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REVIEW **Open Access**

Engineering hypoimmune stem cell-derived beta cells

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Abstract
In type 1 diabetes (T1D), autoimmune targeting destroys insulin-producing β cells in the pancreas, creating a chronic state of insulin deficiency. Islet transplantation presents a regenerative cell therapy approach that can re-establish insulin production and intrinsic glycemic control. However, islet transplantation is currently limited by a lack of cadaveric human islet donors and a requirement for life-long immune suppression following transplant. Developments in stem cell maturation and differentiation protocols have enabled production of insulin-producing cells 'on demand', thereby addressing the pancreatic donor tissue shortage. Continued reliance on immune suppression to avoid graft rejection, however, can result in opportunistic infection and malignancy, thus remaining a major obstacle for wide-spread application of insulin-producing β cell transplantation. As such, there has been significant interest in identifying alternative strategies for avoiding graft rejection without immune suppression including encapsulation and co-transplantation of accessory immunomodulating cells. However, these approaches are limited by incomplete immune isolation as well as concerns over maintenance of effector function and graft survival in vivo, respectively. Genetically engineering hypoimmune stem cell-derived β cells has thus emerged as a promising strategy for improving islet transplantation outcomes. These approaches leverage our understanding of pathways involved in immune regulation to selectively protect the transplanted insulin-producing cells without affecting systemic immune function. This review will summarize recent bioengineering approaches for generating hypoimmune stem cell-derived β cells. It will also discuss relevant safety concerns and potential genetic targets for future investigation that take inspiration from the development of immune evasive primary islets and chimeric antigen receptor (CAR) T cells.

Keywords Pancreatic beta cells, Islet transplantation, Cell therapy, Hypoimmune, Genetic engineering

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Introduction

Type 1 diabetes (T1D) is a chronic disease that is characterized by autoimmune targeting and destruction of pancreatic β cells resulting in absolute insulin deficiency [1, 2]. T1D constitutes approximately 2–10% of all diabetes cases globally, although the incidence is increasing annually and estimates of prevalence in Europe are greater than 15% [3–5]. The current standard of treatment for the past century, exogenous insulin injection, does not prevent progression of diabetes and predisposes patients to hypoglycemic events [6, 7]. There is thus a significant interest in islet transplantation as an alternative and regenerative therapy option for T1D.

Islet transplantation, however, is currently limited by a requirement for life-long immune suppression which increases the risk of cancer and development of serious infection [8]. This was emphasized by a recent clinical trial (ClinicalTrials.gov identifier: NCT04786262) where all β cell transplant recipients experienced significant adverse effects related to immunosuppression [9]. Additionally, a lack of donor tissue limits the application of islet transplantation to patients with severe hypoglycemic unawareness [10]. Research on the development and maturation of pancreatic progenitors into islet cells has enabled the differentiation of stem cells into insulin-producing β cells [11]. This advancement promises to provide a theoretically infinite supply of β cells, thereby overcoming the donor shortage and making this therapy option more feasible.

However, reliance on immune suppression for successful engraftment remains a major obstacle for islet and insulin-producing β cell transplantation. Strategies to avoid rejection of islet grafts without immune suppression have included immune evasion by encapsulation [12], as well as co-transplantation with bone marrow-derived mesenchymal stem cells (MSC) [13, 14], regulatory T (Treg) cells [15, 16] and CAR Treg cells [17]. A further benefit of accessory cell co-transplantation is that it provides their additional biological activity, such as pro-angiogenic signaling and anti-apoptotic effects, which can be leveraged to overcome the physiological challenges of the desired implantation site [18, 19]. Furthermore, invasiveness of accessing the bone marrow for stem cell harvesting, a potential limitation with regards to clinical translation, may be overcome by utilization of adipose tissue which has been identified as a less invasive and higher yield source of MSCs [20, 21]. However, the survival and maintenance of immunomodulatory activity of accessory cells following co-transplantation have been called into question which may limit these approaches [22–24]. Furthermore, islet encapsulation is intrinsically limited by a requirement to simultaneously reject immune cell infiltration while not restricting nutrient exchange [25]. The physical barrier may restrict blood vessel ingrowth making nutrient exchange dependent on diffusion thus delaying the response to changes in glucose levels [26]. It has also been demonstrated that islet encapsulation may be insufficient at limiting diffusion of proinflammatory cytokines [27] and antibodies [28]. Additionally, the foreign body response (FBR), a normally protective fibrotic immune reaction that encapsulates foreign material that cannot be cleared by phagocytosis, may further isolate the islet cell-carrying device from the recipient's tissue and circulation [29]. There is thus a requirement for more targeted and robust strategies for immune evasion of islet grafts.

Along this line, there has been a recent interest in engineering hypoimmune stem cell-derived islet cells to enable graft survival without immune suppression. These strategies mainly focus on modifying the expression of cell-surface ligands on the transplant tissue that either stimulate (e.g., human leukocyte antigen [HLA] class I) or inhibit (e.g., programmed death-ligand 1 [PD-L1] and CD47) host immunity. A further notable advantage of generating immune evasive islets is that they may also be less susceptible to targeting in the islet antigen-primed host environment [30]. The potential that these approaches hold is recapitulated by ongoing clinical trials using immune evasive, stem cell-derived islets (NCT05210530, NCT05565248; results not published). Notably, these developments have recently enabled the groundbreaking first-in-human trial of genetically modified allogeneic islets with positive six-month clinical results [31] (Fig. 1).

While promising, there is currently a lack of consensus regarding what ligand modifications are most effective for developing hypoimmune islets. This review will outline the genetic engineering strategies that have been reported for stem-cell derived β cells to reduce graft rejection at both the integration and maintenance phase (Fig. 2, [32–39]). With regards to future perspectives, additional genetic targets are discussed which take inspiration from developments in designing hypoimmune primary islets and CAR T cells as well as relevant ethical and safety concerns of transplanting immune evasive stem cell-derived β cells. Ultimately, these strategies aim to address the major obstacles currently limiting islet transplantation and contribute to making this a more feasible and widespread therapy option for T1D.

Integration phase (induction of immune protection)

Success of islet transplantation is highly dependent on sustaining islet health with the accumulation of insults reducing graft outcomes. Islet quality diminishes beginning in the donor prior to procurement due to an increase in circulating inflammatory cytokines after brain death [40]. The isolation procedure also presents a source

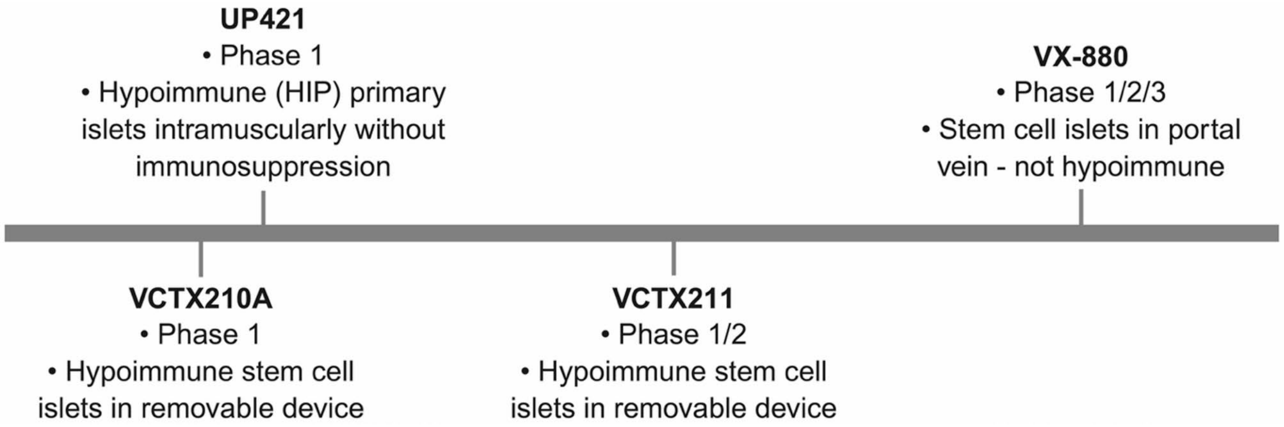


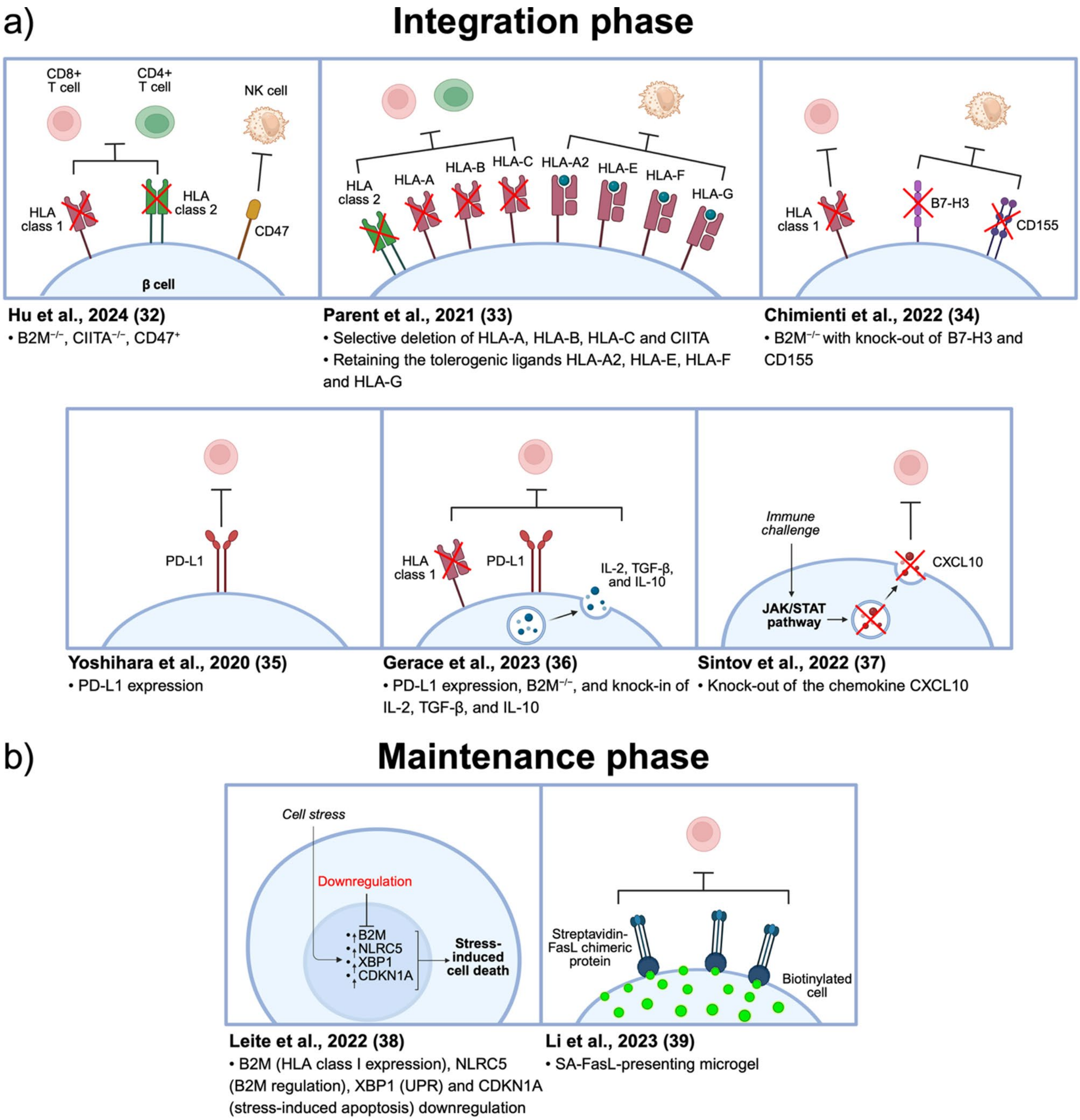
Fig. 1 Overview of ongoing clinical trials for stem cell-derived and/or hypoimmune islets. All trials are being conducted in T1D patients. ClinicalTrials.gov identifiers are NCT05565248 (VCTX210A), NCT06239636 (UP421), NCT05210530 (VCTX211) and NCT04786262 (VX-880). The figure was created using BioRender

of islet injury due to separation from the vasculature and extracellular matrix resulting in ischemia and anoikis-mediated damage, respectively [41]. Furthermore, there is a significant acute post-operative loss of graft tissue due to inflammatory processes. For example, islet injection into the hepatic portal vein, the current clinical standard, elicits an instant blood-mediated inflammatory response (IBMIR) which is triggered from interaction between tissue factor on the islet cell surface and factor VIIa [42, 43]. This elicits a coagulation response, complement activation, cytokine release and leukocyte recruitment resulting in substantial graft loss [44]. Alternative implantation sites that avoid IBMIR have been investigated, such as under the kidney capsule and into the omentum. However, these sites are affected by immune rejection, poor long-term survival and limited robust clinical testing [45]. This rejection response may furthermore be heightened in T1D patients due to presence of islet antigen-primed immune cells that result from the underlying autoimmune disease [46]. The subcutaneous site has also been investigated in this regard and is attractive as it enables graft monitoring and retrieval. However, a relatively low oxygen tension renders islets hypoxic which is not conducive to transplant success. In the context of islet transplantation, graft failure results in a reoccurrence of insulin dependence and necessitates re-transplantation.

Thus, to achieve successful engraftment and integration, the acute immune response must be overcome. Currently, immune suppression protocols enable sufficient graft survival to achieve functional outcomes. Although the additional benefit conferred by immune suppression, namely inhibiting autoimmunity in T1D patients, has been discussed in the literature [43], significant negative systemic effects have been well established and highlight the need for alternative solutions. As such, genetic modification of ligands expressed on β cells that are associated with immune activity may serve as a more targeted approach for achieving graft acceptance. This section will review stem cell-derived β cell-associated immune-modulating ligands and the effect of their genetic modification on graft survival.

HLA editing

Major histocompatibility complex (MHC), which is also called HLA in humans, is a genetic locus that encodes key cell-surface proteins that mediate immune surveillance and activation. While HLA class II is only expressed on antigen-presenting cells (mainly dendritic cells and macrophages) to present exogenous antigen, the universally expressed HLA class I mediates intracellular antigen presentation which is recognized by CD8 + T cells thereby initiating cytotoxic killing. HLA class I consists of a heavy alpha chain and a β_2-microglobulin (B2M). Disrupting cellular immunogenicity against transplanted tissue has been achieved by downregulation of either of these subunits [47, 48] as well as the transporter associated with the antigen processing (TAP) family of proteins [49] which is involved in peptide processing and loading of HLA in the endoplasmic reticulum (ER). This strategy has also been combined with knock-out of the transcription factor class II MHC transactivator (CIITA) which results in the downregulation of HLA class II [50]. However, universal depletion of HLA class I results in a 'missing-self' signal that stimulates natural killer (NK) cell-dependent lysis [51]. NK cell activity ultimately depends on a balance of inputs from cell-surface activation and inhibitory receptors [52]. These immune cells respond to antibody-coated pathogens via Fc (CD16) receptors as well as neoplastic or virally infected cells that either downregulate HLA class I to avoid T cell cytotoxicity or express stress-related factors on the cell surface. Avoiding NK cell alloreactivity has proven to be a challenge due to the high degree of heterogeneity of receptor



Hu et al., 2024 (32)
- B2M$^{-/-}$, CIITA$^{-/-}$, CD47^{+}

Parent et al., 2021 (33)
- Selective deletion of HLA-A, HLA-B, HLA-C and CIITA
- Retaining the tolerogenic ligands HLA-A2, HLA-E, HLA-F and HLA-G

Chimienti et al., 2022 (34)
- B2M$^{-/-}$ with knock-out of B7-H3 and CD155

Yoshihara et al., 2020 (35)
- PD-L1 expression

Gerace et al., 2023 (36)
- PD-L1 expression, B2M$^{-/-}$, and knock-in of IL-2, TGF-β, and IL-10

Sintov et al., 2022 (37)
- Knock-out of the chemokine CXCL10

Leite et al., 2022 (38)
- B2M (HLA class I expression), NLRC5 (B2M regulation), XBP1 (UPR) and CDKN1A (stress-induced apoptosis) downregulation

Li et al., 2023 (39)
- SA-FasL-presenting microgel

Fig. 2 Schematic overview of strategies for bioengineering hypoimmune β cells. Immune evasive strategies that have been tested aim to overcome graft rejection **a** in the acute integration phase or **b** in the maintenance phase. The figure was created using BioRender

expression both within [53] and between [54] individuals. Nonetheless, strategies for achieving immune evasion of stem cell-derived tissue grafts have targeted HLA class I, utilizing additional strategies to avoid NK cell stimulation. Techniques that have been investigated in this regard include B2M$^{-/-}$ pluripotent stem cells (PSC) with co-expression of the minimally polymorphic HLA-E [55], overexpression of the "don't eat me"-signalling ligand CD47 [56], HLA-C retention [57], as well as CIITA$^{-/-}$ with expression of PD-L1, HLA-G and CD47 [58].

Application of these immune-evasion principles has been tested by selective deletion of HLA class I alleles (HLA-A, HLA-B and HLA-C) and knock-out of CIITA in human PSCs (hPSC) while retaining the tolerogenic ligands HLA-A2 as well as HLA-E, HLA-F and HLA-G [33]. These modifications yielded functional insulin-producing cells with protection against NK cell and T cell-mediated allorejection [33]. Chimienti et al. furthermore identified B7-H3 and CD155 as major ligands on iPSC-derived β cells involved in NK stimulation [34]. Compared to B2M$^{-/-}$ iPSC-derived β cells, simultaneous knock-out of B7-H3 and CD155 reduced CD16^{+} NK cell-mediated cytotoxicity and, notably, HLA-E-insensitive CD16^{dim} NK cell-dependent lysis [34]. While these results demonstrated a strategy for improving immune evasion including NK cell-mediated cytotoxicity, stem cell line-specific NK cell-stimulating ligands remain an important consideration that may alter the effectiveness of this approach. This study was furthermore limited by a focus on acute rejection [34]. Further investigation is thus necessary to elucidate the long-term immune response to these strategies for evading NK cell-mediated rejection.

Similarly, Hu et al. have reported promising results with B2M$^{-/-}$, CIITA$^{-/-}$, CD47^{+} insulin-producing hypoimmune (HIP) cells [59]. Allogeneic HIP primary islets (human [60] and rhesus macaque [32]) and induced pluripotent stem cell (iPSC)-derived pseudo-islets (human [32]) were successfully engrafted and re-established glycemic control in immunocompetent diabetic humanized mice and rhesus macaques, respectively. Furthermore, these hypoimmune cells did not elicit T cell or NK cell activation or cytotoxicity in tests conducted on blood samples retrieved from rhesus macaques allogeneic HIP cell transplant recipients [59]. Hu et al. also showed that IgM and IgG antibody production was not elevated in these samples [59]. Notably, the Schrepfer group recently reported a milestone first-in-human clinical study using HIP cells without immunosuppression (NCT06239636). Donor islets were dissociated into single cells and genetically modified using clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 12b (Cas12b) editing to knockout B2M and CIITA as well as lentiviral transduction of CD47 [61]. The HIP-modified islet cells (UP421) were then transplanted intramuscularly into the forearm of a 37-year old T1D patient [61]. The group has reported survival and function of the transplanted islet cells 6-month post-operatively without any adverse events attributed to the allograft [31]. This groundbreaking achievement, which demonstrated both safety and efficacy of this regenerative therapy, is a significant step in making islet transplantation a more feasible treatment option for T1D patients and is a testament to the potential hypoimmune bioengineering strategies have as an alternative to immunosuppression.

PD-L1 editing

PD-L1 plays a critical role in the immune checkpoint by interacting with the inhibitory receptor programmed death-1 (PD-1) on T cells thereby decreasing T cell activity [62]. Expression of PD-L1 has thus been investigated as a strategy for immune evasion and has been demonstrated to reduce islet allograft rejection [63]. Yoshihara et al. expressed WTN4, which is involved in islet maturation and insulin production [64, 65], in human iPSC generating mature insulin secreting cells [35]. PD-L1 expression in these cells protected against both xenograft rejection in STZ-diabetic C57BL/6 mice and allorejection in humanized non-obese diabetic severe combined immunodeficiency (NOD-SCID) mice [35]. These findings suggest that WTN4 expression in human iPSCs may be a strategy for producing mature stem cell-derived islets and that PD-L1 expression may improve graft survival by reducing graft rejection. However, results regarding the efficacy of PD-L1 expression in stem cell-derived islets for immune protection have been mixed. A combination of HLA deletion (B2M$^{-/-}$) and PD-L1 expression, which has been shown to reduce CD8 + T cell activation in human PSC-derived β cells [66], was demonstrated to be insufficient at preventing long-term allograft rejection of human embryonic stem cell (hESC)-derived β cells [36]. However, additional knock-in of the tolerogenic cytokines IL-2, TGF-β, and IL-10 resulted in improved allogeneic transplant survival, euglycemia and enhanced Treg recruitment [36]. Thus, while PD-L1 expression has demonstrated promise for reducing graft rejection of stem cell-derived β cells, this approach alone may be insufficient without additional immune-evasion strategies. It is therefore evident that further research is warranted to identify the combinations of genetic modifications most adept at generating fully immune-evading stem cell-derived β cells.

Cell signaling editing - pro-inflammatory chemokine CXCL10 editing

Another strategy that has been investigated with the goal of protecting stem cell-derived β cells from immune rejection is targeting the signalling pathway which mediates inflammatory immune cell recruitment (i.e., chemotaxis) to the graft tissue. For example, Sintov et al. used single-cell RNA sequencing and whole-genome CRISPR screening to identify JAK/STAT type II interferon as a major pathway augmented following immune challenge in hESC-derived β cells [37]. Knock-out of the chemokine CXCL10, which is downstream of the JAK/STAT pathway, yielded hypoimmunogenic islet cells with improved survival following allotransplantation into humanized mice [37]. CXCL10 is a chemokine that regulates inflammatory chemotaxis and, thereby, graft rejection following transplantation [67]. Notably, these CXCL10-deficient islets were protected from both T cell and NK cell-mediated rejection in vitro [37]. However, a limitation of this study, which used peripheral blood mononuclear cell (PBMC)-injected NOD mice as recipients for the modified hESCs, is that only engraftment of T cells, and not NK cells, was successful in this animal model [68].

Investigation into the effect of CXCL10 knock-out on NK cell-mediated rejection in vivo is thus required to determine its applicability in clinical trials.

Improving implant longevity (maintenance of immune protection)

The road to achieving and maintaining euglycemic control is long and arduous for islet grafts as a result of a relentless exposure to insults and stressors. Once the integration phase is overcome, islet grafts remain exposed to chronic inflammation mediated predominantly by activated lymphocytes which is a significant barrier to maintaining long term islet graft survival [69]. The median longevity of islet grafts in the clinical context is approximately 5.9 years [70]. Strategies that reduce the susceptibility of stem cell-derived β cells to chronic stressors may improve long term maintenance of islet graft survival and function as well as reduce the requirement for re-transplantation. To this end, approaches that have been investigated include modification of cell stress signaling pathways as well as induction of local immune privilege at the implant site.

Cell signaling editing - stress pathways

β cell stress following transplantation contributes to a decrease in function and survival of the transplant graft. This cell stress is triggered by a variety of factors including glucotoxicity [41], inflammatory cytokine signaling [40], the islet isolation process (in the context of primary islet transplantation) [71] and hypoxia prior to vascularization of the implant tissue [72]. Along these lines, Leite et al. characterized the stress response of β cells to factors inducing inflammatory, metabolic and ER stress [38]. Metabolic stress in transplanted islets is largely a result of the increased demand for insulin production due to chronic hyperglycemia [73]. ER stress is a response to elevated protein (i.e., insulin) demand outweighing production capacity resulting in an unfolded protein response (UPR) and, ultimately, cell death [74]. The upregulated genes that were identified, B2M, NLRC5, XBP1 and CDKN1A, are involved in HLA class I expression, B2M regulation, the UPR, and stress-induced apoptosis induction, respectively [38]. Downregulation of these genes protected stem cell-derived β cells from stress-induced cell death in vitro [38]. While this approach does not directly target immune evasion, it may serve as an adjunct to hypoimmune engineering strategies that improves the robustness of β cell grafts against the stresses of transplantation and thereby improve implant longevity. This technique could also take advantage of the interplay that exists between cell stress and immune susceptibly. For example, a major target of NK cell cytotoxicity are cells presenting stress-induced factors. Thus, decreasing cell stress in the graft tissue may decrease the susceptibility of the transplanted β cells to immune-mediated rejection. Furthermore, by decreasing activation of the UPR, these modifications may simultaneously enhance insulin production, thereby improving graft function.

Cell signaling editing - FasL

Another approach to bridging the gap from tolerance induction to maintenance is achieving localized immune privilege at the implant site. Accordingly, research along these lines has included investigation of molecules, such as Fas ligand (FasL), that are expressed in naturally occurring immune privileged sites like the eye and testis [75, 76]. FasL is a tumor necrosis family protein that interacts with the death receptor Fas on the target cell surface [77]. It contributes to immune privileged sites by binding to its receptors on Fas-expressing immune cells. This sets off a signaling cascade to induce apoptosis, thereby limiting the local immune response as well as preventing inflammation and tissue damage. With regards to islet transplantation, transgenic expression of a streptavidin-FasL (SA-FasL) chimeric protein, which involved biotinylating cells such that the SA-FasL chimeric protein would bind to biotin on the cell surface, has been shown to result in long-term graft survival without chronic immune suppression [78, 79]. Thus, this strategy protected islets from graft rejection beyond the induction phase and into the maintenance phase. Achievement of immune privilege was confirmed by successful secondary engraftment of unmodified (non-FasL expressing) islets into the same site following primary graft destruction [78]. This response was dependent on FoxP3 + Tregs, a finding which is further supported by similar studies in which SA-FasL-presenting microgels improved islet graft survival [39, 78, 80, 81]. However, contradictory results have reported elevated neutrophil-mediated rejection of allotransplanted transgenic FasL-expressing murine islets [82]. A possible explanation for this is that matrix metalloproteases can cleave FasL, which is normally cell surface bound, with soluble FasL having been demonstrated to act as a chemokine for neutrophil recruitment [83]. This explanation is supported by the successful islet engraftment using the SA-FasL chimeric protein approach which expresses only the extracellular binding domain of FasL lacking the metalloproteinase cleavage site [78]. Nevertheless, concerns over the potential for neoplastic development in induced immune privileged sites may limit the clinic applicability of this approach. Furthermore, the dependence on Tregs may exclude combining this method with chemokine and pan T cell inhibition strategies. As such, while this approach highlights the importance of realizing the immune modulating potentials of naturally occurring systems, such as immune privileged sites, further research is required to address the efficacy and safety concerns of expressing FasL in transplanted stem cell-derived β cells.

Future perspectives

Broader scope of application

1. Relevance to additional patient populations

The promise of hypoimmune stem cell-derived β cells extends beyond its potential to revolutionize treatment for T1D patients. A further patient population to whom these bioengineering developments may be of particular relevance is those with type 2 diabetes (T2D). This recognition has emerged as a result of the All New Diabetics in Scania (ANDIS) classification system which more accurately represents the underlying aetiologies of different diabetes subtypes [84]. The ANDIS classification system includes severe insulin-deficient diabetes (SIDD), a subtype of T2D which is characterized by the absence of anti-glutamic acid decarboxylase (GAD) antibodies in addition to several other clinical parameters including early onset, low insulin secretion and elevated hemoglobin A1c (HbA1c) [85, 86]. Crucially, although SIDD is genetically distinct from T1D, these patients often present clinically with symptoms resembling T1D, including low levels of C-peptide, severe hyperglycemia and an elevated risk of diabetic ketoacidosis [86, 87]. *Verhoeff et al.* discuss the potential of islet transplantation as a therapy in these insulin-sensitive patients who at present continue to be diagnosed with T2D based on the current classification system [86]. However, the authors highlight that clinically defining SIDD exclusively on the presence of anti-GAD antibodies neglects the potential contributions of other antibodies indicated in T1D, such as anti-islet antigen 2 (IA-2) and anti-zinc transporter 8 (ZnT8) antibodies[86]. Nevertheless, given the severely depleted capacity for insulin production, a re-evaluation of SIDD and other insulin sensitive T2D sub-types as an indication for islet transplantation may be appropriate and should be considered. This was supported by a pilot clinical study reporting the autologous transplant of endoderm stem cell-derived islet tissue into a T2D patient with impaired islet function resulting in significantly improved glycemic control [88]. This was further highlighted by reports of similar improvements in HbA1c levels in T1D and T2D patients receiving simultaneous or successive kidney and pancreas transplantation [89, 90]. As such, further research should continue to investigate how the impact of the bioengineering strategies discussed in this review may extend beyond the treatment of T1D and their potential for improving therapies for other diseases such as SIDD.

2. Applying hypoimmunogenic bioengineering strategies to other cell types

Given the pancreas donor shortage as a severe bottleneck for islet transplantation, researchers have looked to alternative sources of insulin-producing cells, in addition to stem cells, that may enable us to overcome this current limitation. In this vein, there has been significant interest in the possibility of utilizing xenogeneic β cells which would significantly increase the supply of transplantable tissue. Though promising, the robust rejection response mounted against grafted tissue from another species renders xenotransplantation a highly challenging endeavour. Nevertheless, progress has been made in this regard including the knockout of the three main xenoantigens against which humans have preformed xenoreactive antibodies (Gal, Neu5Gc and Sda) as well as expression of human genes that offer immunological protection [91, 92]. Notably, developments in this field enabled the first-in-human porcine heart xenotransplantation [93]. Although the xenograft recipient died 2 months postoperatively, this milestone clinical report demonstrated the progress being made in xenotransplantation [93].

In addressing the challenges associated with xenotransplantation, there is significant interest in identifying the optimal donor species. With respect to liver xenotransplantation, pigs are considered the gold standard source given their relative anatomical and genetic similarities to humans, availability as well as ability to be genetically modified [94]. Similarly, neonatal pigs are seen as an attractive source for xenogeneic islets given that the primary protein structure of porcine and human insulin differs at only a single amino acid [95]. Furthermore, their advantages include a lower cost for both animal housing prior to islet isolation (less than 2 weeks) and the isolation procedure itself compared to fully grown pigs, being highly proliferative in vivo as well as potentially more robust to cell stresses including hypoxia [96–98]. However, their diminished insulin production capacity necessitates a maturation period and a great number of animals is required to account for lower islet isolation yields [97, 99]. The potential for neonatal porcine islet xenografts to improve glycemic regulation in T1D patients has been demonstrated in clinical trials (e.g., NCT01739829) which reported maintenance of HbA1c and a reduction in hypoglycemic events [100]. Given the particular need for immune evasion in xenotransplantation, genetic modification approaches that render these cells hypoimmune could contribute to improved transplant outcomes. However, in addition to concerns about the risk of zoonotic disease transmission, xenotransplantation has sparked significant ethical discussions regarding issues such as animal welfare, human and animal identity as well as a weighing of the risks and benefits to transplant recipients [101, 102]. Nonetheless, it is evident that the relevance of hypoimmunogenic bioengineering strategies extends beyond stem cell-derived β cells to other cell types, such as neonatal porcine islets, which may contribute to overcoming the donor shortage.

Safety considerations

To enable clinical translation, the ultimate goal with regards to treating T1D, considerations over the safety of the transplant tissue will be of primary importance. For example, genetic modifications that decrease immune cell activity may result in decreased monitoring and control of local infection [103]. Misra et al. discuss the possibility of this leading to pathogen-mediated destruction of the islet graft or yield an infection-prone immune privileged site [104]. Furthermore, while stem cells show promise for overcoming the pancreatic donor tissue shortage, safety concerns have been raised over incomplete maturation of stem cells resulting in uncontrolled proliferation and neoplastic transformation [105]. The aforementioned stem cell-based genetic engineering strategies could enable bypassing of cell cycle checkpoints and allow such aberrant growth to go undetected from immune surveillance, increasing concerns over the safety of this strategy [106]. A potential safety mechanism that has been investigated to minimize this risk involves implementation of a drug-inducible cell suicide switch. This has been shown to enable targeted killing of ESCs [107] while genetically engineered hypoimmune islets have been effectively eliminated using the CD47-targeting antibody magrolimab [60]. By enabling targeted and on-demand destruction of graft tissue, this strategy may improve the safety of stem cells for use in islet transplantation.

Similarly, transplantation within a removable device would enable graft retrieval if necessary [105]. However, a relevant consideration for this approach is that islets are sensitive to the physical and mechanical properties of their microenvironment and exhibit improved function when these properties resemble those of the native pancreas [108]. Delivery devices could potentially provide a milieu with sub-optimal stiffness characteristics compared to implantation directly into natural tissue, thus affecting transplantation outcomes. The success of implantable devices has furthermore been fraught by the foreign body response. This immune-mediated reaction is triggered by the unsuccessful phagocytosis of a foreign object by macrophages resulting in a persistence of the pro-inflammatory M1 phenotype and fusion into foreign body giant cells (FBGC) [109]. Cytokine and chemokine secretion from recruited lymphocytes also induce fibroblast α-smooth muscle actin (α-SMA) expression resulting in a switch to the soluble collagen-secreting myofibroblast phenotype [110]. Ultimately, this results in the generation a hypopermeable, dense collagen-rich fibrotic capsule surrounding the implanted device [111]. In the context of islet transplantation, the isolation of the graft from the recipient's vasculature decreases the cells' viability by impeding nutrient exchange and waste removal as well as β cell function by impairing glucose monitoring and endocrine secretion [112]. Various strategies have been investigated to reduce the FBR of implantable devices including coating of biomaterials with anti-inflammatory molecules [113], alteration of physical properties [114] and selection of low-fouling polymers, such as poly(ethylene glycol) (PEG), which demonstrate reduced protein adhesion [115]. Our lab has previously demonstrated that mechanical actuation of an implantable soft dynamic reservoir resulted in a reduction of the fibrotic capsule thickness and number of α-SMA + myofibroblasts [116]. While encapsulated cell damage due to mechanical actuation is a potential limitation of this strategy, alternative approaches such as actuation around the periphery of the device may address this concern [117]. Although continued research is necessary to validate the efficacy of these strategies, they may serve to improve the safety profile of hypoimmune stem cell-derived β cells.

Translational feasibility of bioengineered stem cell-derived β cells

To enable clinical translation of the bioengineering strategies for stem cell-derived β cells discussed herein, considerations of scalability and feasibility will be crucial. Developments in Vertical-Wheel bioreactors have enabled mass manufacturing of iPSCs with a higher expansion potential compared to 2D culture methods [118]. This scalable 3D culture technique has furthermore been used to manufacture iPSC-derived islets with significantly improved yields and reduced cost [119, 120]. Moreover, the ongoing Vertex clinical trial (NCT04786262) demonstrated that stem cell-derived islets can be manufactured at scale under Good Manufacturing Practice (GMP) conditions [9]. By improving cell yields while reducing the cost of production, these developments promise to facilitate the scalability and clinical implementation of bioengineered stem cell-derived β cells which will contribute to making islet transplantation a more feasible treatment option.

Ethical considerations

While discussions in the literature regarding the optimal source of insulin-producing cells for islet transplantation often revolve around immunological challenges, cell manufacturing scalability and practicality, there is also significant contention about the ethical implications of using each cell type. As previously discussed, the consideration of using xenogeneic cells, such as from pigs, raises moral concerns regarding animal welfare and the risk of zoonotic disease transmission [121]. *de Jongh et al.* furthermore discuss that conflicting regulations in different European countries regarding xenotransplantation may limit access to animal cell-containing therapies to certain jurisdictions, disadvantaging patients in countries where this is not permitted [122].

However, while progress in scalable and cost-effective manufacturing of human stem cell-derived islets, such as by using Vertical-Wheel bioreactors, may reduce the scarcity of resources and thereby contribute to making access more equitable, the use of human cells poses unique ethical challenges. These include concerns about regulating the process of organ donation, equitable access to this regenerative therapy as well as ownership of modified human cells. As emphasized by the European Union in a directive outlining the standards of procurement and distribution of human cells, the process of donor selection and tissue acquisition must be founded on the principles of "voluntary and unpaid donation" [123]. Strict regulation and enforcement of these guidelines will be essential to avoid coercion or oppression as a means to increase the number of donor organs. Ethical concerns also arise regarding the potential limitations in access to these therapies based on financial means. For example, in less developed countries, patients with T1D often have significantly poorer outcomes due to a lack of access to insulin and other medications [124]. These concerns are particularly relevant given the anticipated high cost of novel therapies, particularly those involving genetic modifications [122]. Limiting access to those with the financial means may contribute to widening pre-existing socioeconomic disparities. Additionally, questions have been raised about whether modified human cells should be patentable and how this affects ownership of the cells [121]. These concerns relate to the possibility of biotechnology and pharmaceutical companies profiting from donated genetically modified human cell products whereas those who donated the tissue do not [121].

Finally, concerns have also been raised about the importance of accurately depicting the risk-benefit ratio for prospective transplant recipients. Providing a comprehensive outline of the pertinent risks may be challenging given the complexity of the procedure and the unknown long-term effects of genetically modified stem cell-derived grafts [122]. Nonetheless, patients must be made aware of the potential for complications following a transplantation procedure to ensure they can make informed, autonomous decisions, with particular care being taken in this respect for desperate patients who may misjudge these risks [125]. While there are undoubtably many ethical, legal and socioeconomic challenges facing genetically modified stem cell-derived islets as a widespread T1D therapy, this should act as a catalyst for continued debate, in collaboration with patients and policymakers, on the responsible development, distribution and regulation of new treatments.

Considerations of using autologous stem cells

Although allogenic stem cell-derived β cells overcome the donor shortage which currently limits islet transplantation as a therapy option for T1D patients, allograft recognition and rejection necessitates life-long immune suppression. Autologous stem cells for personalized cell therapy have thus been discussed as a potential solution that could remove concerns over allograft rejection and thus negate the need for immune suppression. Methods have been developed to differentiate stem cells derived from T1D patients into insulin-producing β cells that did not demonstrate functional deficits following transplantation into immune-compromised mice [126]. However, reducing the cost barrier for using stem cells in islet transplantation will likely necessitate large scale stock production which may not align with personalized strategies such as using autologous stem cells where on demand production is inherently required. Furthermore, autologous stem cell transplantation would not prevent progression of the autoimmune disease and would likely require additional immune modifying strategies to prevent autoimmune-mediated rejection of the graft tissue.

Potential future genomic targets

Research on genetic modification has shown promise for improving immune evasion. Nonetheless, continued research will be necessary to elucidate additional genomic targets that may improve upon current strategies or target other branches of the immune system involved in graft rejection. In this regard, candidates for investigation may include RNLS, which encodes the enzyme renalase that is involved in stress pathway signaling and the downregulation of which has been demonstrated to protect β cells from autoimmune destruction [127], and Vitamin D receptor, the activation of which has been demonstrated to reduce β cell stress by regulating expression of inflammatory response genes [128]. Expression of the chemokine CCL22, which is involved Treg recruitment, is also being investigated in stem-cell derived β cells as a strategy to dampen the graft rejection response [129]. Similarly, Barra et al. demonstrated immune-protection of stem-cell derived β cells, expressing a unique cell-surface bait protein, by co-engineered bait-specific CAR T cells [130]. While these approaches have shown promise for protecting β cells by leveraging naturally occurring tolerogenic and anti-inflammatory mechanisms, more research is required to establish their effectiveness for providing protection against acute and chronic graft rejection in the clinical setting.

The limitations observed in each of the investigated approaches are a testament to the complexity of the immune system and an indication that combinations of these modifications will likely be necessary to achieve long-term graft survival without immune suppression. As such, further development will be required to see how these will work synergistically with HLA and PD-L1 strategies. Moreover, research should continue into investigating other methods that may enable more robust immune evasion of islet cells. In this regard, the identification of additional genetic targets for future investigation should take inspiration from the advancements made in the development of hypoimmune non-stem cell-derived β cells and CAR T cells, which are discussed below.

1. Lessons from strategies for genetically engineering immune-evasive, non-stem cell-derived insulin-producing cells

Consideration of genetic modifications that have been investigated for immune evasion of transgenic primary islets and non-stem cell-derived insulin-producing cells may offer insight into additional targets for improving graft survival of stem cell-derived insulin-producing cells. These have included strategies leveraging the intrinsic immune regulatory pathways involved in T cell activation. Inactive T cells express CD28 which interacts with B7 (CD80/CD86) on antigen presenting cells (APC) or target cells. These induce expression of the anti-apoptotic Bcl-X_L which reduces T cell anergy and increases proliferation and survival. This pathway, which is known as signal 2, is required in addition to recognition of antigen in the context of HLA (signal 1) and cytokine stimulation (IL2 and IL15), to avoid activation induced cell death (AICD) of T cells. Overactivation of the immune system is attenuated by expression of CLTA4 on activated T cells which competes with CD28 for binding with B7. As such, targeting CTLA4 and an inhibitory B7-like molecule (B7-H4) by genetic modification of non-stem cell-derived insulin-producing cells has shown promise for immune protection.

CTLA4-Ig (abatacept) is a soluble fusion protein of the external domain of human CTLA-4 and the constant Fc region of IgG1 which blocks the interaction of CD80/CD86 with their receptors, thereby attenuating T cell activation [131]. The immune-modulating potential of CTLA4-Ig has been highlighted by prolonged xenogeneic islet survival [132] as well as improved treatment of rheumatoid arthritis in humans [131]. Furthermore, islet xenografts from transgenic pigs expressing LEA29Y (belatacept), a modified version of CTLA4-Ig with higher CD80/CD86 affinity [133], into humanized mice recipients established euglycemia and protected islets from graft rejection [134]. Transgenic expression of single-chain anti-CTLA-4 Fv, a membrane-bound CTLA4-binding molecule, in NOD mice prevented T cell activation and induction of autoimmune diabetes [30]. CTLA4-Ig transfection of porcine islets also did not affect glucose stimulated insulin secretion (GSIS) results, suggesting that this immune evasion strategy did not impact endocrine function [135]. Notably, CTLA4-Ig transfection of allogeneic MIN6 cells transplanted subcutaneously significantly prolonged graft survival [136]. While promising, CTLA4-Ig has also been demonstrated to significantly decrease generation of regulatory T cells (Treg) [137], although another study found no difference [138]. This may limit the success of CTLA4-Ig where treatment outcomes depend on induction of immune tolerance. Nonetheless, these results demonstrate the potential for targeting the intrinsic T cell signaling pathways to modulate the cellular immune response and improve transplantation outcomes.

A further target for genetic modification that has shown promise for immune protection in non-stem cell-derived insulin-producing cells is B7-H4 (also called B7x or B7S1). This protein is a negative regulator of T cell activity, inducing cell cycle arrest as well as inhibiting cytokine production and cytotoxicity [139]. Allogeneic islets from transgenic C57BL/6 mice over-expressing B7-H4 in a β cell-specific manner transplanted under the kidney capsule had prolonged allograft survival and established euglycemia [140]. Similar results were demonstrated with allogeneic transplantation of B7-H4 over-expressing NIT1 (insulinoma cell line) cells [141].

Finally, an additional approach for improving islet graft survival is the transgenic expression of heme oxygenase-1 (HO-1). This intracellular enzyme is involved in physiological catabolism of hemoglobin in senescent red blood cells [142] in addition to serving cytoprotective anti-inflammatory and anti-oxidative functions [143]. HO-1 is thus particularly relevant in the context of transplantation where the graft tissue experiences perioperative ischemia which can affect transplant outcomes [144]. HO-1-transduced human islets were protected from cytotoxicity [145] and had improved graft survival [146]. This treatment approach is supported by similarly improved allograft survival for islets treated with HO-1-inducing cobalt and iron protoporphyrins (CoPP and FePP, respectively) [147]. CoPP, for example, has been shown to increase phosphorylation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) which is involved in the pro-survival ERK signalling pathway and increased HO-1 expression [148]. In summary, immune evasion and improved graft outcomes have been demonstrated by modifying non-stem cell derived insulin producing islet cells through expression of CTLA4, B7-H4 and HO-1 [144]. While these strategies are attractive because they leverage intrinsic immune regulatory and cytoprotective pathways, they are limited by a lack of extensive clinical testing. Nonetheless, they may provide a source of promising genetic targets for genetically engineering stem cell-derived β cells that are immune evasive and more adept at overcoming peri-operative stresses.

2. Lessons from strategies for genetically engineering immune-evasive CAR T cells

Strategies for engineering immune isolated stem cell-derived β cells can also take inspiration from the developments made in the same vein for allogeneic CAR T cell therapies. For example, disruption of the immune synapse, a stable interaction required for receptor interaction with immune cells and triggering of downstream activation pathways, has been investigated for immune evasion of CAR T cells. Knock-out of the adhesion molecules that mediate the immune synapse, intercellular adhesion molecule 1 (ICAM-1; CD54) and lymphocyte function-associated antigen 3 (LFA-3; CD58), in B2M$^{-/-}$ CAR T cells reduced NK cell-mediated lysis in addition to protection against cytotoxicity without altering the cells' effector function [149]. This strategy's potential for use in islet cells is supported by reports demonstrating prevention of islet allograft rejection using anti-ICAM-1 antibodies [150]. Several studies have also focused on preventing antibody-dependent cellular cytotoxicity (ADCC), a process that bridges the innate and adaptive immune responses. Expression of a truncated version of the high-affinity Fc receptor CD64 [151] as well as the IgG-cleaving enzyme IdeS [152] both demonstrated protection against ADCC. The importance of these studies is that ADCC is involved in mediating chronic rejection [153], a significant contributor to the current limitation of clinical islet graft survival duration [154]. Accordingly, consideration of the immune rejection response beyond the acute rejection phase will likely be critical for improving clinical outcomes. Furthermore, transplantation into the autoimmune environment of a T1D patient predisposes the grafted insulin-producing cells to be targeted by the islet antigen-primed host immune system [46]. Mitigating ADCC in combination with previously discussed strategies may therefore be a step towards improving long-term islet graft survival.

Critical assessment of hypoimmunogenic bioengineering strategies

Significant progress has been made in identifying immune and cell stress pathways involved in graft failure. Genetic modification of these pathways has yielded promising results for improving transplant outcomes, which, in combination with developments in scalable and cost-effective stem cell manufacturing, instill confidence in the potential of fully hypoimmune stem cell-based therapies without the need for immunosuppression. However, the limitations of current preclinical studies highlight the complexity of the immune system and indicate that combinatorial approaches with synergistic mechanisms are more likely to be successful. The following discussion will critically assess the strengths and limitations of the presented genetic engineering approaches as well as potential combinatorial effects (Table 1).

HLA knockout is a thoroughly investigated method for reducing T cell recognition with strong preclinical and clinical evidence. Similarly, PD-L1 overexpression is also well validated for inhibiting T cell cytotoxicity. However, a key limitation of these methods is that they do not provide protection against NK cell-mediated targeting and are thus insufficient in isolation. Expression of CD47 as well as maintaining expression of select atypical HLA sub-types (such as HLA-E and G) have been demonstrated as solutions to prevent NK cell-mediated killing. Although CD47 is a broad "self" signal that is not NK cell specific and primarily functions in inhibiting phagocytosis of macrophages and dendritic cells, preclinical and clinical use supports its potential for enabling immune evasion of bioengineered stem cell grafts. The positive six-month clinical results of transplanted HIP islets, which combine HLA knockout with CD47 expression, without immunosuppression are a further testament to this [31]. Alternative strategies to avoid T cell cytotoxicity include knockout of the pro-inflammatory chemokines CXCL10 and CCL22, the immune synapse adhesion molecules ICAM-1 and LFA-3, as well as FasL and CTLA4-Ig expression which induce T cell apoptosis and anergy, respectively. However, concerns have been raised that indiscriminate targeting and killing of T cells, including those with a tolerogenic phenotype, increases the risk for cancer immune evasion. Similarly, high expression of B7-H4, a T cell inhibitory ligand, shows potent immune-suppressive effects in tumor biology, raising concerns over long-term safety and necessitating rigorous tumorgenicity testing [155].

Finally, strategies that modulate peri- and post-operative cell stress pathways (including XBP1, CDKN1A, RNLS, Vitamin D receptor and HO-1) are attractive because they aim to bolster the resilience of grafted cells, thereby improving their potential to thrive. However, the complexity of the molecular cascades involved, which may include pleiotropic and other unknown downstream effects, must be taken into consideration and necessitates scrutinous investigation to ensure safety. Moreover, these approaches do not directly target ligands involved in graft rejection and thus do not by themselves enable immune evasion. However, by mitigating the negative impact of the various sources of transplant-related cellular insults on cell health, these strategies may improve graft outcomes. These strategies may furthermore indirectly improve the robustness of transplanted cells to

Table 1 Strategies for engineering hypoimmunogenic and cell stress-resilient cells

Strategy	Mechanism	Strengths	Limitations/risks	Preclinical and clinical evidence/stage of testing
Inhibiting HLA-mediated antigen presentation (B2M$^{-/-}$, CIITA$^{-/-}$ and TAP$^{-/-}$); atypical HLA sub-type expression	Inhibits antigen presentation-dependent T cell activation; expression of atypical HLA sub-types (e.g., HLA-E and G) provides inhibitory signal for NK cells	Strong T cell evasion; potential to also avoid NK cell cytotoxicity	Requires additional steps to prevent NK cell targeting; removing capacity for antigen presentation (risk of undetected infection and neoplastic transformation)	Robust preclinical evidence and first-in-human clinical testing of hypoimmune human islets in combination with CD47 expression without immunosuppression [61]
PD-L1 expression	Ligand for PD-1; reducing T cell activity	Targeted T cell inhibition; reversible with PD-1 blockade	Insufficient in isolation against NK cell targeting; concerns about tumorigenicity	Preclinical in vitro and in vivo studies; protected against allograft and xenograft rejection in mouse models [35]
CD47 expression	"Don't eat me" signal; mitigates NK cell targeting	Synergistic effect with HLA knockout; CD47-targeting antibodies (e.g., magrolimab) act as cell suicide switch(60)	Concerns about tumor immune evasion	Preclinical and clinical evidence in first-in-human clinical testing of hypoimmune human islets in combination with HLA knockout without immunosuppression [61]
CXCL10 or CCL10 knockout	Removes pro-inflammatory chemokines	Synergistic; targets the non-redundant chemotaxis pathway	Does not inhibit other chemokines or tissue-resident leukocytes	Preclinical in vivo mouse studies [37, 129]
Stress pathway modification (including XBP1, CDKN1A, RNLS, Vitamin D receptor and HO-1)	Rescue decreased cell health due to peri-operative cell stresses	Synergistic strategy improving robustness of cells; reduced stress-associated immune-activating ligand expression	Modification of complex pathways with potential for pleiotropic effects	Preclinical in vitro and in vivo mouse studies [38, 127, 128, 146]
FasL expression	Ligand for Fas receptor on T cells; inducing external apoptosis pathway	Targeted killing of infiltrating Fas-expressing T cells; may enable long-term islet graft survival	Concerns of tolerogenic T cell targeting and tumorigenicity	Preclinical in vivo mouse studies [78]
B7-H4 expression	Negative regulator of T cell activity	Potent inhibition of T cells	Tumor immune evasion concerns; limited research	Early preclinical in vivo mouse studies [140]
ICAM-1 and LFA-3 knockout	Inhibit formation of the immune synapse	Protect against NK cell targeting	Concerns of tumor immune evasion; limited research	Early preclinical in vivo mouse studies with CAR T cells [149]
CD64 and IdeS expression	High affinity Fc receptor and IgG-cleaving enzyme, respectively; prevents ADCC	Protect against ADCC, which may improve long-term graft survival	Potential for tumor immune evasion; limited research	Early preclinical in vivo mouse studies with CAR T cells [151, 152]

immune-mediated rejection, such as by reducing cell stress-related markers which are recognized by NK cells. In summary, continued research will be necessary to identify molecular targets and their combinations that are most adept at enabling immune evasion and reducing cell stress while ensuring rigorous safety testing.

Conclusion

Replacement of exogenous insulin injections by islet transplantation as the gold standard therapy for T1D is currently limited by donor tissue availability. While stem cell-derived β cells may overcome pancreatic shortages, a continued reliance on systemic immune suppression to sustain β cell graft survival is associated with significant morbidity in transplant recipients. As such, recent bioengineering strategies have been investigated that leverage our understanding of the acute and chronic rejection response as well as intrinsic immune regulatory pathways. While considerable advancements have been made, inconsistent or contradictory results in testing these approaches, which are a testament to the complexity of the immune system, indicate that a combination of these strategies is likely required to induce and maintain graft survival of hypoimmune stem cell-derived β cells. Future investigations into these targets should therefore take inspiration from the genetic engineering approaches that have demonstrated promising results in immune evasive primary islet cells as well as CAR T cells. By overcoming key obstacles currently limiting the translational potential of islet transplantation, the development of hypoimmune stem cell-derived β cells may be a catalyst for making this promising, regenerative therapy option more widely available to T1D patients.

Abbreviations

T1D	Type 1 diabetes
CAR	Chimeric antigen receptor

MSC	Mesenchymal stem cells
Treg	Regulatory T cells
HLA	Human leukocyte antigen
PD	Programmed death
PD-L1	Programmed death-ligand
B2M	β2-microglobulin
CIITA	Class II MHC transactivator
TGF	Tumour growth factor
XBP1	X-box binding protein 1
FasL	Fas ligand
SA-FasL	Streptavidin-FasL
IBMIR	Instant blood-mediated inflammatory response
MHC	Major histocompatibility complex
TAP	The antigen processing
ER	Endoplasmic reticulum
PSC	Pluripotent stem cells
HIP	Hypoimmune
iPSC	Induced pluripotent stem cells
hPSC	Human pluripotent stem cells
NOD-SCID	Non-obese diabetic severe combined immunodeficiency
hESC	Human embryonic stem cell
CRISPR	Clustered regularly interspaced short palindromic repeats
JAK/STAT	Janus kinase/signal transducers and activators of transcription
PBMC	Peripheral blood mononuclear cell
UPR	Unfolded protein response
FBR	Foreign body response
FBGC	Foreign body giant cells
α-SMA	α-smooth muscle actin
PEG	Poly(ethylene glycol)
GMP	Good Manufacturing Practice
APC	Antigen presenting cells
AICD	Activation induced cell death
CLTA4	Cytotoxic T-lymphocyte associated protein 4
HO-1	Heme oxygenase-1
CoPP	Cobalt protoporphyrins
FePP	Iron protoporphyrins
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
ICAM-1	Intercellular adhesion molecule 1
LFA-3	Lymphocyte function-associated antigen 3
ADCC	Antibody-dependent cellular cytotoxicity

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