OF MICE AND MEN:

Strategies for humanization in preclinical immuno-oncology research

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Immuno-oncology (IO) research requires special models in which therapeutic targets within the human immune system are faithfully recapitulated. Researchers selecting appropriate *in vivo* models for preclinical applications face real challenges, including trade-offs on cost, availability, quality, and validated, predictable similarity to human systems of interest. And while the mouse model addresses several of the challenges, perhaps the biggest challenge of the mouse model is that the mouse immune system differs significantly from patients in both species-specific genetic variants and environmental factors.

There are three ways to make the mouse an appropriate *in vivo* model for IO studies. The first approach is to engraft a human immune system into an immunodeficient host to generate mice with a humanized immune system (HIS mice). A second approach is to genetically humanize mice by replacing targeted mouse immune system genes with their human homologs (humanized genetically engineered models – humanized GEMs). And, third, the influence of the microbiome on immune system development can be controlled through gnotobiotic techniques, including patient-derived fecal transfer into germ-free mice (humanized microbiome mice). Taken together, these approaches offer a wide variety of different strategies to develop and optimize translatable and flexible IO mouse models. We discuss these three approaches in turn in this paper.

Approach 1.
Human Immune System (HIS) Mice

The ability to engraft a mouse with a human immune system was first made possible by the development of the super combined immunodeficient (SCID) mouse by Bosma and colleagues in 1983.[1] The lack of an adaptive murine immune system (B and T cell deficiency) allowed for human cell engraftment, albeit at low levels mainly due to the activity of the residual murine innate immune system.[2]

Over the next three decades, the need for improved immune system engraftment expedited the development of mouse models with increasingly impaired innate immunity. The current "super" immunodeficient mouse strains combine several factors to achieve high levels of immunodeficiency. For example, the NOG mouse combines the non-obese diabetic (NOD)/SCID background with genetic deletion of the signaling domain of the interleukin 2 receptor common gamma chain to provide a high level of immunodeficiency and successful human cell and tissue engraftment.[3-7] These super immunodeficient models have facilitated the development and production of HIS mice as a translational model for exploring therapies that depend on the presence of a functional human immune system (e.g., checkpoint inhibitors and/or T-cell engaging bispecifics) that would otherwise not be viable in other murine models.[6]

Faced with diverse offerings in current HIS model production, researchers need to understand fully each selection criteria (e.g., origin of cells to engraft and host model). There is no single best model; all have clear advantages and disadvantages. The weight of the selection criteria for the appropriate HIS model and engraftment source should be based on the experimental question. The two most essential questions in determining a HIS model are: what type of human immune cells are required to be present and what degree of functionality is required of those essential cells within a given timeframe?

The origin of the cells for engraftment can impact the type of human immune cells, as well as their degree of functionality in a HIS model. There are also logistical and resource-based questions that apply to both from a researcher standpoint. The two most commonly used approaches for human immune system engraftment rely on injections of either human hematopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs) into an immunodeficient murine host.[8] We consider the PBMC option first.

PBMCs provide for an easily accessible, inexpensive, and mature human immune cell engraftment choice for HIS models. However, PBMC-based HIS models have a substantial limitation in that these mature human immune cells will attack mouse tissues, a process known as graft versus host disease (GVHD). This process affects studies in several ways: first, PBMC HIS studies are limited in duration; typical studies need to be completed within 3 weeks of PBMC engraftment before the onset of clinical disease in the murine host. Second, the influence of the GVHD inflammatory process, which begins early after engraftment, may confound the interpretation of results for therapeutics targeted toward the immune system.

HSC engraftment provides several clear advantages when the presence and function of human immune cells for a prolonged study window is required. HSCs can originate from many different donor sources but the most widely used, due to increased accessibility, is umbilical cord blood (UCB).[8] Since these engrafted HSCs are immature, the development of GVHD is largely mitigated, as self-reactive T cells are removed during clonal selection in the mouse thymus.[2] For example, HSC-engrafted NOG mice can retain engraftment for their entire lifespan with no evidence of GVHD.[3] This allows for HSC-based HIS models to be employed in studies for much longer than PBMC-based HIS models – many months or up to a year, as compared to 3-4 weeks with PBMC. This is especially important when studying slowly growing tumor sources, such as patient-derived xenografts (PDXs).

For HSC-based HIS models, host selection will determine the presence and function of the immune cells available for study. Although HSCs have the potential to develop into all lineages of the adaptive (T and B cell; lymphoid) and innate (macrophage, neutrophil, etc.; myeloid) immune system, this is highly dependent upon the host ›

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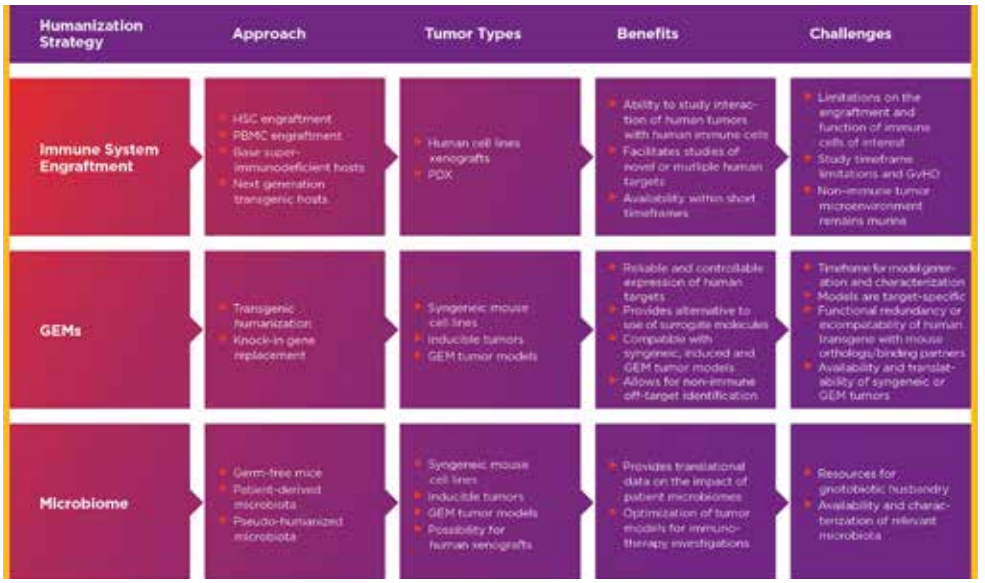

chosen for engraftment. In NOG or other similar super immunodeficient mice, there is almost a complete preference for lymphoid lineage development (i.e., T and B cells), with relatively few myeloid or NK cells present, following HSC engraftment.[2,9] This limitation may be overcome by the use of next generation transgenic animals that produce the human cytokines required for myeloid or NK cell lineage differentiation. Examples of these include NOG mice expressing hGM-CSF/hIL-3 for myeloid development;[10] hIL-6 for monocyte development,[11] hIL-2 to support CAR T cell function;[12,13] and hIL-2 or hIL-l5 for NK cell development.[14] Base super immunodeficient animals are sufficient for general T cell studies, including many checkpoint inhibitor strategies, while second generation cytokine transgenics should be considered for approaches involving alternative immune cell populations and mechanisms such as antigen presentation and antibody-dependent cellular cytotoxicity.

HIS models have wide utility in immuno-oncology research and offer an important tool to study xenografts in the context of a human immune system. There are many factors that should be considered in using these models in research, and new options for host selection are increasing the applicability of HIS models in drug discovery and development.

Approach 2. Humanized GEMs

Instead of engrafting a human immune system into a mouse, there are instances when a more targeted approach may be beneficial. With humanized GEMs, a human gene is inserted into the mouse genome, thus providing a druggable target for *in vivo* studies. The rationale behind this approach is to address species-specific differences between mice and humans that can make certain therapeutic approaches challenging to study; this is especially the case for biologics, which may differentially recognize mouse vs. human targets due to high-binding specificity. Traditional approaches, used in the development of the first immunotherapies, relied upon antibody *surrogates* that recognized mouse targets for preclinical *in vivo* research. The problem with this approach is two-fold: first, this requires the development and production of a molecule that is completely separate from the clinical candidate, and second, the use of a surrogate may miss important off-target effects of a clinical candidate molecule. Generating a humanized GEM can help to overcome these issues and provide a model in which to directly test therapies against specific immune system targets.

The genetic humanization strategy decision is dependent on the biology of the target in question, coupled with resource and time constraints. Unlike humanized HIS models, humanized GEM models are often not immediately available and must be engineered, a process that can be both time-consuming and expensive. However, once these models are established, they can be used extensively in syngeneic tumor experiments to test immune-oncology therapeutics in mice, without the need for immune cell engraftment.

There are several different technologies available to generate humanized GEMs: transgenesis and targeted gene replacement or 'knock-in'.

Genetic humanization via transgenesis can be accomplished through *random*, *targeted* or *conditional-targeted* approaches. These have the advantages of being able to select founder lines with optimal human transgene expression levels and are technically more straightforward, faster and inexpensive compared to humanization via 'knock-in'.[15-17]

- *Random transgenesis* is often disadvantaged by the inadvertent co-expression of the human transgene with an endogenous mouse gene, variation in the number of transgenes inserted, interruption of endogenous mouse genes, and a non-endogenous expression pattern. These outcomes are often unpredictable and require extensive characterization of random transgenics.
- *Targeted transgenesis* directs gene insertion toward a permissive non-syntenic region within the murine genome, such as *Collagen* or *Rosa26* gene loci[18] This permits the precise positioning of a single human transgene copy within the murine genome, and can minimize many potential deleterious

consequences, such as unstable multi-copy insertions, positional effects due to random transgene insertion, and disrupting the expression of other endogenous gene(s).

- *Conditional targeted transgenesis* differs from the targeted approach in that it includes inducible elements (e.g., tet, Cre, etc.) that permit greater control of transgene expression. All of these approaches generate a GEM that co-expresses the human transgene along with the endogenous murine ortholog and may need to be bred onto a null background for the gene of interest to abrogate any undesirable interference due to residual expression of the murine ortholog. This extra step can be avoided by employing the knock-in approach to replace the murine gene of interest directly with its human ortholog.

Genetic humanization via 'knock-in' can be performed by either the insertion of a mini-gene (human cDNA coding sequence) or an entire gene replacement, both of which replaces the mouse coding sequence with its human ortholog. This approach is advantageous in simultaneously inactivating the expression of the murine gene while allowing the expression of the human ortholog to be controlled by endogenous regulatory elements. This approach is technically more challenging than transgenesis but may more faithfully recapitulate the expression pattern and regulatory elements of the human target within a mouse host.

In summary, a wide range of molecular methods and technologies are available to generate humanized GEMs, which have their specific advantages and disadvantages. In addition, important caveats that are often inherent in any experimental GEM should be noted, one of which is that the generation, development, characterization, and validation of any GEM may be time- and resource-consuming, and very often requires highly technical expertise and financial support. Another is the potential heterogeneity in the phenotypes of GEMs, including differential tissue or cell type expression or the generation of additional phenotypes that are not related to the transgene, but instead are due to genome-integration effects, whereby the transgene may affect the expression of neighboring genes or epigenetically alter a larger region in *cis*. Therefore, there is no single best approach for all different applications of any humanization project, and the various strategy options should be appraised based on each project, experimental hypothesis, available resources, and subsequent utilization of the humanized GEM.

Approach 3. Humanized Microbiome Mice

Recent studies have identified the gut microbiome as a key determinant in responsiveness to checkpoint inhibitor therapies for cancer.[19-21] These landmark studies demonstrated not only that patients could be stratified into responders vs. non-responders based on their microbiome profiles, but also that patient responses to immunotherapy could be transferred to mice via fecal microbial transplantation. Certain microbiome components, such as commensal Bifidobacteria, are also known to greatly influence how mice respond to immunotherapies in cancer models.[22] The microbiome, or the entirety of the microbial inhabitants on and within the body, plays a powerful role in immune system development and the subsequent efficacy of immunotherapeutics. While this is a relatively new way of considering humanization, it is becoming clear that the presence of a human immune cell or a specific human therapeutic target may not be sufficient to faithfully model the immune system as it exists in patient populations, and that the microbiome should be considered.

Laboratory mice are generally raised under highly aseptic conditions, free from most pathogens and opportunists. As a consequence, laboratory mice tend to have minimal diversity in their microbiomes and blunted immune system development.[23] These factors make mice very different from patients and can present a challenge in the translatability of research results. Could humanizing the microbiomes of laboratory mice improve preclinical immuno-oncology research or provide insight into specific patient groups or novel therapies?

The first approach in creating a humanized microbiome mouse is to use patient- or volunteer-derived microbiota (i.e., fecal samples), which are transplanted into germ-free mouse recipients to create humanized microbiome mice.[19-21] This strategy has been demonstrated several times in the literature but is limited by the reliability and consistency of donor sources and the extent to which a human microbiome can establish itself within a mouse and induce immune maturation. Nevertheless, the ability to model patient responses to immunotherapies does make this approach attractive.

Pseudo-humanization, in contrast, relies on recapitulating the functional diversity of the human microbiome within the mouse, without specifically sourcing it from a human. For this approach, the microbiome source can be either a diverse mouse microbiome (e.g., derived from a wild or pet store mouse) or an artificial bacterial community designed to stimulate the mouse immune system.[23,24] These approaches have the promise to increase the translatability of preclinical research in a reproducible and controllable fashion.

This microbiome approach to humanization is in its infancy, but there are several possibilities for how the microbiome could be incorporated into immuno-oncology preclinical research. First, clinical samples or microbiology support would be required to source and characterize these microbiomes. Second, specialized gnotobiotic techniques would be required to generate and house these types of humanized mice. It should be noted ›

that each of these may require novel technical expertise and resource investment to enable these types of studies.

Conclusion

There are many ways to consider humanizing a mouse for preclinical immuno-oncology. These advanced approaches are required to meet the expanding need for translatable research solutions and to accelerate progress toward understanding therapeutic mechanisms and developing novel therapies. In this paper, we discussed three in vivo models and considered the pros and cons when selecting which option to use for a given application. We stress that the selection should be made in context of the various available options, the suitability of an option for each project, experimental hypothesis, available resources, and subsequent utility of the model relative to a project's goals. ■

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