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Development of humanized mouse with patient-derived xenografts for cancer immunotherapy studies: A comprehensive review

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Abstract

Immunotherapy has revolutionized cancer treatment, however, not all tumor types and patients are completely responsive to this approach. Establishing predictive preclinical models would allow for more accurate and practical immunotherapeutic drug development. Mouse models are extensively used as in vivo system for biomedical research. However, due to the significant differences between rodents and human, it is impossible to translate most of the findings from mouse models to human. Pharmacological development and advancing personalized medicine using patient-derived xenografts relies on producing mouse models in which murine cells and genes are substituted with their human equivalent. Humanized mice (HM) provide a suitable platform to evaluate xenograft growth in the context of a human immune system. In this review, we discussed recent advances in the generation and application of HM models. We also reviewed new insights into the basic mechanisms, preclinical evaluation of onco-immunotherapies, current limitations in the application of these models as well as available improvement strategies. Finally, we pointed out some issues for future studies.

KEYWORDS
human specificity, humanized mice, immunology, immunotherapy, patient-derived xenografts

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1 | INTRODUCTION

Cancer immunotherapy utilizes host immune system to eradicate tumor cells. Systematic pre-clinical cancer immunotherapy is dependent on selecting, or preferably, developing appropriate animal models. Among various animal models, humanized mice (HM) have been extensively utilized for in vivo studies of human cancer immunology and immunotherapy. Different models are eligible for evaluating new anti-cancer therapies namely patient-derived xenografts (PDXs), humanized PDX and genetically engineered mice.[1]

The pre-requisite of a successful immunotherapy is a functional immune system of the patient since these methods recruit the host immune cells to combat growing tumors. This limits our ability to test the efficacy of these approaches in conventional experimental models. Early murine studies have evaluated the efficacy of anti-CTLA4 immunotherapy targeting the CTLA-4 receptor of a mouse model of fibrosarcoma and ovarian cancer.[2,3] Favorable results of this investigation promoted the production of human antibodies which was further tested in cynomolgous monkey as the only identified species with cross-reactivity.[4] Nevertheless, testing human specific antibodies in cynomolgous monkeys had its pitfalls: complete cross-reactivity could not be obtained and predictive data for human clinical usage was not provided. Therefore, deploying HM models would facilitate the evaluations on the interaction between immune system and tumors and, also, would yield more clinically reliable results.[5,6] HM are the best mouse model for cancer immunotherapy studies and are consisted of three elements: (a) immunodeficient host mice, (b) human immune cells and (c) human tumor cells. In current review, we explain recent advances in the "humanization" of mouse models which improve their application in the study of immunology and immunotherapy. Moreover, we discuss limitations of using these models and strategies that can remove these limitations. Finally, we explain how these improvements shape the future of employing HM models in cancer studies.

2 | AN OVERVIEW ON IMMUNODEFICIENT MICE

The main challenge in the engraftment of human cancer cells in immune-competent rodents is the xenogenic immune rejection. Table 1 shows the evolution of different immunodeficient mice, although modifications are needed. The major improvement in deploying *scid* mouse model was backcrossing of the *scid* mutation to non-obese diabetic (NOD/Lt) strain background which associated with lower NK cell and myeloid function and, as a result, enhanced human engraftment of immune cells.[7] Moreover, backcrossing with NOD mice introduced a receptor that is highly homologous to a human equivalent called signal regulatory protein alpha (Sirpα). Murine macrophages (MQ) express Sirpα which is able to bind to counterpart CD47, a "don't eat me" signal protein, on human immune cells and subsequently inhibit phagocytosis.[8] Another remarkable

TABLE 1 Immunodeficient mouse strains for human immune system engraftment

Mouse model	Strain/Characteristics	Life span	T	B	NK	DC	MQ	Com.	References
Nude	Spontaneous mutation of Foxn1 causing lack of thymic tissue	>18 mo	−	+	+	+	+	+	117
scid	*CB17-Prkdc*$^{scid-/-}$ Defect in DNA protein kinase, no functional rearrangement of antigen-specific receptors	<12 mo	−	−	+	+	+	+	118
NOD-scid	NOD.CB17-Prkdc $^{scid-/-}$ Expression of the scid mutation on the NOD strain background	<10 mo	−	−	FI	FI	FI	−	119–121
NSG	NOD. Cg-*Prkdc*^{scid} *IL2rg*^{tm1Wjl}*/SzJ* NOD-scid combined with IL2rg$^{-/-}$	>18 mo	−	−	−	FI	FI	−	11,122
NOG	NOD *cg-Prkdc*^{scid}*Il2rg*^{tm1Sug} Similar to NSG, with truncated IL2rg (enables binding but not signaling of cytokines)	>18 mo	−	−	−	FI	FI	−	39,123
NRG	NOD. Cg- Rag1^{tm1Mom} IL2rg^{tm1Wjl} NOD, RAG1$^{-/-}$ and IL2rg$^{-/-}$ combined	ND	−	−	−	FI	FI	−	12,15,20
BRG	BALB/c Rag2^{null} IL-2Rgc^{null} interbreeding of NOG and BALB/c-*Rag2*^{null}	ND	−	−	−	FI	FI	−	14,124
BRGS	BALB/c Rag2^{null} IL-2Rgc^{null} NOD.sirpa BRG mice with integration of the NOD/Lt Sirpa polymorphism	ND	−	−	−	FI	FI	−	125,126

Note: Abbreviations: Com., complement; DC, dentritic cell; FI, function impaired; Foxn1, forkhead box protein; IL2Rgc, interleukin-2 receptor γ-chain; MQ, macrophage; ND, not determine; NK, natural killer; NOD, non-obese diabetic; NSG, NOD-scid combination; Prkdc, protein kinase DNA activated, catalytic polypeptide; Rag, recombination activating gene; scid, severe combined immunodeficiency; SIRPa, signal regulatory protein a.

milestone was the introduction of a mouse strain knocked-out in the interleukin (IL)-2 receptor common gamma chain (IL2Rγ) gene[9] that not only this mice would have functionally impaired adaptive immune system but, more importantly, disabled NK cell development.[10] The combination with NOD-*scid* (NSG)[11] mice and RAG (NRG)[12] mice revolutionized human cell engraftment. Similar to NSG, NOG mice have NOD-*scid* background with truncated IL2γc gene which enables binding but not signaling of cytokines.[13] Another improvement of human engraftment was achieved by interbreeding of NOG and BALB/c-*Rag2*^{null} which generated BRG mice. In addition, integration of NOD/Lt Sirpα polymorphism into BRG mice further refined human cell reconstitution.[14] Successful engraftment of human hematopoietic immune cells is achieved in NSG and NOG and provided a suitable animal models for initial immunologic studies of immunotherapy.[15] According to preliminary studies immune reconstitution is not yet optimal. In this review, we aimed to study novel approaches that improve hematopoietic reconstitution in the host mice for studies.

3 | PATIENT-DERIVED XENOGRAFTS AND CELL-DERIVED XENOGRAFTS

Immunodeficient mice grafted with human cancer cells could be classified as PDXs and cell-derived xenografts (CDXs) based on the type of samples or human cells used in transplantation.[16] CDXs are particularly useful in high through put screening assays and genetic modifications. Although CDXs come with some limitations like the selective proliferation of clonal cells.[17] In comparison with CDXs, PDX mouse models maintain more characteristics of their parental malignancy and thereby are stronger tools for investigating the effects of targeted therapy or chemotherapy.[18] In order to produce PDXs, fresh human tumor tissues are implemented into an immunodeficient mice in which the chance of rejection is lower. The size of tumor tissues is no larger than 2 mm^3 and are implemented into the mice subcutaneously or orthotopically meaning at the same site of the tissue-of-origin. Normally the immunodeficient mice used for the generation of PDXs have combined T/B/NK cell deficiency and/or macrophage tolerance for human cells like NOD/SCID and NSG/NOG mice.[16] In addition, PDXs is especially beneficial for in vivo screening of targeted therapies using single-mouse schedule.[19] Following such an approach not only decreases the number of mice and costs of evaluation but also allows for identifying the best treatment in a panel of PDXs and validating the efficacy of tested therapies in the selected target-specific tumors. Thereby, these mouse models perfectly represent the original patient tumor which would serve as a more reliable platform to predict therapeutic outcomes. As an example, Dr Sidransky conducted a research on 237 patients with different tumor types using PDX mice and validated that these models are able to faithfully conserve the genetic profile of primary tumor.[20] While such pre-clinical studies have yet to be developed for immunotherapies, investigating chemo-/radio- and targeted therapies in HM are of particular interest.

3.1 | Limitations of PDX and CDX models

One of the major drawbacks of both PDX and CDX mouse models for human oncogenesis is that the process of oncologic transformation from normal cells into malignant cells is missing. More importantly, generation of a PDX model is time consuming and can take as long as 6 (or more) months. Adding to this, certain tumor types are difficult to establish as PDX models such as prostate cancer which might be due to innumerable unknown factors in the development of prostate tumor. And as for tumors with genetic heterogenicity, if the genetic heterogeneity is not all represented in the dissected tumor that is passaged these tumors cannot always be recapitulated in serial passages.

4 | HUMANIZATION FOR XENOGRAFT

By far, several types of HM have been employed in cancer research. Basically, mice are considered humanized after being engineered to express certain human proteins that are relevant to tumor growth.[21] Needless to say, the ultimate goal of humanization is to develop mice that are fully competent to human immune system and are able to mount proper anti-cancer immune responses. Thereby, allowing for more accurate interpretations of therapeutic interventions. This objective requires implementing malignant and immune cells, ideally from the same donor, into an environment customized as fully compatible between graft and the host; which ensures that neither rejection of human cells by mouse immune cells nor human immune cell toxicity on the host would occur. Upon successful implementation, human leukocyte precursors are ultimately engrafted and receive full trophic support within the host. As mentioned earlier, only immunodeficient mice are suitable for this purpose. Three widely used mouse strains are (a) NSG mice which are characterized by complement deficiency (preventing lysis of human cells by mouse complement) and loss-of-function mutation of Sirpα (decreasing the phagocytosis of human $CD47^+$ cells by mouse macrophages, (b) *scid* phenotype lacking of T lymphocytes and B lymphocytes as a result of mutations in the Prkdc (protein kinase, DNA activated, catalytic polypeptide) gene and (c) strains with mutation in IL-2 receptor common γ chain (IL2rg) featuring profound NK cell deficiency[11,19] (Table 1).

There are three types of HM developed by two sources of human immune cells: PBMC and human $CD34^+$. (a) Hu-PBL (peripheral blood lymphocytes), (b) Hu-$CD34^+$ (also called Hu-SCR) and (c) BLT mice (bone marrow–liver–thymus) (Figure 1). Each of these HM has their own advantages and limitations. In the following, the process of generating these HM is discussed. Table 2 compares the different features of HM models.

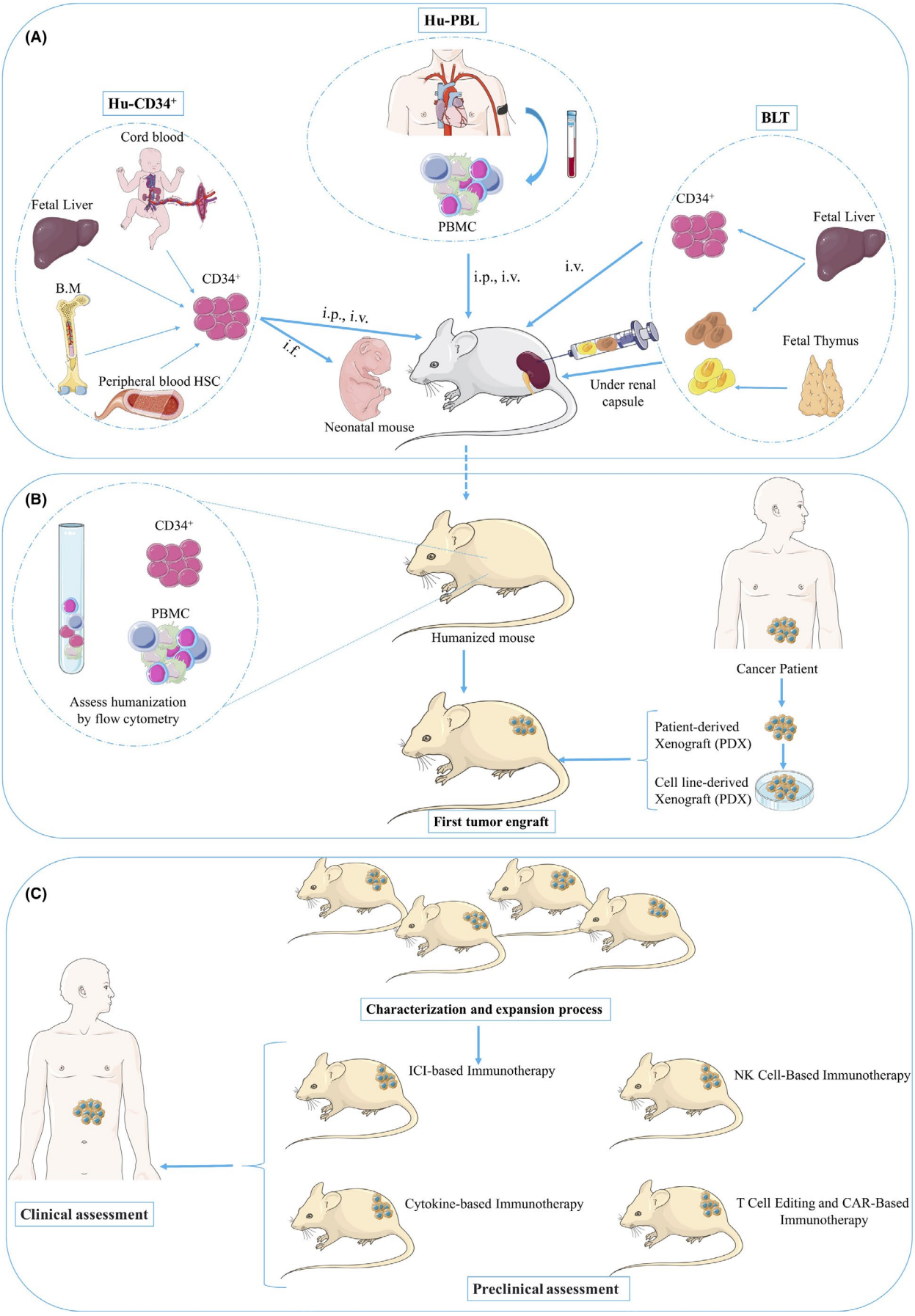
(A)
Hu-PBL
PBMC
Hu-CD34+
Cord blood
Fetal Liver
B.M
CD34+
Peripheral blood HSC
i.p., i.v.
i.f.
i.p., i.v.
i.v.
Neonatal mouse
Under renal capsule
BLT
CD34+
Fetal Liver
Fetal Thymus
(B)
CD34+
PBMC
Assess humanization by flow cytometry
Humanized mouse
Cancer Patient
Patient-derived Xenograft (PDX)
Cell line-derived Xenograft (PDX)
First tumor engraft
(C)
Characterization and expansion process
ICI-based Immunotherapy
NK Cell-Based Immunotherapy
Cytokine-based Immunotherapy
T Cell Editing and CAR-Based Immunotherapy
Preclinical assessment
Clinical assessment

FIGURE 1 The major steps in the production of humanized mice. A, Demonstrates the humanization process of immunodeficient mice. Hu-PBL: intravenous (iv) or intraperitoneal (ip) injection of peripheral blood mononuclear cells to an adult immunodeficient mouse. Hu-CD34$^+$: IV, IP or intra-femoral (if) injection of human CD34$^+$ HSCs derived from umbilical cord blood, bone marrow, fetal liver or peripheral blood HSCs into irradiated neonatal or adult immunodeficient mice. BLT: engraftment of human fetal thymus and liver fragments under the renal capsule of the kidney in irradiated adult immunodeficient mice and IV injection of human CD34$^+$ HSCs from the autologous fetal liver. B, Engraftment of human immune system to mouse models is monitored by flow cytometry to determining the percentage of differentiated human cells in the peripheral blood of the mice. Then Cell line-derived xenografts or patient-derived xenografts can be implanted into immunodeficient mice (First tumor engraft). C, Upon characterization and expansion of the first tumor-xenograft mice, the immunotherapy of interest may be conducted. Findings are then translated and applied to the adapted therapy of the patient. BLT, bone marrow–liver–thymus; B.M, bone marrow; Hu-PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cell

4.1 | Hu-PBL model

Immunodeficient mice reconstituted with PBMCs and tumors, are called Hu-PBL models which are the simplest version of humanization. Generation of Hu-PBL models starts by isolating PBMCs using Ficoll-Hypaque gradient centrifugation by which mainly neutrophils are removed. Beside the mature human leukocytes in PBMC inoculum, a few HSCs exist which are unable to colonize the murine host because of the lack of a proper microenvironment.[22] Moreover, human B lymphocytes and myeloid cells are observed at low levels which may be due to the lack of the human cytokines required for their survival.[23,24] PBMCs could be transplanted into adult mice intravenously, intraperitoneally, or intrasplenically. The major limitation of this model is that it leads to graft-versus-host disease (GVHD).[13,23,25] The onset of GVHD is directly associated with the degree of human T cell, in particular CD4$^+$ T cell, engraftment as well as prior sublethal irradiation.[23,26–28] Therefore, therapeutic-relevant outcomes evaluation is limited to the weeks after PBMC injection and before the onset of GVHD.[13,23] Generating these mouse models have allowed for the identification and characterization of ICIs antibodies.[28,29] Furthermore, these "immune-avatar" mice can be utilized to investigate the immune-mediated effects of antibodies targeting cancer cell antigens and allow for the infiltration of patient-derived tumors by lymphocytes.[30]

4.2 | Hu-CD34$^+$ model

Injection of CD34$^+$ HSCs isolated from bone marrow (BM), cord blood or fetal liver of the patient allows for generation of various types of human immune cells in the murine host and triggers tolerance against mouse tissues. These cells can be injected intravenously, intraperitoneally, intrafemorally and also intracardially and intrahepatically into neonatal or adult immunodeficient mice[11,13,31] (Figure 1). Several factors determine the success of engraftment such as HSCs source, route of injection, strain, age and sex of the recipient. For instance, in newborn or mice up to 4 weeks of age T cell development occurs faster compared with adult mice.[32] In a study, Brehm *et al*[15] evaluated engraftment outcome of different mouse strains and routs of injection in adult or neonatal mice and showed that transplantation into newborn NOD-scid IL2Rγ- and NOD-Rag1- IL2Rγ-mice resulted in higher levels of human immune cell engraftment compared with BALB/c- Rag1- IL2Rγ- mice. Generation of Hu-CD34$^+$ mice starts with irradiation of mice that are 5-12 weeks of age in order to help HSCs engraftment. Then, human CD34$^+$ cells are transplanted into irradiated mice. Around 10-12 weeks of age, engraftment of the human HSCs in the murine host can be confirmed by assessing for differentiated human CD45$^+$ cells (leukocyte common antigen) in the Peripheral blood of the mice using flow cytometry.[33] If the mice have more than 25% human CD45$^+$ cells in their peripheral blood then the engraftment of human immune system is considered successful. Now the HM could be inserted with specific PDXs and an immunotherapeutic agent could be subsequently applied for testing. Alternative methods to irradiations are busulfan [34] and antibody-mediated deletion of mouse progenitor cells.[35] In the same context some mouse strains like the NOD, B6.SCID *Il2rγ*$^{-/-}$*Kit*$^{W41/W41}$ (NBSGW) mice support the transplantation of HSCs without irradiation.[36,37]

Although in the Hu-CD34$^+$ models all human hematopoietic lineages are represented, but not all are functionally fully developed.[13] For instance, the majority of the human B cells are immature CD5$^+$ B cells, mainly because at the transition phase the process of B cell differentiation is blocked and eventually results in the accumulation of B cell precursors in the spleen.[38,39] Similarly, the differentiation of the myelomonocytic lineage is impaired and monocytes are phenotypically immature.[40] In addition, CD4$^+$ T lymphocytes display memory phenotypes, and both T and NK cells have functional impairment.[39,41] Mouse thymus supports human T cell development; however, the question of major histocompatibility complex (MHC) restriction is yet to be elucidated. According to Halkias *et al*[42] human thymocytes show similar behavior in mouse and human thymic environments. Also, they can interact with both HSCs and mouse tissue in HIS mice thymus. Adding to this, Watanabe *et al* demonstrated that the mouse thymic environment, and not the mouse I-A MHC molecule, is crucial for the development of human T cells, suggesting that the human CD4$^+$ T repertoire is restricted by human MHC class II molecules and murine MHC. However, these animals have very poor human thymopoiesis. Mentioned limitations may restrict the value of the Hu-CD34+ model in studies of human immunology and immunotherapy.[43]

4.3 | BLT model

In the BLT model, implemented human fetal liver and thymus create a human thymic microenvironment that promotes human T

TABLE 2 Summary and comparison of different humanized mouse models

	Hu-PBL	hu-HSC	BLT	References
Method, cell source and mice used	i.p. injection of human PBMC. SCID, NOD-SCID, NSG, NOG	Intrahepatic injection of $CD34^+$ HSC into newborn mice within 72 h of birth Intravenous injection of $CD34^+$ HSC into adults. $Rag1^{-/-}$ $gc^{-/-}$, $Rag2^{-/-}$ $gc^{-/-}$, NSG, NOG	Co-implantation of thymic fragments and human fetal liver under kidney capsule with iv injection of autologous $CD34^+$ HSC. $Rag1^{-/-}$ $gc^{-/-}$, $Rag2^{-/-}$ $gc^{-/-}$, NOD-SCID, NSG	11,14,39,43
Preconditioning	−	Sub lethal irradiation	Sub lethal irradiation	43
Human B cell	+	+	+	124,127,128
Human T cell	+	+	+	124,129
Human NK cell	−	−/+ with IL15 or Flt3L	−	61,124
Human macrophages	−	+	+	56,59
Human dendritic cells	−	+ specially with Flt3L	+	56,62
Neutrophils	−	−/+ with IL3, GM-CSF and M-CSF	+	56,127
Primary immune response	−	+ Humoral and cellular. IgM and weak IgG	+ Humoral and cellular. IgM and weak IgG	123,130
Advantages	Easy to prepare and Fast to establish T cells are HLA restricted and functional such as memory T cells	Easy to prepare Multilineage hematopoiesis Primary immune response Mucosal engraftment	Multilineage hematopoiesis Primary immune response Presence of human thymus Human HLA-restricted Mucosal engraftment	11,13,14,124,129,131,132
Disadvantages	More prone to GVHD No primary immune response Lack B and myeloid cell engraftment No multilineage hematopoiesis Just suitable for experiments below 3 mo	Low NK and IL-15/ IL-15Rα requirement to increase function No human HLA restriction Immune cells differentiate more than 10 wk	Poor class switching Possibility of GVHD Surgery needed Requires human fetal tissue Immune cells differentiate more than 10 wk	11,13,14,129,131,132

Note: B.M, bone marrow; BLT, bone marrow–liver–thymus; Flt3L, Flt3 ligand; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte/ macrophage colony-stimulating factor; GVHD, graft versus host disease; HLA, human leukocyte antigen; HSC, hematopoietic stem cell; Hu-PBL, peripheral blood lymphocytes; IgG immunoglobulin G; IgM immunoglobulin M; IL, interleukin; IL-15Rα, interleukin-15 receptor α; NOD, non-obese diabetic; NSG, NOD-scid combination; PBMC, peripheral blood mononuclear cell; Rag, recombination activating gene; scid, severe combined immunodeficiency.

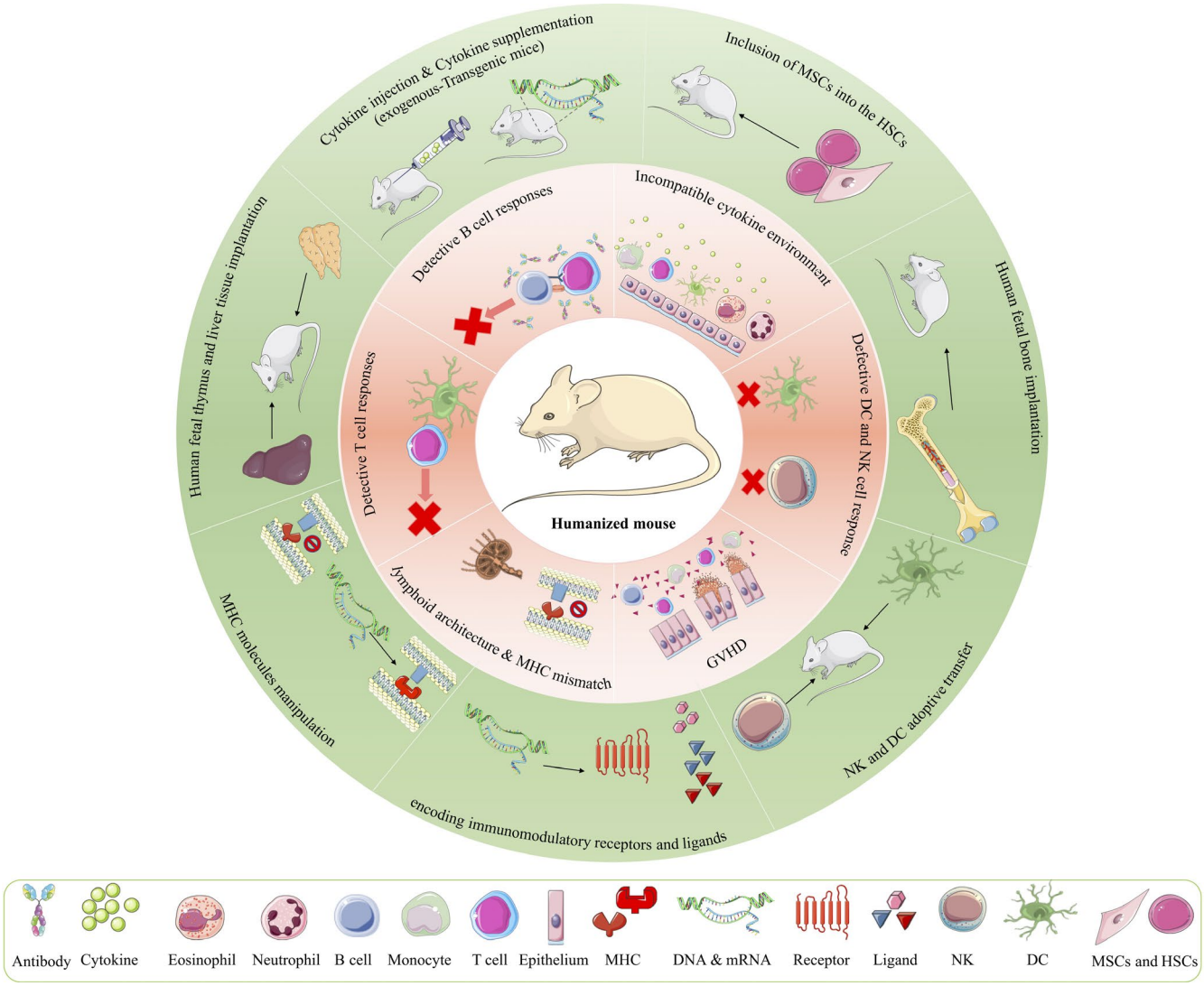

FIGURE 2 Schema showing the areas that require development and optimization in HM model. The pink ring represents immunological limitations in HM and the green ring provides the possible improvement strategies. CAR, chimeric antigen receptor; DC, dendritic cell; MSCs, mesenchymal stem cells; HSCs, hematopoietic stem cells; MHC, major histocompatibility complex; NK, natural killer; GVHD: graft-versus-host disease

cell development and selection.[44] In order to achieve this purpose, pieces of human fetal thymus and liver are transplanted under the kidney capsule. Then, autologous CD 34$^+$ cells that are isolated from the liver are injected which allow HLA restriction.[45] By doing so, human thymopoiesis takes place in engrafted human thymic tissues. More importantly, evidences have shown that BLT models are able to generate potent human immune responses since these animals have the potential to reject allogenic and xenogeneic grafts, trigger HLA-restricted antigen-specific human T cell reaction, and produce antigen-specific human IgM and IgG antibodies with subclass switching upon immunization or xenograft implantation.[46–48] The advantage of this approach would be exclusive positive selection of human T cells in thymus, but on the other hand, high-affinity T cells against mouse MHCs are not eliminated. As a consequence, the incidence of GVHD in these models is higher than other CD34+HSC engrafted models. However, there are some studies indicative of decreased GVHD in BLT models. As an example, mouse dendritic cells (DCs) can migrate into human thymic grafts in BLT models and take part in educating thymic human T progenitor cell and reduce the incidence of GVHD. In addition, pipetting of human thymic grafts before transplantation and cryopreservation can remove existing human T cell progenitor cells which would further alleviate GVHD.[49–51] Implementation of mesenchymal stem or progenitor cells could further improve the BLT models since they are capable of creating BM environment.[52]

Preferably, HM should be generated with the immune system from which PDXs will be produced. Even so, current mouse models are providing a platform for further development of fully personalized and humanized mouse models that can be used for cancer immunology and immunotherapy research. There are several strategies that support the improvement of HM production which will be discussed in the following.

5 | IMPROVING HUMANIZED MICE BY OVERCOMING CURRENT LIMITATIONS

Improving current HM models to better represent the human immune system would permit assessment of new biological therapies. Developing humanized have progressed during years; however, some aspects need to be improved like incidence of GVHD, incomplete engraftment of immune cells, lack of human cytokines and growth factors. On the other, HSCs derived from cancer patients are not optimal for repopulating mice. Some areas that require more development for better recapitulating human immune responses are as follow: (a) Innate immune cell development and function, (b) B cell maturation and antibody responses, (c) secondary lymphoid organ development, (d) New robust renewable sources of human cells and tissues for grafts, (e) Development of robust human HLA-restricted T cell responses, (f) Increase engraftment rate of HSCs, (g) Reduce GVHD, (h) Infrastructure for increasing community access of HM model. Figure 2 summarizes some of the limitations ahead of these improvements as well as some of their related solutions. In the following, we will discuss about some of the improvement strategies for refinement of HM.

5.1 | The role of cytokines and growth factors in upgrading HM

Cytokines signaling in the environment of engrafted HSCs and differentiating immune cells directly affects the orderly maturation and thereby trafficking into the tumors on the organism.[53] Several studies focusing on improving the cytokine environment within HM is ongoing. It has been revealed that integrating a population of mesenchymal stem cells (MSCs) into the HSCs destined for engraftment may also modify the eventual reconstitution of the myeloid cell lineage within the HM. According to Chen, Huang, and Womer, co-culturing of HM BM cells with MSCs, which have shown the evidence of immunoregulatory function and have the potential to produce cytokines and growth factors, improves the viability of newly differentiating DCs.[54] Preliminary studies by Shultz *et al*[11] by NSG model revealed that administration of IL-7 increased the production of T cells in HM. Chen *et al*[55] have shown that administration of IL-5 and Flt-3/Flk-2 cytokines, that are encoded on plasmids expressed in hepatocytes, enhanced the levels of NK cells, while granulocyte-macrophage colony-stimulating factor (G-MCSF) and IL-4 elevated DCs and macrophage colony-stimulating factor (M-CSF) was able to increase the number of macrophages and monocytes detectable within the HM.

Despite the positive effects of addition of these cytokines on immune cell differentiation and expansion, non-physiological concentration of them within HM would misdirect cell development and trafficking. Different genetic backgrounds of HM have been genetically engineered to express IL-3, M-CSF, G-MSCF, Thpo or Sirpα. In order to improve the expression of these critical cytokines, Rongvaux *et al*[56] produced strains of Rag $2^{(-/-)}$-Il2γ^{null} mice called MITRG characterized by targeted knock-ins of the human genes encoding IL-3, M-CSF, GM-CSF and Thpo. After the engraftment of human $CD34^+$, these MITRGs produced T and B cells, as well as functional NK and myeloid cells, that had the capacity to infiltrate cell-line derived melanoma tumors and change their growth through a vascular endothelial growth factor (VEGF)-dependent mechanism. Wunderlich *et al*[57] developed another transgenic humanized strain. They produced the NSG-SGM3 (NSGS) mouse, which expresses human SCF, GM-CSF, and IL-3, to facilitate the study of acute myeloid leukemia via increased production of mature myeloid cells. Another strain, NOG–IL-2 Tg mouse, was developed by Katano *et al*, through inserting a human IL-2 transgene into a NOG background. The HM generated from this strain produced a diverse set of NK cells with the ability to target both introduced leukemia and lymphoma cells.[58]

In the same context, in the NOD background, NSG SGM3 engineered HM were developed by some modifications on NSG mice to express human SCF (c-kit ligand), GMCSF and IL-3 genes which were encoded by cDNA constructs that randomly integrate and are driven by a CMV promoter.[59] In addition, NOG-EXL were developed by engineering NOG mice to ubiquitously express human GMCSF and IL3 genes under control of the SRa promoter.[60] Furthermore, Flavell's group developed SRG-15 engineered HM which are BALB/cRag2$^{-/-}$ IL2rg$_c^{-/-}$ knock-in for human SIRPα and IL-15. These HM showed increased development and function of NK cells, $CD8^+$ T cells, and tissue-resident innate lymphoid cells.[61]

Although generation of mouse strains bearing these transgenes can help in creating a functional myeloid lineage, but they are relatively difficult to breed and their development complicate the generation of the large cohorts necessary for immune therapy cancer studies. Different types of DC exist in mice which are not homologous human DCs. Also, in HM the development of DCs and their maturation are not optimal. In order to overcome this, novel HM models based on the BALB/c *Rag2* $^{(-/-)}$ *Il2rg*$^{(-/-)}$ *Flt3*$^{(-/-)}$ (BRGF) and NOD. Cg-Rag1^{tm1Mom}Il2rg$^{tm1Wjl/SzJ}$ Flk2/Flt3$^{-/-}$ (NRGF) mice containing a mutated receptor tyrosine kinase Flk2/Flt3 were produced. Development of human DCs in BRGF and NRGF mice are improved upon exogenous administration of human Flt3 ligand (Flt3L) after HCT which results in the marked increase of human NK and T cell population.[62,63]

5.2 | MHC modification and limiting GVHD

Myelopoiesis, a process by which different population of leukocytes are generated, is inevitable and associates with pathology in animal models of GVHD. $CD11c^+CD14^+$ is the dominant donor-derived population of leukocytes in GVHD. It is observed that GVHD-isolated macrophages are able to stimulate greater activation and proliferation of allogenic T cells, secret higher levels of inflammatory cytokines in steady-state and mediate direct toxicity. These observations accentuate the function of human macrophages and the potential to prevent and treat GVHD by exploiting their functionality.[64] Another important

player in the development of GVHD are T lymphocytes. According to a recent study on a humanized mouse model, donor monocytes are able to activate host skin-resident T cells and result in GVHD-like dermatitis. The phenomenon suggests a pathogenic role in development of acute GVHD by host tissue-resident T cells.[65]

Several studies are focusing on the improvement of human T cells and preventing GVHD in HM; achieving this purpose will qualify HM with functional T-cell receptors (TCRs) that are able to interact with the matched HLA complexes on antigen-presenting cells. In order to do so, different strategies have been tested based on the genetic manipulation of the MHC molecules. As for Hu-CD34+ mice, defective T cell function was somewhat associated to the mismatch between human and mouse MHCs. Conducting this refinement relies on the substitution of the mouse MHC I and II by different haplotypes. Danner *et al*[66] used NOD-Rag1-IL2$\gamma^{-/-}$ mice that expressed human HLA-DR4 allele and demonstrated that upon engraftment with HLA-matched HSCs, the immune system in these mice was reconstituted with high numbers of functional B and T cells and also was capable of appropriate response to immune challenge. Patton *et al*[67] showed that when NSG mice expressing human allele HLA.A2.1 were engrafted by $CD34^+$ cells from a HLA.A2.1 matched donor, they were not as efficiently humanized as NSG controls. They postulated that this could be due to the alloreactivity between mouse and human peptide antigens bound to HLA proteins.[6]

In another study by Kim *et al*[68] a transgenic NRG mouse called "DRAG" was produced that could express HLA-DR4. This strain of mice which was transplanted with HLA-$DR4^+$ HSCs was able to develop higher number of $CD4^+$ T cells and also higher concentration IgG and IgM. Similarly, BRGSA2DR2 mice, which are generated from BRGS mice and are able to express human HLA-A2 and DR2 transgenes, revealed faster development of $CD4^+$ and $CD8^+$ T cells and higher concentration of IgG.[69] Lone's team developed a mouse called "HUMAMICE" that is a combination of both murine MHC deficient and HLA transgene expressing mice. This engineered mouse lacks T and B cell as a result of rag mutation, NK cells due to IL2Rgc and has no residual cytolytic activity because of perforin KO.[70] Transplanting HLA-matched PBMCs in HUMAMICE reconstituted with human immune cells was not followed by signs of GVHD. Moreover, these mice develop functional human T and B cell as evidence of vaccination with Hepatitis B virus (HBV) showed production of HBV-specific antibodies.[70] Major limitation ahead of this approach is the difficulty to find HSCs that express a particular combination of HLAs.

Recently by using CRISPR/Cas9 in NOG mice, Ka *et al* established a novel beta-2 microglobulin (B2m) KO mouse model. A modified dKO (dKO-em) mouse model is established by crossing B2m KO mice with I-Ab KO mice. dKO-em mice showed high engraftment efficiency as well as no signs of GVHD after the transfer of human PBMC. Moreover, engrafted human PBMCs significantly survived longer in the peripheral blood and spleens of dKO-em mice, compared with dKO-tm mice. Thus, dKO-em mice may count as a promising model for preclinical investigations of novel therapeutics for human diseases.[71]

5.3 | Other improvement strategies

Generally, HM mice undergo myeloablative conditioning before implementation of human HSCs in order to provide the required space in the host BM niche for the substitution of human HSCs engraftment.[11] Each strain of mice reacts differently to the irradiation. For instance, the *scid* mice are more sensitive to radiation-induced DNA damage compared with $Rag1^{null}$ or $Rag2^{null}$ mice.[12] As mentioned earlier, the *c-kit* (CD117) mutant mouse was found to be a suitable host for human HSCs engraftment which requires no prior irradiation. Given that c-kit plays an important role in HSCs maintenance and differentiation, NSGW41 mice that carry the w41 mutation in c-kit, show reduced HSCs numbers which lead to lower competition and better engraftment of human HSCs.[36,72]

A growing number of engineered mice are being commercially developed by modifying immunocompetent mice to express one or more fully human genes. Also, by generating "humanized" knock-ins mice encoding negative or positive immunomodulatory receptors and ligands such as CD47, Programmed death-ligand 1 (PD-L1), B and T lymphocyte attenuator (BTLA), CD137, T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), lymphocyte-activation gene 3 (LAG-3), inducible T-cell costimulatory (ICOS), glucocorticoid-induced tumor necrosis factor receptor (GITR), OX40, OX40L [43]. These mice are particularly utilized for the investigation of checkpoint combination therapy. Mice expressing "humanized" programmed cell death 1 receptor (PD-1) or CTLA-4 molecules have been also beneficial for separating efficacy and autoimmunity induced by anti-CTLA4 antibodies[73] or characterizing a clinical candidate anti-PD-1 antibody.[74]

As for autoimmunity complications, Khosravi-Maharlooei *et al* investigated the role of thymus in development of multi-organ autoimmunity in HIS mice. They observed that autoimmunity was developed earlier in HIS mice with a native mouse thymus than thymectomized mice with a thymocyte-depleted human thymus graft. Structural defect in the native mouse thymus correlated with impairment in the negative selection of transgenic TCR expressing thymocytes with the capacity to recognize self-antigens. It appears that disease developed in an indirectly and without recognition of antigens on recipient mouse MHC. Even if human thymus grafts have normal structure and negative selection, failure in tolerating human T cells that recognize mouse antigens being presented on HLA molecules may explain the development of autoimmunity.[75] This suggests generating methodologies that bypass human autoimmunity in the next generation of HIS mice.

6 | PRE-CLINICAL EXPERIENCES OF HUMANIZED MICE

6.1 | ICI-based and monoclonal antibody therapy

Humanized mice engrafted with tumors are being used to better understand how checkpoint blockade interacts with the immune

system and also to test the efficacy and effects of immunomodulation agents. Given the relatively simple accessibility and handling of human PBL samples, the Hu-PBL model is widely utilized in evaluating the interactions between human immune cells such as T cells and NK cells as well as human tumors in vivo.[76] For instance, in a study by Ignacio Melero *et al* it was observed that this model is useful for investigating the effect of human PD-1 (Nivolumab) and CD137 (Urelumab) antibodies in in vivo T cell-mediated anti-tumor responses.[28] Furthermore, Wang *et al*[77] reported that PD-1 targeted immunotherapy can be installed in Hu-CD34$^+$ humanized NSG mice having CDXs and PDXs partial HLA matched human tumor which indicates the value and efficacy of the Hu-CD34$^+$ model for cancer immunotherapy investigation. Unusual long serum half-life of IgGs and Fc domains are due to their rescue and recycling via the neonatal Fc receptor (FcRn). There is a significant difference between rodent and human FcRn reactivity, rendering wild type rodents an inadequate model for studying monoclonal antibody therapy. To overcome this problem with the advance of genetic engineering, mouse models have been established expressing human FcRn, and lacking mouse FcRn protein like NSG FcRn-/- hFcRn Tg model.[78]

One of the major limitations associated with using antibodies to target checkpoint inhibitors is the incidence of cytokine release syndrome (CRS). As demonstrated by the TGN1412 (anti-CD28) clinical trial, screening these antibodies in HM and non-human primate may not necessarily represent the response of a human immune system.[79] To address this issue, two humanized mouse models were developed for the detection of a CRS. NRG-AB^0 or NSG mice were engrafted with PBMC and injected with muromonabCD3(120), or TGN1412.[80] Utilizing this approach in Hu-CD34$^+$ and BLT models would allow evaluating innate immune cell reactions to antibodies targeted against these cell populations.[81]

As more cancer immunotherapy treatments are tested in clinical trials, an association between immune-mediated tumor killing responses and CRS are observed by clinicians. Due to these observations, pre-clinical studies that are capable of recapitulating CRS in HM are gaining more interest. In a recent study, HM were engrafted with a diffuse large B cell lymphoma (WSU-DLCL2) and treated with either novel CD20-T-cell bispecific antibody (TCB) or obinutuzumab (anti-CD20 monoclonal Ab).[82] CD20-TCB which contains two CD20 binding domains and one CD3e domain, incited more extensive cytotoxic responses than obinutuzumab. Moreover, administration of CD20-TCB was associated with enhanced expression of inflammatory cytokines which indicates that CRS responses were not generated by obinutuzumab treatment. Such pre-clinical experiments on HM highlight the value of them in testing protocols that are designed to maximize both efficacy and safety. The key component of pre-clinical assessments is the question related to immunotherapy mediated toxicity, especially in the context of CRS, and also the requirement for a related reliable assay. As an example, recently a group at the US Food and Drug Administration (FDA) reported the testing of CRS using monoclonal antibody therapies that are known to elicit strong cytotoxic response in the BLT-HIS mice.[83] Models that can accurately and reliably predict the induction of CRS by immune therapeutics are scarce. Recently Chunting Ye *et al* reported the development of a HM model based on the NGS mouse to investigate CRS in vivo. NSG-MHC-DKO, PBMC-engrafted NSG and NSG-SGM3 mice were employed in order to study cytokine release in response to treatment with monoclonal antibody immunotherapies. Results showed that among the three mouse models, PBMC-engrafted NGS models are quick, sensitive and reproducible platform for screening novel therapeutics for CRS.[84] More recently, others developed a model for predicting CRS while minimizing GVHD by spleen mononuclear cells (SPMCs). They reported that NSG mice reconstituted with PBMC-and SPMC better predicted OKT3-mediated CRS. The SPMC model allows generation of large experimental groups while NSG-dKO mice are able to mitigate the limitation of early GVHD.[85]

6.2 | NK cell-based and cytokine-based immunotherapy

Another promising cellular immunotherapy for cancer is adaptive NK cell therapy. Recently some progression has been made in stimulating NK and NKT anti-tumor activity utilizing HM models in various cancers such as glioblastoma, colorectal, ovarian and pancreatic cancer.[86–89] Moreover, cytokine therapy has caught attentions in the efforts to elicit NK and NKT cell antitumor activities. In a study, human neuroblastoma cell line and human NKT cells expanded ex vivo were injected into HSC-engrafted NSG mice.[90] It was observed that NKT cells resided within the tumor associated macrophages (TAMs) in the tumor microenvironment. However, the survival and function of NKT was inhibited by CCL20 secreted by TAMs favoring tumor growth. Lie *et al* showed that transducing NKT with Il-15 before transferring into mice led to decreased tumor growth as a result of increased NKT survival and suggested a role for IL-15 cytokine therapy.[90] Immune therapy with IL-15 was utilized to expand the NK cell populations of Hu-CD34$^+$ NSG mice implemented with human breast cancer and the result was enhanced proportions of activated CD56+CD27− NK cells.[91]

Beside IL-15, the effect of IL-12 in stimulating the immune system to attack tumor cells has also been investigated in humanized tumor-bearing mice. As an example, NHS-IL12 is an antibody-IL12 fusion protein targeting the naked histones/DNA complexes that are found in necrotic tissues such as tumors.[92] NHS-IL12 was utilized in conjunction with antibody-complexed IL12 (IL2MAB602) or IL-7 (FcIL7) in the Hu-SRC-SCID model of rhabdomyosarcoma.[93] NHS-IL12/IL2MAB206 enhanced tumor infiltrates of NK cells, T cells and macrophages.[94]

6.3 | T cell editing and CAR-based immunotherapy

One of the strategies of targeting immune system to upgrade its anti-tumor activity is redirecting T cell specificity via transgenic TCR or chimeric antigen receptor (CAR) engineered T cell therapy. CAR-T cells are MHC independent and thereby can be redirected

to any target of interest.[95] Recently, using CAR-T therapy in HM have been employed to broaden the scope of cancer treatment as well as optimizing the safety and efficacy of CAR manipulation.[96] For instance, CARs targeting mesothelin for mesothelioma, CD44v6 for AML and multiple myeloma and ROR1 for mantle cell lymphoma have been tested in NSG humanized mice.[93,97,98] Adding co-stimulatory motifs in the CD3ζ in the intracellular signaling domain increases CAR signaling. A CAR without co-stimulatory receptor (CCR) is designed for a single antigen while incorporating CCR increased the specificity of CAR for a second antigen. Humanized mice have been extensively used to assess and compare the functions of CCR such as ICOS, CD27, CD28 AND 4-1BB.[99,100] It is documented that CARs incorporated with co-stimulatory domains are more effective in targeting tumors. In a Hu-PBL model, combining PSCA-CAR and PSMA-CCR prostate antigens eradicated tumor cell lines that expressed both antigens and shaped antitumor immune response to PSCA+PSMA- tumors.[101] Jakobsen *et al* reported that Hu-PBL model could be employed to assess the efficacy of Bi-specific TCR-anti-CD3 regimen for the treatment of LAGE1- and NY-ESO-1- positive human tumors.[76] Another approach to test the safety of CARs is using mRNA transfection, as opposed to viral transduction, as a mechanism of CAR generation. The advantage of this method is that it does not involve the integration of DNA into the genome, thereby removing the possibility of genomic editing. MRNA transduction has been utilized in developing anti-CD20 NK cells in Hu-PBL model targeted to Non-Hodgkin's Lymphoma[102] or anti-mesothelin CAR T cells in a Hu-PBL model targeted to mesothelioma.[93]

Another targeted CAR-based therapy is engineering T cells to express anti-CD19 CARs. Patients with B cell malignancies showed positive response to CD19-targeted CAR-T cell therapy.[103–105] However, many showed severe adverse reaction or relapse after therapy largely due to toxicities of unknown mechanisms.[105,106] Development of pre-clinical models would be beneficial in investigating the underlying mechanisms of relapse and toxicity. PDX models engrafted with human B acute lymphocyte leukemia (ALL) revealed to be useful in evaluating CD19-targeted human CART cell therapy, however, these models either lack host immunity or involve allo- and/or xeno-immune responses.[107] On the other hand, leukemic HM models have genetically-identical (autologous) primary B-ALL and a functional human immune system which make them a better model for CD19-targeted CAR T cell therapy.[108] Another favorable feature of HM model is that the anti-CD19 CAR-expressing human T cells are also autologous to the human components (either normal or malignant human cells) and tolerant to the mouse antigens, thereby do not elicit xeno responses against mouse antigens or allo responses against human.

Furthermore, BLT mice can be modified to as a TCR transgenic HM for studies of human T cell adaptive immunotherapy.[109] A melanoma antigen (MART-1)-specific TCR transgenic HM model is developed by cotransplanting autologous human $CD34^+$ FLCs transduced with lentiviral vectors containing HLA-A*0201 restricted MART-1 specific TCR genes and HLA-A*0201^+ human fetal thymic tissues into sub-lethal irradiation pre-conditioned NSG mice.[110] Upon employing this model it was revealed that anti-melanoma effects mediate by adaptive transfer of human MART-1 TCR^+ T cells was remarkably improved by adding rapamycin for MART-1 TCR^+ human T cell expansion in vitro and simultaneous supplementation with human IL-15 in vivo.[109] Recently, other uses of HM have also shown that exosomes derived from phosphoantigen-expanded Vδ2-T cells (Vδ2-T-Exos) contained MHC class I and II, CD80, CD86, TRAIL, FasL and NKG2D. Administration of Vδ2-T-Exos could effectively control BV-associated tumors in Rag2-/-γc-/- and HM. Given that the expansion of Vδ2-T cells and ex vivo preparation of autologous Vδ2-T-Exos from cancer patients in large scale is challenging, the antitumor activity of allogeneic Vδ2-T-Exos was explored in humanized mouse cancer models.[111]

Our focus was on pre-clinical experiments of immunotherapy but HM revolutionized the diagnostic and therapeutic approaches. Moreover, HM are providing a suitable platform for studies of human infectious disease like human immunodeficiency virus, GVHD, regenerative medicine, allergies, and immunity.

7 | FUTURE

Humanized mice models are powerful tools for immunotherapy research in the era of cancer immunotherapy. Despite advances in establishing HM, they do not entirely recapitulate a functional human immune system. Thus, efforts for improving HM are ongoing. Scientists from different biomedical disciplines are testing innumerable strategies such as reducing graft rejections, boosting human cell reconstitution, improving human-specific responses and supporting critical immune cell subsets. Moreover, there are some noticeable obstacles which need to be solved soon. For example, if the HM is engrafted with an immune system from one person and the tumor from another person, then the formed immune response might be as the result of tissue incompatibility, rather than being reflective of the tested treatment. One of the solutions for the issue of MHC incompatibility is using induced pluripotent stem cell (iPSC) technology. This method allows for the use of patient-specific iPSC which reduces the chance of tissue incompatibility and also provide a renewable source of autologous cells. Still, more comprehensive and functional immune systems need to be generated in HM. More specifically, there is an ongoing need to identify new approaches providing the platform for autologous experiments of engrafted immune cell and diseased tissue from the same individual. Thereby, allowing for more accurate understanding of disease progression and treatment efficacy.

Considering the complexity of T cell development, even if it is possible to recreate the human thymic environment, establishing the same TCR repertoire of that particular patient seems very unlikely. Eventually, the utmost purpose is the formation of specific antitumor immune responses by these human-derived T cells. Analyzing immunosuppressive cells such as T regulatory cells and M2 macrophages could also help in drawing the whole picture of the interplay

between the patient's immune system and the tumor that has managed to grow.

More recently, it has been reported that microbiota, particularly in the gut, affects the efficacy of cancer immunotherapy. Given that germ-free mice lack microbes, researchers have established human microbiota-associated mice developed from these mice by fecal microbiota transplantation.[112,113] Importance of the microbiota calls for further research on the effect of microbiota on biological responses. Another challenge in the development of HM is the incomplete cross-compatibility between the murine stroma and transplanted human hematopoietic cells. Recently, complementary strategies have been developed to supplement in vivo xenotransplantation models such as in vivo utilization of three-dimensional human BM organoids and ex vivo deployment of bioreactor models.[114] Cancer-associated fibroblasts are normaly observed within the stroma of various cancers, including lung, breast, colon, and pancreatic carcinomas.[115] Recently reported that, in preclinical mouse models, fibroblast-activating protein a targeting OMTX705 represents a novel a model for cancer immunotherapy study.[116] In conclusion, as humanized PDX are evolving and refining to better represent the human biological system, they are considered as an appropriate platform in personalized medicine and cancer immunotherapy.

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