

The Potential for Immunogenicity of Autologous Induced Pluripotent Stem Cell-derived Therapies*

Published, JBC Papers in Press, December 20, 2013, DOI 10.1074/jbc.R113.509588

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Induced pluripotent stem cell (iPSC) technology offers the promise of immune-matched cell therapies for a wide range of diseases and injuries. It is generally assumed that cells derived from autologous iPSCs will be immune-privileged. However, there are reasons to question this assumption, including recent studies that have tested iPSC immunogenicity in various ways with conflicting results. Understanding the risk of an immune response and developing strategies to minimize it will be important steps before clinical testing. Here, we review the evidence for autologous iPSC immunogenicity, its potential causes, and approaches for assessment and mitigation.

The remarkable discovery that ectopic expression of four transcription factors can reprogram somatic cells to a pluripotent state opened new avenues of research into human disease and regenerative medicine (1, 2). Scientists immediately recognized that these induced pluripotent stem cells (iPSCs)² represent a potential source of autologous cell therapies that could avoid the issues of immunogenicity associated with allogeneic sources such as human embryonic stem cells (hESCs) or donated tissue (3–5). The possibility that cells derived from autologous iPSCs might themselves be immunogenic received little attention until recently, although it had been discussed in a few forward-looking reviews (6–8). This idea was thrust into the spotlight by a provocative paper by Zhao *et al.* (9), who reported immune rejection of syngeneic (genetically identical) iPSC-derived teratomas in mice. However, these results have since been challenged in several high-profile articles that we will discuss in this minireview. The first, by Araki *et al.* (10), actually provided additional evidence for syngeneic iPSC-derived cell immunogenicity, despite its title and conclusions. However, in a more recent challenge, Guha *et al.* (11) utilized a clinically relevant approach and found no evidence for immune rejection of syngeneic cells differentiated from iPSCs. Most

recently, Morizane *et al.* (12) performed autologous iPSC-derived neural cell transplantation in a primate model and found minimal evidence of chronic immune response in the brain. These latter two studies are very encouraging, although confirmatory work is still required.

The possible causes of an immune response to autologous iPSC-derived cells are varied and include immaturity of transplanted cells, genetic and epigenetic changes due to reprogramming or culture adaptation, effects of xenogeneic or non-physiological culture reagents, and expression of gene-corrected proteins. These mechanisms are unlikely to result in MHC mismatch, so potential immune responses are predicted to be less intense than those in the organ transplant setting (8). Still, even low-intensity immune responses could jeopardize engraftment and survival of iPSC-derived cell therapies. Although it is impossible to fully model human immune responses preclinically, we think it will be important for researchers to assess iPSC immunogenicity prior to testing in patients. In cases in which immunogenicity is predicted, such as gene-corrected cells to replace a missing or defective protein, investigators must consider strategies to minimize an immune response.

Evidence for Autologous iPSC Immunogenicity

The possibility that autologous iPSC-derived cells might provoke an immune response was not widely considered prior to a publication by Zhao *et al.* in 2011 (9). In this study, the authors showed that transplanted iPSCs were frequently rejected by syngeneic mice as measured by teratoma formation, regression, and T cell infiltration. In contrast, syngeneic ESC-derived teratomas rarely, if ever, provoked an immune response. The frequency of iPSC rejection was greatly reduced by the use of a nonviral episomal reprogramming method, although these episomal iPSCs (EiPSCs) were still rejected at a frequency of 10–20%, and a majority of growing teratomas were infiltrated by T cells. Although intriguing, these results were met with some skepticism due to the focus on undifferentiated iPSC transplantation, an approach that would never be utilized in the clinic (13, 14).

The first challenge to the findings of Zhao *et al.* (9) was published by Araki *et al.* in 2013 (10). In this study, the authors found a similar frequency of syngeneic integration-free iPSC-derived teratoma rejection but, unlike Zhao *et al.*, found a comparable level of rejection of ESC-derived teratomas. This suggests that teratoma rejection may be related to the expression of genes related to pluripotency or teratomas rather than anything iPSC-specific. Importantly, the authors also tested the immune response to cells terminally differentiated from iPSCs and ESCs and found that both skin grafts and bone marrow were very rarely rejected in the syngeneic setting. However, these cells were differentiated and matured in chimeric mice prior to isolation and transplant, another scenario that lacks clinical relevance. *In vivo* differentiation may have resulted in the elimination of immunogenic cells over the course of development. The authors did attempt a clinically relevant experiment, transplantation of cardiomyocytes differentiated *in vitro* from iPSCs, but

* This is the third article in the Thematic Minireview Series "Development of Human Therapeutics Based on Induced Pluripotent Stem Cell (iPSC) Technology."

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² The abbreviations used are: iPSC, induced pluripotent stem cell; hESC, human embryonic stem cell; EiPSC, episomal iPSC; DA, dopaminergic; MHC-I, MHC class I; NK, natural killer; Neu5Gc, N-glycolylneuraminic acid; MLR, mixed lymphocyte reaction; CFSE, carboxyfluorescein diacetate succinimidyl ester; ELISPOT, enzyme-linked immunosorbent spot; TEC, thymic epithelial cell.

TABLE 1

Summary of iPSC immunogenicity data reported in four recent studies

+, immune rejection reported; −, minimal or no immune rejection reported; NT, not tested.

Syngeneic cells injected	Zhao <i>et al.</i> (9) ^a	Araki <i>et al.</i> (10) ^a	Guha <i>et al.</i> (11) ^b	Morizane <i>et al.</i> (12) ^c
Undifferentiated iPSCs	+	+	−	NT
Undifferentiated ESCs	−	+	−	NT
<i>In vivo</i> differentiated iPSCs	NT	−	NT	NT
<i>In vitro</i> differentiated iPSCs	NT	+	−	−

^a All cells were injected subcutaneously.^b All cells were injected into the subcapsular renal space.^c All cells were injected into the brain (putamen).

observed significant levels of T cell infiltration into the graft. These results, along with their observations of teratoma rejection, do not support the overall conclusion that syngeneic iPSC-derived cells display only limited immunogenicity.

More recently, Guha *et al.* (11) published a second challenge to the findings of Zhao *et al.* (9) using a clinically relevant approach. In sharp contrast to the Zhao and Araki studies (9, 10), Guha *et al.* reported 100% graft survival of transplanted syngeneic PSCs (virally reprogrammed iPSCs, EiPSCs and ESCs). Although they did detect some T cell infiltration into the grafts, it did not result in rejection. The authors also performed the crucial experiments of differentiating cells *in vitro* into three different lineages and transplanting them into syngeneic hosts. They saw no evidence of an immune response as measured by graft survival and T cell infiltration. Furthermore, cells isolated from these grafts did not provoke a secondary T cell response either *in vitro* or *in vivo*. These results are reassuring and an important step in demonstrating the immune privilege of *in vitro* differentiated iPSCs.

Most recently, Morizane *et al.* (12) published a study comparing the immune response to autologous and allogeneic iPSC-derived neural cell transplantation in the primate brain. The authors differentiated iPSCs *in vitro* toward midbrain dopaminergic (DA) neurons, the cell type lost in Parkinson disease. They found that greater numbers of T cells and microglia surrounded and infiltrated allogeneic transplants than autologous transplants. Interestingly, a limited T cell response was observed to autologous DA neurons derived from retrovirally reprogrammed iPSCs, whereas those derived from EiPSCs elicited none. These data reinforce the notion that viral integration of reprogramming factors can result in immunogenicity. One limitation of the study is that it permitted only extensive analysis of immune response at the time of animal death, 3–4 months post-transplantation. They attempted to monitor immune responses longitudinally using positron emission tomography and measurement of cytokines in the blood and cerebrospinal fluid, but results were variable and correlated poorly with histological data. Although it is impractical in the primate model due to the number of animals required, it would be informative to look at additional time points to rule out an acute immune response to autologous transplants of iPSC-derived cells. Although Morizane *et al.* demonstrated an advantage of an autologous approach over an allogeneic one, there were still a significant number of allogeneic DA neurons surviving at 3–4 months post-transplantation in the absence of immunosuppression. This is consistent with clinical observations of long-term survival of allogeneic fetal DA neuron transplants in Parkinson disease patients who

received only short-term immunosuppression or none at all (15–17).

What might account for the different results and conclusions of these four studies (Table 1)? Despite their presentation, the undifferentiated syngeneic iPSC transplantation results of the Zhao and Araki studies (9, 10) are not so different, as both reported similar rates of teratoma formation, a significant rate of teratoma regression, and T cell infiltration. The major difference is that Araki *et al.* reported a similar immune response to syngeneic ESCs. This may have been because Zhao *et al.* tested only one ESC line, which may not have been representative, whereas Araki *et al.* tested five. On the other hand, Guha *et al.* (11) reported 100% teratoma formation from all types of PSCs tested, with the only apparent difference being the site of transplantation: subcapsular renal space *versus* subcutaneous space in the previous two studies.

This may account for the difference in teratoma formation rate, as the subcapsular renal space is smaller and more highly vascularized than the subcutaneous space. Thus, the 10–20% failure rate of teratoma formation reported by Zhao *et al.* (9) and Araki *et al.* (10) may have been due to insufficient vascularization or cell-cell contact rather than rejection *per se*. This is supported by the fact that all three studies reported T cell infiltration into teratomas after establishment, but only the two that transplanted subcutaneously reported <100% teratoma formation, as well in previous work (18). Of course, these teratoma experiments do not reflect the path of clinical translation, which will involve transplantation of cell types differentiated *in vitro* from iPSCs. This scenario was modeled by Araki *et al.*, Guha *et al.* (11), and Morizane *et al.*, with the first reporting immunogenicity of syngeneic iPSC-derived cardiomyocytes and the latter two reporting little or no immunogenicity of differentiated cell types of three lineages. Notable differences between these experiments were, again, the sites of transplantation: the heart, subcapsular renal space, and brain, respectively. The importance of this variable is evidenced by the consistent rejection of allogeneic cells transplanted into the subcapsular renal space by Guha *et al.* compared with the survival of significant numbers of allogeneic DA neurons transplanted into the primate brain by Morizane *et al.* Also different were the differentiation protocols, which for Guha *et al.* involved FACS for both positive and negative markers. Their data suggest that some type of selection step may be important for avoiding potential immunogenicity of immature or aberrant cells, in addition to the universally appreciated threat of teratoma formation by residual PSCs.

Potential Causes of iPSC Immunogenicity

To assess and prevent iPSC immunogenicity, it is important to recognize its potential causes. We group these into four categories (for a thorough review, see Tang and Drukker (8)). The first potential cause is immaturity of cells differentiated from iPSCs *in vitro*. Directed differentiation of PSCs into mature cell types represents a substantial challenge for the field of regenerative medicine across many therapeutic areas. There are a number of human cell types that, to date, can be differentiated only to immature phenotypes *in vitro*, including cardiomyocytes (19), hematopoietic stem cells (20), hepatocytes (21), and pancreatic β -cells (22). An immature phenotype poses two risks for immune response, the first being low MHC class I (MHC-I) expression. Natural killer (NK) cells target cells with low MHC-I levels, and although differentiation of iPSCs causes these levels to rise, they may not reach those of adult tissue. An early proof-of-concept study of autologous iPSC therapy for sickle cell anemia in a mouse model required repeated administration of an NK cell-depleting antibody to enhance engraftment of hematopoietic progenitors (23). Low MHC-I expression by these progenitors may have triggered NK cell attack, limiting engraftment. Another risk of an immature phenotype is expression of embryonic or fetal proteins. These antigens may not have been present during immune system education to go through negative selection in the thymus, leaving them susceptible to T cell attack. This potential is demonstrated by cancers that re-express embryonic or fetal antigens that are targeted by the immune system (24, 25). This mechanism may account for the immune responses generated toward teratomas in the articles reviewed above.

A second potential cause of iPSC immunogenicity is genetic and epigenetic changes that arise from reprogramming or adaptation to culture conditions. Recent studies have demonstrated that reprogramming to pluripotency is incomplete and that iPSCs carry an epigenetic memory of their tissue of origin that affects gene expression and can restrict differentiation potential (26–30). There have also been reports that the reprogramming process induces genetic mutations in coding regions (31, 32). Theoretically, both epigenetic and genetic abnormalities could result in autologous iPSC immunogenicity. Epigenetic memory for the cell type of origin could result in aberrant surface antigen expression when iPSCs are differentiated into other cell types. Similarly, changes in cell surface proteins due to genetic mutations could also induce an immune response. In addition, *in vitro* culture itself has been shown to result in genetic instability in PSCs, most commonly chromosomal amplification, including copy number variation (33–35). These genetic abnormalities could result in not only immunogenicity but also carcinogenicity.

A third potential cause is culturing of iPSCs, or their differentiated progeny, with xenogeneic or non-physiological culture reagents. The danger of using xenogeneic culture reagents was demonstrated by Martin *et al.* (36), who showed that hESCs take up the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) from mouse cell feeder layers and animal serum-containing culture media. This represents a risk because humans have circulating antibodies to Neu5Gc (37). Several

groups have since developed xeno-free culture conditions for reprogramming and differentiation that reduce or eliminate Neu5Gc expression, although these methods are costly and can be technically challenging (38–40). In addition, a recent article reported that xeno-free culture media containing high levels of ascorbate induced epigenetic activation of CD30, a cell surface antigen and biomarker for malignantly transformed cells (41). This demonstrates that the risk is not limited to xenogeneic culture reagents, and new media formulations should be tested for biological effects on cultured cells, including abnormal surface antigen expression.

A fourth potential cause of iPSC immunogenicity is gene correction to restore proper expression of missing or dysfunctional proteins. Genetic diseases may be amenable to treatment with iPSC-derived cells, but only if the underlying mutation is corrected in these cells. However, the expression of proteins that the patient's immune system has never been exposed to, or only in a truncated form, may prompt an immune response. This risk is apparent in the clinical use of enzyme replacement therapies for lysosomal storage diseases as well as hemophilia A and B, in which neutralizing antibodies to the replacement protein can limit therapeutic efficacy (42).

Other potential causes of immune response to any type of cell transplant also apply to autologous iPSC-derived cells. For example, not all transplanted cells will survive, and cell death can elicit an acute inflammatory response, followed by release of intracellular proteins that can trigger an adaptive immune response (43).

Preclinical Assessment of iPSC Immunogenicity

Potential cellular and humoral immune responses to iPSC-derived cells can be assessed by *in vitro* and *in vivo* assays. *In vitro* assays may employ methods to assess the susceptibility of the cells to host T cell immune response. These methods include mixed lymphocyte reaction (MLR), carboxyfluorescein diacetate succinimidyl ester (CFSE) assay, and enzyme-linked immunosorbent spot (ELISPOT) assay. In MLR assays, peripheral blood mononuclear cells from the graft recipient serve as responders, which are co-cultured with donor stimulating cells (*e.g.* iPSC-derived cells), and T cell proliferation is measured by [³H]thymidine incorporation. In renal transplantation studies, an MLR between donor and recipient lymphocytes is predictive of rejection of the graft (44). T cell proliferation can also be analyzed by CFSE assay. CFSE is a dye that passively diffuses into cells and binds to intracellular proteins. Upon cell division, each daughter cell receives an equal portion of CFSE, halving the fluorescence intensity as measured by FACS. On the basis of this decrease in fluorescence, the number of cell divisions can be determined and hence a measure of proliferation. The ELISPOT assay for IFN- γ is an important tool for post-transplant monitoring of T cell reactivity and is also useful in pre-transplant immune risk assessment. For example, Augustine and Hricik (45) showed that pre-transplant measurement of recipient T cell alloreactivity to donor antigen via the INF- γ ELISPOT assay correlates with acute rejection after kidney transplantation.

Additional *in vitro* immunogenicity assays measure cytokines and chemokines secreted by iPSC-derived cells that could

influence the cellular immune response generated by the host following transplantation. Okamura *et al.* (46) used supernatants from cultures of undifferentiated hESCs and hESC-derived oligodendrocyte precursor cells to assess soluble immunomodulatory factors. In addition, a flow-based combinatorial antibody profiling method, such as the commercially available BD FACSTM CAP, can provide further *in vitro* characterization of iPSC-derived cells and tissues for potential humoral response (47).

Because *in vitro* immune assays do not fully recapitulate the *in vivo* responses to a cellular graft, more clinically relevant *in vivo* assays should also be pursued. Immunodeficient and immunocompetent mouse models have been utilized to assess the immune response to ESCs and their derivatives in syngeneic and allogeneic hosts. Three recent studies discussed above investigated *in vivo* immunogenicity of mouse iPSCs and their derivatives (9–11). In one of these, Guha *et al.* (11) assessed whether iPSC-derived cells are susceptible to secondary immune response by CFSE assay. T cells were isolated from the spleens of syngeneic and allogeneic iPSC-derived cell transplant recipients, and their proliferation *in vitro* was analyzed by CFSE assay, revealing very low levels of T cell proliferation in response to syngeneic iPSC-derived cells *versus* high levels in response to allogeneic cells. *In vivo* cellular immune response to iPSC-derived cells or tissues can also be analyzed by an *in vitro* T cell cytotoxicity assay. T cells are isolated from the spleen of the transplant recipient, which can either be a syngeneic host (for mouse iPSCs) or a humanized immunodeficient mouse (for human iPSCs). These T cells are co-cultured with the iPSC-derived cells or tissues to determine whether the isolated T cells can directly kill annexin V-labeled iPSC-derived cells *ex vivo*. Another method for assessing *in vivo* immunogenicity of transplanted cells is bioluminescence imaging, which has been used to show that transplanted xenogeneic hESCs transplanted into immunocompetent mice survived only 7–10 days after primary injection and only 3 days after repeat injection (48).

Strategies to Overcome Immunogenicity

Although Guha *et al.* (11) and Morizane *et al.* (12) provided reassuring data that autologous iPSC-derived cells may not be immunogenic, the studies require confirmation, and there are still reasons for concern. In particular, it is unclear whether iPSC-derived cells that have not been fluorescence-activated cell-sorted or have gone through *ex vivo* manipulation will be devoid of immunogenicity in sites outside the brain. In theory, the immunogenicity of transplanted cells could be addressed by conventional immunosuppressive drugs. However, due to their toxicity and the associated risk of malignancy, they are not a desirable option for autologous iPSC-derived cell therapies (49). Biological therapies with monoclonal antibodies provide an alternative approach. For example, Pearl *et al.* (50) used bioluminescence imaging to show that monoclonal antibody-mediated co-stimulation/adhesion blockade of host T cells can result in long-term engraftment of hESC and human iPSC grafts in murine models. However, this approach has not yet been attempted in the non-human primate or human setting.

Based on recent advances in the stem cell field, several immunological approaches have the potential to improve the accept-

ance of the iPSC-derived grafts. One possible solution is to take advantage of the pluripotency of iPSCs to generate not only therapeutic cells but also immature dendritic cells expressing neoantigens to which tolerance is required. The rationale comes from studies in which administration of immature monocyte-derived dendritic cells, pulsed with keyhole limpet hemocyanin or influenza matrix peptide, led to non-responsiveness to these antigens in healthy volunteers due to regulatory T cell mechanisms (51). Another critical area of investigation into strategies to induce donor-specific tolerance is rejuvenation of the thymus (52). The thymus is the main organ responsible for establishing immune tolerance via elimination of autoreactive T cells. iPSCs are a potential source of replacement thymic epithelial cells (TECs) that could be used to induce tolerance to an iPSC-derived graft. By allowing generation of a T cell repertoire tolerant to stem cell self-antigens and neoantigens, co-transplantation of stem cell-derived TECs could potentially prevent immune rejection of other transplants derived from the same cell line, which would have a major impact on stem cell-based therapies. Two recent studies describe progress in generating TECs from human PSCs, although work remains to improve their maturity and functionality (53, 54). Other approaches include development of immune-privileged PSC derivatives capable of blocking the activation of co-stimulatory receptors responsible for immune recognition. This could be accomplished by genetic “knock-in” of ligands of potent inhibitory receptors expressed by T cells (*e.g.* CTLA4 or PD-1) or by targeting inhibitory pathways that mediate immunosuppression (*e.g.* indoleamine 2,3-dioxygenase or HLA-G) (55–57).

Creation of a “haplobank” of iPSC lines homozygous for a range of HLA types representative of different geographical populations and ethnic groups could simplify HLA matching, provide matches for a reasonable percentage of a target population, and extend iPSC-derived therapies beyond the autologous setting. According to one estimate, an iPSC bank from 150 selected homozygous HLA-typed volunteers could match 93% of the United Kingdom population with a minimal requirement for immunosuppression (58). Similarly, due to limited diversity of the Japanese population, as few as 50 such lines could potentially match 90% of the population (59). However, more diverse populations will require more lines (60).

Advancing to Clinical Studies

Many challenges remain for advancing iPSC technology to the clinic, although pioneering approaches are moving forward. The California Institute for Regenerative Medicine, the state’s stem cell agency created by the citizens of California in 2004, is currently investing over \$250 million in 109 total awards using iPSCs. Twenty of those awards are focused on translational research to generate proof-of-concept data and select candidate therapeutics for further preclinical development. One award, to Alfred Lane at Stanford University, is focused on development of gene-corrected iPSCs to treat dystrophic epidermolysis bullosa, a highly morbid, blistering skin disease in children (61, 62). The approach utilizes homologous recombination in patient-derived iPSCs to correct the mutant collagen VII (*COL7A1*) gene, followed by differentiation into skin kera-

tinocytes. The expression of corrected functional protein has the potential to prompt an immune response, which will be an important consideration in moving toward the clinic. Using a different approach, Masayo Takahashi from the RIKEN Center for Developmental Biology in Japan recently received regulatory approval to conduct the first clinical study of iPSC-derived cells in humans (63, 64). This study will test the safety of autologous iPSC-derived retinal pigmented epithelial sheet transplant into the retinas of a small number of patients with the neovascular form of age-related macular degeneration, which can lead to severe vision impairment and blindness. Although the normal eye is thought to be an immune-privileged site, inflammation associated with age-related macular degeneration can lead to a breach of the blood-retina barrier, allowing an influx of immune cells. Therefore, the potential for an immune response to the transplanted autologous iPSC-derived retinal pigmented epithelial cells should not be discounted.

Another autologous iPSC-based clinical approach is being pursued by Advanced Cell Technology in Santa Monica, CA (62). The goal is to differentiate iPSCs into functional platelets and test their efficacy in blood-clotting disorders. Platelets are non-nucleated cells and thus do not have oncogenic potential, diminishing the primary safety concern surrounding pluripotent cell-based therapies. However, platelets do express cell surface proteins that can prompt immune response, including clinical refractoriness to platelet transfusion, so a lack of immunogenicity for this approach should also not be taken for granted (65).

The potential of PSC-derived cells to form tumors after transplantation is one of the most significant risk considerations for clinical entry. One of the major factors influencing tumor development is immune recognition of aberrant, immature, or undifferentiated cells. An immune-privileged transplant, which is the primary goal of autologous iPSC approaches, risks extending that privilege to aberrant tumorigenic cells existing within the transplant and underscores the importance of fully understanding the complex interactions of PSC immunogenicity (66).

Conclusions

The widely presumed immune privilege of autologous iPSC-derived cell transplants has recently become a topic of debate in the literature. Although two of the most recent contributions provide encouraging evidence in favor of immune privilege, the debate should not be considered settled. There are several potential causes of iPSC-derived cell immunogenicity, and results may depend on the cell preparation protocol and site of transplantation. We believe that preclinical work in this area should continue, using differentiation and transplantation methods that reflect the envisioned clinical applications as closely as possible. Although the predictive value of preclinical models of human immune response may be limited, methods are available to address many of the potential causes of iPSC-derived cell immunogenicity. As autologous iPSC-derived cell transplants advance into the clinic, it is important that trials be conducted with the awareness that even cells derived from autologous tissue may be immunogenic. Initial trials should be designed to investigate the possibility of immunogenicity while

ensuring the safety of participants to the greatest extent possible.

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