

Humanized Mouse Xenograft Models: Narrowing the Tumor–Microenvironment Gap

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Abstract

Cancer research has long been hampered by the limitations of the current model systems. Both cultured cells and mouse xenografts grow in an environment highly dissimilar to that of their originating tumor, frequently resulting in promising treatments that are ultimately clinically ineffective. The development of highly immunodeficient mouse strains into which human immune systems can be engrafted can help bridge this gap. Humanized mice (HM) allow researchers to examine xenograft growth in the context of a human immune system and resultant tumor microenvironment, and recent studies have highlighted the increased similarities in attendant tumor structure, metastasis, and signaling to those features in cancer

patients. This setting also facilitates the examination of investigational cancer therapies, including new immunotherapies. This review discusses recent advancements in the generation and application of HM models, their promise in cancer research, and their potential in generating clinically relevant treatments. This review also focuses on current efforts to improve HM models by engineering mouse strains expressing human cytokines or HLA proteins and implanting human bone, liver, and thymus tissue to facilitate immune cell maturation and trafficking. Finally, we discuss how these improvements may help direct future HM model cancer studies. *Cancer Res*; 76(21); 6153–8. ©2016 AACR.

Introduction

Humanized mice: innovative research tools

A barrier to the adequate study of human disease is the availability of suitable animal models. Many model systems either cannot propagate the disease in question or provide a foreign milieu, not representative of the conditions in humans (1). To address these challenges, chimeric systems designed to incorporate relevant human genes or tissues into a disease model organism have been developed. Genetically modified yeast, flies, and mice have greatly facilitated medical research. More recently, the human immune system has been partially reconstituted in mice. These "humanized mice" (HM) aim at harboring an immune environment capable of more accurately reflecting that present in human diseases (2). HM variations have proven key in the study of allograft rejection and autoimmune diseases, as well as in research investigating transmissible diseases, such as the human immunodeficiency virus and viral hepatitis, among others (3, 4).

HM models have become feasible as a result of the identification of increasingly immunocompromised strains of mice into which a human immune system could be successfully engrafted (5). Athymic nude mice lack T cells, so are incapable of mounting

an immune response against implanted tissues. NOD mice lack innate immunity. The subsequently identified SCID mice lack both T and B cells and can be successfully engrafted not only with human tissues but also with hematopoietic cells. Their utility is somewhat limited by the presence of natural killer (NK) cells and by leaky expression of T and B cells as the mouse ages. This leakiness has been eliminated in the RAG-1 and RAG-2 mice, although NK cells remain. The NSG (NOD/SCID-IL2 $\gamma^{-/-}$) strain, produced by the targeted mutation of the IL2 receptor γ -chain locus in a previously bred NOD/SCID strain, lacks T cells, B cells, macrophages, NK, and NKT cells and has become an increasingly used platform for HM development (6).

In this review, we will address the state of the art of the development and utilization of HM for cancer research, with a particular focus on the challenges facing HM and their implementation.

State of the Art in Cancer Research

The complexity of the cancer microenvironment

The uncontrolled cell division emblematic of cancer is highly adaptive to the selective conditions imposed by the surrounding immune microenvironment (7). Tumor cell lines quickly evolve to the conditions present in a tissue culture environment. Even when patient tumor tissue is implanted directly into traditional xenograft mouse models, the immunodepleted murine environment often produces different experimental outcomes to treatment than those seen in the patient (8). The interplay between the rapidly dividing cancer cells and the surrounding stromal tissue is a critical factor in tumor growth and metastasis, as well as in treatment efficacy (9).

The relationship between the tumor and the stromal immune cells is particularly complex (10). As cancer cells divide, they recruit cells to contribute to the infrastructure of the growing tumor, mainly consisting of fibroblasts, endothelial, and

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circulating immune cells (11). Although chemokines from the cancer cells are responsible for the development of the stroma, signals from the stroma also affect subsequent tumor growth. Dysregulated gene expression in the cancer cells results in aberrant surface antigen presentation that would normally target these cells for attack by circulating T cells and NK cells. Cancer cells produce cytokines to pacify nearby immune cells and escape immune surveillance. The chemokine CCL22 can attract T regulatory cells (Treg), which produce cytokines, such as TGF β and IL10, to downregulate the activity of nearby immune cells. Likewise, many cytokines, including CCL2, HIF-1 α , and VEGF, recruit circulating monocytes and myeloid-derived suppressor cells into tumors, where they typically differentiate into tumor-associated macrophages (TAM; ref. 12). Many TAMs quickly convert from an M1 macrophage anticancer phagocytic phenotype into M2 macrophages that suppress immune activity of other cells, leading to a pro-cancer effect. In addition, cancer and/or antigen-presenting cells express ligands that inhibit checkpoint receptors in effector immune cells, such as the programmed cell death 1 receptor (PD-1), and cytotoxic T lymphocyte-associated protein 4 (CTLA4). Inhibition of these interactions leads to continuous T-cell activation and has resulted in novel immunotherapies, such as ipilimumab (targeting CTLA4), nivolumab (PD-1), and pembrolizumab (PD-1), which have proven effective as cancer treatments (13).

Because these therapies harness the patients' immune cells to fight their tumors, they rely on the existence of a functional human immune system. As such, it has been relatively difficult to test their effectiveness using conventional experimental models. Early studies examining the efficacy of an anti-CTLA4 immunotherapy targeted the murine CTLA4 receptor in mouse fibrosarcoma and ovarian cancer models (14). The effectiveness of this treatment encouraged the production of a human antibody, which was subsequently tested in the cynomolgus monkey, as no other species with cross-reactivity could be identified (15). Similarly, the potential effectiveness of anti-PD-1 therapies was first investigated in mice using antibodies specific to murine PD-1 (16), and human antibodies were later examined *in vitro* and in cynomolgus monkeys (17). The use of cynomolgus monkeys to test human-specific antibodies has not been without shortcomings, due to instances where such antibodies did not fully cross-react or provide predictive data for human clinical use. Such work would be simplified if HM could be deployed to help investigate the interaction between a tumor and the immune system to develop clinically effective treatments (18).

Humanized mice to propel cancer biology research

Currently, there are several types of HM being employed in cancer research. In their most basic form, mice can be considered humanized after being engineered to express specific human proteins considered relevant to tumor growth (19). HMs can also be generated by the direct injection of human peripheral blood. In one early cancer study, Tang and colleagues injected purified human blood lymphocytes from donors infected with Epstein-Barr virus into SCID mice and monitored these animals for indications of human B-cell lymphoma (20). In other cases, mice are classified as humanized after the implantation of human stromal tissue together with the tumor tissue. For example, Bankert and colleagues orthotopically injected freshly biopsied ovarian tumor cell aggregates, consisting of tumor cells as well as associated fibroblasts and blood cells, into NSG mice (21). The resulting tumors retained a population of human fibroblasts and

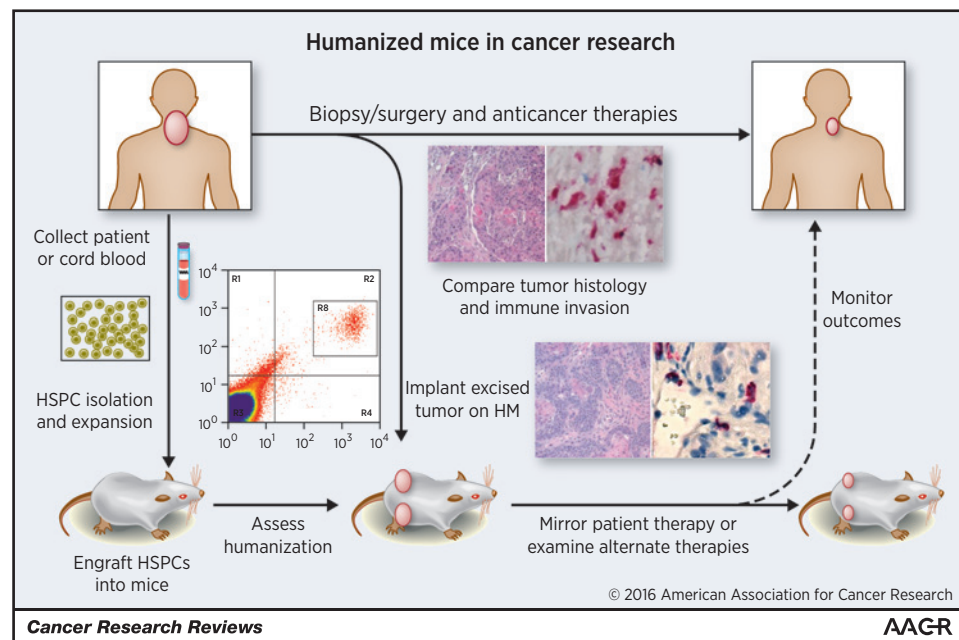
functional lymphocytes. Likewise, during their creation of a breast cancer tumor bank in NOD/SCID mice, DeRose and colleagues found that the coimplantation of human mesenchymal stem cells (MSC) stimulated angiogenesis throughout the resulting tumors and promoted their growth (22). These cells can alter the tumor microenvironment, making it temporarily more physiologically similar to that found in a cancer patient.

It is becoming increasingly common to create HM by engrafting CD34⁺ human hematopoietic stem and precursor cells (HSPC) isolated from fetal cord blood or other tissues into the marrow of sublethally irradiated immunocompromised mice to allow them to develop into a functional immune system (23). These precursor cells engraft into the damaged bone marrow and, once established, begin to divide into differentiated progeny, including T cells, B cells, macrophages, and other cells capable of interacting with xenograft tumor tissues. In an early study of this type, Seitz and colleagues implanted rhabdomyosarcoma cell lines onto the flanks of previously humanized NSG mice (24). Tumors originating in the HM were more necrotic, which the authors speculated may have been due to an interaction with the human immune system. Wege and colleagues simultaneously injected purified HSPCs together with cells from a breast cancer cell line into the livers of newborn NSG mice (25). Analysis of the mouse tissues after tumor growth showed evidence of a specific tumor-stimulated immune response. HMs have also been used to examine leukemia (26). In this case, CD133⁺ cells, thought to be precursors to even CD34⁺ cells, were injected directly into the bone marrow of NOG (a strain similar to NSG) mice. After evidence of humanization, the mice were injected with human T-cell leukemia virus type 1-producing cells and monitored until they developed clinical symptoms of leukemia.

We recently generated an HM model system by the engraftment into the bone marrow of NSG mice of HSPCs that were stabilized and expanded *in vitro* (Fig. 1; ref. 27). The addition of purified Tat-MYC and Tat-Bcl2 fusion proteins to the cultured HSPCs drives MYC-dependent cell division and inhibits apoptosis, while the fused HIV-Tat transduction domain eliminates the need for any genetic modifications to the expanding cells (28). These fusion proteins are subsequently degraded in cells; MYC is not detectable within 72 hours of transduction, even though cellular response to its presence endures. Transduced HSPCs also retain the capacity to differentiate into either myeloid or lymphoid cells.

In these experiments, the introduction of Tat-MYC and Tat-Bcl2 to cultured cord blood cells increased the number of CD34⁺ cells by an average of 16.6-fold over that of unsupplemented cultured cord blood cells after 12 days and enabled the creation of larger cohorts of isogenic HM than would have otherwise been possible (28). Analysis of the HM bone marrow showed that HSPCs were capable of prolonged and continuous expansion and could be isolated and reinjected into a new cohort of mice without loss of replicative potential. Within 8 to 10 weeks, differentiated T cells, B cells, and myeloid cells could also be identified in the bone marrow, lymphoid organs, and peripheral blood of HM, indicating that the expanded HSPCs retain their ability to recapitulate both lineages of the human immune system. When patient-derived head and neck cancers were implanted on the flanks of HM, these immune cells invaded and reprogrammed the stromal microenvironment, altering the expression of genes governing the extracellular matrix, the immune response, and the epithelial-mesenchymal transition, so that their expression was more similar to that found in the patient in whom the tumor originated. Other

Figure 1.
Schema showing the creation of HM from cord or patient blood, implantation of patient tumors, and comparative analysis of therapy.



features associated with human tumor growth but absent in traditional xenograft models, such as lymphangiogenesis and chemokine signaling, were also detectable and quantifiable in tumors implanted on these HMs.

Although this HM model is capable of reproducing many attributes of the relationship between a growing tumor and its associated immune cells, it is under continuous development. Tumors were not equally infiltrated by stroma and immune cells, and the percentage of CD34⁺ cells within the bone marrow of the HM was variable. Myeloid cells were detected in the peripheral blood of the animals, but no TAMs infiltrating the tumor cells were initially identified. Further refinement of this HM, including variations to HSPC harvesting and processing and the use of unmodified HSPCs, is ongoing.

Humanized mice to inform cancer therapies

A fundamental property of an HM model is the ability to mount an anticancer immune response. During subsequent studies with these HMs, we evaluated the interaction between the engrafted immune system and implanted head and neck tumors to palliative doses of radiation. In a recent study, Sanmamed and colleagues injected Rag 2^{-/-}-IL2γ^{null} mice with peripheral blood mononuclear cells (PBMC) from a gastric cancer patient (29). The xenoreactivity of the T cells within this blood induced xenograft-versus-host disease (a phenotype similar to what is normally observed in GVHD) within the mice. This condition was exacerbated when the mice were treated with nivolumab and urelumab (an antibody specific to the cellular CD137 antigen, also involved in lymphocyte activity). When tumor tissue from the same cancer patient was implanted on additional mice, application of these agents allowed the autologous T cells to attack, slowing its growth.

Additional immunomodulatory cancer targets are also being studied. Chang and colleagues humanized an orthotopic renal cell carcinoma (RCC) model mouse with PBMCs to examine the effectiveness of an antibody targeting the carbonic anhydrase IX protein expressed in RCC, showing that this antibody primed

T cells and inhibited cancer growth (30). Finally, while examining a novel immunotherapy against melanoma, Hu and colleagues generated HMs from CD34⁺ HSPCs engineered to express an HLA class I-restricted melanoma antigen (MART-1)-specific T-cell receptor (TCR) gene (31). Mature T cells harvested from these mice were observed to attack melanoma cells *in vitro* in an antigen-specific manner, and after transfer into melanoma-bearing NSG mice, prolonged the survival of these animals.

HM systems capable of generating or hosting differentiated hematopoietic cells with proven immune activity seem appropriate to examine the efficacy of existing as well as experimental immunotherapies. As demonstrated by Hu and colleagues, it is also possible to modify the HPSCs used to humanize mice, further expanding their utility in the development of new classes of therapeutics.

Overcoming Current Humanized Mouse Limitations

Recapitulation of the immune system

There are many aspects of HM systems where improvements are required to ultimately ensure a bona fide human tumor micro-environment. The processes and cytokine signals governing HSPC differentiation and immune cell maturation within the HM are incompletely understood. The importance of HLA compatibility between the immune cells and the implanted tumor and its impact on a tumor-specific immune response is also unclear. Many of these challenges, the desired improvements, as well as the steps taken to incorporate them into the model and a description of the resulting modified mouse platforms, are discussed below and summarized in Table 1.

The engrafted HSPCs and differentiating immune cells rely on environmental cytokine signaling for orderly maturation and subsequent trafficking into the tumors on the organism (32). The chemokines present in the NSG are capable of undertaking some but not all of these roles (33), as the IL2γ mutation within this strain is essential for IL2, IL4, IL7, IL9, IL15, and IL21

Table 1. Challenges limiting the utility of HM model

Limitation	Solution	Resultant HM features
Incompatible cytokine environment prevents complete human immune cell differentiation	<ul style="list-style-type: none"> - IL7 injection - Cytokine expression from introduced plasmids - Inclusion of MSCs within HSPC bone marrow-engrafted cells - Transgenic mice expressing necessary human cytokines 	<ul style="list-style-type: none"> - Improved T-cell production (23) - Improved NK cell, monocyte, and macrophage differentiation (34) - Improved DC viability (35) - Increased immune cell differentiation and functionality (36–38)
Incomplete T-cell maturation and thymic restriction	<ul style="list-style-type: none"> - Human fetal thymus and liver tissue implantation - Transgenic mouse strains expressing transgenic HLA alleles autologous to engrafted HSPCs 	<ul style="list-style-type: none"> - Higher proportion of mature and functional T cells (41) - In some cases, a higher proportion of appropriately functional T (and B) cells (43, 44)
Incomplete B-cell differentiation and activation Antigen-specific immune response	<ul style="list-style-type: none"> Human fetal bone implantation - MSC-mediated immune modulation - Immunoregulation by isolated DCs 	<ul style="list-style-type: none"> Higher proportion of mature and functional B cells (42) - Suppressed immune rejection (45) - Elimination of GVHD; regulated immune invasion of tumors (46)
HSPC and tumor compatibility	<ul style="list-style-type: none"> - Autologous HM systems 	<ul style="list-style-type: none"> - Elimination of nonspecific tumor attack due to mismatched HLA antigens (47)

NOTE: The first column identifies immune features not well recapitulated in the HM. The second column provides possible solutions, and the third column outlines resultant features of the improved model.

signaling. Several groups are actively engaged in studies to improve the cytokine environment within HM. Early work by Shultz and colleagues with NSGs showed that the administration of IL7 enhanced the production of mature T cells in HM (23). Chen and colleagues demonstrated that supplemental administration of IL15 and Flt-3/Flk-2 cytokines (encoded on plasmids expressed in hepatocytes) resulted in elevated levels of NK cells, while granulocyte macrophage colony-stimulating factor (GM-CSF) and IL4 were able to increase the dendritic cell (DC) population, and macrophage colony-stimulating factor (M-CSF) was able to increase the number of monocytes and macrophages detectable within the HM (34). More recently, it has been observed that the incorporation of a population of MSCs into the HPSCs destined for engraftment can also alter the eventual reconstitution of the myeloid cell lineage within the HMs. Chen and colleagues demonstrated that coculture of HM bone marrow cells with MSCs, which produce growth factors and cytokines and have a previously described immunoregulatory function, improved the viability of newly differentiating DCs (35).

Although their addition improves immune cell differentiation and expansion, nonphysiologic concentration of these cytokines within the HM can misdirect immune cell development and trafficking. In an effort to refine expression of these critical cytokines Rongvaux and colleagues generated Rag2^{-/-}-IL2γ^{null} mouse strains featuring targeted knock-ins of the human genes encoding M-CSF, IL3, GM-CSF, and Thpo (36). After human CD34⁺ cell engraftment, these MITRG (and the similar MISTRG, containing an additional *SIRPα* transgene) mice produced T and B cells, along with functional NK and myeloid cells, which were able to infiltrate cell line-derived melanoma tumors and alter their growth via a VEGF-dependent mechanism. Other transgenic humanized strains have also been created. Wunderlich and colleagues developed the NSG-SGM3 (NSGS) mouse, which expresses human SCF, GM-CSF, and IL3, to facilitate the study of acute myeloid leukemia through increased production of mature myeloid cells (37). Katano and colleagues produced the NOG-IL2 Tg mouse by inserting a human IL2 transgene into a NOG background. They observed that HM created from this strain produced a diverse set of NK cells, capable of targeting both introduced leukemia and lymphoma cells (38). Although these systems can help recreate a functional myeloid lineage, mouse

strains bearing these transgenes can be relatively difficult to breed, complicating the generation of the large cohorts necessary for immune therapy cancer studies.

A key aspect in HM models is the fidelity of T- and B cell-mediated adaptive immune responses. Following their formation in the bone marrow, immature T cells are directed to the thymus, where those capable of appropriate interaction with the MHC antigens presented to them there are allowed to mature. T-cell progenitors undergo thymic selection, including death by neglect, negative selection, and positive selection, to become mature T cells. Although functional T cells have been identified in HM, other reports have shown that these T cells are minimally proliferative, become quickly anergic and are particularly frail within the peripheral tissues of the HM (39, 40). Likewise, B-cell progenitors formed in the bone marrow of the HM frequently fail to mature completely within the spleen (40). Although these cells can produce IgM in response to introduced antigens, they are incapable of antigen-specific IgG responses.

To overcome these obstacles, Joo and colleagues engrafted human fetal liver and thymus tissue beneath a kidney capsule of Rag2^{-/-}-IL2γ^{null} mice, which were subsequently humanized with fetal liver-derived *ex vivo*-expanded CD34⁺ cells (41). Several weeks after humanization, they were able to identify functionally mature T cells within these animals. Further work by this group also showed that coimplantation of fetal bone into these mice could simultaneously improve B-cell development and function (42). Although this bone-liver-thymus (BLT) model of humanization has advantages, the creation of the human organoids needed to generate a specific immune response makes it challenging to produce the relatively large HM cohorts needed for therapeutic cancer studies.

An alternative to the creation of BLT HM is the construction of transgenic strains expressing a common HLA allele in their thymus. After engraftment with HSPCs with the same histocompatibility allele, this mouse thymus should become more hospitable to T-cell maturation. Studies examining this hypothesis have met with mixed success. Using NOD/Rag1-IL2γ^{-/-} mice expressing the human HLA-DR4 allele, Danner and colleagues showed that after engraftment with HLA-matched HSCs, the immune system in these mice contained high numbers of functional T and B cells, capable of appropriate response to immune challenge (43). Most

recently, Patton and colleagues reported that NSG mice expressing the human allele HLA.A2.1 and engrafted using CD34⁺ cells from an HLA.A2.1 matched donor could not be as efficiently humanized as NSG controls (44). They speculate that this may be due to alloreactivity between the human and mouse peptide antigens bound to the HLA proteins.

Recapitulation of controlled immune cell activity

In addition to ensuring that the engrafted HSPCs differentiate and function properly within an HM model, and that mature lymphoid and myeloid cells must be present and capable of mounting an antigen-specific immune response, they must not attack incompatible mouse stromal tissues indiscriminately. Activated immune cells, such as T cells, NK cells, B cells, and M1 macrophages, as well as immunosuppressive cells, including Treg cells and M2 macrophages, should be present and representative of their distribution within a patient tumor. Conversely, these cells must not preferentially migrate into and damage mouse tissues or organs.

Several strategies have been adopted to help ensure a controlled and coordinated immune response in the HMs. Recently, Coulson-Thomas and colleagues demonstrated that MSCs isolated from umbilical cord blood are capable of suppressing immune rejection when transplanted in mouse tissues (45). The engrafted MSCs can inhibit the invasion of inflammatory cells and induce Treg cell maturation. Subsequently, Roth and Harui showed that isolated DCs could also perform an immunoregulatory role in an HM model (46). When NSG mice were engrafted with isolated peripheral blood lymphocytes (PBL) and autologous DCs (immune cells specialized to present foreign antigens to activate T cells), they did not exhibit symptoms of GVHD. When human prostate cancer cells were injected into these mice, the PBLs invaded the resultant tumors in a manner similar to that seen in human prostate cancers and altered their rate of growth.

Future Directions

Although continued investigation will almost certainly help increase the potential of all of these variant humanized models in cancer research, an accurate modeling of the patient's immune response during treatment demands that the immune system in the HM is compatible with both its host environment and with implanted tumor tissues. An immune response occurring in HM engrafted with an immune system from one person and implanted with the tumor from another may be the result of tissue incompatibility and may not be specifically reflective of the treatment being evaluated. This has been the environment in which the vast majority of xenograft research on HM has been conducted. Although some researchers have observed features associated with GVHD, these conditions do not preclude cancer growth (29). Most often, when HMs are created from the engraft-

ment of carefully purified CD34⁺ cells, few (if any) mature xenoreactive T cells are introduced into the mice. The T cells that differentiate within the engrafted bone marrow mature within the mouse and seem to display few xenoreactive tendencies. When tumor tissue is implanted on the mouse, it seems to be recognized as foreign and attacked accordingly. The weakened state of the human immune system may prevent it from completely rejecting the xenograft, although we have observed that, in general, tumors implanted on well-humanized mice tend to grow more slowly than on comparative NSG animals. Little has been published about the growth of tumors in HLA-mismatched systems, and it remains an area of active research.

One possible solution to this problem would be the creation an HM xenograft model in which the HSPCs and the subsequently implanted tumor tissue come from the same cancer patient. This has, to our knowledge, not been reported yet. Recently, Werner-Klein and colleagues successfully humanized NSG mice using CD34⁺ HSPCs isolated from biopsied bone marrow of breast, lung, prostate, or esophageal cancer patients (47). This study did not report on the subsequent engraftment of any of these patients' tumors onto their HM.

In a truly active autologous HM system, the engrafted immune system should mirror that of the cancer patient while remaining hospitable to the mouse. Even in this case, however, it remains possible that human T cells in HM models selected on mouse thymic epithelial cells may not be identical to their normal counterparts, which are usually selected on human thymus. On the other hand, the infinite repertoire of TCRs generated by V(D)J recombination during T-cell development almost warrants the existence of tumor-specific T cells. Given the complexity of T-cell development, even if recreation of the human thymic environment were possible in a mouse, it is unlikely to reestablish the exactly same TCR repertoire of that particular human patient. In the end, the critical issue is whether these human-derived T cells can mount specific antitumor immune responses. In such a situation, mechanisms of immune evasion by the tumor can be accurately elucidated. In addition to tumor invasion by active T cells, B cells, and M1 macrophages, immunosuppressive cells, such as M2 macrophages and Treg cells, can also be analyzed to generate a complete picture of the interplay between a patient's tumor and the immune system in which it has managed to grow. In addition, potentially therapeutic treatments, including novel immunotherapies, can be tested on the matched HM to determine their potential benefit to the patient.

Disclosure of Potential Conflicts of Interest

Y. Refaeli is the chief executive officer at Taiga Biotechnology. No potential conflicts of interest were disclosed by the other authors.

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