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Immunotherapy for Type 1 Diabetes  
– Preclinical and Clinical Trials

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1. Introduction

According to the American Diabetes Association (http://www.diabetes.org/diabetes-basics/diabetes-statistics), 2-3 million individuals in the United States have type 1 diabetes and about 1 in every 400 to 600 children and adolescents was affected by the disease in 2007. Similar to other autoimmune diseases, allergies, and asthma, the incidence of type 1 diabetes is on the increase at an alarming rate in industrialized countries for unknown reasons. The disease is immune-mediated and thought to be caused by a combination of genetic and environmental factors that ultimately leads to loss of insulin secreting beta cells in pancreatic islets and high levels of blood glucose. High sugar levels and autoimmunity cause acute complications, such as ketoacidosis, as well as a wide variety of late complications, for example, atherosclerosis, retinopathy, kidney failure, neuropathy, and infection. Injection of insulin is a life saving treatment rather than a cure, and individuals suffering from type 1 diabetes and receiving insulin still show an unacceptable 15 year reduction in life expectancy and complications of the disease according to the Juvenile Diabetes Research Foundation (JDRF) (http://www.jdrf.org.uk/page.asp?section=163&sectionTitle=FAQs+about+type+1+diabetes).

The requirement for insulin injection can be alleviated by transplantation of pancreas or pancreatic islets, but the procedure is limited by the low number of donors, the out lived usefulness of immunosuppression, and the pre-existing autoimmunity which still cause ~85% of islet transplants to fail 10 years post-transplantation.

The mammalian immune system is the main line of defense to protect from attacks by infection, cancer, and pathological autoimmunity. Leukocytes normally do not destroy host tissues or cells because immune cells targeting self-antigens are either deleted in the thymus and bone marrow, i.e., central tolerance; or they are under the control of regulatory T cells in peripheral tissues, i.e., peripheral tolerance. However, defects in either of these immune control systems can result in pathological autoimmunity and large bodies of evidence indicate that defects in central as well as peripheral tolerance can contribute to type 1 diabetes (Geenen et al., 2010). Type 1 diabetes is an organ-specific autoimmune disease resulting from the selective destruction of pancreatic insulin secreting beta cells by autoreactive CD4 and CD8 effector T lymphocytes which normally play an important role in self-protection by killing infected and tumor cells. On the other hand, CD4 and CD8 T
lymphocytes also include regulatory cells that control the activity of effector cells, and can be drawn upon to ameliorate type 1 diabetes. In the past decade, these regulatory cells, especially CD4 T regulatory cells, have become a major immunotherapeutic target for the treatment of type 1 diabetes and other autoimmune diseases. In addition, antigen-presenting cells like dendritic cells, which present protein-derived antigens to T lymphocytes on their major histocompatibility complex molecules, have also received much attention not only because they can contribute to disease onset, but also because they can be used to induce T regulatory cells (Paul, 1999, Nikolic et al., 2009).

In addition to direct cell-to-cell interaction, immune cells modulate immune responses by secreting small molecular proteins, called cytokines, into their environment. Cytokines promote the development and differentiation of immune cells with different functions and therefore play an important role in directing immune responses. Several cytokines have been used alone and together with other immunotherapeutics for treatment of type 1 diabetes in animal model systems. One of the largest groups of cytokine is the interleukin group, which consists of more than 30 different proteins with interleukin-1, interleukin-2, interleukin-4, interleukin-10, and interleukin-15, among others, being potential targets for treatment. Other cytokines like tumor necrosis factor-alpha can also be used for type 1 diabetes treatment.

Antibodies are another important group of therapeutic molecules that have been investigated for type 1 diabetes. Antibodies, or immunoglobulins (Ig), are large Y-shaped proteins produced by B lymphocytes found in blood and other bodily fluids of vertebrates where their function includes binding to protein antigens and neutralizing pathogens. In the case of type 1 diabetes, the therapeutic antibodies that have been developed target self-antigens synthesized by cells that participate in the inflammatory response and cause the disease.

Under the effect of both genetic and environmental factors, immune cells like CD4 T lymphocytes and CD8 cytotoxic T lymphocytes infiltrate pancreatic islets resulting in different levels of insulitis (Figure 1). Insulitis ultimately results in the selective destruction of pancreatic beta cells as the result of regulatory dysfunction of CD4 T lymphocytes, and/or of dendritic cells and other cell types.

![Graphic Representation of Mouse Islets with Lymphocyte Infiltration](image)

**Fig. 1. Graphic Representation of Mouse Islets with Lymphocyte Infiltration.** A. Normal islet; B. Islet partially infiltrated by lymphocytes; C. Islet fully infiltrated by lymphocytes.

Since there is no cure for clinical type 1 diabetes, novel immunotherapeutic approaches aimed at preserving and restoring functional endogenous beta cell mass as well as protecting transplanted islets are needed to rebuild immune tolerance to beta cells and improve the condition of type 1 diabetic patients. One of the animal models closest to
human type 1 diabetes and most often used for studying the disease is the non-obese diabetic (NOD) female mouse, which is a spontaneous diabetes model that has helped to dissect the various stages of disease progression in human (Zhang et al., 2008, Ridgway, 2003). A variety of immunotherapies are being investigated in non-obese diabetic mice to treat type 1 diabetes. They can be divided into four main categories: first, non-antigen-specific therapies such as antibody therapies targeting effector cells; second, antigen-specific therapies such as protein and DNA vaccines targeting diabetic autoantigens; third, other immunotherapies such as Bacille Calmette-Guérin (BCG), vitamin D3, and fourth, combinational therapies which combine antigen specific and non-specific therapies (Figure 2). Bench-to-bedside translation of these different approaches has already identified a number of promising therapies, which will be the main focus of this chapter. The strong impetus to develop prophylactic and therapeutic approaches for the disease is reflected by the activity of organizations like TrialNet (http://www.diabetestrialnet.org/) and the Immune Tolerance Network (http://www.immunetolerance.org/). TrialNet is an expansion of the Diabetes Prevention Trial (DPT-1) network, but with an emphasis not only on diabetes prevention but also on trials to prevent further destruction of islet beta cells in patients with type 1 diabetes.

2. Antibody therapies

Intervention studies using antibodies for type 1 diabetes in the 1980s demonstrated a potential for preserving insulin C-peptide level which serves as an indication of insulin secretion by beta cells. However, they were eventually abandoned due to the adverse side effect profiles of the agents being used (You et al., 2008). Pilot studies of new immunosuppressive and immunomodulatory agents with decreased side-effect profiles have recently shown promise in preserving C-peptide in new-onset type 1 diabetic patients. Some of these agents are specifically designed monoclonal antibodies targeting immune effector cells, e.g., anti-CD3, anti-CD20, anti-CD25 (daclizumab), and anti-thymocyte globulin are being tested in clinical trials for type 1 diabetes. Other antibodies, such as anti-CD154 (MR1) have been tested in the female non-obese diabetic mouse.

The first anti-CD3 antibody used clinically was a murine monoclonal IgG2 antibody, OKT3, which was identified while antibodies were investigated as lymphocytic mitogens (Kaufman & Herold, 2009). CD3 is a protein complex found on the cell surface of T cells and is involved in transduction of signals originating from the antigen receptor to start a cascade of events initiating activation of the T cell. The binding between anti-CD3 antibody and CD3 on T cells can inhibit lysis of targets by T cells. Along with potent mitogenic activity, OKT3 was found to be a potent inducer of cytokines, specifically, tumor necrosis factor-alpha, interleukin-2, and interferon-gamma. Among these induced cytokines, interleukin-2 is necessary for T regulatory cell proliferation and tumor necrosis factor-alpha and interferon-gamma are considered as regulatory cytokines. These characteristics made anti-CD3 a potential therapeutic agent for both transplantation medicine and anti-tumor activity in the clinic. Unfortunately, the toxic side effects of OKT3 became clear after patients receiving the drug immediately developed chills, fever, hypotension, and in some cases, dyspnea. The toxicity was thought to be associated with a cytokine, specifically tumor necrosis factor-alpha from T cells in response to the drug. This effect was attributed to the anti-CD3 mediated cross-linking of T cells, i.e., the anti-CD3 antibody causing linkage of T cells bearing CD3 molecules and cells bearing the Fc (fragmental crystallizable region of the
antibody molecule) receptor. This cross-linking is thought to activate both the T cell and the Fc receptor-bearing cells, leading to the massive release of cytokines. These toxic side effects severely limited the clinical application of anti-CD3 to type 1 diabetes.

Fig. 2. **Immunotherapies for Type 1 Diabetes.** Without immunotherapy (top), pro-diabetic islets ultimately become diabetic islets after destruction of insulin-secreting beta-cells. Immunotherapies for type 1 diabetes can protect pro-diabetic islets from further destruction and permit them to regain function. A variety of immunotherapies, either alone or in combination, has been shown to stop pathological autoimmunity and protect islets to ameliorate type 1 diabetes.

To circumvent these problems, new antibodies were engineered to reduced Fc receptor binding after amino acid substitutions in the Fc portions of the antibody (Kaufman & Herold, 2009). Although reduced Fc receptor binding was predicted to eliminate T cell activation and cytokine release, the new anti-CD3 antibodies are still mildly mitogenic, i.e., T cell proliferation can be shown *in vitro* and even mild cytokine release has been observed. Nevertheless, it is important to note that the new anti-CD3 monoclonal antibodies differ greatly from OKT3 in their induction of T cell activation *in vivo*, and that the toxic side effects have been significantly reduced. In addition, the anti-CD3 antibodies derived from mouse generally are fully humanized to minimize the potential immunogenicity associated with heterogenic antibody isotype between mouse and human.

The modified anti-CD3 antibodies have been shown to be as effective as the full-length molecule but with reduced morbidity (Chatenoud et al., 1994). Using this treatment, 64–80% of treated diabetic non-obese diabetic mice returned to a euglycemic state without
glycosuria while none of the non-obese diabetic mice treated with control immunoglobulin recovered. In addition, the new antibodies could also protect transplanted allogeneic pancreatic islets in diabetic non-obese diabetic mice (Chatenoud et al., 1997). The mice could still reject allogeneic skin grafts and remain resistant to adoptive transfer of diabetogenic T cells, suggesting that their immune system was intact and that only beta cell specific immune responses were affected. However, results indicated that treatment of 4- and 8-week old mice did not prevent diabetes, but did protect 12-week-old mice, which indicated that the anti-CD3 was only effective when used after the onset of the disease.

Humanized non-Fc receptor binding anti-CD3 monoclonal antibodies include otelixizumab, teplizumab, and visilizumab. Teplizumab was shown to preserve C-peptide production up to five years after a single course of twelve days of treatment of new-onset type 1 diabetic patients in phase I/II clinical trials (Herold et al., 2009). Insulin is synthesized in the pancreas firstly as pro-insulin consisting of the A, C, and B peptide moieties. Mature active insulin is obtained after excision of the C peptide and folding/assembly of the A chain and B chain. Thus levels of C-peptide can be used as an indication of insulin secretion activity by β-cells. There may be several therapeutic mechanisms involved: induction of regulatory T cells, increased proportion of CD8+ T cells, increased expression of Foxp3 in CD8+ T cells, as well as induction of apoptotic cells. Similarly, otelixizumab could preserve residual beta-cell function for at least 18 months in patients with recent onset type 1 diabetes who were under treatment for six consecutive days (Keymeulen et al., 2005). However, anti-CD3 based approaches have suffered a setback because of the announced suspension of Phase III clinical trials for type 1 diabetes by MacroGenics and Eli Lilly as well as by Tolerx and GlaxoSmithKline due to lack of efficacy (GEN news, 2010; GSK Press Release, 2011). Previously, ulcerative colitis Phase II/III trials of visilizumab (trade name Nuvion) by PDL BioPharma Inc. had also been stopped due to inefficacy and poor safety profile (Lawler, 2009), which constitutes another setback.

Anti-CD25 IgG1 antibody (daclizumab) is another humanized recombinant monoclonal antibody which targets T cells. It is approximately 10% murine and 90% human, and activates T regulatory cells in vivo since the antibody inhibit initial T cell proliferation and differentiation but not initial activation (Egan et al., 2001). When used with the immunosuppressants rapamycin and tacrolimus, daclizumab maintains normal islet function in human recipients of transplanted allogeneic islet grafts. This glucocorticoid-free regimen appears to be highly beneficial for islet survival and function, as all patients were insulin independent (Shapiro et al., 2000). Daclizumab has also been used with success in human pancreas transplantation (Sutherland et al., 2001). Monoclonal antibodies against CD154, also known as CD40 ligand, have also been used for treatment of type 1 diabetes. The CD154 protein is primarily synthesized by activated CD4 T cells, and binds to the CD40 receptor on antigen-presenting cells. CD40 receptor engagement induces activation through co-stimulation in antigen-presenting cells like dendritic cells, macrophages and B cells. Anti-CD154 monoclonal antibodies will bind to CD154 on the surface of CD4 T lymphocytes, and block stimulation of antigen-presenting cells (Howard & Miller, 2004). The application of anti-CD154 antibodies was shown to prevent expansion of CD40 expressing T cells, later called T helper 40 cells, which are highly pathogenic in the non-obese diabetic mouse model and human diabetes (Waid, et al., 2004). In addition, anti-CD154 monoclonal antibodies induced islet allograft tolerance involving a dominant mechanism associated with intragraft regulatory cells, and prevented autoimmune diabetes in non-obese diabetic mice, possibly because it inhibits effector T cells by expending regulatory T cell (Rigby et al., 2008).
Anti-thymocyte globulin (ATG) consists of antibodies from horse and rabbit against human T-lymphocytes. Its administration as intraperitoneal injections of 500 micrograms antibodies stops new-onset diabetes and induces long-term tolerance in non-obese diabetic mice (Simon et al., 2008). Treatment was associated with increased frequency and activity of CD4^+CD25^+ T regulatory cells, as well as altered alteration of dendritic cell profile and function (Bresson and von Herrath, 2011). However, the treatment was efficacious only when administered late in the prediabetic phase (12-week of age) or after recent-onset. Another report showed that the antibodies failed to protect diabetes in a more stringent virus (RIP-LCMV) induced diabetes model due at least partially to the inability to maintain or increase a sufficient CD4^+CD25^+ T regulatory cell frequency.

Human clinical trials of anti-thymocyte globulin suggest that short term therapy (9 mg/kg followed by 3 consecutive doses of 3 mg/kg/day intravenously) in eleven recent onset patients, i.e., within 4 weeks of diagnosis and with residual post-glucagon C-peptide levels still over 0.3 nmol/l, contributed to the preservation of residual C-peptide production and to lower insulin requirements in the first year following diagnosis (Saudek et al, 2004). Nevertheless, significant adverse effects consisting mainly of transient fever and moderate symptoms of serum sickness were observed during the first month of treatments. Two other clinical trials are ongoing, which include a phase II trial to determine whether thymoglobulin treatment can halt the progression of newly diagnosed type 1 diabetes when given within 12-weeks of disease diagnosis (Gitelman, 2007); and a phase I/II trial to determine if giving a combination therapy consisting of Thymoglobulin (anti-thymocyte globulin) and Neulasta (granulocyte colony-stimulating factor, GCSF) given to established type 1 diabetes patients is safe and preserves insulin production (Haller, 2010).

Although type 1 diabetes is considered a T cell-mediated disease, recent data have indicated a role for antigen presentation by B lymphocytes in disease pathogenesis. In fact, anti-CD20 monoclonal antibody treatment of 5-week-old non-obese diabetic female mice reduces B cell numbers by approximately 95%, decreases insulitis, and prevents diabetes in >60% of mice (Xiu et al., 2008). Furthermore, anti-CD20 monoclonal antibody treatment of 15-week old female non-obese diabetic mice significantly delays diabetes onset. A recent clinical trial delivering a four-dose course of anti-CD20 antibody (rituximab) 3 months after diagnosis of diabetes onset showed a significant improvement in beta cell function in 23% of patients at 1 year (Pescovitz et al., 2009). No beneficial effects were noted in placebo-treated subjects. These patients also showed significant improvements in clinical parameters like hemoglobin A1c level, which is a indicator of average blood glucose level, and decreased insulin use. After 3 months, however, there was a parallel decline in beta cell function in the drug and placebo treated subjects.

Clearly, antibody therapies can be effective in controlling diabetes. However, they cannot always be combined with other therapies in the clinic. For example, clinical trials of the combined application of daclizumab (anti-CD25) with either intensive insulin therapy or mycophenolate mofetil reported failure in preserving beta-cell function in clinic (Rother et al., 2009b; Gottlieb et al., 2010), and the anti-diabetic effects of anti-CD3 are negated by rapamycin in non-obese diabetic mice (Valle et al., 2009). In addition, antibodies act systemically in a non-specific manner and can interfere with normal immune function. For example, anti-CD20 and anti-CD3 monoclonal antibodies have caused treatment-related adverse events in some patients during clinical trials (Pescovitz et al., 2009; Keymeulen et al., 2005; Herold et al., 2005). The recent announcement by Biogen and Roche that clinical trials of an anti-CD20 monoclonal antibody for rheumatoid arthritis and systemic lupus had
to be stopped due to death from opportunistic infection illustrates further the potential risks of certain monoclonal antibody treatments for autoimmune diseases.

3. Cellular and cytokine therapies

The main sign of pathosis in diabetic pancreatic islets is leukocyte infiltration or insulitis (Figure 1). Studies of islet-infiltrating cells have yielded clues to new therapeutic approaches by identifying immune cells and their associated molecules, such as cytokines, to suppress the disease. Preclinical and clinical trials of cellular therapies and cytokine therapies are currently ongoing, and will be discussed in this section.

3.1 Cellular therapies

Type 1 diabetes-associated deficiencies have been observed in several immune cell populations, such as CD4 T cells, CD8 T cells, B cells, dendritic cells, macrophages and NK cells in both non-obese diabetic mice and humans (Sgouroudis & Piccirillo, 2009; Luczyński et al., 2009). Regardless of whether diabetes is the result of increased effector cell activity or decreased regulatory cell function, the goal of cellular and many other therapies is to increase the regulatory function of cells like T regulatory lymphocytes and dendritic cells. Both cell types are important not only for therapeutic purposes, but are also suspected to play a determining role in the development of diabetes. T regulatory cellular therapies are currently at the preclinical stage and human T regulatory cell expansion strategies performed in vitro are under development.

T regulatory cells play a vital role as suppressive cells that regulate and control the effector arm of the immune system. In the past decade, an overwhelming body of literature has confirmed that CD4+CD25+FoxP3+ and other T regulatory cells comprise a dominant mechanism regulating autoimmunity as well as responding to infection and cancer. These findings are further confirmation that T regulatory cell dysregulation is implicated in autoimmune disorders like type 1 diabetes. In the non-obese diabetic model system, the defects of T regulatory cells result in the loss of control of autoaggressive T cells and diabetes progression (Tang & Bluestone, 2006). In humans, defects in polyclonal regulatory T cell have also been proposed as a mechanism by which individuals develop type 1 diabetes (Kukreja et al., 2002). Here T regulatory cell numbers decrease in child (age 9.4 ± 2.16 years) and adult (age 45.2 ± 9.7 years) patients. Another report showed that while the T regulatory cell numbers remain normal, they are associated with functional deficiency in adult patients (age 32.3 ± 6.8 years) (Lindley et al., 2005). Therefore, control of pathological autoimmunity through the induction of functional T regulatory cells is a highly promising approach.

Surprisingly, central tolerance mechanisms associated with regulatory cells are generally intact in non-obese diabetic mice (Feuerer et al., 2007), and the frequency and function of single positive CD4+FoxP3+ T regulatory cells in the thymus of these animals is comparable to that of diabetes-resistant C57/BL6 mice (Tritt et al., 2008). The results suggest that a defect in the regulatory T cell population most likely happens in the peripheral immune system after a certain age. T regulatory cells are diverse in their phenotype and mechanisms of function, and as such investigators are developing various types of induced T cell precursors (e.g. antigen targeting specificities) with a variable degree of regulatory potential. It is possible to induce and expand islet antigen-specific T regulatory cells in vitro from both non-obese diabetic mice and humans. T regulatory cells from non-obese diabetic mice could
be efficiently expanded in vitro using interleukin-2 and beads coated with a recombinant islet peptide mimic, a MHC class II molecule, and anti-CD28 monoclonal antibody. The expanded cells expressed normal surface markers like CD4, CD25, Foxp3, CD62L, GITR, and CTLA-4 in mice; and CD4, CD127\textsuperscript{lo}, CD25, and FOXP3 in humans. Once activated by the islet specific antigen, the T regulatory cells could suppress T effectors with other specificities both in vitro and in vivo. This feature is important for controlling type 1 diabetes in which numerous self-antigens are targeted by pathogenic effector cells. Importantly, the islet antigen specific T regulatory cells were more efficient than non-specific polyclonal T regulatory cells in suppressing autoimmune diabetes (Masteller et al., 2005; Putnam et al., 2009).

Results from T regulatory cell analysis of type 1 diabetic patients are varied, with either decreased cell frequency, unaltered T regulatory cell frequency with marked decrease in suppressive activity in vitro, or no difference compared to healthy controls (Kukreja et al., 2002; Lindley et al., 2005; Putnam et al., 2005). These variable results could be caused by different factors including different methods of T regulatory cell isolation and purification, the lack of functional tests for islet specific T regulatory cells in the blood, or simply different disease etiologies. Furthermore, studies in murine models indicate that T regulatory cells exert their function within the target organ undergoing autoimmune attack rather than solely in the lymph node draining sites. Thus, subtle functional differences in the T regulatory cell pool within sites of inflammation may not be adequately reflected in the peripheral blood and lymph node compartments, which are the main source of samples for human clinical data (Sgouroudis et al., 2009). A remaining question is whether changes in T regulatory cell function are the cause of diabetes onset or the uncontrolled activation and expansion of diabetogenic T effector cells are the cause of diabetes onset.

Similar to cells in mice, human T regulatory cells can be expanded in vitro. T regulatory cells from recent onset type 1 diabetic patients were expanded using anti-CD3 and anti-CD28 coated microbeads and interleukin-2 cytokine. Importantly, suppressive function, lineage markers such as FOXP3, and cytokine productions were similar to the T regulatory cells from healthy control subjects (Putnam et al., 2009). A phase I clinical trial of CD4\textsuperscript{+}CD127\textsuperscript{lo/-}CD25\textsuperscript{+} polyclonal T regulatory cells expanded using anti-CD3/anti-CD28 beads plus interleukin-2 started in 2010. The primary purpose of the trial is to assess the safety and feasibility of intravenous infusion of ex vivo selected and expanded autologous polyclonal regulatory cells in patients with type 1 diabetes to support dose selection for a future efficacy trial. The study also aims to assess the effect of T regulatory cells on beta cell function as well as on other measures of diabetes severity and the autoimmune response underlying type 1 diabetes (Gitelman & Bluestone, 2010).

In addition to T regulatory cells, dendritic cells can be cultured in vitro for in vivo applications. Dendritic cells are known as the most potent antigen-presenting cells and play a vital role in the control of immune homeostasis. Dendritic cells initiate T cell-mediated immunity and maintain immune tolerance in the periphery through activation of T regulatory cells and other mechanisms. Dendritic cells are the only professional antigen-presenting cells, as their main function is to prime naive T cells. They are the exclusive antigen-presenting cell subset capable of potentiating T regulatory cell proliferative and suppressive functions both in vitro and in vivo. As such, dendritic cells are at the crossroad of pathogenesis and therapy of autoimmune diseases like type 1 diabetes. Together with T regulatory cells, dendritic cells have been a main target for both in vitro and in vivo approaches to treat type 1 diabetes. The exact mechanism of tolerance induction by dendritic
cells in diabetes remains to be established, but it likely includes inhibition and killing of effector T cells and induction of regulatory T cells (Feili-Hariri et al., 2006; Nikolic et al., 2009).

A dendritic cell clinical phase I safety trial is currently being conducted with type 1 diabetic patients (Phillips et al., 2009). The proposed studies describe a randomized trial to evaluate the safety of a new diabetes-suppressive cell vaccine, consisting of autologous monocyte-derived dendritic cells treated ex vivo with antisense phosphorothioate modified oligonucleotides targeting the primary transcripts of the dendritic cell co-stimulatory molecules CD40, CD80, and CD86 (immunoregulatory dendritic cells; iDC). Fifteen patients exhibiting fully-established, insulin-dependent type 1 diabetics, without any diabetes-related complications, infectious disease, or other medical anomaly, have been enrolled to establish safety of the approach. 7/15 patients have been administered autologous control dendritic cells and 8/15 patients have been administered immunoregulatory dendritic cells. The study is anticipated to be complete within a twelve month period.

Solely expanding cell population in vitro may not be sufficient as a therapy for an organ specific autoimmune disease, since it will be a systemic treatment and may be inducing significant off-target effects, such as general immuno-suppression that will compromise beneficial immune responses to infections and cancers. In addition, current methodologies are limited in terms of the capacity to isolate and expand a sufficient quantity of endogenous antigen specific human cells for therapeutic intervention at low cost. Even a T regulatory cell induced with antigen specificity possesses bystander suppressive function (Brusko et al., 2010; Masteller et al., 2005), and the induced antigen specific T regulatory cells could quickly revert to Foxp3 negative CD4 T cells in a Foxp3 transgenic mouse model (Koenecke et al., 2009). Therefore, significant improvements may have to be made before cellular therapies can be safely applied to humans.

### 3.2 Cytokine therapies

Several immunotherapies for type 1 diabetes have used cytokines and other molecules such as fusion proteins targeting the costimulatory pathway (http://en.wikipedia.org/wiki/Costimulation). For example, cytokines like interleukin-2 provides vital survival signals to regulatory cells and can trigger the death of effector T cells, and impede interleukin-15 driven expansion of memory cells. In addition, interleukin-4, tumor necrosis factor-alpha, interferon-alpha, and lymphotoxin cytokines can exert selective effects upon crucial lymphocyte subset populations in vivo that may also enable translation into potent therapies. Preclinical trials and a few clinical trials of cytokine therapies will be addressed in this section.

Pro-inflammatory cytokines may impair islet viability and function by activating inflammatory pathways. Interleukin-1 beta is a master inflammatory cytokine that is synthesized early during inflammation by a wide variety of cells. Interleukin-1 receptor-deficient non-obese diabetic mice have a reduced development incidence of diabetes (Thomas et al., 2004). Anakinra, which is a clinically approved recombinant human interleukin-1 receptor antagonist, can block the effects of interleukin-1 beta on rat islet cultured in vitro, and inhibits the activation of interleukin-1 beta dependent inflammatory pathways to protect islets from apoptotic impairment (Schwarznau et al., 2009). In addition, short term treatment with anakinra resulted in reduced ability of mononuclear cells to traffic to sites of inflammation in a small group of patients with type 1 diabetes. This suggests that
mechanistic studies from large scale trials using interleukin-1 blockade in type 1 diabetes should focus on changes in monocyte trafficking and the interleukin-8 pathway (Sanda et al., 2010). Moreover, an adenoviral vector encoding interleukin-1 beta receptor antagonist together with another anti-inflammatory factor, i.e., hepatocyte growth factor, can reduce apoptosis of transplanted islets, and improve their survival in streptozotocin induced diabetes leading to lower blood glucose levels, as well as increased serum insulin and C-peptide levels (Panakanti & Mahato, 2009). Nevertheless, initial clinical data do not suggest that interleukin-1 blockade alone can prevent or reverse type 1 diabetes (Donath & Mandrup-Poulsen, 2008).

Interleukin-2 is another significant cytokine which has an important role in promoting T regulatory cell survival. Defective interleukin-2 or interleukin-2 receptor signaling in CD4 T-cells of type 1 diabetic subjects contributes to decreased persistence of FOXP3 expression that may impact establishment of tolerance (d’Hennezel et al., 2010). Therefore, interleukin-2 could be used as a therapeutic target for the restoration of Foxp3+ regulatory T cell function in organ specific autoimmunity. Indeed, administration of a low dose of interleukin-2 in prediabetic non-obese diabetic mice results in restoration of CD25 expression, survival of intra islet T regulatory cells and prevention of type 1 diabetes, which are associated with enhanced synthesis of T regulatory cell associated proteins and suppression of interferon-gamma (Grinberg-Bleyer et al., 2010). Moreover, a cytolytic interleukin-2 and Fc fusion protein binding specifically to the interleukin-2 receptor is capable of suppressing induced diabetes in non-obese diabetic mice (Zheng et al., 1999). Co-administration of interleukin-2 and rapamycin to 10-week old non-obese diabetic mice synergistically prevents diabetes development for 13 weeks post therapy. Furthermore, the treatment could also synergistically protect transplanted syngeneic islet in diabetic non-obese diabetic mice most likely due to the decreasing numbers of leukocytes, which were associated with increasing apoptosis of these cells, and a shift from T helper-1 (pathogenic) to T helper-2 and T helper-3 (protective) lymphocytes (Rabinovitch et al., 2002).

It has been hypothesized that manipulating the levels of interleukin-2 or interleukin-15 available to activated effector and regulatory T cells may provide a means to govern the balance of cytopathic T cells and regulatory T cells in vivo. Interleukin-15 is a cytokine that can induce massive apoptosis of recently activated pathogenic cells but not regulatory T cells. The combined therapy of interleukin-2 and immunoglobin fusion protein, mutated interleukin-15 and immunoglobin fusion protein plus rapamycin restores euglycemic state in recent-onset diabetes, and prolongs transplanted syngeneic islet survival in diabetic non-obese diabetic mice. It also decreases inflammatory gene expression in pancreatic draining lymph nodes and other tissues (Koulmanda et al., 2007). Based on the results of these preclinical studies, the Immune Tolerance Network is conducting a trial of interleukin-2 and rapamycin in a Phase I clinical trial with new onset type 1 diabetic human patients starting 2007 (http://clinicaltrials.gov/ct2/show/NCT00525889).

In addition to interleukin-1, both tumor necrosis factor-alpha and interferon-gamma have a direct cytotoxic effect on beta cells, and are postulated to be a direct cause of pancreatic islet beta cell destruction. Nevertheless, tumor necrosis factor-alpha has a more complex role in diabetes progression, and sometime appears more like a regulating instead of a pathological molecule. Tumor necrosis factor-alpha may prevent development of insulitis and diabetes, and even the adoptive transfer of diabetes by lymphocytes into young non-obese diabetic mice (Jacob et al., 1990). Moreover, neutralization of tumor necrosis factor-alpha accelerated diabetes in older mice, but prevented disease at a younger age. Tumor necrosis factor-alpha
or its agonists selectively kill autoreactive CD8, but not CD4 T cells derived from the blood of human type 1 diabetic patients (Ben et al, 2008). Most recently, a report from a small pilot trial (Phase I/II) of newly diagnosed pediatric patients found that administration of the soluble tumor necrotic factor receptor, etanercept, resulted in lower hemoglobin A1c level, and increased endogenous insulin production, suggesting preservation of beta-cell function in diabetic patients (Mastrandrea et al., 2009).

Interferon-alpha administered orally to non-obese diabetic mice caused decreased insulitis, increased mitogen-induced production of interleukin-4, interleukin-10 (T helper-2 like cytokines), and interferon-gamma secretion in splenocytes. The administered dose was 10 units daily from 9-week old, and suppressed diabetes from 100% diabetic mice in controls down to 10% in 24-week old mice. In addition, adoptive transfer of thirty million splenocytes intraperitoneally into 8-week old non-obese diabetic mice suppressed diabetes (Brod et al., 1998). In Phase I clinical trials of interferon-alpha, 10 newly diagnosed type 1 diabetic patients (ages 10-25, daily or every other day for 1 year) received an oral dose of 30,000 units of the cytokine within 1 month of diagnosis. The treatment induced at least a 30% increase in stimulated C-peptide levels at 0, 3, 6, 9, and 12 months compared to baseline (Brod et al., 2001). A phase I/II trial of safety and efficacy treated 31 new-onset patients (ages 3-25, daily for 1 year), and found that patients in the 5,000 units treatment group maintained increased beta-cell function 1 year after study enrollment compared with individuals in the placebo group (Rother et al., 2009a). In contrast, the effect was not observed in the 30,000 units treatment group.

Other cytokines, such as interleukin-4 and interleukin-10, have been reported to suppress type 1 diabetes at varying levels in animal models when delivered as part of a plasmid or virus vector (Wolfe et al., 2002; Goudy et al., 2001). Nonetheless, similar to antibodies, cytokine approaches are also systemic and associated with undesirable side-effects because of non-specificity in vivo.

4. Protein vaccine immunotherapies

It is now well established that loss of tolerance to beta-cell self-antigens, i.e., autoantigens, plays a determining role in the pathogenesis of type 1 diabetes. Autoantigens are self-molecules that become the target of the immune system under non-homeostatic conditions, which result from the combinations of genetic abnormalities with unknown environmental factors that ultimately lead to loss of immune tolerance for self-antigens. The autoantigens associated with type 1 diabetes covered in this chapter are heat shock protein 60, insulin, and glutamic acid decarboxylase 65, because they are therapeutic targets in ongoing clinical trials for type 1 diabetes. Nevertheless, there are others autoantigens like tyrosine phosphatase IA2, which is a target of humoral response in humans similar to insulin and glutamic acid decarboxylase 65, as well as the islet-specification efflux transporter znT8 and chromogranin A (Wenzlau et al., 2007; Stadinski et al., 2010), which will not be covered in this chapter.

The goal of autoantigen delivery as an immunotherapy is to re-establish at least some degree of immune tolerance activity for a target tissue to suppress pathological inflammation and ameliorate disease (Ludvigsson, 2009). This is not a new concept as it was used early in the 20th century to treat allergies to normally harmless foreign antigens (Krishna & Huisssoon, 2011). In recent years, immunoregulatory vaccination, also sometimes referred to as “negative” or “inverse” vaccination, has become increasingly attractive as an
immunotherapy principally because of the revival of the suppressor T cell concept, now named T regulatory cells. We know that T regulatory cells like CD4+CD25+Foxp3+ cells that are natural, i.e., thymus derived, or adaptive, i.e., induced in the periphery, are fundamental for immune tolerance because deleterious mutations in the Foxp3 gene causes systemic autoimmunity in both mice and humans (Mercer & Unutmaz, 2009). Accordingly, it is anticipated that approaches that modulate T regulatory cells activity will provide the means to mimic the immune system and finally control inflammation in a tissue specific manner. Two particularly apparent properties of T regulatory cells raise the hopes that development of T regulatory cells inducing immunoregulatory vaccines could lead to potent means of controlling inflammation (Tang & Bluestone, 2008). The first property is by-stander suppression, which is the capacity of T regulatory cells induced by a specific antigen to directly suppress effector cells induced by other antigens at the site of inflammation. The second property is infectious tolerance, which is the ability of a T regulatory cell clone to induce T regulatory cells of different antigen specificities. Therefore, T regulatory cells are naturally equipped to amplify in a targeted manner an immunoregulatory response induced by an immunoregulatory vaccine.

The first immunoregulatory vaccine to be successfully translated to the clinic was a peptide vaccine. Peptide vaccines are short polymers of amino acids derived from full-length proteins characterized as autoantigens. They are chosen based on their ability to bind as epitopes to major histocompatibility complex molecules on antigen-presenting cells. The bound peptide is then presented to the receptor of a restricted number of T lymphocytes, preferably T regulatory cells in the case of type 1 diabetes. The advantage of a peptide vaccine compared to the full-length parent protein is that it can focus the desired immune response by activating only a small number of T cell clones instead of multiple clones that may not be all relevant. The disadvantage of a peptide vaccine is that its activity will vary depending on the MHC molecules synthesized by a given individual. In this case, polypeptide vaccines are more advantageous because they ensure that all treated patients will present an epitope. Both peptide and polypeptide vaccines have been used with varying outcomes in type 1 diabetes.

4.1 Heat-shock protein 60 peptide vaccine (Diapep277)
Diapep277 was the first peptide vaccine successfully used in clinical trials for type 1 diabetes. Irun Cohen and colleagues identified peptide p277 derived from heat shock protein 60 and found that subcutaneous injection of 100 micrograms of the peptide in incomplete Freund’s adjuvant inhibited the development of spontaneous diabetes in non-obese diabetic mice (Elias & Cohen, 1995). The main function of eukaryotic heat shock protein 60 is as a mitochondrial chaperone that assists in protein folding and translocation in the mitochondrial matrix (Fischer et al., 2010). From an immunological standpoint, mammalian heat shock proteins like HSP60 can also act as damage associated molecular patterns that are released or presented by dying cells and can activate antigen-presenting cells of the innate immune system (Chen et al., 1999). Heat shock proteins activate macrophages and dendritic cells through Toll-like receptor 4, which belongs to a class of membrane-bound proteins that act as sensors for immune cell activation, and promote proinflammatory effector immune responses. Paradoxically, heat shock proteins can also have T cell mediated anti-inflammatory effects through Toll-like receptor 2, and DiaPep277 peptide functions through a Toll-like receptor 2-mediated mechanism (Eldor et al., 2009). Heat shock protein 60 has been found to be a possible autoantigen in type 1 diabetic children and murine models. It is found located on the membranes of the beta cell insulin
secretory granules in pancreatic islets, and shares significant homology to bacterial heat shock protein 65. Epitope scanning of heat shock protein 60 with antibodies identified the peptide DiaPeP277 with the amino sequence VLGGGVA LRV PALDSLTPANED as an immunodominant epitope in type 1 diabetic children (Brudzynski et al., 1992; Horváth et al., 2002).

As mentioned previously, in contrast with full-length heat shock protein 60, DiaPeP277 has no effect on Toll-like receptor 4 and activates anti-inflammatory effectors through Toll-like receptor 2. Toll-like receptor 2 promotes cell adhesion, inhibits migration, and modulates cytokine secretion resulting in a deviation to a T helper-2 cytokine profile that is associated with a shift from an inflammatory to a regulatory response. Indeed, the peptide inhibits diabetes by shifting T cell responses from a T helper-1 to a T helper-2 like activity as indicated by the presence of pep277-specific antibodies of the IgG1, but not of the IgG2a isotype, and production of interleukin-4 and interleukin-10 in spleens of non-obese diabetic mice (Elias et al., 1997). The peptide also causes a decrease in the number of interferon-gamma producing islet T cells, which is concomitant with increased islet numbers and the arrest of type 1 diabetes when administered to 12-week old non-obese diabetic mice (Ablamunits et al., 1998). Moreover, the peptide induces islet protective T cells that can be adoptively transferred to protect non-obese diabetic-SCID mice from diabetes (Ablamunits et al., 1999).

Clinical trials of the peptide vaccine DiaPeP277 have been ongoing and results have been reported since 2001. Patients were treated with 0.2, 1, and 2.5 mg doses of the peptide (Pfleger et al., 2010). The results showed that increased T helper-1 related cytokine responses (interferon-gamma) were associated with lower beta cell function whereas T helper-2 (interleukin-5, interleukin-13) and T regulatory activity (interleukin-10) related cytokine responses were positively associated with beta-cell function in adults and children. DiaPeP277 also acts as an inhibitor of human T cells response. The signal transduction cascade induced by the p277 peptide involves suppression of both cytokine signaling 3 (SOCS3) expression and signal transducer and activator of transcription 3 (STAT3) (Zanin-Zhorov et al., 2005). This inhibition of T cell mediated inflammation by the peptide is due to down-regulation of T cell chemotaxis and reduced secretion of proinflammatory cytokines. A randomized, double blind, phase Ib/II study of DiaPeP277 tested its safety and efficacy, which was undertaken with recent onset type 1 diabetes patients with remaining insulin production (Huurman et al., 2007). Forty-eight adult patients were administered subcutaneous injections of 0.2, 1.0 or 2.5 mg DiaPeP277 (12 patients per dosage) at entry, 1, 6, and 12 months, or they received four placebo injections (12 patients). C-peptide levels decreased in all groups with the exception of patients receiving the 2.5 mg dose, and decreased C-peptide production was attenuated in treated patients versus placebo. The main conclusion was that the treatment was safe, and may have a beneficial effect on C-peptide levels over time, although this was not supported by lower hemoglobin A1c levels as an indicator of diabetic control or daily insulin requirement.

Another phase II trial studies the effects of DiaPeP277 in 2 stages (Lazar et al., 2007). In the first stage, 17 patients received four injections of 1 mg DiaPeP277 at months 0, 1, 6, and 12, and 18-month, and preservation of endogenous insulin secretion was observed up to 18 months. In the second stage, which was only for those who completed stage 1 including placebo with C-peptide above 0.1 nmol/L, patients continued treatment with DiaPeP277 (six patients, 1 mg DiaPeP277 at months 0, 3, 6, and 9), and those switched from placebo to DiaPeP277 (thirteen patients, 1 mg DiaPeP277 at months 0, 3, 6, and 9) manifested a trend towards a greater preservation of beta-cell function compared to five patients maintained on
and seven patients switched to placebo (Raz et al., 2007). Conversely, a trial with recent onset diabetic children (7-14 years old) showed no beneficial effect in preserving beta-cell function or improving metabolic control.

Phase III trials of DiaPep277 are currently ongoing. The first Phase III study has begun in 2005 in 40 centers worldwide (Fischer et al., 2010). The inclusion criteria include type 1 diabetes for less than 3 months, ages 16 – 45, and C-peptide > 0.22 nmol/L. The treatment regimen includes nine injections of 1 mg DiaPep277 or placebo over 21 months and 3 additional months of follow-up. At the end of recruitment in September 2009, 457 patients were randomly assigned to one of the groups and results are expected in September 2011. After an interim analysis, the Independent Data Monitoring Committee (IDMC) concluded that there were no safety concerns and that the study could be continued without modification. In addition, there is a clear treatment effect in different sub-group populations. Two additional phase III trials are ongoing and will end in August and December of 2013.

Immunological studies of the effects of DiaPep277 in human patients have revealed that the treatment is immunologically effective, specific and safe, when comparing T cell responses to specific antigen DiaPep277, related autoantigens heat shock protein 60, glutamic acid decarboxylase 65, and non-related third party antigen tetanus toxoid, between treatment and placebo (Huurman et al., 2008). Cytokine production in response to therapy was dominated by interleukin-10, and decreasing autoantigen specific T cell proliferation was associated with beta cell preservation. The investigators concluded that declining or temporary T cell proliferation in response to DiaPep277 may serve as an immunological biomarker for clinical efficacy.

4.2 Insulin peptide vaccines

Insulin is a hormone which is mainly responsible for regulating sugar and fat metabolism in the body. Insulin permits cells in the liver, muscle, and fat tissue to take up sugar from blood and store it as glycogen in the liver and muscle. Chronic high blood glucose, or hyperglycemia, can cause severe complications by damaging and impairing tissues via molecular mechanisms like protein glycosylation (Aronson, 2008). Since the onset of diabetes is the result of low levels of and ultimately no insulin secretion from pancreatic beta cells, injection of the insulin protein has been used as a replacement therapy to treat diabetes after its beneficial effects were discovered by Canadian scientists Frederick Grant Banting and Charles Best in 1921.

The current standard means of delivering insulin as replacement therapy is subcutaneous injection because of its lower cost and ease of self-delivery compared to other methods. Other routes such as oral administration and intramuscular injection are not chosen because of loss of insulin function in the digestive tract and rapid dispersion of the hormone, respectively. These limitations are not a concern when using insulin as a vaccine because only structural aspects of the molecule are needed, i.e., antigenicity instead of function. Various clinical trials have been conducted to test whether delivering insulin protein as a vaccine via different delivery routes can prevent or ameliorate type 1 diabetes. The Diabetes Prevention Trial network (DPT-1) screened 103,391 healthy individuals who were under 45 years of age, islet antibody positive, and relatives of type 1 diabetic patients. Individuals at high risk of developing type 1 diabetes, i.e., at least 50% probability of developing type 1 diabetes within 5 years, received insulin both short-term intravenously and long-term...
subcutaneously (Schatz & Bingley 2001). In addition, individuals at moderate risk of developing type 1 diabetes, i.e., 25-50% probability of developing the disease within 5 years, received oral delivery of insulin. Results showed that subcutaneous injection of insulin did not slow type 1 diabetes onset, and that oral insulin did not delay or prevent the disease. Further studies of a subgroup with higher insulin autoantibody levels indicated that although oral insulin did not reduce insulin autoantibody levels, there was a possible delay in diabetic progression for approximately 4.5 years (Skyler et al., 2005, Barker et al., 2007). Accordingly, larger trials with more participants and similar standards of the oral insulin study are ongoing (Skyler 2008).

In addition to insulin polypeptide, the B:(9-23) B chain peptide or its altered form contains a major epitope recognized by the immune system and could delay or prevent diabetes when administered subcutaneously and intranasally in non-obese diabetic mice (Daniel & Wegmann, 1996; Kobayashi et al., 2007). A phase I/II clinical trial of insulin B-chain in incomplete Freund's adjuvant (IBC-V501), i.e., non-inflammatory, was conducted using a single intramuscular injection in a small group (12 patients) with recently diagnosed type 1 diabetes (Orban et al., 2010). After two years, the patients developed robust insulin-specific humoral and T cell responses including insulin B-chain specific CD4 T regulatory cells, but did not show statistically different levels of C-peptide compared to the control group. Nevertheless, the results are meaningful because there is a growing body of evidence suggesting that autoimmunity observed in type 1 diabetes is the result of an imbalance between autoaggressive and regulatory cell subsets. Therefore, therapeutics that supplement or enhance the existing regulatory T cell subset could be beneficial.

Over the years, multiple studies have shown that mucosal administrations of insulin orally and intranasally retard development of autoimmune diabetes in the non-obese diabetic mice (Bergerot et al., 1994; Harrison et al., 1996). Accordingly, a trial using intranasal delivery of insulin (Humulin) to 38 children (median age 10.8 years) at risk for type 1 diabetes was undertaken as part of the Melbourne Pre-Diabetes Family Study in Australia (Harrison et al., 2004). The results suggested that intranasal insulin induced immune changes consistent with mucosal tolerance to insulin, and did not accelerate loss of β-cell function. Conversely, additional trials with 224 infant and 40 sibling relatives with HLA-DQB1 susceptibility allele genotypes and two or more autoantibodies at three university hospitals in Finland did not prevent or delay type 1 diabetes (Näntö-Salonen et al., 2008).

Altogether, results from these different clinical trials indicate that the pre-clinical success of insulin as an immunoregulatory vaccine in non-obese diabetic mice has not yet successfully translated in humans. Interestingly, evidence suggests that insulin may be the initiator autoantigen in type 1 diabetes, in other words, loss of tolerance to insulin could be the trigger of the disease (Harrison, 2008). Hypothetically, this loss of tolerance mechanisms for insulin could explain the difficulty in inducing tolerance in humans using therapeutic insulin vaccines. On the other hand, insulin may be a target of choice to prevent disease when mechanisms of tolerance to insulin are still in place in pre-diabetic individuals.

4.3 Glutamic acid decarboxylase 65 protein vaccine

In contrast with clinical trials using insulin polypeptide and peptide vaccines, the first human trials with glutamic acid decarboxylase 65 autoantigen as a therapeutic vaccine have shown beneficial therapeutic results (Ludvigsson & Linköping Diabetes Immune Intervention Study Group, 2009). Glutamic acid decarboxylase 65 is an enzyme that catalyzes the synthesis of
gamma-aminobutyric acid (GABA), which acts as a neuroinhibitor as well as an immunoregulatory molecule. Several observations suggest that glutamic acid decarboxylase 65 may have a critical early role in mediating islet beta cell destruction. Detection of anti glutamic acid decarboxylase antibodies in the sera of prediabetic patients is a reliable predictive marker for the progression to overt diabetes, and anti glutamic acid decarboxylase reactivity can be detected in non-obese diabetic mice very early in the disease process (Ludvigsson & Linköping Diabetes Immune Intervention Study Group, 2009; Tisch et al., 1994). Evidence for a role for the protein in disease etiology came from experiments reporting that delivery of glutamic acid decarboxylase 65 either intravenously or intrathymically into female non-obese prediabetic mice can prevent insulitis and diabetes (Kaufman et al., 1993; Tisch et al., 1993). Additional reports showed that intravenous delivery of glutamic acid decarboxylase 65 once every three days for a total of four injections of 200 micrograms from age 12-week can prevent diabetes from 70% in controls down to 20% in treated animals at week 35 through induction of glutamic acid decarboxylase 65 specific CD4 T regulatory cells (Tisch et al., 1998). Furthermore, injection of glutamic acid decarboxylase 65, but not heat shock protein pep277 or insulin B-chain, can increase survival of syngeneic islets transplanted into diabetic mice through modulation of T-helper 1/T-helper 2 balances (Tian et al., 1996).

The first results of phase I/II clinical trials using subcutaneous delivery of 4, 20, 100, and 500 micrograms of alum-formulated human recombinant glutamic acid decarboxylase 65 to eight patients with Latent Autoimmune Diabetes in Adults (LADA) were reported in 2005. Data showed that only the 20 microgram dose could increase both stimulated and fasting C-peptide levels and T regulatory cells from baseline over the 24-week period. A 5-year follow up study found that the 20 microgram dose, and to a lesser extent the 4 and 100 microgram doses, could maintain C-peptide levels compared to the placebo group (Agardh et al., 2005; Agardh et al., 2009).

In addition, seventy children and adolescents aged 10–18 years with recent onset type 1 diabetes participated in a phase II trial (Ludvigsson et al., 2011). Participants received either a subcutaneous injection of 20 microgram of the alum formulated glutamic acid decarboxylase 65, or placebo at baseline and 1 month later. Although there was no statistical significant differences in fasting C-peptide levels between the glutamic acid decarboxylase 65 and the placebo groups, those patients who were treated within 6 months of diabetes diagnosis had fasting C-peptide levels that decreased significantly less in the glutamic acid decarboxylase 65 group after 4 years compared with the placebo group. These results are significant because they indicate that, in contrast with Diapep277, glutamic acid decarboxylase vaccination is applicable to diabetic children who represent a large segment of the population with type 1 diabetes. However, in May 2011 it was reported that a European Phase III study with the antigen did not meet the primary efficacy endpoint of preserving beta cell function at 15 months, although a small positive effect was seen.

With regard to immune responses induced by alum-formulated glutamic acid decarboxylase 65 in human, a reduced percentage of IgG1 and increased IgG3/IgG4 antibodies were detected in treated children after 3 months, which suggested a T helper-2 deviation in the immune system. In addition, levels of IA-2A, IgE and tetanus toxoid antibodies as well as glutamic acid decarboxylase enzyme activity were unaffected, which suggested specificity of the treatment (Chéramy et al., 2010; Ortqvist et al., 2010). Importantly, injection of the glutamic acid decarboxylase 65 protein vaccine enhances the percentage of CD4+CD25highFOXP3+ T regulatory cells, and induces secretion of interleukin-5, interleukin-
10, and interleukin-13 correlating with the expression of CD4+CD25highFOXP3+ cells 21 to 30 months after treatment in 35 patients aged 10–18 years (Hjorth et al., 2011). Vaccination with polypeptides/peptides to prevent and treat type 1 diabetes appears to be well-tolerated and safe in humans; however, the possibility of adverse events cannot be completely ignored. For example, insulin allergy occurs in less than 1% of diabetic patients treated with insulin peptide (Zhang et al., 2008). In these patients, different methods have been used for the treatment of insulin allergy including use of different insulin or insulin formulations. Allergic reactions range in severity from erythema and pruritus to life-threatening anaphylaxis. Indeed, vaccines inducing T helper-2 like responses can induce lethal anaphylaxis in non-obese mice (Overbergh et al., 2003, Pedotti et al., 2003), and may be less preferable compared to vaccines inducing regulatory cells like Foxp3 and T regulatory-1 T lymphocytes.

5. DNA vaccine based immunotherapies

DNA vaccines generally consist of bacterial plasmid DNA engineered to synthesize an antigen and other gene products after injection into a recipient. Compared to polypeptide/peptide vaccine immunotherapies, DNA vaccines bear several unique advantages, such as rapid development and standardized production, lower cost of storage, and synthesis over time of the chosen antigen in its native conformation. The first DNA vaccines were designed to induce immunogenic responses to pathogens and cancer but have increasingly been applied to induce immune tolerance for autoimmune diseases like type 1 diabetes. DNA vaccines and other gene-based vaccines belong to a third generation of vaccines following live and attenuated whole organism vaccine and recombinant protein vaccines. Recent reports of beneficial results in different clinical trials indicate that DNA vaccination is reaching a stage where we are likely to see accelerated development of a therapeutic future of DNA vaccines for a variety of diseases. In the case of type 1 diabetes, early results using a DNA vaccine encoding insulin have shown promise in clinical trials, confirming the notion that DNA vaccines may be particularly well suited for promoting immune tolerance in humans compared to effects desired for infectious diseases and cancer. In addition, DNA vaccines encoding heat shock protein 60 or 65 and glutamic acid decarboxylase 65 have also shown efficacy in mice and are reviewed in this chapter.

5.1 Heat shock protein 60 or 65 DNA vaccines

As mentioned previously, Pep277 derived from mammalian heat shock protein 60 has shown protective effects in both pre-clinical and clinical trials. With regard to DNA vaccination, two 100 microgram intramuscular injections of plasmid DNA coding for mammalian heat shock protein 60 into 4-week old non-obese diabetic mice can prevent cyclophosphamide accelerated diabetes, i.e., 30% of treated mice develop diabetes compared with 60% diabetic in vector treated controls (Quintana et al., 2002). Disease prevention is associated with reduced T cell proliferation, an increase in interleukin-10 and interleukin-5 secretion, and a decrease in interferon-gamma secretion, which suggests a shift from a T helper-1 like toward a T helper-2 like immune response. Furthermore, plasmid DNA encoding mycobacterial 65-kDalton heat shock protein caused decreased insulitis when injected intramuscularly in three doses (100 micrograms each) administered at 2-week intervals into 6- to 8-week-old, streptozotocin-induced diabetic C57BL/6 mice (Santos et al., 2009). The treatment was associated with the appearance of a
regulatory cell population in the spleen, with higher production of interleukin-10 in spleen and islets, and with a decreased infiltration of CD8 T lymphocytes in the islets. The same DNA vaccine with the same dose and delivery reduced the occurrence of diabetes from 100% to 33% in 28-week old non-obese diabetic mice when injected at 4-week of age, and was associated with a reduction in CD4 and CD8 T cells infiltration, appearance of CD25 cells, and increased levels of interleukin-10 in the islets (Santos et al., 2007).

5.2 Insulin DNA vaccines
Insulin-encoding plasmid DNA is the only type of DNA vaccine that has been tested in both preclinical and clinical trials for type 1 diabetes. The initial report demonstrating feasibility of this concept used a virus-induced diabetic mouse model system to show that intramuscular inoculation of plasmid DNA encoding the insulin B chain reduces the incidence of diabetes (blood glucose > 350 mg/dl) from 100 to 50% (Coon et al., 1999). DNA vaccination induced CD4 T regulatory cells that reacted with the insulin B chain, secreted interleukin-4, and locally reduced autoreactive activity of cytotoxic T lymphocytes in the pancreatic draining lymph nodes. The DNA vaccine also protected non-obese diabetic mice reducing diabetes onset from 80% down to 25% depending on the presence of interleukin-4 (Bot et al, 2001).

Rodent animals synthesize two isoforms of insulin, I in islets and II in both islets and thymus that are the products of non-allelic genes while humans have only one form of insulin. The pancreatic beta cells synthesize proinsulin before converting it to functional insulin (Sizonenko & Halban, 1991). Intranasal delivery of plasmid DNA encoding mouse proinsulin II (3-50 micrograms doses over a 2-week interval starting at 4 weeks of age) together with an anti-CD154 antibody (3 doses of 300 microgram over 2-week interval from 4 weeks of age) prevented type 1 diabetes by reducing the incidence in 40-week old mice from 100% diabetic animals down to 0%. On the other hand, intranasal delivery of the DNA vaccine alone did not prevent disease, but did induce regulatory T cells (Every et al., 2006). In contrast to prototypic CD4+ CD25+ T regulatory cells, the CD4 T regulatory cells induced by the proinsulin DNA vaccine alone were both CD25+ and CD25-, and were not defined by markers such as glucocorticoid-induced TNFR-related protein (GITR), CD103, or Foxp3.

Another report showed that co-delivery of a DNA vaccine encoding human proinsulin (50 microgram) and insulin peptide (100 microgram) intramuscularly twice over 2-week intervals starting when the mice were 6 weeks of age prevents diabetes in non-obese diabetic mice until 24 weeks of age, but not the DNA or peptide vaccine alone (Zhang et al., 2010). Results also indicated that the induction of transforming growth factor-beta producing CD4+CD25- islet specific T regulatory cells was observed only in the co-immunization group, but not in the DNA or the protein vaccine alone group, which confirmed a synergistic effect.

Among the most promising reports of insulin DNA vaccination is a plasmid DNA vaccine encoding mouse proinsulin II, which reduces the incidence of diabetes in non-obese diabetic mice when administered intramuscularly to prediabetic 8-week old mice, and to diabetic mice older than 12-week old with blood glucose > 170 mg/dL (Solvason et al., 2008). The efficacy of the vaccine was improved by increasing the level of expression of insulin, frequency of dosing, dosage, and subcellular localization modification of the autoantigen to the intracellular compartment instead of secretion. In the prophylactic setting, the DNA vaccine decreased the incidence of diabetes from 80% in the control group down to 45% in 25-week old mice receiving weekly administration of 50
micrograms of the vaccine. The treatment caused increased numbers of interferon-gamma-secreting cells and a decrease in insulin autoantibodies. In the therapeutic setting, the DNA vaccine reduced progression to overt diabetes from 100% in the control groups down to 25% in treated mice (observation made at 25 weeks post treatment initiation). Treatment consisted of weekly delivery of 50 microgram of the vaccine for a total of 9 injections. The treatment induced increased numbers of insulin-specific interferon-gamma-producing T cells and levels of interleukin-10, which suggested induction of T regulatory-1 cells. Adoptive transfer experiments indicated that the protection was not mediated by induction of CD4^+CD25^+ T regulatory cells.

Importantly, a similar vaccine was used in the only human trial of a DNA vaccine for diabetes conducted to date (Gottlieb et al., 2008). The plasmid DNA vaccine (BHT-3021) has undergone a Phase I/II trial using four doses of plasmid DNA, i.e., 0.3, 1, 3 and 6 milligrams, administered intramuscularly once a week for 12 weeks. The interim results for the 1 mg dose showed pancreatic beta-cell preservation, demonstrated by a mean 17% increase in C-peptide levels with BHT-3021 by week 15 after enrollment, whereas placebo patients experienced a mean 42% decrease in C-peptide. Evidence for immune tolerance was suggested by a mean 17% reduction in anti-insulin antibodies, and 25% reduction in anti-glutamic acid decarboxylase 65 antibodies by week 15 after enrollment, whereas placebo patients experienced a mean 6% and 4% increase, respectively. The most recent report of the trial claimed that BHT-3021 preserves C-peptide levels for at least six months and one year in some of the patients from the point of initiation of the therapy (Garren, 2009). These results together with its favorable side-effects profile appear to be comparable to those reported with anti-CD3 monoclonal antibody and the glutamic acid decarboxylase 65 protein vaccines for type 1 diabetes.

5.3 Glutamic acid decarboxylase DNA vaccines

DNA vaccines encoding glutamic acid decarboxylase 65 are currently at the preclinical stage. The first report of a beneficial effect in non-obese diabetic mice showed that plasmid DNA encoding wild-type intracellular or engineered secreted glutamic acid decarboxylase, i.e., a fusion of the interleukin-2 signal peptide with a truncated form of human glutamic acid decarboxylase 65, causes decreased insulitis compared to plasmid vector alone when delivered intramuscularly, and is accompanied by higher secretion of interleukin-4 by splenocytes (Liu et al., 1999). A subsequent report indicated that only the DNA vaccine encoding secreted glutamic acid decarboxylase could suppress cyclophosphamide-accelerated diabetes in 4-week old female non-obese diabetic mice with a tendency to increase T helper-2 like activity when 2 x 400 micrograms were delivered intramuscularly over 2 days (Filippova et al., 2001). Another report published the same year corroborated the notion that secretion of glutamic acid decarboxylase encoded by a DNA vaccine is important to ameliorate diabetes in mice (Weaver et al., 2001). In this report, plasmid DNA was engineered to encode a secreted fusion protein of a truncated form of glutamic acid decarboxylase 65 and an IgG Fc fragment as well as interleukin-4. Intramuscular injection of 50 micrograms of the vaccine effectively prevented diabetes in non-obese diabetic mice treated at early (4-week old, 3 times weekly) or late (12-week old, 4 times weekly) preclinical stages of diabetes. Diabetic onset reduction went from 75% in controls down to 25% at week 50+ and from 70% to 20% at week 55+. Protection was dependent on the vaccine-encoded interleukin-4 and endogenous interleukin-4, and was associated with induction of glutamic acid decarboxylase 65 specific
regulatory T helper-2 cells (Tisch et al., 2001). However, when the same vaccination strategy was applied using insulin, the vaccine encoding insulin B chain/IgG Fc fusion protein and interleukin-4 caused accelerated progression of insulitis and diabetes, which was correlated with an increased number of interferon-gamma secreting T cells in response to insulin B chain specific peptides (Weaver et al., 2001).

In addition, a study reported that a DNA vaccine encoding full-length intracellular human glutamic acid decarboxylase 65 could prevent spontaneous diabetes when delivered at week 4 or week 10 of age using intramuscular injection of 2 x 50 micrograms in non-obese diabetic mice (Balasa et al., 2001). Notably, disease prevention was associated with CD28/B7 costimulation because co-expression of B7-1 or B7-2 and glutamic acid decarboxylase 65 by the same DNA vaccine abrogated protection.

With regard to DNA vaccination and the effects of interleukin-4, a virus-induced murine diabetes model was used to study the relationship between different endogenous expression levels of islet autoantigen in beta-cells and the efficacy of DNA vaccination (Wolfe et al., 2002). Lower expression levels of a model autoantigen in beta-cells support immune regulation resulting in induction of autosuppressive regulatory cells characterized by increased interleukin-4 production (T helper-2 like). In contrast, higher levels of the autoantigen favor T helper-1 like autoaggressive responses characterized by increase interferon-gamma generation. Immunization with a DNA vaccine coding the autoantigen and interleukin-4 reduced the risk of augmenting autoaggression and thus increased the safety margin of this immune-based therapy.

DNA vaccines encoding secreted glutamic acid decarboxylase and anti-inflammatory interleukins have also been applied to transplantation for type 1 diabetes. Survival of syngeneic neonatal pancreata transplanted under the kidney capsule of non-obese diabetic mice is promoted by intramuscular injection of a DNA vaccine encoding the secreted glutamic acid decarboxylase 65/IgG Fc fusion and interleukin-4 plus interleukin-10 (Seifarth et al., 2003). The treatment consisted of 50 micrograms of the vaccine delivered weekly for four weeks from the age of 10 weeks with transplantation performed one week after the final DNA vaccination. The DNA vaccination protected the syngeneic islet transplanted mice from 100% diabetic mice in controls down to 20% diabetes incidence in treated animals at 30 weeks of age, 15 weeks post transplant, but required co-delivery of both interleukin-4 and interleukin-10. Increased islet survival correlated with a marked reduction in interferon-gamma reactivity that is glutamic acid decarboxylase 65 specific, and an increase in interleukin-10-secreting T cells. These results made apparent the increased difficulty in protecting exogenous syngeneic islet compared with endogenous islets, and the need for more stringent conditions of vaccination in the transplantation setting.

Intramuscular injection has traditionally been used for DNA vaccination because it permits delivery of larger amounts of DNA, and is commonly used in the clinic. Nonetheless, other routes of delivery may be more advantageous to induce tolerogenic responses. A report compared intramuscular, intradermal, and oral delivery of plasmid DNA coding for the intracellular or secreted form of glutamic acid decarboxylase for prevention of diabetes in a 4-week-old non-obese diabetic mouse model system (Li & Escher, 2003). Results showed that both intradermal and oral deliveries were more effective than intramuscular delivery for delaying the disease, and cytokine-specific ELISpot analysis indicated that immune responses induced by the DNA vaccines were more dependent on the cellular localization of glutamic acid decarboxylase antigen than on the delivery route. In contrast, ELISA indicated that intradermal delivery of DNA was most likely to induce a T helper-2 like response.
In addition to route of delivery, the method used to administer a DNA vaccine can be beneficial by directly improving immune responses and permitting lower vaccine dosage. For example, dermal delivery of plasmid DNA using gene gun technology, which consists in shooting microscopic metal particles covered with the vaccine, can improve protection from diabetes. In this regard, gene-gun delivery of 1 microgram of a DNA vaccine encoding the secreted glutamic acid decarboxylase 65/IgG Fc fusion polypeptide into 10-week old non-obese diabetic mice was compared with intramuscular injection of 50 micrograms of the same vaccine (Goudy et al., 2008). Results showed that in both cases gene expression peaked at week 8 post deliveries, and was maintained until at least week 35 with more than 40% higher expression from the gene-gun delivery. However, only gene-gun delivery could protect from diabetes with 90% diabetic in controls down to 50% diabetic at 35 weeks of age that was associated with induction of interleukin-4 secreting CD4 T cells. In contrast, intradermal gene-gun administration of plasmid-DNA encoding intracellular glutamic acid decarboxylase 65 to 3-week old non-obese diabetic mice does not suppress diabetes in non-obese diabetic mice (Joussemet et al., 2005). The different results might be attributed to the different subcellular localizations of the autoantigen.

So far in this section we have described how DNA vaccines can be engineered to enhance tolerogenic-like immune responses by co-delivering cytokine-encoding DNA, engineering subcellular localization of a target autoantigen, and choosing an effective route and method of delivery. The results obtained by different laboratories illustrate the promising potential of DNA vaccination as a safe, low-cost and patient-friendly means to treat autoimmune diabetes and other immune-mediated inflammatory disorders. Yet, as with all immunotherapies that seek safe means of improving the life of diabetic individuals, there is a pressing need to improve treatment efficacy. We strongly believe that one of the solutions to this problem is to mimic how the immune system maintains immune tolerance in peripheral tissues. DNA vaccination is particularly well-suited to achieve this goal because of the ability of plasmid DNA to deliver genetic instructions directly in situ for a limited time span and with low levels of danger signals known to activate proinflammatory immune responses. Here, we briefly discuss vaccine-induced apoptosis as a possible means to mimic physiological immune tolerance and to approach the “Holy Grail” of immunotherapy, namely, the ability to suppress inflammation in a homeostatic manner (Figure 3).

Apoptosis is a constantly on-going form of cell death that produces fifty to seventy billion dead cells on a daily basis in the average human adult (Reed, 1999). Upon a given intrinsic or extrinsic signal, cells initiate the process of apoptosis and become membrane-bound cellular fragments, or apoptotic bodies, which are rapidly engulfed and processed by surrounding living cells. For many years it was believed that these apoptotic bodies had little effect on the immune system. Today, it is becoming increasingly clear that apoptotic cells play a fundamental role in both establishing and maintaining peripheral immune tolerance as they not only serve as a source of self-antigens to maintain immune tolerance, but also recruit antigen-presenting cells, secrete anti-inflammatory cytokines, and display tolerogenic molecules (Birge and Ucker, 2008). The remarkable capacity of apoptotic cells to induce either tolerogenic immune responses or immunogenic responses depending on signals received makes them attractive candidates to intervene in many disorders like infectious diseases, cancer, and autoimmune diseases.
Fig. 3. Possible events following intradermal injection of a pro-apoptotic DNA vaccine coding for secreted pancreatic autoantigen glutamic acid decarboxylase (GAD). The plasmid DNA vaccine can transfect a variety of cell types at the chosen site of vaccine delivery, e.g., fibroblasts and keratinocytes in the case of intradermal injection. Dendritic cells (DC) recruited by vaccine-induced apoptotic cells can uptake and process GAD-containing apoptotic cells induced by the vaccine as well as vaccine-encoded secreted GAD, and present GAD on major histocompatibility complex class I and II molecules. The dendritic cells then migrate to lymph nodes and spleen where they can induce tolerogenic immune responses.

An important feature of pro-apoptotic DNA vaccination is that it permits the manipulation of physiological apoptosis both de novo and in situ. This is important because apoptotic cells synthesize a variety of immune molecules with levels that are most likely physiologically relevant in the context of a microenvironment. The concept of immunological microenvironment is also crucial to immune responses induced by dendritic cells, which are equipped to sense and act upon changes in their immediate vicinity. Therefore, induction of apoptosis by DNA vaccination could be a way to have access to homeostasis and maintain non-responsiveness to self.

The first report of DNA vaccines designed for pro-apoptotic immunoregulation, i.e., anti-inflammatory, used plasmid DNA coding for the pro-apoptotic BAX protein and intracellular or secreted glutamic acid decarboxylase, to prevent diabetes in the non-obese diabetic mouse (Li et al., 2004). Results indicated that intramuscular injection of the BAX cDNA recruited
dendritic cells carrying vaccine-encoded protein in both spleen and lymph nodes. Furthermore, delivery of 2 x 150 micrograms plasmid DNA coding for secreted glutamic acid decarboxylase and BAX at a 3 days interval into 4-week old mice could prevent diabetes, i.e., reduce the incidence from 93% in controls down to 47% in treated animals. In contrast, the vaccines coding for BAX or secreted glutamic acid decarboxylase DNA alone or intracellular glutamic acid decarboxylase and BAX did not prevent diabetes. Notably, ELISA results indicated that co-delivery of BAX suppressed T helper-2 like activity which indicated that another cell type was responsible for disease suppression. Indeed, a subsequent report showed that delivery of both secreted glutamic acid decarboxylase and BAX were required to induce CD4+CD25+FoxP3+ cells with contact dependent regulatory activity that was independent of transforming growth factor-beta and interleukin-10 (Li et al., 2006).

Importantly, additional studies revealed that increased CpG methylation of the DNA vaccine together with delivery of secreted glutamic acid decarboxylase and BAX DNA acted synergistically to ameliorate recent onset of diabetes in non-obese diabetic mice (Li et al., 2010). Mice receiving a weekly intradermal injection of 50 micrograms of the vaccine over eight weeks following early diabetes ameliorated diabetes from 90% diabetic in controls down to 20% in treated mice at 40 weeks of age. It is hypothesized that increased CpG methylation of plasmid DNA makes the DNA vaccine appear more mammalian-like to the immune system, as it is known that bacterial DNA has low levels of CpG methylation that can act as an inflammatory signal (Krieg, 2002). Taken together these results indicate that apoptosis-inducing DNA vaccination is a promising approach for treatment of type 1 diabetes.

5.4 Comparing DNA vaccines and polypeptide/peptide vaccines

Compared to polypeptide/peptide vaccine, the main advantages of DNA vaccines are: 1) known process of manufacturing, i.e., plasmid DNA can be isolated using a standard procedure while different polypeptides may require different protocols that have to be optimized for a specific antigen; 2) Cost-effective shipment and storage because plasmid DNA does not require refrigeration; 3) A more sustained expression of the antigen in its native conformation, or shape, instead of a purified antigen that can adopt different non-native conformations; and 4) Expression of the whole protein rather than specific epitopes in the case of peptides to ensure delivery of full antigenic signals that can be recognized by different major histocompatibility complex molecules in an outbred human population.

6. Other immunotherapies

There are currently other ongoing immunotherapies, such as the applications of Bacille Calmette-Guérin (BCG), Vitamin D3, nicotinamide, immunosuppressants, nanoparticles, and antisense oligonucleotides, etc., which have certain effects in suppressing type 1 diabetes in non-obese diabetic mice. Bacille Calmette-Guérin is a vaccine that is prepared from a strain of attenuated live bovine tuberculosis bacillus that has lost its virulence in human, which has been used as a vaccine to prevent tuberculosis. Although it has shown efficacy in animal models, clinical trials in recent onset diabetic children have been disappointing (Elliott et al., 1998; Allen et al., 1999).

Several clinical trials of vitamin D3 have been conducted since the 1990s, and results showed either temporary effects (Pitocco et al., 2006; Li et al., 2009), or no effects (Walter et al., 2010;
Nicotinamide is a molecule belonging to the vitamin B group, i.e., vitamin B3, and has anti-inflammatory effects \textit{in vivo}. The first report of clinical trials on type 1 diabetes was published in the mid 1980s (Vague et al., 1987), followed by multiple trials conducted worldwide with complex results. When using 25 mg/kg or 1.2 g/m\textsuperscript{2} body surface/day of the vitamin B, most of the recent reports showed that nicotinamide has no protective effect on type 1 diabetes in new-onset patients or high-risk relatives (Pitocco et al., 2006; Skyler, 2008), even though it induces decreased spontaneous and \textit{in vitro} autoantigen-induced interferon-gamma secretion in high-risk relatives who develop type 1 diabetes and may play a role in immune regulation (Hedman et al., 2006). Only one of the reports showed that nicotinamide treatment results in higher C-peptide values at 3 months and lower insulin requirement at 1 year in pancreatic interleukin-2 accumulated diabetic patients post 1 year treatment (Chianelli et al., 2008).

The immunosuppressant cyclosporin A was employed in the first trials showing effects of immune therapies on T1D. Continuous cyclosporin A treatment initiated soon after diagnosis eliminated the need for exogenous insulin (Bougneres et al., 1988; Stiller et al., 1984). Nevertheless, the lack of lasting effects and renal toxicity of the drug diminished enthusiasm for this approach and other broad-spectrum immune modulating agents such as azathioprine and prednisone (Bougneres et al., 1990; Silverstein et al., 1988).

The Major Histocompatibility Complex (MHC) genomic region is found in most vertebrates and encodes protein molecules playing an important role in immunity and recognition of antigens by T cells. It has been shown that nanoparticles loaded with diabetes relevant peptide-major histocompatibility complexes prevent and treat diabetes when administered intravenously in non-obese diabetic mice (Tsai et al., 2010). The treatment prevented diabetes from 75\% in control down to 25\% in 30-week-old non-obese diabetic mice (4-week old mice received 7.5 mg every 2 weeks until the 3rd injection and every 3 weeks thereafter), and restored normoglycemia in diabetic mice (blood glucose higher than 11 mM mice received 7.5 mg twice a week for 5 week). The treatment expanded CD8+ regulatory T cells which suppressed local presentation of autoantigens in an interferon-gamma, indoleamine 2,3-dioxygenase, and perforin dependent manner. Furthermore, adoptive transfer of CD8+ but not CD4+ splenocytes suppressed diabetes and restored normoglycemia in a humanized diabetic mouse model.

Antisense oligonucleotides are single strands of DNA or RNA that are complementary to chosen sequences of target messenger RNAs. Antisense DNA oligonucleotides for messenger RNAs coding for CD40, CD80, and CD86 were delivered subcutaneously into 5- to 8-week old non-obese diabetic mice using 50 micrograms of a 1:1:1 mixture of each antisense oligonucleotides administered weekly for eight consecutive weeks (Phillips et al., 2008). The treatment prevented disease in 25 \% of mice compared to 100 \% diabetes in control animals. A similar treatment was given to diabetic mice with blood glucose higher than 300 mg/dL three times a week maintained blood glucose lower than 200 mg/dl for 50+ days compared to higher than 200 mg/dL in control mice. The treatment decreased CD40, CD80, and CD86 cell surface expressions on dendritic cells in spleen, and augmented Foxp3+ T regulatory cells numbers with hyporesponsiveness to self-antigen but not to alloantigen. In addition, spleen T-cells adoptive transfer from treated mice could suppress diabetes, confirming the induction of regulatory T cell activity.
7. Combinatorial approaches - the future of immunotherapy?

Combination immunotherapies are increasingly being considered, since none of the immunotherapies alone have reported thus far long term remission of type 1 diabetes (Li et al., 2008; Luo et al., 2010; Bluestone et al., 2010). This is especially true in view of the announcements in 2011 of failure of the anti-CD3 and glutamic acid decarboxylase protein vaccine therapeutic phase III trials for type 1 diabetic patients. Type 1 diabetes is an autoimmune disease correlated with multiple autoantigens and autoantibodies, and possible dysfunction in several cell types and associated cytokines. Therefore it is reasonable to anticipate a variety of synergistic effects that may be induced by combination therapies, as demonstrated in animal model systems. For example, a novel combination treatment with anti-CD3 epsilon specific antibody and intranasal delivery of proinsulin peptide can reverse recent onset diabetes in non-obese diabetic mice and a virus-induced diabetic mouse model with much higher efficacy than with monotherapy using anti-CD3 or the peptide alone (Bresson et al., 2006). Protection is associated with expansion of CD25$^+$ Foxp3$^+$ and insulin specific T regulatory cells producing cytokines, such as interleukin-10, transforming growth factor-beta, and interleukin-4. In addition, these cells can transfer dominant tolerance to immunocompetent recent onset diabetic recipients, and suppress heterologous autoaggressive CD8 T cell responses.

As mentioned previously, another synergistic effect was reported with prime boosting using DNA vaccine encoding proinsulin plus insulin protein vaccine to prevent new onset diabetes in non-obese diabetic mice. The induction of the transforming growth factor-beta producing CD4$^+$CD25$^+$ islet specific T regulatory cells against the onset of diabetes was observed only in the combination therapeutic group, but not in the monotherapy groups (Zhang et al., 2010). Standard clinical complex therapeutic protocol for controlling allo organ transplant rejection may be used as an example of combinatorial therapy where various drugs can be used in combination or alone at different times to increase allograft survival. Single therapy alone has its limits, ranging from targeting a single arm of the immune process, lower efficacy, and higher possible adverse effect due to higher dose requirement. Combination therapies could be used to overcome these problems. The combinations could include antibody or cytokine therapy combined with polypeptide/peptide and DNA vaccine, DNA vaccination combined with polypeptide/peptide vaccine and cellular therapy, as well as other combinations (Figure 4).

Considering the number of approaches that have been developed for the treatment of type 1 diabetes over the years, there is a significant number of possible combinations of different therapies. Yet we cannot exclude the possibility that platform technologies that provide access to a wide array of possible gene-based therapeutic enhancements could still perform satisfactorily on their own at lower cost. Our work with pro-apoptotic DNA vaccination does indicate that combining different properties of DNA vaccines alone can result in potent synergistic effects (Li et al., 2010). In addition, different combinations of autoantigens and vaccine technologies could still yield significant therapeutic improvements in the clinic. For example, the fact that GAD65 polypeptide appears to be a better therapeutic vaccine than insulin polypeptide/peptide vaccines combined with the promising results of the DNA vaccine encoding pro-insulin suggests the possibility that GAD65 might be a better autoantigen than insulin for therapy of T1D, and that plasmid DNA could improve efficacy of vaccination compared to an equivalent protein vaccine. Therefore, a DNA vaccine coding for GAD65 could be particularly beneficial for treatment of T1D.
Fig. 4. Hypothetic Models of Combinatorial Immunotherapies for Type 1 Diabetes.
Because of their increased safety compared to other approaches, antigen-based vaccines are the most likely to be applied to type 1 diabetes prevention. Combinatorial approaches for disease prevention could use prime-boosting with DNA and polypeptide vaccines. A body of evidence has shown the beneficial effects of this type of approach for infectious diseases and cancer, and initial results suggest that it is also applicable to type 1 diabetes. Antibodies like anti-CD3 may not be readily applicable to diabetes prevention for reasons of safety and efficacy, but could be used as induction therapy followed by prime-boost with DNA/polypeptide vaccines.

Clearly, the immunotherapeutic tools that have been generated over the past decade offer renewed hope for type 1 diabetic patients, as well as for the increasing number of individuals suffering from other chronic inflammatory disorders. We expect that in the near future, the development of novel therapeutic and preventive approaches, novel methods of delivery, and a better understanding of immunological mechanisms translated from animal models to human clinical studies and practices, will render the possibility of immunotherapy for type 1 diabetes a clinical reality.

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Type 1 Diabetes - Pathogenesis, Genetics and Immunotherapy
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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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