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Autoantigen-Specific Immunotherapy

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1. Introduction

The incidence of type 1 diabetes (T1D) is increasing dramatically and new treatment modalities are needed urgently. Arising from autoimmune destruction of the pancreatic β-cells, T1D manifests itself when less than 10-20% of functional β-cells remain in the islets and is characterized as a disorder of glucose regulation due to the insufficient production of insulin. Insulin is a hormone secreted by pancreatic islet β-cells and its main function is to move blood sugar into cells where it is stored and later used for energy. In T1D, the β-cells are destroyed by the patients' immune cells leading to limited or no insulin production. Without enough insulin, glucose accumulates in the bloodstream instead of entering the cells, causing symptoms of hyperglycemia. Chronic hyperglycemia can lead to many serious complications. Insulin replacement therapy is the current standard for treatment involving injection of recombinant insulin. Normal pancreatic insulin secretion is exquisitely sensitive to the minute-to-minute changes in blood glucose and glucose-stimulated insulin secretion (GSIS) and cannot be mimicked precisely by exogenous insulin injections. Thus, while insulin treatment successfully prolongs the life of affected individuals, it fails to prevent some of the serious complications of T1D that adversely affect quality of life and ultimately lead to significant morbidity and mortality. An alternative promising therapy is islet cell transplantation, but this suffers from several drawbacks including limited pancreatic islet tissue from cadaveric donors, the requirement for lifelong immunosuppression, and the increased risk of infection due to non-specific suppression of the immune system.

To cure T1D, researchers are pursuing combined immunological and biological strategies for restoring β-cell mass. Strategies for increasing β-cell mass include promoting endogenous pancreatic β-cell regeneration, reprogramming non-pancreatic cells into insulin-producing cells (IPCs), and generating unlimited autologous pancreatic β-cells from induced pluripotent stem (iPS) cells of the T1D patients. At the same time, there is considerable interest in approaches for modulating and suppressing autoimmunity to pancreatic β-cells and surrogates of IPCs. An individual's pancreatic β-cell mass is tightly regulated according to insulin demand, reflecting a balance between the rate of β-cell replication, regeneration/reprogramming, and the rate of β-cell apoptosis. Research efforts are focusing on how best to expand, reprogram, or generate β-cells or their surrogates from pancreatic stem/precursor cells, non-pancreatic adult/stem cells, or the iPS cells of T1D patients for β-cell replacement therapy. However, without preventing ongoing autoimmune

destruction of β-cells, the regenerated, reprogrammed, or replaced β-cells or their surrogates may suffer the same fate as the endogenous pancreatic β-cells.

The loss of pancreatic β-cells in T1D is a T cell-mediated autoimmune process orchestrated by CD4+ and CD8+ T cells specific for β-cell antigens. Pathogenic diabetogenic T cells and autoantibodies to β-cell specific autoantigens have been found in T1D patients and non-obese diabetic (NOD) mice, a well-studied animal model of T1D. These T cells employ a variety of mechanisms to destroy β-cells. Moreover, genetic susceptibility factors for T1D mainly affect the ontogeny and survival of immune cell subsets at the level of the thymic selection and in the peripheral tolerance. T1D is an organ-specific autoimmune disease where the immune tolerance to the β-cells is broken and the immune system attacks and eliminates these β-cells. So, restoration of self-tolerance to β-cells and suppression of β-cell autoimmunity combined with increasing β-cell mass are the key components for β-cell replacement therapy.

Experimental evidence indicates that the immune system can be modulated and immune tolerance to β-cells can be restored in experimental animal models of T1D. Self-tolerance can be re-established centrally or peripherally by broad-based immunoregulatory strategies, including the inhibition or deletion of lymphocyte subsets and/or the use of agents proposed to induce or re-establish immune tolerance via activation of regulatory T (Treg) cells. An example is the use of nonmitogenic anti-CD3 or antithymocyte globulin. However, the induction of antigen-specific tolerance to β-cell antigens is a highly desirable objective because only the pathogenic autoreactive T cells are inactivated whereas the remainder of immune system remains unperturbed. Many autoantigens have been identified and implicated in relation to T1D. Well-established autoantigens include insulin (Palmer et al., 1983), glutamic acid decarboxylase 65 (GAD65) (Baekkeskov et al., 1990), insulinoma associated-2 antigen (IA-2) (Bonifacio et al., 1995), and heat shock protein (HSP) (Birk et al., 1996), as well as newly discovered β-cell-specific autoantigens zinc transporter-8 (ZnT8) (Wenzlau et al., 2007), pancreatic duodenal homeobox factor 1 (PDX1) (Li et al., 2010), and chromogranin A (CHGA) (Stadinski et al., 2010). These markers of T1D are useful for diagnosis as well as for prediction prior to disease onset and may prove useful for intervention by restoring immune tolerance[See table-1].

Antigen-specific immunoregulatory therapies are currently being evaluated for preventing and treating T1D and they include autoantigen therapy, dendritic cell (DC)-based strategies, and adoptive transfer of Treg cells. This chapter will review recent work on the antigen specific immunotherapy of T1D including strategies for tolerance induction.

2. The importance and feasibility of antigen specific immunotherapy

Nonspecific and specific approaches have been exploited by immunologists to control the autoimmune response in T1D. They include deleting pathogenic T cells and activating regulatory T cells to induce immune tolerance against β-cells. Non specific approaches included using immunosuppressive drugs such as steroids or cyclosporin A as well as some monoclonal or polyclonal antibodies. Polyclonal anti-lymphocyte antisera and anti-CD3 or anti-CD4 monoclonal antibodies have been used to cure early diabetic disease in murine models for T1D (Maki et al., 1992; Chatenoud et al., 1994; Makhlouf et al., 2004). Anti-lymphocyte antisera and anti-CD4 antibodies are strong immune suppressors and principally act on depleting the autoaggressive T cells. In contrast, anti-CD3 antibodies function as immune modulators (Chatenoud et al., 2007) since they can maintain long term

Autoantigen	Protein	Function	Antigen specific immunotherapy in humans
(Pre)(pro)insulin	Precursor and processed product	Hormone	??
GAD65	Gutamic acid decarboxylase - Mr 65,000 isoform	GABA producing enzyme	Alum-formulated GAD65 preserves beta-cell function
IA-2	Islet antigen-2	tyrosine phosphatase-like protein - unknown function	To be tested
ZnT8	Zn T8 transporter	One of several Zn transporters but specific to the beta cells	To be tested
PDX1	pancreatic and duodenal homeobox 1	a transcriptional activator of several genes, including insulin	To be tested
DiaPep277	HSP60	chaperone	DiaPep277 preserves beta cell function.

Table 1. Islet autoantigens in type 1 diabetes

immune tolerance through inducing adaptive Tregs, interfere with T cell activation, and induce anergy/apoptosis in activated cells. Based on results from the mouse model, the humanized anti-CD3 antibodies (teplizumab and otelixizumab) were explored in phase II trials. It was found that anti-CD3 antibody treatment could preserve endogenous insulin secretion and reduce HBA1c levels and insulin requirement in the treated group during a two year period in recent onset T1D. Recent follow-up studies indicate that the beneficial effects extend over a period of up to 5 years following a single treatment (Cernea et al., 2010). However, some patients develop "flu-like" symptoms due to significant cytokine release by antibody-bound T cells, and recurrent Epstein-Barr viral infections (Keymeulen et al., 2010). It is well known that steroid and and cyclosporin A therapy have very serious side effects. The common problem of antibody-based immunotherapies is that they can't discriminate between normal versus autoimmune effector T cells. The safety concerns and adverse effects of antigen nonspecific interventions, as well as the lack of permanent remission of disease with any agent tested to date have prompted interest in antigen specific interventions that might modulate the autoimmune aspect of the disease. Compared with nonspecific immune-approach, antigen-based immunotherapy allows selective targeting of disease-relevant T cells, while leaving the remainder of the immune system intact. Numerous studies have demonstrated that antigen specific immunotherapy can be highly effective in preventing and suppressing autoimmunity in rodent models of T1D and other autoimmune diseases (Harrison et al., 2000).

The concept of antigen-specific tolerance has been apparent for some years. The presence of self-reactive lymphocytes in the blood of healthy individuals (Burns et al., 1983) implies that self-antigen-specific regulatory mechanisms are physiological and prevent pathological autoimmunity. Antigen-specific tolerance operates directly on effector T cells (via apoptosis or anergy) and via Treg cells that secrete anti-inflammatory cytokines or compete with effector T cells at the level of the antigen presenting cell (APC). Many factors determine the induction of antigen specific tolerance including: 1) characters/properties of antigen, dose/concentration, physical form (soluble or aggregated, intact protein or peptide),

chemical composition, affinity for MHC molecule, content of CD4+ and CD8+ T cell epitopes and 'immunodominance', purity and contaminants (e.g. endotoxin), and adjuvants; 2) route of administration; 3) level of co-stimulation; and 4) antigen-specific precursor cell frequency and duration of treatment (Harrison et al. 2000). High-dose soluble peptide or monomeric protein delivered by the intraperitoneal (i.p.), subcutaneous (s.c.), or intravenous (i.v.) route induces clonal deletion or clonal anergy (Liblau et al., 1997). Delivering antigen through mucosal routes, for example oral and naso-respiratory routes, could induce mucosal immune tolerance that suppresses subsequent systemic priming to the antigen (Faria et al., 1999). Oral tolerance means the specific suppression of immune responses to an antigen by prior administration of the antigen by the oral route. Oral tolerance is an active immunologic process that is mediated by more than one mechanism and is dose-dependent. Low doses have been shown to induce differentiation of regulatory T cells capable of downregulating T cells specific for distinct antigens within a given tissue. This phenomenon of bystander suppression in oral tolerance is associated with the induction of Th2 and TGF-β-secreting Tregs (Anderton et al., 1999) whereas higher doses favor the induction of clonal anergy or deletion (Chen et al., 1995).

T cells recognize antigen peptide via the T cell receptor (TCR) and produce a spectrum of responses that range from activation to anergy or cell death. In the late 1980s, peptides were modified to interfere with the MHC/peptide/TCR complex to prevent autoaggressive T cell activation (Adorini et al., 1988). Later on, this type of peptide was termed an altered peptide ligand (APL). In APLs, TCR contact residues in an immunodominant self-epitope are altered to impair the T cell strength of signal. So, APLs could induce anergy by engaging less than optimal numbers of TCRs. APLs have been applied therapeutically in animal models of autoimmune diseases and in the clinical trial (Harrison et al. 2000). One recent paper reported that proinsulin-derived APLs (prAPLs) modulate the cytokine signature of proinsulin-reactive T cells at a micromolar range by increasing anti-inflammatory cytokines, including TGF-β1 (van et al., 2010). The results suggest that APLs do not act as antagonists *in vivo* but mediate bystander suppression, probably by the generation of Treg cells.

Treg cells have an ability to regulate immune response by non specific bystander suppression in response to specific antigen that is recognized locally at the site of the lesion or in the draining lymph node. During the disease process of animal models and human T1D, T cell autoimmunity progressively spreads intra- and inter-molecularly among β-cell autoantigens such as insulin, GAD65, HSP, and IGRP (van Belle et al., 2011). Bystander suppression resolves the dilemma of multiple autoantigens in human autoimmune diseases by obviating the need to know if the antigen used to induce tolerance is the major or primary pathogenic autoantigen (Tang et al., 2008). Therefore, antigen-specific approaches use only one antigen to tolerize against multiple autoreactivities. In the long term, Treg cells create a regulatory environment by producing TGF-β and/or by substantially changing DC functions to promote new Treg cell production. These processes promote bystander suppression and are critical to the creation of infectious tolerance that can spread beyond the local tissue and exert long-lasting influence over the immune system. In contrast, tolerance-based on anergy or deletion is limited to each individual antigen.

Antigen-specific therapy is logically appealing and demonstrably effective in experimental autoimmune disease animal models. This proof-of-concept and its safety make it an attractive approach for treatment of human autoimmune disease including T1D.

3. Antigen specific immune tolerance induction strategies

Immunotherapy for T1D has been attempted at two main stages of the disease process - prior to clinical onset but after the appearance of islet autoantibodies (secondary prevention) and immediately after diagnosis (intervention) (Staeva-Vieira, T. et al. 2007) .

Prevention of T1D, meaning intervening early during the development of disease, holds greater promise for success because, in this stage, organ damage has not progressed very far and β-cells are still capable of self regeneration. Successful prevention of T1D depends on: 1) a good prediction/identification of at-risk individuals and 2) a safe intervention that causes no harm in those individuals who would have never developed T1D. In T1D, identifying the individuals at risk for developing disease can be obtained through screening islet autoantibodies, combined with genetic markers in the HLA region, and additional risk factors (Siljander et al., 2009). Moreover, metabolic assessments can also be included to identify late-stages of disease development, at which there is already some loss of glycemic control. Thus, it is now feasible to 'stage' individuals according to various modeled rates of progression to T1D (Staeva-Vieira et al., 2007). Currently, preventive trials for T1D include two main classes, antigen- or non-antigen-specific. Compared with a non-antigen specific-approach, an antigen-specific immune tolerance holds more promise and advantage. Many antigen-specific prevention trials have been conducted in recent years. Of note is the oral insulin trial used in relatives of T1D patients following screening of 103,391 first- and second-degree relatives of patients with T1D. Of them, 97,273 samples were analyzed for islet cell antibodies and 2,523 underwent genetic, immunological, and metabolic staging to quantify risk of developing T1D. This trial concluded that it is possible to identify individuals at high risk for T1D and enroll them in a large, multisite, randomized, and controlled clinical trial (Skyler et al., 2005).

Immune intervention for recent onset T1D aims to prevent or reverse the disease by blocking autoimmunity, thereby preserving/restoring β-cell mass and function and decreasing the likelihood of both hypoglycemia and long-term complications. It is a clinically feasible approach because potential subjects are readily identified and efficacy can be evaluated within a much shorter time frame. Currently, large quantities of evidence show that it is possible to reverse clinically established T1D. Firstly, evidence shows that loss of β-cells is a gradual process and that newly diagnosed patients still have 10-20% of functional β-cells remaining within the islets. Therefore, the reversal of hyperglycemia could be obtained through (1) promoting endogenous regeneration and replication of remaining β-cells, (2) reducing metabolic stress on the remaining β-cells, (In support, it is known that early and intensive insulin therapy in patients with recent onset T1D can clearly reduce the loss of remaining β-cell capacity (Shah et al., 1989).), (3) changing immune environment from producing proinflammatory (IFNγ) to anti-inflammatory (IL-10) cytokines (Alizadeh et al., 2006), and (4) suppressing recurrent β-cell autoimmunity. In support, it was found that anti-CD3 antibody could induce durable regression of recently diagnosed T1D in NOD mice by restoring self-tolerance following short-term treatment (Chatenoud 2003). According to this evidence, it is very clear that interventions in recent onset T1D have to be operational within a small and clearly defined window, which might also vary on an individual basis. In this stage patients still have sufficient remaining β-cell mass that can be assessed by C-peptide measurements (Staeva-Vieira, T. et al.2007). In order to maintain or restore critical mass of viable and functional β-cells in T1D patients for developing immune intervention, many approached are being explored. They include (1) stimulation of β-cell regeneration/proliferation in vivo through hormone and growth factor treatment, (2) direct

conversion by in vivo reprogramming of adult pancreatic exocrine cells to transform into β-cells, (3) isolation and in vitro differentiation of pancreatic progenitors, embryonic stem cells, or extrapancreatic adult stem cells, and (4) induction of iPS cells from adult-differentiated cells through targeted reprogramming with soluble factors (5) islet transplantation (Guo T et al. 2009). As soon as these approaches for increasing viable and functional β-cells become readily available, immune intervention will be feasible for recent onset T1D patients and even for late stage patients.

4. Antigen-specific immunotherapy approaches

4.1 Autoantigen therapy

Antigens may behave as immunogens that provoke an adaptive immune response or as tolerogens that induce a state of specific immunological unresponsiveness (immune tolerance) to subsequent challenging doses of the antigen. Several factors determine the immunogenic versus tolerogenic capacity of an antigen, including its molecular form, the nature of APCs, the dose, the route of administration and local response environment (Anderton et al., 1999). Many attempts have been made to induce or restore tolerance in T1D by the use of candidate autoantigens.

Insulin

Insulin and proinsulin molecules have been identified to play a primary role in the initiation of the autoimmune process that ultimately leads to destruction of β-cells and onset of clinical T1D for both humans and the NOD mouse. Insulin autoantibodies usually precede the development of T1D and can be utilized to assist in disease prediction. Transplanted T cell clones recognizing insulin, both CD4+ and CD8+, can transfer disease to young mice or immunodeficient animals. Specific insulin peptides reacting with these clones have been identified (Gottlieb et al., 2002). Based on knowledge about insulin immunity in T1D, insulin specific tolerance induction approaches for prevention and immune intervention have been explored in T1D animal models and patients[See table-2].

Group	Antigen	Route	Protection from diabetes	Reference
BB rat (DP, DR)	Insulin, high dose continuous	Subcutaneously	Yes	41,42
BB rat (DP, DR)	Insulin	Oral	No	42
NOD mouse	Insulin	Subcutanously	Yes	22
NOD mouse	B chain	Subcutanously	Yes	44
NOD mouse	B9-23	Subcutanously	Yes	26,27
NOD mouse	B9-23	Intranasally	Yes	23,26,27
NOD mouse	B10-24	Intranasally	Yes	45
NOD mouse	Proinsulin	DNA	Yes	51
LCMV mouse	Insulin B chain	Oral DNA	Yes	47-49
PVG.RT1u Rat	B1-18	Intrathymic	Yes	50
Human, new onsets	Insulin	Oral	No	56
Human, high risk prediabetic	Insulin, low dose continous	Subcutaneous	No	55
Human, new onset disbetes	APL of B9-23	Subcutanous	?	38
Human, medium-risk prediabetic	Insulin	Oral	?	55

Reference: Gottlieb PA, Eisenbarth GS. Insulin-specific tolerance in diabetes. Clin Immunol. 2002, 102(1):2-11

Table 2. Insulin Immunotherapy of Type 1 Diabetes [Gottlieb PA, 2002-p2]

In NOD mice, reports demonstrated that delivery of insulin (intravenously, subcutaneously, orally, or intranasally), proinsulin, or insulin peptides could delay the onset and reduce the incidence of T1D (Chatenoud 2010). Further study investigating the protection mechanism indicated that administration of insulin could induce active tolerance against T1D (Aspord et al., 2002). Insulin-derived mutated proinsulin peptide B24-C36 was shown to induce anti-diabetogenic regulatory T cells that block the adoptive transfer of T1D and protect the NOD mice from T1D (Fierabracci 2011).

Based on the success in animal models, clinical trials of oral or nasal insulin have been conducted in humans. These trials can be divided into prevention trials and intervention trials. One human prevention trial has included a double-blinded crossover safety study conducted in 38 individuals with antibodies to one or more islet antigens (insulin, GAD65, or IA-2) and the results showed intranasal insulin does not accelerate loss of β-cell function in individuals at risk for T1D and induces immune changes consistent with mucosal tolerance to insulin (Harrison et al., 2004). This is the first report that showed intranasal insulin was immunotherapeutic and retarded progression to clinical T1D. However, another double blind prevention trial with nasal insulin obtained contradictory findings. In this trial, 224 infants and 40 siblings positive for two or more autoantibodies received short-acting human insulin or placebo once a day intranasally. Median duration of the intervention was 1.8 years. The results showed that, in children with HLA-conferred susceptibility to T1D, administration of nasal insulin immediately following detection of autoantibodies could not be shown to prevent or delay T1D. The prevention efficacy of oral insulin was also tested in 388 prediabetic patients who were first- and second-degree relatives of T1D patients and were also classified as having increased risk for developing T1D by genetic, immunological, and metabolic staging (Skyler et al., 2005; Sosenko et al., 2006). It was concluded that oral insulin did not delay or prevent T1D. However, according to subgroup analysis, it appeared that there might be a potential benefit in T1D prevention in those subjects with higher autoantibody levels. Reasons for failure may include insufficient dosing as well as the fact that by the time an individual is identified with autoantibodies, the disease process is well established and difficult to reverse. Moreover, the dose, frequency of mucosal antigen administration, and timing of antigen delivery are critical determinants of tolerance induction and subsequent suppression of T cell mediated autoimmune disease.

Intervention trials were also performed in patients with recent onset T1D with insulin. In one trial, 52 adults with recent onset T1D were given nasal insulin. It was found that nasal insulin did not retard loss of residual β-cell function, but the evidence showed that it could induce immune tolerance to insulin and it provided a rationale for its application to prevent T1D in at-risk individuals (Fourlanos et al., 2011). Two studies including about 100 patients each tested the use of oral insulin at a limited dose range and they didn't observe clinical efficacy (Chaillous et al., 2000; Pozzilli et al., 2000). A large phase II clinical trial with NBI 6,024 (an altered peptide ligand of the 9–23 insulin B chain peptide) showed there is no efficacy of APL treatment in patients at the stage of overt disease (Walter et al., 2009). It did not cause significant changes in insulin requirement, metabolic control, hypoglycemic and hyperglycemic events, autoantibody concentrations, or CD4+ and CD8+ T cell numbers. The lack of response may reflect a fundamental defect in the proposed mechanism of action or inadequacy of exposure (dose), frequency, or timing of injected peptide.

GAD65

GAD65 is one of the major T1D-related autoantigens recognized by self-reactive T cells. T cells specific for GAD65 are among the first to enter inflamed pancreatic islets and may be important for the initiation of autoimmunity (Kaufman et al., 1993). Moreover, GAD65 is also an early target of autoantibodies during the initiation of T1D. Immune therapies targeting GAD65 have been tested in both animal models and in human T1D patients [See table-3].

Group	Antigen	Route	Protection from diabetes	Reference
NOD mice	GAD65	Intraperitoneally	Yes	Peterson 1994 and 1997
BB rats	GAD65	IV	No	Pleau 1995
NOD mice	GAD65 peptide	Nasal	Yes	Wang 2009
NOD mice	GAD65 peptides	Intranasal	Yes	Tian 1996
NOD mice, diabetic	GAD65	IV	Yes	Tisch 1998
NOD mice	DNA	Intramuscular	Yes	Tisch 2001
NOD mice	rVV-GAD65	Intraperitoneally	Yes	Jun 2002
NOD mice, diabetic	DNA	Intramuscular	Yes	Goudy 2008
Human, onset diabetes	GAD-alum (Diamyd)	Subcutaneously	Yes	Agardh 2005
Human, onset diabetes	GAD-alum (Diamyd)	Subcutaneously	Yes	Ludvigsson 2011 and 2008

Table 3. GAD65 Immunotherapy of Type 1 Diabetes

In NOD mice, administration of GAD65 can prevent and block autoimmune destruction of pancreatic β-cells and subsequent need for exogenous insulin replacement. It was found that oral administration of a fusion protein composed of cholera toxin B subunit (CTB) and GAD65 peptide could significantly reduce pancreatic islet inflammation and delay the development of T1D through generating regulatory T cells and induction of immunological tolerance (Gong et al., 2010). 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 CD4+ Treg cells in GAD-treated mice suggests a major role for bystander suppression in the induction of tolerance by treatment with this autoantigen (Tisch et al., 1998).

Based on the above convincing data from NOD mice, autoantigen immunotherapy with GAD65 was tried in human. GAD-Alum (Diamyd Therapeutics) is an adjuvant-formulated vaccine incorporating recombinant human GAD65 which is the specific isoform of GAD expressed in human pancreatic β-cells and a major antigen targeted by autoreactive T lymphocytes in T1D. It was shown to be safe and to preserve residual insulin secretion in patients with late onset T1D of adulthood (Agardh et al., 2005). A subsequent phase II trial in recent onset T1D showed significant preservation of residual insulin secretion and a GAD-specific immune response, both humoral and cell mediated immune response (Agardh et al., 2009). Moreover, Alum-formulated GAD65 has been given subcutaneously in two injections with one month apart to recent onset T1D patients with positive GAD65 autoantibodies. The injections were found to preserve residual β-cell function without treatment related serious adverse events (Agardh et al., 2005). Further study showed that two injections of GAD-Alum to children and adolescents with T1D could induce GAD specific CD4+CD25high forkhead box P3 (Foxp3)+ regulatory T cells and secretion of Th2 and regulatory cytokines (Hjorth et al., 2011). These data support a long-lasting immunomodulatory effect of GAD-Alum treatment. Follow-up after 5 years completed in

2008 still show a significantly beneficial effect of the Diamyd (Morales et al., 2011). According to these promising clinical data, Phase III studies in children with recent onset T1D are ongoing along with a study (DIAPREV-IT) aimed at testing whether Diamyd® may prevent the clinical onset of T1D in non-diabetic children with GAD65 autoantibodies and at least one more islet autoantibody (Larsson et al., 2011). Also, Diamyd Medical is currently conducting two clinical Phase III studies on T1D: one in Europe and one in the United States. Here, the formulation is crucial to Dyamid's GAD drug because *1)* adjuvant reduces the required quantity of antigen by maximizing its immunogenicity and *2)* aluminum salts preferentially induce a humoral rather than cellular immune response. Immune readouts show an increase in FoxP3 and transforming growth factor-β in cells from GAD-Alum-treated patients compared with placebo after 15 months (Ludvigsson 2009). In summary, GAD 65 vaccination has shown encouraging results with preservation of residual insulin secretion in 10- to 18-year-old type 1 diabetic patients with recent onset. In patients with short T1D duration, the effect was quite pronounced. These effects were reached with a very well tolerated treatment. Future studies will show if the good effect seen so far can be confirmed. There is a hope that GAD vaccination will cause remission or even cure and prevention of T1D seem to be a realistic possibility (Morales et al., 2011).

DiaPep277

HSP60 is a ubiquitous protein that is part of a highly conserved family of intracellular chaperones and is also located in the mitochondria and mature insulin–secretory granules of pancreatic β-cells with an important regulatory role in the innate immune system and considered an important autoantigen in T1D (Birk et al., 1996). Although HSP60 autoantibodies are not yet considered a diagnostic tool for T1D, the evidence suggests this autoantigen may be important in disease development. Therefore, vaccine strategies that are based on HSP60 as a T1D autoantigen have been tested in mouse models and human T1D patients [See table-4].

Group	Antigen	Route	Protection from diabetes	Reference
NOD mice, prediabetic and diabetic	DiaPep277	Subcutaneous	Yes	Elias 1995 and 1997
STZ-C57BL/KsJ diabetes mice model	DiaPep277	Subcutanous	Yes	Elias 1996
NOD mice, prediabetic	DiaPep277	Intraperitoneally	Yes	Elias 1991
NOD mice, prediabetic	DiaPep277	Trans inoculations	Yes	Jin 2008
BB-DP rats, Prediabetic	DiaPep277	Oral	Yes	Brugman 2004
NOD mice, Prediabetic	Pep277	Nasal	Yes	Liang 2010
NOD mice, , prediabetic and diabetic	Pep 277	several	No	Bowman 2002
Human, on-set	DiaPep277	Subcutaneous	Yes	Raz 2001 and 2007
Human, on-set	DiaPep277	Subcutaneous	Yes	Huurman 2008 and 2007
Human, paediatric on-set	DiaPep277	Subcutaneous	?	Schloot 2007
Human, on-set	DiaPep277	Subcutaneous	?	Schloot 2007
Children, on-set	DiaPep277	Subcutaneous	No	Lazer 2007

Table 4. DiaPep277 Immunotherapy of Type 1 Diabetes

DiaPep277 is a 24 amino acid synthetic peptide derived from the C 31 terminus of the human HSP60. Compared with HSP60, DiaPep277 activates T cell TLR2 receptors but has no effect on macrophage TLR4 receptors. So, DiaPep277 only mediated anti-inflammatory effect on T cells and lacked pro-inflammatory effect on innate immune cells. In NOD mice, nasal administration of a diabetogenic peptide Pep277 showed significant anti-inflammatory immune response (Liang et al., 2010). Moreover, neonatal oral administration of DiaPep277

combined with hydrolysed casein diet could protect against T1D in BB-DP rats. All the results showed that DiaPep277 could significantly prevent T1D development and arrest the progression of insulitis in mice that have already become hyperglycemic (Fierabracci 2011). These promising experimental results led to clinical trials with DiaPep277. It was found that DiaPep277 treatment for patients with recent onset T1D could prevent further β-cell loss (Raz et al., 2001; Raz et al., 2007). Specific intervention with DiaPep277 re-establishes the balance in the immune system by enhancing the Th2-type cytokine production and inducing a strong signal for the development of Th2 cells. Phase II clinical trials in adult subjects with T1D have shown suggestive evidence of better preservation of C-peptide and cessation of destruction of β-cells with DiaPep277 (Raz et al., 2001). Moreover, 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). Diapep277 was more pronounced in patients with a high β-cell reserve at the start of the treatment. The phase III study has began in 40 medical centers worldwide and the results are expected in 2011 (Eldor et al., 2009). If DiaPep277 proves to be efficacious, it will cause a paradigm shift in the treatment of T1D from treating the subsequent insulin deficiency to addressing the initial autoimmune process that is at the core of the disease (Eldor et al., 2009).

In summary, efficacy of autoantigen therapy has been disappointing. Based on preclinical models, several factors are emerging as critical in determining the outcome of Autoantigen immunization most notably dose, route, adjuvant, and frequency of administration. Studies have shown that too frequent antigen administrations, as well as very high dosages, do not result in optimal induction of immune regulation and tolerance (Peakman M et al. 2010). Another challenge for developing effective autoantigen therapy is to know the role that β-cell autoantigens play in islet inflammation and restoration of immunological tolerance, for example, how DCs recognize autoantigens and how autoantigen exposed DCs guide T cell development into immunosuppressive or autoreactive T cell subsets. The other important questions that need to be answered include precisely which autoantigen epitopes should be targeted and whether the use of a single antigen is sufficient in order to induce immune tolerance and stop an ongoing T cell response to multiple autoantigens. Moreover, only blood samples are readily available from patients to evaluate efficiency of antigen immunotherapy. The question is if peripheral T cell populations reflect the T cell population infiltrating the target organ. Another important concern is if immune deviation and functional dominance of regulatory responses are reflected by changes in peripheral antigen-specific T cells. More-sensitive assays are necessary to develop to evaluate the impact of immunotherapy on disease progression through analysis of peripheral cells. All these issues need to be addressed by performing autoantigen specific immunotherapy studies in future clinical trials.

4.2 Dendritic cell (DC)-based immunotherapy

4.2.1 Brief overview of DC biology

Dendritic cells (DCs) are a special subset of leukocytes able to alert the immune system of the presence of infections and are responsible for the activation and the control of adaptive immune responses. There is also mounting evidence that DCs establish and maintain immunological tolerance. Indeed, DCs can prevent, inhibit, or modulate T cell mediated

effector responses through a variety of mechanisms including T cell anergy, T cell deletion, immune deviation (i.e. polarization of T cell cytokine profiles), and the expansion or induction of Tregs. The induction/expansion of FoxP3+ Tregs is one of the major mechanisms by which DCs maintain immune tolerance (Steinman et al., 2003).

DCs are a heterogeneous mix of the distinct leukocyte subsets that have different functions and homing patterns. In humans, there are two major and intrinsically different subpopulations of DCs: myeloid DCs (myDCs) and plasmacytoid DCs (pDCs). These two DC subsets recognize different microbial pathogens by expressing distinct repertoires of toll-like receptors (TLR) and induce different types of innate and adaptive immune responses depending on environmental factors. Much evidence supports the presence of DCs with tolerogenic properties in both myDCs and pDC subsets (Gregori 2010). Both myDC and pDCs develop from hematopoietic bone marrow progenitor cells that turn into DC precursors (pre-pDCs) upon encounter of Flt3-ligand (Flt-3L). Pre-DCs migrate from the bone marrow to the bloodstream where they circulate as immature DCs characterized by low expression of HLA class II and co-stimulatory molecules, high endocytic activity, and low T cell activation potential. Immature myDCs and pDCs encounter pathogens and become activated mature DCs to prime CD4+ and CD8+ T cells (Liu et al., 2010). In addition to their potential to become activated DCs, immature myDCs and pDCs in the steady state are also critically involved in homeostasis and in promoting and maintaining peripheral tolerance, primarily via the induction of CD4+ Tregs. Of note, the DCs with tolerogenic function not only comprise immature DCs, but also include other DCs covering a spectrum of different maturation states. The induced CD4+ Tregs include the naturally occurring Tregs (nTregs) and the adaptive Tregs. Thymic-derived nTregs are defined by the constitutive high expression of FoxP3 (Sakaguchi et al., 2010). Adaptive Tregs composed of different Treg subsets are generated in the periphery in tolerogenic microenvironments and suppress T cell responses via immunomodulatory cytokines. The best characterized are the IL-10-producing type 1 regulatory T (Treg1) cells that depend on IL-10 for their generation and functions (Allan et al., 2008).

According to current understanding of DC development, protocols have been set up for *in vitro* propagation of large numbers of activated and tolerogenic DCs using defined growth factors as well as biological, genetic, and pharmacological modifications (Morelli et al., 2007). Depending on the agent used for tolerogenic DC induction, the resulting DCs are equipped with defined tolerogenic molecules which determine their ability to promote different subsets of Treg cells. Tolerogenic DCs secreting high levels of IL-10 in the absence of IL-12 are primarily involved in the induction of adaptive IL-10-producing Treg1 cells. FoxP3-inducer DCs express low levels of co-stimulatory molecules and express indoleamine 2,3-dioxygenase which allows them to secrete TGF-β and retinoic acid and to promote FoxP3+ Treg cells (Gregori 2010). According to these prerequisites, it can be postulated that depending on the type of Tregs required for promoting tolerance toward a given antigen, different protocols for *in vitro* generation of tolerogenic myDC with the desired phenotypic and functional characteristics could be designed. In general, tolerogenic potential of DCs were determined by low-expression of co-stimulatory molecules, microenvironmental factors (in particular, immunosuppressive cytokines), expression of death-inducing ligands, (in particular Fas ligand (FasL)), and inhibition of gene transcription regulatory proteins (e.g. NF-kB) (Morelli et al., 2007). The development of culture methods for generating large numbers of DCs that can be pharmacologically or biologically manipulated opens important new perspectives for the development of DC-based cell therapy protocols.

4.2.2 DC-based therapeutic approach

DCs are critical for both preventing and perpetuating autoimmunity, reflecting their dual roles in regulating the immune system, i.e., maintenance of T cell tolerance and induction of pro-inflammatory immune responses. The defects in immune regulation by DCs may play a central role in T1D (Anderson et al., 2005). In T1D, abnormalities in DC phenotype and function in both NOD mice and humans may result in the skewing response toward pathogenic Th1 cells (Feili-Hariri et al., 2006). The purpose of using tolerogenic DC therapy for T1D is to re-balance dysregulated immune responses that have already been ongoing for some time. A number of studies have shown that therapy with various forms of DCs can protect mice from T1D development. For example, DCs generated from the bone marrow of NOD mice by culturing DCs in granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-4, and fetal bovine serum (FBS) could prevent T1D in some recipients when administered to young (5-week-old) NOD mice (Feili-Hariri et al., 1999). Transfer of DCs *ex vivo* stimulated with IFN-γ down-modulates T1D in NOD mice (Shinomiya et al., 1999). However, one study showed that only DCs from pancreatic lymph nodes (panLNs), but not DCs from other anatomical sites, could protect from disease, presumably because panLN-derived DCs would present β-cell-derived antigens after adoptive transfer (Clare-Salzler et al., 1992). This study indicated that autoantigen exposed DCs have better protection effects.

4.2.2.1 Exposure of therapeutic DCs to antigen *ex vivo*

The original observation that DCs from the panLNs could prevent T1D when transferred to NOD mice, while those from other sites could not, suggests the potential importance of the incorporation of β-cell antigens into DC-based therapeutics for this disease (Mukherjee et al. 2010). The von Herrath group explored this approach in a rat model (Haase et al., 2005). They utilized the rat insulin promoter (RIP)-LCMV model of T1D in which disease is induced upon LCMV infection. BM-DCs were generated and pulsed with a viral peptide recognized by CD8+ T cells. It was found that the antigen pulsed DCs have stronger ability to inhibit development of T1D than the unpulsed DCs. This study suggests that disease relevant antigen exposed tolerogenic DCs have therapeutic benefit for T1D. Next, autoantigen pulsed DCs were explored for treating T1D in NOD mice. It was found that adoptive transfer of insulin peptide- pulsed, immature DCs could protect NOD mice from T1D whereas unpulsed DCs had no effect on T1D development (Haase et al., 2010). In another study, immature DCs derived from BM were pulsed with antigen-specific apoptotic bodies from the β-cell line NIT-1 cells and the administration of these cells to NOD could decrease T1D incidence significantly and correlated positively with insulitis reduction (Marin-Gallen et al., 2010). All these results show that DC therapy is capable of preventing T1D in an antigen-specific manner in animal model and DCs expressing disease-associated antigens constitutes a promising strategy to prevent T1D. The mechanism for preventing and delaying development of T1D with autoantigen exposed DCs is inducing expansion of regulatory T cells (Cheatem et al., 2009).

4.2.2.2 Targeting of antigens to DCs *in vivo*

Loading antigen ex vivo to DCs is challenging and faces many problems including labor-intensive procedure for each patient, high cost, and limited DC number (Tacken et al., 2007). Thus, many studies have begun to explore the utility of *in vivo* delivery of β-cell antigens to DCs for the prevention and treatment of T1D.

Many receptors used in targeting DC studies belong to the C-type lectin receptor (CLR) family, a family of calcium-dependent lectins that share primary structural homology in their carbohydrate recognition domain (CRD). Through their CRD, the CLRs bind to specific self or non-self sugar residues. Many of these lectins were shown to be associated with antigen uptake by DCs.Approaches to targeting CLRs fall into two categories: based on the binding of natural receptor ligands and exploited antibodies against the receptor. CD205 belongs to type I CLR family and study indicated that it is a suitable target for in vivo antigen-targeting studies. CD205 recycles through late endosomal or lysosomal compartments and mediates antigen presentation. (Tacken et al. 2007).

Much evidence shows that DEC-205 is involved in antigen processing and presentation. In mice, DEC205 expression is relatively DC restricted: it is highly expressed by mature DCs (mDCs), thymic epithelium, at low levels by B cells, and at very low levels by T cells and granulocytes. Therefore, DEC205 is an excellent target to study *in vivo* DC targeting. The therapeutic potential of DEC-205-mediated antigen delivery has begun to be explored in mouse models of T1D (Mukhopadhaya et al., 2008; Bruder et al., 2005). In Mukhopadhaya A et al's study, autoantigen peptide-linked anti-DEC-205 could lead to deletion of autoreactive CD8+ T cells even in the context of ongoing autoimmunity in NOD mice and induce subsequent immune tolerance to β-cells. This study provides support for the development of DC targeting of self antigens for treatment of chronic T cell-mediated autoimmune diseases. Bruder D et al. utilized the INS-HA/TCR-HA transgenic mouse model for T1D. It was found that T1D was prevented in most animals when treated with HA peptide linked anti-DEC-205. Further analysis showed that these HA-specific CD4+ T cells from anti–DEC-HA–treated mice exhibited increased expression of FoxP3, cytotoxic T-lymphocyte–associated antigen-4, and the immunosuppressive cytokines interleukin-10 and TGF-β. The findings indicate that targeting of the HA antigen to immature DCs in vivo leads to a relative increase of antigen-specific $FoxP3^+$ regulatory T cells that suppress the development of T1D. Another recent related study showed that targeting a mimotope peptide to the endocytic receptor DEC-205 on DCs in NOD mice induces efficient conversion of pancreatic β-cell-reactive BDC2.5 CD4+ T cells into long-lived FoxP3+ Treg cells (Petzold et al., 2010). These results suggest that promoting antigen-specific Treg cells in vivo might be a feasible approach in T1D with DC in vivo targeting.

4.2.3 Future challenges for DC based approaches

4.2.3.1 Challenge for exposure of DCs to antigen *ex vivo*

Adoptive cellular therapy with tolerogenic DCs has potential applications not only in human autoimmune diseases but also for allogeneic tissue transplantation and hypersensitivity. One big challenge is to generate tolerogenic DCs with a stable phenotype which are resistant to maturation mediated by pro-inflammatory mediators.

Currently, DCs for clinical use are generated from peripheral blood monocytes of the patients. However, the number of monocytes obtained from the patients is limited and the potential of monocytes to differentiate into DCs varies depending on the blood donor. Thus, the issue of limited cells is a serious obstacle for DC therapy. ES cells and iPS cells have pluripotency and unlimited propagation capacity and may be an ideal cell source for DC-therapy. Several groups have developed methods to generate DCs from ES cells or iPS cells (Menendez et al., 2005).

Developing tolerogenic DC-based therapies also requires optimizing the following conditions. (1) To define the best source of DC precursors for the *in vitro* induction of tolerogenic DCs, i.e. peripheral blood monocytes *vs* bone marrow CD34+ cells. Studies performed in non-human primates indicate that bone marrow cells are a good source of tolerogenic DCs (Steinman et al., 2003). (2) To determine the disease stage, route of delivery, and dose (Lo J et al., 2006**)** of DC transfer. Because the autoimmune response leading to TID in humans takes place for several years before hyperglycemia develops, it may be important to determine a critical stage for initiation of DC-based therapy. The study from NOD mice indicates that prevention initialized at early stage has a positive effect. Future studies are needed to establish whether DC-based therapies block disease onset at advanced stages of islet destruction or even reverse acute onset T1D. For the route of DCs administration, until now, there is no solid evidence to show which administration route is the best. It was noted DCs have different migration in different administration route. For example, subcutaneous injected DCs home mainly to the draining lymph nodes whereas intravenous administration mainly goes to the spleen, lungs, and liver. Concerning the injection dose, the effect of the number per treatment has not been comparatively evaluated. Of note, the lifespan of DCs in vivo is limited to about 2-3 weeks. Therefore, it could be envisaged to perform repetitive injections unless a single injection would be sufficient to generate a microenvironment suitable for sustaining *de novo in vivo* induction of tolerogenic DCs (Morelli et al., 2007).

4.2.3.2 Challenge for targeting DC *in vivo*

When developing DEC-205-mediated therapeutic strategies for T1D, the choice of antigen is a major consideration. Until now, the antibody can be modified to include an antigen and it proves difficult to combine multiple antigens. As mentioned before, multiple antigens are targeted by T cells in both NOD mice and T1D patients (DiLorenzo et al., 2002). Particularly in humans, it is unclear which of these are the most 'important', i.e. critical for disease initiation and/or progression. However, accumulating evidence suggests that insulin and GAD65 are key targets of pathogenic T cells in T1D in both NOD mice and humans and that they may be candidates for targeting to DEC205 in T1D.

DCs have two functions, maintaining tolerance to self and inducing immunity. In the context of activation stimuli such as those found during inflammation, infection, or tissue destruction, DCs induce prolonged T cell activation and in steady state, they induce tolerance. This character of DCs was also shown in the Hawiger D study. It was found that HEL linked anti-DEC 205 could induce immune tolerance under physiological conditions but induce prolonged T cell activation and immunity against HEL when performed in conjunction with an agonistic anti-CD40 antibody. (Hawiger et al., 2001). It indicated antigen delivery to DCs in the context of an inflammatory environment may lead to exacerbation of a pathogenic autoimmune response rather than tolerance induction. So, when considering the translation of such a strategy to humans, the potential remedy should be considered. (Mukherjee et al. 2010)

Another potential concern is that CD205 expression in humans is less DC restricted than in mice, and targeting constructs might also be endocytosed by several other cell types for example B cells, T cells, monocytes, macrophages, and natural killer (NK) cells (Erbacher et al., 2009). It still needs to be confirmed if targeting these cells has any side effects. On the whole, many issues need to be resolved before translating DC based approaches into human T1D prevention and treatment.

4.3 Adoptive transfer of Tregs

4.3.1 Brief overview of Tregs

Tregs play a crucial role in the maintenance of immune homeostasis. The two best studied types of CD4+ regulatory T cells are the FoxP3+ Tregs and Th1 cells secreting IL-10. Large quantities of evidence show that CD4+ regulatory T cells play a protective role in autoimmune disease (Spoerl et al., 2011).

CD4+FoxP3+ Tregs account for approximately 5-10% of the total CD4+ T cells in the periphery, most prominently in the lymphoid organs such as the spleen and lymph nodes, with a small subset found in the non-lymphoid tissues. FoxP3+ Tregs suppress both innate and adaptive immune cells through several mechanisms and maintain tolerance and immune homeostasis. They exert suppression by cell contact-dependent mechanisms (for example, functional modulation by means of CD39, CD73, and LAG-3, or killing of APCs or responder T cells by means of granzyme and perforin) as well as mechanisms mediated by soluble factors (for example, secretion of immunosuppressive cytokines such as IL-10, TGF-β, IL-35, and galectin-1), or deprivation of cytokines (for example, IL-2) necessary for the expansion and/or survival of responder T cells (Zhang et al., 2009). Moreover, Tregs have two key characters that are very important for their translational application; (1) Bystander suppression: When Tregs are first activated through their respective TCRs to induce suppressive activity, but once activated, the cells are capable of suppressing responses in a non-specific manner. (2) Infectious tolerance: The transferred Tregs generate an immunoregulatory milieu that persists long after the removal of the eliciting cell population. This occurs as new regulatory cell populations are activated by antigen in the context of tolerogenic APCs and Tregs. These two characteristics allow Tregs of single or limited-antigen specificity to establish a broad and stable immunoregulatory effect and constitute the quintessential Treg phenotype (Brusko et al., 2008b). Phenotypically, CD4+FoxP3+ Tregs express multiple cell surface markers such as CD25, CTLA-4, GITR (glucocorticoid-induced tumor necrosis factor receptor), OX40, CD103, CD39, and CD73, many of which are also expressed by activated CD4+ T effector cells (Kang et al., 2007). Thus, these markers are not Treg specific.

CD4+FoxP3+ Tregs are not a uniform cell population, but consisted of those that developed in the thymus (nTregs) and those that are derived from T effector cells in the periphery (adaptive or induced Tregs, iTregs). nTregs are shown to arise as a committed lineage in the thymus and bear a diverse TCR repertoire against a broad range of self antigens. iTreg cells are induced under particular conditions of antigenic stimulation both in vitro and in vivo. Furthermore, iTregs are particularly unstable and tend to lose FoxP3 expression more easily than nTregs, which is likely related to epigenetic differences in the FoxP3 gene in both subsets. Indeed, certain non-coding regions in the FoxP3 gene in nTregs have been shown to be completely demethylated while such regions are often methylated in iTregs (Curotto de Lafaille et al., 2009). Such differences may be important in therapeutic manipulation of nTregs and iTregs (Spoerl et al., 2011).

Another important development about Tregs is that many protocols were developed for isolating and expanding nTregs for therapeutic applications from healthy individuals and patients (Brusko et al., 2008a). This provides important tools and principles for translating Treg therapies into clinical treatments for patients with T1D and other autoimmune disorders.

4.3.2 Tregs in the pathophysiology of T1D

A growing body of evidence suggests that an imbalance in the immune system plays a major role in the pathogenesis of autoimmune disease (Brusko et al., 2008b). Tregs play a major role in controlling the activity of self-aggressive T cells that were not deleted in the thymus and are one of the key factors for maintaining homeostatic balance in the immune system (Chatenoud et al., 2001). Defects in the number and function of Tregs and a resistance of effector T cells to Treg-mediated suppression could each contribute to failed T cell regulation. Each of these defects contributes to the development of autoimmune disease including T1D (Buckner 2010).

A role for nTreg cells in T1D is evident in individuals with IPEX, in which the absence of nTreg cells results in enhanced susceptibility to T1D. In NOD mice, reduced CD4+ Treg cell frequencies or function represent a primary predisposing factor to T1D (Sgouroudis et al., 2009). The adoptive transfer of Tregs could suppress disease through reducing effector Th1 T cells and macrophages and inhibiting effector T cell cytokine and chemokine production (Tonkin et al., 2009). However, Tritt M et al. have contradicting reports as they couldn't detect the defect of cellular frequency of FoxP3+ nTreg cells in the lymphoid tissues. However, the nTreg cell functional potency and intra-pancreatic proliferative potential declines with age, in turn augmenting diabetogenic responses and disease susceptibility (Tritt et al., 2008). On the whole, strong evidence shows that complete absence of Tregs precipitates T1D and the disease can be prevented by the adoptive transfer of additional Tregs (Sgouroudis et al., 2009).

Most studies investigating the role of Tregs in human T1D showed contradictory results in the peripheral blood frequency and suppression function of these cells between T1D and control subjects when the expression of transcription factor FoxP3 was used for their identification (Alonso et al., 2009). One reason is that FoxP3 is an unreliable marker for Tregs in humans, as it is also expressed in activated effector T cells. Moreover, unlike mice, the frequency of Tregs in islet couldn't be obtained from human. The results obtained from blood samples might not indicate the involvement of Tregs in the pathophysiology of T1D. Moreover, the method used to isolate Tregs may influence the degree of suppression observed in vitro. This effect may also account for some of the discrepancies in the reported functions of these cells in T1D (Brusko et al., 2008b). The above data from mice and human indicated central importance of Tregs in the pathogenesis and potentially the treatment of T1D.

4.3.3 Therapy of T1D with Tregs

In the mouse animal model, the adoptive transfer of CD4+ CD25+ Treg cells has been shown to protect from T1D (Salomon et al., 2000; Tonkin et al., 2008; Tonkin et al., 2009). According to published results, the adoptive Treg cells include polyspecific and autoantigen specific. Now the evidence is very clear that antigen-specific Tregs are more effective in several autoimmune syndromes when compared with polyclonal populations (Brusko et al., 2008b). According to a study by Wu AJ, polyspecific Tregs had to be transferred regularly (twice weekly) in high doses and mice developed T1D after Treg transfer had been stopped (Wu et al., 2002). This makes this approach difficult to be performed in human patients. The other concerns about polyspecific Tregs include the efficacy and safety of these Tregs related to their polyspecificity and a paucity of information about their stability, function, and fate in vivo. So, autoantigen-specific Tregs would have more advantages in terms of safety and

efficacy because they could specifically recognize antigens from the target tissue to achieve tissue specific immunoregulation. Several studies already showed natural CD4+ CD25+ Tregs from the NOD mouse specific for an islet antigen were more protective than their polyspecific Tregs (Tang et al., 2004; Tarbell et al., 2004; Tarbell et al., 2007). Compared with poly specific Tregs, relatively small numbers of islet autoantigen-specific Tregs prevent T1D in NOD mice. Moreover, it was found that adoptive transfer of islet specific Tregs showed considerable efficacy in ameliorating ongoing T1D in NOD mice (Tarbell et al., 2007). Until now, not many treatments were shown to interfere with disease pathophysiology at later disease stages in the NOD mouse. Therefore, adoptive autoantigen specific regulatory T cells should have great potential therapeutic benefit in humans.

For developing adoptive autoantigen specific regulatory T cell therapy, in vitro expansion of this unique cell population becomes very important. Several research groups already developed the expansion protocol of autoantigen specific Tregs from peripheral blood in vitro (Dromey et al., 2011; Tang et al., 2004; Tarbell et al., 2004). Because proinsulin and GAD65 usually are targets of autoreactive T cells in human T1D (Harrison 2008). Tregs specific for these autoantigens are being attempted to expand in vitro and they will have potential application for adoptive immunotherapy of T1D.

4.3.4 Future challenges for Treg therapy

Translating Tregs into the clinical setting still faces several challenges. Firstly, until now, no unique cell surface markers were identified for human Tregs. Because activated effector T cells also express CD25, there exits high frequency of activated effector T cells contamination present in the CD4+ CD25+ fraction with present Treg isolating kits. So, for the isolation of human Tregs, the combination of the surface markers CD4+ CD25high CD127low results in a population which is enriched in Tregs with fewer recently activated effector T cells (Brusko et al., 2008b). Second, another major challenge in antigen-specific Treg therapy lies in the isolation of sufficient quantities of antigen-specific T cells. In order to obtain enough cell number for adoptive transfer, in vitro expansion of this population is necessary. Although several groups already developed protocols for expanding autoantigen specific Treg cells, the current technology may not provide a robust method for isolating and expanding a sufficient quantity of these cells for use in the clinic. The next most important factor will be the stability of the Treg differentiation phenotype. Recent animal studies provide evidence for functional heterogeneity and lineage plasticity within the Treg compartment (Addey et al., 2011). In the healthy immune system, the majority of natural Tregs are relatively stable, but the study showed that 10-15% of "stable" Treg cells were found to lose FoxP3 expression after adoptive transfer into lymphopenic hosts (Zhou et al., 2009). There are two fates for Tregs that lose FoxP3 in lymphopenic hosts: death or de-differentiation. One study showed that half of the Tregs transferred into lymphopenic hosts did not die but rather began producing IL-2 and IFNγ and became effector T cells with the same antigen specificity (Komatsu et al., 2009). These reverted autoreactive effector cells will aggravate the autoimmune damage.

The studies described above suggest a critical challenge in the transition of Treg-based cell therapies from animal studies to human clinical trials. Knowledge of the purity, stability, and phenotypic characteristics of cell therapy products will be essential prior to their introduction into patients.

5. The importance of combination therapy

After several attempts to develop potent immunotherapy to cure T1D, the results are disappointing. Because of the multifaceted nature of this disease, a monotherapy will not be sufficient to combat T1D. The best approach is to use a combination therapy. Two major goals have to be accomplished: (1) the rapid blockade of the immune system and dampening of any auto-reactive responses without strong side-effects and (2) the regeneration of a critical β-cell mass in order to maintain euglycemia without repetitive insulin injections (Bresson et al., 2007). Each of these goals can be achieved by different means, but the combination of different therapies is very necessary for successful prevention and treatment for T1D. It should be noted that the ideal combination therapy would utilize two or more agents whose mechanisms of action are complementary and that have already been demonstrated as safe T1D monotherapies in humans.

5.1 Combination of anti-CD3 Ab with antigen-specific approach

The first and key step for treatment of T1D is dampening the auto-reactive responses and maintaining permanent tolerance to islet cells. In order to obtain this goal, the combination of immunotherapy approaches including nonspecific immunoregulatory and antigen-specific tolerance strategies are necessary. It was found that efficacy of antigen-specific therapy can be greatly enhanced when combined with anti-CD3 in recent onset T1D. A previous study (Bresson et al., 2006) showed that combination treatment with anti-CD3 specific antibody and intranasal administration of proinsulin peptide can reverse recent onset T1D in murine T1D models with much higher efficacy than with monotherapy using anti-CD3 or antigen alone. Further in vivo analysis showed that expansion of insulin-specific CD25+Foxp3+ Tregs producing IL-10, TGF-β, and IL-4 was strongly enhanced. These cells could transfer dominant tolerance to immunocompetent recent onset T1D recipients and suppressed autoaggressive CD8 responses. Moreover, the combination using anti-CD3 antibody and GAD65-expressing plasmid was shown to reverse recent onset T1D in RIP-LCMV-GP mice models (Bresson et al., 2010). Thus, the efficacy of antigen-specific therapy to induce Tregs and long-term tolerance can be greatly enhanced by addition of anti-CD3 treatment. The potential reasons include: (1) It was found that Tregs were not able to suppress activated autoaggressive T cells within the islets and PLNs (Nagler-Anderson et al., 2004; Tarbell et al., 2004). That is the reason why antigen-specific therapies were only effective during the prediabetic phase in animal models. Anti-CD3 antibody could directly decrease numbers of autoaggressive T cells in mice as described in the study (Herold et al., 2002) and create a systemic immunomodulatory milieu to facilitate the islet antigen–specific induction of Tregs. (2) Anti-CD3 has been shown in some settings to directly promote the generation of Tregs. In NOD studies by Chatenoud and others, anti-CD3 increased the number of CD4+CD25+ cells as well as systemic TGF-β production, which was required for therapeutic efficacy (Chatenoud et al., 2007). According to these results, an ideal combination therapy will consist of an islet antigen-specific immune tolerance induction, such as autoantigen immunization, coupled with a systemic drug that dampens islet destructive immunity but does not affect Tregs. Anti-CD3 treatment is suggested as a good candidate for this. In summary, the combination therapies with anti-CD3 Ab and antigen specific tolerance induction constitutes a great hope towards the development of safer and more effective immuno-interventions and would be excellent candidates for clinical studies in humans.

5.2 Combination of immunotherapy and β-cell regeneration

Since β-cell mass decreases over time, patients with long-standing T1D are very likely to have lost most of their insulin producing cells. Thus, treatments that only target the autoreactive immune system would not obviate the need for insulin injections in these patients. So, improving β-cell function at the time of diagnosis is an important determinant of response to treatment with immune therapy, and therefore strategies that improve β-cell mass and/or function may have a positive effect on response to immune treatment. Much evidence showed that reduced β-cell mass can eventually be restored endogenously. It has been demonstrated that β-cells can replicate, differentiate, or transdifferentiate from various endocrine or non-endocrine cells. Specifically, certain peptides and growth factors such as gastrin and glucagon-like peptide 1 (GLP1) can increase β-cell mass and restore normoglycaemia in animal models of T1D in the absence of immunosuppressants (Suarez-Pinzon, WL et al. 2008). So, it is necessary and feasible to develop combination therapy including improving β-cell regeneration and immunotherapy. Sherry NA et al. reported the efficiency of such combination therapy. They found that treatment with glucagon-like peptide-1 receptor agonist enhances remission of T1D in NOD mice treated with anti-CD3 Ab by enhancing the recovery of the residual islets (Sherry et al., 2007). Also, it was demonstrated that exendin-4 synergistically augments the remission-inducing effect of anti-lymphocyte serum in NOD mice (Ogawa et al., 2004). These results suggested that this combinatorial approach may be useful in treatment of patients with recent onset T1D. Accordingly, a new phase II study about combination of regenerative agents (lansoprazole and sitagliptin) and Diamyd are recruiting patients. Currently, this is the only clinical trial combining a treatment to control the autoimmune attack with autoantigen vaccine and a treatment to help the body generate more insulin. It is believed that combinations like this represent a very promising approach to the future cure for T1D

6. Conclusion remark

Antigen specific tolerance induction is the targeted therapy for prevention or early reversal of T1D. Ideally the tolerance therapy would specifically target β-cell antigens and create a local tolerance environment for inhibiting ongoing autoimmune attack against β-cells and contributing to β-cell regeneration.

Development of safe and effective prevention of T1D is becoming a major worldwide public health goal. While many preventive modalities have succeeded in animal models of T1D, prevention of human T1D remains elusive. With further understanding of genetic and environmental factors that determine the relapsing-remitting course of β-cell destruction and autoimmune mechanisms of β-cell destruction, primary prevention with antigen specific immune tolerance induction will likely be the optimal approach for the prevention of T1D in high-risk groups.

As patients develop autoimmunity, β-cell function declines. At this stage, efficacy of antigen-specific therapies is limited. The experience accumulated from previous clinical trials indicated that combination therapies may enhance efficacy while lowering risk. It is likely that one day combination therapy including systemic immune modulators, antigen specific immune tolerance induction, and a compound with positive effects on β-cell proliferation will become the standard of care for newly diagnosed T1D.

7. References

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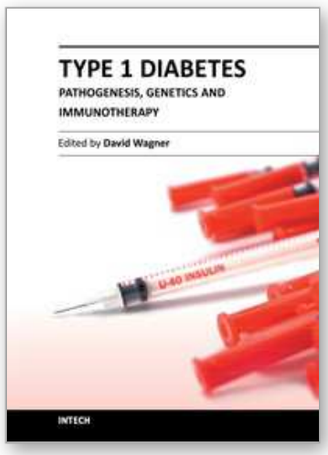

Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy
Edited by Prof. David Wagner

ISBN 978-953-307-362-0
Hard cover, 660 pages
Publisher InTech
Published online 25, November, 2011
Published in print edition November, 2011

This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology and pathogenesis of this disease is essential. A number of experts in the field have covered a range of topics for consideration that are applicable to researcher and clinician alike. This book provides apt descriptions of cutting edge technologies and applications in the ever going search for treatments and cure for diabetes. Areas including T cell development, innate immune responses, imaging of pancreata, potential viral initiators, etc. are considered.

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