino, 2004). Another approach is to use lineage-specific differentiation cytokines to drive human stem and progenitor cells ex vivo (Ishikawa *et al.* 2005; van Hensbergen *et al.* 2006). One more method is to co-inject HSC with human mesenchymal stem cells (MSC), somatic stem cells, or cytokine-transduced stromal cells to provide a stromal environment (Ramasamy *et al.* 2007; Ringden *et al.* 2006; Pearson *et al.* 2008).

Other obstacles in the first generation immunodeficient mice were the the development of murine T and B cells on aging (referred to as 'leakiness'), high occurrence of thymomas and hence short life-span, and the inability to generate human T cells (Bosma 1992; Custer *et al.* 1985; Ito *et al.* 2008a; Pearson *et al.* 2008; Shultz *et al.* 2005). The most recent breakthrough has been the development of mice carrying a mutation in the IL2Rγ chain (Il2rg or Il2rgc) gene, leading to severe impairments in innate and adaptive immunity, including complements, and differentiation and function of APCs (Cao *et al.* 1995; DiSanto *et al.* 1995; Ohbo *et al.* 1996). Combining these with NOD/Shi-*scid* or NOD/LtSz-*scid* phenotype has resulted in NOG and NOD/LtSz-*scid IL2rg* mice, respectively, leading to extreme immunodeficiency derived from the three parental strains of mice: (a) reduced innate immunity derived from the NOD strain, (b) lack of functional T and B cells due to the *scid* mutation, and (c) NK cell, DC, and other unknown deficiencies resulting from inactivation of the *IL-2Rγ* gene.

Lymphoid organs, including thymus and LNs, in both NOG and NOD/LtSz-*scid IL2rg null* mice are immature, atrophic and rudimentary. NOG mice can live as long as conventional inbred mice under strict specific pathogen-free conditions (Pearson *et al.* 2008) whereas half of the NOD/LtSz-*scid IL2rg null* mice die within one year (Shultz *et al.* 2003). The incidence of thymoma in NOG mice is much lower than that in NOD/LtSz-*scid IL2rg null* mice (Ito *et al.* 2008a; Prochazka *et al.* 1992). Similarly, little leakiness is observed in NOD/Shi-*scid* mice, with no leakiness at all in NOG mice (Ito *et al.* 2008a). These mice show fairly complete human immune systems, including thymocytes and peripheral mature T and B cells, myeloid cells, myeloid and plasmacytoid DCs, platelets, and erythrocytes (Ito *et al.* 2002; Shultz *et al.* 2005; Traggiai *et al.* 2004). T cells that develop in these mice have a diverse TCR repertoire and direct antigen-specific IgM and IgG responses after immunization with T-dependent antigens (Shultz *et al.* 2007) in newborn and adult recipients, and by different routes of injection (Ito *et al.* 2008a). Therefore, these mice are closest to being "humanized."

Applications of humanized mice

Hematopoiesis

Transplantations of immunodeficient mice are the most reliable methods to evaluate stages of human hematopoiesis

and the function of human HSCs (Shultz *et al.* 1995; Larochelle *et al.* 1996). However, despite their ability to produce all mature lineages (Galy *et al.* 1995; Yahata *et al.* 2006), HSCs have limited self-renewal capacity in this setting (Ema *et al.* 2005), possibly due to the lack of a special niche (Ando *et al.* 2008). Stem cell niches and key molecules which regulate niche function have been identified in mice (Arai *et al.* 2004; Calvi *et al.* 2003; Kiel *et al.* 2005; Sugiyama *et al.* 2005; Zhang *et al.* 2003), but this may not translate to humans. *In vitro*, MSCs can give rise to cells that produce a number of cytokines and extracellular matrix proteins, and express cell adhesion molecules, all of which contribute to the hematopoietic microenvironment (HME) (Conget and Minguell, 1999; Majumdar *et al.* 1998; Pittenger *et al.* 1999). However, little is known about their in vivo phenotypic and functional characteristics. Although MSCs accelerate the hematopoietic recovery of cotransplanted human HSCs (Koc *et al.* 2000; Noort *et al.* 2002), and donor MSCs may exist in recipient BM (Bensidhoum *et al.* 2004) and reconstitute HME (Muguruma *et al.* 2006), there is no concrete evidence that the transplanted MSCs indeed engraft and function directly

Self-renewal, multilineage differentiation, frequency, surface marker expression, homing, hierarchy, and dynamic behavior have been demonstrated in *scid* mouse repopulation assays (Bhatia *et al.* 1997, Guenechea *et al.* 2001; McKenzie *et al.* 2006; Peled *et al.* 1999). However, in NOD-*scid* mice, $CD34^+$ cells appear to reconstitute only B-lymphoid and myeloid cells and not T cells, although it can be enhanced by exogenous addition of human IL-7 (Hiramatsu *et al.* 2003; Shultz *et al.* 2005). On the other hand, NOD-*scid/β2m*$^{-/-}$ *or RAG2* $^{-/-}\gamma c^{-/-}$ mice demonstrate human T cell development and functionality (Hiramatsu *et al.* 2003; Ishikawa *et al.* 2005; Shultz *et al.* 2005; Traggiai *et al.* 2004; Yahata *et al.* 2002). A lymphoid-like structure develops in the spleen and contains human macrophages and DCs (Watanabe *et al.* 2007), but follicular DCs are not observed (Ito *et al.* 2008a). T cell areas and B cell follicles are observed in the spleen, and B cells differentiate first, followed by development of T cells (Ito *et al.* 2008a). The mice produce antigen-specific IgM, but not much IgG, even with multiple immunizations (Traggiai *et al.* 2004; Legrand *et al.* 2006), possibly due to the overwhelming repopulation of B1 type B cells (Matsumura *et al.* 2003), which are known to produce mainly IgM (Kipps, 1989).

Human T cells generated in these transplanted mice include a fairly normal ratio of $CD4^+$ and $CD8^+$ cells with broad TCR diversity, regulatory and γδ cells (Ito *et al.* 2008a). Mature T cells exit the thymus and home to secondary lymphoid organs, and stages of T cells are consistent with T cell development in humans (Hiramatsu *et al.* 2003, Matsumura *et al.* 2003, Yahata *et al.* 2002). However, human T cells are educated by mouse thymic stroma in these mice. In addition, myeloid lineage cells are not well generated: erythrocytes, neutrophils and platelets are too few in number, although some fully matured erythrocytes can be observed in newborn NOD/LtSz-*scid IL2rg null* mice (Ishikawa *et al.* 2005; Nakamura *et al.* 2006).

As far as NK cell development is concerned, even though differentiated human NK cells arising from the graft express most of the cell surface antigens and activation functions found with fresh human NK cells (Huntington and Di Santo ,2008), inhibitory receptors and their MHC ligands (Anfossi *et al.* 2006; Yokoyama and Kim, 2006) are absent. Another factor to consider is the unavailability of human IL-7 and IL-15, which are required for NK cell generation and survival (Kennedy *et al.* 2000; Huntington and Di Santo, 2008; Vossehenrich *et al.* 2006).

Adaptive immunity

Initial studies to induce human antibody production in immunodeficient mice implanted with human PBMC (Abedi *et al.* 1992; Mosier *et al.* 1988; Sandhu *et al.* 1994) were unsuccessful because of graft versus-host disease (GVHD) (Ito *et al.* 2008b). GVHD was reducely greatly by decreasing the number of engrafted PBL, but antigen-specific antibodies were almost undetectable (Sandhu *et al.* 1994). In NOD-*scid* or NOD-*scid/β2m*$^{-/-}$ or NOG mice, the developed human $CD4^+$ or $CD8^+$ T cells express mature T cell markers and produce cytokines when stimulated with ionophores or superantigens, or via TCR ligation (Saito *et al.* 2002; Yeoman *et al.* 1993). However, spenic T cells from immunized mice lack the ability to produce IL-2 or proliferate. Further, although serum IgM and IgG levels are high, the level of antigen-specific IgG is extremely low (Ishikawa *et al.* 2005; Ito *et al.* 2008b). This rudimentary adaptive immunity is presumably because human T cells are positively selected by the murine MHC in the thymus of these mice (Ishikawa *et al.* 2008). To overcome the impediment, one can express human MHC transgenically (Faulkner *et al.* 1998; Friese *et al.* 2006) or engraft human lymphoid tissues (e.g., embryonic thymus) to direct the selection of human T cells. Indeed, when adult LNs or embryonic liver, thymus, or skin are engrafted into NOG mice which are then immunized with antigen, human immune cells proliferate and are activated efficiently with given antigens (Carballido *et al.* 2000; Ishikawa *et al.* 2008). In addition, in *NOD-scid-IL2Rγ null* mice, significant amounts of IgG and IgM specific to T-dependent antigen are detectable, suggesting functionality and effective class switching (Ishikawa *et al.* 2008). Recent studies also show the generation of functional T cell subsets specific to a viral pathogen (Jaiswal *et al.* 2009; Shultz *et al.* 2010). Of great clinical significance is the ability to easily generate completely human monoclonal antibodies for therapeutic use (Becker *et al.* 2010).

Infectious Disease

Humanized mice are very useful in the investigation of human-specific infectious diseases, including viral infections caused by human immunodeficiency, herpes (human cytomegalovirus, Epstein-Barr virus), hanta, Chikungunya, hepatitis C, dengue and other viruses, as well as parasites such as *Plasmodium*. *Scid* mice coimplanted with human fetal thymus and liver tissues show high susceptibility to human immunodeficiency virus type 1 (HIV-1) infection (Aldrovandi *et al.* 1993; Namikawa *et al.* 1988), and can be used to assess efficacy of anti-HIV compounds (McCune *et al.* 1990; Rabin *et al.* 1996). However, direct injection of virus into the implant is required because of the low numbers of circulating $CD4^+$ T cells, and no antiviral immune response is observed. *Scid* mice injected with PBMC from healthy adults can also be

used (Mosier *et al.* 1991; Nakata *et al.* 2005), but these mice develop GVHD (Sandhu *et al.* 1995), and both T cell phenotype and coreceptor usage by HIV-1 do not reflect that in humans (Mosier *et al.* 1993; Nakata *et al.* 2005). Nevertheless, some immune responses are induced in these mice (Gorantla *et al.* 2005). Further, MHC-restricted anti-HIV-1 T cell responses can be elicited by using autologous skin that contains tissue DC (Delhem *et al.* 1998). Similarly, inactivated HIV-1-pulsed, human monocyte-derived DC (MDDC) can elicit partially protective anti-HIV-1 antibody production (Santini *et al.* 2000). The lack of a suitable HME in these mice can be overcome by transferring PBMC together with inactivated HIV-1-pulsed autologous MDDC directly into the mouse spleen (Yoshida *et al.* 2003). This reduces excessive GVHD, produces larger yields of human T cells, elicits a protective T cell immunity, and the sera from the immunized mice contain a soluble HIV-1 suppressive factor produced by human antigen-specific $CD4^+$ T cells (Yoshida *et al.* 2005).

The HIV-1 replication occurs at high levels and the virus persists for a long time without any GVHD in the human hematopoietic cell reconstituted NOD/Shi-*scid* mice (Koyanagi *et al.* 1997a; 1997b). NOG (Nakata *et al.* 2005; Watanabe *et al.* 2007) and *Rag2 null Il2rg scid* mice (Baenziger *et al.* 2006; Gorantla *et al.* 2007; Ince *et al.* 2010; Sun *et al.* 2007) have long-lasting infection of human cells by HIV-1 as well as HIV-specific human immune responses. Plasma viral RNA and antigen load, stabilization of viremia, cell tropism, quasispecies generation, $CD4^+$ T cell depletion and sometimes syncytium formation all recapitulate what happens in humans, including late-stage disease. However, no robust IgG or T cell responses are detected, dampening the excitement about testing vaccines and antivirals (Koyanagi *et al.* 2008).

The NOG mice are susceptible to infection with Epstein-Barr virus (EBV), a causal agent of epithelial carcinomas and B cell lymphomas, and human T-lymphotropic virus type I (HTLV-I), which causes adult T-cell leukemia/lymphoma (ATL). EBV DNA can be detected in the peripheral blood and spleen cells, EBV^+ B lymphoblastoid cells can be isolated from NOG mice, and specific immunity can be elicited (Melkus *et al*. 2006, Mosier *et al.* 1989; Shultz *et al.* 2010). The NOG mice develop clinical signs following inoculation of ATL cells, with massive infiltration of cells in various organs (Dewan *et al.* 2006; Imada *et al.* 1995), providing a model for testing targets for ATL therapy (Dewan *et al.* 2003; Dewan *et al.* 2006; Mori *et al.* 1999).

Immunodeficient mice can also be used to reproduce the pathogenic process and clinical manifestations of disease, especially in the case of agents which do not cause any disease in laboratory animals. For example, immunodeficient mice expressing HLA molecules and lacking endogenous MHC class I molecules, are able to produce symptoms of Lassa fever. Further, the exacerbation of disease following depletion of human T cells revealed the hitherto unknown possibility of immunopathology in this disease (Flatz *et al.* 2010). Work with dengue virus has also shown recapitulation of human-like disease and genotype-dependent severity (Bente *et al.* 2005; Mota and Rico-Hesse, 2009) as well as specific antibody and T cell responses (Jaiswal *et al.* 2009). Other examples include symptoms of paralysis induced by poliovirus infection (Horie *et al.* 1994; Ito *et al.* 2002) and induction of streptococcal impetigo (Scaramuzzino *et al.* 2000).

Cancer

Immunodeficient mice have been used to study cancer biology for over three decades (Giovanella and Fogh, 1985; Shultz *et al.* 2007), but the early strains (*nude* and *scid*) could only support a limited number of tumors (Friese *et al.* 2006; Hudson *et al.* 1998). The development of NOD-*scid* or NOD/Ltz-*scid IL2rg* mice has permitted the study of many primary human lymphomas and leukemias as well as liver metastatic models (Dewan *et al.* 2004; Dewan *et al.* 2005; Ishikawa *et al.* 2007; Suemizu *et al.* 2007).

Hematogenous metastasis models, which mimic events that occur after cells enter the blood, are useful to understand mechanisms of human gastrointestinal, hepatic and pulmonary cancers (Nakamura and Suemizu, 2008). Several models of liver metastasis have been established in *nude* mice (Bresalier *et al.* 1987; Nomura *et al.* 2002), but it is unlikely that such a large number (> 106) of cells would enter the liver simultaneously and form metastatic foci in patients (Nakamura and Suemizu, 2008). The incidences of liver metastases in NOG mice are very high and reproducible, and the lesions are dose dependent over a wide range of inoculum, but are better produced by the elimination of NK cells (Nakamura and Suemizu, 2008). Lymphatic metastasis models are difficult to develop as the cancers need to first invade the lymphatic vessels and metastasize to regional LNs, whose structures are not developed in immunodefieicnt mice. On the other hand, dissemination in body cavities, a late event of malignancy, is much easier to develop, but it gives limited information for targeting therapy for early-stage cancer (Nakamura and Suemizu, 2008). Several human melanoma cell lines are reported to metastasize in *nude* mice (Cornil *et al.* 1989; Iliopoulos *et al.* 1989), albeit at low rates. On the other hand, metastasis is high in NOG and NOD/Shi-*scid* mice (Nakamura and Suemizu, 2008).

The concept of tumor stem cells, small number of cells with the capacity to self-renew and differentiate to give rise to tumor (Shultz *et al.* 2007), has also been validated with humanized mice (Passague *et al.* 2003; Reya *et al.* 2001; Warner *et al.* 2004) for myeloma, and breast, brain and pancreatic cancers (Al-Hajj *et al.* 2003, Li *et al.* 2007, Pilarski and Belch, 2002; Singh *et al.* 2004). This concept could be critical in treating early stage cancers, rather than targeting reduction of tumor mass (Nakamura and Suemizu, 2008). Molecular mechanisms of carcinogenesis have also been explored with these and transgenic mice (Mitsumori *et al.* 1997; Nakamura and Suemizu, 2008).

Stem Cells and Regenerative Medicine

The *scid* mice have been used extensively as models for human stem cell transplant (Greiner *et al.* 1998). BM cells possess the capacity to generate hepatocytes, hematopoietic

cells, epithelial cells, cardiomyocytes as well as functional pancreatic beta cells (Camargo *et al*. 2004; Fujino *et al.* 2007; Harris *et al.* 2004; Hess *et al.* 2003; Ianus *et al.* 2003; Ishikawa *et al.* 2006; Ishikawa *et al.* 2008; Krause *et al.* 2001; Ma *et al.* 2006; Petersen *et al.* 1999; Wang *et al.* 2005). Several investigators have also reported the differentiative capacity of human MSCs in vivo (Ishikawa *et al.* 2008; Sato *et al.* 1999; Toma *et al.* 2002). Other studies include reconstitution of human endometrium, resulting in induction of human sexual hormone-dependent menstrual cycle (Masuda *et al.* 2007; Matsuura-Sawada *et al.* 2005), and therapeutic evaluation of thrombopoietics (Nakamura *et al.* 2006). Recognition that transdifferentiation and/or cell fusion of transplanted HSC also occurs in damaged tissues has led to intense investigation in this area (Ishikawa *et al.* 2008; Prockop *et al.* 2003; Shultz *et al.* 2007).

Autoimmunity

The study of autoimmune diseases has benefited extensively by animal models, where, the genetic basis for the disease can be identified, the genome can be altered, and therapies can readily be tested without ethical concerns (Pearson *et al.* 2008). Humanized mice have been used for the study of autoimmune type 1 diabetes, thyroiditis, and rheumatoid arthritis (Shultz *et al.* 2007). In diabetes, PBMC from diabetic individuals adoptively transferred to CB17-*scid* mice led to the detection of autoantibodies to islet components, but no infiltration or beta cell destruction (Petersen *et al.* 1993). In studies of the adoptive transfer of human T cell clones with specificities to islet autoantigens into NOD-*scid* mice, infiltration, but not islet cell destruction was detected (van Halteren *et al.* 2005). The use of newer models based on the *IL2rg null* mutation and transgenic expression of human HLA molecules should permit the direct study of human autoreactive T cells *in vivo*.

Drug metabolism and pharmacology

Metabolism of pharmacological compounds is mainly mediated by the liver, whose enzymes, including cytochrome P450, display tremendous heterogeneity as well as species-specificity. Transgenic immunodeficient mice in which endogenous hepatocytes are depleted through genetic alterations causing hepatocyte damage and human hepatocytes are transplanted to repopulate the liver (Katoh and Yokoi, 2007; Meuleman *et al.* 2005; Strom *et al.* 2010) serve as excellent models to study receptor specificity, metabolism, toxicity and excretion (Cheung and Gonzalez, 2008; Kamimura *et al.* 2010; Yoshizato and Tateno, 2009).

The road ahead

There are three broad limitations in humanized mice: (a) many human-specific molecules are absent, (b) remaining innate immune mechanisms present obstacles to engraftment, and (c) the architecture of the lymphoid system remains undeveloped (Ito *et al.* 2008a; Pearson *et al.* 2008). These can be forfended by (a) the introduction of human genes that code for growth or differentiation factors, (b) the depletion of macrophages, DCs, mast cells, neutrophils, etc., or their differentiation factors, and (c) the introduction of HLA genes (Ito *et al.* 2008a).

The expression of human-specific genes, especially the HLA genes (Camacho *et al.* 2004), is of great importance for thymic selection. However, the expteme polymorphism of HLA means that multiple HLA genes may need to be introduced, by using, for example, artificial chromosomes (Chen *et al.* 2009; Kuriowa *et al.* 2002). Additional molecules such as cytokines and growth factors should facilitate the development, differentiation, and survival of a functional human immune system. Genes related to inflammatory responses and allergic reactions, e.g., IL-4 and IL-5, may be useful in developing disease models (Wills-Karp, 1999).

Both genetic approaches and exogenous administration of reagents can be used to further depress innate immunity. Better engraftment has already been achieved through the ablation of remaining cells, either partially or completely, by various techniques (Heyman *et al.* 1989; Jung *et al.* 2002; Kamogawa *et al.* 1993; Saito *et al.* 2001; Santini *et al.* 1998). For example, transgenic expression of simian diphtheria toxic receptor can selectively deplete DCs, macrophages, and other cells in combination with exogenous administration of diphtheria toxin (Bennett *et al.* 2005; Duffield *et al.* 2005; Jung *et al.* 2002; Matsumura *et al.* 2004), to which mouse cells are relatively insensitive (Cha *et al.* 2003).

Poor secondary lymphoid organ development is another concern in humanized mice. Appropriate lymphoid architecture requires interaction of follicular DCs with lymphocytes and other nonlymphoid cells for full development (Fu *et al.* 1997a; 1997b). Engraftment of human stromal cells, artificial thymic stroma or biocompatible scaffolding may facilitate restoration of the lymphoid structure (Poznansky *et al.* 2000; Suematsu and Watanabe, 2004). In addition, transduction of BM or thymic stromal cells with factors that are needed for human hematopoiesis or lymphocyte development may prove useful (Brenner *et al.* 2004). Additional molecules, such as adhesion and homing molecules expressed on tissues in the host, may overcome some of the issues associated with homing of human immune cells. The genes for lymphotoxin-related molecules and chemokines that are necessary for reconstruction of human secondary lymphoid organs may also be targets for introduction (Fu *et al.* 1997b; Suemetsu and Watanabe, 2004).

Distant metastasis of malignant neoplasms consists of entrapment in vessels, invasion of the basement membrane, destruction of the vasculature, and entry into blood or lymph (Nakamura and Suemezu, 2008). Metastatic models only reflect late events, i.e., after adhesion or entrapment in organs. Models for initial steps or local invasion mechanisms at the primary lesion of the cancer must be developed (Nakamura and Suemizu, 2008). Further, developments in in vivo imaging techniques could help in the study of events as they happen (Masuda *et al.* 2008).

Recent advances have allowed us to identify genes responsible for self-renewal of stem cells (Seita *et al.* 2007; Yamazaki *et al.* 2007), and their introduction into mice may make it possible to maintain human stem cells for a long time. As far as stem cells and regenerative medicine are concerned, there is a clear need for targeting research into replacement

of tissues constituting organs or areas of extensive loss. In addition, inflammatory and autoimmune disorders, which involve immunological disturbances, need to be addressed separately as both regenerative approaches and normalization of immune system may be required.

One other issue that needs to be addressed is the husbandry of immunodeficient mice. Infections with microbes including mouse hepatitis virus and *Staphylococcus aureus* in *nude* mice and *Pneumocystis carinii* (*P. carinii*) in *scid* mice are a constant problem (Nomura *et al.* 2008). Ordinary rearing environment can cause mortality in NOG mice. For example, contamination of transplated human tumors with *Pseudomonas aeruginosa* and spread to the rearing environment via drinking water, researchers, or caretakers has been observed (Nomura *et al.* 2008). Indeed, *nude* and *scid* mice show resistance to *P. aeruginosa and P. pneumotropica* but NOD-*scid* and NOG mice die (Nomura *et al.* 2008). In addition, the intestinal flora, e.g., E. coli, of NOG mice has been found to change because of stress caused by transport or changes in rearing temperature (Nomura *et al.* 2008). These incidents confirm that higher-level control, e.g., germ-free breeding techniques and rearing facilities, is indispensable for the generation and maintenance of extremely immunodeficient mice (Nomura *et al.* 2008).

Conclusions

The ability to experimentally manipulate in small animal models each incremental step in complex human biological processes offers great promise for rapid advances. Humanized mice having human cells, tissues, or organs, should facilitate the study of human disease pathogenesis and help develop therapeutics and prophylactics. While there is no alternative to the accuracy of information obtained from human clinical trials, these mice should allow derivation of valuable preclinical information, and substantially reduce the cost and time associated with extensive clinical trials. Humanized mice also provide a platform to attempt novel interventions in regenerative medicine and cancer treatment before their application to the clinic.

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