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Tolerance and Autoimmunity in Type 1 Diabetes

Valentina Di Caro[1,2], Nick Giannoukakis[1] and Massimo Trucco[1]
[1]*Division of Immunogenetics, Department of Pediatrics, School of Medicine, University of Pittsburgh,*
[2]*Rimed Foundation,*
[1]*USA*
[2]*Italy*

1. Introduction

A functional immune system is able to distinguish between foreign antigens expressed by pathogens and self-antigens expressed by the body. The absence of a pathological response to self-antigens (e.g. tolerance) is dependent on a number of events that occur both centrally and peripherally. Central tolerance is induced at sites of lymphocyte development such as thymus and bone marrow for T cell and B cell respectively. On the other hand, peripheral tolerance occurs at sites of antigen recognition and processing, and includes secondary lymphoid as well as non-lymphoid tissues. Failure of central and/or peripheral tolerance can lead to increased development and expansion of pathogenic effector T cells and subsequent initiation and progression of autoimmunity.

Type 1 Diabetes (T1D) is an autoimmune disease due to a chronic inflammation in the pancreas that leads to the destruction of insulin-producing β-cells. The β-cells are selectively destroyed via both direct and indirect mechanisms by different immune cell types. Studies in animal models and humans have demonstrated that T cells play a major role in β-cell death. However, other cell types are present in the pancreatic infiltrate and in the pancreatic lymph node, where the initial presentation of islet antigen by dendritic cells (DC) to islet antigen specific T cells occurs. Besides different DC subsets, B cells and natural killer (NK) cells also contribute, with different roles, to β-cell destruction. This suggests a strong crosstalk between the immune cells that are involved in pathogenesis and those involved in immune regulation.

Herein, we will describe the autoimmune processes that result in clinical manifestation of this disease and we will discuss the immunologic basis supporting possible new therapeutic interventions.

2. The breakdown of self-tolerance in Type 1 Diabetes

T1D is the most common autoimmune disorder in childhood but the disease may become manifest at any age, even in adults. In the past decade, the incidence of T1D has increased considerably among children under the age of 15 years in most developed countries and, if the present trend continues, the current incidence is predicted to double in European children younger than 5 years, by 2020 (Patterson et al., 2009).

Despite a plethora of data in rodent models of the disease, the etiology and pathogenesis of T1D in humans is largely unknown. The onset of the disease and clinical/diagnostic signs are preceded by a long non-clinical phase during which an aggressive autoimmune reaction is proposed to be taking place. Clinical T1D is the result of end-stage insulitis, and it has been estimated that at the time of diagnosis only 10–20% of the β-cells are still functioning.

Studies in the non-obese diabetic (NOD) mouse, a mouse model that spontaneously develops autoimmune diabetes, have highlighted the critical role of adaptive immune responses in the pathogenesis of the disease. Initial β-cell death occurs physiologically in NOD mice, at 2-3 weeks of age, during tissue remodeling and β-cell metabolic changes or it could occur by injury mediated, for example, by viral infections (Turley et al., 2003). Such β-cell death leads to activation of DC, priming and expansion of specific β-cell-autoreactive T cells, initially in the pancreatic draining lymph nodes and subsequently in the pancreas itself. Ultimately, this chronic process ends with enough β-cell mass destruction to need insulin therapy.

It is now well established that a specific genetic constitution is required to develop diabetes. The most important genes contributing to disease susceptibility in humans are located in the HLA class II locus on chromosome 6. Additionally ten other genes or genetic regions have been associated with T1D (Morel et al., 1988; Todd et al., 2007). Nevertheless a relatively small proportion, less than 10%, of individuals with HLA-conferred diabetes susceptibility progress to clinical disease. This implies that additional factors, very likely environmental, are needed to trigger and drive β-cell destruction in genetically predisposed individuals.

Several models illustrate hypotheses on the outcome of the interplay between genetic and environmental factors. The linear β-cell decline hypothesis originally postulated by Eisenbarth remains the most widely referenced benchmark model for T1D (Eisenbarth, 1986). According to this model, genetically susceptible individuals at some point in time encounter certain environmental agents that trigger islet autoimmunity leading to a linear decay in β-cell mass, development of autoantibodies, hyperglycemia, and eventually complete loss of C-peptide. While this view provides an explanation for the sequence of events observed during the course of T1D, it does not integrate factors contributing to the variability along the time axis during the prediabetic phase. Some authors argue that disease progression in T1D is not a linear process, but rather proceeds at variable steps in patients (Chatenoud & Bluestone, 2007). As mentioned before, there is an effect of specific genetic polymorphisms on disease susceptibility but, on the other hand, predisposing DNA sequence variations may by themselves never lead to T1D, or require some degree of environmental insult (viral infection) to culminate in hyperglycemia. Today a more detailed version of the nonlinear model depicting T1D as a "relapsing-remitting" disease has been proposed (Bonifacio et al., 1999; von Herrath et al., 2007; van Belle et al., 2011). Specifically, this model posits that a disequilibrium between autoreactive effector T cells and T regulatory cells could develop over time and eventually lead to a decline in β-cell mass. Whereas the net balance shifts to islet autoimmunity, this effect is temporarily counteracted by the β-cells' proliferative response, perhaps resulting in a late transient phase of reduced insulin requirement called the "honeymoon phase". In an attempt to fit the role of infectious agents into this temporal T1D model, Von Herrath and colleagues introduced the "fertile field" hypothesis (von Herrath et al., 2003). The fertile field is described as a time window that follows viral infection. It can vary depending on the type, anatomical location, and duration of the virus-induced inflammatory response. This fertile field would allow autoreactive T cells to expand and lead to full-blown autoimmunity and clinical T1D (Figure 1).

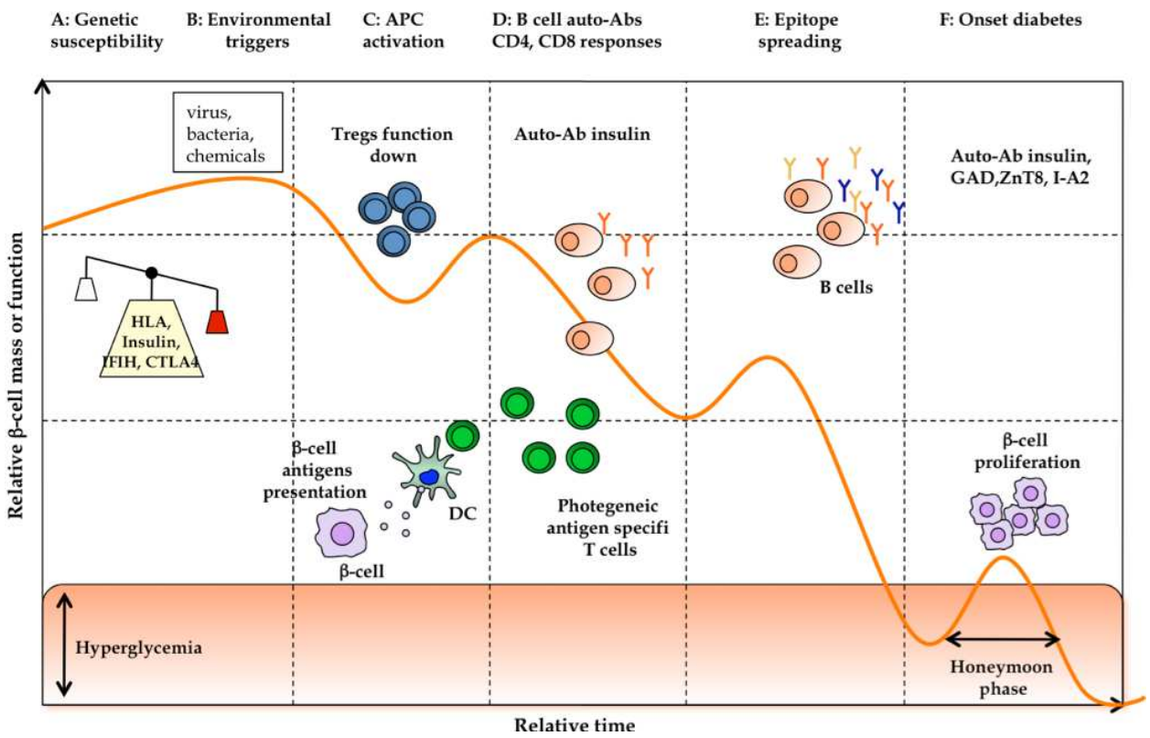

Fig. 1. How T1D might arise. The figure represents the β-cell mass or function (represented by the orange line) as well as the different immunological phases (columns with alphabetized tabs on top) that occur in the pancreas and peripherally. Once the orange line of β-cell function falls into the red zone, the individual is clinically diagnosed with T1D. Initially, a concurrence of genetic susceptibility and an environmental trigger sets an individual up for developing diabetes by causing β-cell death. In the pancreas, β-cell upregulate IFN and subsequently MHC class I. This exposes β-cell to attack by pathogenic antigen specific T cells. Consequently, the released β-cell antigens are picked up by resident APC and transferred to the pancreas-draining lymph nodes. Meanwhile in the periphery, a proinflammatory environment favors effector T cell responses over Treg function. β-cell antigens presented in this proinflammatory context and with CD4 help initiate conversion of B cells into plasma cells and the appearance of insulin autoantibodies . Also, autoreactive CD8 T cells are stimulated to proliferate and migrate into the pancreas. The stress induced by this second wave of β-cell killing causes some β-cell to stop insulin production. The killing also causes the release of new β-cell antigens that are picked up by APCs, including migrated B cells, which get shuttled to the pancreatic lymph node. This engages new antigen-specific clones of CD4 and CD8 T cells and B cells in a process called epitope spreading. Surprisingly, the autoimmune inflammation can also stimulate some β-cell proliferation so that the β-cell mass temporarily increases. The fluctuation between destructive autoreactive responses and β-cell proliferation may create a cyclical relapse-remitting profile of β-cell mass (orange line). Eventually, the autoreactive response wins though, and T1D is diagnosed when only 10 –30% of functional β-cell remains. The remission after clinically diagnosed diabetes is termed the honeymoon phase, a temporary state of relative self-sufficient insulin production.

3. Humoral β-cell autoimmunity

Human and murine T1D studies have shown that the appearance of autoantibodies is the first detectable pre-clinical sign of emerging β-cell autoimmunity. There are four disease-related autoantibodies that have been shown to predict clinical T1D (Knip et al., 2002). These include classical islet cell antibodies (ICA), insulin autoantibodies (IAA), and autoantibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and the protein tyrosine phosphatase-related IA-2 molecule (IA–2A). Insulin is the first antigenic target detectable during the early progression of diabetes (Nakayama et al., 2005), although most autoantibodies are targeted against the β-cells themselves and other β-cell secreted proteins (Atkinson & Eisenbarth, 2001). Recently, ZnT8, a pancreatic β-cell specific zinc transporter, has been identified as a candidate autoantigen associated with T1D (Wenzlau et al., 2007). During the progression of T1D, a process of autoantigen epitope spreading occurs. Epitope spreading provides an explanation of how the immune system is capable of recognizing increasing numbers of autoantigens in correlation with increased T1D disease severity (von Herrath et al., 2007). Epitope spreading begins with the immune system recognizing and mounting an immune response against a single antigen, which is recognized via a single epitope. Over time, new antigens can be recognized, and previously recognized antigens can be differentially processed by antigen presenting cells to generate multiple epitopes for a single antigen (Morran et al., 2010).

The number and titer of detectable autoantibodies, rather than the specificity of the autoantibody, is unequivocally related to the risk of progression to overt T1D both in family studies and also in surveys based on general population cohorts. In family studies positivity for three to four autoantibodies is associated with a risk of developing clinical T1D in the range of 60–100% over the next 5–10 years (Barinas-Mitchell et al., 2004; Pietropaolo et al., 2005; Barker, 2006).

Islet specific autoantibodies are, however, considered more diagnostic than causative in T1D. It is generally accepted that the destruction of the β-cells is mediated by cellular immune responses. This is supported by the following facts: (a) T cells are present in insulitis; (b) disease progression is delayed by immunosuppressive drugs directed specifically against T cells; and (c) circulating autoreactive T cells can be detected in patients at clinical presentation of T1D (Roep, 2003).

4. Immune cell crosstalk in Type 1 Diabetes

4.1 T and B lymphocytes

Studies in NOD mice have shown that autoreactive T cells are released into the circulation because of faulty presentation of self-antigens by disease-susceptible MHC molecules that prevent negative selection in the thymus (Trucco, 1992; McDevitt, 2001). Central tolerance can be broken even in the presence of disease-resistant MHC molecules. Indeed, it has been demonstrated that disruption of thymic expression of a single tissue-specific gene self-molecule, as insulin for diabetes, is sufficient to trigger autoimmunity toward the specific tissue (Figure 2) (Fan et al., 2009).

In pre-diabetic mice, insulin specific T cells are the predominant component of islet-infiltrating T cells. Multiple $CD4^+$ and $CD8^+$ T cell clones, targeting different insulin epitopes, have been isolated (Wegmann et al., 1994) demonstrating that T1D development depends on both $CD4^+$ and $CD8^+$ T cells. Moreover, T1D can only be transferred to

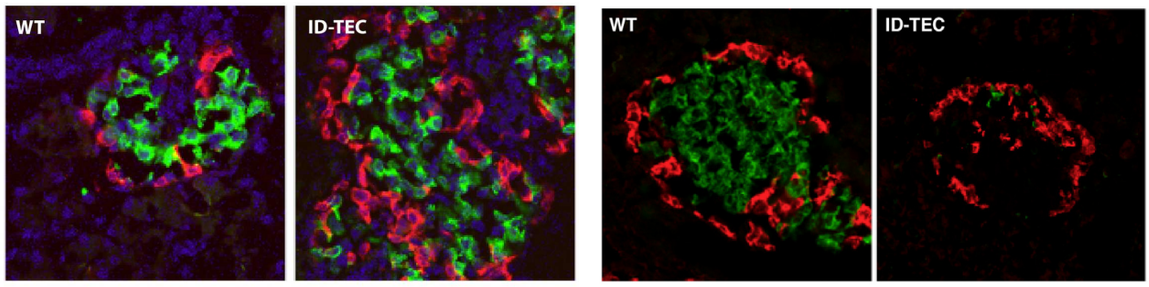

Fig. 2. Transgenic mice that do not express insulin in the thymus (ID-TEC) develop diabetes within 3 weeks. A) Normal islet development of transgenic mice at day 1 after birth; B) 4 week after birth only a small number of β-cells are still present in the islets. Pancreatic section stained using anti-insulin (green), and glucagon (red) antibodies (Fan et al., 2009).

immunocompromised syngeneic recipients by a combination of splenic $CD4^+$ and $CD8^+$ T cells from donor NOD mice but not by either T cell subset alone (Phillips et al., 2009).

There are several ways in which autoreactive T cells can mediate β-cell death. $CD8^+$ T cells may kill pancreatic β-cells through MHC class I mediated cytotoxicity, and both $CD4^+$ and $CD8^+$ T cells produce cytokines, such as interferon-γ (IFNγ), that induce expression of the death receptor Fas (CD95) and chemokine production by β-cells. Activation of Fas by Fas ligand (FasL)-expressing activated T cells can initiate β-cell apoptosis. Chemokine production by β-cells results in further recruitment of mononuclear cells to the site, thereby enhancing inflammation (Eizirik et al., 2009). In addition, IFNγ can activate macrophages and induce increased pro-inflammatory cytokine production, including interleukin-1β (IL-1β) and tumour necrosis factor (TNF). β-cells express high levels of IL-1 receptor and seem to be more sensitive to IL-1β-induced apoptosis than other endocrine cells in the islet. This crosstalk between T cells and macrophages undoubtedly exacerbates the immune-mediated stress on β-cells and contributes to their destruction. IFNγ, IL-1β and TNF also induce the expression of reactive oxygen species (ROS) including nitric oxide by β-cells, and ROS have the potential to mediate apoptosis.

Although T cells have a pathological role in T1D onset, there is also evidence supporting a role for a subset of T cells, the T regulatory cells (Tregs), able to prevent β-cell death.

Tregs play an indispensable role in maintaining homeostatic balance within the immune system. Tregs are involved in mediating normal immune responses against pathogens and terminating such responses when they are no longer required, as well as in preventing autoimmunity. Phenotypically, most Tregs express the surface marker CD25, the high affinity interleukin 2 (IL-2) receptor ligand-binding α chain, and Foxp3, an intracellular transcription factor (Fontenot et al., 2003). Because of that they are identified as $CD4^+CD25^+Foxp3^+$ cells. Both CD25 and Foxp3 coordinate Treg development and function. In the thymus, IL-2 is critical for the development of Tregs, while, in the periphery, it has been shown that interleukin 7 (IL-7) can complement potentially limiting amounts of IL-2 in promoting Treg survival and functional fitness (Di Caro et al., 2011).

Many studies in the NOD mouse strain have demonstrated the role of $CD4^+CD25^+Foxp3^+$ Tregs in the maintenance of self-tolerance. Indeed, depletion of CD25-expressing T cells results in a marked acceleration of T1D and $foxp3^{-/-}$ NOD mice display an increased

incidence and earlier onset of the disease compared to wild type mice (Brunkow et al., 2001). In humans, patients with IPEX syndrome, who have a mutation in the *FOXP3* gene, develop endocrine autoimmune disease including T1D (Bennett et al., 2001). Tregs can control or limit the activation of $CD4^+$ and $CD8^+$ T cells at various stages such as differentiation and/or proliferation during priming in the draining lymph node, inhibition of IL-2 production or trafficking to the pancreas.

T cells are clearly of pivotal importance for T1D development, but there are also data suggesting an involvement of B-lymphocytes in initiation and progression of the disease. Recently, it was demonstrated that B cell depletion in NOD mice, either through gene targeting or antibody treatment, impaired the development of T1D (Hu et al., 2007) .

The investigation of the roles of B cells in autoimmune inflammatory diseases has focused mainly on the ability of B cells to secrete autoantibodies. More recently, B cells have been identified as important sources of pro- and anti-inflammatory cytokines, for example IL-6 and IL-10. B cells can either provide a quantitatively or functionally dominant source of cytokines. Moreover, they can have a role as antigen-presenting cells that maintain islet antigen-specific T cell activity (Hu et al., 2007; Pescovitz et al., 2009).

4.2 Innate immune cells

As islet antigen-specific T cells can differentiate into either pathogenic effector T cells or regulatory T cells, many studies have investigated the role of innate immune cells in T1D, as these cells usually determine a specific type of immune response. Innate cells producing pro- or anti-inflammatory cytokines define the milieu in which islet antigen specific T cells are activated and whether a deleterious or protective immune response occurs in the pancreas (Figure 3).

Macrophages are one of the two major antigen-presenting cells in islet infiltrates of NOD mice. It has been shown that inhibition of the macrophage influx into the pancreas, by blocking adhesion-promoting receptors on those cells, inhibited the development of T1D (Hutchings et al., 1990). *In vitro* and *in vivo* studies in mice and rats showed that the deleterious effect of macrophages on β-cells can be mediated through the production of TNF and IL-1β (Arnush et al., 1998; Dahlen et al., 1998). Interestingly, pro-inflammatory macrophages can be detected in pancreatic islets before T cell infiltration, as well as in NOD/*scid* (severe combined immunodeficient) mice, which lack functional B and T cells. Macrophages have been shown to produce IL-12 (Alleva et al., 2000) and to promote efficient differentiation of diabetogenic $CD8^+$ cytotoxic T lymphocytes (CTLs) leading to T1D onset (Jun et al., 1999). More recent data suggest that recruitment of macrophages to islets is mediated by the secretion of CC-chemokine ligand 1 (CCL1) and CCL2 by $CD4^+$ T cells and pancreatic β-cells, respectively (Cantor & Haskins, 2007; Martin et al., 2008). Macrophages recruited to the pancreas produce IL-1β, TNF and ROS that can cause β-cell death, revealing an additional role for macrophages in the destructive phase of T1D. Finally, TNF and IL-1β-producing macrophages have been observed in pancreatic islet infiltrates from patients with recent-onset T1D (Ueno et al., 2007). Together, these studies support a pathogenic role for macrophages in both the initiation and destruction phases of T1D at least in the mouse.

NK cells mediate early protection against viruses and are involved in the killing of infected cells and tumours. NK cells are both cytotoxic and producers of cytokines, particularly IFNγ. Thus, NK cells could contribute directly and indirectly to the destruction of β-cells.

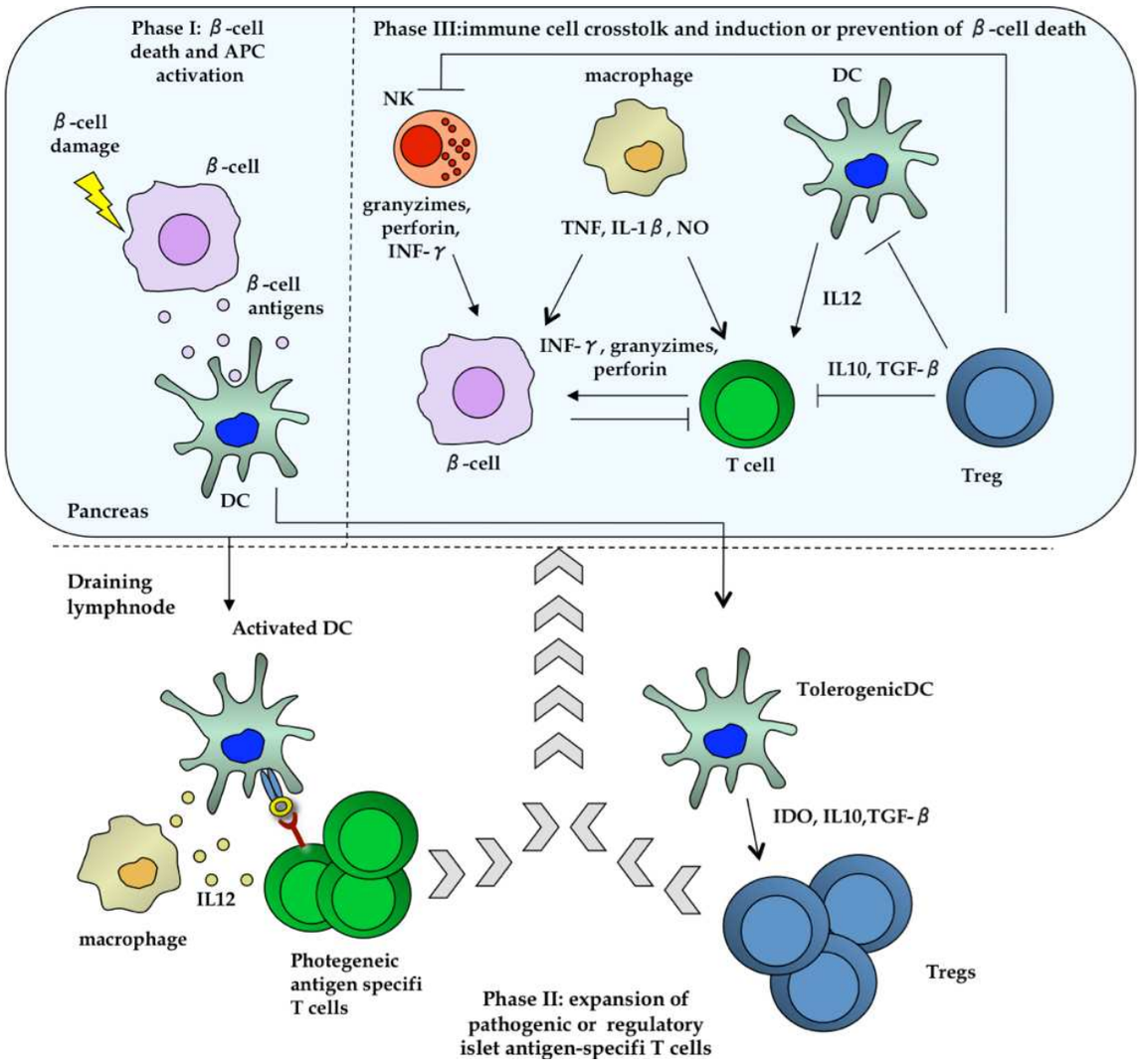

Fig. 3. Cellular and molecular mechanisms in the development or prevention of T1D. The initiation phase of T1D takes place in the pancreas, where DCs capture and process β-cell antigens. β-cell damage can occur by 'natural' apoptosis or after viral infections. Activated DCs prime pathogenic islet antigen-specific T cells after migration to the draining lymph nodes and macrophages promote this activation through IL-12 secretion. The activation of islet antigen-specific T cells can be inhibited by DCs through various mechanisms, such as expansion of Tregs through production of IDO, IL-10 and TGFβ. In the pancreas, β-cells can be killed by diabetogenic T cells and NK cells through the release of interferon-γ (IFNγ), granzymes and perforin, as well as by macrophages through the production of TNF, IL-1β and nitric oxide (NO). β-cell damage can be inhibited by Treg cells that inhibit diabetogenic T cells and innate immune cells through IL-10 and TGF-β. Tolerogenic DCs stimulated by NK cells could also control diabetogenic T cells through IDO production. Lastly, β-cells can inhibit diabetogenic T cells by expressing PDL1. This complex crosstalk between innate and adaptive immune cells results in the development or the prevention of T1D. (Figure adapted from Lehuen et al., 2010).

NK cells have been detected in the pancreas of patients with T1D and in T1D mouse models (Dotta et al., 2007; Alba et al., 2008; Brauner et al., 2010). Moreover, several reports have described a correlation between the frequency and/or activation of NK cells with the destructiveness of the pancreatic infiltrate (Poirot et al., 2004; Feuerer et al., 2009). NK cells isolated from the pancreas of diabetic mice have a more activated phenotype, proliferate more and spontaneously produce higher levels of IFNγ, which promote the effector function of diabetogenic $CD4^+$ T cell, and express CD107a on their cell surface, a marker of granule exocytosis, reflecting their cytotoxic function (Gur et al., 2010). Interestingly, NK cells were observed in the pancreas in NOD mice before T cell infiltration and in the pancreas of NOD–*Rag* mice, which lack mature B and T cells, suggesting that they could have a sentinel role in the pancreas.

Besides macrophages and NK cells, an important role in the pathogenesis of an autoimmune response is played by DCs. DCs are a heterogeneous population of antigen presenting cell that check tissue homeostasis, initiate T cell mediated immunity and control the maintenance of the immune tolerant state. It is known that patients with a congenital DC deficiency develop autoimmune diseases (Ohnmacht et al., 2009). This highlights their role in mediating peripheral tolerance. DCs, depending on their subset and function, can activate Tregs. It has been shown that they mediate peripheral tolerance by inducing T cell depletion or anergy and expansion of antigen specific Tregs (Ueno et al., 2007).

Studies aimed at elucidating the role of DCs in T1D have outlined beneficial as well as detrimental roles of this cell type in the autoimmune process. In the NOD/BDC2.5 transgenic mouse model, it was demonstrated that DCs prevent the inflammation process in the pancreas by producing indole 2,3-dioxygenase (IDO), a tryptophan catabolizing enzyme that arrests T cell proliferation (Saxena et al., 2007). However, in the same model, it was shown that IFN type 1 is more intimately involved in the initiation of the destructive autoimmunity and is correlated with the increased DC expression in the pancreatic lymph nodes (Li et al., 2008). Alternatively, these two opposite findings might point to a dual role of DC in the autoimmune process most probably depending on the stage of DC maturation and capacity to activate specific immunomodulatory cell types.

5. Immunotherapy to induce immunotolerance

Individuals with T1D develop hyperglycemia due to insufficient insulin production by β-cells in the pancreas. To prevent the rise of blood glucose to pathological levels, T1D patients have to receive a life long treatment with recombinant insulin. Despite insulin supplementation, rapid excursion of glucose levels, in these patients, increases the risk for severe complications such as cardiovascular diseases, nephropathy and neuropathy. Insulin replacement therapy cannot match the precision of endogenous insulin secretion, for this reason new treatments that, ideally, can cure the disease or at least delay/prevent the onset are needed.

The new emerging therapies for T1D, aimed at regulating the autoimmune response largely involve broad based immunoregulatory strategies, including the inhibition or deletion of lymphocytes subsets and/or the use of agents that induce or re-establish immune tolerance via activation of regulatory cells (Chatenoud, 2003; Luo et al., 2010).

5.1 Immunosuppressive drugs

Several randomized clinical trials (RCT), based on preclinical study in animal models, have been performed to test the effect of different immunosuppressive drugs on diabetes

patients. Cyclosporin A (CSA) was employed in the first trials showing effects of immunosuppressive therapies on T1D. Continuous CSA treatment initiated soon after diagnosis eliminated the need for exogenous insulin (Bougneres et al., 1990; Carel et al., 1996). However, the lack of lasting effects and renal toxicity of the drug diminished enthusiasm for this approach. Indeed, in considering immunosuppressive therapies we have to remember that these drugs increase the risk of developing infections and malignancies and that some of them have been shown to inhibit β-cell regeneration (Nir et al., 2007). Within the multitude of immunosuppressive drugs, we are now focusing our overview on those drugs that are of particular interest because of their low levels of side effects and/or because they are able to induce Tregs or tolerogenic DCs.

5.1.1 Anti T-lymphocyte Globulin (ATG)

ATG is a very potent immunosuppressive drug. It depletes almost the entire T cell population in treated patients and is primarily used as inductive treatment after solid organ transplantation or in acute rejection settings in transplant patients. Since ATG is a polyclonal non-human protein mixture, common side effects include fever and serum sickness including arthralgia, rashes and lymphadenopathy. Administration over a longer period increases the risk for immunoproliferative disorders, which is why only short-term treatments are considered. A pilot trial involving new-onset T1D patients has shown a reduction of insulin requirement (Eisenbarth et al., 1985). In a more recent study, ATG (Fresenius) retarded the loss of C-peptide in new-onset patients without the need for continuous drug administration (Saudek et al., 2004) but additional studies are being performed to confirm these findings.

5.1.2 Anti-CD3

One of the most potent treatments at reversing new-onset diabetes in NOD mice is therapy with anti-CD3 mAb (Chatenoud et al., 1994). Chatenoud et al. showed that an intravenous treatment with anti-CD3 mAb resulted in a long-lasting restoration of normoglycemia in 80% of treated NOD mice. The treatment was given for only 5 days indicating that continuous administration might not be required to reach a beneficial effect through restoration of the immune balance in favor of endogens tolerance. These studies also showed that treatment was only effective if it was given shortly after the onset of hyperglycemia (Chatenoud, 2003). These results in NOD mice have led to trials in humans using humanized Fc-engineered monoclonal anti-CD3 antibodies. So far, two antibodies have been tested in diabetic patients, hOKT3 g1 Ala-Ala (Teplizumab) (Herold et al., 2005) and ChAglyCD3 (Otelixizumab) (Keymeulen et al., 2005), and both have shown positive results in patients with T1D in terms of C-peptide preservation and reduction of insulin requirements (Herold et al., 2002). Additionally, sustained C-peptide levels for approximately 2 years and in some cases up to 5 years were observed (Herold et al., 2005, Keymeulen et al., 2005). The side effects of anti-CD3 treatment were predominantly headaches, fever and arthralgia. Moreover gastrointestinal symptoms and most importantly transient EBV-viremia with symptoms of acute mononucleosis were observed. All patients however recovered spontaneously. The mechanism of action of this treatment has been extensively investigated. It can be demonstrated that anti-CD3 treatment modulates the T cell receptors in a way that renders T cells blind to antigens, induces T cell anergy, blocks the IL-2 signaling pathway, and induces apoptosis (Chatenoud & Blustone, 2007).

Interestingly, it has also been shown that Tregs are less susceptible to anti-CD3 induced apoptosis; at least when administered in low doses, thus leading to higher numbers of T regulatory cells under the generalized $CD3^+$ T cell lymphopenia. Taken together, these data have made anti-CD3 antibody a possible candidate for future combination therapies. However, the anti CD3 based phase III clinical trial by Lilly didn't meet the target of the trial and the use of anti-CD3 is no longer being pursued by this commercial entity (http://www.fiercepharma.com/press_releases/macrogenics-and-lilly-announce-pivotal-clinical-trial-teplizumab-did-not-meet-primary).

5.1.3 Anti-CD20

B cells are implicated in the pathogenesis of diabetes. Hu et al., (2007) and Xiu et al., (2008) have shown that diabetes can be prevented in NOD mice by depleting B cells with anti-CD20 mAb before and at the time of onset of hyperglycemia (9–12-week-old mice) and can even reverse disease in about 30% of animals treated at the first appearance of hyperglycemia. Interestingly, cotransfer of B cells from the successfully treated mice diminished the rate of adoptive transfer of disease via T cells, suggesting a possible role for

Agent	Target mechanism	Phase/ ID	Details	Reference
Cyclosporin A	Immune suppression	Completed	Remission successful during treatment but severe side effects	Bougneres et al., 1990, Carel et al., 1996.
Teplizumab Anti CD3 (hOKT3) g1 Ala-Ala	T cell immunomodulation and treg generation by anti Cd3 mAb	Phase III	Primary end point not achieved	
Otelixizumab Anti CD3 (ChAgly CD3)	T cell immunomodulation and treg generation by anti Cd3 mAb	Phase II	6 day treatment: better maintenance of C-peptide levels, reduced insulin requirement out to 18 mo	Chatenoud et al., 1994; Keymeulen et al., 2005.
Rituximab (Anti-CD20 mAb)	B cell depletion	Phase II	Preservation of C-peptide levels for 3/6 months	Prescovitz et al., 2009; Hu et al., 2007.
ATG	T cell depletion generate Treg population	Phase II	Could cause cytokine release syndrome	Simon et al, 2008.

Table 1. Summary of immunotherapy approaches in T1D using antibodies.

activation of ''regulatory'' B cells. Others have shown that IL-10-producing B cells can be induced in mice depleted of $CD20^+$ B cells (Yanaba et al., 2008).
In a recent phase II clinical trial, depletion of B cells using an anti-CD20 mAb (Rituximab), has shown modest (23%) but significant improvement in β-cell function 3 months after diagnosis and overall at 1 year, in antibody-treated compared to placebo-treated subjects (Pescovitz et al., 2009). There were also significant improvements in clinical parameters including glycated hemoglobin A1c, C-peptide level and insulin use. Side effects that appear frequently are mostly related to the administration itself and decrease over the course of the therapy. However the patients eventually returned to hyperglycemia as B cells reappeared to a great extent (69%) by the end of the year and the C-peptide level started to decline.
This study could ultimately prove that there is a role for B cells in disease pathogenesis, which is scientifically of great interest. However, B cell depletion in this setting does not appear to mediate a significant deceleration of disease progression.

5.2 Anti-inflammatory treatments

5.2.1 Cytokine and cytokine receptor-directed therapies

Cytokine and cytokine receptor-directed therapies are also in development for treatment of T1D. Human insulitis shows a considerably greater infiltration of innate immune cells such as macrophages and NK compared to NOD insulitis (Itoh et al., 1993; Dotta et al., 2007). Moreover, innate mediators (TNF-α, IL-1, and type 1 interferons) were among the first molecules shown to have direct cytotoxic effects on β-cells and were postulated to be the direct cause of β-cell killing (Rabinovitch et al., 1990). Possibly because of its innate role in activating adaptive immune responses, it was not surprising that IL-1 receptor-deficient NOD mice had reduced development of diabetes (Thomas et al., 2004). Treatment with the IL-1 receptor antagonist (Anakinra) was shown to improve glucose control in patients with T2D (Donath & Mandrup-Poulsen, 2008). Interestingly, the drug mechanism appeared to involve a beneficial effect on β-cells, reflected by an increase in the insulin:proinsulin ratio. β-cells may be a source of IL-1, particularly in response to glucose, suggesting a destructive cycle in which hyperglycemia induces expression of the inflammatory mediator resulting in immune activation and further β-cell destruction. Initial preclinical data do not suggest that IL-1 blockade alone will prevent or reverse T1D, but this could be an important target of a combination strategy.
TNF-α is considered to play an active role in the pathogenesis of T1D. TNF-α is directly cytotoxic to β-cells, suggesting this cytokine as an additional possible target for immune therapy. In a small pilot trial in children and adolescents early after diagnosis (< 30 days on average), the use of a TNF antagonist (Etanercept) resulted in preservation of residual insulin secretion compared with placebo (Mastrandrea et al., 2009). C-peptide loss was reduced, as well as a decrease in insulin needs. However there are contrasting data about targeting the TNF-α pathways. Indeed, it has been shown, *in vitro*, that selective $CD8^+$ autoreactive Tcell death induction can be activated by TNF-α, suggesting the use of a TNF agonist instead of a TNF antagonist (Ban et al., 2008). The discrepancy could be due to the timing of TNF-α blockade/ TNF-α administration.

5.3 Antigen specific strategies

Establishment of a simple strategy that results in the emergence of antigen-specific regulatory T cells and the induction of tolerance to autoantigens is a desirable goal. It would

ultimately stop the autoimmune process without inducing some of the major side effects that have been observed, for example, in chemical and antibody-based immunosuppressive treatments. Moreover, individuals at risk could be treated prior to significant destruction of β-cell mass and clinical onset of disease. However, the risk of boosting autoreactivity should never be underestimated. As outlined earlier, several autoantigens have been described in T1D; insulin and GAD65 are believed to be the major autoantigens that drive the autoreactivity. Consequently they have been studied most intensively in terms of inducing tolerance in humans.

5.3.1 Insulin

Several clinical trials target insulin because it is the initiating antigen in the NOD model and is also a major autoantigen in human T1D (Nakayama et al., 2005; Fan et al., 2009). There have been a number of human new-onset trials using insulin therapy. In the immunotherapy diabetes (IMDIAB) trial, a total of 82 patients with clinical T1D were randomized to receive oral insulin or placebo (Pozzilli et al., 2000). At a 1-year follow-up, there was no difference between the insulin-treated and the placebo-treated groups with respect to mean C-peptide secretion, requirement for insulin therapy, or IgG insulin antibodies. Furthermore, in patients younger than 15 years, a tendency for low C-peptide at 9 and 12 months was observed in the oral insulin group, suggesting acceleration in the decline of β-cell function. These results are consistent with those seen in murine models where oral insulin was shown not to reverse new-onset diabetes (Fousteri et al., 2007). Interestingly, if nasal insulin therapy is used in combination with anti-CD3 therapy, a significant benefit in reversing recent-onset diabetes is then achieved in two animal models of autoimmune diabetes (Bresson et al., 2006). Expansion of insulin-specific Treg cells producing IL-10, TGF-β, and IL-4, and possibly their modulation of antigen-presenting cells in local draining lymph nodes, were proposed as likely mechanisms. These findings should provide the basis for using combinatorial therapies in future trials for humans with recent-onset diabetes.

A recent phase I study using a single intramuscular injection of human insulin B chain in incomplete Freund's adjuvant in 12 subjects with recent-onset diabetes showed that this therapy led to the development of lasting (at a 2 year follow-up) insulin B chain-specific Tregs (Orban et al., 2009). This study provides the basis for testing this modality of insulin B chain therapy in a larger T1D trial to determine the effect on glycemic level. Another ongoing phase I–II clinical trial of subcutaneous BHT-3021, a plasmid encoding proinsulin, is testing the safety, dose, and preliminary efficacy of this therapeutic modality in recent-onset T1D patients

5.3.2 Glutamate decarboxylase 65

Immune therapies using GAD65 have also been tested in both animal models and human T1D. Interestingly, the initial antigenic region is confined to a few epitopes near the C terminus of the GAD protein but later spreads intramolecularly to other GAD determinants, followed by further intermolecular spreading to other β-cell antigens. Consequently, tolerance induction by intravenous or intrathymic injections of GAD in female NOD mice at 3 weeks of age eliminates the anti-GAD T cell responses, as well as subsequent spreading of the cascade of T cell responses to other β-cell antigens and the development of insulitis or clinical diabetes (Tisch et al., 1993). Intravenous injections of GAD during the later stages of

disease still effectively blocked disease progression in prediabetic mice and protected syngeneic islet graft survival in diabetic NOD mice (Tian et al., 1996). The identification of Tregs in GAD-treated mice suggests a major role in the induction of tolerance by treatment with this autoantigen, which raises the question of whether GAD is targeted early in T1D (Tisch et al., 1998).

Agent	Target mechanism	Phase/ ID	Details	Reference
Anakinra (IL1 antagonist)	Anti-inflammatory and improve β-cell survival	Phase II/III	Recruiting	Pickersgill et al., 2009
Etanercept (TNF-α blockade)	Anti-inflammatory	Phase II/III	Low HbA1C and insulin need, increased C-peptide	Mastrandrea et al., 2009
Insulin in IFA	Tolerance vaccination to insulin B chain	Phase I/II	Ongoing	Orban et al., 2009.
BHT-3021	Tolerance vaccination to insulin	Phase I/II	Reduce insulin Ab titers, preserved C-peptide and reduce HbA1c	Gottlieb, 2009.
GAD-Alum	Tolerance to GAD65 skewing Th1 to Th2	Phase II Phase III	Preservation of residual insulin secretion, GAD specific humoral and cellular response, Ongoing in Europe and USA	Agardh et al., 2009; Ludvigsson et al., 2008
Diap277	Induction of Tregs via TLRs	Phase III	Phase I: preserved C-peptide Phase II: no effect in T1D adults and children Phase III: recruiting	Raz et al., 2001; Lazar et al., 2007; Schloot et al., 2007

Table 2. Summary of immunotherapy approach in T1D using autoantigens, cytokines or cytokine-specific antibodies.

Promising preclinical data in the NOD model prompted two clinical trials using alum-formulated human recombinant GAD65. A phase II safety and dose-finding trial conducted in patients with latent autoimmune diabetes in adults (LADA) (Agardh et al., 2005) showed the approach to be safe, and administration of two subcutaneous doses led to an increase of fasting and stimulated C-peptide at 24 weeks compared to baseline, a benefit that was associated with an increase in $CD4^+CD25^+$ Treg cells. In a second trial, in recent-onset T1D children between 10 and 18 years of age, a slower decline of fasting and stimulated C-peptide in the GAD-alum group was observed compared to the placebo (Ludvigsson et al., 2008). More importantly, the protective effect of GAD-alum was preferentially seen in those who received treatment within 6 months of diagnosis, suggesting that the autoimmune process is more susceptible to GAD-based modulatory therapy if initiated at an earlier stage.

5.3.3 Heat shock protein

Early controversies existed as to whether heat shock proteins (hsp) were true autoantigens implicated in the pathogenesis of T1D (Atkinson & Eisenbarth, 2001). However, extensive preclinical studies using the hsp60 peptide p277 demonstrated efficacy of peptide vaccination in halting disease progression in NOD mice (Elias et al., 1991; Elias & Cohen, 1995). p277 treatment appeared to promote Th2-type cell responses with upregulation of IL-10 and IL-13 and downregulation of IFN-γ (Elias et al., 1997; Jin et al., 2008). p277 also has inhibitory effects on the innate immune system via signaling through TLR-2, leading to inhibition of inflammatory lymphocyte chemotaxis (Nussbaum et al., 2006). The equivalent of human hsp60 p277 is a 24 amino acid synthetic peptide derived from the C terminus of the human hsp60, termed DiaPep277. Several phase I and II clinical trials in human T1D patients have been completed in Europe, and phase III trials are underway. A phase II trial was conducted in patients with established T1D but with residual β-cell function (Huurman et al., 2007) and used a range of doses of subcutaneously administered DiaPep277. Results showed a trend of dose-dependent preservation of stimulated C-peptide secretion. Three additional trials were conducted in new-onset T1D patients (Raz et al., 2001; Lazar et al., 2007; Schloot et al., 2007). Two of these trials enrolled adult TID patients, whereas the third enrolled pediatric T1D patients. The adult trials showed significantly better preservation of insulin synthesis as measured by C-peptide production in the treated groups compared with placebo, but this effect was not seen in the pediatric trial. Similar results were observed in one other trial performed in pediatric patients (Schloot et al., 2007), although in children with less aggressive disease progression based on genetic background, there appeared to be a trend to better preserved C-peptide at the end of the study period. In summary, phase II trials with DiaPep277 have shown some promise in preserving residual β-cell function, which appears to be less effective in patients with more aggressive disease. A phase III trial is underway with results expected in later 2011.

6. Cell therapy in type 1 diabetes

Cellular adoptive-transfer-based approaches have shown significant promise preclinically in the NOD model, both in prediabetic and postdiabetic stages. The idea is to compensate a presumed deficiency in tolerogenic cells or tolerogenic cell/molecular signaling pathways by transferring cell types with immunomodulatory capacity. Specifically, both *ex vivo* expanded Tregs or induced $CD4^+CD25^+Foxp3^+$ Tregs (iTreg) have been shown to control ongoing autoimmunity and either prevent progression to diabetes or protect syngeneic islet

grafts and/or allow β-cell recovery, thus inducing diabetes remission in NOD mice (Tang & Bluestone, 2006; Weber et al., 2006; Luo et al., 2007; Godebu et al., 2008). It is unclear whether antigen specificity is critically important in this approach because both nonspecifically-expanded polyclonal or induced Tregs and islet antigen-specific Tregs have shown efficacy in controlling the disease. Additionally, it also appears that Tregs of one antigen specificity may be sufficient in controlling ongoing autoimmunity that is probably caused by autoaggressive T cells of multiple islet antigen specificities (Tarbell et al., 2004; Luo et al., 2007). Clearly delineating these characteristics of Treg adoptive-transfer therapy will have significant impact on the design of future clinical trials using this modality.

Another strategy for enhancing Treg numbers *in vivo* is by DC-based therapy. It has been shown that direct injection of either DC from pancreatic draining lymph nodes or β-cell antigen-pulsed immature DC protects prediabetic NOD mice from developing overt diabetes, possibly through the *in vivo* induction of Treg cells (Clare-Salzler et al., 1992; Lo et al., 2006). However, direct *ex vivo* DC therapy carries the potential risk of their acquiring an activated phenotype upon adoptive transfer, leading to immune activation to some antigen(s) rather than tolerance.

6.1 Diabetes-suppressive dendritic cells

Methods to stably maintain DC in an immature state, defined by low levels of surface costimulatory proteins that include CD80, CD86 and CD40, by downregulating these proteins or blocking their interaction with their ligands, are at the forefront of tolerogenic biologicals like the CTLA4-Ig protein. These strategies result in tolerance to allografts and prevention of autoimmune disease. We have considered two strategies to maintain DCs in a stably-immature state. The first involves *ex vivo* treatment with short double-stranded decoys of the NF-kappaB transcription factor and the second involves *ex vivo* treatment of DCs with antisense oligonucleotides (AS-ODN DC) targeting the primary transcripts of CD40, CD80 and CD86 concurrently. Both DC products are able to prevent and to reverse new onset T1D (Ma et al., 2003; Machen et al., 2004; Trucco & Giannoukakis, 2007; Giannoukakis et al., 2008). These preclinical studies have led to a recently completed phase I trial using autologous *ex vivo*-engineered DC from established diabetic patients (clinicaltrials.gov identifier NCT00445913), conducted at the University of Pittsburgh Medical Center (UPMC), to determine safety as a primary end-point (Figure 4).

Mechanistically, functionally immature DCs, with low to absent costimulatory molecule expression, mediate peripheral tolerance by inducing T cell anergy and the expansion of antigen specific $Foxp3^+$ $CD25^+$ $CD4^+$ Treg (Ueno et al., 2007).

In our approach, AS-ODN DCs promote Treg cell survival through IL-7 signaling in addition to impaired provision of CD40, CD80 and CD86 costimulation (Harnaha et al., 2006). AS-ODN DCs, but not control DC, produce IL-7, in response to a secondary action of the antisense oligonucleotides on Toll Like Receptor (TLR) signaling. It is known that CpG oligonucleotides, like the AS-ODN we use to make tolerogenic DCs, can activate TLR signaling and confer an immunoregulatory phenotype to DCs (Roberts et al., 2005; Jarnicki et al., 2008) and are thus useful for treatment of autoimmune conditions (Ho et al., 2005). It is possible that the oligonucleotides act in a sequence-nonspecific manner when interacting with TLRs, TLR9 in particular, based on conformation and higher order multistrand structures (Guiducci et al., 2008; Kindrachuk et al., 2008). For example, certain multimer formations or conformations would induce non-MyD88 signaling pathways, whereas others

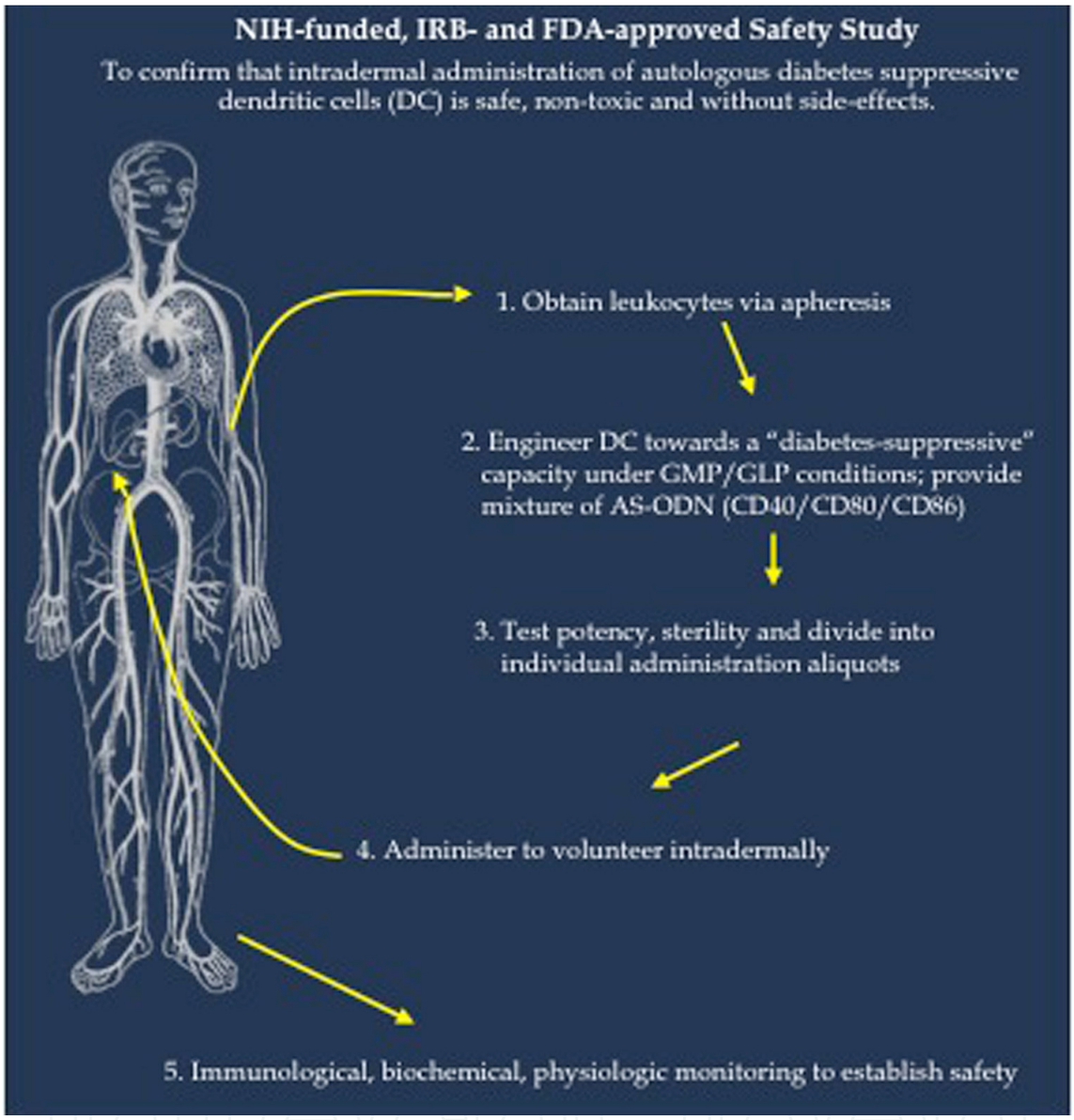

Fig. 4. DC-based clinical trial for T1D. Schematic of the procedures involved in the phase I clinical trial recently completed at the University of Pittsburgh to prove the safety of the DC-based vaccine. (Giannoukakis et al., 2008)

would recruit MyD88. We propose a model where AS-ODN treatment results in a coordinate downregulation of CD40, CD80 and CD86 and induction of IL-7 production via non-MyD88 TLR signals. At this time it is unclear which of the DNA-sensing TLRs transduces the AS-ODN effects. TLR3, TLR7, TLR8 and TLR9 are all equally possible, although the effect of chloroquine on IL- 7 production would suggest an endosomal TLR with TLR9 being the most likely candidate (Figure 5) (Di Caro & Giannoukakis, unpublished data). Indeed, the data indicating that CpG oligonucleotide-triggered TLR9 signaling confers immunosuppressive capacity to DC that can treat autoimmunity *in vivo* strengthens our hypothesis (Ho PP et al., 2005).

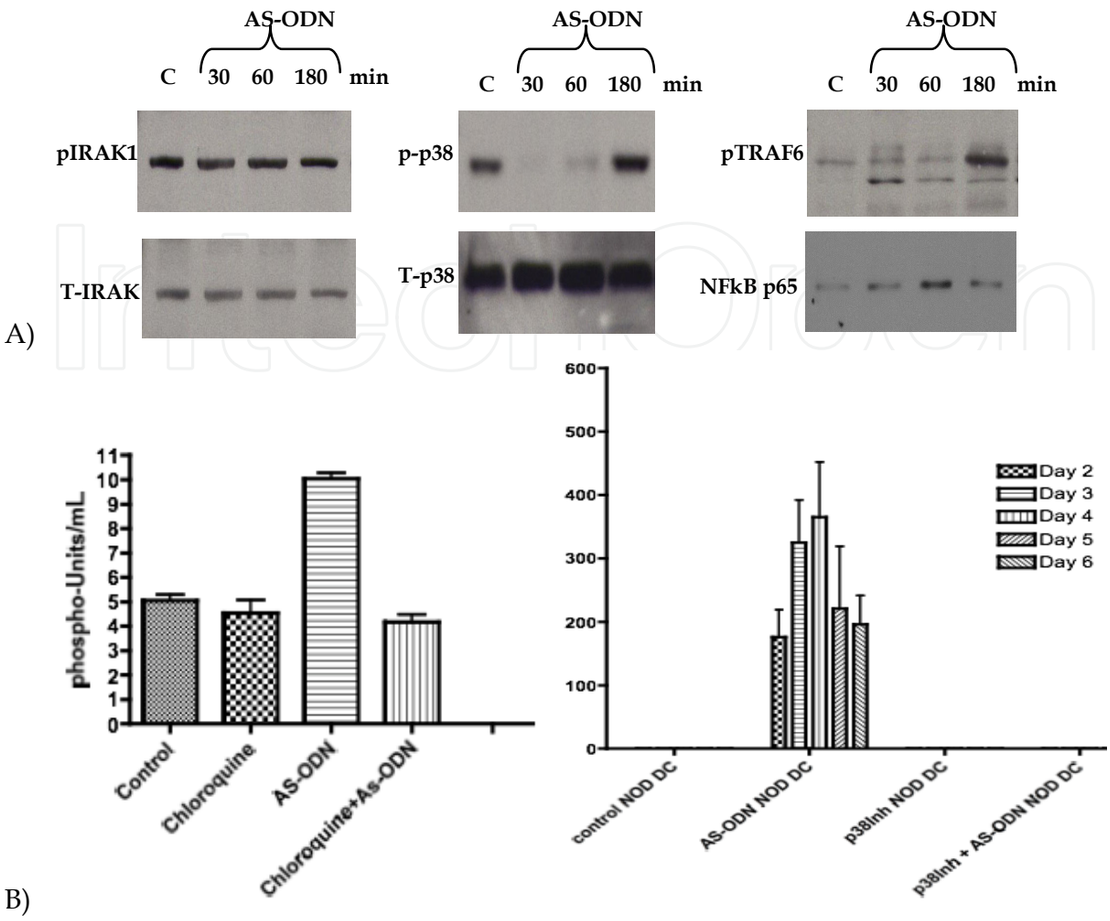

Fig. 5. AS-ODN treatment of DC *in vitro* activates TLRs signals leading to IL-7 production. A) Western blot analysis of protein extracts from DC treated with AS-ODN for CD40, CD80 and CD86 over time, using the indicated antibodies, shows activation of NFkB after 1 hour and activation of p38 MAP kinase and TRAF6 after 3 hours. B) p38 MAP kinase phosphorylation in AS-ODN DC in the presence of chloroquine, a specific inhibitor of endosomal TLR signaling (e.g. TLR9), is decreased, as demonstrated by LUMINEX-based nuclear transcription factor analysis. C) inhibition of p38 phosphorylation, using the p38 MAP Kinase inhibitor SB203580, shows a complete abrogation of IL-7 production for each of the 7 days of generation of the AS-ODN DC.

Recently, we identified a novel $CD127^+$ $CD25^+$ $Foxp3^+$ T cell subpopulation that expresses the IL-7 receptor (CD127) and has immunosuppressive activity (Figure 6). More interestingly, exposure of this novel T cell subpopulation to IL-7 *in vitro* results in the phenotypic maturation of $CD127^+$ $CD25^+$ $Foxp3^+$ T cells to the classical $CD25^{HIGH}$ $Foxp3^+$ Treg (Figure 7) (Di Caro et al., 2011).

IL-7 production by the AS-ODN DC could serve to mature the $CD127^+$ $Foxp3^+$ cells into powerfully suppressive $CD25^{HIGH}$ $Foxp3^+$ Tregs, and maintain their survival for a longer time period, especially when the IL-2 concentration in the lymphoid environment is expected to be limiting, given the competition among $CD25^+$ Tregs and $CD25^+$ effector T cells for this critical cytokine. Furthermore, the apparent biregulation of cell surface CD25

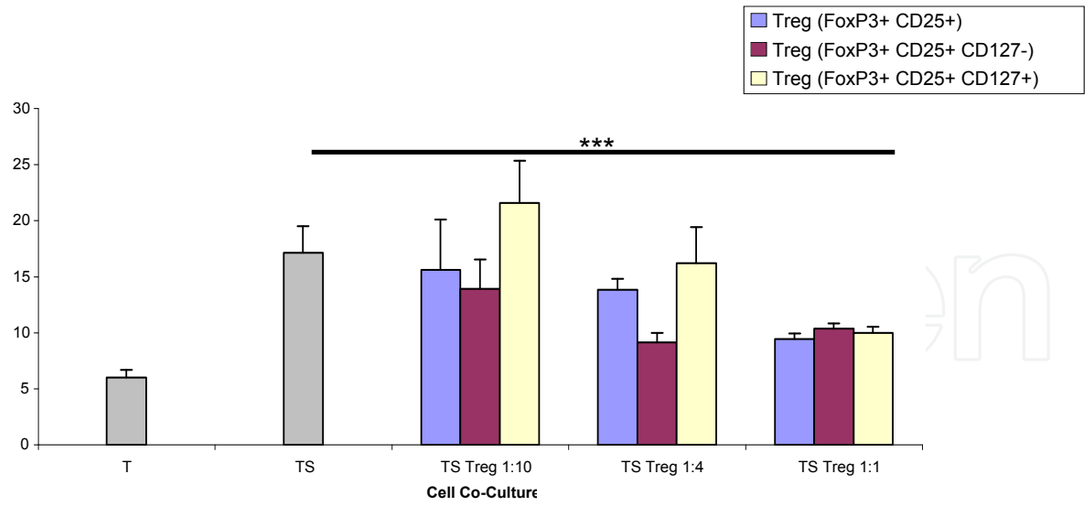

Fig. 6. CD127⁺ CD25⁺ Foxp3⁺ T cell are functionally suppressive *in vitro*. Highly enriched, flow sorted CD4⁺CD25⁺CD127⁺ splenic T cells, isolated form FoxP3 promoter-GFP transgenic mice, are suppressive when added to a co-culture of syngeneic T cells and allogeneic, irradiated, splenocytes (Di Caro et al., 2011).

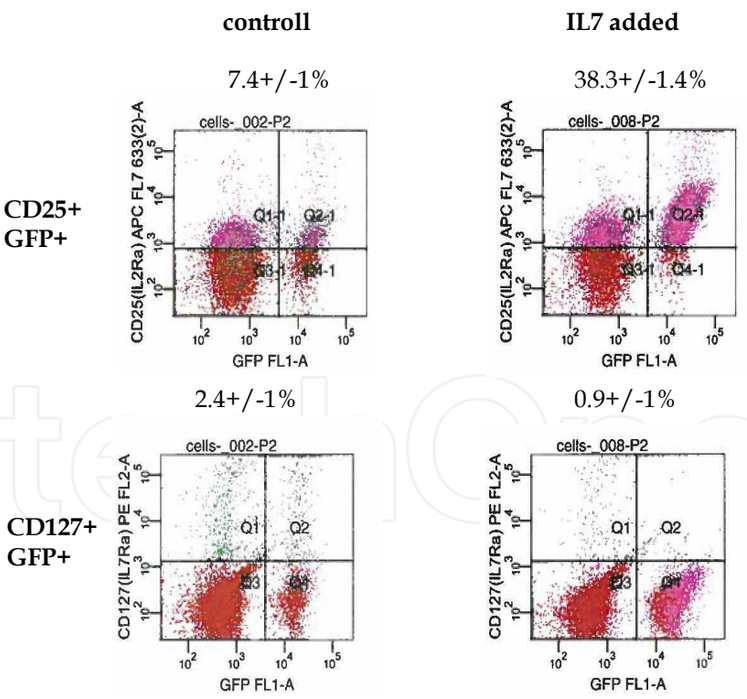

Fig. 7. IL-7 promotes an increase in prevalence of CD127-CD25⁺Foxp3⁺ cells. Incubation of splenic CD4⁺GFP⁺ T cells from Foxp3 promoter-GFP transgenic mice with IL-7 overnight results in an increase in CD25⁺GFP⁺ T cells, whereas IL-7 downregulates the prevalence of CD127⁺ GFP⁺ cells (Di Caro et al., 2011).

and CD127 on Tregs in response to their respective ligand availability and signaling, in peripheral lymphoid organs, could have two functions; Treg maintenance and suppressive competency. IL-7 could best serve Tregs under homeostatic conditions in the periphery where IL-2 production would be low. This would maintain a pool of CD4+ $CD25^{HIGH}$ Tregs as some type of "memory" Treg population. In contrast, in an environment where IL- 2 would be acutely produced at high levels (i.e. vigorous proliferation of autoreactive T-cells), Tregs would compete as well as the effector T cells for IL-2 and therefore, IL-7 might not be as relevant.

Through these mechanisms and others yet unknown, tolerogenic DC could modulate and restore the balance of pro and anti-inflammatory components of the immune system. Our data and the work carried out by other groups highlights the relevance of using tolerogenic DC to treat autoimmune diabetes as well as other tissue specific autoimmune disorders.

7. Conclusion

T1D most likely results from a combination of genetic susceptibility and exposure to an environmental trigger. The main effector mechanism is clearly an autoimmune reaction, which is also evident at time of clinical diagnosis. A better knowledge of the causes that lead to T1D is critical for prevention as well as for developing new therapies. Early detection is also required to maximally preserve the remaining β-cell mass, because the ability to secrete even small amounts of insulin can make disease control easier and help minimize the complications due to chronic inadequate glycemic control.

Much of our current understanding of T1D comes from the NOD mouse model, this autoimmune diabetes model, so far, has been useful to discover and develop treatments even if some of them were not as successful in humans (e.g. the anti-CD3 therapy).

Today the landscape of possible treatment has been changed by the prospect that T1D progression may be blocked by the active stimulation of tolerance induced by autoantigen-specific Tregs or tolerogenic DCs. The ultimate goal of autoimmune therapy is to silence the immune attack against self without sacrificing the patient's protective immune response to pathogens. This will most likely be achieved by a therapy that combines a nonspecific immune suppressant and the induction of Tregs/ tolerogenic DCs. Regardless of the tolerogenic method employed for therapy, we think that early intervention in T1D patients is critical to prevent ongoing islet destruction and to establish an ideal microenvironment to allow the recovery of a normal β-cell mass from endogenous progenitor cells. The chances for disease prevention will be improved by the identification of biomarkers identifying patients at risk as early in the disease process as possible.

Major efforts on several fronts are still required to fully realize the benefits of the technological and scientific advances in autoimmune diabetes research even if substantial improvements in the cure of T1D patients were indeed promoted.

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