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STEM CELLS: POLICIES FROM THE BENCH TO THE CLINIC (G MOLL AND N DRZENIEK, SECTION EDITORS)

Immune Editing: Overcoming Immune Barriers in Stem Cell Transplantation

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Abstract

Purpose of Review Human pluripotent stem cells have the potential to revolutionize the treatment of inborn and degenerative diseases, including aging and autoimmunity. A major barrier to their wider adoption in cell therapies is immune rejection. Genome editing allows for tinkering of the human genome in stem and progenitor cells and raises the prospect for overcoming the immune barriers to transplantation.

Recent Findings Initial attempts have focused primarily on the major histocompatibility barrier that is formed by the human leukocyte antigens (HLA). More recently, immune checkpoint inhibitors, such as PD-L1, CD47, or HLA-G, are being explored both, in the presence or absence of HLA, to mitigate immune rejection by the various cellular components of the immune system.

Summary In this review, we discuss progress in surmounting immune barriers to cell transplantation, with a particular focus on genetic engineering of human pluripotent stem and progenitor cells and the therapeutic cell types derived from them.

Keywords Cell replacement therapy · Genome editing · Immune evasion · Tolerance · Graft rejection · Hypoimmunogenic stem cells

Introduction

Immune Barriers

Regenerative medicine has come a long way since the derivation of the first human pluripotent stem cells (hPSC) [1]. As a community, we have become better at sourcing stem cells, differentiating them into therapeutic cell types and transplanting them to cure different diseases [2–4]. To unlock the full potential of stem cell therapies, we need to overcome the immune barrier to transplantation. The human immune system is incredibly discerning in distinguishing between self and non-self, which could be viral or bacterial proteins, malignant cells, and, of course, cells from a genetically non-identical donor. Genetic differences between the donor and the recipient are recognized as alloantigens if they have never been encountered by the host's immune system before (as opposed to autoantigens) and may prompt allograft rejection [5]. Based on the nature of the genetic polymorphism and how/when they present themselves to the immune system, three types of alloantigens can be distinguished that, together, define the immune barrier (Fig. 1A).

Human Leukocyte Antigens (HLA)

Initially recognized as the main drivers of skin graft rejection [6–8], the major histocompatibility genes, or human leucocyte antigen (HLA) genes in humans, allow the immune system to differentiate between self and non-self. HLA are glycosylated surface proteins that present peptides to T cells. It is the origin of these very peptides bound to HLA that provides immune cells with information about the presence of invading pathogens or the malignant transformation of a cell. HLA are encoded by two highly polymorphic gene families,

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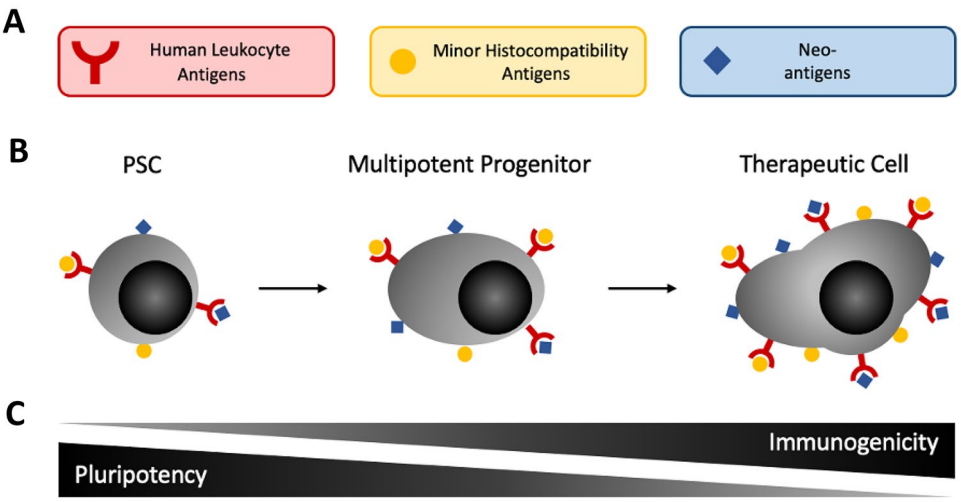

Fig. 1 Immune barriers to stem cell transplantation. **A** Different types of alloantigens. Inborn and acquired genetic differences contribute to the unique immunological fingerprint of stem and progenitor cells from different donors. Human leukocyte antigens (HLA) are the immunodominant barrier to cell and tissue transplantation. Minor histocompatibility antigens (miHA) can vary in their expression from cell type to cell type. Neoantigens (NA) can accumulate during prolonged culture and pose a risk of rejection even of cells of autologous origin. **B** Immunogenicity of pluripotent stem cells (PSC) and their derivatives. While PSC and multipotent progenitor cells (MPC) typically have relatively low HLA surface expression, postmitotic differentiated cells display high HLA class I surface levels. Moreover, differentiated cells reveal unique combinations of miHA as well as co-stimulatory and co-inhibitory ligands (not displayed) and may acquire NA over time, resulting in a unique immunological fingerprint for each cell type. **C** Immunogenicity and pluripotency are inversely correlated

HLA class I and class II on chromosome 6p, the major histocompatibility locus (MHC). As of July 2022, 34,422 different HLA alleles have been described in the human population that encode more than 20,000 distinct proteins (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/). HLA that are recognized as foreign by cytotoxic T cells can trigger acute graft rejection [9]. They can also act as the target for donor-specific antibodies (DSA) that trigger hyperacute graft rejection [10]. Matching of the HLA makeup of donor and recipient is, therefore, highly desirable in order to reduce the risk for graft rejection. Matching can be facilitated by banking of HLA homozygous stem cell lines, and prescreening for DSA can further reduce the risk for hyperacute graft rejection.

Minor Histocompatibility Antigens (mHA)

It is known, both from the bone marrow transplantation literature, and more recent animal studies using induced pluripotent stem cell (iPSC)-derived grafts that HLA-matching alone is not sufficient to prevent chronic graft rejection [11]. There are other polymorphic proteins in the human genome, both within and outside of the MHC locus on chromosome 6p, that can be recognized as foreign [12, 13]. The most prominent of these so-called minor histocompatibility antigens (miHA) are encoded on the human Y chromosome (H-Y antigens). A female cell line is, therefore, compatible with both, male and female recipients, while a male donor line would be recognized as foreign by a female recipient that has never encountered H-Y antigens before. A prerequisite to act as a miHA is that the peptides containing a polymorphism can be presented on HLA. Both HLA class I and class II have been found to present miHA [14]. MiHA can also arise by posttranslational modification. A prominent example being the ABO blood group antigens. More than 100 autosomal miHA have been described, yet their contribution to chronic graft rejection is highly variable, which most likely reflects the fact that miHA are tissue- and cell-type specific [12, 13].

Neoantigens (NA)

The observation that even grafts derived from autologous iPSC are rejected points toward the de novo acquisition of alloantigens [15]. The accumulation of genetic changes during prolonged culture, incomplete reprogramming, and subsequent untimely expression of fetal antigens may all result in antigens that appear new to the immune system (neoantigen, NA). NA arise spontaneously by mutations in the coding sequences of genes, and their spontaneous nature makes them impossible to predict [16]. In contrast to HLA and germline-encoded miHA, NA thus form an acquired immune barrier. Recently, the work of Deuse et al. has demonstrated that even a single nucleotide polymorphism (SNP) in the mitochondrial DNA of iPSC can be detected by an otherwise genetically identical animal and trigger immune rejection [17].

It had been noted early on that hPSC express comparatively low levels of HLA class I molecules when compared to their more differentiated progeny [18, 19]. The belief that they are, therefore, protected from immune rejection has, however, been debunked by multiple animal studies, which revealed that allogeneic stem cells and stem cell xenografts are still being rejected [20]. In fact, over time, hPSC accumulate NA and acquire tissue- and cell-type-specific miHA upon differentiation. A consequence of this is that as stem cells differentiate into progenitors, and then into mature cell types, they become intrinsically more immunogenic, as illustrated in Fig. 1B and C.

Progress in the Genome Editing Field

In the decade since the inception of CRISPR/Cas9 technology, the gene-editing field has advanced at a rapid rate and has undoubtedly revolutionized our capacity to engineer the genome of hPSC with unprecedented efficacy [21, 22]. A major advantage of hPSCs with regard to genome engineering is that one can easily generate single cell-derived clonal lines that can be quality controlled and selected for their potential to differentiate into a particular therapeutic cell type. Moreover, changes introduced at the stem cell level will carry over to their differentiated progeny (Fig. 2). Edits introduced into hPSC with the intent of changing their immunogenicity can broadly be divided into three different categories that we outline in more detail in the subsequent sections: 1) attempts to overcome the major histocompatibility barrier by targeting HLA genes either globally or more selectively to allow for easier matching of banked cell lines with respective transplant recipients (Fig. 2A); 2) tolerance induction by either overexpressing checkpoint inhibitors or immunosuppressive cytokines to establish a local immunosuppressive environment conducive to cell engraftment (Fig. 2B); and 3) a combination of the two aforementioned strategies (Fig. 2C).

The promise and challenges of therapeutic genome editing have been extensively discussed elsewhere [23, 24] Rather than focus on each technical advance in the gene-editing space itself, here we analyze how these tools have been leveraged for tweaking the immunogenicity (or "immune editing") of stem cells thus far and later posit how we see them being utilized further to overcome the immune barriers to transplantation and may allow for long-term engraftment of hPSC and their derivatives.

Overcoming the Major Histocompatibility Barrier

Targeting HLA Class I

Mismatches in HLA class I genes between the donor and the recipient are among the main drivers of acute and hyperacute graft rejection [5]. With three highly polymorphic HLA class Ia genes (*HLA-A*, *-B* and *-C*) that occur as different alleles, one from each parent, HLA matching, and similarly genome editing of potentially 6 different alleles becomes highly challenging. Twenty four thousand seven hundred three different HLA class I alleles (that translate into 14,137 different proteins) have so far been described in the human population (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/).

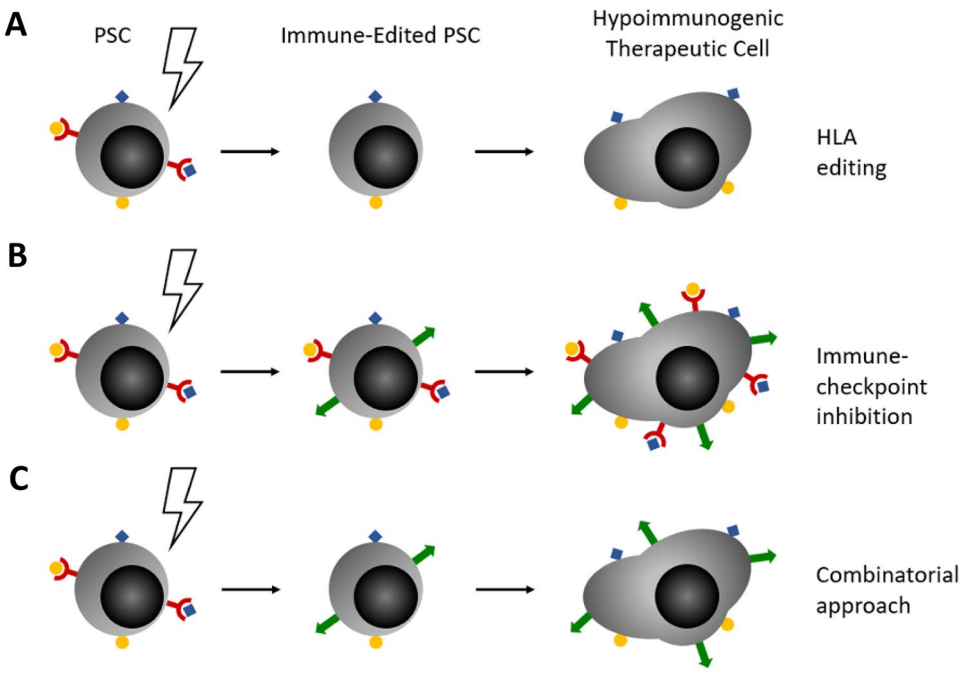

Fig. 2 Immune editing of pluripotent stem cells (PSC). Genome editing at (but not limited to) the PSC stage is indicated by a flash symbol. **A** HLA editing. **B** Immune checkpoint inhibition. **C** Combinatorial approach

There is, however, a relatively simple way to remove HLA class I from the cell surface by targeting the accessory chain, β-2-microglobulin (B2M), which is required for proper folding and surface trafficking of all HLA class I molecules [25, 26]. Indeed, inactivation of B2M is a common mechanism in cancer to evade immune surveillance [27, 28] and has been documented in ~30% of all checkpoint therapy-resistant melanoma cases [29]. Learning from what nature has already figured out, various genome editing tools have been employed to inactivate the *B2M* gene in hPSC (Table 1), which reflects the rapid evolution of the genome editing field over the last decade. The tools employed range from more traditional HDR-based methods [30, 31•] to TALENs [32, 33] and, more recently, CRISPR/Cas9-based tools [34, 35, 36••]. Expectedly, it has been found that B2M-deficient cells evade CD8+ T cell responses and are not subject to DSA binding [30, 31•]. B2M-deficient hPSC are viable and have been differentiated into a variety of cell types including cardiomyocytes [35, 36••], EC [36••, 37], vSMC [38••], megakaryocytes and platelets [33], iNKT [39], T cell [40•], and, most recently, sc-β cells [41••].

Removing HLA class I molecules from the cell surface, however, comes at a cost; while transplanted cells devoid of HLA may go undetected by CD8+ cytotoxic T cells, the lack of inhibitory signals provided by HLA (missing self) will render them a target for natural killer (NK) cells [42–45]. Early on, expression of the next to invariant HLA class Ib molecules HLA-E and HLA-G has been explored to counteract NK cell responses [31•, 46]. In the absence of B2M, HLA-E/G-B2M fusion constructs have been shown to traffic to the cell surface and to protect HLA-deficient hPSC and their progeny from NK cell attack in vitro [31•, 40•, 47]. While the inhibitory role of HLA-E on NK cells is well-established, it is less clear how much HLA-E contributes to allorecognition. There is evidence that HLA-E can present other peptides than the class Ia leader peptide [48], and HLA-E-specific antiviral T cell clones have been described recently [49, 50]. Given there are different subsets of NK cells that vary significantly between different individuals, it is not clear how many different NK cell ligands may have to be included to fully protect HLA-deficient cells from NK cell lysis. The deletion of B2M raises concerns about whether the function of engineered cells would be negatively affected. Indeed, B2M has been shown to interact with several other proteins of the MHC superfamily, including cluster of differentiation 1 (CD1), the homeostatic iron regulator (HFE), the neonatal Fc receptor (FCGRT), and major histocompatibility complex class I-related gene protein (MR1) [51–53]. Whether B2M inactivation will impact a therapeutic cell's function would have to be tested empirically for each cell type.

As an alternative strategy to B2M inactivation, several groups have targeted individual HLA alleles to increase the immunocompatibility of banked hPSC lines [54, 55]. More recently, multiplexing (the use of several sgRNAs at the same time) has been used to simultaneously excise selected HLAs from the genome of hPSC [38••, 56••, 57]. Han et al. [38••] were able to delete all six alleles of the three polymorphic HLA-A/B/C genes, while leaving the genes encoding the invariant HLAs (E/F/G) intact [38••].

Inspired by the HLA makeup of fetal trophoblasts, Xu et al. deleted the genes encoding the highly polymorphic HLA-A and -B genes, while maintaining HLA-C expression [56••]. This "HLA-C-retained" strategy may facilitate matching of banked iPSC lines; however, HLA-C can still present foreign peptides derived from the donor cells. While this can be seen as beneficial in clearing viral infections, it puts the transplant at risk of being rejected if miHA or NA are presented by HLA-C. Indeed, HLA-C-restricted T cell clones for certain viruses such as HCMV have been isolated [58]. During pregnancy, HLA-C is the only polymorphic HLA expressed by fetal trophoblasts, presumably mitigating the conflict between rejection of the hemi allogeneic embryo and clearing viral infections [59]. Pregnancy complications have, indeed, been linked to mismatches between maternal and fetal HLA-C [60].

Stem cell–derived pancreatic β (sc-β) cells have been differentiated from hESC that retained only the expression of one HLA-A allele, HLA-A2 [61]. HLA-A2 is the most common HLA-A allele in Caucasians, and the resulting sc-β should, therefore, be immuno-compatible with a large fraction of this population. The authors show that deletion of individual HLA alleles can protect sc-β from T cell-mediated rejection, and that genome editing did not affect sc-β cell differentiation and function. Moreover, retention of HLA-A2 did allow for HLA-E surface trafficking and reduced NK cell rejection [61]. A downside to this strategy is, however, that self-peptides can still be presented by HLA-A2 and thus, while circumventing alloimmunity, the cells may still be a target of ongoing autoimmunity.

Targeting HLA Class II

With more than 9700 different alleles described thus far in the human population, HLA class II genes contribute significantly to the major histocompatibility barrier (https://www.ebi.ac.uk/ipd/imgt/hla/about/statistics/). This diversity of the HLA class II surface proteins does not only make them a target for DSA, but they can also present miHA and NA to potentially cytotoxic CD4+ T cells [12, 62]. The most polymorphic HLA class II genes–HLA-DP, -DQ, -DR–are each composed of discrete alpha and beta chains that are encoded by individual genes, which would render editing quite cumbersome if one were to consider targeting each gene individually. HLA class II expression can be prevented by targeting the HLA Class II Transactivator (CIITA), a

Table 1 Immune-editing strategies to engineer "off-the-shelf" universal stem cells, multipotent progenitor cells, and their terminally differentiated hypoimmunogenic progeny

Knockout	Transgene	Cell type	Reference
B2M (AAV vector)	None	ESC	Riolobos et al. (2013), Mol Ther [30]
HLA-A (ZFN)	None	ESC	Torikai et al. (2013), Blood [54]
CIITA (TALEN)	None	ESC	Ding et al. (2013), Cell Stem Cell [66]
B2M (TALEN)	None	ESC	Lu et al. (2013), Stem Cell Rev and Rep [32]
B2M (TALEN)	None	iPSC (megakaryocyte, platelets)	Feng et al. (2014), Stem Cell Reports [33]
CIITA (CRISPR-Cas9)	None	ESC	Veres et al. (2014), Cell Stem Cell [67]
None	PD-L1, CTLA4-Ig (HPRT1 locus)	ESC (fibroblast, CM)	Rong et al. (2014), Cell Stem Cell [68•]
None	HLA-G (PiggyBac)	ESC	Zhao et al. (2014) Stem Cell Res [46]
B2M (CRISPR-Cas9)	None	HSC, T cells	Mandal et al. (2014) Cell Stem Cell [34]
B2M (HDR)	None	ESC	Wang et al. (2015) Stem Cells Transl Med [69]
B2M (AAV vector)	HLA-E/B2M fusion (HDR, B2M locus)	ESC	Gornalusse et al. (2017), Nat Biotech [31•]
B2M, CIITA (CRISPR-Cas9)	None	iPSC (CM)	Mattapally et al. (2018), JAHA [35]
HLA-B (CRISPR-Cas9)	None	iPSC (EC, MSC, chondrocytes)	Jang et al. (2019), Exp and Mol Medicine [55]
B2M, CIITA (CRISPR-Cas9)	CD47 (lentivirus)	iPSC (EC, CM)	Deuse et al. (2019), Nat Biotech [36••] Deuse et al. (2021), PNAS [70]
HLA-A, -B, -C, CIITA (CRISPR-Cas9)	PD-L1, HLA-G, CD47 (AAVS1 locus)	ESC (vSMC, EC)	Han et al. (2019), PNAS [38••]
HLA-A, -B, CIITA (CRISPR-Cas9)	None (HLA-C retained)	iPSC (CM, CD43+ cells) (EC, vSMC) (megakaryocyte, platelets) (CM, CD14+ monocytes)	Xu et al. (2019), Cell Stem Cell [56••] Luo et al. (2020), Cell Stem Cell [71] Suzuki et al. (2020), Stem Cell Reports [72] Kitano et al. (2022), Mol. Therapy [73]
B2M, CIITA (CRISPR-Cas9)	None	HECFC (EC)	Merola et al. (2019), JCI Insight [37]
None	Pdl1, H2-M3, Cd47, Cd200, Fasl, Serpinb9, Ccl21, Mfge8 (lentivirus)	mESC (neuron, muscle, CM, EC, DE)	Harding et al. (2019), BioRxiv [88•]
B2M (CRISPR-Cas9)	HLA-G/B2M fusion (HDR, B2M locus)	ESC (CM)	Shi et al. (2020), Stem Cells [47]
None	PD-L1 (lentivirus)	iPSC (Sc-β)	Yoshihara et al. (2020), Nature [85]
RNLS	None	iPSC (Sc-β)	Cai et al. (2020), Nat Metabolism [98]
HLA-B, -C, CIITA (CRISPR-Cas9)	None (HLA-A retained)	ESC (Sc-β)	Parent et al. (2021), Cell Reports [61]
HLA-A, -B, -DR (CRISPR-Cas9)	None (HLA-A, -B hemizygous)	ESC	Kim et al. (2021), Stem Cell Rev and Rep [57]
B2M, CIITA (CRISPR-Cas9)	None	HSC (NKT)	Li et al. (2021), Cell Reports Medicine [39]
B2M, CIITA, CD155 (HDR, CRISPR-Cas9)	HLA-E/B2M fusion (lentivirus)	iPSC (T cells)	Wang et al. (2021), Nat Biomed Engineering [40•]
B2M (CRISPR-Cas9)	PD-L1, HLA-E/B2M fusion; IL-10, TGF-ß, and IL-2 mutein (GAPDH locus)	ESC (Sc-β)	Gerace et al. (2022), BioRxiv [41••]

B2M beta-2-microglobulin, *HLA* human leukocyte antigen, *CIITA* class II transactivator, *ESC* embryonic stem cells, *HSC* hematopoietic stem cell, *iPSC* induced pluripotent stem cells, *EC* endothelial cells, *vSMC* vascular smooth muscle cells, *CM* cardiomyocytes, *AAV* adeno associated virus, *HDR* homology-directed repair, *DE* definitive endoderm, *HECFC* human endothelial colony-forming cells

bona fide transcriptional master regulator, which is necessary and sufficient for the concerted expression of all HLA class II genes [63]. Although its expression is mostly limited to professional antigen-presenting cells (APC), such as macrophages, dendritic cells, B cells, and activated T cells, which are all bone marrow derived, other cell types, mostly of the mesoderm lineage, have also been described to express HLA class II [64]. Vascularized grafts, in particular, pose a risk of rejection, since endothelial cells (EC) can upregulate HLA class II upon inflammatory conditions, such as in the presence of IFN-γ [65].

From the perspective of a cell engineer, the question then remains whether it is necessary to edit *CIITA* in cells that do not express HLA class II. With an eye on potential off-targets, a rule of thumb should be "the fewer edits, the better." Therefore, if a cell type, such as neurons or sc-β cells, does not express HLA class II, it should not be necessary to edit out the *CIITA* gene, although, for some cell types such as smooth muscle cells or cardiac fibroblasts, which are of mesodermal origin, it cannot be ruled out that they will express HLA class II under certain conditions, which would prompt T cell-mediated rejection if not taken care of.

Similar to *B2M*, inactivation of *CIITA* has been accomplished in hPSC early on using different editing modalities including TALENs [66] and CRISPR/Cas9 [67]. CIITA-deficient hPSC have been differentiated into a variety of cell types, including cardiomyocytes [35, 36••, 56••], EC [36••, 37, 38••, 71], vSMC [38••], megakaryocytes and platelets [72], and, most recently, CIITA-deficient iPSC have been differentiated into sc-β [61], T and NKT cells [39, 40•], and CD14+ monocytes [73].

Although the consequences of *CIITA* deletion have not yet been fully investigated in every cell type, loss-of-function mutations in humans are somewhat tolerated. They have collectively been grouped as bare lymphocyte syndrome (BLS) [74]. Affected individuals are overall healthy but suffer from recurring upper respiratory infections, which is thought to reflect the lack of CD4+ T cells. While there seem to be no overt developmental defects, it is not clear for every cell type whether disruption of *CIITA* will interfere with a particular cell's function. As determined by CHIP-Seq, it is estimated that there are more than 400 binding sites of CIITA in the human genome, yet the number of genes that are regulated by CIITA outside of the MHC locus seems to be fairly limited [75, 76]. This can, however, change under different environmental conditions, e.g., during viral infection. A recent report has described a new function for CIITA as a restriction factor limiting Ebola and Corona virus infection via expression of the p41 isoform of the invariant chain (Ii) [77].

As an alternative to compromising CIITA's function, it can be envisioned that other components of the HLA class II transcriptional network could be targeted, such as RFX5, RFX-AP, and -ANK, which, together with CIITA, form the HLA class II enhanceosome that drives HLA class II expression [74]. Yet again, the outcome of inactivation of these rather pleiotropic transcription factors would have to be assessed for every therapeutic cell type with regard to differentiation efficiency and functionality. Recently, a study from Korea has demonstrated that it is feasible to excise individual HLA class II genes (HLA-DR), with the intention to increase compatibility between the donor and the recipient [57]. Multiplexing may allow extending this strategy to include other HLA class II genes as has been demonstrated for the deletion of multiple HLA class I genes [38••].

Tolerance Induction

While targeting the major histocompatibility–HLA barrier–has become feasible and has been achieved using different gene-editing strategies, other underlying genetic differences between the donor and the recipient such as miHA and NA may still be detected by the immune system if presented indirectly by the recipient's APC. Other immune-editing strategies are thus being developed that employ the overexpression of immune-inhibitory ligands, or tolerogenic factors, of various types. The tolerogenic factors highlighted in Table 1 can be broadly classified into factors that a) directly inhibit cells of the adaptive immune system (T, B cells), b) target innate immune cells (MF, NK cells), and c) induce a local immunosuppressive microenvironment, such as immunosuppressive cytokines. Such secreted factors can impact other immune effector cells directly or result in induction or recruitment of suppressor cells such as regulatory T cells (Treg).

So far only a limited set of well-known inhibitory ligands has been explored for their protective effect in stem cells and their derivatives. The genes selected for overexpression have, in large part, been inspired by cancer immune evasion [78] and the immune interaction at the placenta [79]. HLA-E and HLA-G are monomorphic HLA class Ib genes expressed on placental trophoblast, where HLA-G is thought to control T and NK cell responses at the maternal-fetal interface [80, 81]. Expression of programmed death ligand 1 (PD-L1), the ligand for PD-1, an inhibitory receptor expressed predominantly on activated T cells but also on NK cells and subsets of macrophages, is coopted by trophoblasts and several cancer types. CD47, a molecule also highly expressed on placental syncytiotrophoblast and upregulated in certain cancers, interacts with macrophage receptors to deliver a "don't eat me" signal [82, 83].

One of the pioneering studies explored the two well-characterized immune checkpoint inhibitors–PD-L1 and CTLA4–for their potential to protect hESC and their derivatives from immune rejection in a humanized mouse model [68•]. CTLA4-Ig, a soluble form of the CTLA4 ectodomain,

which outcompetes binding of the activating co-stimulatory receptor CD28 to its ligands on DC, was knocked into the *HPRT1* locus of hESCs in combination with or without the checkpoint inhibitor PD-L1 to investigate the combined protective effect. These cells were subsequently differentiated into fibroblasts and cardiomyocytes and then transplanted into a humanized mouse model. The double knock-in line showed decreased activation of transplanted immune cells, as measured by immunofluorescent staining of infiltrating T cells up to 8 weeks post-transplantation.

Deuse et al. (2019) investigated whether a CD47 knock-in is an effective alternative to HLA-E or -G for overcoming the missing self-reaction [36••, 84]. B2m$^{-/-}$, Ciita$^{-/-}$, CD47 transgenic murine, and human PSCs, and their EC and cardiomyocyte progeny, were investigated for their immunogenicity in vivo following transplantation into allogeneic mice and a humanized NSG-SGM3 mouse model in the case of the human cells. CD47-overexpressing cells survived in vivo, as measured with bioluminescent imaging through the 50-day experiment. Transgenic human cells also demonstrated decreased activation of NK cells when compared with controls, as measured by IFN-γ secretion.

Han et al. [38••] inserted a construct containing CD47, PD-L1, and HLA-G at the AAVS1 safe-harbor locus into an HLA-A/B/C −/− background. Differentiated cells were then investigated in a variety of in vitro assays with T cells, NK cells, and macrophages, and in vivo following transplantation into immune-deficient mice reconstituted with pre-sensitized human CD8+ T cells [38••].

Recently, a PD-L1-overexpressing human sc β-like cell was reported to survive xenograft rejection in an immunocompetent diabetic mouse model [85]. Cells transduced with a PD-L1-encoding lentiviral vector showed improved glycemic control through 50 days following transplantation when compared with the wild-type control. Decreased immune infiltrates and engrafted cell survival were demonstrated, using flow cytometric analysis of recovered grafts. The result that PD-L1 can suppress all components of the immune system in a xenograft context is surprisingly powerful and may be explained by the broader functions beyond T cell co-inhibition that have recently been described, for example, by signaling through PD-1 expressed on macrophages [86, 87]. The results from Rong et al. [68•] in which the PD-L1 only line is rejected is not immediately reconcilable with this study. A higher level of PD-L1 expression as a consequence of the use of a lentiviral vector rather than a conventional knock-in into the *HPRT* locus could be a potential explanation. A most recent report has also observed rejection of sc-β cells endowed with PD-L1 in both the presence or the absence of the HLA barrier [41••]. Those differences are most likely a reflection of differences in experimental settings and, in particular, the respective mouse models being used.

A more expansive combinatorial strategy, in which eight immunomodulatory candidate transgenes were overexpressed, has also been investigated in a mouse allograft context [88•]. Piggybac transposon-mediated integration of Pdl1, Cd47, H2-M3 (a putative HLA-G ortholog), Ccl21, Fasl, Serpinb9, Cd200, and Mfge8 into a single mouse ESC line was accomplished. While the cells were able to form teratoma in fully immunocompetent animals, a few caveats with respect to the cell type and transgene expression levels make it difficult to draw strong inferences about the immune-editing strategy and the contributions of the individual components that were overexpressed. The cells were transplanted as stem cells, which are intrinsically less immunogenic than their progeny. Moreover, transplantation of highly proliferative cells can confound the extent to which cell survival itself can be measured. Interestingly, the modified cell line was able to protect co-transplanted human ESCs from immune rejection presumably by establishing an immunoprotective niche [88•]. This concept has recently been extended by Gerace et al., who overexpressed three immunosuppressive cytokines (TGFβ, IL-10, and IL-2 mutein that preferentially drives the amplification of Treg) and observed a survival benefit of the modified cells over the course of 9 weeks [41••]. The authors speculate that edited and unedited versions of their sc-β cell preparations could be mixed at different ratios in order to avoid compromising a therapeutic cell's function by heavy genomic engineering. Another group has recently demonstrated that mixing in an entirely different accessory cell type into their sc-β cell preparation–modified mesenchymal stem cells (MSC) that express the two checkpoint inhibitors–PD-L1 and CTLA4-Ig–provided a long-term survival benefit in trans for over 100 days without the use of systemic immune suppression [89].

Deletion of Co-stimulatory Ligands

The flip side to introducing checkpoint inhibitors and other inhibitory ligands is the deletion of co-stimulatory ligands in stem cells and their differentiated progeny that are required for maximal activation of immune cells. In the absence of such co-stimulatory signals, immune cells enter an anergic state as opposed to being triggered, which is a crucial safety checkpoint behind peripheral tolerance [90]. While the expression of the classical costimulatory ligands B7-1 and B7-2 is fairly limited to professional APC, other activating ligands are rather expressed under inflammatory or other stress conditions that mark virus-infected or, otherwise, aberrant cells for destruction by NK cells [42]. Deletion of the stress signals MICA/B has recently been reported, [91] which could potentially help to evade NK cell responses. CD155 or poliovirus receptor (PVR), another costimulatory ligand for NK, has recently been deleted in HLA-deficient

iPSC that were successfully differentiated into T cells [40•]. The expression of co-stimulatory molecules may also differ between stem and progenitor cells and their differentiated progeny and will certainly also differ between therapeutic cell types. Certain stem cell-derived cell types have been found to be poor stimulators in in vitro antigen presentation assays, e.g., vSMC [36••] or sc-β [41••]. Recently, an elegant CRISPR/Cas9 screen using hundreds of "DNA-barcoded" solid tumor cell lines has identified other activating and inhibitory ligands that are subverted by cancer cells to inhibit NK activation [92]. In conclusion, the right combination of checkpoint inhibitors to express and which co-stimulatory molecule to remove will have to be determined empirically for each therapeutic cell product.

Other Immune Barriers

Overshooting Inflammatory Response and Fibrosis

Other immune barriers might not be that obvious at first and are much harder to address using genome engineering. The mere act of transplantation will inevitably induce tissue damage and subsequent inflammation, resulting in and being exacerbated by neutrophil and macrophage infiltration of the graft. The role of innate immune cells other than NK cells in transplant rejection has only recently been appreciated and is still under active investigation [93, 94]. Uncontrolled fibrosis, tissue scarring, and occlusion of vascular grafts, in particular, can further compromise transplant function and viability.

Cell Stress and Exacerbated Cell Death

Overcoming barriers to successful engraftment can have a huge impact on the costs of a cell therapy. For example, excessive cell death due to activation of stress pathways during transplantation of pancreatic β cells impedes their clinical translation [95]. Interfering with those stress pathways could potentially improve cell survival during and post transplantation when a great number of cells are lost due to stress-induced cell death. Cell death can also expose NA that may result in an exacerbated immune attack [95]. For pancreatic β cells, it has been established that an upregulation of HLA class I in T1D patients is correlated with increased CD8+ T cell infiltration [96]. Leite et al. demonstrate that interfering with certain stress-related genes and genes involved in antigen presentation provided enhanced protection from cell death in vitro [97]. Recently, a genome-wide CRISPR screen in a diabetic mouse model has identified RNLS as a modifier of β cell vulnerability in vivo and as a potential target to avert β cell loss following transplantation into diabetic patients [98].

Chronic Graft Rejection

With long-term survival of transplanted cells in mind, chronic graft rejection becomes the next barrier to durable engraftment. While T cell-mediated acute rejection can be well managed using immunosuppressive drugs (or by removing the HLA barrier using genome editing), it is the chronic rejection that, in the long term, causes loss of transplanted cells and tissues. Chronic graft rejection is mediated by antibodies that have been generated against miHA and NA. If such antigens are expressed on the cell surface, for example, of EC, they can bind DSA that then activate the complement system or NK cells and elicit complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC), respectively. Such humoral responses (as opposed to T cell-based cellular immunity) are difficult to model in vitro, and the most informative preclinical animal models have historically been minipigs used to study xenograft transplantation [99]. The current record in genome engineering is held by a minipig strain with modifications in 13 genes and 42 different alleles introduced to overcome the xenobarrier [100].

Prior Immune History

Another factor to consider is the prior immune history of the recipients and their immune status–a combination of DSA and immune memory cells–at the time of transplantation. Prescreening for DSA is already routine in bone marrow transplantation and can reveal a prior history of exposure. Immune memory can be a barrier to transplantation, especially upon repeated dosing with allogeneic cells but may be circumvented by DSA prescreening and careful profiling of the immune cell repertoire. In addition, the contribution of innate immune memory to transplant rejection might aggravate inflammatory responses during a secondary transplant [94].

Conclusions and Future Perspectives

While overcoming the immune barriers to stem cell transplantation may have sounded like a pipe dream until recently, genome editing technology applied to stem cells is quickly making it a reality. Already, the major histocompatibility barrier–represented by HLA class I and II genes–has been taken down using different genome editing approaches. Moreover, strategies have been developed to overcome the "missing self" dilemma of HLA-deficient cells, and engineering efforts have started to also address other immune cells that participate in graft rejection such as NK cells and macrophages.

Removal of HLA can, however, only partially overcome the barrier posed by miHA. While miHA cannot be presented by a therapeutic cell in the absence of HLA, they can still be presented indirectly by APC in the draining lymph node and drive the generation of DSA that can trigger ADCC and complement activation if miHA are expressed at a transplant's cell surface. WGS, in combination with exome sequencing, could come up with a personalized immunogenicity score with regard to the genetic differences between the donor and the recipient. This could assist in both identifying a suitable donor cell line that is matched to the greatest extent with the recipient's genetic diversity and, at the same time, serve as a roadmap to the cell line's further editing to increase compatibility.

Both prolonged culture and genome editing using CRISPR/Cas9-based technologies can introduce NA and pose the risk of cellular transformation. Indeed, it has been found that the majority of hPSC lines harbor mutations in p53 [101]. More recently, it has been noticed that the genotoxic stress resulting from double-stranded breaks during genome editing can enrich for cells with a deficient p53 pathway, hence increasing their neoplastic potential [102]. Of course, a careful assessment of off-targets, genetic stability, and NA load is warranted before clinical use of any engineered cell line or its progeny, and including a suicide switch that will allow ablation of the transplant in case of adverse events may help to address safety concerns [103, 104].

Emerging 2nd-wave CRISPR/Cas9-based technologies such as base editing [105–107] and prime editing [24] that do not rely on double-stranded breaks may help to further de-risk stem cell engineering. Base editing in particular may lend itself to multiplexing–targeting multiple genes at once–with greater efficacy and less toxicity [108]. Base editing of both *B2M* and *CIITA* has recently been reported in primary T cells to decrease the immunogenic burden of allogeneic CAR T cells [109, 110]. It is conceivable that base editing will be used in future studies to adjust the number of miHA or NA load in a given stem cell line to decrease their immunogenicity score.

We see great scope for mining of other tolerogenic factors and to test these more comprehensively. The molecules employed thus far (Table 1) represent only a small selection of candidate genes that can be investigated [111], and recent single-cell analyses of tumor infiltrating T cells keep adding candidates to the list that tumors exploit to keep immune cells in check [112]. PD-L1, for example, is only one molecule in the B7 family that can suppress effector mechanisms of immunity, including VISTA (B7-H5), VTCN1 (B7-H4), CD276 (B7-H3), and the members of the butyrophilin B7-like family [113–115]. Immunotherapies targeting several of these pathways are in clinical development; the results of which can be used to hone in on candidates that have validated data in humans. Recently, sc-RNA sequencing has identified new ligands expressed in the placenta that could also be investigated for their protective effects [116]. Conversely, characterization of the receptor profile of primary immune cells of a recipient of a cell therapy would allow for greater mechanistic understanding of protection, for example, of how the killer Ig-like receptor (KIR) repertoire on NK cells impacts the effectiveness of strategies to inhibit the missing-self reaction [117].

Last but not least, the rapid technical advances in the genome editing field have fueled a boom in cell engineering not only in academic centers. In industry, the goal is to drive down the cost of production and, therefore, make these, in the beginning very costly, treatments more widely available to any patient in need [118]. While there has been some progress in circumventing hyperacute and acute graft rejection, it is still not clear how a cell product will look that will withstand immune rejection in the long term. Factors that weigh into the design of a donor cell line are the unique immunostimulatory landscape of a particular therapeutic cell type, the recipient's immune status at the time of transplantation, as well as how long the transplanted cells are expected to persist in the recipient. Next-generation cell products will thus most likely reflect a combination of banking of carefully curated immune-compatible cell lines that are further edited to transform them into "off-the-shelf" products compatible with a wider range of patients.

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Compliance with Ethical Standards

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- • Of importance
- •• Of major importance

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