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Immunogenicity of *in vitro* maintained and matured populations: potential barriers to engraftment of human pluripotent stem cell derivatives

Chad Tang¹, Irving L. Weissman¹, and Micha Drukker¹

¹Institute of Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

Abstract

The potential to develop into any cell type makes human pluripotent stem cells (hPSCs) one of the most promising sources for regenerative treatments. Hurdles to their clinical applications include *i*) formation of heterogeneously differentiated cultures, *ii*) the risk of teratoma formation from residual undifferentiated cells, and *iii*) immune rejection of engrafted cells. The recent production of human isogenic (genetically identical) induced PSCs (hiPSCs) has been proposed as a “solution” to the histocompatibility barrier. In theory, differentiated cells derived from patient-specific hiPSC lines should be histocompatible to their donor/recipient. However, propagation, maintenance, and non-physiologic differentiation of hPSCs *in vitro* may produce other, likely less powerful, immune responses. In light of recent progress towards the clinical application of hPSCs, this review focuses on two antigen presentation phenomena that may lead to rejection of isogenic hPSC derivatives: namely, the expression of aberrant antigens as a result of long-term *in vitro* maintenance conditions or incomplete somatic cell reprogramming, and the unbalanced presentation of receptors and ligands involved in immune recognition due to accelerated differentiation. Finally, we discuss immunosuppressive approaches that could potentially address these immunological concerns.

Introduction

Major and minor histocompatibility complex (MHC and mHC, respectively) antigens belong to a large and diverse group of molecules involved in immune recognition and graft rejection. Classical graft rejection responses stem primarily from structural differences between donor and host antigens, most prominently those belonging to the MHC family (reviewed in [1]). Recent experiments have demonstrated that differentiated human embryonic stem cells (hESCs) express MHC class I (MHC-I) molecules [2,3]. As such, hESC derivatives are expected to promote allojection responses similar to those observed following organ transplantation [4]. With advancements toward production of patient-

Correspondence to: Micha Drukker.

Contact Information:

Chad Tang: cztang@stanford.edu

Irving L. Weissman: irv@stanford.edu

Micha Drukker: dmicha@stanford.edu, Phone 650-725-5808, Fax 650-498-6255

specific hPSCs by parthenogenesis [5], somatic cell nuclear transfer of oocytes [6], and induction of pluripotency [7], rejection based on MHC mismatches may become technically avoidable. Here, we discuss experiments indicating that aberrant antigens and unbalanced presentation of immunologic signals that develop due to *in vitro* maintenance and differentiation may promote immune responses even against grafts derived from isogenic hPSCs. We primarily discuss immunologic hurdles relevant to hiPSC derivatives, since hiPSC lines may become a main source of patient-matched grafts. Discussions of immunologic considerations for allogeneic hPSC transplantation are covered elsewhere [8,9].

We first discuss improper immune antigen presentation by hPSCs as a result of long-term maintenance *in vitro*. At least four possible sources and mechanisms are involved in incorporation and production of aberrant immune antigens, including: i) animal-derived [10,11] and ii) non-physiologic media compounds [12] (i.e. high concentrations of hormones or antibiotics), iii) genetic abnormalities that lead to formation of atypical antigens [13,14], and iv) incomplete reprogramming of somatic cells [15]. The second concern that we review here is rejection of hPSC progeny that are immunologically immature. Interactions of the immune system with somatic cells during fetal development and following birth gradually shape the presentation of activating and inhibiting signals required for immune surveillance [16]. Such fine-tuning of immune ligand presentation is unlikely to take place during the rapid course of hPSCs differentiation *in vitro*. These non-physiological conditions may produce somatic derivatives expressing residual embryonic antigens and/or exhibit an imbalanced repertoire of surface ligands necessary for immune cell inhibition.

Importantly, evidence highlight here indicate that both phenomena may lead to immune rejection and that isogenic and allogeneic hPSC derivatives are equally at risk. Still, it is likely that immune responses against aberrant antigens and immunologically immature cells will be more indolent than rejection processes targeting mismatched histocompatibility antigens expressed by allogeneic hPSC derivatives. We therefore also discuss milder immunosuppression regimes that could potentially attenuate these anticipated weaker rejection processes. Since the number of studies directly examining our hypothesized rejection is relatively small, we go on to propose experiments that should provide greater understanding of the immunological properties of hPSC-derived isogenic grafts.

Maintenance of hPSC may result in aberrant antigen presentation

The reliance on animal products for propagation of hPSCs raises the concern of xenoantigen incorporation (reviewed in [17]). For example, Martin et al. showed that hESCs present the non-human sialic acid N-glycolylneuraminic (Neu5Gc) when co-cultured with mouse embryonic fibroblasts (MEFs) and animal serum [10]. Uptake of Neu5Gc by human cells results in the substitution of N-acetylneuraminic acid (Neu5Ac), a glycan frequently added to human surface proteins [18]. Humans are unable to synthesize Neu5Gc [19]. However, most individuals develop glycan targeting antibodies as this glycan is introduced through animal and bacterial products [18]. This raises a concern for rejection of hPSC derivatives by anti-Neu5Gc antibodies as exposure of hESCs to human sera with such antibodies has resulted in complement deposition and cell death [10]. Notably, a follow-up study by Cerdan

et al. challenged these data by showing that complement fixation via Neu5Gc targeting antibodies did not result in significant hESC death, although the tested cell lines expressed Neu5Gc [20]. Although these studies have been critical in establishing proof-of-concept for the risks associated with xenoantigen presentation by hPSCs, they were limited to direct complement-mediated lysis assays *in vitro*, and did not include exposure of hESCs to antibodies and immune cells *in vivo*. Therefore, conclusion of the immunological consequence of Neu5Gc incorporation awaits further analysis. Since it is likely that other xenoantigens are incorporated into hPSCs *in vitro*, we propose that ensuring transplantation safety requires broader xenoantigen characterization efforts.

To circumvent the possibility of xenoantigen incorporation, several laboratories have developed methods to derive and culture hPSCs without animal products [21,22,23,24]. Culture protocols utilizing human serum, defined medias, human feeder layers, and/or synthetic polymeric surfaces have been shown to be effective [22,24]. However, none of these conditions have yet to be fully optimized or broadly adopted. Standard hPSC culture conditions, which include MEFs and animal sera, remain widely used and are considered better suited for long-term support of hPSC self-renewal [25].

Importantly, high concentration of media constituents may also alter the antigen signature of hPSCs and their derivatives. For example, CD30 is expressed by hESCs that are cultured in the presence of animal-free knock-out serum due to high ascorbate levels [12]. The concept that changes in culture conditions may alter antigen presentation by hPSCs has also been further supported by Newman and Cooper who reported that both hESCs and hiPSCs exhibit lab-specific gene expression signatures [26]. These results highlight the need to extensively characterize and optimize the effects of media formulations on antigen presentation by hPSCs as they may lead to changes in immunogenicity.

Genomic rearrangements of hPSCs have been described by a number of studies and may also lead to antigenic changes. Draper et al. have characterized a gain of chromosome 12 and a 17q segment in H1, H7, and H12 hESC lines following long-term culture [13]. Other groups have described additional chromosomal lesions in other cell lines [14,27]. Cells bearing genetic abnormalities are likely selected in culture when the modifications provide survival and growth advantages [13,14,27]. Werbowetski-Oglivie et al. have demonstrated this correlation by producing two genetically abnormal hESC sublines through prolonged culturing [28]. These lines were found to exhibit an order of magnitude increase in the frequency of tumor-initiating cells and significantly higher proliferation capacity [28]. Genomic rearrangements in hPSCs have also been shown to correlate with aberrant surface antigen presentation. For example, Herszfeld et al. discovered that karyotypically abnormal hESCs exhibit elevated CD30 levels [29]. The amplification of the CD30 gene in hESCs indicates that this marker may provide a survival/growth advantage, as previously shown in Hodgkin's lymphoma cells [30]. Although a direct link between genetic abnormalities and increased immunogenicity has yet to be demonstrated, given the frequency and number of genetic lesions reported, we hypothesize that hPSC subclones may exhibit disparate immunological properties.

Reprogramming of somatic cells into hiPSCs may also results in aberrant antigen presentation due to partial transcriptional memory retained from the epigenetic signature of the original tissue [15]. These marks were shown to correlate with disparities in gene and miRNA transcription compared to hESCs even after prolonged culturing period [31,32,33], although a different study reported contrary results [34]. To test whether these transcriptional disparities increase the immunogenicity of iPSC Zhao et al. compared the survival of transplanted mouse isogenic iPSCs with isogenic ESCs. They found that the isogenic transplants derived from mouse iPSCs stimulate immune responses, while those derived from isogenic mouse ESCs did not [32]. Importantly, they confirmed that retroviral integration were not the cause of the heightened immunogenicity as iPSCs produced through episomal (non-integrative) plasmid exhibited the same level of immunogenicity. Epigenetic abnormalities have also been reported in hESCs that were derived from parthenogenetic tumors and in SCNT-derived ESCs and fetuses. For example, Stelzer et al. reported that differentiated parthenogenetic hiPSCs exhibit altered trophoctoderm, liver, and muscle gene transcription profiles [33]. Animals produced through SCNT have also been found to exhibit faulty expression of developmental genes due to incomplete reprogramming (reviewed in [35]). Taken together, these data indicate that epigenetic alternations can lead to elevated immunogenicity of hPSCs.

We therefore propose to systematically compare hPSC derivatives with the equivalent somatic cells *in vivo*, to detect differences in surface antigen presentations. Functional assays should follow to evaluate the clinical consequences of antigenic discrepancies. Such characterization efforts are already underway including those coordinated through the International Stem Cell Initiative Consortium, which have determined preliminary census properties of hPSCs by characterizing dozens of hESC lines [36,37], and culture [38] and storage conditions [39].

Transplantation of non-matched hPSC lines elicits alloreactive immune responses

To discuss potential rejection pathways of isogenic hPSC lines, we first introduce the known interactions of hPSC allografts with the immune system. Classical MHC-I molecules are ubiquitously expressed heterodimers consisting of a single human leukocyte antigen (HLA)-A, B, or C chain and a β 2-microglobulin molecule that together present a short peptide sequence sampled from intracellular proteins. The interactions of T-cell receptors (TCRs) with MHC-I molecules are one of the fundamental mechanisms distinguishing peptides as self or non-self. In transplantation settings, a small fraction of the hosts' TCRs interact with donor's MHC-I molecules, leading to maturation of cytotoxic T-cells and consequently the development of immune responses [1].

The expression of MHC-I molecules is developmentally regulated, increasing from low levels on pluripotent cells to higher levels throughout fetal development [16,40,41,42,43]. Multiple studies have shown that MHC-I molecules are similarly expressed at low levels on human [2,3,44,45,46] and mouse [2,47,48] PSCs and increase on differentiated derivatives, albeit ultimate expression levels are lower than somatic cells. Although MHC-I presentation by ESC-derivatives is lower than somatic cells, these levels have been shown to promote T-

cell recognition [44,49,50]. In concurrence with these *in vitro* studies, multiple reports have indicated that T-cells also mediate acute rejection of PSCs and their derivatives in mice [46,47,50,51,52]. Other studies, however, presented evidences that some PSC derivatives are not targeted by T-cells [45,53]. Ultimately, these studies indicate that long-term exposure of almost any PSC line or their derivatives to T-cells would ultimately elicit sufficient sensitization for an immune attack. In contrast, in the case of isografts derived from hPSCs (e.g. derived from patient specific hiPSCs), the full MHC match would prevent the development of a T-cell mediated acute immune response. This principle was previously demonstrated by transplantation of SCNT-derived PSC progeny into isogenic animals [54]. In this case, despite mitochondrial antigen mismatches (mitochondria are primarily derived from the ova cytoplasm [55]), T cell response was not observed [56].

Although the primary focus of allojection studies has been the direct cytotoxic response mediated via CD8 T-cells, recent studies have highlighted the involvement of CD4 helper T-cell subsets in graft rejection and survival. It has been shown that hESC transplants survive better in CD4 null compared with CD8 null mice, yet ultimately both strains rejected the human xenografts [52]. Lui et al. also showed that ablation of CD4 T-cells via systemic anti-CD25 antibody treatment permits survival of mouse ESC grafts in immunocompetent CB/K mice [57] and that inhibition of CD4 T-cells severely dampened the CD8 T-cell activity [58].

These data highlight that host T-cells would likely become central mediators for rejection of differentiated hPSCs, either directly through activation of cytotoxic CD8 T-cells or through indirect exposure of transplanted alloantigens to CD4 T-cells. The fact that MHC and mHC alleles would match in the hPSC isograft setting would cancel many of the immunologic barriers imposed by allogenic transplantation. However, as discussed above, the expression of aberrant antigens even by isogenic cells is likely to promote isograft rejection by the host's T-cells. In addition, as outlined below, retention of embryonic antigen expression by isografts derived from hPSCs may also promote T-cell mediated rejection.

Retention of embryonic antigens may lead to T-cell mediated rejection of isogenic hPSC derivatives

T-cell variability is driven by random rearrangements of the V(D)J region of the *TCR* gene. A diverse array of T-cells is generated in this fashion, of which some specifically recognize self-antigens. These auto-reactive clones are normally depleted through apoptosis in the thymus [59]. To allow tolerance towards somatic antigens expressed outside the thymus, medullary epithelial and dendritic thymic cells express the *AIRE* gene which induces transcription of somatic genes [60]. During human development, the fetal thymus becomes capable of rudimentary support of T-cell selection by approximately 7 weeks gestation [61] and produces the first mature T-cells during week 8 [62]. Since thymic development occurs well over a month after the last pluripotent and early germ layer progenitors have differentiated, T-cells reactive to early embryonic antigens may exist in adults [61]. Therefore, embryonic proteins and glycans expressed by hPSC progeny may elicit immune responses, unless they are ectopically expressed in the thymus by *AIRE*. Partial list includes TRA-1-81, TRA-1-60, SSEA-3, SSEA-4, and SSEA-5 glycans [63,64,65,66]. These glycans

exist as post-translational modifications on hPSC proteins and lipids, producing isoforms specific to early embryonic development [63,67,68]. Since immune responses are known to take place against primitive oncofetal antigens expressed by tumors [69,70,71,72], it is probable to assume that the thymus does not support negative selection of all T-cells that are specific for embryonic antigens. For example, developmental pluripotency associated 2 protein (DPPA2), which is normally restricted to the placenta and testis is expressed by a subset of ovarian cancers, and is known to elicit immune responses [72]. This notion is further supported by studies utilizing ESCs as part of a vaccination protocol to promote immune responses against tumors, including those of the colon [69] and lung [73].

The known T-cell response against embryonic antigens raise a concern that even isogenic hPSC derivatives would be targeted if embryonic antigens were not entirely removed. Since it is unlikely that the non-physiological differentiation environments of hPSCs *in vitro* would precisely recapitulate normal development, some of the embryonic antigens may persist on differentiated progeny. In such a scenario, therapeutic products would induce T-cells responses. The likelihood that this pathway will affect transplantation outcomes is currently difficult to predict because the extent to which embryonic antigens are expressed by hPSC progeny is not known. Still, the detection of immune responses against embryonic antigens [72,73] and the discovery of aberrant DNA methylation patterns in hiPSCs [15] indicate the high probability of immune response towards residual embryonic antigens. We therefore recommend conducting thorough characterization of retained embryonic antigens on differentiated cells intended for transplantation followed by functional evaluation of their immunogenicity.

Natural Killer cell-mediated lysis of hPSC derivatives

As implicated by their name, NK cells are poised to lyse cells, particularly, those exhibiting aberrantly low MHC-I levels, a phenomenon often resulting from viral infections [74,75]. NK cells monitor MHC-I molecules through the NKG2 and KIR receptors. NK cells then integrate inputs from additional activating and inhibitory stimuli to determine whether the cytotoxic threshold has been reached [75,76]. This mode of immune surveillance has led to the development of the missing-self hypothesis [77].

Similarly to fetal cells, hPSCs and their early derivatives maintain low levels of HLA molecules relative to somatic cells [2,3,42]. The reduced MHC-I presentation, which is arguably an important mechanism that prevents recognition of fetal cells by T and B cells [45], may promote rejection by NK cells [54,78,79,80,81]. During pregnancy, low MHC-I expression by fetal cells does not lead to rejection as a specialized subset of CD56⁺ NK cells interact with the trophoblasts in the endometrium, promoting quiescence of maternal NK cells [82]. In transplantation settings, however, if the engrafted hPSC derivatives will present low MHC-I levels, there exists the probability that they will be probed and lysed by circulating NK cells. Derivatives of parthenogenetic hESCs, in particular, may be at high risk of NK cell-rejection, as lines that are derived from unfertilized metaphase II (MII) oocytes harbor only one set of chromosome homologs [5]. Since MII lines are capable of expressing only one allele of each HLA-gene, such cells are more likely to promote NK cell response due to their inherently reduced HLA expression [83].

Similarly to T-cells, certain NK cells undergo selection or “education”. Although this process, termed licensing, is not as well understood as T-cell education, multiple studies have indicated that NK cells initially express an array of inhibiting and activating receptors and their interactions with self-MHC molecules on somatic cells determine whether they mature to become functionally competent [74,75] (for a comprehensive review refer to Orr et al. [76]). As with all lymphocytes, NK cell “education” can begin only after the development of the immune system, which occurs at a relatively advanced gestational stage. It is likely that the early developmental age of hPSC derivatives may not allow for their participation in NK cell education.

Our initial analysis has demonstrated that hESC derivatives express low levels of inhibitory classical HLA-I and non-classical HLA-Ib molecules (HLA-E), and that the levels of these molecules increase during differentiation. In addition, we observed only a basal response of activated NK cells towards undifferentiated and differentiated hESCs [2]. NK cells have been reported to be inhibited by hESC derived mesenchymal cells, at least partially by expressing HLA-G, a protein which plays a pivotal role in promoting maternal NK cell tolerance towards the placenta [53]. Since HLA-G is primarily expressed by trophoblasts [84], it is likely that these cells represent placental mesenchyme. Conversely, other reports have indicated that ESC derivatives are targeted by NK cells [54,78,79]. For example, Preynat-Seauve et al. reported that neural cells derived from hESCs are lysed by both T and NK cells [79]. Such discrepancies in NK cell responses towards hPSC derivatives could emanate from differences in NK cell subtypes utilized for *in vitro* lysis assays and their degree of activation. Differences in NK cell properties between mouse and human could also account for some of these disparities, especially since some of the studies have examined the xenogeneic response of mouse NK cell towards hPSC derivatives. Given the complexity of NK cell-target interactions and the existence of stimulatory or inhibitory signals it is not surprising that uncertainty exist as to whether low MHC expression on hPSC-derivatives leads to an increase [46,50,81] or decrease [45,85,86] in NK cell activity. In addition, each differentiated progeny type may profoundly differ in its capacity to promote NK response.

Take together, the uncertainty regarding the interaction of NK cells and hPSC derivatives leads us to stress that clinical advancements require extensive characterization of NK cell responses towards individual cellular products. Due to the inherent limitations of *in vitro* assays, development of more clinically relevant *in vivo* assays should be pursued. In addition, since patient hiPSC derivatives are expected to become a major source for isogenic transplants, their susceptibility to NK cell lysis should be rigorously analyzed. It is also worth noting that it is currently unknown whether therapeutic populations derived from hPSCs are able to reach and maintain expression of MHC-I molecules at adult-like levels, including the various different HLA-I subtypes. We therefore conclude that clinical applications of hPSC derivatives require fundamental analyses of their interactions with NK cells, especially in the context of long-term engraftment.

Additional mechanisms modulating immune responses towards differentiated hPSCs

Recent studies have indicated that PSCs are capable of modulating immune responses [16,87] in similar ways to embryos *in vivo* [82]. Yachimovich-Cohen et al. have found that both mouse and human ESCs secrete Arginase I, which inhibits lymphocyte proliferation and TCR expression [88]. This immunosuppressive activity has been shown to be important for pregnancy [89], particularly for protecting fetal-derived trophoblasts from maternal T-cells [90,91]. Other studies have also described the ability of ESCs to inhibit immune cells via secretion of TGF- β [92] and by activating the hemoxygenase I enzyme, which produces anti-inflammatory molecules including biliverdin and carbon monoxide [93].

Additional studies proposed that ESC directly suppress immune cells through Fas ligand (FasL, CD95L) presentation [86,94,95,96], a molecular which acts by inducing T-cells apoptosis through interaction with the Fas receptor (CD95). The functions of FasL have been extensively studied in the context of immune-privileged sites such as the placenta, where it plays an important role in inhibiting maternal immune reactions [87,97]. Although the expression of FasL by PSCs is inline with their early embryonic origin, contradictory evidence has indicated that functional FasL is not present in mouse [98] or human [44] ESCs.

It is important to note that the pathways by which undifferentiated hPSCs modulate immune responses are probably not relevant for clinical translations as transplantation of undifferentiated cells is undesirable since they produce teratomas [66,99]. Hence, FasL or Arginase I could provide immune protection only if they are expressed by the differentiated therapeutic progenies, a possibility which has not been extensively tested thus far. Therefore, we propose to focus future studies on the potential immunomodulating effects of differentiated cells.

Mild immunosuppression is expected to improve engraftment of isogenic hPSC derivatives

As hPSCs and their derivatives may activate immune responses in allogeneic and even isogenic hosts, the development of immunosuppression treatments to mute these responses is also under investigation [52,79,100]. For example, Swinjinberg et al. have shown that dual treatment with the potent clinical drugs tacrolimus and sirolimus enable survival of mouse ESCs in immunocompetent mice while monotherapy with either drug was ineffective [52]. Notably, a caveat of such aggressive immunosuppression may be the inhibition of graft maturation and function, as highlighted by Preynat-Seauve et al, who showed that cyclosporine and dexamethasone inhibited neuroprogenitor cell differentiation from hESCs [79]. Since T and NK cell response towards tissues generated from isogenic PSC sources is expected to be mild, we propose that gentler conditioning regimes, such as those provided by antibody perturbations should be developed to prevent isogenic tissue rejection.

Several studies have thus far reported encouraging results for the utility of monoclonal antibodies (mAbs) targeting immune cell receptors to inhibit T [51,58,100] and NK [78,79]

cell cytotoxicity. Robertson et al. induced tolerance towards undifferentiated mouse ESCs utilizing non-depleting mAbs against the T-cell co-receptors CD4 and CD8 [51]. Similarly, Lui et al. showed that inhibition of CD4 T-cells alone through an anti-CD25 mAb is sufficient to induce tolerance towards undifferentiated mouse ESCs [57]. Grinnemo et al. and Pearl et al. went on to show that costimulation blockade of T and dendritic cells utilizing anti-CD40L, CTLA4, and LFA-1 mAbs enabled teratoma formation from hESCs in immunocompetent mice [58,100]. In addition, mAbs against the NKG2D receptor were shown to inhibit NK-cell mediated PSC lysis *in vitro* [78,79,81].

An important caveat of the majority of the mAb conditioning studies performed thus far is that they utilized teratoma growth as their primary assay. Teratomas are poor surrogates to assess the engraftment potential of functional and clinically applicable progenitors. Notably, numerous cellular properties, including the molecules involved in immune recognition, are altered during differentiation. It is therefore difficult to predict the survival of differentiated progeny while assaying engraftment of undifferentiated cells. Hence, we highlight that it is essential that continual immune modulation research focus more on enhancing the engraftment of functional hPSC derivatives.

As an alternative for immunosuppression, immunoprivileged sites, including the anterior chamber of the eye, the brain, and the testes [101] could serve as “safe havens” for protecting hPSC-derivatives from rejection. Immune-privileged sites are characterized by a number of mechanisms that disable or suppress immune effector cells [101]. The anterior chamber of the eye is lined by secretory cells that produce various cytokines that suppress helper T-cell activity and promote FAS expression, which in turn, promotes T-cell apoptosis [101,102]. The brain presents a different set of mechanisms to dampen immune cells, including the endothelial blood-brain barrier, which inhibits entry of immune cells including monocytes and T-cells [103]. Since, many routes of hPSC rejection involve T-cells, which are largely inhibited in immunoprivileged sites, these sites are expected to enhance survival. Still, the full extent to which immunoprivileged sites offer protection for hPSC-progeny remains to be functionally tested.

Conclusions

Since the derivation of hESCs by Thompson et al. [104], the field of PSC biology and their potential clinical utility has been in a constant state of advancement and change. Although the antigenicity and immunogenicity of hPSCs were initially extensively studied, this research slowed following the demonstration that genetically-matched hiPSC lines could be prepared from somatic cells. However, this view was based on the assumption that MHC matching will by itself suffice to prevent immune response towards hiPSC derivatives.

In this review, we highlight the possibility that isogenic hPSC-derived transplants should not be considered non-immunogenic although they are genetically identical to an original tissue donor. Unlike conventional matched organ transplants, differentiated hPSCs are propagated and matured *in vitro* in an artificial environment that promotes non-physiological proliferation and accelerated specification towards committed cells. The propagation of hPSCs in the presence of animal products may lead to presentation of xenogenic epitopes,

and the artificial nutrient environment *in vitro* may promote aberrations in cell surface antigen presentation. In addition, extensive propagation *in vitro* may select for genetically abnormal hPSC clones expressing deviant surface molecules. Finally there is an incompletely understood yet real chance that incompletely reprogrammed iPSCs may result in increased immunogenicity. These factors alone may significantly contribute to elevated immunogenicity of differentiated hPSCs. Compared with normal development, differentiation *in vitro* is substantially faster and likely does not allow for complete removal of embryonic antigens and induction of adult epitopes including optimization of the MHC-I levels. Such aberrant immunological signatures could activate cytotoxic T and NK cells which undergo a complex and developmentally regulated education process from which embryonic antigens are likely excluded. Therefore, retention of embryonic antigens on PSC-derivatives may lead to T and/or NK cell response. In regards to expression of immunosuppressive molecules by hPSCs it is currently unclear whether these factors persist to later stages of differentiation, and therefore, their relevance to transplantation tolerance is uncertain. In summary, these data indicate that differentiated isogenic hPSCs could potentially promote rejection processes unless quality controls and immunosuppression measures are taken.

Nevertheless, we predict that the immune reactions against isogenic hPSC derived transplants will be subtler compared to allogeneic responses directed at MHC and mHC mismatches. Therefore, we anticipate that immune reactions against isogenic hPSC-derived transplants could be dampened successfully via targeted therapy. Promising therapies include specific mAbs against receptors found on subsets of immune effector cells, rather than global immunosuppression employed during the course of allograft transplantations. However, we stress that arriving upon immunologic solutions requires a deeper understanding of the mechanisms underlying rejection and tolerance in addition to a better understanding of hPSC biology. The subject of immunogenicity of isogenic hPSC derived graft therefore deserves more attention with special emphasis on rigorous *in vivo* analysis before we can confidently and successfully utilize these cells for regenerative purposes.

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