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Research paper

Precise immune tolerance for hPSC derivatives in clinical application

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ABSTRACT

Human pluripotent stem cells (hPSCs) promise a foreseeing future for regeneration medicine and cell replacement therapy with their abilities to produce almost any types of somatic cells of the body. The complicated immunogenicity of hPSC derivatives and context dependent responses in variable transplantations greatly hurdle the practical application of hPSCs in clinic. Especially for applications of hPSCs, induction of immune tolerance at the same time increases the risks of tumorigenesis. Over the past few years, thanks to the progress in immunology and practices in organ transplantation, endeavors on exploring strategies to induce long term protection of allogeneic transplants have shed light on overcoming this barrier. Novel genetic engineering techniques also allow to precisely cradle the immune response of transplantation. Here we reviewed the current understanding on immunogenicity, and efforts have been attempted on inducing immune tolerance for hPSC derivatives, with extra focus on modifying the graft cells. We also glimpse on employing cutting-edge genome editing technologies for this purpose, which will potentially endow hPSC derivatives with the nature of wide spectrum drugs for therapy.

1. Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have the ability to differentiate into all types of cell. hESCs and hiPSCs share substantial similarities in terms of pluripotency and differentiation, but are derived from different origins. hESCs are established from the inner cell mass of human blastocyst [1], while hiPSCs from somatic cells by reacquiring pluripotency, either through ectopically expression of a set of transcription factors or by chemical based regimens [2,3]. In defined culture system, hPSCs can differentiate into all three germ layers [4–6], as well as various types of functional cells of a specific lineage. These derivatives together with the process of obtaining them, provide a valuable system in exploring human embryonic development, modeling genetic diseases *in vitro* and developing cell based therapy [7]. Upon transplantation into disease models such as Parkinson disease [8], Huntington disease [9], Amnesia [10], or heart failure [11,12], the hPSC derived functional entities exhibit remarkable effectiveness and reasonable safety. In most of the cases, transplantations were carried out in experimental settings to verify the functionality of hPSC derived cells, which did not take the possible immune incompatibility into consideration. Although immune tolerance strategies for organ transplantations to certain degree work in allogeneic cell transplantation, advantages of cell therapy are comprehensively compromised by these systematic immune suppressions.

hPSCs are once thought to have low immunogenicity because of low level expression of HLA class molecules or co-stimulation molecules [13]. Yet, this happens mostly in experimental settings of an unrealistic circumstance. Since immune response is an adaptive reaction between graft and host, specific gene expression and epigenetic abnormality of the graft in local niche could potentially lead to immunologic rejection regardless of the cell types. iPSCs in theory should be immune compatible to the individual where they are generated, but depending on types of cell graft, approaches of reprogramming or tissue origins from which iPSCs are generated, there remains variable immune response upon autogenic transplantation of iPSCs or their derivatives [14].

Abbreviations: AAV, adeno-associated virus; ATIIC, lung alveolar epithelial type II cell; B2M, b-2-microglobulin; BAC, bacterial artificial chromosome; CIITA, class-II MHC transactivator; CP hESC, hESC lines constitutively expressing CTLA4Ig and programmed death-ligand 1 (PDL1); CTLA4Ig, cytotoxic T lymphocyte antigen 4 fused with immunoglobulin; HSCs, hematopoietic stem cells; HDR, homology directed repair; hESC, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; HLA, human leukocyte antigen; KD, knockdown; KO, knockout; mAbs, monoclonal antibodies; MHC, major histocompatibility complex; MKPs, megakaryocyte progenitors; MPCs, mesenchymal progenitor cells; MSCs, mesenchymal stromal cells; NK, natural killer; PD-L1, programmed death ligand-1; PG, parthenogenetic; TALEN, transcription activator-like effector nuclease; TAP1, antigen presentation 1; TAPBP, TAP-associated glycoprotein; Tol-DC, tolerogenic dendritic cells; ZFN, zinc finger nuclease

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Further operations on the iPSCs, such as correcting defects of iPSC for autologous based therapies, alterations of the functional genes or introducing of extra DNA fragments into genome may also be immunogenic, triggering de novo immunologic responses. PSCs established through somatic nuclear transfer (SCNT) is another example which should be immune compatible to the nuclear donor, however, because of the mismatched mitochondria, they can trigger certain levels of host immune response as well [15]. Inflammatory environment and cytokines in the recipients are strong triggers of immune response and upon transplantation, HLAs are usually upregulated by these complicated stimulations, which induce immune rejection to the cell transplants [16]. Thus, it remains challenging in the stem cell field to overcome immune rejection for fruitful cell replacement therapy.

Based on current understanding on hPSCs, their immunogenicity and responses that are induced upon transplantation, it is possible to tackle this question either through repressing immune system of the host, or through modifying stem cells to produce immuno-compatible cells for transplantation. Like that in allograft organ transplantation, immunosuppressive drugs can be used to block the immune rejection to hPSC derivatives at multiple steps of the immune response. However, long term use of immunosuppressive agents could generate severe side effects, especially the possible tumor formation or serious infection [17]. With some success in animal models, safer and more effective approaches to induce non-systemic immune tolerance are on their way to clinic. Here, we reviewed updates of how native immune system recognizing and reaction to the transplanted hPSC derivatives, and how novel strategies are developed to induce precise immune tolerance for effective and safe cell transplantation therapy. Importantly, we introduce how the stem cell community employs the fast-developing genome editing technologies to engineer human embryonic stem cells escaping immune surveillance without causing systemic immune repression [18–20].

2. Immune rejection to graft of hPSC derivatives

Mammalian immune system recognizes “self” and “non-self” through a built-in machinery derived during development. Both pathogens and transplanted cells/tissues, or alteration of native cells can activate innate immunity or/and acquired immunity. MHC molecules present fragments of a protein (epitope) to the cells surface, which could be identified as “self” or “hostile”, to T cell. In the context of cell transplantation, both cytotoxic T lymphocytes ($CD8^+$ T cell) and natural killer cell (NK cell) take responsibility to mediate attacks of donor cells. $CD8^+$ T cells of recipient recognize specific antigens presented by class I major histocompatibility complex (MHC-I) molecules. Class II MHC molecules which normally expressed by Antigen Presenting Cells (APCs) can trigger $CD4^+$ T cell to secrete proinflammatory cytokines such as Interferon-γ (IFN-γ), Tumor Necrosis Factor α(TNF-α), Interleukin 12(IL-12) and Interleukin 17(IL-17), and in turn enhance the $CD8^+$/NK cell performance.

Discoveries of the major histocompatibility complex (MHC), known in humans the human leukocyte antigen [21] significantly valued the practice of transplantations [21]. HLAs are mapped to multiple genetic locus of chromosome 6 and are main molecular targets of allograft rejection by host immune system. The class I HLAs, including HLA-A, HLA-B, HLA-C, are expressed on almost all nucleated somatic cells and contain β2 subunits that can only be recognized by $CD8^+$ T cells, a process called cellular immunity. The class II HLAs are HLA-DR, HLA-DQ, and HLA-DP, which are mostly expressed on APCs (macrophage or dendritic cells) [22]. By interacting with $CD4^+$ molecules on surfaces of helper T cell, HLAs class II participate to the establishment and augmentation of the adaptive immunity (Fig. 1). HLAs seem to play a critical role in graft rejection of stem cell transplant, especially when MHCs are upregulated [23], either because of the stages of differentiation, inflammatory cytokines stimulation [22], or particular situation such as teratoma formation [24].

Undifferentiated hPSCs, proved firstly in hESCs express very low level of MHCs, which can protect themselves from being recognized by native T cells in recipient. Directly targeting MHC expression in PSCs might be able to produce HLA-class-I-knockdown hESC lines, which was once thought inducing less immune response upon transplantation of the differentiated derivatives [25]. However, this was proved not the case and graft cells are instead susceptible to be recognized and eradicated by NK cells. Low level of MHC-I on cell surface would lead to NK cell recognition and NK cell-mediated killing, in which Inhibitory killer immunoglobulin-like receptors (KIRs) are actively involved. In normal cells, interaction of KIRs and the appreciated MHC-I molecules protect these cells from cytotoxicity. Without MHC-I expression identifying “self”, the hPSCs will not be recognized by KIRs and become vulnerable to NK cells [26]. Indeed, in the NK-deficient SCID beige mice, teratoma grow much faster than that in normal mice [27], indicating the effective NK-mediated rejection. Similarly, transfusion of iPSC-derived hematopoietic progenitors also causes NK-mediated immune rejection [28]. Thus, destroying the interaction between MHCs and T cell might from one aspect reduce the rejection, it cannot guarantee long-term survival of PSC derivatives [29,30].

In adaptive immune response, beside the primary antigen-specific recognition between T-cell receptor (TCR) and MHCs, a secondary confirmation is needed for full activation of T cells, the so called “co-stimulatory signal”. Varies co-stimulatory molecules coordinately and strictly control the intensity and range of immune response before its “over-heating”. Undifferentiated human PSCs express low levels of co-stimulation molecules but unlike class I MHCs, neither incubation with proinflammatory cytokines nor differentiation increases their expressions [31]. Short-term blockage of co-stimulatory signals extends survival time of the hESC derived pancreatic endoderm grafts [32]. Programmed cell death protein 1 (PD1) and B7 homolog 1 (B7H1; also, known as PDL1) are proved to play an important role in T cell activation, and antibodies targeting these proteins have shown satisfactory therapeutic potentials. In summary, interplay between graft and recipient generates complicated outcomes in stem cell therapies in which the immune recognition and immune responses play critical roles. Graft cells are harshly interrogated by host immune system, and in the presence of co-stimulation signals, mismatched MHCs expressed on PSCs or their derivatives will be directly presented to T cells. NK cells, as well, participate in the immune rejection of graft cells in a MHC independent manner.

3. Immunogenicity of iPSCs

iPSCs hold great promise in “personalized medicine” because of their potential as “customized” cell sources for individual therapy. iPSCs are directly derived from somatic cells, meaning they maintain both the nuclear and mitochondrial genomes and bear MHCs compatible to individuals from whom the iPSCs are generated, thus their derivatives theoretically are recognized as “self” upon autologous transplantation. A patient who suffering from age-related macular degeneration became the first recipient who received autologous iPSC-derived retinal pigmented epithelium in her 70 s [33]. Although this trial greatly stirred the iPSC as well as the stem cell field, it also raised concerns on risks of the reprogramming-related mutations and a reconsideration of the overall advantages of autologous versus allograft transplantations using iPSC derived cells. In this regard, a public bank of HLA-typed iPSCs would be a more practical approach for HLA-matched cell and tissue transplantation.

Because multiple factors affect the immunogenicity of iPSCs which could potentially trigger rejection [34], the concept of iPS cell-based personalized medicine remains controversial [34,35]. Infiltrating T cells were found in graft of undifferentiated iPSCs but not in that of iPSC derived differentiated cells upon transplantation into syngeneic mice [36,37]. Such immunogenicity might be negligible and without clinical significance, at least in the corresponded tissues that receive

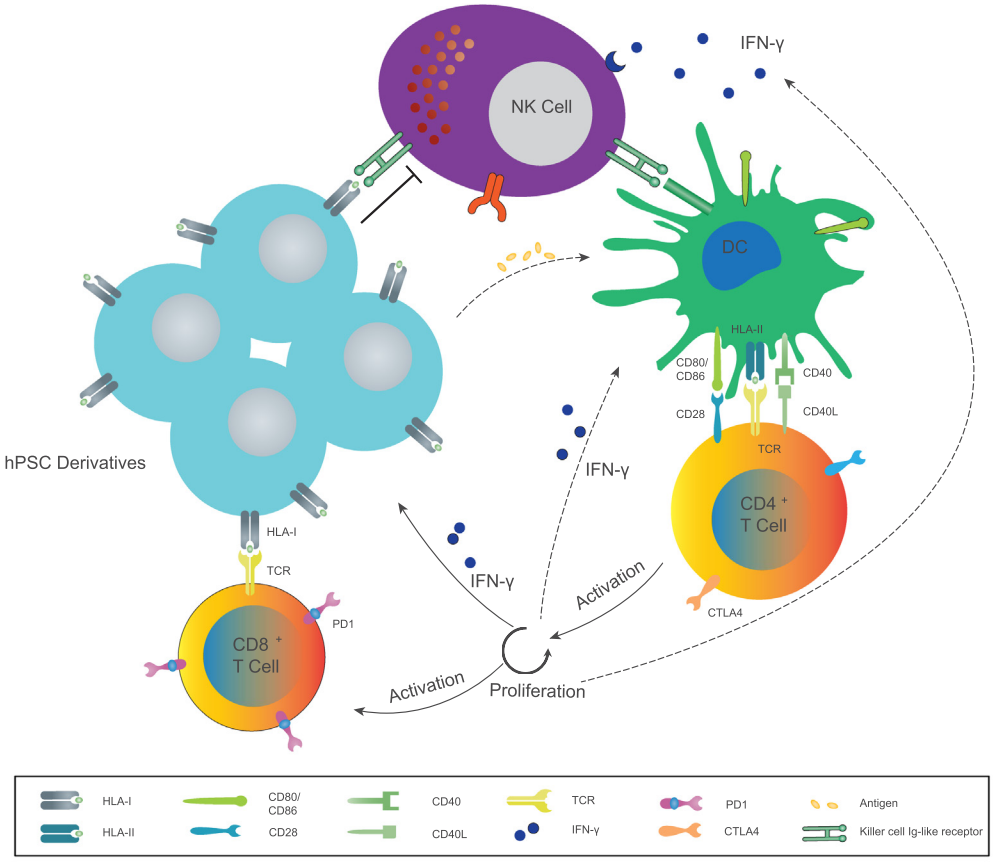

Fig. 1. Overview of the response to hPSCs/hPSC derivatives in recipient. $CD8^+$ T cell recognize HLA-I and kill the donor cells directly, meanwhile antigens presented by APCs could trigger $CD4^+$ T cell to secrete proinflammatory cytokines like IFN-γ, which could further amplify immune response. HLA-I could also prohibit NK cell activity, low level of HLA-I would lead to NK cell-mediated killing. Abbreviations: HLA-I, class-I HLA; DC, dendritic cells; hESCs, human embryonic stem cells; HLA-II, class-II HLA; IFN-γ, Interferon-γ; NK, natural killer.

iPSC-derived skin cell, hepatocytes and neurons [38]. In nonhuman primate models, iPSC-derived mesodermal stromal-like cells functionally integrated into the recipients [39], and autologous iPSC-derived dopamine neurons trigger only minimal immune response following transplantation in a nonhuman primate Parkinson's disease model [40]. Thus, in spite of the promise of iPS cell-based autologous or syngeneic transplantation, there remains questions about how transplant microenvironment contributes to the immunogenicity of iPSCs and how to systemically fine-tune the immune response for successful therapy.

On the other hand, generation of iPSCs usually involves ectopic expression of core factors through either viral or non-viral based approaches which also affect the immunogenicity. For the viral system, different types of virus distinctively alter the expression pattern of these factors during the reprograming. Similarly, cell types that used to generate iPSCs could contribute to the various residual gene expression in the iPSCs [41]. To further evaluate the immunogenicity of different types of iPSC-derived cells in a context of human immune system background, humanized mouse models that bear human T and B cells as well as NK cells have played important roles. In these humanized mouse models, immune response appears to the specific graft cell type, even these cells are all derived from the same iPSC line [14]. Thus, abnormal expression of immunogenic antigens in iPSC and their derivatives inevitably trigger discernable immunologic rejections which need additional investigations [42]. Nonetheless, epigenetic abnormalities in early reprogramming process could be ameliorated by repetitive subculture and subsequent passaging [43]. In addition, not all iPSC clones are identical that highlights the necessity of evaluating each iPSC line individually for transplantation purpose. Again, more work is needed to clarify why these epigenetic abnormalities can occur, how these abnormalities cause defective differentiation and whether they have functional consequences. Not until these questions are solved, the clinical translation of the promising iPS cell-based therapy will get success.

4. Cell-based tolerance

4.1. Allogeneic cell transplantation of HLA matching iPSCs

Banking human PSCs will supply sufficient diversity of HLA types which is much more practical to ensure the prompt cell availability to public. Taylor et al. used UK population as a model to predict how large an ESC band required to meet the demand. It turns out that the 150 most useful homozygous HLA types have the potential to provide a match for 93% of the UK population [46]. iPSCs are particularly suitable for such a purpose due to without significant ethical concerns. It would become possible to select the one that maximally matches the HLA type of a given individual from a pool of iPSC lines, and differentiate that iPS cell line into the desired cell types for cell therapy. Several groups have initiated banking clinical grade iPSC lines [44,45]. Completely matching of both HLA-class I and II molecules between graft and recipient is not practical, thus, locis that mostly contribute to the rejection, such as HLA-A, -B, and -DR are selected for priority. It should be kept in mind that even HLA-A, -B, and -DR matches, allografts may also be rejected because of mismatches of other HLAs. Finally, concerns remain that abnormal expressions profiles generated from the hPSC differentiation may also be immunogenic.

Such a concept has been proven by the functional integration in MHC-matched nonhuman primates of iPSC-derived cardiomyocytes [47]. Long-term graft survival without immunosuppression might be possible if the iPSC derived cells are transplanted back to certain regions in the body of the autologous recipient. For instances, in *cynomolgus* monkeys that received cell sheets of autologous iPSC-derived retina pigmented epithelium exhibit no evidence of immune rejection for nearly 1 year. However, in monkeys that received allogeneic transplantation, fibrous tissue was found in the eye with leakage of fluorescein, signs of rejection. These work together indicate that for iPSC based allogeneic transplantation, immunosuppression drugs will

be needed anyway [48]. Thus, it will be critical to carefully select less immunogenic iPSC lines and continually monitor for any side-effect after transplantation.

4.2. Regulatory T cells or dendritic cells to repress immune response

Regulatory or repressive immune cells, such as FoxP3$^+$ regulatory T cells (Tregs) [49], tolerogenic dendritic cells (Tol-DC) [50] or myeloid derived suppressor cells (MDSC) [51] are important players to induce or maintain immune tolerance. It is worth noting that Tregs, as well as MDSC derived from recipient rather than from donor could induce immune tolerance [52]. Tol-DC, on the other hand, from either donor or recipient could lead to immune repression [53], allowing large scale production of such immune tolerogenic DCs for therapeutic purposes [54]. Hematopoietic stem cells (HSCs) differentiated from MHC-mismatched ESCs, once optimized, may also provide an effective approach for inducing tolerance [55].

Thymusderived or naturally occurred Tregs expressing high level of FOXP3 are selected in thymus and responses to self-antigens, creating a tolerogenic micro-environment in the periphery. Boosting Tregs for immunotolerance in cell transplantation is thus a promising and practical strategy [56,57]. Tregs suppress immune reaction through cytotoxic T lymphocyte antigen 4 (CTLA4), which inhibits APC activity and prevent effector T cells from activation. Binding of CTLA4 to the costimulation molecules CD80 and CD86 also activates the expression of indoleamine 2,3dioxygenase (IDO), which in turn exhausts essential amino acid tryptophan and causes death of the cytotoxic T cells [58]. Tregs can also produce IL10, which can inhibit APC activity and promote the conversion of T cells into T regulatory type 1 (Tr 1) cells [59].

Dendritic cells (DCs), but not immature DCs (iDCs) as major antigen-presenting cells activate naive T cells and trigger immune responses. iDCs, nevertheless produce IL-10 and can repress immune response [60]. In addition, iDCs express very low level of MHC class II and costimulatory molecules on their cell surface, also facilitate the induction of T cell tolerance [61]. Tol-DCs that induce immune tolerance in organ transplantation might as well work for cell replacement therapy [62]. DCs have been successfully differentiated from hPSCs [63], and donor iPSC-derived immature DC acquiring alloantigen could significantly prolong the survival of skin grafts in mice [64]. Yet, the differentiation of hPSCs into Tol-DC remains not efficient enough for clinical application, and any undifferentiated cells or abnormally expressed tumorigenic factor could potentially become triggers of the immune rejection. Differentiation of specific types of regulatory cell from stem cells, such as HSCs, may also provide an approach for inducing tolerance. Islet allografts in NOD mice are well tolerated if the mice also received HSCs differentiated from MHC-mismatched ESCs.

5. Genetic engineering for immune tolerance

Ideal tolerance is to establish a circumstance in clinical settings free of using immunosuppressive drugs. While neither banking PSCs nor immunosuppressive immunocytes fulfill that requirement so far, additional approaches are under extensive exploration. Since occurrence and extent of immune rejection are closely regulated by cell surface molecules and their ligands [65], it would in principle be possible to induce immune tolerance by blocking the interactions of these molecules, such as genetic modifying hPSC lines before differentiating them to functional derivatives. The latest generation of genome editing technologies, such as zinc finger nuclease (ZFN) [66], transcription activator-like effector nucleases (TALEN) [67] or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) [18], allow precisely targeting a given genome locus by invoking the Homology directed repair (HDR) or Non-homologous end joining (NHEJ) (Table 1). These technologies value the cell based therapy at least in several aspects. As a set of powerful tools, CRISPR system helps a lot in further interrogating the molecular mechanism underlying immune recognition, and is efficient to identify new immune regulators through genome-wide screening [68]. This system also allows for the precise insertion of genes of interest into hPSC genome without random integration of foreign DNA fragments [69], avoiding risks of genome abnormality.

5.1. Targeting Class I MHCs in hPSCs

Reducing major histocompatibility complex antigen expression in murine ES cells allows for immune escape [13], so as in hESCs [25]. The allograft from hESCs with HLA-knock down survived for up to 42 days in immunocompetent BALB/c mice, while those controls could survive for 10 days only. NK cells in these studies appeared to play minor roles in immune rejection. In other conditions such as ischemia in a coronary artery ligation model, similar outcomes were observed [70].

HLA-A was once knocked out using ZFN for generation of a universal hPSCs line fulfill any individual, yet information of post-transplantation outcome is missing [71]. Since HLA molecules cannot be present properly without B2M, another way to disrupt the HLAs expression is to target beta-2 microglobulin (B2M), the light chain of class I HLAs (Fig. 2A). Knock out B2M utilizing TALEN could generate HLA-ABC-negative platelets matching large population of people [72]. The B2M^{null} hESCs induced minimal T cell activation with few CD8$^+$ T cell-mediated killing (less than 1%) [73]. Upon transplantation of B2M$^{-/-}$ hESCs into muscles of BALB/c mice, they as well induce modest T cell infiltration compared to their wide type counterpart hESCs [74]. Knocking out antigen presentation 1 (TAP1) and TAP-associated glycoprotein (TAPBP) in hESC via TALEN to disrupt the antigen presentation of MHC-I also reduce immunogenicity significantly [75].

As MHCs identify “self” in biology system, thus eliminating class I HLAs alters the identity of the donor cells, which could potentially cause unpredicted side-affects in transplantation. Autogenic class-I HLA molecules could also function as inhibitory ligands for NK cells, and those cells with low levels of class-I HLA may become vulnerable to NK cell induced rejection at certain circumstances. IFN-g treatment up-regulated HLA-I expression during the differentiation of hPSCs and significantly suppressed NK cell-mediated killing. However, for B2M^{null} hESCs, IFN-g treatment could not protect these cells from NK cell-mediated lysis and more infiltrating KLRA1$^+$ NK cells were observed in B2M^{null} hESCs implants than that in the control group [73]. In summary, appropriate level of HLA-I expression in hPSC or their derivatives is crucial for initiating NK cell and T cell mediated immune responses.

5.2. Lessons learned from fetomaternal tolerance

Although lack of HLA-I expression usually initiates NK killing process in most cases, exceptions exist in scenarios such as during fetal development, in which the maternal immune system could be successfully repressed with low expression of HLA-I at the maternal-fetal interface [76]. Among a number of signals or molecules that contribute to such a special fetomaternal tolerance, HLA-G, a non-classical MHC class I molecule is proved to be essential in maternal acceptance of the fetus.

HLA-G is expressed in placental trophoblast cells, and unlike classic HLA-I molecules, it has limited ability to present intracellular peptide to T cells or APCs. HLA-G exerts overall negative immune regulatory functions at the maternal-fetal interface through induction of immunosuppressive regulatory T Cells [77,78] or inhibition of natural killer cell cytotoxicity [79]. hESCs express certain level of HLA-G but downregulate its expression during blastocyst development [80]. BMP4 treatment could increase the HLA-G expression in trophoblast cells derived from hESCs [81]. Other HLA-G expressing cells that are derived from hESC, such as hESC derived mesenchymal progenitor cells (MPCs), as well medicate resistance to NK cell killing [82]. In parthenogenetic embryonic stem cell (PG hESCs)-derived neural stem cells, the allele-specific expression of HLA-G molecules also protect PG hESCs derived

Table 1
Abbreviated list of strategies for molecule-based immune tolerance induction in hPSCs.

Cell type	Molecule	Strategy	Evaluation methods	Ref.
hESC	HLA Class I	HLA-I K_D by siRNA or/and intrabody	Xenogeneic Transplantation Allogeneic cytotoxic killing *in vitro*	[70]
hiPSC hiPSC-derived MKPs and Hemogenic Endothelial-like Cells	B2M	KO via TALEN	No immunogenicity evaluation	[72]
RUES2-modified hESC RUES2-modified hESC derived cardiomyocytes	B2M	90% K_D by siRNA	T-lymphocyte activation *in vitro*	[99]
hESC hESC-derived embryoid bodies	B2M	KO via AAV delivery	Allogeneic cytotoxic killing *in vitro*	[100]
hESC hESC-derived ATIICs	B2M	KO via sequence disruption by targeting vector	Allogeneic cytotoxic killing *in vitro* Transplantation into NK^+ SCID mice	[73]
hESC	B2M	KO via TALEN	allogeneic immune response *in vitro* Xenogeneic Transplantation	[74]
hESC	HLA-A	KO by ZFNs targeting	No immunogenicity evaluation	[71]
hESC	TAP1 TAPBP	KO via TALEN	allogeneic immune response *in vitro*	[75]
hESC hESC-derived fibroblasts and dendritic cells	CIITA	KO via TALEN	No immunogenicity evaluation	[71]
hESC hESC-derived epidermal progenitor cells	Mutant HLA-G	Ectopic expression via random integration	NK cell mediated killing in *vitro*	[84]
PG hESC PG hESC-derived neural Stem Cells	HLA-G	Induced during differentiation	NK cell mediated killing in *vitro*	[83]
hESC-derived pancreatic endoderm	CD28 CD40	CTLA4Ig/Anti-CD154 blockade	Transplantation into humanized mice model Xenogeneic Transplantation	[32]
hESC hESC-derived fibroblasts, cardiomyocytes	CTLA4Ig PD-L1	Knock-in via BAC-based target system	Teratomas formation Transplantation into humanized mice model	[86]

neural stem cells from NK-Induced Apoptosis [83]. Ectopic expression of HLA-G as well decreases the immunogenicity of hESCs and their epidermal derivatives *in vitro* [84] (Fig. 2B). Although the detailed mechanism of HLA-G in hESC immunogenicity and in immune repression followed transplantation remains unclear, the fetomaternal tolerance does provide an excellent clue to establish immune tolerance in cell replacement therapy.

5.3. Co-stimulation and accessory molecule blockade

An alternative approach to induce immunosuppression following transplantation of hPSC derivatives is to block the signaling between co-stimulation or accessory molecules and their ligands. In mouse ESC derived embryonic bodies, antibodies for CD4 and CD8 have been proven to be effective to protect allograft from the immune system of recipient [29]. Short term blockage using a combination of cytotoxic T-lymphocyte-associated antigen 4 (CTLA4)-Ig, anti-CD40 ligand (anti-CD40L), and anti-lymphocyte function-associated antigen 1 (anti-LFA-1) could prolong the survival of allogeneic and xenogeneic ESC engraftment [85]. As for hPSCs, co-stimulation blockade via CTLA4Ig and anti-CD40L mAbs (MR-1) exhibit significantly improved functional integration of the hESC-derived pancreatic endoderm [32]. As that in chemical drug induced immune suppression, systemic administration of these Abs inevitably produces aforementioned severe side effects.

To fully take advantage of co-stimulation and accessory blockade to induce safer and local immune protection, it seems reasonable to incorporate these blocking signals into hPSCs and their derivatives, thus these cells express the immunomodulatory molecules themselves and create a tolerogenic microenvironment inside the recipient, without causing unnecessary systemic immune suppression. For this goal, hESC lines constitutively expressing CTLA4Ig and programmed death-ligand 1 (PDL1) (CP hESC) have generated by inserting a copy of the combined constructs using bacterial artificial chromosome (BAC)-based targeting strategy [86] (Fig. 2C). CTLA4Ig compete with CD28, yet with higher affinity to bind to CD80 and CD86, the primary co-stimulatory pathway for T cell activation [87]. PDL1 as the ligand of programmed cell death 1 (PD1), is one of the immune checkpoints for T cells inhibition [88]. Upon injection of these CP hESC into humanized mice model that mimic human immune response, the CP hESC derived teratomas are protected from allogeneic immune rejection. While most mice that received CP hESC injection formed teratoma and none showed $CD4^+$ T cells infiltration, those received wild type hESCs formed smaller teratoma with substantial tissue necrosis. In addition, CP hESC-derived fibroblasts or cardiomyocytes could successfully induce a local immunosuppressive microenvironment after being transplanted into the hind leg muscle [86]. CP hESCs so far have the best shot to become the universal immune compatible hESCs in the future. Nevertheless, functional integration and long-term safety need to be further evaluated in models with fully functional or more robust active immune system.

5.4. Alternative approaches for immune tolerance

Several other strategies have been proposed based on different mechanisms for suppressing host immune response. For example, HLAs class II trans-activator (CIITA), if knocked out in hESCs using TALEN ($CIITA^{-/-}$ hESCs), could differentiate into fibroblasts and DCs that can block CD4 T cell recognition which are promising for therapy, although further transplant experiments are needed to further prove this [89]. Local indoleamine-2,3-dioxygenase (IDO) and Arginase I, has been proven to be effective to induce immunosuppression. After transplant of hESC-derived mesenchymal stromal cells (hESC-MSC) into the experimental model of arthritis, they can induce host-derived IDO and ameliorate the collagen-induced arthritis (CIA) [90]. Arginase I from PSCs, as well is able to block T cell proliferation and activation, thus local Arginine starvation in theory could suppress T cell response as well [91]. Biocompatible and biodegradable micro-matrix is also promising for localized immunosuppression, which was demonstrated lately with improved graft survival of the encapsulated pre-differentiated aggregates [92].

6. Genetic modified hPSCs for precise immunosuppression

Immuno-tolerogenic hPSCs such as CP hESCs are capable of inducing local immune suppression by forming a tolerogenic microenvironment in the recipient without systematic administration of immune suppressive drugs or cells. However, while these cells are

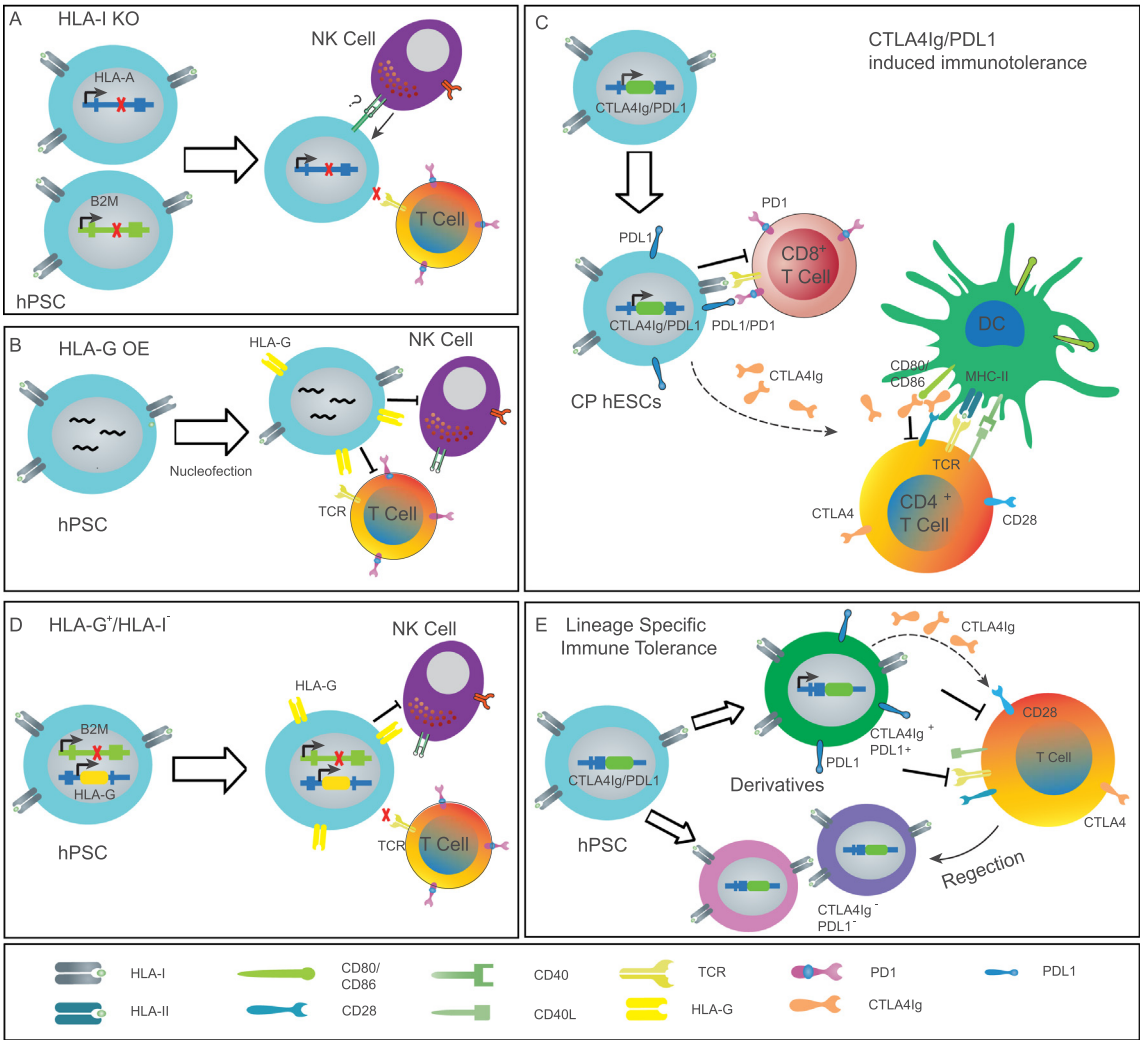

Fig. 2. Strategies to induce immune tolerance. (A) Disruption of HLA-I expression makes hPSCs lose "self" identity, reduce T cell ignorance but susceptible to NK cell-mediated killing. (B) Non-classical HLAs class I molecule, HLA-G, ectopically expressed in hESCs shows resistance to NK cell killing. (C) Expression of negative regulators CTLA4Ig and PDL1 through specific site integration lead to local immunosuppression. (D) Proposal for inducing immune tolerance by combining HLAs class I elimination and HLA-G overexpression, potentially reduce NK and T cell based immune rejection. (E) Proposal for inducing lineage specific immune tolerance by putting CTLA4Ig/PDL1 under lineage specific endogenous promoters to establish a lineage specific expression pattern, potentially lower the risk of tumor formation.

potentially immune tolerated with improved survival upon transplantation, they also increase the risk of tumor formation because of the immune tolerance they induced. Indeed, escaping from the immune surveillance could potentially increase the risk of overgrowth of residual hPSCs, if there exist, in the allograft, or cause tumorigenesis by expression of the tumorigenic or tumor-promoting genes during differentiation, for example, SURVIVIN, MYC and E2F2. Although chemical compounds preventing tumor formation are potentially helpful [93], they remain beyond acceptable in practice because of the dosage issue. In order to eliminate the residual hPSCs and cells that express tumorigenic genes, engineered hPSCs with controllable and selectable elements are in urge need of developing.

One approach is to engineer a suicide gene under the control of a small molecule. Incorporating the suicidal gene herpes simplex virus thymidine kinase (HSVTK) into the constitutive HPRT locus of the immune tolerance CP hESCs could lead to self-elimination upon administration of the FDA approved drug ganciclovir (GCV) [94]. Caspase-9 (iC9) dimerization induced by a specific chemical inducer (AP1903) could initiates a caspase cascade that eliminates the residual iPSCs, which could also be employed for hESC engineering [95].

Without using any drugs and inducers, one potential solution might be putting CTLA4Ig and PDL1 under the control of lineage specific endogenous promoters, like Tuj1 for neurons, AAT for hepatocytes or PDX1 for islet cells using "gene trap" technology to produce lineage specific but not regular immune tolerance (Fig. 2E). This way the CTLA4Ig/PDL1 are expressed only if the hESC are completely differentiate into the preferred cell lineage, and only the functional differentiated cells will be protected by the expression of CTLA4Ig/PDL1. Those cells, either less or undesired differentiated, are supposed to be rejected by the native immune system. This could potentially establish the idea of precise immune tolerance or lineage specific immune tolerance induction. Besides, through multi-step genetic engineering with CRISPR/Cas9, hPSCs with more sophisticated genetic modification become possible. For example, overexpression of HLA-G in HLA-I knockout hPSCs could potentially escape from T cell infiltration while resistant NK cells killing (Fig. 2D).

7. Conclusion

Immune responses are inevitable because of the complicated immunogenicity of hPSCs and various factors that affect such responses. Even in immune privileged sites such as brain, breakdown of the blood-brain barrier at the diseased sites may initiate rejection [96]. Thus, to value a much larger population with hPSCs, the hurdle of immune

rejection needs to be lifted. The overall benefit versus risk should be carefully considered for each individual in the context of the particular disease. Systematic immune suppression with immunosuppressant or co-stimulatory or accessory blockers are valuable for cell transplantation therapy [97,98], but fails to be satisfactory because of the toxicity and side-effects, particularly for those nonlethal but progressive diseases, which needs long-term treatment. With the increasing understanding of immunogenicity and immune response, and the state-of-art technologies of genetic engineering, it is possible to curdle the immune response toward the direction we wanted, and generate immune-compatible hPSCs free of tumorigenic risks, which will easily get accepted and functional integrated into the recipient. It is optimistic to predict that curing disease with cell transplantation based therapy might be within reach.

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