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Pluripotent Stem Cells: Immune to the Immune system?

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SUMMARY

Embryonic stem cells (ESC) which were initially characterized as immune privileged subsequently have proven susceptible to immune recognition. Induced pluripotent stem cells (iPSCs) have been proposed as an autologous source of pluripotent cells; however, more recent data now suggest that even autologous iPSCs may be targets of immune rejection. With numerous clinical trials on the horizon, it is imperative that the immunology of pluripotent stem cells (PSCs) be definitively understood.

Keywords

Embryonic stem cell; induced pluripotent stem cell; immune tolerance; stem cell transplantation; graft rejection

INTRODUCTION

In embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), regenerative medicine has found a promising solution to the problem of repairing tissues and organs with irreversible damage. Protocols have been developed to harness pluripotency to generate cardiomyocytes, pancreatic beta cells, neurons, hematopoietic progenitor cells, and other cell types for potential clinical applications (1). However, due to its nascent state, the field of regenerative medicine has been plagued by unrealistic timelines, ethical controversies, regulatory challenges, and practical hurdles to therapy. In terms of temporal progress, human (h)ESCs were first isolated and grown in cell culture 15 years ago; however, results have only been published from one trial involving transplantation of hESC-derived cells (2). In this trial, hESC-derived retinal pigment epithelium (RPE) cells were transplanted into patients with dry age-related macular degeneration and Stargardt's macular dystrophy to evaluate safety and tolerability (2). hESC-RPE cells were deemed safe in human subjects and data regarding efficacy will hopefully be reported in the near future. In the world's first hESC clinical trial, hESC-derived oligodendrocyte precursor cells were transplanted into

patients with neurologically complete traumatic spinal cord injury, but the trial was placed on hold in 2009 due to concerns from the FDA regarding microscopic cysts found in treated animals(3). The trial was cleared to proceed in 2010, but was discontinued months later for financial reasons. This is an example of the regulatory and practical hurdles challenging the clinical translation of ESC therapy. Another formidable but often overlooked obstacle is the host immune response towards transplanted ESCs. To bypass histocompatibility barriers, the transplantation of somatic cells reprogrammed into pluripotent stem cells has been proposed. However, recent data suggest that even iPSCs contain antigens distinct from histocompatibility antigens that will incite an immune response despite autologous transplantation (4). With multiple clinical trials involving ESCs and iPSCs on the horizon, it is imperative to understand the distinct immunogenic profiles of ESCs and iPSCs. This understanding will dictate the immunosuppressive requirements to optimize pluripotent stem cell engraftment and whether or to what extent autologous transplantation of iPSCs will be exempt from such requirements.

ESCs may possess only a fragile state of immune privilege

Classifying hESCs as being either capable of immune evasion or being targets of immune recognition is a flawed proposition, as the immune privilege here is likely a continuum, rather than a matter of absolutes. A mixed leukocyte reaction (MLR) is an *in vitro* method to assay whether a “responder” T cell reacts/proliferates in response to a “stimulator” cell. MLR data suggest that undifferentiated hESCs do not induce proliferation of allogeneic human peripheral blood lymphocytes (hPBLs) and that hESCs can actually decrease the proliferation of hPBLs in response to allogeneic dendritic cells (DCs) (5). *In vivo* results show that mouse (m)ESCs transplanted into syngeneic recipients will successfully engraft; by contrast, allogeneic transplantation results in immune rejection, suggesting an alloantigen-directed immune response (6). Repeat transplantations of allogeneic mESCs stimulate an accelerated secondary immune response, indicating that allogeneic ESCs can stimulate the formation of immunologic memory (7). These *in vivo* results suggest that the MLR experiments are not predictive of ESC immunogenicity. However, ESCs may possess diminished immunogenicity compared to some fully differentiated tissues. For instance, in some murine models, long-term acceptance of transplanted ESCs is achieved with significantly less immune conditioning than that required for skin allograft acceptance (8). For example, the administration of non-depleting anti-CD8 and/or CD4 monoclonal antibodies (mAb) can induce tolerance to mESCs-derived tissues across minor histocompatibility (mH) and fully allogeneic barriers, whereas anti-CD4 and CD8 mAbs cannot induce tolerance to conventional tissues, such as skin grafts, across even minor histocompatibility barriers (8). As skin grafts are known to incite an exceptionally strong immune response, future studies comparing the immune rejection of transplanted ESCs to other graft types (e.g., cardiac and renal) may provide quantifiable information regarding the immunogenicity of ESCs to help guide the choice of optimal immunosuppressive agent(s) in future clinical trials.

Consequences of dynamic expression of major histocompatibility complex antigens

Which processes underlie the inability of ESCs to stimulate MLR reactivity and possibly engraft across allogeneic barriers with decreased immune conditioning requirements? One possible mechanism is through limited expression of major histocompatibility complex (MHC) molecules. Undifferentiated hESCs express very low levels of MHC-I and undetectable levels of MHC-II antigens (9). Underlying limited MHC expression is the low expression of antigen processing machinery (APM) such as the transporter associated with antigen processing (TAP1/2) and tapasin (TPN), and consequently ESC-derived endogenous peptides cannot bind to MHC-I dimers and the MHC-I heavy and light chain complexes are prevented from leaving the endoplasmic reticulum (10). However, ESC-MHC-I expression

can be increased by either creating inflammatory conditions via IFN- γ exposure, or by permitting spontaneous differentiation towards embryoid bodies (EB), which represent a 3-dimensional amalgam of different cell types (9). Considering the rapid kinetics with which MHC expression responds to environmental cues, epigenetic modifications likely also play a role in this process. To support this, the treatment of undifferentiated hESCs with epigenetic inhibitors (5-azacytidine, trichostatin A) has been shown to increase the expression of MHC-I and APM genes(10). Interestingly, neither spontaneous differentiation nor exposure to IFN- γ *in vitro* has been documented to induce MHC-II expression (8, 9). MHC-II expression may be repressed by epigenetic mechanisms, as methylation arrays indicate that in hESCs, the MHC-II genes (HLA-DP, -DQ and DR) and their transcription factor MHC class II transactivator are hypermethylated (10). A consequence of limited MHC-II expression is that ESC-derived antigens are unlikely to be recognized through direct antigen presentation, which refers to host T cells recognizing peptides presented by donor antigen presenting cells (APC) bound to donor MHC molecules (Figure 1a). Direct antigen recognition requires ESC-derived grafts to contain either APCs or hematopoietic cells capable of differentiating into APCs. By contrast, indirect recognition requires donor-derived antigens be shed and recipient APCs to collect and present these antigens to recipient T cells via recipient MHC. In the long run, indirect recognition of ESCs is likely to predominate, specifically involving the presentation of ESC-derived antigens by recipient APCs to CD4⁺ T cells. The *in vivo* immunogenicity of ESCs thus may appear greater than their *in vitro* immunogenicity because after transplantation, ESC death and release of antigens could lead to increased indirect antigen presentation and subsequent immune activation. However, considering the diversity of cell types derived from ESCs, it is likely that multiple mechanisms of immune recognition are activated in transplant recipients. For example, non-hematopoietic cells, such as vascular endothelium, can activate direct allorecognition independent of alloantigen presentation by professional APCs (11). Therefore, hESC-derived non-hematopoietic cells such as endothelial cells may also activate the direct pathway of antigen recognition even in the absence of hematopoietic differentiation.

Potential processes by which ESCs may modulate the host immune response

Prior attempts to identify mechanisms of hESC-mediated immune modulation have been largely unrevealing. While rat ESC-like cells have been reported to engraft permanently in allogeneic hosts without any immunosuppressive conditioning, perhaps due to their expression of Fas ligand (FasL) (12), analysis of hESCs indicates that they lack FasL expression (13). hESCs also fail to express CTLA-4, another negative regulatory protein that has been investigated as a possible source of ESC immune privilege (13). Despite the limited evidence for a cell contact-mediated mechanism, there is support for soluble factors that might contribute to ESC-mediated immunosuppression. For example, conditioned media obtained from hESC culture significantly decreases T cell proliferation and IFN- γ production in response to known stimulators of T cell activation (14). This T cell inhibition is thought to occur via consumption of L-arginine by hESC-arginase I because supplementing the conditioned media with L-arginine abolished the inhibition of IFN- γ secretion in a dose-dependent manner (14) (Figure 1b). Interestingly, one study found that immune modulation did not require intact hESCs, as the cellular protein extract alone was sufficient to actively inhibit T cell proliferation in allogeneic MLRs and to diminish the ability of DCs to stimulate allogeneic T cells (15). Although this conclusion is drawn from *in vitro* scenarios, the significant cell death and lysis following transplantation of hESCs could potentially expose the host immune system to hESC-derived cellular proteins. The identity of the associated proteins is still unknown, but a potential mechanism by which hESC protein extracts may exert an immunomodulatory effect is through their prevention of DC functional maturation, as DCs exposed to hESC total protein extract have diminished

expression of cytokines (IL-12p40) and costimulatory molecules (CD80, HLA-DR, and CD83) (15) (Figure 1c). Finally, hESCs could also facilitate their own engraftment by establishing a microenvironment favoring the polarization of naïve T cells towards a regulatory phenotype (Figure 1d). More evidence from future studies will be needed to clarify this possibility.

Taken together, the *in vitro* data suggest potential mechanisms for ESC-reduced immunogenicity distinct from differential expression of MHC-I, and these mechanisms thus may still function after MHC-I expression increases upon differentiation. However, a challenge unique to ESCs is that their immunogenic properties are dynamic and likely variable depending on the identity of the ESC-derived cell type. Because the studies discussed above were conducted at various stages of differentiation, whether the ascribed immune modulatory properties are retained by each specific ESC-derived cell type remains to be investigated.

Prospects for immune conditioning to achieve successful ESC engraftment

Although ESCs are theoretically capable of modulating immune rejection, they are in the end inducers of an immune response that will prevent engraftment across histocompatibility antigens without immunosuppression. A number of conventional immunosuppressive agents previously tested to prolong ESC survival include calcineurin inhibition (tacrolimus), mTOR inhibition (sirolimus), and anti-proliferative agents (mycophenolate mofetil) (16). Unfortunately, no combinations of these agents have been observed to prolong transplanted hESC survival in animal models past 28 days (16). More extensive regimens (e.g., whole body irradiation or high dose cyclophosphamide) may successfully induce functional engraftment; however, the significant toxicity associated with these regimens would have to be balanced against the morbidity and mortality of the disease attempting to be cured. Interestingly, biologic therapies have generated more promising results for ESC engraftment than conventional immunosuppressant agents. Our group has demonstrated in mice that brief blockade of costimulatory and adhesion pathways via mAbs, which interferes with the CD28:B7, CD154:CD40, and LFA-1:ICAM1 pathways, can promote long term engraftment of ESC and iPSC grafts, as well as their differentiated derivatives such as hESC-derived endothelial cells and iPSC-derived neuronal progenitor cells (17). The characteristics of ESCs which favor prolonged graft acceptance in the setting of costimulation-adhesion blockade remain unclear, but may reflect the numerous immune modulatory processes previously discussed. However, these results have only been tested in murine hosts, and it is well documented that immune tolerance is more difficult to induce in large animal models such as non-human primates (18) and in patients, which have not yet been tested.

If future non-human primate studies produce promising results, costimulation and adhesion blockade may become an increasingly feasible option, with FDA-approved drugs (abatacept and belatacept) targeting the CD28:B7 costimulation pathway for use in patients with rheumatoid arthritis and following renal transplantation (19) (20). Antibodies targeting CD154 (CD40L) are currently unavailable for patient use due to thromboembolic complications observed in the first clinical trials of these agents (21). Fortunately, anti-CD40 antibodies have not been associated with thromboembolism, and are currently being developed for clinical transplantation. Efalizumab, an anti-LFA-1 antibody, was approved for treatment of psoriasis; however, long-term (years) treatment increased the risk of developing progressive multifocal leukoencephalopathy, resulting in its voluntary withdrawal from the market (19). Alternatively, adhesion blockade with anti-VLA4 (FDA-approved for multiple sclerosis) is still available and is being actively investigated for its potential to prevent rejection after allo-transplantation. The choice of immunosuppressive agent will also be dictated by the intended application. The risk of side effects needs to be

weighed against practical considerations for individual patients, such as projected lifespan of therapeutic cells and the severity of disease condition.

As ESCs can differentiate into hematopoietic stem cells (HSCs), the prospect of mixed chimerism-induced tolerance has been proposed (Figure 2). In both murine and swine models, stable mixed hematopoietic chimerism associated with bone marrow transplant, induced after non-toxic, non-myeloablative pre-transplant conditioning, has been shown to induce immune tolerance, both to the transplanted HSCs and to donor-derived cellular and solid organ transplants (22). Potential obstacles to hESC-mediated mixed chimerism include an unproven ability of hESCs to undergo definitive hematopoiesis, which is a technical problem likely to be solved in the future; and dependence on the direct administration of HSCs to the bone marrow because of hESC-derived HSCs' inherent inability to home in on their proper niche, likely reflecting the hESC tendency to support primitive instead of definitive hematopoiesis (23). Additionally, stable mixed-chimerism remains difficult to achieve in both non-human primates and patients with low-intensity pre-transplant conditioning. While increasing the intensity of the conditioning regimen (which would increase the likelihood of stable engraftment) would be appropriate for life-threatening clinical conditions, widespread use of ESC-derived chimerism-based strategy may require novel strategies to induce stable mixed chimerism after minimal pre-transplant conditioning.

Non-immunosuppressive strategies for pluripotent stem cell engraftment

Cellular (compared to solid organ) transplantation lends itself exceptionally well to pre-transplantation engineering by altering the expression of immunomodulatory factors to generate hypoimmunogenic ESCs (Figure 2). Possible approaches include enhancing cell-to-cell mediated immune suppression by overexpressing CTLA-4 or FasL. Prior experiments overexpressing FasL in solid organ transplantation have generated organ-specific conclusions, leading to prolonged survival of renal, pulmonary, and hepatic grafts but also accelerating rejection of cardiac and pancreatic grafts (24). Cell contact-independent mechanisms of immune suppression may be enhanced through overexpression of the immunosuppressive cytokines TGF β or IL-10. Additionally, the expression of serine protease inhibitor 6 (Serp16), an inhibitor of cytotoxic T lymphocyte (CTL) derived granzyme B, may be increased to generate ESCs resistant to CTL killing (25). Lastly, decreasing MHC-I expression in ESCs may be especially powerful due to ESCs' lack of MHC-II and costimulatory molecule expression (26). Major hurdles to the successful translation of this graft-engineering approach include meeting regulatory requirements and the need to ensure patient safety, as these cells will be genetically modified, which increases their risk of tumorigenicity.

Bypassing histocompatibility barriers through induced pluripotency

The seminal discovery that terminally differentiated somatic cells could be induced towards a state of pluripotency opened the possibility of creating patient-specific regenerative medicine as autologous iPSCs can theoretically bypass barriers of histocompatibility (Figure 2). However, a recent study by Zhao et al. indicates that despite histocompatibility matching through syngeneic transplantation, mouse (m)iPSCs can still incite a T cell dependent immune response of sufficient intensity to prevent teratoma formation (4). An important question that must be answered is what antigens are being targeted by the syngeneic immune system? The genes *Zg6*, *Hormad 1*, and *Cyp3a11* are candidates because the overexpression by mESCs of each of these genes is sufficient to prevent teratoma formation in syngeneic recipients (4). Future efforts should clarify whether these results are generalizable to all iPSCs or unique to the iPSC line tested, and whether similar antigens are present in human iPSCs. Additionally, questions as to whether these antigens will persist throughout all stages of iPSC differentiation and whether additional antigens will emerge during differentiation

remain unanswered. Finally, the approach by which reprogramming factors are delivered to somatic cells to create iPSCs likely has consequences on immunogenicity. Future studies will clarify the impact of viral vectors (e.g., retrovirus and lentivirus), which integrate the reprogramming factors into host genomes compared to non-integration methods (e.g., Sendai virus, minicircles, episomal plasmids, and mRNA).

If common antigens identified amongst iPSCs are capable of preventing successful therapy, a theoretical way this immunogenicity may be circumvented is by establishing antigen-specific tolerance through the directed differentiation of iPSCs to immature DCs. Considering the inherent tolerogenicity of immature DCs, autologous administration prior to cell replacement therapy in a non-inflammatory setting that does not promote maturation of DCs could establish donor-specific Treg cells. The advantage of this approach is that iPSCs can provide both the immature DCs expressing the antigens with the desired tolerance and the therapeutic graft cells, which, acting as a constant source of antigens, would reinforce tolerance. To support feasibility, pluripotent stem cells have been successfully differentiated towards DCs (27), and administering only immature donor-derived DCs has been shown to be sufficient for skin grafts to be accepted across a mH barrier in a murine model (28).

In addition to immunogenicity, there are feasibility barriers to clinical iPSC therapy. There will be a very stringent level of required regulatory approval before an iPSC line is used clinically, particularly due to the risk of teratoma formation (29). This will require vigorous validation and quality control of the cell lines. If the iPSC line is derived on an individual patient-specific basis, these practical restraints may prove prohibitive in terms of both time and cost for their practical delivery to critically ill patients. For example, it may take too long for the *in vitro* derivation of patient-specific iPSCs to deal with the acute nature of many of the disease processes (e.g., myocardial infarction or stroke). These problems may be best approached by creating an iPSC bank. A prior estimate based on hESCs suggests that ~150 cell lines would offer sufficient HLA diversity to provide a match for the majority of the population (30). This prediction was based on criteria used clinically for kidney and heart transplants with matching for blood group and for three of nine MHC loci with immune suppression used to overcome residual immunogenicity. Unfortunately, this number may be inaccurate because a failure to consider mH antigen disparity. However, if a greater number of cell lines can be used, individuals that possess the desired HLA type may be more easily identified and serve as iPSC donors.

Conclusion

Understanding how pluripotent stem cells interact with the immune system and why they may be tolerogenic can lead to the identification of novel immunosuppressive mechanisms and approaches. Although pluripotent stem cells may be capable of immune modulation, immune interventions will be required for successful therapy as pluripotent stem cells are rejected in its absence. Current immunosuppressive strategies include traditional immune suppression, creation of a pluripotent stem cell bank, and possibly costimulation and adhesion blockade in the near future. For regenerative medicine to fulfill its initial grand promise to revolutionize modern medicine, the immunogenic barrier must be successfully and comprehensively addressed.

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Figure 1. Processes which may diminish the host immune response towards ESCs
 Potential mechanisms include: **(A)** Limiting direct presentation of ESC derived antigens: Limited MHC-II expression by ESCs favors the indirect pathway of antigen recognition wherein ESC-derived antigens (orange) are either shed from the surface or collected from dying/apoptotic ESCs by recipient APCs (blue) and re-presented to recipient T cells. In the direct pathway (left side of panel), T cells engage with APCs of donor origin (red) and recognize the donor APC's MHC-II molecule and/or donor-derived antigen. **(B)** Expression of ESC-arginase I depletes L-arginine (orange circles) from the local environment, which renders responder T cells unresponsive, possibly through downregulation of the TCR-associated CD3-ζ chain (green). **(C)** hESC protein extracts (pink circles) may prevent the maturation of dendritic cells by decreasing the expression of CD80, HLA-DR, and CD83, as well as decreasing the production of IL-12p40 (yellow circle), which presumably will decrease the expression of IL-12 and IL-23. **(D)** Future studies may clarify whether ESCs can influence the polarization of naïve T cells towards a regulatory phenotype; the question marks indicate that the mechanism remains to be clarified.

Figure 2. Approaches to achieve successful engraftment of pluripotent stem cells
Potential approaches include: **(A)** Immunosuppression with calcineurin or mTOR inhibitors (tacrolimus and sirolimus). **(B)** Patient-specific regenerative therapy by harvesting and reprogramming somatic (skin) cells into iPSCs, then differentiating the iPSCs into the therapeutic cell type and performing an autologous transplantation with the possible inclusion of immunosuppression or immature DCs expressing the antigens to which tolerance is desired. **(C–D)** Central tolerance can be achieved through mixed hematopoietic chimerism. Pluripotent stem cells (PSCs) from an allogeneic donor are differentiated into HSCs, which will engraft in the bone marrow of the recipient. The HSCs produce precursors of APCs and T cells, which migrate to the host thymus, where donor APCs delete donor-reactive T cells through negative selection. Recipient and donor cells are blue and red, respectively. **(E, F)** Induction of peripheral immune tolerance by costimulatory and adhesion blockade (anti-CD40L, CTLA4Ig, anti-LFA-1). **(G)** Generation of hypoimmunogenic PSCs by decreasing the expression of MHC-I, increasing the expression of immunosuppressive cytokines IL-10 (yellow) and TGF-β (pink), or increasing the expression of Fas L (ligand), which can induce apoptosis of reactive T cells upon engagement with CD95.