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The development and improvement of immunodeficient mice and humanized immune system mouse models

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Animal models play an indispensable role in the study of human diseases. However, animal models of different diseases do not fully mimic the complex internal environment of humans. Immunodeficient mice are deficient in certain genes and do not express these or show reduced expression in some of their cells, facilitating the establishment of humanized mice and simulation of the human environment *in vivo*. Here, we summarize the developments in immunodeficient mice, from the initial nude mice lacking T lymphocytes to NOD/SCID rg^{null} mice lacking T, B, and NK cell populations. We describe existing humanized immune system mouse models based on immunodeficient mice in which human cells or tissues have been transplanted to establish a human immune system, including humanized-peripheral blood mononuclear cells (Hu-PBMCs), humanized hematopoietic stem cells (Hu-HSCs), and humanized bone marrow, liver, thymus (Hu-BLT) mouse models. The different methods for their development involve varying levels of complexity and humanization. Humanized mice are widely used in the study of various diseases to provide a transitional stage for clinical research. However, several challenges persist, including improving the efficiency of reconstructing the human B cell immune response, extending lifespan, improving the survival rate of mice to extend the observation period, and improving the development of standardized commercialized models and as well as their use. Overall, there are many opportunities and challenges in the development of humanized immune system mouse models which can provide novel strategies for understanding the mechanisms and treatments of human disease.

KEYWORDS

immunodeficient mouse, humanized immune system mouse, nude mouse, NOD mouse, NOD/SCID mouse

Immunodeficient mice

The development of immunodeficient mice occurred in four main stages. The first stage included nude mice that are simply deficient in T lymphocytes owing to abnormal thymus development (1). However, the application of nude mice in many diseases remains limited because of their low relative degree of immunodeficiency. The second stage included mice with severe combined immunodeficiency (SCID), carrying a mutation of the *Prkdc* gene (2, 3). SCID mice are deficient in T and B lymphocytes, but retain natural killer (NK) cells and show "leakage" (4). The SCID mutation was then introduced into non-obese diabetic (NOD) mice with NK cell defects to obtain NOD/SCID mice (5), forming the third stage of immunodeficient mice. However, these mice exhibit a high frequency of spontaneous thymic lymphoma and short life cycles, as well as partial NK cell activity. Therefore, their application as a humanized animal model has remained limited (5). To improve this situation, the fourth stage of immunodeficient mice, NOD/SCID rg^{null} mice, was developed by knocking out the IL-2 receptor gamma chain (IL-2 rg); these knock-out mice had a higher rate of human-cell implantation without leakage or spontaneous thymomas, and are currently the gold standard immunodeficient mouse model (6). The characteristics of different immunodeficient mice are summarized in Figure 1.

Nude mice

Nude mice are the earliest immunodeficient mouse model, first reported by Flanagan in 1966 (1). Owing to an allele mutation on chromosome 11, a resultant defect in the *Foxn1* gene prevents normal thymus development (7), thereby leading to a mature T lymphocyte deficiency. The main immunoglobulin in these mice is IgM (8), with little or no IgA. As such, they do not show a rejection reaction to allogeneic tissue (9). The commonly used strains include BALB/c-nu, Swiss-nu, NC-nu, and NIH-nu, all of which are widely used in the study of immune diseases and tumors (10). However, as they still retain B cells and NK cells, they cannot completely accept human immune cell engraftment, and so cannot be used as an ideal humanized mouse model (11).

SCID mice

In 1983, researchers found CB-17 inbred mice that carried a recessive mutation of a single gene on chromosome 16, which led to the abnormal recombination enzyme activity of the sequence encoding the mouse lymphocyte antigen receptor gene VDJ, due to which immunoglobulin, T, and B lymphocyte receptors could not be synthesized effectively (3). This mutation obstructs the repair and recombination of T and B cell receptors and seriously affects the differentiation and maturation of these cells, resulting in the lack of mature T and B cells and low immunoglobulin levels in the peripheral blood or lymphoid organs of SCID mice (2). However, the NK cells and macrophages in SCID mice function normally (12, 13). Furthermore, "leakage" was observed (4, 14, 15), meaning that 2 to 23 percent of the mice showed recovery of T and B lymphocytes with increasing age (16). As SCID mice are highly sensitive to radiation, Rag^{null} mice were generated by the knockout of recombinant activated genes Rag1 (15) or Rag2 (17) to reduce their radiosensitivity. Rag1 and Rag2 induce V(D)J rearrangement of TCR and immunoglobulin genes by producing DNA double-strand breaks. Homozygous mutations in these genes result in the inability to produce mature T and B cells and produce the same SCID-like phenotype (18). Similar to the SCID mutation, mice with the Rag mutations lack mature T and B lymphocytes. Contrastingly, this mutation does not repair spontaneously. Nevertheless, these mice also allow limited human cell and tissue engraftment because of highly active NK cells (19–21).

NOD/SCID mice

In 1980, researchers obtained nonobese diabetic (NOD) mice *via* inbreeding and selective breeding, with pathological characteristics and changes similar to those in human diabetes (22). NOD mice have defects in their innate immune system, with low NK cells and macrophage activity, and an absence of circulating complement. Introducing the SCID mutation into the genetic background of NOD mice was hypothesized to result in NOD/SCID mice with simultaneously defective adaptive and innate immunity (23). Indeed, researchers successfully introduced the SCID mutation into NOD mice in 1995. The resulting NOD/SCID mice showed functional loss of T and B lymphocytes and other immune cells, as well as defective NK cell function, resulting in a higher degree of immune deficiency than in the previously noted mouse models (5). Human B cell reconstruction in nude mice and SCID mice was poor. In one study, NOD/SCID mice injected with 1×10^5 human $CD34^+$ cells showed that humanized B cells from different organs showed different stages of maturation, with immature IgM^-IgD^- $CD24^{hi}$ $CD38^{hi}$ B cells predominating in the bone marrow and mature $CD5^+$ IgM^+ IgD^+ $CD24^{int}$ $CD38^{int}$ $CD19^+$ B cells predominating in the spleen and peripheral blood.

Compared with SCID mice, human tumors and immune cells had better survival status in NOD/SCID mice (23). The NOD/SCID mice had the following characteristics (1): low NK cell levels, with significantly reduced killing function; (2) complement C5 deficiency, resulting in inhibition of

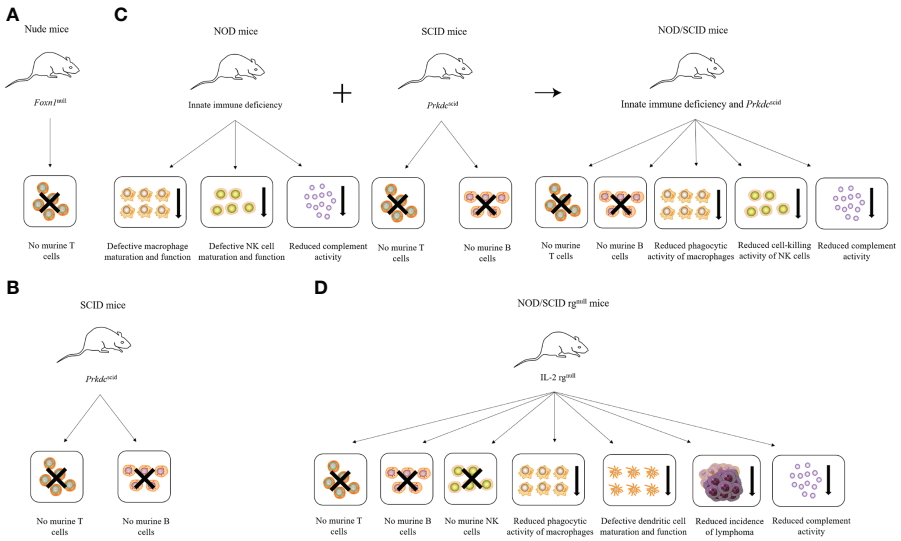

FIGURE 1
Characteristics of different immunodeficient mice. **(A)** Nude mice lack T cells due to *Foxn1* mutation. **(B)** SCID mice lack T and B cells due to *Prkdc* mutation. **(C)** NOD mice combined with SCID mice produce NOD/SCID mice, which lack T and B cells and have reduced phagocytic activity of macrophages, the cell-killing activity of NK cells, and complement activity. **(D)** NOD/SCID rg^{null} mice lack T, B, and NK cells and have reduced phagocytic activity of macrophages and complement activity, defective dendritic cell maturation and function, and reduced incidence of lymphoma due to the loss of the IL-2 receptor γ chain.

complement activation;(3) defective IL-1 secretion in lipopolysaccharide-induced macrophages. These characteristics enabled the generation and survival of human cells and grafts in NOD/SCID mice at higher levels. However, this model remained unsuitable, owing to certain defects, including radiosensitivity, which only allows a small radiation dose. T and B cell leakage also occurred in older mice, and their average life span was only 8 months. Furthermore, the NOD gene mutation in NOD/SCID mice increased the probability of spontaneous thymic lymphoma, resulting in a short life cycle of such mice along with partial NK cell activity, limiting its application as a humanized animal model (5).

NOD/SCID mice are not as commonly used to generate humanized mice because they require a higher dose of HSCs for efficient engraftment, compared with more deficient mouse strains like NOD/SCID rg^{null} mice and they developed thymic lymphomas shortening their lifespan. Despite these disadvantages, the model is still in use because of its unique characteristics. For example, it has been shown that NOD/SCID mice better support the development of human gut-associated lymphoid tissue (GALT) structures due to the presence of the common gamma chain. Therefore, when more robust human GALT structures are needed, NOD/SCID BLT mice may be preferred (24). One study also showed enhanced human cell reconstitution in the GALT of BLT mice. This study, using HIV infection of humanized mice (BLT) as a model of heterosexual transmission, demonstrated that blocking lymphocyte egress from lymph nodes prevented viremia and infection of the gut (25).In addition, NOD/SCID mice transplanted with HSCs are specifically used to generate mice that possess human myeloid and B cells but are devoid of human T cells following the transplant to study certain aspects of EBV and HIV infection (26, 27).

NOD/SCID rg^{null} mice

The IL-2 receptor gamma chain, also known as the common cytokine receptor gamma chain, is a key component of high-affinity receptors for cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. The development and maturation of T and B lymphocytes and NK cells require the participation of some of these cytokines. The loss of the IL-2 receptor gamma chain hinders the development of T and B lymphocytes as well as NK cells and severely weakens the innate and adaptive immune systems of mice (28). When IL-2 rg^{null} was combined with SCID, $Rag1^{null}$, or $Rag2^{null}$ mutations, the resulting NOD/Shi-SCID IL-2^{null} (NOG) (29), NOD/LtSz-SCID IL-2^{null} (NSG) (30), and NOD-$Rag1^{null}$ IL-2 rg^{null} (NRG) (31) mice were deficient in T and B lymphocytes as well as NK cells (13). These mice completely lost the ability to mount an adaptive immune response and showed serious defects in the innate immune system; which are the main requirements for immunodeficient mice for the construction of humanized mouse models (6).

NOD/SCID rg^{null} mice can be divided into NOG and NSG mice according to the mutation of the IL-2 receptor gamma chain. The major difference between the NSG and NOG strains is that the IL-2 rg targeted mutation used to develop the NSG strain is a complete null so that no IL-2 rg is expressed, effectively hindering cytokine binding, whereas the IL-2 rg mutation in the NOG strain produces a protein that is expressed and will bind cytokines but cannot transduce the signal (32). NOG and NSG mice were found to be the best models for human cell and tissue transplantation, with a higher transplantation success rate than either SCID or NOD/SCID mice (33, 34). Another important advantage of NOG and NSG mice is the absence of leakage and spontaneous thymomas, which may also be related to the lack of active IL-2 rg. Moreover, several immunodeficient mouse strains, such as NSGB2m and NSG-SGM3, have been derived by gene modification based on NSG mice. These mice are more advantageous in xenotransplantation (35, 36). One study comparing the implantation rate of human cells in the peripheral tissues of NSG mice with that in NOD/SCID mice showed a significantly higher implantation rate of human cells in NSG and NOG mice than in NOD/SCID mice. In addition, the implantation rate of human cells in the bone marrow of NSG mice was higher than that in the other strains, especially in females (37). Therefore, NSG mice are good candidates for generating humanized immune system mouse models. Another study examined the recovery of the immune system in humanized mice after the transplantation of human hematopoietic stem cells in NSG mice. The results showed that T, B cells, monocytes, macrophages, and neutrophils were developed to normal human levels in these mice. Moreover, the phagocytic ability of monocytes and macrophages, and the secretion ability of inflammatory factors under TLR4 stimulation also developed to normal human levels (38).

Signal regulatory protein α (SIRPα) is a transmembrane protein that contains three Ig-like domains within the extracellular region. SIRPα is expressed in macrophages, myeloid cells, and neurons, and interacts with its ligand CD47 *via* respective IgV-like domains, where the NOD strain has specific polymorphism. CD47 is a member of the immunoglobulin (Ig) superfamily that is ubiquitously expressed in hematopoietic as well as non-hematopoietic cells. The cytoplasmic region of SIRPα has immunoreceptor tyrosine-based inhibitory motifs (ITIMs), and the cell surface binding of CD47 with SIRPα on macrophages provokes inhibitory signals *via* phosphorylation of ITIM of SIRPα (39), preventing their phagocytic activity (40–42). A recent study showed that transgenic expression of mouse CD47 into the $CD34^{+}CD38^{-}$ human fetal liver cells significantly enhanced the human cell engraftment into BALB-RG mice (43). Based on these results, it is assumed that the binding of NOD-SIRPα with human CD47 produces signals for mouse macrophages not to engulf human HSCs, presumably making the strain permissive for human HSC engraftment (44). The important question was whether the NOD-specific highly efficient human cell engraftment *in vivo* could be explained solely by the NOD-SIRPα polymorphism. In one study, Yamauchi et al. established a C57BL/6-$Rag2^{null}$IL-$2rg^{null}$ (C57BL/6-RG) line harboring the NOD-type SIRPα. The results clearly show that the replacement of the C57BL/6-type SIRPα with the NOD-type SIRPα is sufficient for the C57BL/6-RG strain to be endowed with the xenotransplantation capability at least equal to NOD-RG mice. Thus, they successfully segregated the genetic abnormality responsible for efficient human cell engraftment from multiple genetic abnormalities in the NOD strain (45). The simplified humanized mouse system established by the new C57BL/6-$Rag2^{null}$IL-$2rg^{null}$NOD-SIRPα (BRGS) strain should be very useful to improve xenotransplantation strategies in studies on human cell biology. In one study, Di Santo et al. induced the expression of thymic-stromal-cell-derived lymphopoietin (TSLP) in a BALB/c $Rag2^{-/-}$IL-$2rg^{-/-}$$SIRPα^{NOD}$ (BRGS) human immune system (HIS) mouse model. The resulting BRGST HIS mice developed a full array of LNs with compartmentalized human B and T cells. Compared with BRGS HIS mice, BRGST HIS mice have a larger thymus, more mature B cells, and abundant IL-21-producing follicular helper T (TFH) cells, and show enhanced antigen-specific responses. Peripheral human B cells in HIS mice retain an immature, transitional phenotype with elevated expression of CD24 and CD38. In BRGS and BRGST HIS mice, they observed this predominant population of $CD24^{hi}CD38^{hi}$ immature B cells in the bone marrow, liver, and spleen. In contrast, mature $CD24^{lo}CD38^{lo}$ cells were the dominant human B cell subset in LNs of BRGST mice. Although they did not observe notable differences in these different B cell subsets between the two models, the total numbers of mature $CD24^{lo}CD38^{lo}$ B cells in LNs were significantly increased in BRGST HIS mice compared with those in BRGS mice (46).

Humanized mouse models constructed by engrafting peripheral blood mononuclear cells have mainly revealed the presence of human T cells (47, 48). However, in stem cell transplant models, B-cell reconstitution is efficient with T-cell engraftment lagging (49). Although both HSC-infused newborn and adult mice were highly reconstituted with human B cells, the development of B cells was arrested in an early stage and did not suffice for reconstitution of human immunoglobulins (natural antibodies) in serum, other than IgM (50). Impairment of human T and B cell function in HSC reconstituted IL-2 rg^{null} genetic stocks has been attributed to the lack of expression of human leukocyte antigens (HLA) in the mouse thymus since HLA molecules are required for the development of human T cells that in turn, are essential for stimulation of B cells towards immunoglobulin class switching and antibody secretion (51, 52). In one study, Danner et al. generated NOD.Rag1KO.IL2RγcKO mice expressing HLA-DR4 molecules under the $I\text{-}E^{d}$ promoter infused as adults with HLA-DR-matched human hematopoietic stem cells generating a new strain of NOD.Rag1KO.IL2RγcKO mice expressing HLA-

DR*0401 molecules (DRAG mice). The presence of these HLA-DR4-IE transgenes allows irradiated DRAG mice to be engrafted with HLA-DR-matched hematopoietic stem cells; resulting in humanized T-cell and B-cell populations. The HLA-DR4 expressing mice reconstituted serum levels (natural antibodies) of human IgM, IgG (all four subclasses), IgA, and IgE comparable to humans, and elicited high titers of specific human IgG antibodies upon tetanus toxoid vaccination (53). In another study, Ito et al. generated transgenic mice with HLA-DRA-IEα and HLA-DRB1*0401-IEβ chimeric genes. The HLA-DRA-IEα/HLA-DRB1*0401-IEβ molecules rescued the development of $CD4^+$ T cells in major histocompatibility complex (MHC) class II-deficient mice, but T cells expressing Vβ5, Vβ11, and Vβ12 were specifically deleted (54).

These various types of mice are suitable for constructing various humanized mouse models for studying tumors, hematological diseases, infectious diseases, immune diseases, and metabolic diseases (29).

Development of humanized immune system mice

Humanized immune system mice can be divided into three groups according to the method used for immune system reconstruction: humanized-peripheral blood mononuclear cells (Hu-PBMCs) or humanized-peripheral blood lymphocytes (Hu-PBLs), humanized hematopoietic stem cells (Hu-HSCs) and humanized bone marrow, liver, thymus (Hu-BLT) mouse models. The different construction methods and characteristics of humanized mice are shown in Figure 2 and Table 1.

Hu-PBMCs/PBLs mouse model

In this model, human peripheral blood mononuclear cells (PBMCs) or peripheral blood lymphocytes (PBLs) are transplanted into mice *via* a caudal vein or peritoneal injection (48, 55, 56). In general, 50–80% of human $CD45^+$ cells can be detected in the blood and spleen of mice. Human $CD3^+$ T cells are usually detected in the first week after transplantation, forming an ideal model for studying mature effector T cells (57). Small numbers of B cells, myeloid cells, and other immune cells are also detected in these mice (57, 58). In this model, human memory B cells can produce antibodies after antigen stimulation, but cannot produce a primary immune response. Human immune cells can survive for several weeks after transplantation and are effective to some extent. They can be efficiently infected with HIV, HBV, EBV, HSV, HCMV, KSHV, etc., and play an important role in allogeneic immune response and viral immunity studies (59–65). In the early stage of establishing the humanized mouse model, human peripheral blood mononuclear cells or peripheral blood lymphocytes were injected into nude mice. Since nude mice are only deficient in T lymphocytes, they cannot completely accept human PBMCs or PBLs, resulting in immune rejection (2). In later work, PBLs were directly transplanted into SCID mice to construct a humanized PBLs-SCID mouse model. In this model, a multi-lineage humanized immune system can be obtained, and long-term reconstruction can be maintained. However, the main problem with this model is the fatal graft-versus-host disease (GVHD) caused by the MHC mismatch between human T cells and mouse immune cells (35, 66). GVHD symptoms usually appear 4–6 weeks after injection of human PBMCs, with a short observation window of limited use (58, 67). However, this window can be prolonged and ameliorated by using NSG (NOD/SCID IL-2Rγ $C^{-/-}$) or RG (BALB/C $Rag2^{-/-}$ IL-2R γ $C^{-/-}$) mice with deletion of the MHC-I or II genes (68). The Hu-PBLs model is the simplest and most economical humanized mouse model because of the easy availability of human PBLs. However, it also has several shortcomings, such as the low and unstable level of human lymphocyte reconstruction, lack of a normal lymphoid tissue structure and a follicular germinal structure in the spleen, EBV-associated lymphoproliferative disease after massive injection of human cells, and xenograft rejection.

Hu-HSCs mouse model

In this model, the immunodeficient mice were irradiated with a sublethal dose to destroy the hematopoietic function of the autologous bone marrow in mice; human $CD34^+$ hematopoietic stem cells (HSCs) were then transplanted into these immunodeficient mice through the vein or femoral artery (HSCs *via* intrahepatic injection as pups and *via* tail vein injection as adults) (69–71). Human HSCs in mice (such as NOG, NSG, etc.) developed into T cells, B cells, and NK cells, and formed bone marrow sources of inhibitory myeloid-derived suppressor cells (MDSCs), and other immune cells (72). The number of human $CD45^+$ T cells usually reaches 25–60% in peripheral blood at 4 weeks after implantation (72). As these immune cells develop from transplanted HSCs and are tolerant to the mouse host, GVHD usually does not occur. The stable period can be as long as 10–12 weeks (73), allowing the study of HIV (74), EBV (75), other infection models, and hematopoietic system development. HSCs can be obtained from bone marrow, umbilical cord blood, or peripheral blood after mobilization using granulocyte colony-stimulating factor (G-CSF) (76). In the early stage of developing this model, human $CD34^+$ hematopoietic stem cells were transplanted into NOD/SCID mice, which could reconstruct lymphocyte proliferation, but the resulting T cells were dysplastic. NSG, RG, or NOG (NOD/Shi-SCID IL-2Rγ $C^{-/-}$) mice now allow better implantation of human cells (77, 78).

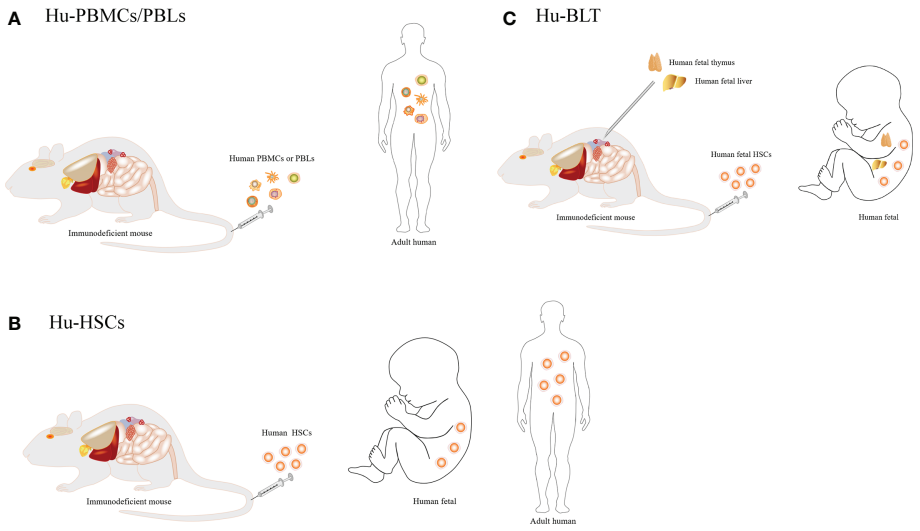

FIGURE 2
Different construction methods of humanized mice. **(A)** Injection of human peripheral blood mononuclear cells or lymphocytes. **(B)** Injection of human CD34$^+$ hematopoietic stem cells. **(C)** Co-transplantation of human fetal thymus and fetal liver into the renal capsule of mice, involving injection of hematopoietic stem cells from the fetal liver or bone marrow of the same individual into mice.

Hu-BLT mouse model

The precursor of this model was the SCID-hu mouse model, which involved surgical implantation with a human fetal thymus and liver under the renal capsule of SCID mice (79, 80). However, this model had obvious shortcomings, such as a low level of human cell reconstruction, unstable development of human T cells, and short survival time (81). Human fetal thymus was also transplanted into SCID-hu mice; and although human T cells developed in the thymus, the level of humanized development was low and unstable. However, when human HSCs were transferred into the hu-HSCs mouse model, a large number of B cells and myeloid cells were generated, but human T cells were completely lacking. In one study, the advantages of SCID-hu mice and Hu-HSCs mice were combined. Human fetal thymus and fetal liver tissue blocks were transplanted from

TABLE 1 Different construction methods and characteristics of humanized mice.

Models	Construction methods	Advantages	Disadvantages
Hu-PBMCs/PBLs	Injection of human peripheral blood mononuclear cells or lymphocytes	1 Sample is easy to obtain and the transplantation method is simple 2 Efficient and stable transplantation of T cells	1 Lack of B, NK, and other immune cells 2 Possible induction of GVHD 3 The massive injection of human cells results in EBV-associated lymphoproliferative disease
Hu-HSCs	Injection of human CD34$^+$ hematopoietic stem cells	1 Multiple line of hematopoietic cell development, including T, B, myeloid, and NK cells 2 Less GVHD	1 Limited sample sources 2 Lack of T cells (NOD/SCID mice)
Hu-BLT	Co-transplantation of human fetal thymus and fetal liver into the renal capsule of mice, involving injection of hematopoietic stem cells from the fetal liver or bone marrow of the same individual into mice	1 Better T, B, myeloid, and NK cell development 2 Can produce the human mucosal immune system and secondary lymphoid tissue and mount an adaptive immune response 3 Human T cells are educated on human MHC (HLA-restricted) in the human thymic organoid	1 Limited sample sources 2 Possible induction of GVHD

Hu-PBMCs/PBLs, humanized-peripheral blood mononuclear cells/peripheral blood lymphocytes; Hu-HSCs, humanized hematopoietic stem cells; Hu-BLT, humanized bone marrow, liver, thymus; GVHD, graft-versus-host disease.

NOD/SCID mice irradiated with a sublethal dose under the renal capsule, and $CD34^+$ HSCs were isolated from the homologous fetal liver by tail vein injection to construct Hu-BLT mice (82). The major difference between the BLT and the SCID-hu mouse model is the additional reconstruction of hematopoietic stem cells from the same fetal liver in the BLT model. The complete range of T cells, B cells, NK cells, DCs, monocytes, macrophages, and other human immune cells can be found in Hu-BLT mice *in vivo*. Furthermore, they can produce a human adaptive immune response, thereby constituting the most effective mouse model of human immune system reconstruction (83).

The Hu-BLT mouse model (NOD/SCID mice) also shows a human mucosal immune system, and secondary lymphoid tissue, and mounts adaptive human immune responses, such as the production of IgM, IgG, and other immunoglobulins. Therefore, the immune response of humanized BLT mice to implanted exogenous tissues or cells is more similar to the natural response of the human body. NOD/SCID mice were used in the early BLT model, whereas NSG, NOG, or RG mice are used in the improved model (82, 84). The use of human grafts can result in the production of more T cells, B cells, macrophages, NK cells, and DCs. In one study, the generation of humanized BLT mice by the co-transplantation of human fetal thymus and liver tissues and $CD34^+$ fetal liver cells into NOD/SCID rg^{null} mice allows for the long-term reconstitution of a functional human immune system, with human T cells, B cells, dendritic cells, and monocytes/macrophages repopulating mouse tissues (85). As T cells in human thymus tissue have a high affinity for the MHC of mice, the Hu-BLT model may exhibit a graft-versus-host reaction after 20 weeks of implantation. Humanized mice constructed in the TKO-BLT model, (Rag2, IL-2YC, and CD47 triple gene knockout) did not develop GVHD at 45 weeks, and showed a better effect than the existing BLT mouse model (86).

BLT humanized mice are now widely used for studying tumors, immunology, infectious diseases, regenerative medicine, stem cell therapy, and other research areas (87–89). BLT mice have made a lot of contributions to the study of HIV infection. Using HIV vaginal infection of humanized mice as a model of heterosexual transmission, Deruaz et al. demonstrate that blocking the ability of leukocytes to respond to chemoattractants prevented HIV from leaving the female genital tract (25). In one interesting study, intravital microscopy was used to observe changes in humanized mice after the intervention. Usmani et al. show by intravital microscopy in humanized mice that perturbation of the actin cytoskeleton *via* the lentiviral protein Nef, and not changes to chemokine receptor expression or function, is the dominant cause of dysregulated infected T cell motility in lymphoid tissue by preventing stable cellular polarization required for fast migration (90). Smith et al. have developed a method to quickly propagate established BLT mice by the secondary transfer of bone marrow cells and human thymus implants from BLT mice into NSG recipient mice. In this way, they were able to expand one primary BLT mouse into a colony of 4–5 propagated BLT mice in 6–8 weeks. These propagated BLT mice reconstituted human immune cells, including T cells, at levels comparable to those of their primary BLT donor mouse. They also faithfully inherited the human immune cell genetic traits from their donor BLT mouse, such as the HLA-A2 haplotype that is of special interest for studying HLA-A2-restricted human T cell immunotherapies. This method provides an opportunity to overcome a critical hurdle to utilizing the BLT humanized mouse model and enables its more widespread use as a valuable preclinical research tool (91). Vatakis et al. used the BLT humanized mouse as a stem cell-based gene therapy tumor model. They use genetically modified human HSCs to construct the thymus/liver implant followed by injection of transduced autologous human HSCs. This approach results in the generation of genetically modified lineages. After the intervention, the regression of the tumor was observed by positron emission tomography (PET) (92). In conclusion, the BLT mouse model has many advantages in human disease research, but its complex construction process needs to be further optimized.

The improvement of humanized immune system mouse models

The humanized mouse models can be further improved by irradiation or chemical pre-treatment, deletion of mouse autoimmune cells, injection of human cytokines, construction of viral vectors, high-pressure injection of gene expression plasmids, and construction of genetically engineered mice. The methods for improving humanized immune system mouse models are summarized in Table 2.

Irradiation or chemical pretreatment

Immunodeficient mice could be irradiated or pretreated with chemical reagents to provide more "space" for humanized construction. One study compared the efficiency of transplantation and found that human immune cells could survive better in NOD/SCID mice when 2–3 Gy pre-radiation was performed before injection of human HSCs (93). Furthermore, a single dose (35 mg/kg) of Busilvex can achieve the same transplantation effect as 3.5 Gy irradiation (66). Therefore, these pretreatments may result in increased concentrations of growth factors and chemoattractants and reserve a certain amount of space for the development of human HSCs and immune cells in immunodeficient mice.

Depletion of auto-immune cells in mice

The innate immunity of immunodeficient mice limits the regeneration of human immune cells. Mouse NK cells can be depleted by using CD122 or IL-2R antibodies (94). Another

TABLE 2 Improvement of humanized immune system mouse models.

Treatment	Results	Reference
Irradiation and chemical reagents	Busilvex (35 mg/kg) → Mouse immune system ↓	(66)
	Irradiation (2–3 Gy) → Mouse immune system ↓	(93)
Knock out of mouse autoimmune cells	Anti-asialo GM1 antibody → Mouse NK cells ↓	(29)
	CD122 antibody → Mouse NK cells ↓	(94)
	IL-2R antibody → Mouse NK cells ↓	(94)
	Cl2MDP → Mouse macrophages ↓	(94)
Injection of human cytokines	Human G-CSF → Human dendritic cells, monocytes, and neutrophils ↑	(38)
	Human IL-7 → Multi-lineage human cell differentiation	(95)
	Human SCF, IL-3, GMCSF, TPO → Human myeloid cells and lymphocytes ↑	(96)
	Human FLT3L → Human dendritic cells ↑	(97)
Construction of viral vectors	Adenoviral vectors overexpress human IL-15 → Human NK cells ↑	(98)
	Lentiviral vectors overexpress human IL-7 → Human T cells and B cells ↑	(99)
Injection of gene expression plasmids	Human IL-15 and Flt3l gene expression plasmid → Human NK cells ↑	(100)
Genetic engineering	Human M-CSF gene knock-in → Human monocytes and macrophages ↑	(41)
	Human M-CSF, IL-3, GM-CSF, TPO gene knock-in →Humanized myeloid and NK cells ↑	(101)
	Human SIRPαand TPO gene knock-in → Human hematopoietic engraftment levels ↑	(102)
	Human TPO gene knock-in → Multi-lineage human immune cells and platelets ↑	(103)
	Human SIRPα gene knock-in → Phagocytosis of macrophages ↓	(104)
	Human SCF, GM-CSF, and IL-3 gene knock-in → Human myeloid cells ↑	(105)
	Human SF, IL-3, and GM-CSF gene knock-in → Humanized myeloid cells ↑	(106)
	Human SCF and KITL gene knock-in → Humanized myeloid cells ↑	(106)
	Human M-CSF, IL-3, GM-CSF, TPO, SIRPα gene knock-in → Human myeloid and NK cells ↑	(107)
	Human M-CSF, IL-3, SIRPα, TPO, GM-CSF, and IL-6 gene knock-in →More susceptible to SARS-CoV-2 infection	(108)

"↑/↓" in humanized immune system mouse models represent an increase or decrease respectively compared with non-intervention control.

approach to deplete NK activity is the use of anti-asialo GM1 antibody injection before HSC transfer (29). Liposome-encapsulated dichloroethylene diphosphonate (Cl_2MDP) can deplete mouse macrophages and facilitate better reconstruction of the human immune system (94, 109). Therefore, when a particular cell type needs to be focused, specific elimination of antibodies may be a good choice.

Injection of human cytokines

With the development of immunodeficient mice and the improvement of reconstitution levels, the role of cytokines has attracted wide attention. In one study, a significant increase in neutrophils, monocytes, and DCs was obtained by injecting human G-CSF into NOG mice (38). Injection of human IL-7 into NOG mouse models was found to promote multi-lineage cell differentiation, achieving a reconstruction effect equivalent to that obtained with umbilical cord blood stem cells (95). In NOD/SCID mice injected with human SCF, IL-3, GMCSF, and TPO for two weeks, the development and differentiation of lymphocytes and myeloid cells were significantly improved (96). Furthermore, in NOD/SCID mice injected with human FLT3L, the number and function of DCs were significantly increased after four weeks (97). In summary, cytokines can greatly promote the construction of humanized mice. Further studies should focus on finding more suitable cytokines for the construction of humanized mice.

Construction of viral vectors

With advances in molecular biology, viral vectors have become a common tool, which can transfer the required genetic material into cells, to achieve the effect of foreign gene expression. In one study, injection of human IL-15 or overexpression of human IL-15 using adenoviral vectors was found to promote NK cell development and maturation (98). Lentiviral vectors carrying the human IL-7 gene have been used to overexpress human IL-7 in $Rag2^{-/-}$ γ $C^{-/-}$ mice; the serum level of human IL-7 was maintained at a high level during the observation period of up to six months in these mice. Il-7 overexpression significantly increased the proportion of T and B cells in peripheral blood, but had little effect on the overall immune reconstitution and did not affect the differentiation of T cell subsets (99).

Injection of gene expression plasmids

The high-pressure injection is a common technique for gene overexpression *in vivo*. A study on humanized mice generated *via* high-pressure injection of IL-15 and Flt3l expression vector found that the reconstruction of NK cells was significantly increased. Furthermore, these NK cells showed normal expression of activated receptors and inhibitory receptors, which could be induced to cause liver damage and could kill target cells *in vitro*, demonstrating that the reconstructed NK cells were functional (100).

Genetic engineering

Mouse models genetically engineered from immunodeficient mice are more stable. Mice repopulated with human hematopoietic cells are a powerful tool for the study of human T and B cells *in vivo*. However, existing humanized mouse models are unable to support the development of human innate immune cells, including myeloid cells and NK cells. In one study, Rongvaux et al. describe a mouse strain, called MI(S)TRG, in which human versions of four genes (human M-CSF, IL-3, GM-CSF, and TPO) encoding cytokines important for innate immune cell development are knocked into their respective mouse loci. The human cytokines support the development and function of monocytes/macrophages and natural killer cells derived from human fetal liver or adult $CD34^+$ progenitor cells injected into the mice. Human macrophages infiltrated a human tumor xenograft in MI(S)TRG mice in a manner resembling that observed in tumors obtained from human patients (101).

Human $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) can reconstitute a human hemato-lymphoid system when transplanted into immunodeficient mice. Although fetal liver-derived and cord blood-derived $CD34^+$ cells lead to high engraftment levels, engraftment of mobilized, adult donor-derived $CD34^+$ cells has remained poor. Saito et al. generated so-called MSTRG and MISTRG humanized mice on a $Rag2^{-/-}$IL-$2rg^{-/-}$ background carrying a transgene for human SIRPα and human homologs of the cytokine macrophage colony-stimulating factor, TPO, with or without IL-3 and granulocyte-macrophage colony-stimulating factor under murine promoters. They transplanted mobilized peripheral blood (PB) $CD34^+$ cells in sublethally irradiated newborn and adult recipients. Human hematopoietic engraftment levels were significantly higher in bone marrow (BM), spleen, and PB in newborn transplanted MSTRG/MISTRG recipients as compared with non-obese diabetic/severe combined immunodeficient IL-$2rg^{-/-}$ or human SIRPα-transgenic $Rag2^{-/-}$ IL-$2rg^{-/-}$ recipients. Furthermore, newborn transplanted MSTRG/MISTRG mice supported higher engraftment levels of human phenotypically defined HSPCs in BM, T cells in the thymus, and myeloid cells in non-hematopoietic organs such as liver, lung, colon, and skin, approximating the levels in the human system. Similar results were obtained in adult recipient mice (102).

In addition, in one study, human TPO knock-in mice were constructed using $Rag2^{-/-}\gamma C^{-/-}$ mice, resulting in an increased level of humanized reconstruction, multi-lineage immune cell development and differentiation, and increased platelet counts (103). SIRPα inhibits the phagocytosis of macrophages physiologically (110) and plays an important role in the maintenance of hematopoietic stem cells, red blood cells, and platelets (101). In one study, the phagocytic activity of macrophages was significantly inhibited by knock-in human SIRPα in $Rag2^{-/-}$ γ $C^{-/-}$ mice (104). In another study, the expression of human monocytes and macrophages in bone marrow, spleen, peripheral blood, lung, liver, and the abdominal cavity was significantly increased by knock-in of human M-CSF into $Rag2^{-/-}$ γ $C^{-/-}$ mice and their migration, phagocytosis, and activation were enhanced (41). Human SCF, GM-CSF, and IL-3 have also been expressed in NSG mice using transgenic technology, to form NSG-SGM3 mice. The reconstruction level of myeloid cells, especially dendritic cells, is significantly improved in these mice (105). In addition, NSG-3GS mice were also constructed by knock-in of human SF, IL-3, and GM-CSF into NSG mice. Humanized myeloid cells were significantly increased in these mice (106). Similarly, myeloid cells were significantly increased by the knock-in of human SCF and KITL in NSG mice (106). On this basis, a study was conducted integrating human M-CSF, IL-3/GM-CSF, TPO, and SIRPα in $Rag2^{-/-}\gamma C^{-/-}$ mice which promoted the increase of human myeloid and NK cells (107).

Humanized mice are also irreplaceable in COVID-19 research. Severe COVID-19 is characterized by persistent lung inflammation, inflammatory cytokine production (111–113), viral RNA, and sustained interferon (IFN) response all of which are recapitulated and required for pathology in the SARS-CoV-2 infected MISTRG6-hACE2 humanized mouse model (based on the $Rag2^{-/-}$ $IL2rg^{-/-}$129xBalb/c background supplemented with genes for human M-CSF, IL-3, SIRPα, TPO, GM-CSF, and IL-6 knocked into their respective mouse loci) of COVID-19 with a human immune system (108). In this study, Sefik et al. show that SARS-CoV-2 infection and replication in lung-resident human macrophages is a critical driver of the disease (108). In summary, the genetic engineering of humanized mice plays a unique role in modeling and studying specific diseases.

Conclusions and future prospects

To the present day, immunodeficient mice have undergone development from Nude mice to SCID, NOD/SCID, and NOD/SCID rg^{null} mice, and their immunity level has gradually increased. To better simulate human diseases, researchers have constructed the human immune system in immunodeficient mice, and the humanized immune system mouse model provides a powerful tool for studying human diseases. However, there are still many limitations of the various humanized mouse models, and further improvements are needed to truly recapitulate the human immune system. One major hurdle is the scarcity of sources of human cells and tissues, in particular, those obtained from fetal samples carry ethical restrictions. One possible solution to this is induced pluripotent stem cell (iPSC) technology, which enables the use of patient-specific iPSCs allowing a renewable source of autologous cells without immune rejection. The second obstacle is that in humanized mice, secondary lymphoid structures are either missing or disorganized, curtailing essential humoral responses, resulting in impairments for both class switching

and affinity maturation post-immunization. To overcome this, lymphoid tissue inducer cells should be introduced without affecting IL2rg receptors. Alternatively, immunodeficient mice can be engrafted with both fetal liver and cells that support fetal liver cell growth from the same clinical donor and supplemented with cytokines, to ensure that the differentiation and maturation of HSCs can take place to improve functional immune cells including macrophages, follicular DC, and T helper cell reconstitution. The third obstacle is that an absence of essential human cytokines hinders optimal HSC engraftment, differentiation, and maturation of functional immune cells. To tackle this issue, mouse models can be hydrodynamically boosted with plasmids encoding cytokines. Despite this improvement, the binding of human cytokines may be hindered by residual mouse cytokines or may induce mouse cells to proliferate and displace the engraftment of human cells due to the cross-reactivity between human and mouse cytokines. Eliminating this problem would require the absolute depletion of murine cells or the introduction of high-affinity human-specific cytokines and growth factors. The fourth hurdle is that human cell engraftment is being negatively affected by mouse cells (red blood cells and innate immune cells) that were not completely depleted during the construction of immunodeficient mice. To improve this, additional gene knock-outs could be added to current strains of immunodeficient mice to further reduce mouse red blood cells, granulocytes, and macrophage functions. However, because of the low human erythrocyte engraftment, excessive reduction of mouse red blood cells might result in anemic mice which have short lifespans, are weak, and are not suitable for experiments. A long-term solution would be to optimize and increase the engraftment rate of human red blood cells in humanized mice so that all traces of mouse red blood cells can be removed. The fifth is the irreproducibility of mouse studies when donors are different for each "batch" of mice. This may be the most important and challenging task for the development of humanized mouse models. Indeed, there is a significant lack of evaluation criteria for donors including clinical data of patients in different disease states and the quantity and quality of their donated specific immune cells. For humanized mouse models, various systemic characteristics are still needed to comment on the development of a successful model. In summary, despite great progress and advances, there are still many limitations to the various humanized mouse models, and further improvements are needed to truly recapitulate the human immune system.

Author contributions

JC and SL wrote the manuscript. ZX and QRP designed the figures. XW, KS, and SW designed the tables. LY, FG, H-FL, and QJP revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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