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"Humanized Mouse Models for Evaluation of PSC Immunogenicity"

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Abstract

New pluripotent stem cell (PSC)-derived therapies are advancing to clinical trials at an increasingly rapid pace. In addition to ensuring that the therapies function properly, there is a critical need to investigate the human immune response to these cell products. A robust allogeneic (or autologous) immune response could swiftly eliminate an otherwise promising cell therapy, even in immunosuppressed patients. In coming years, researchers in the regenerative medicine field will need to utilize a number of *in vitro* and *in vivo* assays and models to evaluate and better understand human PSC immunogenicity. Humanized mouse models—mice engrafted with functional human immune cell types—are an important research tool for investigating the mechanisms of the adaptive immune response to PSC therapies. This article provides an overview of humanized mouse models relevant to the study of PSC immunogenicity and explores central considerations for investigators seeking to utilize these powerful models in their research.

Introduction

In the two decades since James A. Thomson's seminal publication describing the isolation and characterization of human embryonic stem (ES) cells, tremendous progress has been made in validating Thomson's prediction that "standardized production of large, purified populations of euploid human [ES] cells…will provide a potentially limitless source of cells for drug discovery and transplantation therapies."(Thomson et al., 1998) The term pluripotent stem cells (PSCs) describes both ES cells and induced pluripotent stem cells (iPSCs). There are now multiple, robust, reproducible protocols for the directed differentiation of PSCs into highly purified cell populations, including various subtypes of cardiomyocytes(Lian et al., 2013) endothelial cells,(Zhang et al., 2017) and neurons(Yuan et al., 2011) that accurately mimic their corresponding primary human cells. As capabilities for scale-up and cGMP manufacturing of these cells have been optimized, a number of PSC-based cellular therapies are now being tested in Phase I clinical trials (Schwartz et al., 2012). The pre-clinical regulatory pipeline contains several additional cell therapy candidates. While the progress and prospects of these trials are indeed promising, to achieve the long-term goal of truly *curative* PSC cellular therapies, researchers not only must generate large quantities of pure and highly functional cells but also must ensure that these cells are not rejected by recipients' immune systems. A robust immune response can swiftly destroy even

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functionally ideal cell preparations.(Harper et al., 2015; Tittelbach-Helmrich et al., 2014) This review will discuss the use of advanced humanized mouse models for studying PSC immunogenicity, including important considerations for optimization of transplant grafts.

The Importance of PSC Immunogenicity Studies

Two primary goals for translational PSC researchers are to create reparative functional cell therapies that mimic healthy primary cells and to ensure that those cells will engraft and function for the life of the transplant recipient. Our lab and others are actively studying PSC immunogenicity with these goals in mind; however, beyond improving long-term transplantation engraftment, there are additional important reasons to seek a better understanding of PSC immunogenicity, including patient quality-of-life. Traditional organ transplants rely upon immunosuppressant drugs to prevent allorejection and graft loss, and this reliance will presumably apply to allogeneic PSC cell therapies as well. Anti-rejection drugs can have powerful adverse effects in the long term, such as increased risk of infection and malignancy.(Duncan & Wilkes, 2005) Moreover, approximately 30–35% of graft loss in kidney transplant patients is related to non-adherence, i.e., patients choosing to stop taking immunosuppressants.(Sketris, Waite, Grobler, West, & Gerus, 1994; Zhu, Zhou, Zhang, Zhang, & Lin, 2017) By better understanding the innate and adaptive immune responses to purified PSC cell types, we may be able to decrease, or in certain clinical contexts mitigate, the need for these drugs. Additionally, the study of PSC-centric immune interactions will enable development of new iterations of genetically engineered hypo-immune cells. Such cells could provide a single, “universal” donor source of PSC cell therapies, which thousands of patients in theory will tolerate with minimal need for immunosuppression. (Deuse et al., 2019; Gornalusse et al., 2017) Lastly, the donor- and patient-specific iPSC-derived cells for use in “transplant modeling” studies will provide us with valuable insights that will allow us to better design treatments for both PSC cellular therapies and traditional solid organ transplants.

Methods for Assessing PSC Immunogenicity

Over the past 60 years in the field of transplantation immunology, a number of *in vitro* and *in vivo* assays useful for PSC immunogenicity studies have been developed and refined. Well-established *in vitro* assays include the mixed lymphocyte reaction (MLR)(Morris et al., 2015) for assessment of the proliferative T cell response to alloantigens; the crossmatch assay for determination of donor-specific antibody activity (Manna, Halpin, Campbell, & Hidalgo, 2015); and other highly specific HLA-typing assays, such as sequencing-based typing, which allow for highly refined donor:recipient matching.(Philogene et al., 2020; Vazirabad et al., 2019) For example, these assays enable studies correlating the effects of HLA disparities with transplantation outcomes. Several *in vivo* models have been used to study the systemic immune response to cell and organ transplants. Despite the well-described differences between humans and mice with regard to genetics, physiology, and especially immunobiology,(Mestas & Hughes, 2004) a variety of inbred mouse strains, including those with various transgenes and/or other useful modifications,(Jaenisch & Mintz, 1974; Rongvaux et al., 2014; L. D. Shultz et al., 2005) continue to play a key role in advancing many fields of study, including transplantation. Non-human primate and swine

models offer more relevant physiology and other benefits, but both models have significant financial costs. Further, they lack the full suite of research reagents available to researchers working with human or mouse cells. While important insights can be gained from mice and other non-human models, including answers to research questions that cannot be ethically explored in human subjects, effective translation of human PSC therapies to the clinic in a manner that avoids allorejection will require human-focused immunogenicity models and assays. Humanized mice are an attractive research model that offers insight into human adaptive immunity with the convenience of a mouse host. Data from these models can be coupled with *in vitro* assays to gain a fuller picture of the human immune response to PSC therapies. In turn, this work will bolster confidence in the potential for favorable clinical trial outcomes using PSC therapies. The remainder of this discussion will focus on humanized mouse models and their optimal utilization in studies of human PSC immunogenicity.

Humanized Mice: Background and Benefits

For the purposes of this article, we define “humanized mice” as mice that 1) have been bred to have minimal *mouse* adaptive and innate immunity and are therefore receptive to transplantation of xenogeneic tissues, and 2) have been subsequently engrafted with human hematopoietic tissues, resulting in mature human immune cells in the mouse’s circulation and tissues. In the literature, these models also are called “human immune system (HIS) mice” or “human immune mouse (HIM) models.” Humanized mice are well established in the literature for assaying the human immune response to numerous therapeutic interventions. They have been used extensively in HIV and other infectious disease studies, (Honeycutt et al., 2018; Ma et al., 2011) for research into human lymphopoiesis,(Khosravi-Maharlooei et al., 2019; Mold et al., 2010) and for PSC immunogenicity studies.(Brown et al., 2018; Zhao et al., 2015) These models can be a powerful tool for assessing both *in vivo* human immune response to PSC grafts, primarily by histological analysis of immune infiltration into transplanted grafts, but also via analysis of systemic T cell repertoire phenotype and function.(Khosravi-Maharlooei et al., 2019; Kooreman et al., 2017) Other aspects of the human immune system, such as B cell and NK cell function, are less robust in conventional humanized mouse models. Thus, special consideration should be given to mouse strain selection prior to starting experiments that require extensive evaluation of these cell populations.

Humanized mice as described above can possess additional attributes by way of genetic modification of the mouse host strain, such as KIT modifications to obviate the need for irradiation-based myeloablation,(McIntosh et al., 2015) or the addition of human HLA transgenes(Strowig et al., 2009) and/or transgenic human cytokines in the mice to enhance the immune repertoires within the animals. (Hanazawa et al., 2018) These attributes are not directly related to the tissue sources used for humanization. However, the mouse host strain may interact more synergistically with the humanizing tissue to affect the character and function of the chimeric human immune cells, e.g., by providing human cytokines to influence the development of certain cell subpopulations in a manner specific to the developmental status of the tissue. Many of the above-mentioned host strain modifications are useful for questions relevant to only certain research fields and may not be generally

applicable to all PSC-related projects. Therefore, prior to conducting any experiments, it is crucial to determine the most suitable model for the research questions being studied.

Humanized Mice Varieties and Recommendations for PSC Studies

A number of versions of humanized mouse models exist, including novel iterations currently under development in our lab and elsewhere.(M. Khosravi-Maharlooei et al., 2020; Rongvaux et al., 2014) To select the most appropriate model for individual PSC immunogenicity studies, researchers must 1) have a thorough understanding of the different types of models that are available, 2) know what types of research questions each model is useful for answering, 3) know the strengths and limitations of each type of model, and 4) be aware of proper methodological considerations. Extensive reviews describing the various types of humanized mice have been published elsewhere in recent years.(Allen et al., 2019; Leonard D. Shultz, Brehm, Garcia-Martinez, & Greiner, 2012) Here, we focus on models that are particularly relevant and useful for PSC immunogenicity studies. Table 1 describes the key attributes of each model, including common alternative names seen in the literature (TABLE 1).

During the experimental planning phase, the researcher should take several steps to ensure selection of the appropriate model, and its proper use.

Step 1: Choice of Mouse Host Strain

As mentioned above, the humanizing tissues and the mouse host strain both impact humanization. The specific roles of mouse vs tissue are often conflated in the literature. An example would be a claim that a certain hematopoietic stem/progenitor cell [HSPC] population or source results in a specific degree of myeloid engraftment, when those results would differ significantly, depending on whether the HSPCs were injected into a baseline immunodeficient animal vs an animal also harboring transgene(s) for human cytokines. It is therefore important to choose carefully both the appropriate tissues and mouse strains for your experiments. We have discussed this topic in previous publications(B. E. McIntosh & M. E. Brown, 2015; Simpson & Brown, 2020). We recommend that every researcher who is beginning to work with humanized mice consult the article by Shultz et al. (2012) in *Nature Reviews Immunology* for an introduction to these models, including a detailed description of potential mouse host strains.(Leonard D. Shultz et al., 2012) For PSC studies, we recommend NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac (NOG), NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG), and C.Cg-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Sug}/JicTac (BRG) based strains (the parent strains, and those with additional genetic modifications needed for particular experiments, e.g., NSG vs NSG-SGM3 (Bryce et al., 2016)). We do not recommend creating humanized mice using first-generation immunodeficient mouse strains such as the C.B-*Igh-1*^{b}/IcrTac-*Prkdc*^{scid} (SCID without other modifications) or B6.129S7-*Rag1*^{tm1Mom}/J (RAG knock-out without other modifications) mice. Often, mouse strains are selected by individual labs based on historical usage or personal preference, and may not be the best choice for modern PSC experiments. It is therefore important to survey the literature regularly for the most recent iterations of mouse strains being used by researchers in the field. Conversely, as described below with thymectomized NSG mice, it is also important to evaluate whether the potential

benefits of using promising new humanized mouse iterations outweigh the costs for a lab's own particular research.

Another key consideration with regard to mouse host strains is the use of irradiation for myeloablation. This has been discussed in more detail by us and others.(Cosgun et al., 2014; Brian E. McIntosh & Matthew E. Brown, 2015) Briefly, irradiation is required in most strains of immunodeficient mice to obtain robust human HSPC engraftment. This effect can also be achieved with chemical myeloablative treatments, such as Busulfan or Treosulfan, prior to humanization.(Weaver, Boyne, Pang, Chimalakonda, & Howard, 2015) Irradiation, while effective, produces a host of adverse effects on the hematopoietic, gastrointestinal, and neurological systems of the mice, which can cause experimental complications and premature death in the animals. Our lab has extensively used the NOD.Cg-*Kit*$^{W-41J}$ *Tyr*$^{+}$ *Prkdc*^{scid} *Il2rg*^{tm1Wjl}/ThomJ (NBSGW) host strain, which is similar in engraftment potential to the NSG, but does not require irradiation, as described in more detail in our previous publications.(Brown et al., 2018; McIntosh et al., 2015) There is a large body of humanized mouse literature using irradiated mouse strains, but evaluation of irradiation and its effects on biology relevant to a particular lab's individual research questions should be carefully considered prior to the start of experiments.

Step 2: Humanized Mouse Model/Method Selection

The choice of humanization method will be dictated by 1) the type of tissue available to the research team (or humanized mouse core/company providing mice), 2) the required experimental timeframe, 3) the type of immune cells needed for the study, and 4) the project's budget.

Peripheral Blood Leukocyte Humanized (PBL-hu) Mice.—PBL-hu mice were among the first humanized mice described in the literature.(Mosier, Gulizia, Baird, & Wilson, 1988) These mice are created by injecting large numbers of adult peripheral blood leukocytes (PBLs) (~10–15 $\times 10^6$) into immunocompromised mice, unlike the models described below, which use human HSPCs instead of PBLs. PBL-hu mice do not require myeloablation for durable engraftment; however, T cells will emerge as quickly as within 1 week post-injection when irradiation is used, and a few weeks after injection in non-irradiated mice. Note: irradiated mice will rapidly succumb to graft vs host disease (GVHD), giving irradiated PBL-hu mice an extremely short experimental window (~1–2 weeks). Non-irradiated PBL-hu mice are slower to engraft, but the experimental window prior to GVHD onset can extend over the course of 3–4 weeks. PBL-hu mice can be very useful for short-term transplantation studies,(Ling et al., 2015) especially when adult peripheral blood is the only cell source available for humanization and HSPCs and/or liver/thymus tissues are unavailable. However, as mentioned above, these mice succumb to GVHD, and the associated inflammatory environment is not ideal for certain experimental applications. Additionally, this model should only be used for T cell-focused studies. If HSPCs and/or thymus tissue is available, and if longer experimental windows are needed, we recommend using either the HSC-hu, BLT type or NeoThy humanized mice models described in the next section.

Hematopoietic Stem Cell Humanized (HSC-hu) Mice.—HSC-hu mice are created by direct injection of HSPCs, typically using magnetic bead-sorted CD34+ HSPCs from human cord blood, bone marrow, mobilized blood, or fetal liver. This cell population contains some true hematopoietic stem cells (HSCs) but is technically a mixed population enriched with HSPCs; the “HSC” name is historical in nature. The cells engraft into the bone marrow of the mice and go on to develop into various human immune subsets. If cells are injected into newborn pups, it is possible to see human T cell development in these animals. However, the T cells will not be MHC-restricted, as they will be positively and negatively selected only on murine thymus tissue devoid of human MHC. This approach can also be done using HLA-transgenic mice, which allows for some degree of human MHC interaction in the murine thymus and in the periphery. Still, the developing T cells will not encounter a full complement of MHC molecules (i.e., HLA-A, B, C, DR, DP, DQ) unless they are specifically added to the mouse strain. To date, there have been no published descriptions of creating a transgenic mouse harboring six HLA loci, and even if it were to be achieved, there would still be an absence of human tissue-associated antigens expressed via AIRE in the thymic epithelial cells, which is required for development of self-tolerance in humans.(Perry et al., 2014) Therefore, the most robust humanized mouse models for PSC immunogenicity studies are the BLT type and NeoThy, described in the next section.

Bone Marrow Liver Thymus (BLT) Type Mice.—BLT type mice are created by injecting HSPCs, similar to the HSC-hu mouse described above, but they also include a surgical transplantation of human fetal thymus tissue and sometimes a fragment of fetal liver under the kidney capsule of an NSG or similar immunodeficient mouse strain. (J. M. McCune et al., 1988) In recent years, it has become common to omit the fetal liver fragment. Similar levels of engraftment are seen, and this allows for cryopreservation of the more durable thymus fragment, enabling tissue banking and more flexibility with experiment start dates.(Kalscheuer et al., 2012) These mice allow for the *de novo* development of T cells in the presence of mouse antigens, which is thought to minimize the incidence of GVHD and concordant premature death of the mouse host. An additional modification introduced by Kalscheuer et al. was the use of anti-CD2 antibody to deplete passenger thymocytes in the thymus fragment, further reducing the incidence of GVHD by reducing/eliminating mature thymocytes in the transplanted thymus fragment, which did not develop in the presence of mouse antigens.

The addition of human thymus tissue in this model is critical for of human MHC-restricted T cells. During normal human thymopoiesis, HSPC-derived T cell progenitors undergo both positive and negative selection in the thymus to ensure they differentiate into T cells that both have functioning human MHC-restricted T cell receptors (TCRs) and exhibit minimal self-reactivity (i.e. autoimmunity). Thus, the inclusion of human thymic tissue in the BLT model (and the NeoThy model, mentioned below) ensures proper development of a human T cell repertoire in the mice. An important consideration is that the murine physiological environment exposes developing T cells to murine MHC molecules, in the periphery and potentially via residual murine thymic epithelium. This could potentially result in some proportion of T cells being murine MHC-restricted, in addition to the desired human MHC-restricted population. Unfortunately, quantifying the heterogeneity of these populations is

challenging, if not impossible. Two strategies to eliminate the influence of murine MHC restricted T cells, are to use MHC class I and II knock-out immune deficient host strains, (Brehm et al., 2019) or to perform a thymectomy procedure i.e. surgically removing the disorganized thymic residual tissue,(M. Khosravi-Maharlooei et al., 2020) as described by in The Next Generation of Humanized Mice section below.

BLT mice have been widely used in HIV and other infectious disease studies and have been instrumental in advancing the understanding of human adaptive immunity in many research areas,(Joseph M. McCune & Weissman, 2019) including PSC immunogenicity studies. (Deuse et al., 2019; Zhao et al., 2015) However, a lingering concern with the model relates to the use of fetal tissue for humanization. Namely, the T cells that result in these animals, while effective for a number of functional studies, may be developmentally immature with regard to their regulatory phenotype.(Mold et al., 2010) Recent studies have also questioned whether the balance of naïve vs memory T cells in the BLT model is representative of a typical adult patient.(Kooreman et al., 2017) Nonetheless, the BLT type model is appropriate for PSC immunogenicity studies, and it will continue to be the gold standard against which the next generation of humanized mice will be compared.

NeoThy Humanized Mice.—The NeoThy humanized mouse is the newest iteration of the BLT type model. The NeoThy was developed at the University of Wisconsin-Madison as an alternative to the BLT type model, using a similar humanization strategy but incorporating non-fetal tissues (cord blood CD34+ HSPCs and neonatal thymus from living cardiac surgery patients) instead of cadaveric human fetal tissue.(Brown et al., 2018) This model has similar attributes to BLT type models when comparing commonly accepted metrics relevant to typical use of these types of models, including those for PSC immunogenicity studies. For example, the phenotype, frequency, and function of the T cell and other immune cell subtypes are similar between the two models. However, we have noted significantly increased frequencies of CD45RA+ CD4+ T cells in BLT type mice vs the NeoThy. Our observations align with a previous report indicating a skewing towards this naïve T cell phenotype in the BLT type model.(Kooreman et al., 2017) Our preliminary study did not explore all aspects relevant to the many potential uses of the two models, and future studies are warranted to determine which types of research questions are most appropriate for which model.

Our lab and others are actively exploring the possibility that the more developmentally mature neonatal tissues used in the NeoThy may affect the resultant T cells within the animals. As this research progresses, we expect to find areas of research that may be better suited to one or the other model (e.g., studies of fetal T cell function may benefit from use of the BLT type model) and areas where either model would be appropriate. We expect that the results of these rigorous comparative studies will help clarify the best specific roles for each model and which aspects of each need further improvement in next generation models. For PSC studies, we recommend using either type of model (depending on availability to your research team) as both harbor functional T cells capable of mediating allo- and xenograft rejection, and both models have been used to monitor immune cell infiltration of PSC grafts.

An important final note regarding humanization methods for PSC immunogenicity studies: for some applications, it may be beneficial to investigate NK cell mediated immune responses to PSC cell therapies. For example, one promising area of research involves using various genetic engineering approaches to create hypoimmunogenic PSC lines, e.g., knock-out of MHC class I and or class II genes. These modifications can enable the cells to evade T cell mediated lysis and donor-specific antibody binding, but they may result in NK cell-mediated alloimmunity due to the "missing self" response unless additional genetic modifications are made.(Deuse et al., 2019; Gornalusse et al., 2017) Conventional BLT type and NeoThy mice do not have robust long-term engraftment of NK cells, due to the constraints of the mouse host strains, and therefore cannot typically be used to assess the NK cell-mediated response to novel gene edits. To date, there have been reports of achieving human NK cell chimerism in certain humanized mouse models via modification of mouse host genetics, (Matsuda et al., 2019; Rongvaux et al., 2014) but more work needs to be done to validate and make available these custom humanized models for widespread use. A promising future direction will be to use novel mouse host strains capable of sustaining human NK cells in combination with BLT type and/or NeoThy humanization techniques, to allow for robust immunogenicity studies investigating simultaneous NK, T cell, and other immune cell mediated immune responses.

Step 3: Assessing Human Chimerism, Transplant Date, and Experiment Duration

The optimal date to assess human chimerism (i.e., successful humanization) will be dictated by the type of model chosen. For example, we typically sample the peripheral blood of BLT type and NeoThy type humanized mice around Weeks 12–16 to verify human engraftment. At this time point, many of the animals will begin to show human T cell chimerism (in addition to other immune cell subsets). To date, there are no universally accepted threshold standards for human chimerism in these models. We recommend that prior to transplantation studies, mice should have at least 20% human CD45+ cells (as determined by via flow cytometry, and calculated by dividing the hCD45+ percentage of cells by the sum of the total human and mouse CD45+ percentage of cells). For models assessing T cell mediated immunity, the human CD3+ percentage (of the total human CD45+ cells) should be at least 2%. Once these frequencies are achieved, we typically see an increase in frequency over the course of the experiment. We recommend assaying human chimerism at/near the start of transplantation studies (e.g., Week 12–16 post-humanization), as well as upon completion of the study. Additionally, depending on the study duration, it may be advisable to sample the peripheral blood of the mice throughout, to monitor for any changes in human immune chimerism that may impact experimental outcomes. Duration of the "experimental window" will vary based on the individual study requirements, but in our experience, immune cell infiltration can be noted in PSC-derived cell grafts in NeoThy humanized mouse models after 2–4 weeks ((Brown et al., 2018), unpublished data).

Additional Considerations for Humanized Mouse/PSC Immunogenicity Studies

When studying the immune response to PSC cell therapies in humanized mice, after choosing the proper humanized mouse model for your study as described above, graft

preparation, characterization, and the transplantation method are all critical for experimental success. *In vitro* preparation of PSC-derived grafts, method of graft construction, and anatomical site of implantation are crucial. Consideration of these factors will result in the most informative transplant model possible for investigating physiological systems, disease processes, and/or developmental courses of pre-clinical PSC cell therapies.

Cell Graft Format

The primary goal when creating *in vitro* cell therapies is to enable the most robust cell type, tissue, and/or organ with regard to function and structure, in the context of its unique anatomical location and microenvironment. The transition from an *in vitro* culture to an *in vivo* location can place stress on cells as they move from a fully controlled setting to a highly variable and completely novel environment. Prior to transplantation, graft preparations must be optimized to be accurate models of the target system and to ensure both the short and long-term viability of the graft's constituent cells in an *in vivo* transplant setting, such as humanized mice and patients. (Laflamme et al., 2007)

An important consideration for cell therapy preparation is whether to use a two-dimensional (2D) vs three-dimensional (3D) PSC cell preparation and/or a combination of the two. (Pineda, Nerem, & Ahsan, 2013) 2D cultures predominate in the literature, and are suitable representations of many *in vivo* tissues (e.g., epithelium).(Kesimer et al., 2009) 2D cultures can be easily dissociated into single cell suspensions for direct injection into humanized mice (for example, as described with endothelial cells),(Kooreman et al., 2017) and are typically straightforward to characterize by flow cytometry and other *in vitro* assays. On the other hand, 3D cell preparations are becoming more common for a number of studies. They more accurately reproduce biochemical and biomechanical microenvironments vs 2D culture,(Pampaloni, Reynaud, & Stelzer, 2007) can recapitulate organogenesis,(Sasai, 2013) and can be more feasible for transplant.(Duval et al., 2017; Sasai, 2013) 3D cell preparations, which are often referred to as organoids or spheroids, also offer the convenience of a macroscopically visible and tractable graft with an inherent structure that does not necessarily rely on a extracellular scaffolds and/or successful incorporation of individual cells into *in vivo* tissues. Organoids have been developed for multiple systems, including but not limited to cardiac, brain, and pancreas; transplant methodologies have been developed for many of these systems. (Daviaud, Friedel, & Zou, 2018; Dutta, Heo, & Clevers, 2017; Mattapally et al., 2018) Thus, this is a powerful approach for developing and transplanting more complicated and more physiologically accurate cell preparations, and may be suitable for humanized mouse experiments.

Another graft type consists of cells embedded in an extracellular matrix (ECM) and/or synthetic scaffold, which, like 3D organoid grafts, can be used to better represent *in vivo* microenvironments and provide structure for a cell preparation to improve graft viability post-transplant. Extracellular matrices can be xenogeneic, allogeneic, or autologous. They can be developed by decellularizing tissues, leaving behind solely the ECM to be repopulated with PSC-derived cells of interest.(Badylak, 2004; Badylak, Freytes, & Gilbert, 2009; Sackett et al., 2018) Synthetic matrices are polymers meant to mimic ECM proteins and/or peptides by providing biological support to the cells within them.(Silva & Mooney,

2004) Transplant methodologies have begun to incorporate both biological and synthetic matrices because of the added benefit of being able to better control graft structure and cellular composition.(Stendahl, Kaufman, & Stupp, 2009; Vegas et al., 2016)

The use of single cell suspensions, directly injected in heterotopic locations has also been reported in humanized mouse models.(Kooreman et al., 2017) This, and each of the graft formats above, may impact the phenotype and function of the PSC-derived therapy—an important consideration in humanized mice studies beyond the basic need for graft:immune cell interactions. In our lab, we frequently utilize kidney capsule transplantations, as cell boluses and other preparations can be surgically placed into the site, which has a rich vascular supply needed to sustain the graft and provide access to circulating immune cells.

The transplantation of these graft formats will be discussed in more detail in the Transplantation Techniques section below.

Pre-transplant Characterization of Humanizing Cells/Tissues

We recommend that all humanized mouse studies of PSC immunogenicity include the HLA typing (at the A, B, and DRB loci) of the PSC graft donor (see below), as well as of the humanizing tissues used to create the animal. Thymus, cord blood, and/or PBL donors should be HLA typed whenever possible for all of the models described above.

Pre-transplant Characterization of PSC-Derived Grafts

Characterization of the input cell preparation is critical for optimal experimental results. Cell therapies differentiated from PSC cultures often contain cell populations of mixed purities; a positive portion containing the therapeutic cells of interest, and an impure secondary population(s) with non-therapeutic, unknown, and/or tumorigenic potential. It is therefore important to characterize cell preparations for purity just prior to transplantation. For instance, in our hands the GiWi protocol,(X. Lian et al., 2012) a widely used PSC-cardiomyocyte differentiation protocol, typically yields ~85% cardiac troponin T^+ cardiomyocytes. The other ~15% of the population contains cells that are not fully defined yet, and may affect downstream applications, potentially to the detriment of the therapy.(Xiaojun Lian et al., 2012) Moreover, cell-type differences have been associated with major differences in graft survival and immunogenicity in transplants into different anatomic areas, e.g., the CNS.(Praet et al., 2012) Finally, it is especially important when dealing with PSC-derived tissues to determine if any undifferentiated PSCs persist, as it is common for naïve PSCs to develop into teratomas, especially in immune-compromised mice.(Wakitani et al., 2003) Proper pre-transplant characterization will help to prevent such undesirable cellular growths that could negatively affect the graft and/or harm the host. Input graft characterization also enables accurate correlation of cell therapy implantation with functional outcomes in the transplant model. Poorly defined and otherwise unknown cell populations can compromise experiments and yield inconsequential data. A final important reason for pre-transplant graft characterization is that various PSC-derived cell types have been reported to elicit differential immune responses *in vitro* and *in vitro*, including in humanized mouse models. For example, Zhao et al. described a differential immune

response to retinal pigment epithelial cells (less immunogenic) compared with smooth muscle cells (more immunogenic) from the same donor in a BLT type humanized mouse model, independent of anatomical transplant site.(Zhao et al., 2015) Inadvertent transplantation of two different terminally differentiated cell types resulting from the same differentiation protocol may complicate immunogenicity studies.

Lastly, for transplantation immunology studies, it is important to gather and report HLA typing data on the PSC graft donor whenever possible. Even if not directly studied in the project at hand, HLA match/mismatch status may influence experimental outcomes; including this information in research manuscripts is tremendously valuable to others in the field.

Transplantation Techniques

Surgical technique and anatomical transplantation site both help to define the *in vivo* environment encountered by transplanted grafts, and both therefore may need optimization for best results. The first consideration is route of implantation (related to graft format, as mentioned above): injection of singularized cells suspended in buffer or implantation of a tissue or 3-D cell preparation.(DeWard, Komori, & Lagasse, 2014) It is important to balance the ease of transplantation technique with the optimal *in vivo* experience for the therapeutic cell type of interest. For example, injection of individualized cell preparations may be simpler from a technical standpoint, but there are barriers to survival to consider, depending on injection route and buffer. A nutrient-rich buffer may promote cell survival, but it may also be toxic to the mice and/or prevent effective transplantation.(Deak et al., 2010) For all cell injection experiments, we and others have found consistent results using HBSS+1uM HEPES.(Brown et al., 2018; McIntosh et al., 2015) Moreover, the three most common injection routes are subcutaneous (SC), intravenous (IV), and intraperitoneal (IP). SC and IP routes are simple, may be best suited to some cell types, but lack good access to vasculature. (DeWard et al., 2014; López-Iglesias et al., 2011) IV provides direct blood flow access and immediate immune system exposure, but migration of cells to the lung is a major obstacle for many cell types.(Fischer et al., 2009; Schrepfer et al., 2007)

An alternative to injecting PSC-derived cells is to transplant allogeneic 3-D cell preparations, as mentioned above. Location and graft structure are the two most important considerations for this approach. Graft structure is an important factor, as the development of spheroids and scaffolds enables the study of 3D tissues. Determining which 3D graft type is more applicable will depend on the cell type of interest and the transplant location. Location of the graft contributes extensively to differences in viability and engraftment success as well as potential side effects like teratoma formation.(DeWard et al., 2014; Hentze et al., 2009; Sui, Mfopou, Chen, Sermon, & Bouwens, 2013) Access to adequate blood flow, nutrients, and sufficient space is critical for graft survival, thus making sub-renal capsule transplantation common in murine models.(Hentze et al., 2009; Szot, Koudria, & Bluestone, 2007) Moreover, other sites such as the SC or IP spaces are beneficial locations as they have adequate space for large grafts and are simple to access.(DeWard et al., 2014) However, physiologically relevant orthotopic sites that are more difficult to access are also

utilized frequently, to assess function and promote *in vivo* maturation, such as transplanting PSC-derived cardiac spheroids into the murine heart.(Don & Murry, 2013)

The Next Generation of Humanized Mice

The PBL-hu and BLT type humanized mice are the most-cited humanized mouse models in the literature. In recent years, a number of optimizations of these models have been published. For example, in order to prevent the short experimental windows associated with GVHD, the PBL-hu mouse can be created in an MHC I/II knock-out NSG mouse.(Brehm et al., 2018) Another example of optimization of well-established models is our lab's development of the NeoThy humanized mouse model.(Brown et al., 2018) This new iteration of the BLT type model specifically builds on the BLT type model optimization of Kalscheur et al. (Kalscheuer et al., 2012), incorporating anti-CD2 antibody injections and cryopreservation of the neonatal thymus fragment to avoid passenger thymocytes (which can mediate GVDH). Importantly, the NeoThy utilizes more developmentally mature humanizing tissue, which may have beneficial effects on the functionality of the T cells within the model, and also avoids regulatory and ethical restrictions associated with human fetal tissue research.(Mold et al., 2010)

When deciding whether to utilize an existing or next-generation humanized mouse model, it is important to weigh the potential advantages to your research vs the cost and effort of using the new model. Lack of validation can be a downside of new models, which may not be available to the wider research community. Additionally, feasibility of using a model is important. A description of thymectomized BLT type humanized mice was recently published.(Mohsen Khosravi-Maharlooei et al., 2020) The authors describe clear advantages of eliminating human T cells that are inadvertently educated on residual murine thymic tissue. While this is a compelling model that may give a clearer picture of *in vivo* human MHC-restricted T cell function, it remains to be seen whether wide-spread adoption of this model, which requires two invasive surgical procedures for humanization, is possible. On a related note, the authors predominantly used human fetal tissue for humanization, but incorporated pediatric (not neonatal) thymus tissue into one iteration of their model, with mixed results. It was unclear from this portion of their study, which had low n values and did not track the source of the engrafted T cells, whether the more mature pediatric thymus tissue (vs neonatal or fetal tissue used in previously published reports), and/or the cadaveric nature of their tissue specimens, influenced their results. Before making conclusive recommendations about the utility of the thymectomy approach for use in PSC immunogenicity studies, further study of thymectomized mice is warranted, incorporating fetal, neonatal, and pediatric thymus from living and cadaveric donors.

As mentioned above, a key distinction when using humanized mice is certain properties are conferred by the mouse strain, and other properties result from the tissue source. BLT type and NeoThy humanized mice have been documented in multiple host strains.(Brown et al., 2018; Lavender et al., 2013) But it is important to note that injection of HSCs alone can produce a markedly different myeloid compartment, depending on the mouse strain. Promising new host strains such as the MISTRG(Rongvaux et al., 2014) and the those that

incorporate human HLA transgenes (Labarthe et al., 2020) may enable a new generation of models that more accurately represent human patients.

One intriguing next-generation approach for making humanized mice is the incorporation of PSC technology for humanization. All existing humanized mouse models rely on donated human hematopoietic cells and tissues. Beyond the difficulty of obtaining these tissues, the use of small batches of changing materials imparts a high degree of experimental variation to humanization experiments. For example, human fetal tissue for BLT type mice typically results in 20–40 mice per tissue set. If additional mice are needed, then new batches of tissue, with varying genetic background, must be procured. The NeoThy model offers more consistency; one neonatal thymus can provide enough tissue for over 1000 mice before moving on to a new tissue batch.(Brown et al., 2018) Still, HSPCs are a limiting reagent for both types of model. If both thymic epithelial cells and HSPCs could be produced from PSCs, it would in theory be possible to make millions of homogeneous humanized mice from one donor. Multiple groups have published results with PSC-thymic epithelial cells(Sun et al., 2013) and PSC-HSPCs, (Sugimura et al., 2017) but to date, these approaches do not engraft or function robustly enough to serve as a reliable alternative to primary tissue-derived humanized mice. This is a very promising future direction that, if successful, will result in improved humanized mouse models and new cell therapies.

Conclusion

While clinical translation of PSC therapies has made great progress in the last twenty years, immune rejection remains a critical barrier to realizing the full potential of these promising therapies. Humanized mice are a highly valuable *in vivo* model for PSC immunogenicity studies; when coupled with *in vitro* transplant immunology assays, the resulting data can greatly enhance our understanding of the mechanisms of transplant rejection and tolerance (for PSC therapies, as well as for traditional organ transplants). This review serves as a starting point for researchers embarking on critical studies of PSC immunogenicity, and aims to facilitate rapid progress toward effective, beneficial, widespread clinical use of PSC therapies.

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TABLE 1.

Mouse Host Strains and Types of Humanized Mice for PSC Immunogenicity Studies

The four types of humanized mice that we recommend for PSC studies are:

1	**PBL-hu**. (Also referred to as Hu-PBL-SCID, human-PBL-SCID, hu-PBL, SCID-hu-PBL, PBL-SCID). Briefly, these mice are humanized via direction of mature human peripheral blood leukocytes (PBLs).
2	**HSC-hu**. (Also referred to as SRC-hu, Hu-SRC-SCID, hu-CD34, HSC mice). Here, humanization is achieved by injection of purified human hematopoietic stem/progenitor cells, which engraft and develop into various immune cell subsets within the animal.
3	**BLT type**. These mice build upon the HSC-hu mice, and include the surgical implantation of a human fetal thymus fragment under the kidney capsule of the mouse host, allowing for more relevant T cell development and function vs the HSC-hu.
4	**NeoThy**. This model, developed by our team, builds upon the BLT type model, incorporating more developmentally mature neonatal human tissues for humanization (cord blood and thymus) instead of using human fetal tissue.

A Note on In-House vs Outsourcing of Humanized Mouse Model Creation

Depending on one's institution and budget, whenever possible, we recommend that researchers utilize humanized mouse core facilities or commercial vendors for obtaining humanized mice. This allows for the greatest choice in humanization tissue and ensures greater consistency in model production. The humanized mouse core or company should have a demonstrated record of publications using the models of interest. If outsourcing is not an option, we highly recommend that scientists embarking humanization experiments be trained in-person by experienced personnel (if possible), consult the current literature, and contact experienced researchers who have successfully published research using these models.

TABLE 2.

Important Considerations for Using Humanized Mice in PSC Immunogenicity Studies

Experimental Considerations	**Mouse Model Considerations**
Immunogenicity assays	Immunodeficient mouse strain
HLA typing of PSCs, humanization donor tissues	Utilization of proper immunodeficient strain and humanized model type (PBL-hu, HSC-hu, BLT type, NeoThy, Other)
Transplant timing • development of tissues *in vitro*	Utilizing standard humanized mice strains vs genetically modified strains
Optimal experimental window duration • Time for graft immune infiltration/other considerations	Threshold for human chimerism • ≥20% human CD45+ • ≥2% human CD3+ for T cell based studies
Pair *in vivo* humanized mice models with complementary *in vitro* assays	Timeframe for assaying human chimerism • At minimum: start of study (e.g., for NeoThy mice @ 12–16 weeks post-humanization) and end of study
Source of PSCs • iPS vs ES • Genetically modified PSC lines	• Consult literature to stay up-to-date on best models available
Optimal graft preparation: 2D vs 3D	First-generation immunodeficient mice strains (e.g., CB17 SCID) are not recommended for most applications
In vivo environment/transplant site best suited to study tissue type of interest	Benefits of novel mouse models vs feasibility to use/body of reference literature
Transplantation format best suited to experimental questions	Myeloablation via irradiation vs chemical vs genetic manipulation of c-kit?
Characterization of cell populations present in cell preparations (purity)	PBL-hu for short-term studies or those with limited access to HSPCs and/or thymus tissue
Pre- and post-transplant functional characterization of grafts	BLT or NBSGW for PSC studies if at all possible