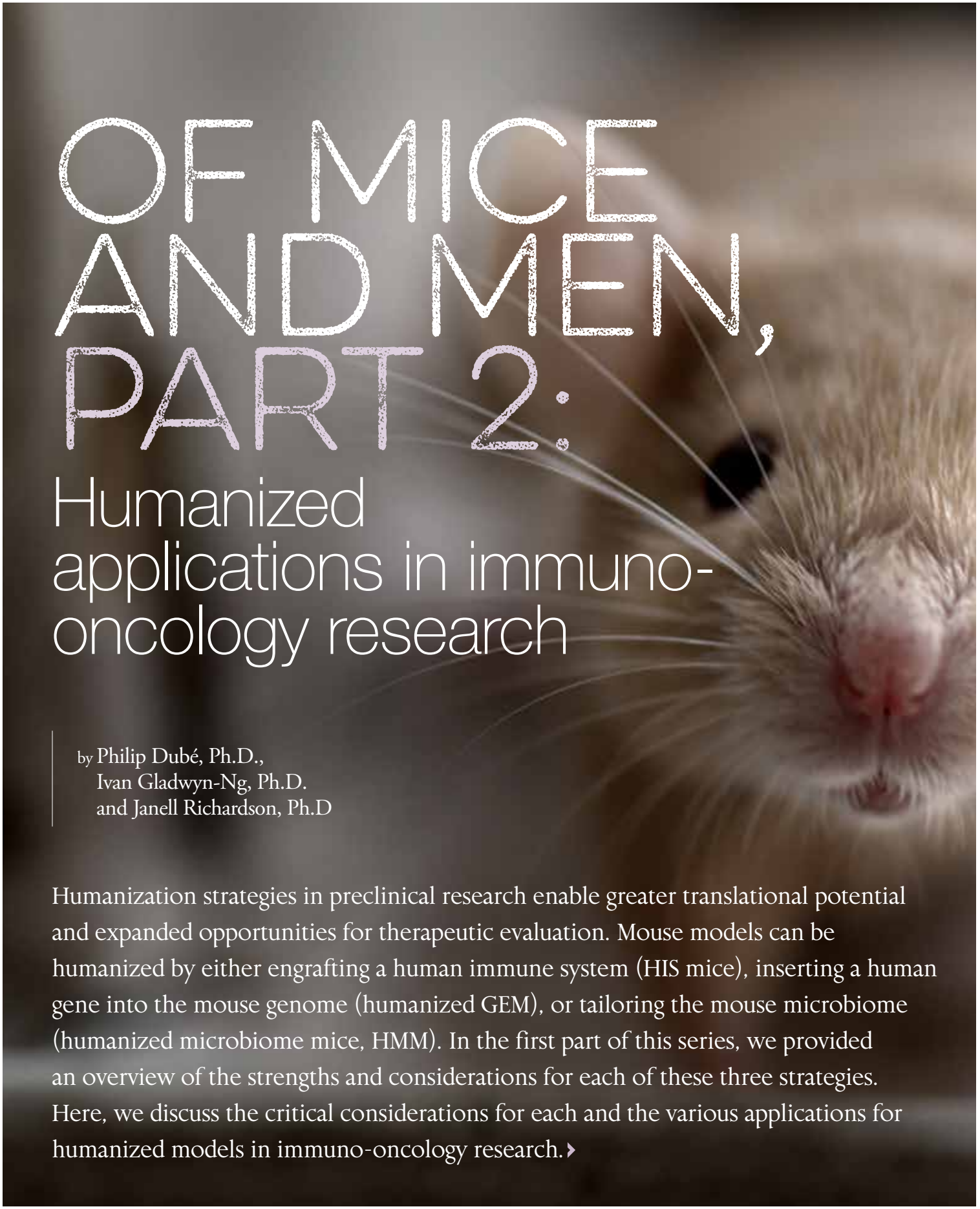

OF MICE AND MEN, PART 2:

Humanized applications in immuno-oncology research

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Humanization strategies in preclinical research enable greater translational potential and expanded opportunities for therapeutic evaluation. Mouse models can be humanized by either engrafting a human immune system (HIS mice), inserting a human gene into the mouse genome (humanized GEM), or tailoring the mouse microbiome (humanized microbiome mice, HMM). In the first part of this series, we provided an overview of the strengths and considerations for each of these three strategies. Here, we discuss the critical considerations for each and the various applications for humanized models in immuno-oncology research.

A Toolbox: Targeting T cells in HIS mice

Current HIS models rely upon super-immunodeficient mice with mutations in the interleukin-2 receptor common gamma-chain locus (IL-2R gamma) that, in combination with a NOD/scid background, render these mice deficient in many aspects of host immunity. The two most widely used models, NOG and NSG (see **Table 1**), are excellent hosts for human cells and tissues.[1-4] Their level of host immunosuppression facilitates human hematopoietic stem cell (HSC) engraftment and enables the generation of HIS mice as a platform for preclinical immuno-oncology research.

The simplest approach for humanization is the engraftment of peripheral blood mononuclear cells (PBMC) into NOG or NSG mice. This offers the fastest engraftment kinetics and provides fully developed adult human immune cells for target evaluation. These PBMC-HIS models have been used widely to study single and combination therapies.[5] However, these studies are limited by the onset of graft versus host disease (GvHD), which occurs concomitant with T cell expansion. For studies that can be undertaken in relatively short timeframes (approximately 2-3 weeks), this does enable investigation into T cell-mediated mechanisms; however, the use of major histocompatibility complex (MHC) knockouts[6] or purified CD8+ T cell preparations[7] may enable longer study durations.

HSC-engrafted HIS models are powerful tools that are becoming increasingly common for studies into T cell-mediated immunotherapy mechanisms. Unlike PBMC models, these HSC-HIS mice enable long-term studies. For example, HSC-engrafted NOG mice (huNOG) may retain human immune cell engraftment for their entire lifespan (up to one year post-engraftment) to enable long-term studies of human T cells with implanted tumors (cancer cell lines or patient-derived xenografts (PDX)). These studies are largely limited to T cells, as HSCs develop almost exclusively towards the lymphoid lineage in either NOG or NSG mice, with the development of relatively few myeloid or natural killer (NK) cells. However, despite this limitation, huNOG and other HSC-engrafted mice have been successfully used to model T cell-dependent mechanisms, such as blocking PD-1, and benefit from long experimental timeframes.[8,9]

Expanding applications for second-generation HIS mice

The development of HSCs within a mouse can be expanded to lineages other than T cells by providing the human cytokines required for alternate lineage(s) determination. (**Figure 1**). The so-called "second-generation" immunodeficient models express human transgenes for expanded immune lineage development for applications requiring specific immune cell subsets other than T cells. These models provide several benefits. First, they allow for myeloid and NK cell engraftment, thus increasing the types of studies possible in the humanized setting. Furthermore, second-generation models may also increase the function of engrafted human immune cells, thus improving the utility and translatability of these models.

Myeloid cell models

Interleukin 3 (IL-3) and granulocyte-macrophage colony-stimulating factor

Table 1: List of Mouse Models and Related Abbreviations Referenced in Text

HIS	Humanized immune system
Humanized GEM	Genetically-engineered model
HSC	Hematopoietic stem cell
NOD	Non-obese diabetic
SCID	Severe Combined Immunodeficiency Disease
TAM	Tumor-associated macrophage
huNOG	Humanized (HSC-engrafted) NOG
PDX	Patient-derived xenograft
NOG	NOD.cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}
NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}
NOG-EXL	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(SV40/HTLV-IL3,CSF2)10-7Jic/JicTac
NSG-SGM3	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl} Tg(CMV-IL3,CSF2,KITLG)1Eav/MloySzJ
IL-6 NOG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(CMV-IL6)1-1Jic/JicTac
IL-15 NOG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(CMV-IL2/IL15)1-1Jic/JicTac
IL-2 NOG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(CMV-IL2)4-2Jic/JicTac
IL-3/GM-CSF	Interleukin 3/granulocyte-macrophage colony-stimulating factor

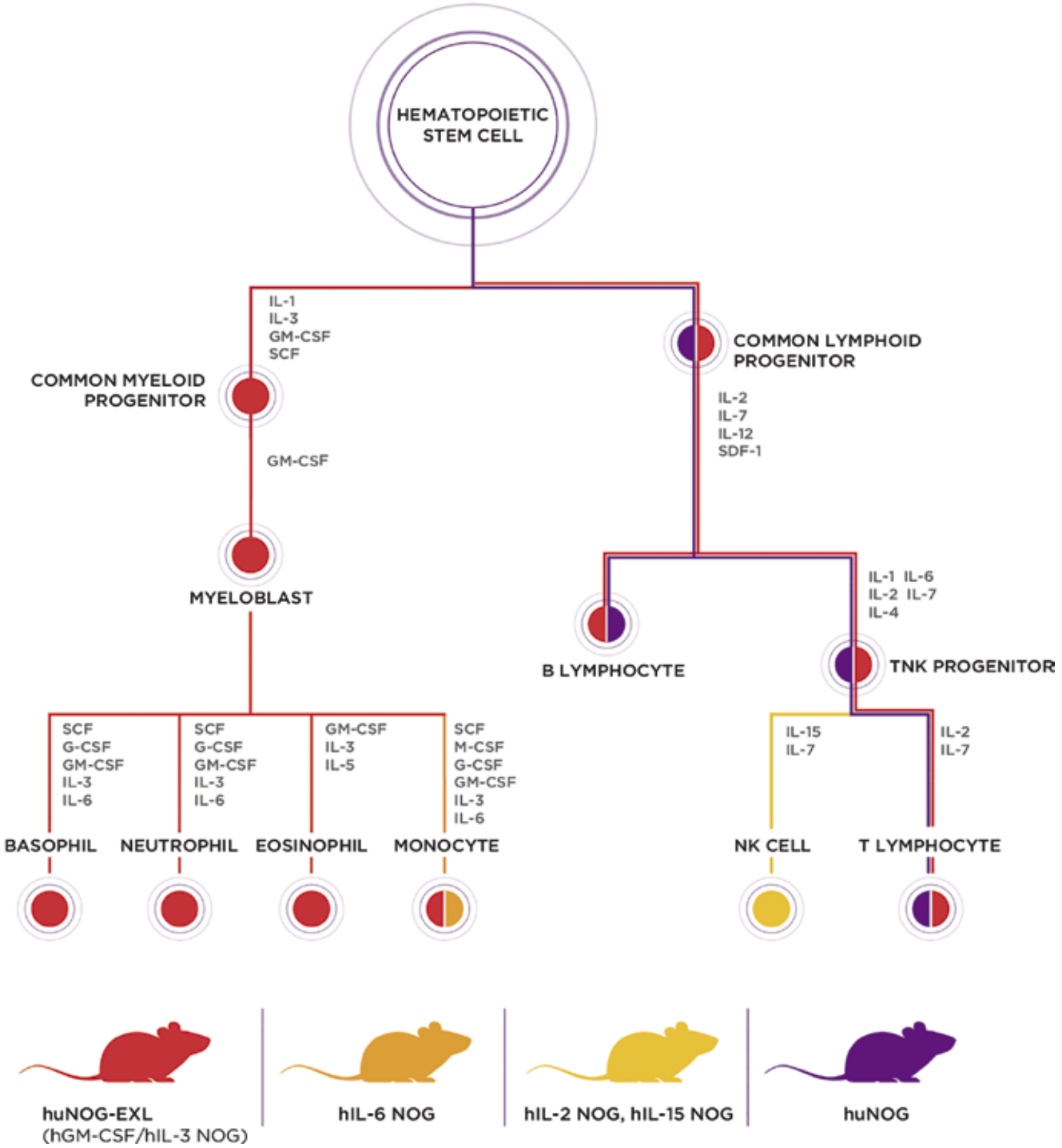

Figure 1: Second-generation NOG mice allow for the engraftment, survival and function of multiple human immune cell lineages *in vivo*.

(GM-CSF) are critical for myeloid lineage specification. Second-generation models expressing these cytokines include NOG-EXL and NSG-SGM3 (see **Table 1**). Each of these models allow for expanded myeloid lineage development, including monocytes and granulocytes, as well as accelerated engraftment kinetics and increased overall human chimerism.[10,11] These models are not specific only to myeloid lineages, but also allow for greater T cell engraftment and the potential to study mechanisms such as antigen presentation and regulatory myeloid pathways. One of the strengths of these models is the ability to study combination therapies alongside T cell-engaging checkpoint inhibition.[12,13]

The IL-3/GM-CSF models illustrate how the characteristics of a transgenic model is dependent upon the chosen transgene

Table 2: Humanization strategies for models and the various approaches, tumor types, benefits, and challenges to aid in selecting the most appropriate model

Humanization Strategy	Approach	Tumor Types	Benefits	Challenges
Immune System Engraftment	▸ HSC engraftment ▸ PBMC engraftment ▸ Base super-immunodeficient hosts ▸ Next generation transgenic hosts	▸ Human cell lines xenografts ▸ PDX	▸ Ability to study interaction of human tumors with human immune cells ▸ Facilitates studies of novel or mutliple human targets ▸ Availability within short timeframes	▸ Limitations on the engraftment and function of immune cells of interest ▸ Study timeframe limitations and GvHD ▸ Non-immune tumor microenvironment remains murine
GEMs	▸ Transgenic humanization ▸ Knock-in gene replacement	▸ Syngeneic mouse cell lines ▸ Inducible tumors ▸ GEM tumor models	▸ Reliable and controllable expression of human targets ▸ Provides alternative to use of surrogate molecules ▸ Compatible with syngeneic, induced and GEM tumor models ▸ Allows for non-immune off-target identification	▸ Timeframe for model generation and characterization ▸ Models are target-specific ▸ Functional redundancy or incompatability of human transgene with mouse orthologs/binding partners ▸ Availability and translatability of syngeneic or GEM tumors
Microbiome	▸ Germ-free mice ▸ Patient-derived microbiota ▸ Pseudo-humanized microbiota	▸ Syngeneic mouse cell lines ▸ Inducible tumors ▸ GEM tumor models ▸ Possibility for human xenografts	▸ Provides translational data on the impact of patient microbiomes ▸ Optimization of tumor models for immunotherapy investigations	▸ Resources for gnotobiotic husbandry ▸ Availability and characterization of relevant microbiota

promoter and the insertion site(s), both of which determine the level of transgene expression. In the case of NOG-EXL, cytokines levels are approximately 35-80 pg/ml, whereas NSG-SGM3 achieves much higher levels of 2000-4000 pg/ml. These different levels of cytokine production impact engraftment rates and stability of the model. Although NSG-SGM3 generally engrafts much quicker than NOG-EXL, the high level of cytokine stimulation may lead to early onset GvHD and increased mortality.[14] While enhanced myeloid engraftment in second-generation models has increased experimental potential for HIS mice, these benefits should be weighed against the potential for morbidity.

Although IL-3 and GM-CSF increase myeloid engraftment, these cells are generally skewed towards cells with granulocytic characteristics. Monocytes, such as regulatory tumor-associated macrophages (TAMs) are increasingly recognized as promising therapeutic targets. Interleukin 6 (IL-6) drives the differentiation of HSCs towards these lineages, and the transgenic IL-6 NOG (see **Table 1**) develops immunosuppressive monocytes, including TAMs, following HSC engraftment.[15] The recent addition of IL-6-driven second-generation models thus makes it possible to generate models with characteristics for testing specific hypotheses within the various types of myeloid cell lineages.

in vivo modelling of human antibody-dependent cellular cytotoxicity mechanisms

Antibody-dependent cellular cytotoxicity (ADCC) is another area of intense interest in immuno-oncology therapeutics. Modelling human ADCC mechanisms *in vivo* can be very challenging, as the human NK cells required for this activity have limited development or survival in the mouse. Interleukin 15 (IL-15) is required for NK cell development, proliferation and activation. The addition of an IL-15 transgenic second-generation model (see **Table 1**) has allowed for enhanced NK cell survival *in vivo* and provides a platform for investigating NK cell-dependent anti-tumor activity.[16]

Models with improved T cell function

While the aforementioned second-generation models focus on improving the differentiation and survival of various human immune cell populations, the presence of an immune cell does not necessarily indicate its function. One example of this is with tumor-infiltrating lymphocytes (TILs); while these cells survive well in a NOG or NSG host, they are not totally functional due to the absence of human IL-2. One approach to overcome this is the administration of exogenous IL-2, which, due to its short half-life, can be expensive and labor intensive. Transgenic IL-2 NOG mice (see **Table 1**) can help to overcome this problem, and this model can recapitulate the clinical responses of patient-derived TILs.[17] Furthermore, and critical to the development of CAR-T therapeutics, IL-2 NOG mice also promote the full function of CAR-T therapeutics *in vivo*.[18]

We summarize these models in **Table 2** (reproduced from Part 1 of this two-part series). Taken together, these second-generation models have increased the possibilities for studying alternate immune cell populations in preclinical immuno-oncology research by expanding the differentiation, survival, and activity of various immune cell lineages. This toolbox represents an expanding resource to generate HIS mice for testing specific therapeutic mechanisms *in vivo*.

Applications for humanized GEMs

Humanized GEM models overcome the species-specific differences between mouse and human and make it possible to test therapeutics against a human target within a syngeneic mouse model. This strategy of adding a human gene to the mouse genome is complex and numerous considerations would determine the overall targeting strategy, including the biological hypothesis of the researcher, the level of homology between the mouse and human loci, costs and timelines. The cost and timeframe for a genetic knock-in project can vary widely, depending on the size of the knock-in and the requirement for the preservation of the human regulatory sequences. In general, transgenics, either by random or targeted insertion, are the most straightforward and easily accomplishable strategy. However, these methods suffer from non-endogenous expression patterns, unpredictable expression levels, and the existence of the endogenous murine locus. The knock-in approach replaces the endogenous mouse gene with the human ortholog, and, while it can overcome many of the challenges with transgenics, is both more technically demanding and resource intensive.

Humanized GEMs need to be tailored towards a specific target but have numerous potential applications that allow for therapeutic investigation of human targets *in vivo*. When engrafted with syngeneic mouse tumors, these humanized GEMs make it possible to use human antibodies to directly assess the efficacy and safety of a particular immuno-oncology therapeutic. The availability of these models is limited, and often requires the custom generation of a model *de novo* for a specific research program. These models are becoming increasingly common for checkpoint inhibitor research, such as the common targets PD-1 and CTLA-4.[19,20] This approach overcomes some of the challenges of HIS models, such as the limitations surrounding immune cell engraftment.

The Future of Safety Applications

Humanized models may potentially have their greatest impact in safety evaluation, both in supplementing non-human primate (NHP) studies, as well as offering unique insights. For these applications, both HIS models and humanized GEMs have been used; HIS models, in particular, may be able to better predict immunotoxicities related to therapies better than either traditional rodent or NHP studies. For example, the severe lymphopenia observed in patients receiving the CD28 agonist, TGN1412, was not predicted in either traditional rodent or NHP models, but have since been replicated in HIS mice.[21,22] The application of humanized mice in safety evaluations is evolving, but now includes myelotoxicity in HIS mice,[23] as well as off-target adverse events in non-immune tissues in humanized GEMs.[24]

The translational promise of microbiome applications

The gut microbiome regulates many aspects of immune function and has recently been identified as a key determinant in immunotherapy responsiveness.[25-27] Modeling the human microbiome *in vivo* would provide several benefits. First, this may increase the translatability of preclinical mouse models. Second, this would enable investigation into microbiome-based therapeutics for immuno-oncology. This new avenue for humanization is in its infancy but holds promise to revolutionize how mouse models are used in immuno-oncology preclinical research.

The importance and the power of microbiome humanization was recently demonstrated in three landmark papers showing that the microbiome was a key determinant in patient responses to immunotherapies.[25-27] These studies used fecal microbiota from cancer patients transplanted into germ-free mice to model how these patient-derived microbiomes affected tumor growth and immunotherapy responses. Interestingly, *patient responsiveness to anti-PD-1 therapy was transferred to recipient mice*, showing that microbiome humanization could recapitulate this major hurdle in immuno-oncology. Using patient-derived microbiota, therefore, offers an opportunity to study immuno-oncology therapeutics in a clinically-relevant manner.

There is currently no standard way to humanize the mouse microbiome, and the literature is filled with numerous strategies and approaches. Certain best practices ›

have been identified that together suggest that a rigorous and reproducible method for microbiome humanization could be possible. Two parameters should be considered for developing a humanized mouse microbiome model:

First, the type of mouse used for microbiome engraftment.

Standard specific pathogen free laboratory mice are largely resistant to microbiome transfer and must be depleted of their endogenous microbiomes, either by germ-free derivation or by antibiotic depletion. While antibiotic depletion is fairly inexpensive and does not require derivation into specialized gnotobiotic isolators, this approach can be ineffective and non-reproducible.[28]

Furthermore, off-target effects of antibiotics make this approach problematic.[29] Germfree mice, in contrast, offer a reliable and reproducible host for microbiome associations. The problem with either of these approaches is that there is a critical window within neonatal development during which the microbiome shapes immune system development, and that microbial association into adult animals leads to aberrant inflammatory responses.[30]

To circumvent these issues, a standard practice is gaining widespread acceptance, in which germ-free mice are first inoculated with the desired microbiome, followed by breeding under gnotobiotic conditions to "naturally" transfer the intended microbiome to offspring at birth. This approach has been routinely used to establish large cohorts of mice with custom microbiome for preclinical applications (Taconic Biosciences, unpublished observations).

Second, the source of the microbiome for inoculation.

Recapitulation of "true human microbiome" within a mouse is challenging due to species-specific genetic and behavioral differences. Patient samples have been used for microbial association, and while some phenotypes can be transferred to mouse recipients, the full spectrum of the human microbiome has not been achieved in a mouse host.[31]

An alternative approach is to inoculate mice with a microbiome not from humans, but one that provides the full functional diversity as seen in patients. One such approach is the use of a microbiome sourced from mice in the wild,[32] which possess a much greater microbiome complexity and subsequent immune system development compared to standard laboratory mice. Regardless of the source, mice with humanized or pseudo-humanized wild microbiomes need to be maintained under strict gnotobiotic conditions to maintain consistency and reproducibility over time.

Conclusions and perspectives

Humanization approaches encompass multiple different methods with their strengths and weaknesses. Immune cell engraftment in HIS mice provides a powerful way to directly study human cells in the *in vivo* setting, whereas humanized GEMs target a single gene, and the microbiome provides a third novel way to consider humanization. Each method has to be selected based on the specific experimental hypothesis and there is no one-size-fits-all for these approaches. These strategies are generally considered separate approaches; however, the possibility of combining these methodologies offers promise to recapitulate human tumor biology more faithfully and contribute to the next generation of preclinical immuno-oncology models. ■

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