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Cell-based interventions to halt autoimmunity in type 1 diabetes mellitus

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Summary

Type 1 diabetes mellitus (T1DM) results from death of insulin-secreting β cells mediated by self-immune cells, and the consequent inability of the body to maintain insulin levels for appropriate glucose homeostasis. Probably initiated by environmental factors, this disease takes place in genetically predisposed individuals. Given the autoimmune nature of T1DM, therapeutics targeting immune cells involved in disease progress have been explored over the last decade. Several high-cost trials have been attempted to prevent and/or reverse T1DM. Although a definitive solution to cure T1DM is not yet available, a large amount of information about its nature and development has contributed greatly to both the improvement of patient's health care and design of new treatments. In this study, we discuss the role of different types of immune cells involved in T1DM pathogenesis and their therapeutic potential as targets and/or modified tools to treat patients. Recently, encouraging results and new approaches to sustain remnant β cell mass and to increase β cell proliferation by different cell-based means have emerged. Results coming from ongoing clinical trials employing cell therapy designed to arrest T1DM will probably proliferate in the next few years. Strategies under consideration include infusion of several types of stem cells, dendritic cells and regulatory T cells, either manipulated genetically *ex vivo* or non-manipulated. Their use in combination approaches is another therapeutic alternative. Cell-based interventions, without undesirable side effects, directed to block the uncontrollable autoimmune response may become a clinical reality in the next few years for the treatment of patients with T1DM.

Keywords: β cells, dendritic cells, macrophages, stem cells, T cells

Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disease afflicting an increasing number of individuals worldwide, characterized by hyperglycaemia and insufficient insulin levels to maintain the metabolic demand. The inappropriate insulin level is due to the specific destruction of pancreatic β cells. Multiple genes, mainly major histocompatibility complex (MHC) class II loci, determine susceptibility to T1DM in both humans and the non-obese diabetic (NOD) mouse model. However, genetic predisposing factors are not sufficient determinants for disease onset. The environment plays an important part in disease progression and may account for the constant increment in the incidence of T1DM [1,2].

Treatment with exogenous insulin is mandatory for T1DM patient survival. Despite improvements in insulin formulation, insulin analogues and different administration regimens, it is sometimes still difficult to achieve tight glycaemic control. The lack of rigorous glucose homeostasis in the long term leads to vascular damage associated with kidney failure, heart diseases, retinopathy and neuropathy. Although clinical evidence has indicated that insulin replacement may ameliorate life-threatening complications of hyperglycaemia its use is by no means considered a cure, but a palliative, which cannot prevent long-term

disease-related complications. Therefore, discovering therapeutics that help to prevent β cell destruction and/or increase its mass is desirable.

The autoimmune process in type 1 diabetes mellitus

T1DM occurs as a result of a chronic and progressive autoimmune destruction of insulin-secreting β cells. The disease remains subclinical until the remaining β cells are unable to maintain glucose homeostasis. Generally, greater than 80% of the β cell mass is destroyed before clinical manifestation.

The disease aetiology is not understood completely, with pathogenesis being primed by invasion of inflammatory cells led by dendritic cells (DCs) and macrophages as a first stage, followed by T and B lymphocytes in NOD mice. Similarly, the same process occurs in humans, with a lower degree of infiltration [3]. It has been suggested that the putative initial events in T1DM occur during organogenesis, when DCs are activated by apoptotic β cells that, in turn, prime autoreactive T cells within pancreatic lymph nodes [4]. The presence of one or a combination of intra- and post-thymic tolerance failures could impair the control of diabetogenic T cell clones, which might then reach the islets.

A presumptive role has been assigned to certain pathogens in human T1DM development. However, pathogens may confer contrasting susceptibility for T1DM onset through the induction of inflammatory or immunoregulatory mediators that, in turn, may alternatively promote or prevent disease [1]. Microbes have the ability to activate or down-modulate the host immune cells through a variety of compounds [lipopolysaccharide (LPS), RNA or DNA] that bind to receptors, mainly Toll-like receptors (TLRs) on the surface of macrophages/monocytes and DCs. These effects are not exclusive to pathogens, as similar immune responses with commensal organisms have been observed. These observations indicate that gut-colonizing microbes interact with the innate immune system and might define T1DM outcome in susceptible individuals [5].

Although the mechanisms and circumstances that initiate T1DM are not understood completely, there is compelling evidence showing dysregulation of both the innate and adaptive components of the immune system. A consensus exists that disease development is the consequence of an orchestrated cross-talk between innate and adaptive immune cells to specifically kill pancreatic β cells (Fig. 1).

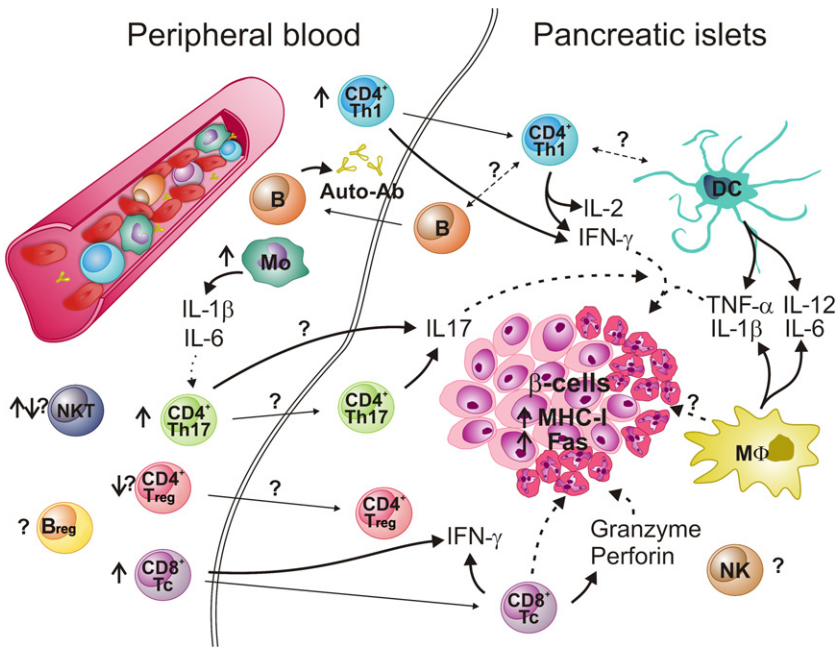

Fig. 1. Schematic diagram of immune cells reported in human type 1 diabetes mellitus (T1DM) pathogenesis. Initial steps of T1DM may be triggered by environmental factors in genetically at-risk individuals. Activated dendritic cells (DCs) prime β cell antigen-specific T cells within pancreatic lymph nodes. T cell activation is promoted by secretion of proinflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-17, IL-12, tumour necrosis factor (TNF)-α and interferon (IFN)-γ. Several infiltrating immune cells contribute to the inflammatory microenvironment: macrophages (MΦ), monocytes (Mo), DC, $CD4^+$-Th (helper) 1 cells and $CD8^+$-Tc (cytotoxic). This inflammatory microenvironment within islets may up-regulate major histocompatibility complex class I (MHC-I) and Fas molecules recognized by infiltrated diabetogenic T cells. Conclusive evidence of the role of natural killer (NK_, NK T, regulatory B cells (B_{reg}) and regulatory T cells (T_{reg}) cells in the naturally occurring disease in humans is still unavailable. This autoimmune process is generally slow, and progression may vary among diabetic individuals. Finally, only a few insulin-producing cells are present in most of the islets.

Cells from the adaptive arm of the immune system in type 1 diabetes mellitus

Both T and B lymphocytes are present within islets at different disease stages. Until recently, B lymphocytes have been given little attention in disease development, although they are critical antigen-presenting cells (APC) in the response against autoantigens [6]. APC such as macrophages and DCs may activate and initiate responses against β cell autoantigens. However, B cells appear to play a unique role in mediating determinant spreading by means of expansion and diversification of T cell clones [7]. B lymphocytes play a broad spectrum of functions and show both regulatory and pathogenic activities in T1DM [8].

The relevance of B cells in T1DM is illustrated by the fact that only few immunoglobulin (Ig)$\mu^{-/-}$ NOD mice, lacking B cells, display spontaneous autoimmune diabetes [9]. Arguing against the importance of B cells, there has been a case report of an X-linked agammaglobulinaemic patient who developed T1DM [10]. B cell targeting and depletion impair disease progression in NOD mice [11]. The infusion of anti-CD20 into newly diagnosed T1DM patients caused B lymphocytes depletion, preserving β cell function during a 1-year period, without major side effects [12]. After 1 year of treatment, loss of C-peptide levels coincided with the partial recovery of B cell numbers. A protocol using repeated infusions of anti-CD20 over a prolonged time-period should be assayed in order to achieve a more durable β cell protection. T lymphocytes, derived from patients who responded to treatment, show higher autoantigen proliferative responses in comparison to those who did not respond and to the control group. This observation highlights the importance of B and T cell interaction and suggests that the lack of autoantibodies enhances long-term T cell responses [13]. However, to what extent this effect contributes to disease progression is a matter of speculation. B cells may induce and maintain lymphoid structures. In fact, ectopic expression of a B cell chemoattractant within the pancreas leads to the formation of tertiary lymphoid structures [14]. Through B cell-targeted interventions for autoimmunity has emerged the notion that B and T cells interact, providing signals to each other, thereby triggering and/or sustaining the disease. Interventions directed to limit the capacity of B lymphocytes to promote determinant spreading deserve to be explored as therapeutics for T1DM.

Depleting B cells (anti-CD22/inotuzumab), in combination with Food and Drug Administration (FDA) clinically approved cytotoxic T lymphocyte antigen 4 (CTLA4)-Ig, prolonged islet survival in a stringent graft model in a mixed allo/autoimmune setting [15].

Conversely, there is evidence of regulatory B cells (B_{reg}) cells able to prevent or delay autoimmune diabetes in NOD mice [11,16,17]. It is believed that impaired frequency or function of these cells could promote autoimmunity. Interestingly, during infusion of autologous co-stimulation-impaired DCs, a relative increment of peripheral blood mononuclear cell (PBMC) $B220^{+}CD11c^{-}$ B cell population has been reported in T1DM patients which contains a putative B_{reg} cell subpopulation [18]. Whether this B_{reg} cell subpopulation has a relevant immunoregulatory role in the autoimmune process of T1DM needs further investigation.

Several findings sustain the pivotal role of activated T cells in T1DM: T cells infiltrate human pancreatic islets before disease onset [19]; early treatment with the T cell immunosuppressant cyclosporin induces T1DM remission in children [20]; disease may be transferred between siblings using diabetic-donor bone marrow cells [21] and β cell-specific T cells have been detected in prediabetic or recent-onset T1DM patients [22].

In early clinical studies, positive results were obtained with the use of humanized versions of anti-CD3 monoclonal antibodies targeting pathogenic T cells and restoring immune self-tolerance. In these short treatment studies, preservation of β cell function was achieved for approximately a 1-year period. Teplizumab administration to recent-onset diabetes patients showed a C-peptide response to a mixed meal in 60% of patients, respectively, to 8% of controls [23]. Otelixizumab, another anti-CD3 antibody, also showed preservation of β cell mass and less insulin needs to be used at higher doses than teplizumab [24]. These encouraging findings in terms of C-peptide response and acceptable adverse effects has led to the carrying out of two Phase III clinical trials: the Protégé Study, using teplizumab, and the DEFEND study (Durable-Response Therapy Evaluation For Early or New-Onset Type 1 Diabetes), employing otelixizumab. The former study failed in terms of the proposed primary end-point of plasma HbA1c level < 6·5% and insulin requirement of < 0·5 U/kg/day [25]. The DEFEND study, employing lower doses of otelixizumab than the original trial, also did not reach the primary end-point. The data obtained from these studies revealed that low doses of anti-CD3 are ineffective; however, higher doses are associated with adverse effects. Therefore, antibody dosing constitutes a critical factor in the design of a clinical trial for T1DM. In this sense, the AbATE study (Autoimmunity-Blocking Antibody for Tolerance in Recently Diagnosed Type 1 Diabetes) had the objective of testing whether or not repeated doses of teplizumab would prolong insulin secretion. Patients in this intensive two-cycle treatment showed greater C-peptide response to a mixed meal at 2 years compared to the control groups. It is noteworthy that a high number of subjects did not complete their full dose due to adverse events [26]. An escalating teplizumab dose over a 14-day treatment period (delay study) is ongoing, with T1DM subjects diagnosed 4–12 months prior to enrolment to test the prevention of loss of insulin secretory capacity. It is probable that anti-CD3 (teplizumab) administration in appropriate dosages may down-regulate

autoimmunity in people at high risk of T1DM development. The At Risk study, sponsored by TrialNet and the National Institute of Diabetes and Digestive and Kidney Diseases, is currently under recruitment to test this hypothesis.

β cell destruction and diabetes progression need the co-existence of both $CD4^+$ and $CD8^+$ T cells. Transfer experiments indicate that both of these T lymphocyte subsets are required equally.

Compelling evidence highlights the importance of $CD4^+$ T cells in the development and progression of T1DM, such as the adoptive transfer of $CD4^+$ T cells eliciting disease in mice [27]. Both T helper (Th) cell differentiation and cytokines milieu are thought to be involved in disease development. During natural disease, Th1 responses spread among β cell antigens and are responsible for diabetes progression. Experimental therapies with a single β cell antigen resulted in Th2-spread responses against several antigens that resulted finally in disease inhibition [28]. However, approaches directed to counteract the disease at advanced stages are more difficult to achieve. Th1 lymphocytes may promote T1DM in NOD mice, while Th2 cells and their hallmark cytokines may ameliorate/suppress disease under certain experimental conditions [29]. A shift from Th1 to Th2 lymphocytes may protect against T1DM progression [30,31]. However, no conclusive evidence is available so far showing that shifts in the cytokine balance towards Th2 may favour disease protection. Studies have indicated that the Th1/Th2 shift appears as a secondary effect, rather than the actual cause of autoimmune diabetes suppression [32].

Naive $CD4^+$ Th cells may differentiate into one of several T cell lineages, including regulatory T cells (T_{reg}), depending on environment stimuli. T_{reg} cells inhibit effector T cells and disease progression is prevented by action of the transforming growth factor (TGF)-β-expanded intra-islet T_{reg} cells [33]. Upon antigen stimulation in a TGF-β-enriched environment, up-regulation of the master transcription factor for induction of T_{reg} cell differentiation, forkhead box protein 3 (FoxP3), occurs. FoxP3-knock-out (KO) mice lack T_{reg} cells and develop a fatal autoimmune pathology [34]. Due to several observations, it could be speculated that autoimmune diabetes might be associated with a reduction in the total number of T_{reg} cells or their function. CD28-KO NOD mice show accelerated insulitis and incidence of diabetes that correlate with their lack of T_{reg} cells [35]. Reports show a normal [36] or reduced [37] frequency of circulating T_{reg} cells in T1DM patients, compared to healthy controls. These discrepancies might be explained by examining different patient cohorts (new-onset or long-standing patients, ethnicity and diagnosis criteria) or the use of non-matched controls. $CD4^+CD25^+$ T cells vary with age [36]. Siblings displaying human leucocyte antigen (HLA) high-risk T1DM haplotypes have a reduced T_{reg} cell frequency in comparison with those carrying the HLA low-risk haplotypes [38].

Defining T_{reg} subtypes either as $CD4^+CD25^+$, $CD4^+FoxP3^+$ or $CD4^+CD25^+FoxP3^+$ might further explain discordant findings. It has been shown that $CD4^+CD25^+FoxP3^+$ T cells correlate with $CD4^+CD25^{high}$ T cells with suppressive function, while suppressor $CD4^+CD25^+FoxP3^-$ T cells correlate with $CD4^+CD25^{low}$ T cells [36]. Putnam and co-workers [39] reported normal T_{reg} suppressive activity, while others [40] found a reduced functionality. T_{reg} cells with intact suppressive activity may fail to inhibit effector T cells (T_{eff}) cells due to resistance to the latter population [41]. Marwaha *et al*. show an increased frequency of $CD4^+FoxP3^+$ T cells in T1DM patients. This population could be divided into $CD4^+CD45RA^-FoxP3^{high}$ and $CD4^+CD45RA^+FoxP3^{low}$ with normal frequency and suppressive activity, and interleukin (IL)-17-secreting $CD4^+CD45RA^-FoxP3^{low}$ T cells with no suppressive activity [42].

T_{reg} cells used as therapeutic vectors remain as promising for the treatment or prevention of diabetes. The efficacy of T_{reg} cell administration in counter-regulating autoimmunity has been confirmed in NOD mice [43]. T_{reg} cells may act as antigen-specific or bystander suppressors avoiding systemic immunosuppression. Long-lasting antigen-specific tolerance by means of T_{reg} cells is a goal to achieve in the treatment of T1DM. Encouraging results have been obtained with T_{reg} cells preventing experimental diabetes. However, diabetes remission for humans is still pending. Recently, in a Phase I clinical trial, rapamycin/IL-2 combination therapy resulted in β cell dysfunction, despite a transient increase of T_{reg} cells, although the immunoregulatory activity of these cells has not been determined in this study [44].

In-vitro expansion of a T_{reg} cell population with suppressive activity from recent-onset T1DM patients has been achieved recently [45]. The reported *ex-vivo* procedure, obtaining a ~1500-fold expansion of polyclonal T_{reg} cells from T1DM patients, laid the groundwork for a Phase I clinical study, currently recruiting participants (Table 1), to assess intravenous infusion of autologous polyclonal T_{reg} cells in T1DM patients. Infusion of *ex-vivo*-conditioned cells has the disadvantage of the need for Good Manufacturing Practice (GMP) in the entire process, as well as the requirement of a highly purified T_{reg} cell fraction without contamination from any other T cell subpopulation, especially T_{eff} cells. To achieve this goal, standardization of the isolation techniques based on T_{reg}-specific markers is a hallmark for success [45]. However, this is a difficult task due to the lack of unique cell surface markers. A major concern about the putative success of adoptive transfer of polyclonal T_{reg} cells is that this approach has shown efficacy only in disease lymphopenic models in which homeostatic proliferation may play a role. Thus, it represents a major drawback for translation to the clinical setting that could be overcome with one dose of a high number of T_{reg} cells and/or repeated infusions over time. Whereas re-establishment of immunological tolerance is feasible to

Table 1. Latest clinical trials employing cell-targeted and -based approaches for type 1 diabetes mellitus (T1DM).

Intervention	Study type	Outcomes	Reference
Targeting specific immune cells			
Protégé study anti-CD3 mAb (teplizumab)	Phase III	Fails to meet primary end-point	[25]
AbATE dose repeated anti-CD3 mAb (teplizumab)	Phase III	↑ C-peptide response at 2 years	[26]
Anti-CD3 mAb (otelixizumab)	Phase III	Fails to meet primary end-point	[109]
Anti-thymocyte globulin	Phase II	Ongoing n.a.	ClinicalTrials.gov Identifier: NCT00515099
Anti-CD20 mAb (rituximab)	Phase II	Preserves C-peptide response; moderate adverse events	[12]
Transplantation			
Vascularized pancreatic graft + kidney	Clinical practice	Normoglycaemia ↓ morbidity/mortality immunosuppression	[110]
Solitary vascularized pancreatic graft	Clinical practice	Insulin independence 60% patients ↓ survival ↑ complications immunosuppression	[111]
Islets	Various studies in selected centres	Immunosuppression Insulin independence 60% patients	[98]
Immune cells			
Anti-sense oligo-modified autologous dendritic cells	Phase I	Safe	[18]
Ex-vivo expanded T_{reg} cells	Phase I	Recruiting patients	ClinicalTrials.gov Identifier: NCT01210664
Autologous stem cell treatment			
Non-myeoablative haematopoietic stem cells transplantation	Phases I/II	Preserve C-peptide response	[102]
Umbilical cord infusion	Phase I	Failed to preserve C-peptide response	[105]
Conditioned lymphocytes by cord blood-derived cells	Phases I/II	Preserve C-peptide response ↓ exogenous insulin ↑ peripheral T_{reg} cells	[106]
Ex-vivo cultured mesenchymal stem cells (Prochymal®)	Phase II	Ongoing n.a.	ClinicalTrials.gov NCT00690066
Combination approaches			
Anti-thymocyte globin plus GCSF	Phases I/II	Recruiting n.a.	ClinicalTrials.gov Identifier: NCT01106157
Haematopoietic stem cells plus GCSF	Phases I/II	Recruiting n.a.	ClinicalTrials.gov Identifier: NCT01285934

↑ Denotes increase; ↓ denotes decrease. GCSF: granulocyte-colony stimulating factor); n.a.: not available; GCSG: granulocyte colony-stimulating factor; T_{reg}: regulatory T cells; mAb: monoclonal antibody; AbATE: Autoimmunity-Blocking Antibody for Tolerance in Recently Diagnosed Type 1 Diabetes study.

achieve with polyclonal T_{reg} cells, risk for pan immunosuppression persists. Alternatively, efforts need to be invested in generating *ex-vivo* expansion of autoantigen-specific T_{reg} cells, a goal that has not yet been reached. However, in planning to execute this task, what antigen/s should be chosen to generate high numbers of T_{reg} cells *in vitro* with potent immunoregulatory activity *in vivo* is a major concern. Unfortunately, if a unique antigen really exists, which antigen triggers the autoimmune process is not known. Therefore, the choice of one autoantigen between several candidates remains a matter of speculation. Adoptive transfer of *ex-vivo*-expanded antigen-specific T_{reg} cells has been successful in animal models; however, translation to the clinic must await further research [46–48].

Anti-CD3 administration to NOD mice has pointed out the importance of T_{reg} cells induction. However, a permanent tolerance state against β cell antigens without undesirable side effects has not yet been achieved, indicating that development of new and safe interventions, such as combinatorial treatments of anti-CD3 and immunoregulatory agents and/or peptides, are required [24,49].

The significance of Th17 cells in several autoimmune disorders has been revealed. Nevertheless, their role in T1DM remains uncertain. Both mRNA and protein levels are expressed in NOD mice pancreata correlating with insulitis [50]. Blockade of IL-17 does not prevent disease [50,51]. Moreover, blockade of this cytokine delays the development and reduces the incidence of autoimmunity in 10 week-old NOD mice, but not in younger mice [52]. Th17 cell transfer to NOD mice did not induce diabetes, regardless of evident insulitis, suggesting that Th17 cells are not essential for initial autoimmunity even though they may take part in disease progression [50]. Th17 cells from BDC2·5 NOD mice induce diabetes after adoptive transference into NOD-severe combined immunodeficient (SCID) recipients, but this occurred along with *in-vivo* conversion of Th17 into Th1 cells. Employing the CD8-driven lymphocytic choriomeningitis virus-induced model of T1DM, IL-17 was not detected during T1DM development [53]. Similarly, no detectable IL-17 producing splenocytes was observed by our group in another rodent model [31]. Circulating β cell autoreactive Th17 cells are more prevalent in T1DM patients than in healthy controls, although their role in human T1DM is not completely known [54]. Anti-CD3/anti-CD28 stimulates high production of IL-17 by $CD4^+$ T cells obtained from PBMCs of T1DM patients [42,55]. The Th17 population is expanded within pancreatic lymph nodes of T1DM patients in comparison with those derived from healthy controls [56]. IL-17 enhances IL-1β/interferon (IFN)-γ and tumour necrosis factor (TNF)-α/IFN-γ apoptotic effects on human β cells, suggesting that this cytokine might contribute to β cell killing [54,55]. However, IL-17 alone possesses no apoptotic activity on β cells. This could be explained by increased IL-17-receptor expression mediated by IFN-γ/IL-1β on β cells [54].

$CD4^+$ T cell participation in islet infiltration and β cell damage is unquestionable. The need for $CD8^+$ T cells has been under debate since antigen-specific T cell receptor (TCR) transgenic $CD4^+$ T cell clones may trigger accelerated insulitis and diabetes in NOD-SCID mice.

Injection of either anti-$CD4^+$ or anti-$CD8^+$ non-depleting antibodies showed inhibition of autoimmune diabetes, demonstrating that both T lymphocyte subsets contribute to disease development, highlighting $CD8^+$ T lymphocyte action [57]. T1DM could be transferred by either $CD4^+$ or $CD8^+$ T cell clones alone, and also by antigen-specific β cell TCR transgenic T cells. These apparently inconsistent results might be explained by the fact that when a high number of islet-specific diabetogenic T cells is transferred adoptively, they could be more efficient disease inductors than polyclonal T cells. $CD8^+$ T lymphocytes infiltrate pancreatic islets of T1DM patients [3]. $CD8^+$ T cell infiltration increases as β cell numbers decrease, and finally disappears at late disease stages. The presence of β cell autoreactive $CD8^+$ T lymphocytes has been reported in peripheral blood [58] and in islet infiltrates [59] of T1DM patients. The fact that $CD8^+$ T lymphocytes kill human β cells *in vitro* further supports the notion that these cells may contribute to T1DM pathogenesis [60]. These observations highlight $CD8^+$ T lymphocytes as relevant effectors in autoimmune diabetes and raise the possibility of using them as therapeutic targets. Thus, ablation of autoreactive $CD8^+$ T cells using toxin-coupled MHC-I tetramer complexes delayed autoimmune diabetes in NOD mice [61].

The scenario in which the adaptive arm of the immune system participates with β cell destruction is complex. Intra-islet production of IFN-γ, IL-1β, TNF-α and IL-17 by activated $CD4^+$ T cells and $CD8^+$ T lymphocytes is a hallmark of this inflammatory process. Increments of proinflammatory cytokines trigger apoptosis of β cells as well as *in-situ* chemokine expression which, in turn, increase islet infiltration by innate immune cells [54,59].

Cells from the innate arm of the immune system in type 1 diabetes mellitus

Innate immune cells such as macrophages, DCs and natural killer (NK) cells have shown both pathogenic and protective functions in T1DM.

NK cells are cytotoxic lymphocytes with important roles in the host defence against pathogens and tumour cells. Upon recognition of altered expression of self-MHC-I molecules they become activated, and secrete cytokines and chemokines. Pancreas infiltration by NK cells increases with disease severity and might have a disease-promoting role in T1DM. NK cells have been found within islets before T cells during disease progression, helping to initiate inflammation and contributing to β cell damage. However, at late disease stages, NK cells from NOD mice became hyporesponsive [62]. Ligands present on β cells promote NK cell activation and β cell death through direct cytotoxicity [63]. Despite their disease-promoting role, they may also exhibit protective functions in models of islet transplantation and prevention [64]. Through multiple activities, NK cells interact with DCs and regulate the adaptive immune response, polarizing and tolerizing diabetogenic T cells. These characteristics point to NK cells as interesting targets in the control of autoimmunity in diabetes.

Several reports have shown normal [65–67], reduced [37,68] or increased [69,70] frequency of circulating NK T cells in T1DM patients compared to healthy controls. Upon activation, NK T cells secrete IL-4 and it is postulated that this could drive to a Th2 profile, conferring protection for autoimmunity [71]. NK T cells express a restricted CD1d invariant TCR and featured NK cell markers. Thus, the lack of reliable markers for detection of NK T cells might explain the inconsistency of the reported circulating levels of NK T cells in T1DM patients. Experimental data in NOD mice suggest a protective role for NK T cells. Activation of NK T cells or over-expression of the invariant TCR

prevented disease onset [72–74], while mice lacking CD1d expression showed accelerated diabetes [75,76].

Monocytes/macrophages are involved intimately in autoimmune-mediated β cell destruction. Monocytes are recruited into islets when the CCL2 chemokine is overexpressed transgenically in β cells and are capable of killing β cells, resulting in diabetes even in the absence of mature T and B cells [77]. The relevance of monocytes is manifested further when depletion after transfusion of diabetogenic T cells results in inhibition of diabetes in NOD mice [78]. Monocytes contribute to proinflammatory cytokine secretion in T1DM. Both basal and stimulated IL-1β- and IL-6-secretion by monocytes are increased in T1DM patients [79]. These cytokines promote *in-vitro* Th17 expansion directly, suggesting that monocytes may be implicated in Th17 differentiation during disease progression [79]. TNF-α secretion by monocytes from T1DM patients is increased upon LPS stimulation, as well as O_2^- production and TLR-2 and TLR-4 expression compared to control subjects [80]. TNF-α-expressing macrophages/DCs are found among the first islet-infiltrating cells when no T cells are yet detected in NOD mice. Moreover, macrophage and DC TNF-α expression is found within NOD-SCID mice islets, suggesting that T lymphocytes are not required at early stages for APC infiltration, but they need a NOD genetic background, as other mouse strains show no infiltration at all. Nevertheless, it is clear that the presence of T cells accelerates infiltration by macrophages/DCs, and subsequently diabetes onset. Biopsies taken from recent-onset diabetic patients reveal that macrophages/DCs infiltrate islets, and show that they are sources of TNF-α and IL-1β [81]. Interestingly, macrophages/DCs infiltrate islets with or without β cells, suggesting that β cell death occurs mainly at the initial stages of diabetes.

How to define macrophages or DCs is a problem still to be solved. Because no markers distinguish DCs and macrophages unequivocally, the existence of DCs as a separate cell type has been argued [82]. With this concept in mind, we will next describe the role of DCs in T1DM based on integrin alpha X (Itgax, CD11c) expression, commonly employed for DC characterization. DCs could be considered as links between the innate and adaptive immune systems, responding to endogenous and exogenous danger signals; they play a pivotal role in the induction and modulation of T-, B- and NK-driven cell responses, establishing either T cell immunity or tolerance. Thymic DCs induce tolerance to self-antigens through clonal deletion of double-positive thymocytes (e.g. central tolerance). Moreover, DCs maintain self-tolerance by modulating T cell responses directly or indirectly through the generation, expansion and activation of T_{reg} cells. The fate of antigen-specific T_{eff} cells may be either T cell death and/or anergy as a result of antigen presentation by tolerogenic DCs. The direct participation of DCs in peripheral tolerance has been established in fully DC depleted mice [83]. Mice devoid of myeloid, lymphoid and plasmacytoid DCs develop autoimmunity spontaneously with inflammation, organ infiltration and elevated numbers of Th1/Th17 cells and antibody production. Similarly, autoreactive $CD4^+$ T cells in this mouse model are primed by B lymphocytes and/or macrophages acting as APCs. Plasmacytoid DCs could promote tolerance, while myeloid DCs would be necessary for autoantigen presentation and T cell activation in NOD mice [84]. The activation state of DCs is critical for their function as being either tolerogenic or inflammatory. Altered frequency or function of DC subsets could contribute to immune imbalance in T1DM. In humans, controversial results have been reported [85–88]. The discrepancies may be attributed to the use of different surface markers for DC characterization, cohort selection of patients and lack of appropriate matched controls [85]. Whether DC subsets, frequencies or functions are altered in T1DM is not completely known, but they seem to change with disease stage, suggesting that they may have diverse roles in autoimmune diabetes.

In-vitro-generated mature DCs can proliferate further and differentiate under the influence of stromal spleen cells, acquiring a regulatory function [89]. A classification of DCs as immature, semi-mature and fully mature DCs, with regard to their roles in T cell tolerance and immunity, respectively, has been proposed [90]. Semi-mature DCs express high MHC-II and co-stimulatory molecules (CD80/86) and release very low levels of proinflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α). They act as tolerogenic by induction of IL-10-producing antigen-specific T_{reg} cells. Semi-mature DCs would maintain tolerance by inducing antigen-specific T_{reg} cells. *In-vitro*-generated and freshly isolated DC subsets have been employed as regulators of self-reactive T cell responses, and are indicated as good therapeutic candidates for the modulation of dysregulated responses against self-antigens.

DCs might be employed therapeutically to treat T1DM. Several methods have been assayed for the generation of immunoregulatory DCs. With the potential to ameliorate autoimmunity, most *in-vitro* methods to generate DCs are focused on their ability to induce Th2 cytokines and impair IL-12p70/IL-1β. The mechanisms through which anti-inflammatory and immunosuppressive agents are able to generate tolerogenic DCs are diverse, and include the use of different drugs, e.g. corticosteroids, eicosanoids/lipids, growth factors, sphingolipids, cytokines, etc. These agents may target DC biology at different levels: development, subset differentiation, activation, maturation, proliferation, antigen uptake/processing/presentation, migration and survival. Indeed, it has already been shown that infusion of *in-vitro*-generated and genetically modified DCs suppressed autoimmune diabetes in the NOD mouse, exploiting the mechanisms of Th2 shift [91].

Cellular immunointervention in T1DM should suppress immune responses exclusively against specific β cell

antigens. Infusion of antigen-specific DCs might be useful to down-regulate immune responses, allowing patients to be immunocompetent against infections. A drawback of DC therapy is potential plasticity *in vivo*. Therefore, efforts are still required to improve the methods employing DCs with the capacity of ameliorating/inhibiting autoimmune diabetes. Small interfering RNA (siRNA) provides a powerful tool for inhibiting endogenous gene expression to modulate immune responses effectively. Genetic intervention using siRNA is a novel strategy to manipulate DCs. Improvements in siRNA delivery will help to develop DCs with specifically sought phenotype and function [92]. This could be a useful approach for autoimmune diabetes treatment, as genetic modification of DCs to target proinflammatory cytokines would generate DCs with Th2 cell polarization capacity or regulatory activity.

Cell replacement therapy

Adult β cells may proliferate *in vivo* via self-duplication of pre-existing cells [93,94]. Whether the same process takes place in humans is difficult to demonstrate. Therefore, cell replacement constitutes an alternative to provide long-term regulated insulin secretion.

Substantial efforts have been made to achieve normoglycaemia by means of human islet transplantation. This strategy may improve glycaemic levels but, disappointingly, for a limited period of time [95,96]. Islet transplantation is limited by the shortage of cadaveric donors and high β cell death after transplantation [97]. Nevertheless, improvements employing a new immunosuppressor cocktail and cytoprotective strategies aiming at increasing β cell viability prior to transplantation have been documented recently [98–100].

Experimental efforts are focused on the potential of pluripotent and multipotent stem cells to differentiate into β-like cells. Although some studies are encouraging, the pathways involving intermediates and transcription factors governing β cell differentiation are not understood fully. It is possible to differentiate human embryonic stem cells into insulin-secreting cells [101]. Feasibility, reproducibility and scalability of these attempts to achieve the final goal of β cell replacement are still questionable.

Therapeutic application of embryonic stem cells is questioned by ethical concerns and teratoma formation. Therefore, alternative sources of stem cells such as multipotent adult-derived mesenchymal stem cells have become an area of interest. Attempts to generate β cells with induced pluripotent stem cells have shown promise not only for cell replacement purposes, but also as a source of diabetic-specific models. Based on the premise of achieving immunological tolerance by infusion of autologous non-myeloablative haematopoietic stem cell transplantation (HSCT) after immune ablation, Couri *et al.* reported that a significant number of patients became insulin free, with no mortality and mild adverse effects, after a mean follow-up period of 29·8 months [102]. This study shows promise for newly diagnosed ketoacidosis-free diabetics, the selected inclusion criterion for individuals enrolled into this trial. Indeed, if the HSCT shows mainly immunomodulatory effects then it is appropriate to use this therapeutic approach at the early disease stages, when sufficient β cells are still available for salvage. However, a randomized controlled trial to confirm the specific beneficial role of the HSCT in T1DM patients excluding the immune ablation effects is warranted [103,104].

Umbilical cord-derived stem cells represent a readily available source of cells that have been assayed as modulators of the autoimmune process in T1DM. Although no adverse effects were reported after 2 years of infusion, this treatment failed to preserve C-peptide in children with T1DM [105].

Recently, a unique infusion of autologous conditioned lymphocytes by cord blood-derived stem cells to T1DM patients achieved reversal of autoimmunity with increments in peripheral T_{reg} cells and less exogenous insulin requirements [106]. Although encouraging results have been obtained, extended post-treatment observations with larger samples are required. Moreover, a critical ethical issue is involved here, and a major question is whether it is reasonable to expose diabetic patients to therapies with some degree of toxicity/risk when palliative treatments, such as administration of exogenous insulin, is relatively effective. Perhaps similar criteria that are applied for those patients eligible for islet transplantation might also be taken into consideration for cell replacement protocols. It is ethically reasonable to propose the consideration of cell replacement in those individuals who develop rapid secondary complications despite intensive medical follow-up, as well as diabetics with autonomic insufficiency associated with an increased risk of death.

Conclusions

T1DM is usually diagnosed by clinical symptoms or late subclinical stages and time after appearance of the first self-reactive T cells. The discovery of biomarkers able to predict with accuracy those candidates who will progress to diabetes before disease onset will help in developing therapies to prevent dysregulated autoimmunity. These tools would allow the implementation of antigen-specific immune cell-based therapies in combination with immunosuppressive drugs at non-toxic doses, thus reducing dependence on non-specific immunosuppression and its side effects. A major drawback is the need for GMP for *ex-vivo* manipulation and reinfusion of cells into human beings. Table 1 shows some of the latest cellular-based interventions and their achievements and failures in human T1DM.

More efforts need to be employed to understand more clearly the developmental mechanisms required to generate

fully functional β cells. Once such a methodology is finally achieved, control of reactive T cells for prevention or the potential recurrence of specific anti-β cell responses will be necessary for the preservation of glucose homeostasis. Interestingly, stem cells have shown immunoregulatory potential, adding a new strategy for regenerative medicine [107].

Improvements in the development of new immunodeficient mice will allow studies to use the human immune system in health and diabetes without intervention in human beings, thus accelerating translational medicine in this field [108].

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Disclosure

The authors declare that there are no conflicts of interest.

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