Published in final edited form as:
Circ Res. 2013 February 1; 112(3): 549–561. doi:10.1161/CIRCRESAHA.111.249243.

Immunogenicity of Pluripotent Stem Cells and Their Derivatives

Patricia E. de Almeida[1,2], **Julia D. Ransohoff**[1], **Md Abu Nahid**[1,2], and **Joseph C. Wu**[1,2,3]

[1]Department of Medicine, Division of Cardiology, Stanford University School of Medicine, Stanford, CA 94305

[2]Institute of Immunity, Transplantation, and Infection, Stanford University School of Medicine, Stanford, CA 94305

[3]Stanford Cardiovascular Institute, Stanford University School of Medicine, Stanford, CA 94305

Abstract

The ability of pluripotent stem cells to self-renew and differentiate into all somatic cell types brings great prospects to regenerative medicine and human health. However, prior to clinical applications, much translational research is required to ensure that their therapeutic progenies are functional and non-tumorigenic, that they are stable and do not de-differentiate, and that they do not elicit immune responses that could threaten their survival *in vivo*. For this, an in-depth understanding of their biology, genetic and epigenetic makeup, and their antigenic repertoire is critical for predicting their immunogenicity and for developing strategies needed to assure successful long-term engraftment. More recently, the expectation that reprogrammed somatic cells would provide an autologous cell therapy for personalized medicine has been questioned. Induced pluripotent stem (iPS) cells display several genetic and epigenetic abnormalities that could promote tumorigenicity and immunogenicity *in vivo*. Understanding the persistence and effects of these abnormalities in iPS cell derivatives is critical to allow clinicians to predict graft fate following transplantation, and to take requisite measures to prevent immune rejection. With clinical trials of pluripotent stem cell therapy on the horizon, the importance of understanding immunological barriers and devising safe, effective strategies to bypass them is further underscored. This approach to overcome immunological barriers to stem cell therapy can take advantage of the validated knowledge acquired from decades of hematopoietic stem cell transplantation.

Keywords

immunogenicity; embryonic stem cells; induced pluripotent stem cells; transplantation; tolerance; patient-specific therapy; stem cell therapeutics

Introduction

Pluripotent stem cells can differentiate into cell types of the three primary embryonic germ layers, and therefore have extraordinary potential for regenerative medicine. James A.

Correspondence to Joseph C. Wu, MD, PhD, Stanford University School of Medicine, Lorry Lokey Stem Cell Research Building, 265 Campus Drive, Room G1120B, Stanford, CA 94305-5454, Ph: 650-736-2246, Fax: 650-736-0234, joewu@stanford.edu.

Disclosures: None.

Thomson[1] and Benjamin E. Reubinoff[2] pioneered the development and differentiation of human embryonic stem (ES) cell lines over a decade ago. Following their discovery, these cells have been under intense investigation as a source of functional cells to augment damaged tissue function and to treat degenerative diseases. In animal models, differentiated ES cells have demonstrated regenerative capabilities in treating spinal cord injury[3], diabetes[4], Parkinson's disease[5], liver failure[6], and myelin disease.[7] Pluripotent stem cells have also generated great excitement for cardiovascular regenerative medicine, as they can be differentiated to functional cardiomyocytes[8–10] and, as a part of biological pacemakers, can be grafted into injured myocardium.[11, 12] Despite these promising results, immunological constraints associated with the transplantation of pluripotent stem cell derivatives have not been adequately addressed and remain one of the greatest obstacles to cell replacement therapy.

It was originally thought that pluripotent stem cells would be capable of evading immune surveillance and rejection due to their low expression of major histocompatibility complex (MHC) class I, MHC class II, and costimulatory molecules,[13] and due to the expression of immunomodulatory molecules such as perforin-deactivating Serpin-6 (endogenous inhibitor of granzyme B)[14] and transforming growth factor-β (TGF-β), which both inhibit T cell proliferation.[15] However, this initial enthusiasm was dampened by evidence that pluripotent stem cells do elicit a donor-specific immune response in immunocompetent mice.[16, 17] Indeed, transplanted allogeneic and xenogeneic ES cells and their derivatives are not immune-privileged, and therefore may encounter the same immunological barriers as any other grafts.

In an effort to minimize immunological rejection of transplanted ES cell derivatives, Taylor and colleagues[18] devised a strategy to create a human ES cell bank from donated surplus embryos with sufficient HLA diversity to provide a HLA match for a reasonable percentage of the population in the United Kingdom. They predicted that a bank of 150 human ES cell lines would provide a full match at HLA-A, HLA-B, and HLA-DR for 20% of potential recipients; a beneficial match (defined as one HLA-A or one HLA-B mismatch only) or better for 37.9% of recipients; and an HLA-DR match or better for 84.9% of recipients (Figure 1). The results were calculated based on a criteria used clinically for kidney and heart transplantation, in which matching of blood group and of three out of nine MHC loci is considered sufficient and acceptable. Predictions such as these, however, have limited clinical value, and it remains unclear what level of disparity in MHC loci would warrant acceptance versus rejection of stem cell-derived grafts in humans. Moreover, recent research has indicated that matching MHC molecules alone is insufficient to guarantee tolerance to *in vitro* differentiated ES cells, as variance at the minor histocompatibility loci alone has been shown to induce rejection.[19] A better understanding of how varying levels of compatibility elicit varying levels of immune responses to pluripotent stem cells and their derivatives *in vivo* is required to validate or revise cell line banking predictions, and would further aid in the progression of stem cell therapy toward clinical translation. Here, we review current knowledge of the immunogenicity of pluripotent stem cells and their progenies, discuss mechanisms of graft rejection, and present possible strategies to prevent immunological rejection.

Hope for Immunocompatible Pluripotent Stem Cell Therapy

Hurdles associated with ES cell-based therapy have led to interest in a more readily accessible alternative with potential to be immunologically matched to the recipient. In 2006, Takahashi and Yamanaka narrowed down a list of transcription factors over-expressed in ES cells to four factors: octamer-binding transcription factor 4 (Oct4), SRY (sex determining region Y)-box 2 (Sox2), Krueppel-like factor 4 (Klf4), and c-myelocytomatosis

viral oncogene homolog (c-Myc). When expressed retrovirally, these transcription factors were capable of reprogramming fibroblasts to an embryonic-like state.[20, 21] Known as induced pluripotent stem (iPS) cells, they have revolutionized the field of stem cell research by demonstrating somatic cell plasticity and offering an appealing solution to the problem of immune rejection for stem cell-derived therapeutics. The derivation of ES-like cells from somatic tissues ignited the possibility of pursuing exciting avenues for patient-specific cell therapy, and as a platform for drug screening and disease modeling.[22–24] Moreover, these cells represent a possible solution to the ethical objections that have been raised against the use of human ES cells.

Initial studies looking at the biology of iPS cells compared to ES cells showed they have similar morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity.[20, 25] Nevertheless, a rapidly accumulating body of work suggests that considerable differences exist between these two pluripotent cell types, including important aspects such as their global gene expression,[26] single cell transcription signature,[27] epigenetic landscape,[28, 29] genomic imprinting,[30] and somatic mutations.[31] These deficiencies represent a significant hurdle to the clinical value of iPS cells as therapeutics. For example, genomic alterations acquired during the reprogramming of somatic cells and also during the differentiation of iPS cells to a desired cell type may increase not only the tumorigenicity of these cells,[32] but also generate potentially immunogenic “neoantigens” that could elicit immune responses even in a MHC-matched context.[33] In support of this premise, a recent study has demonstrated that iPS cells carry a high incidence of duplications on chromosome 12[34], resulting in significant enrichment of cell cycle-related genes. Such aneuploidy may affect the differentiation capacity of iPS cells, and also increase their tumorigenicity and possibly their immunogenicity.[33]

Very limited research has been done to determine whether clinically relevant therapeutic cells derived from autologous iPS cells are non-immunogenic or whether they possess some level of “autogenicity” (ability of a particular autologous substance to provoke an immune response in the body of a human or animal). If proven autogenic, the high costs and the length of time needed to produce adequate quantities of patient-specific iPS cell-derived therapeutics may not justify their use over allogeneic ES cells. As more systematic investigations into the immunobiology of iPS cells begin, the goal of bypassing immunologic barriers—even when transplanted autologously—remains only a possibility rather than a reality. The recent demonstration by Zhao and colleagues[35] that mouse iPS cells are rejected in syngeneic recipients suggests that stringent screening for incompatibilities between the donors and recipients of stem cell-derived cellular therapeutics may be required not only for transplantation of allogeneic cells but also autologous cells.

Immunogenic Molecules of Pluripotent Stem Cells

Major Histocompatibility Antigens

The major histocompatibility complex, termed Human Leukocyte Antigen in humans, consists of glycoproteins encoded by highly polymorphic genes on chromosome 6 that are co-dominantly expressed on the surface of almost all vertebrate cells. MHC encodes the main molecular targets of allograft rejection and MHC-associated incompatibilities between donors and recipients are responsible for almost all acute rejection. MHC is critical for the development of an adaptive immune response against pathogenic and foreign antigens as it contains a groove into which the antigen binds and is presented to T cells. In most species, each class of MHC is represented by more than one locus (polygeny). In humans, the class I loci are HLA-A, -B and –C, which are expressed on every nucleated somatic cell. The class II loci, including HLA-DR, -DQ and -DP, are expressed mostly on antigen presenting cells

(APCs) such as dendritic cells and macrophages. It is unclear why T cells should ever recognize foreign HLA molecules as they do in an allogeneic transplantation setting, but an estimated 1% to 10% of the T cell pool can react with intact allogeneic HLA during direct T cell allorecognition. As with any other tissue type, histocompatibility appears to be an important factor in the rejection of undifferentiated ES cells. ES cell rejection is accelerated when MHC molecules are upregulated during differentiation,[36] interferon (IFN)-γ stimulation,[37] or after teratoma formation.[9, 17, 38–40] These results suggest that ES cells and potentially their progeny can become more immunogenic if transplanted into an environment that promotes upregulation of MHC (e.g., inflammatory environment). HLA matching as a criterion for transplantation of stem cell-derived grafts may reduce the possibility of eliciting an immune response, but may not be sufficient to promote graft acceptance. Manipulation of HLA expression on stem cells has recently shown promise as a strategy for generating hypoimmunogenic grafts.[41] The same strategy has been previously used to facilitate transplantation of hematopoietic stem cells (HSCs).[42] The clinical applicability of this strategy, however, remains questionable, as it requires genetic manipulation to knock down a gene, and may lead to the introduction of genetic variations. Knocking down HLA is yet another double-edged sword, as it can increase the susceptibility of cells to NK cell-mediated killing.[43–45]

Minor Histocompatibility Antigens

Identical HLA phenotype is not sufficient to guarantee graft survival. The role of non-HLA histocompatibility antigens such as minor histocompatibility antigens (miHA) in the context of immunological rejection of pluripotent stem cells and their derivatives remains murky. miHAs are peptides derived from normal cellular proteins that show polymorphism among related and unrelated individuals, and when transplanted, can be sufficiently antigenic to induce $CD4^+$ and $CD8^+$ T cells alloresponses.[46, 47] The importance of miHA in human transplantation is proven by the observation that even MHC-identical sibling pairs can develop T cell-mediated graft failure,[48] severe graft-versus-host disease (GVHD),[49, 50] and graft-versus-leukemia (GVL) effect.[51] Robertson and colleagues[19] transplanted murine ES cell-derived embryoid bodies (EB) in MHC-matched mice that differed in their expression of miHAs. They demonstrated that full concordance at the MHC loci between donor and recipient mice was insufficient to promote acceptance of murine ES cell-derived EB as they were vigorously rejected at a rate similar to fully allogeneic EB. In transplantation of ES or iPS cell-derived therapeutics, natural miHA incompatibilities between donor and recipient may be accentuated by factors such as residual expression of embryonic antigens (e.g., Oct4)[52] present in the graft and ectopic expression of miHAs acquired during reprogramming stress or during *in vitro* culture adaptation (Figure 2).[35, 52] Additionally, pluripotent stem cell-derived therapeutics may incorporate immunogenic miHA as a results of exposure to animal product-containing media and/or to media containing non-physiologic constituents. Epitopes derived from these incorporated neoantigens may augment immunogenicity.[53] For example, a recent study demonstrated that prolonged culture of human ES cells in animal-free knockout serum and high ascorbate levels resulted in ectopic expression of CD30.[54] Incompatible miHA peptides can be presented directly on self-MHC class I to $CD8^+$ T cells that destroy the therapeutic graft or through APCs that process and present miHA peptides to T cells, eliciting an alloresponse.[55] However, the extent and severity by which miHA will influence immune response against pluripotent stem cell-derived therapeutics remains to be determined. If proven important, the optimization of culture conditions and reprogramming technique could prove crucial to the clinical translation of this technology. Also, the identification of potentially immunodominant miHA among pluripotent stem cell-derived progenies would facilitate screening for incompatibilities between donors and recipients and prediction of immunogenicity *in vivo*.

The ABO Blood Group

Primates express ABO blood group antigens that are displayed on the surface of red blood cells, epithelial cells, and vascular endothelial cells.[56] Bacteria colonizing the gastrointestinal tract display carbohydrate structures that are similar to the oligosaccharide structures which comprise the ABO. To confer host protection against gastrointestinal bacteria, there is a natural production of immunoglobulin (Ig)M and IgG antibodies. These naturally occurring antibodies can cause antibody-mediated rejection of ABO-incompatible organ transplants. Recent studies have shown that human ES cells, as well as differentiated hepatocytes and cardiomyocyte-like cells,[57] express ABO antigens.[58] Therefore, the transplantation of an ABO-expressing stem cell derived-graft in an ABO-mismatched recipient could prompt an antibody-mediated hyperacute rejection by activating the complement cascade, thereby eliciting a complement-dependent target cell injury.[59] Information about ABO expression in the various stem cell progenies is required prior to clinical translation, and the use of therapeutics derived from human ES and iPS cells from blastocysts of blood group O should be prioritized.[60]

Killer Immunoglobulin-like Receptors (KIR)

Natural Killer (NK) cells are key innate immune lymphocytes that play a critical role in recognizing self-MHC class I through a unique class of receptors called NK cell receptors (NKRs). While in an autologous setting, NK cells can kill cells that express low HLA class I molecules; in an allogeneic environment, they can kill cells that express HLA class I that are not recognized by their inhibitory KIRs.[61, 62] The importance of the KIR family of NKRs for transplantation and its role in the rejection of MHC-matched organs and cells are becoming increasingly evident, especially for HSC transplantation. The great variations in gene content, gene copy number, and allelic polymorphism within individual KIR genes result in significant diversity in KIR haplotypes among individuals. It remains to be seen whether KIR-matching between donors and recipients will need to be considered prior to the transplantation of stem cell derivatives.

Pluripotent Stem Cells Meet the Immune System: Pathways to Allorecognition

The human immune system evolved in a hostile environment inhabited by pathogens, and consequently has developed productive responses against both pathogens and foreign cells. The best-known cell types responsible for the direct killing of pathogenic cells are cytotoxic $CD8^+$ T cells, also known as cytotoxic T lymphocytes (CTLs), and NK cells. During an infection, both NK cells and $CD8^+$ T cells are activated via antigen-specific receptors and by pro-inflammatory cytokines produced by auxiliary $CD4^+$ T cells (also known as helper T cells) and APCs. The success of transplantation is largely limited by the activation of some of these same mechanisms. CTLs, helper T cells, and NK cells have been shown to hinder the survival of undifferentiated stem cells and embryonic stem cell-derived vascular progenitors *in vivo*,[17, 45, 63] suggesting that these same mechanisms will pose an obstacle for stem cell-based therapy. However, the exact pathway(s) leading to immune reactivity against these cells remains unknown.

The adaptive immune response is usually necessary and sufficient to reject allografts and this also seems to be the case in rejection of stem cells and their cellular derivatives, where T cells emerge as pivotal players.[17, 19, 36] Donor-derived MHC antigens expressed by an allograft almost always trigger T cell allorecognition. T cell receptor (TCR)-mediated recognition of the MHC antigens can occur essentially by two distinct pathways: (1) T cells recognize peptides complexed to donor MHC molecules displayed on the surface of the transplanted cells (direct pathway); or (2) T cells interact with processed donor-derived

peptides bound to MHC molecules on self APCs (indirect pathway) (Figure 3).[64, 65] Increasing evidence supports the role of the indirect pathway in acute and chronic rejection of various grafts,[66, 67] and most recently, in rejection of stem cells. Rejection of pluripotent stem cells has been proposed to involve 3 main developmental stages. The first stage is intra-graft delayed-type hypersensitivity, during which recipient MHC class II-restricted $CD4^+$ T cells recognize alloantigens presented by recipient APCs and release graft-damaging pro-inflammatory cytokines. The second stage is CTL response, during which self-restricted $CD4^+$ T cells help generate CTLs that can recognize intact allogeneic MHC class I molecules. The third stage is alloantibody response, during which alloantigen-primed $CD4^+$ T cells deliver activating signals to B cells. However, considering the many layers of redundancy in the immune system, the pathways that lead to rejection may be diverse.

It was originally proposed that the direct pathway of allorecognition could be easily mitigated by eliminating APCs from the graft prior to transplantation.[68] However, certain types of pluripotent stem cell derivatives such as endothelial cells may elicit an immune response by interacting with T cells in an antigen-specific manner.[69] Endothelial cells are capable of acting as non-professional APCs and, upon upregulation of MHC class II molecules, can present antigens in a MHC-restricted fashion. Consequently, these cells can mediate the direct pathway of allorecognition. Such a pathway of allorecognition has been demonstrated to compromise the survival of allografts.[70, 71] Therapeutic use of ES or iPS cell-derived endothelial cells will likely require addressing this pathway. This example underscores how the mechanisms leading to allorecognition may vary depending on the nature of the graft, further emphasizing the fact the need for graft-specific strategies to adequately manipulate the immune system and prevent rejection.

Several investigators claim that undifferentiated ES cells are resistant to NK cell attack *in vitro* and *in vivo*,[17, 40, 72] while others report that stem cells do express NK cell-activating ligands and are susceptible to NK cell attack.[38, 44] In a study that compared the susceptibility of undifferentiated versus differentiated mouse ES cell-derived cardiomyocytes to NK cell-mediated destruction, undifferentiated ES cells were deemed susceptible to NK cell destruction in a perforin-dependent manner.[44] This was attributed to the expression of the NK cell receptor, natural-killer group 2 member D (NKG2D), and the expression of the intercellular adhesion molecule 1 (ICAM-1). In support of these findings, NKG2D has also been detected in many other mouse pluripotent stem cells.[38, 43] Interestingly, mouse ES cell-derived cardiomyocytes were not susceptible to NK cell killing, even after stimulation with IFN-γ and retinoic acid, which is known to mediate expression of NKG2D ligands.[44] Whether the hypoxic and inflammatory microenvironment of the infarcted heart plays a role in damping the NK cell response remains to be investigated.

Innate *vs* Adaptive Immune Responses to Pluripotent Stem Cells Grafts: Insights From Immunodeficient Animal Models

Pluripotent mouse[72, 73] and human[74, 75] stem cells have been studied extensively in immunodeficient recipients. Although the immunobiology of stem cells was not the primary focus, these studies indirectly provided evidence for the absence of specific immune cells in promoting graft survival, and conversely for their role in clearing teratomas. The pivotal role of T cells in immune rejection of stem cells has been demonstrated using T cell-deficient mice[76, 77] and rats.[39, 78] These studies showed that injection of ES cells is readily followed by teratoma formation. Drukker and colleagues[77] comprehensively investigated immune responses to ES cells using NOD/SCID (T- and B cell-deficient), BALB-nude (T cell-deficient), C57BL/6-$Lyst^{bg}$ (NK cell-deficient), and CBA/CaHN Btk^{xid} (B cell-deficient) mice. Five weeks after transplantation of ES cells, teratoma formation was detected only in the T and B cell-deficient NOD/SCID mice. By contrast, transplantation of human ES cells

to $Lyst^{bg}$ (NK cell-deficient) and Btk^{xid} (B cell-deficient) mice led to vigorous rejection and failure to develop teratoma. These experiments demonstrated that xenorejection of human ES cells is T cell-mediated, and that NK or B cells play only a minor role in this process. The role of the complement pathway has also been investigated using an immunodeficient mouse model. Using complement 3 (C3)-deficient mice, Koch and colleagues showed that the homologous complement delays the formation and growth of mouse ES cell-derived teratomas and can completely prevent teratoma formation when a low number of mouse ES cells are implanted (1×10^5).[79] The authors attributed the susceptibility of ES cells to the complement pathway to low expression of sialic acid. Koch and colleagues also investigated the susceptibility of ES cells to B cells and antibodies using $J_H^{-/-}$ mice (B cell-deficient). Their results confirmed that B cells and antibodies are not critical for immune rejection of ES cells.

The innate immune response to human ES cells[80] and mouse ES cells[38] has also been studied using immunodeficient mice. Among the cells of the innate immune system, NK cells are the best characterized in the context of stem cell rejection. Mouse ES cell-derived teratomas grew significantly faster in SCID/beige mice (T, B, and NK cell-deficient) compared to SCID (T and B cell-deficient).[38] NK cell responses to ES cells have been shown to modulate teratoma growth in syngeneic, allogeneic, and xenogeneic immunological conditions.[43] Upon activation, NK cells have been shown to prevent teratoma formation in 50% of SCID recipients, and teratoma growth, where present, was decelerated.[43] Studies in knockout mice null for the recombination-activating gene-2 and cytokine receptors that contain the common γ-chain ($RAG2^{-/-}\gamma_c^{-/-}$) have implicated NK cells in rejection of hematopoietic stem cells.[81] Conversely, ES cell-derived cardiomyocytes[44] and vascular progenitor cells[45] were not deemed susceptible to NK cell killing. This observation was attributed to the upregulation in MHC-I expression that occurs with differentiation. Conflicting results in susceptibility of NK cell killing described between hematopoietic cells versus pluripotent stem cell-derived cardiomyocytes and endothelial cells suggest that the immune response pathways involved in rejection of the various stem cell derivatives cannot be generalized.

Rejection of Pluripotent Stem Cells in Different Histocompatibility Settings

Xenogeneic

ES cells transplanted xenogeneically at various anatomical sites (e.g., intra-muscularly, under the kidney capsule, subcutaneously, etc.) elicit both innate and adaptive immune responses. Upon intra-muscular injection, macrophages, neutrophils, B cells, and $CD4^+$ and $CD8^+$ T cells infiltrate human ES cell grafts.[17, 36] Evidence indicates that rejection of undifferentiated human ES cells is largely T cell-mediated, while NK cells and B cells play only a minor role.[17] However, in a rat model, NK cells have been implicated in the killing of undifferentiated mouse ES cells.[38] In this study, susceptibility of mouse ES cells to NK killing was attributed to the expression of ligands of the activating NK receptor NKG2D, which was downregulated in differentiated cells. More recently, evidence that implicates the involvement of T cells in the rejection of a clinically relevant cell type was brought up by Pearl et al.,[82] who demonstrated that a regimen consisting of a short-term course of costimulatory blockers that mitigates T cell alloresponses could extend survival of the human ES cell-derived endothelial cells. In another study, human ES cell-derived cardiomyocytes were injected into immunocompetent rats after myocardial infarction and were shown to survive, proliferate, and integrate with host cardiac tissues.[9] In this study, a combination of cyclosporine A and methylprednisone was used as immunosuppressive therapy and the viability of grafted cells were monitored for up to 8 weeks.

Allogeneic

The rejection of allogeneic undifferentiated mouse pluripotent stem cells has been analyzed by several studies, but limited information is available regarding alloresponses to progenies differentiated from these cells. Allogeneic undifferentiated mouse ES cells (2×10^5) injected into ischemic hearts of mice have demonstrated the ability to mobilize innate and adaptive immune responses that resulted in graft destruction 4 weeks post-transplantation.[83] The allograft rejection coincided with infiltration of IFN-γ-producing $CD3^+$ T cells and $CD11c^+$ dendritic cells. In a similar study, a higher number of mouse ES cells (1×10^6) was injected into ischemic hearts, and immune cell infiltration was monitored over time at 1, 2, 4, and 8 weeks post-transplantation. A progressive infiltration of $CD4^+$ and $CD8^+$ T cells as well as macrophages, dendritic cells, and granulocytes was observed, predominantly at weeks 4 and 8 after cell implantation.[40] Similar results were reported by a different study showing that at 5 weeks following implantation into the myocardium, mouse ES cells were rejected, coinciding with lymphocytic infiltrates.[72] The survival of allogeneic mouse ES cells (1×10^6) implanted in the gastrocnemius muscle was also limited to approximately 4 weeks as monitored by longitudinal bioluminescence imaging.[16] Furthermore, this study showed that rejection of allogeneic mouse ES cells was accelerated in mice that had been previously pre-sensitized with mouse ES cells, suggesting that an immunological memory specific to antigens expressed in mouse ES cells had developed. Strategies to abrogate memory T cells may have to be taken into account upon consecutive implantation of stem cell therapeutics. Similar to human endothelial cell progeny, rejection of allogeneic mouse iPS-derived neural progenitor cells has been abrogated by blockers of costimulatory molecules in T cells.[82]

In an attempt to investigate allogeneic immune responses to human ES cells, Drukker et al. utilized a “humanized” mouse model termed “Trimera.”[17] Essentially, Trimera mice are immunodeficient mice reconstituted with human peripheral blood mononuclear cells. Transplantation of undifferentiated human ES cells (1×10^6), or differentiated teratoma fragments, or a teratoma-derived primary cell line, resulted in tumor formation in all three settings. By contrast, Trimera mice were able to completely eliminate Burkitt's lymphoma cells. The authors attributed these findings to the hypo-immunogenic nature of human ES cells and their derivative tissues (Figure 4). This hypo-immunogenic phenotype might limit the activation of direct allospecific T cell responses. Humanized mouse models represent a very promising platform to study human immune responses *in vivo*, but results from studies using these models need to be cautiously interpreted, as there is evidence that aspects of immune responses in this model may be dysfunctional (e.g., defective cytotoxic T cells and NK cells).[84, 85] As a result of the lack of robust humanized mouse models, the allogeneic immune responses to human ES cells remain unknown. The opportunity to address some of these questions in immunologically healthy humanized animal models will contribute greatly to our understanding of immune responses that thus far have impaired the survival of transplanted pluripotent stem cells *in vivo*.

Syngeneic

Numerous studies have demonstrated that transplantation of mouse ES cells into syngeneic recipients leads to teratoma formation.[40, 72] However, the characterization of immune responses to syngeneic and clinically relevant stem cell derivatives is absent or minimal at best. T and B cells as well as macrophages have been shown to infiltrate teratomas in syngeneic mice. While they were incapable of preventing tumor growth, these lymphocyte infiltrates did decelerate tumor growth.[38] Interestingly, teratomas did not develop when a small number of ES cells were implanted.[38, 86, 87] Implantation of 5×10^5 undifferentiated or differentiated syngeneic ES cells has been shown to result in teratoma formation in only 33% and 17% of recipients, respectively. On the other hand, implantation of high numbers of undifferentiated or differentiated cells (2×10^6) produced teratoma formation in 100% of

recipients.[38, 87] These results suggest that when low numbers of cells are implanted, cells either die immediately following transplantation or are rejected. Implantation of a higher number of cells may overcome immune responses due to intense proliferation or stem cell-mediated immunosuppression (e.g., release of TGF-β and IL-10).

A recent study demonstrated that iPS cells are immunogenic and can be rejected even in MHC-matched recipients.[35] $CD4^+$ and $CD8^+$ T cells were implicated in the rejection of syngeneic iPS cell grafts. They attributed their findings to the expression of aberrant genes (i.e., Hormad1, Zg16, and Cyp3a11) in iPS cells. They further validated their results by demonstrating specific T cell responses to Hormad1 and Zg16 *in vitro* and *in vivo* (Figure 5). Hormad1 and Zg16 have been identified as human tumor-associated antigens,[88, 89] though no information was presented regarding the expression of these so-called immunogenic genes in human iPS cells. Although this study presented important information regarding the immunobiology of undifferentiated iPS cells, it did not evaluate whether clinically relevant iPS cell therapeutics possess similar immunogenicity. Another caveat was the use of only one syngeneic murine ES cell line as control. These omissions make it difficult to assess the significance of their findings and impossible to determine: (i) if "autogenicity" is a property exclusive to iPS cells or also exist in autologous somatic cells maintained *in vitro* for similar amount of time and in similar culture conditions; and (ii) whether there are any immunological benefits of using autologous iPS cells versus allogeneic ES cells. Results from this study are not entirely surprising considering that syngeneic iPS cells do express several oncofetal antigens, some of which are already known to be immunogenic (e.g., Oct4).[52] Moreover, these cells are highly susceptible to chromosomal abnormalities due to the reprogramming process itself[33] and culture adaptations.[90] Nevertheless, results from this study suggest that no degree of matching between donors and recipients may be able to prevent rejection of stem cell therapeutics in the absence of immune intervention, and that screening for aberrant antigen expression in pluripotent stem cell therapeutics may be critical for predicting the risk of immunological rejection and immune intervention required.

Prospects for Circumventing Immunogenicity

Evidence obtained from studies that used pluripotent stem cells and their progeny suggests that even full MHC concordance between donor and recipient may not guarantee survival stem cell therapeutics.[19, 43] Some level of immunosuppression or tolerizing regimen will likely be required but it remains unknown the extent of immunological conditioning that will warrant acceptance of stem cell-derived therapeutics. In many transplantation scenarios, life-long immunosuppression has been used to accommodate residual antigen disparities between donor and recipient, ensuring the survival of the therapeutic graft. However, this approach can produce devastating toxic side effects, promote opportunistic infections, and increase patients' susceptibility to malignancies. Because these adverse side effects can outweigh the potential curative benefits of stem cell-derived therapy, strategies to promote long-term tolerance with minimal immunosuppressive therapy are essential.

In a clinical setting, the benchmark for the establishment of tolerance means the complete and successful withdrawal of immunosuppressive drugs. Although tremendous progress has been made in the field of transplantation tolerance during the past half century (Table 1), durable donor-specific tolerance to prolong transplant survival in humans in the absence of immunosuppressants has not been consistently achieved. Mixed chimerism induced by HSC transplantation is known to promote donor-specific tolerance for over 40 years. In mixed chimeric animals, the continued presence of the organ donor's bone marrow-derived cells in the recipient's thymus and peripheral lymphoid tissue promotes and maintains immune tolerance by eliminating T cell clones that react to alloantigens of the graft.[91] However, the

use of HSCs to induce tolerance in the clinical setting has been unfeasible thus far, largely due to the unacceptable toxicity, morbidity, and mortality associated with the conditioning needed to achieve engraftment of allogeneic bone marrow, as well as complications such as GVHD. Significant efforts have been devoted to developing non-myeloablative methods for inducing mixed chimerism.[92, 93] Short-term fractionated total lymphoid irradiation (TLI) has been successfully used to achieve mixed chimerism without GVHD in MHC mismatched mice strains.[94] TLI radiotherapy is non-myeloablative and targets major lymphoid organs such as the spleen, thymus, and peripheral lymph nodes. This conditioning has been shown to promote engraftment of bone marrow cells and to promote immunological tolerance to skin and heart allografts that were transplanted concomitantly with donor bone marrow cells. When combined with anti-thymocyte serum (ATG), TLI has also achieved impressive results in post-transplant conditioning to promote organ allograft tolerance in rats.[95] In humans, Samuel Strober has tested this regimen at Stanford University for induction of tolerance to kidney transplants. Out of 25 kidney transplant recipients conditioned with TLI and ATG, 15 patients had long-term functional grafts with minimal requirement for chronic immunosuppression.[96]

Some clinically available immunosuppressive regimens have been tested for enhancing the survival of undifferentiated human ES *in vivo*. Interestingly, the calcineurin inhibitor Tacrolimus, the target of rapamycin (TOR) inhibitor Sirolimus, and the anti-proliferative Mycophenolate Mofetil have been shown to provide only marginal improvements in the survival of human ES grafts.[36] By contrast, the target-specific suppression of T cells has been much more effective in this context. The use of minimal host conditioning with non-depleting monoclonal antibodies against CD4 and CD8 was shown to induce long-term acceptance of ES cell-derived EBs implanted under the kidney capsule.[19, 97] Costimulatory blockade in combination with a low dose total body irradiation (TBI) (3Gy) has been shown to promote stable mixed chimerism with high levels of engraftment of fully MHC-mismatched HSCs and to enhance tolerance to donor skin grafts.[98] The costimulatory pathway is mediated by interactions of CD28 and CD40 ligand (CD40L, also called CD154) on T cells, and by B7 (CD80 and CD86) and CD40 on APCs. This pathway is of central importance for T cell-dependent immune responses, and its manipulation has improved survival of various grafts.[99, 100] Optimal results have been obtained when CD28 and CD40 were blocked simultaneously.[101, 102] A short-course blockade of the costimulatory receptors CD28, CD40 ligand (CD40L), and lymphocyte associated antigen-1 (LFA-1) on T cells alone has been recently shown to induce long-term acceptance of pluripotent stem cells as well as endothelial and neural progenitors (Figure 6).[107, 108, 103] Interestingly, this regimen was remarkably more efficient in preventing rejection of pluripotent stem cell grafts than the conventional immunosuppressants Tacrolimus and Sirolimus. Despite these promising results, it is important to keep in mind that regimens proved efficient in preventing rejection of undifferentiated ES or iPS cells will not necessarily work for transplanting differentiated stem cell therapeutics.

Though promising in small animal models, the aforementioned drug regimens have not been able to induce donor-specific tolerance to kidney or to skin allografts in non-human primates.[104, 105] Considering the insights gained from various transplantation models in primates, a more holistic approach using different immunomodulatory strategies simultaneously (e.g., costimulatory blockade, TLI, mixed chimerism, etc.) may be better for achieving tolerance with permanent acceptance while reducing or eliminating the need for long-term immunosuppressive therapy. At present, studies focused on immune responses to stem cell therapeutics in primates are still lacking and the immunological conditioning necessary to guarantee survival of stem cell-derived grafts may prove to be much more modest than expected. Addressing these questions will be critical for the advancement of stem cell technology to clinics.

Conclusions

As more diverse pluripotent stem cell derivatives become available, investigators have learned much about their *in vivo* behavior, functional properties, and immunogenicity. A comprehensive screening process for antigen repertoire variations among independent stem cell derived-therapeutics will be vital prior to translation of pluripotent stem cell therapy. Such information, combined with accurate interpretation of data, may facilitate the prediction of immunological responses and the development of risk scoring for optimal donor-recipient matching. Further understanding of the immunological pathways triggered by these putative therapeutic cells combined with efficient tracking of immune response is necessary to drive the development of safe strategies to bypass immune rejection. Progress made in these arenas should accelerate the development and clinical application of transiently administered, well-tolerated treatment regimens that exploit the specificity of the immune system while promoting long-term, rejection-free graft survival. More recently, the possibility of cell transdifferentiation, by directly converting or reprogramming one cell type to another while bypassing a pluripotent intermediate,[106–108] introduces both additional complexity and exciting prospects for regenerative medicine. The investigation into how such an approach compares to pluripotent stem cells in terms of therapeutic repertoire of cell types, and whether transdifferentiated cells are functional or non-immunogenic in the host, will be a new frontier in this fast-paced field.

Acknowledgments

We thank Amy Morris for preparing the illustrations and Dr. Everett Meyer for valuable advice and constructive critical input. Due to space constraints, we are unable to include all relevant and important studies regarding the immunological barriers to pluripotent stem cell therapy; we apologize to those investigators whose valuable work we have omitted in this review.

Sources of Funding: This work was in part supported by National Institutes of Health AG036142, AI085575, HL099117, EB009689 (J.C.W.) and the International Society for Heart & Lung Transplantation (P.E.A.).

Non-standard Abbreviations and Acronyms

APC	Antigen Presenting Cell
C3	Complement Component 3
CD40L	Cluster of Differentiation 40 Ligand
CTL	Cytotoxic T Lymphocyte
CTLA4	Cytotoxic T Lymphocyte Antigen-4
EB	Embryoid Body
ES	Embryonic Stem
GVHD	Graft-Versus-Host Disease
HSC	Hematopoietic Stem Cell
HLA	Human Leukocyte Antigen
IFN	Interferon
Ig	Immunoglobulin
IL-12	Interleukin-12
iPS	Induced Pluripotent Stem

KIR	Killer Immunoglobulin-Like Receptor
Klf4	Krueppel-like factor 4
LFA-1	Lymphocyte Associated Antigen-1
MHC	Major Histocompatibility Complex
miHA	Minor Histocompatibility Antigen
NK	Natural Killer
NKG2D	Natural-Killer Group 2 Member D
NOD	Non-Obese Diabetic
Oct4	Octamer-Binding Transcription Factor 4
SCID	Severe Combined Immunodeficiency
Sox2	SRY (sex determining region Y)-box 2
SSEA1	Stage Specific Embryonic Antigen 1
TCR	T Cell Receptor

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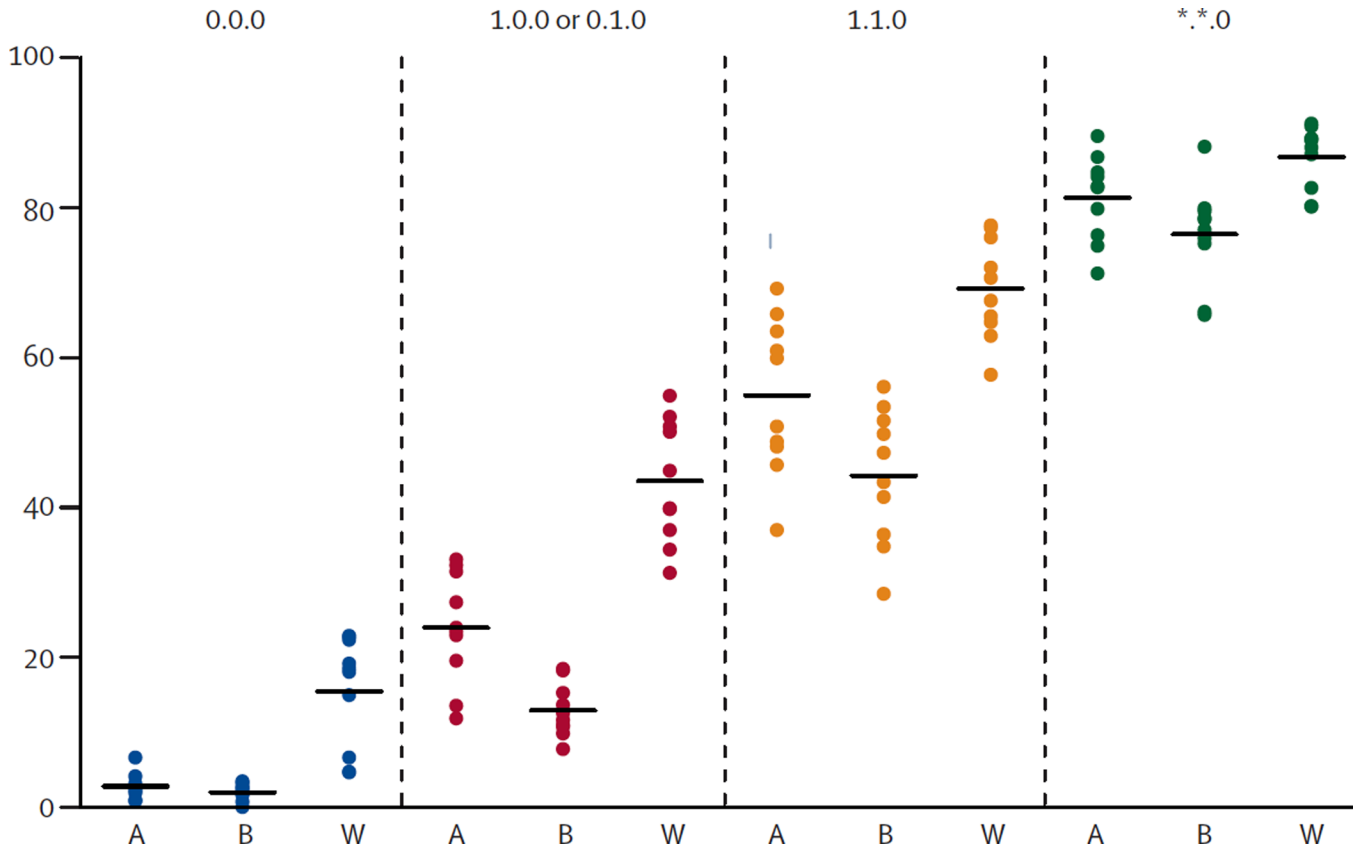

Figure 1.
Percentage of Asian (A, n=797), Black (B, n=441), and White (W, n=5087) patients HLA matched using ten cohorts of 150 cadaveric organ donors. HLA mismatch grades was based on criteria used for allocation of cadaveric kidney donors in the UK: 1) zero HLA-A, HLA-B, and HLA-DR mismatch (0.0.0); 2) zero HLA-DR mismatch with no more than a single HLA-A or HLA-B mismatch (1.0.0 or 0.1.0); 3) zero HLA-DR mismatch with no more than a single HLA-A and a single HLA-B mismatch (1.1.0); 4) zero HLA-DR mismatch (*.*. 0). Reprint with permission.[18]

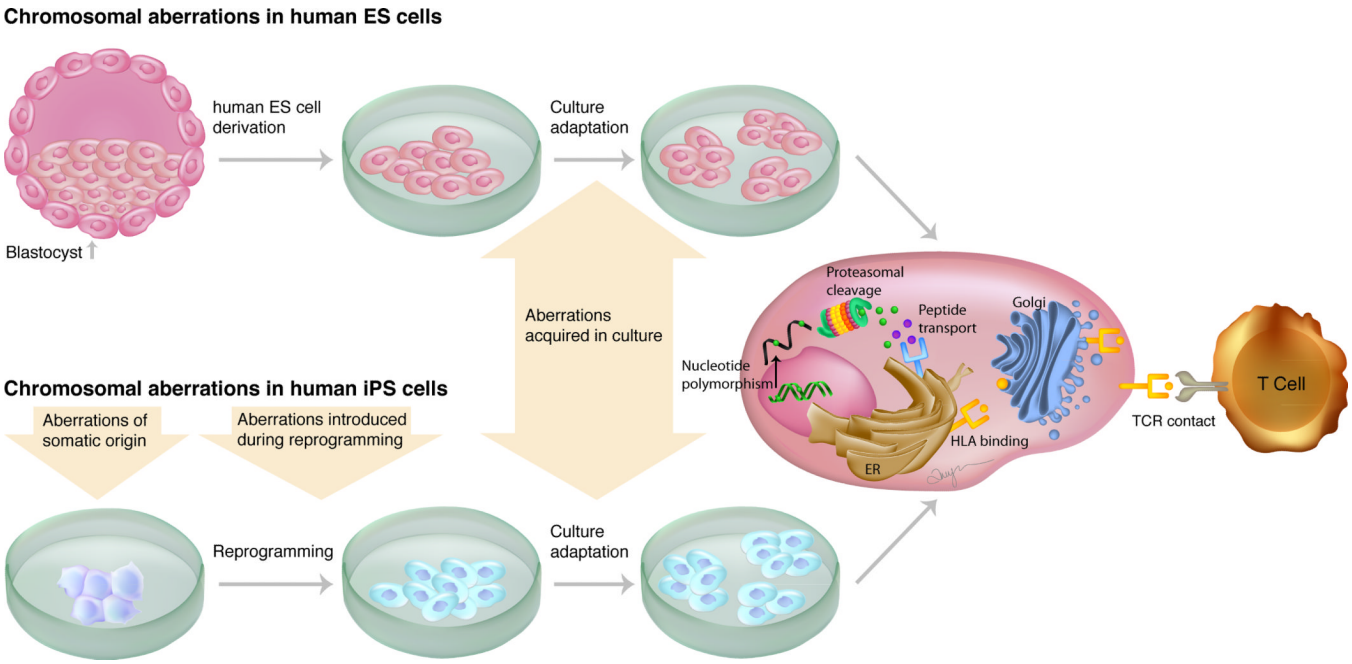

Figure 2.

Mechanisms for generating minor histocompatibility antigens in pluripotent stem cells. Polymorphisms induced in ES and iPS cells can result in expression of proteins and peptides that are distinct from those in the donor cells. Upon proteolytic degradation, these peptides are transported by the peptide transporter into the endoplasmic reticulum (ER), where they can bind to HLA molecules and pass through the Golgi apparatus to be presented at the cell surface as a complex with HLA and be recognized as foreign by donor T cells.

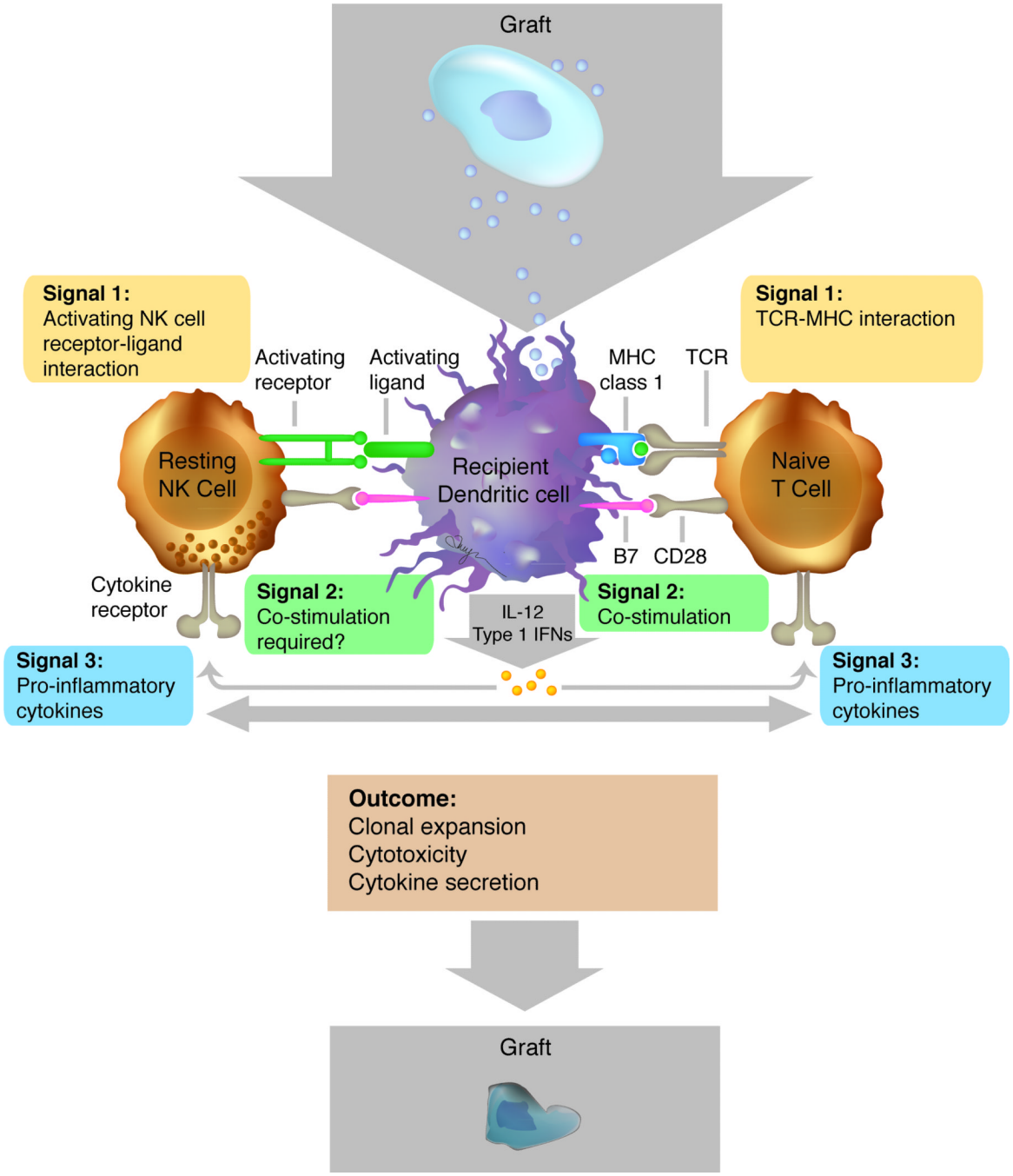

Figure 3.

Simplified schema exemplifying an immune response to a stem cell-derived cellular therapeutic. Dendritic cells acquire antigens from the graft for presentation to T cells and NK cells, which mount specific responses following antigen receptor activation (Signal 1). Upon TCR–MHC interactions, co-stimulation (Signal 2) and pro-inflammatory cytokines (Signal 3), such as interleukin-12 (IL-12) and type I IFNs, can promote the activation and clonal expansion of T cells. Similarly, resting NK cells may also receive signals via activating receptors (Signal 2) and pro-inflammatory cytokine receptors (Signal 3). Activated T cells and NK cells can generate cytotoxic responses against the graft, resulting in rejection. Figure adapted from Sun & Lanier (2011).[109]

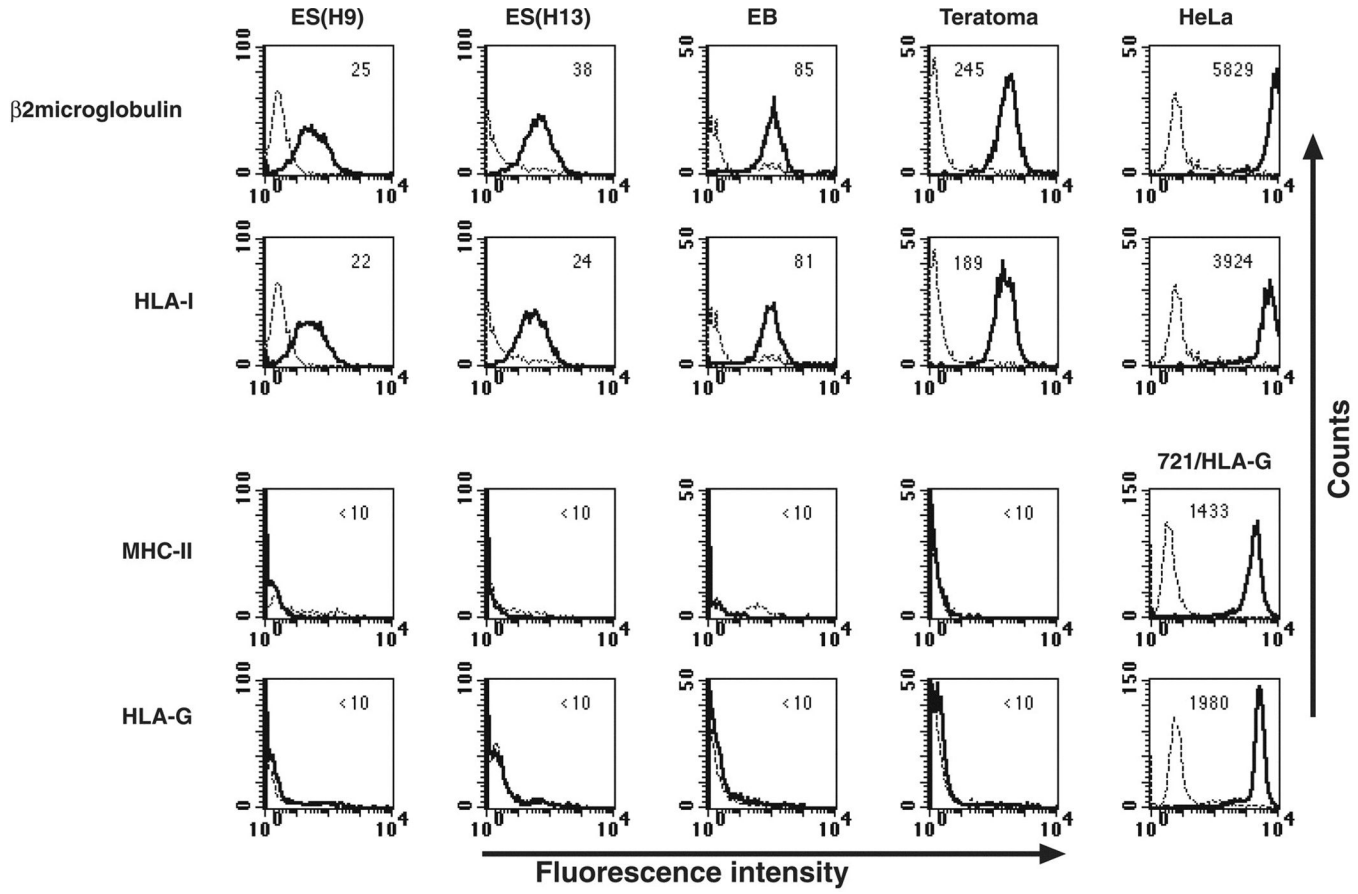

Figure 4.

Mean fluorescence intensity of various HLA proteins in various undifferentiated and differentiated human ES cell lines. The expression of HLA class I, HLA class II (HLA-DP,-DQ,-DR), and the non-classical HLA-I HLA-G was determined in two undifferentiated human ES cell lines (H9 and H13), embryonic bodies from *in vitro* differentiated human ES cells, *in vivo* differentiated human ES cells-teratomas; cervix epithelial cell line (HeLa). Dashed lines represent background control staining and solid lines demonstrate expression of specific antigens. Median fluorescence intensity staining is indicated at the top of each box. Reprint with permission.[37]

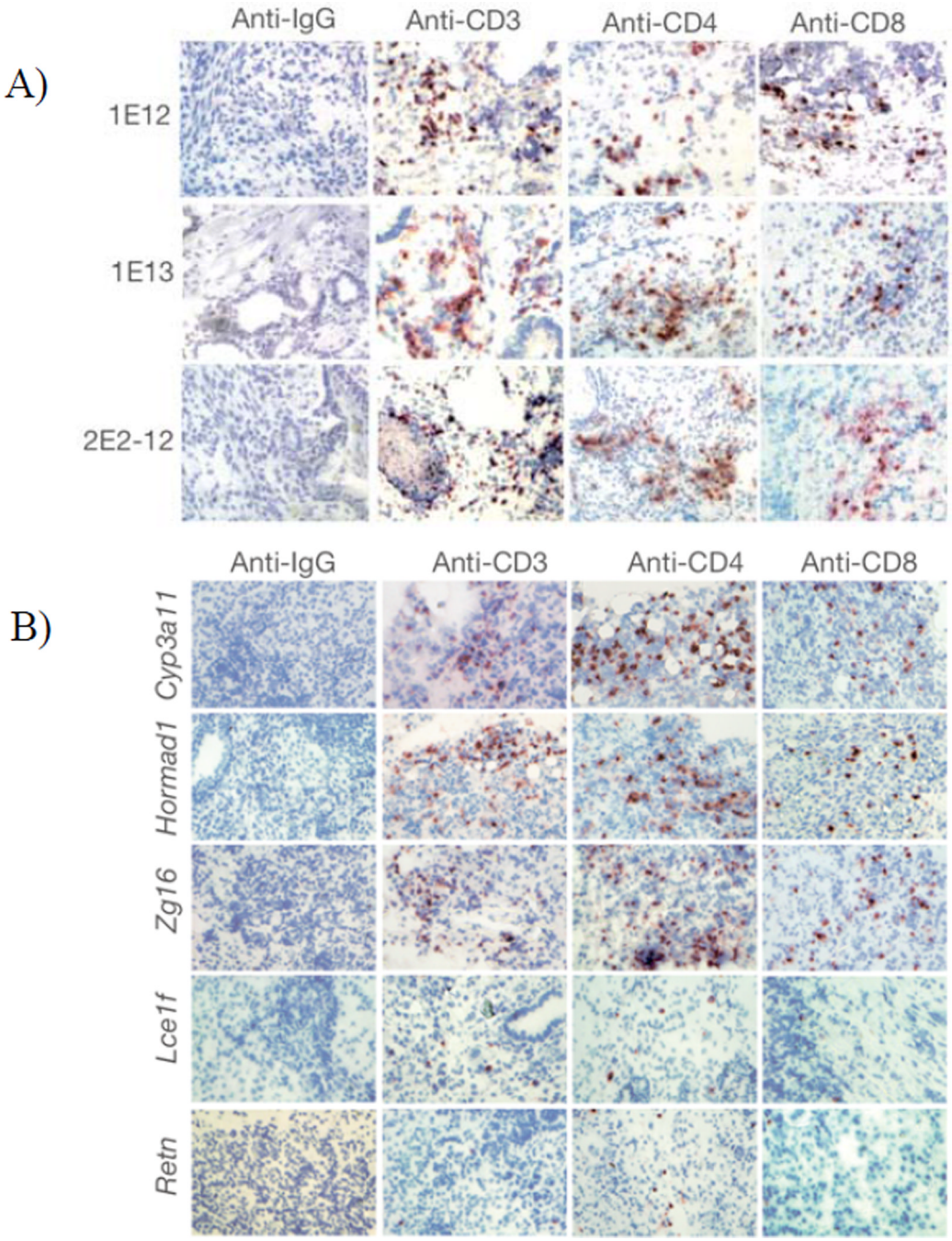

Figure 5.

Extensive infiltration of T cells in regressing teratomas formed by syngeneic iPS and transgenic ES cells. A) T-cell infiltration in teratomas formed by syngeneic episomal-derived iPS cells from two different passages (1E-12, 1E-13) and after LoxP/Cre-mediated deletion of the reprogramming factor expression cassette from the integrated copy of episomal vector (2E2-12). B) Ectopic expression of Cyp3a11, Hormad1, and Zg16 in syngeneic mouse ES cells elicited infiltration of T cells in the teratomas. Few infiltrating T cells were detectable in the teratomas formed by Lce1f- and Retn-B6-expressing mouse ES cells. Reprint with permission.[35]

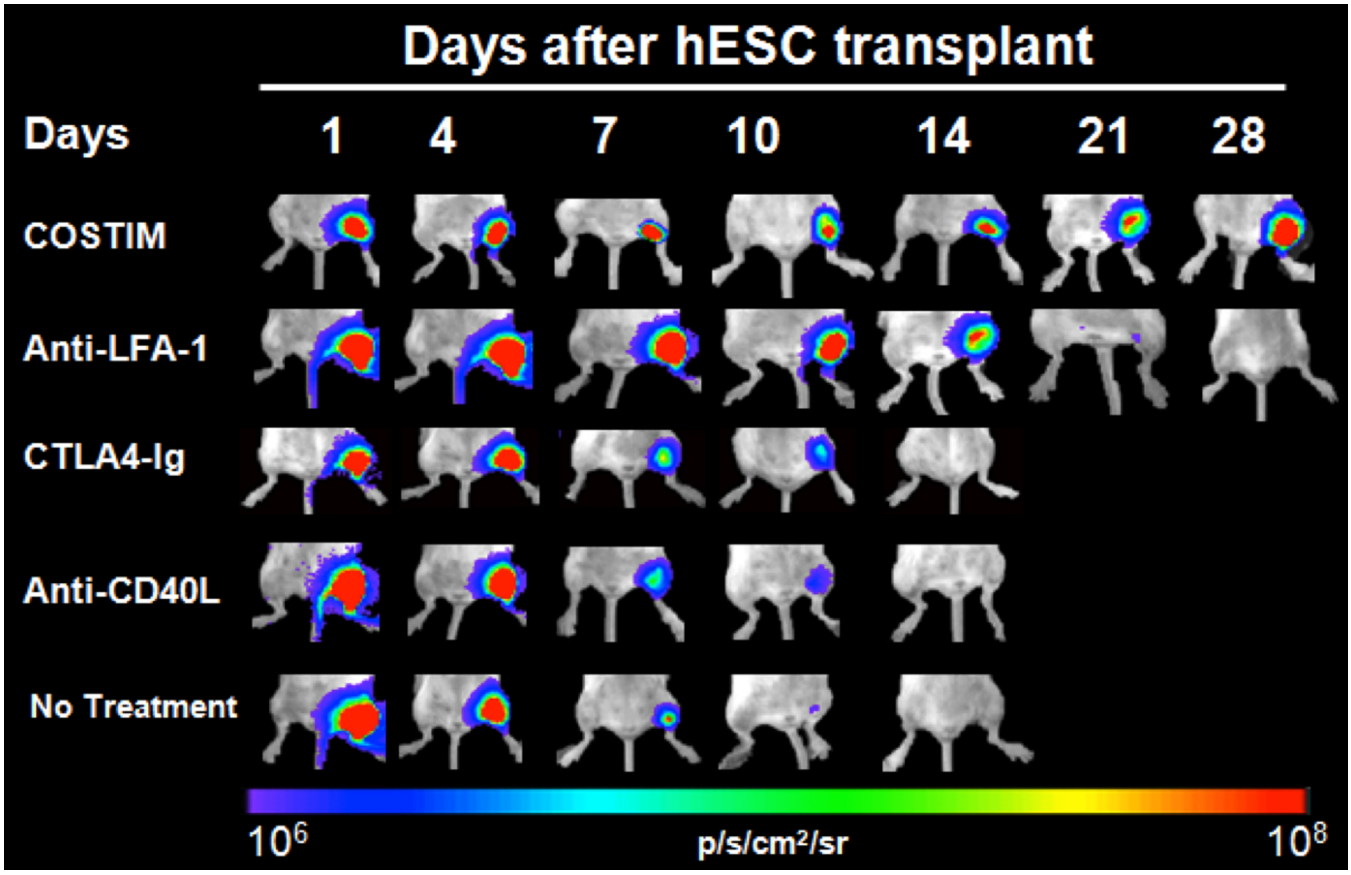

Figure 6.

Longitudinal bioluminescence imaging of human ES cells implanted intramuscularly in mice demonstrating that triple-costimulatory blockade therapy (COSTIM) administered at days 0, 2, 4, and 6 prevented rejection of human ES cells. COSTIM refers to a combination of CTLA4-Ig, anti-LFA-1, and anti-CD40L. COSTIM was remarkably more efficient than monotherapy with anti-LFA-1, CTLA4-Ig, or anti-CD40L. Reprint with permission.[82]

Table 1

Examples of short-course regimens known to promote successful long-term acceptance of allogeneic or xenogeneic stem cells in mice. WBI = whole body irradiation; TI = thymic irradiation.

Tolerance Regimen	Treatment Course	Mechanism of Action	Findings	References
			Indefinite survival of xenogeneic ES cells in testis but not in heart.	
CTLA4-Ig anti-CD40L anti-LFA1	6 to 8 days		Indefinite survival of xenogeneic ES and iPS cells, allogeneic ES and iPS cells in the leg muscle.	82,110
CTLA4-Ig anti-CD40L 3Gy WBI	2 days	Inhibits T cell activation by blocking CD28, CD40L, and LFA1 expressed on T cells.	Prolonged survival of xenogeneic ES cell-derived ECs and allogeneic iPS cell-derived NSCs. Permanent engraftment of fully MHC-mismatched allogeneic HSCs. Tolerance to donor skin.	98,111
Anti-CD40L 200cGy WBI	14 days		Engraftment of fully MHC-mismatched HSCs. Long-term tolerance to donor skin.	112
Non-depleting anti-CD8 & anti-CD4	4 days	Blocks $CD8^+$ and $CD4^+$ T cells from TCR-specific activation. Treg recruitment.	Indefinite survival of allogeneic mouse ES cells and EBs.	19,97
Depleting anti-CD8 & anti-CD4 7Gy TI	3 days	Depletes $CD8^+$ and $CD4^+$ T cells.	Permanent engraftment of fully MHC-mismatched allogeneic HSCs. Tolerance to donor skin.	113