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Peptides and Proteins for the Treatment and Suppression of Type-1 Diabetes

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1. Introduction

Type-1 diabetes (T1D) is an autoimmune disease in which self-reactive immune cells infiltrate the islets in the pancreas to destroy β-cells. One of many possible causes is that self-reactive T cells that are normally eliminated can escape from the thymus along with normal T cells. The escaped T cells can be activated in response to a low level of secondary self-antigens, which can lead to a major step for tissue self-recognition. For activation, T cells interact with antigen-presenting cells (APC) via formation of the immunological synapse, which has a "bull's eye" structure at the membrane interface between both cells (Grakoui et al., 1999). The immunological synapse is composed of two segregated clusters of Signal-1 and Signal-2 molecular complexes. Signal-1 is generated by interaction between T-cell receptors (TCR) and antigen/multi histocompatibility complex-II (Ag/MHC-II). Signal-2 (costimulatory signal) can be delivered by a positive signal via B7/CD28 interactions or a negative signal via B7/CTLA-4 interactions. In addition, the CD40/CD154 costimulatory interaction between APC and T cells was found to induce an inflammatory immune response (Baker et al., 2008; Munro et al., 2007). Cell adhesion molecule interactions such as ICAM-1/LFA-1 interactions have also been categorized as a positive signal (Bromley et al., 2001; Grakoui et al., 1999). The positive costimulatory signal assists the induction of T-cell activation while the negative costimulatory signal suppresses T-cell activation (Bour-Jordan et al., 2011; Manikwar et al., 2011). The formation of the immunological synapse involves translocation of Signal-1 and Signal-2. Prior to the translocation process, Signal-1 is clustered at the periphery and Signal-2 is clustered at the center. Then, Signal-1 and Signal-2 switch places to establish the immunological synapse where Signal-1 is at the center (called central zone supramolecular activation complex or cSMAC) and Signal-2 is at the periphery (peripheral zone supramolecular activation complex or pSMAC) (Bromley et al., 2001; Grakoui et al., 1999).

TCR on T cells recognize self-antigens presented on MHC-II molecules on the surface of APC for activation of self-reactive T cells to initiate autoimmune diseases. In T1D, glutamic acid decarboxylase-65 (GAD65), is one of the important self-antigens in humans, and is a reliable marker in overt diabetes (Tisch et al., 1998). Administration of GAD peptides in complete Freund's adjuvant (CFA) into non-obese diabetes (NOD) mice triggers insulitis and destruction of β-cells to cause diabetes (Liu et al., 1999; Tisch et al., 1999; Yoon et al., 1999). There is a correlation between islet expression of GAD enzymes and the development of T1D. Different types of MHC-II molecules such as I-A^{g7} and I-$A^{g7.PD}$ recognize different

epitopes of GAD65 (Table 1) (Chao et al., 1999). Administration of GAD65/67 antisense gene suppresses GAD enzyme expression and eliminates T1D development in NOD mice (Yoon et al., 1999)

Aly *et al.* suggested that there are several stages in treating patients with T1D (Aly et al., 2005). First, T1D could be treated prior to detection of autoantibodies in patients in high-risk populations. Second, patients could be treated after autoantibody detection but prior to the onset of clinical diabetes. Third, patients could be treated to prevent further destruction of β-islet cells and ameliorate the disease. Finally, treatment could be developed for curing the disease by islet cell transplantation. Many new and novel treatments of T1D have been proposed with the hope of not just treating the symptoms but halting or reversing the disease. Proteins and peptides have been evaluated as therapeutic agents for T1D; they include antibodies to cell adhesion molecules (i.e., anti-ICAM-1, anti-LFA-1, anti-VCAM-1, and anti-VLA-4 monoclonal antibodies (mAbs))(Chowdhury et al., 2002; Moriyama et al., 1996; Tsukamoto et al., 1995), anti-CD3 mAb (Bresson et al., 2006; You et al., 2007), and anti-TNF-α mAb (Ryba et al., 2010). GAD peptides (Tisch et al., 1999) and bifunctional peptide inhibitor (BPI) molecules (Murray et al., 2007) have also been investigated to induce immunotolerance in T1D by altering the balance from effector to regulatory immune cells.

Early diagnosis of T1D is necessary for prevention of irreversible damage to the β-cells. The detection of islet cell antibody (ICA) as a common marker for disease progression is critical for individuals with a high risk of developing T1D (Honeyman et al., 1995). Unfortunately, not all patients with ICA progress to clinical diabetes; therefore, there is still an urgent need to find other markers or factors that can predict the rate of progression of T1D. The presence of certain HLA genes can influence the rate of destruction of β-cells by the immune system. For example, a high risk of acquiring T1D in caucasians has been associated with the expression of DR3-DQ2 and DR4-DQ8 HLA class II. Other identifiers for relatives who are most likely predisposed to T1D are class II antigens DR3, DR4, DQ2, and DQ8. An individual with DR4 within a family has a higher probability to become diabetic compare than an individual with DR3. An additional independent risk factor is class I antigens such as HLA-A24, which are found significantly more frequently in ICA-positive relatives who developed T1D than in those who did not (Honeyman et al., 1995). In Japanese subjects, however, T1D has been correlated with the haplotype of DR4-DQ9, and the presence of A24 has been correlated to T1D in young Japanese patients.

2. Animal models of T1D to evaluate potential peptide and protein therapeutics

The *in vivo* efficacies of potential therapeutic agents for T1D are usually evaluated in spontaneous diabetes or GAD peptide/CFA-induced diabetes in NOD mice as well as in the BioBreeding (BB) diabetic rat model (Aly et al., 2005; Calcinaro et al., 1997). T cells could respond to β-cell antigens such as GAD65, GAD67, peripherin, carboxypeptidase H, insulin, and HSP60; however, not all of these antigens become targets of the immune cells (Tisch et al., 1993). GAD is the initial and criticial antigen for the development of T1D; however, participation of other antigens during the development of diabetes cannot be ruled out. Histology analysis of the pancreas of NOD mice treated with GAD65 shows significant reduction in intra-insulitis. Mice treated with intrathymic injections of GAD65 did not develop diabetes and had significant reduction of IFN-γ production. It should also be noted that IFA or CFA could inhibit the development of diabetes in young NOD mice; this was

due to the change in population of destructive autoimmune cells surrounding the islets. In this case, a higher proportion of IL-4-producing cells compared to IFN-γ-producing cells were around the islets (Calcinaro et al., 1997; Liddi et al., 2000). Environmental factors such as the presence of retrovirus influence the development of T1D in NOD mice. Young NOD mice (below 2 months of age) infected with retrovirus are protected from developing T1D, and female sex hormones increase the incidence of T1D in NOD mice.

Progression of the disease occurs by means of lymphocyte infiltration of the pancreatic islets followed by destruction of insulin-producing β-cells. In NOD mice, early infiltration of leukocytes occurs at 3 weeks of age with intial infiltration of APC followed by $CD4^+$ and $CD8^+$ T cells (Solomon et al., 2004). Then, the β-cell destruction by $CD8^+$ T cells begins to show after 14 weeks (Qin et al., 2004a). Th17 and Th1 cells have been shown to participate in T1D (Bradshaw et al., 2009), multiple sclerosis (MS) (Hedegaard et al., 2008), and rheumatoid arthritis (RA) (Ziolkowska et al., 2000). Naïve CD4 T cells can be converted to Th-17 cells by IL-21 and TGF-β. In T1D patients, IL-1β and IL-6 cytokines produced by $CD14^+CD16^-$ and $CD14^+CD16^+$ monocytes stimulate the production of Th17 cells (Bradshaw et al., 2009). However, the role of Th17 cells has not yet fully elucidated. It has been reported that Th17 cells reverted to a Th1-like profile when transferred into NOD.Scid receipients and treatment with IL-17 neutralizing antibody did not prevent onset of T1D (Bending et al., 2009). NOD mice with depleted macrophages cannot develop T1D because macrophages are necessary for differentiation of β-cell-specific cytotoxic T cells. B cell-deficient NOD mice do not develop T1D because B cells play a role as antigen-presenting cells. The balance between self-reactive and regulatory T cells (T-regs) influences the development and progress of T1D. T-regs that express FoxP3 have a major role in tolerance in T1D as well as other autoimmune diseases (Chen et al., 2005) with no statistical difference between the number of T-regs in NOD and other autoimmune diseased mice (Waid et al., 2008). Adoptive transfer experiments were also used to elucidate the involvement of both $CD4^+$ and $CD8^+$ T cells in the etiology of T1D (Hartemann et al., 1999). The development of T1D is initiated by prolonged insulitis followed by induction of cytotoxic T cells (CTL) for β-cell destruction, which eventually leads to overt diabetes. Knockout Fas or FasL NOD as well as perforin-deficient NOD mice have a lower incidence of T1D, sugesting that apotosis of β-cells is due to the interaction of Fas ligand (FasL) on CTL and Fas on the surface of target cells (Qin et al., 2004a). Using different animal models, there are many efforts to investigate peptide and protein drugs as potential therapeutics for autoimmune diseases by tipping the balance from inflammatory to regulatory immune cells.

3. The role of cytokines and cellular mechanisms

It is not simple to correlate the role of each cytokine in the development of T1D; this is due to the influence of other factors in T1D development (Solomon et al., 2004). Diabetic patients have higher levels of IL-6, TNF-α, TNF-β, IL-1α, IL-1β, IFN-γ, IL-2, and IL-12, and the presence of Th1 cells is evident in T1D patients. Diabetogenic Th1 cells secrete IFN-γ, and the treatment of NOD mice with anti-IFN-γ mAb halts T1D development (Bradley et al., 1999; Hartemann et al., 1999). However, the disease progression does not correlate with the number of IFN-γ-secreting cells, suggesting that β-cell destruction may be independent of the IFN-γ-triggering mechanism. Thus, it supports the idea that $CD8^+$ CTL has an important role in the destruction of β-cells (Hartemann et al., 1999). In the early events of T1D

development, $CD4^+$ Th1 cells enter and accumulate in the pancreas quicker than Th2 cells. This is due to the presence of LFA-1 and $\alpha_V\beta_4$-integrin on Th1 cells and the upregulation of adhesion molecules such as ICAM-1, VCAM-1, PNAd, and MAdCAM-1 on pancreatic vessels (Bradley et al., 1999). There is a high presence of activated $CD4^+$ Th1 cells in islets and splenocytes of diabetic NOD mice; although Th2 cells are present in the islets, they are in a resting state (Hartemann et al., 1999). Adoptive transfer of Th1 T cells into neonatal NOD mice induced T1D, while transfer of Th2 cells did not, confirming the Th1 cells had a more important role than Th2 cells in the destruction of β-cells (Bradley, 1999; Pakala et al., 1997). The production of RNA for multiple chemokines such as lymphotactin suggests the recruitment of Th1 cells into the pancreas during the T1D progress. The increase in MCP-1 chemokine produced by Th1 cells infers the recruitment of macrophages and monocytes into the pancreas (Bradley et al., 1999).

It has been proposed that T-regs have a protective effect to maintain tolerance and that a deficiency in T-regs can lead to T1D in NOD mice (Chen et al., 2005; Lawson et al., 2008). Adoptive transfer of T-regs in NOD mice can suppress the development of T1D by controlling the aggressiveness of autoreactive effector T cells in the target organ (Buckner, 2010). However, T-regs have no influence in the initial priming and activation in the draining lymph nodes. It is interesting to find that there is no significant difference in the number of $CD4^+CD25^{hi}$ cells and the level of FoxP3 in patients with long-standing diabetes compared to control subjects. It was proposed that individuals with newly diagnosed T1D had reduced T-reg function compared to control individuals (Lawson et al., 2008). However, this proposal is still controversial because some studies found that there was no significant difference in the level of $CD4^+CD25^+FOXP3^+$ T-regs between T1D and normal individuals. It is possible that the function and persistence of generating T-regs are lacking in T1D patients (Buckner, 2010).

Th2 cells that secrete IL-4 are also important in suppressing T1D and treatment with IL-4 prevents the development of diabetes in NOD mice (Hartemann et al., 1999; Tisch et al., 1999). On the other hand, Pakala *et al.* have shown that co-transfer of a tenfold excess of Th2 over Th1 cells could not prevent diabetes, indicating that Th2 might not be the only cellular regulator of T1D (Pakala et al., 1997). Therefore, Th2 and T-regs may control the development of diabetes; this is reflected by the involvement of IL-4 and IL-10 in NOD mice (Hartemann et al., 1999; Solomon et al., 2004). Treatment of NOD mice with anti-IL-4 and anti-IL-10 mAbs causes a loss of β-cells along with generation of glucose intolerance (Calcinaro et al., 1997). An increase in the number of IL-4 secreting cells does not always indicate the presence of Th2-suppressive cells in the islets. Besides Th1, Th2 cells could transfer T1D in NOD.Scid mice, and Th2-mediated diabetes had a lower disease incidence and a longer prediabetic phase of the disease (Pakala et al., 1997). It has also been reported that transfer of Th2 cells into young NOD mice induced T1D (Dobbs et al., 2001). The lesions induced by Th2 cells were predominantly due to eosinophilic infiltration, islet necrosis and abscesses, and severe destruction of exo- and endocrine tissue.

4. Potential new therapies for T1D

Many potential therapies derived from peptides and proteins have been developed to control self-reactive T cells and induce the production of regulatory cells for generating immunotolerance (Masteller et al., 2002). Because incomplete or partial signaling in Signal 1 and/or Signal-2 can lead to regulatory responses, many of these theraputic agents are being developed to modulate these signals separately or simultaneously.

4.1 Activity of GAD Peptides to Suppress T1D:

In NOD mice, subpopulations of T cells recognize primary and secondary antigens (Sercarz et al., 1993; Tisch et al., 1999). In early stages of T1D, T cells are associated with the primary antigen causing damage to self-tissue (Lehmann et al., 1993). After the disease is in an advanced stage, T cells recognize secondary antigens; this process is called antigen spreading. B cells are effective promoters of antigenic spreading, and this is a way of increasing the immune response because a minimal number of antigenic peptide epitopes is sufficient to generate a strong immune response. Unfortunately, antigenic spreading could involve cross-reactivity with other antigens such as carboxypeptidase H, insulin, and HSP65, which are normally observed at the late stage of T1D.

Antigen-specific therapy could selectively inactivate autoreactive T cells without altering the remaining immune cells. Administering the whole GAD65 protein prevented the progress of T1D in older NOD mice that already developed insulitis (Tisch et al., 1998; Tisch et al., 1999). GAD65-treated mice had high levels of IL-4 and reduced levels of IFN-γ compared to control animals. It was proposed that prevention of T1D was due to the suppression of Th1 cells and upregulation of Th2 and T-reg cells (Awasthi et al., 2008; Tisch et al., 1998). A shift into the Th2 phenotype could be maintained for a long time, and the capacity of different epitopes to induce Th2 cells depended on the frequency of the clonotype T cells (Tisch et al., 1999).

$P_{208\text{-}217}$ peptide from the GAD sequence (Table 1) suppressed the progress of T1D in NOD mice due to deviation of T-cell differentiation from inflammatory to regulatory cells (Kim et al., 2004; McDevitt, 2004; Tisch et al., 1998). Different GAD65 epitopes are recognized by I-A^{g7} and I-$A^{g7.PD}$, and the selectivity of recognition is due to the specificity of MHC-II for a certain epitope as well as the specificity of TCR recognition of the MHC-peptide complex by a subpopulation of T cells (Chao et al., 1999). It was found that $P_{201\text{-}220}$ and $P_{231\text{-}250}$ peptides have high affinity for DQ8 while $P_{121\text{-}140}$ and $P_{471\text{-}490}$ peptides have poor binding properties to DQ8. The poor binding of peptides $P_{121\text{-}140}$ and $P_{471\text{-}490}$ to class II DQ8 is compensated by high efficiency presentation on B cells (Liu et al., 1999). It was proposed that antigenic peptides with low affinity may elicit a Th2 response to produce IL-4 and IL-5 rather than a Th1 response (Honeyman et al., 1995).

Adoptive transfer of T cells isolated from mice treated with $P_{206\text{-}220}$ and $P_{221\text{-}235}$ peptides from GAD65 (Table 1) suppressed T1D in the recepient mice, and these cells were peptide-specific regulatory cells that coproduced IL-4 and IL-10 (Chen et al., 2003). T1D suppression is presumably due to the inhibition of diabetogenic T cell migration into the lymph nodes. Transgenic mice (G286) expressing a T-cell receptor (TCR) specific for GAD65 epitope 286-300 ($P_{286\text{-}300}$) were used to investigate the role of GAD65-specific T cells in pathogenesis diabetes (Tarbell et al., 2002). During 30 weeks observation, 80% of the non-transgenic developed diabetes with severe insulitis while none of the transgenic mice (G286) developed diabetes with only 10% insulitis. The observed protection in G286 mice may be due to suppression of islet-specific cellular response and not to the absence of a pathogenic repertoire (Tarbell et al., 2002). In addition, $GAD_{206\text{-}220}$ was incorporated in the sequence of an immunoglobulin and tested for the treatment of T1D (Jain et al., 2008). It was shown that treatment with Ig-$GAD_{206\text{-}220}$ molecules leads to proliferation of pancreatic β-islet cells and restored normoglycemia in hyperglycemic mice.

Adoptive transfer of splenocytes from G286 failed to induce diabetes to NOD.Scid mice. Transfer of G286 splenocytes with diabetogenic cells resulted in a significant delay in diabetes transfer (McDevitt, 2003; Tarbell et al., 2002). The development of diabetes could be

Name	Sequence
GAD Peptides and GAD-BPI	**Suppression of Type 1 Diabetes**
$P_{208-217}$	EIAPVFVLLE
$P_{201-220}$	NTNMFTYEIAPVFVLLEYVT
$P_{231-250}$	PGGSGDGIFSPGGAISNMYA
$P_{121-140}$	YVVKSFDRSTKVIDFHYPNE
$P_{471-490}$	VDKCLELAEYLYNIIKNREG
$P_{206-220\ (g7)}$	TYELAPVFVLLEYVT
$P_{221-235\ (g7)}$	LKKMRFIIGWPGGSG
$P_{286-300\ (g7)}$	KKGAAAIGIGTDSVI
$P_{401-415\ (g7)}$	PLOCSALLVREEGLM
$P_{561-575\ (g7)}$	ISNPAATHQDIDFLI
$P_{331-345\ (g7.PD)}$	LVSATAGTTVYGAFD
$P_{456-470\ (g7.PD)}$	WLMWRAKGTTGFEAH
$P_{551-565\ (g7.PD)}$	GDKVNFFRMVISNPA
GAD-BPI	EIAPVFVLLE-(AcpGAcpGAcp)-ITDGEATDSG
PLP Peptide and PLP-BPI	**Suppression of EAE**
$PLP_{139-151}$	HSLGKWLGHPDKF
PLP-BPI	HSLGKWLGHPDKF-AcpGAcpGAcp-ITDGEATDSG
Ac-PLP-BPI-NH_2-2	Ac-HSLGKWLGHPDKF-$(AcpGAcpGAcp)_2$-ITDGEATDSG-NH_2
Ac-PLP-BPI-PEG6	Ac-HSLGKWLGHPDKF-$(C_2H_5O)_3$-G-$(C_2H_5O)_3$-ITDGEATDSG-NH_2
CII Peptides and CII-BPI	**Suppression of Rheumatoid Arthritis**
CII-1	PPGANGNPGPAGPPG
CII-BPI-1	Ac-PPGANGNPGPAGPPG-$(AcpGAcpGAcp)_2$-ITDGEATDSG-NH_2
CII-2	Ac-GEPGIAGFKGEQGPK-NH_2
CII-BPI-2	Ac-GEPGIAGFKGEQGPK-$(AcpGAcpGAcp)_2$-ITDGEATDSG-NH_2
CII-3	Ac-QYMRADEADSTLR-NH_2
CII-BPI-3	Ac-QYMRADEADSTLR-$(AcpGAcpGAcp)_2$-ITDGEATDSG-NH_2

Table 1. The sequence of different types of BPI molecules and antigenic peptides, which suppress EAE, RA, and T1D.

due to faster division or a longer lifespan of pathogenic cells compared to those of protective cells from G286 mice. The pathogenic cells overpowered the G286 cells over time. The *in vitro* activation of $CD4^+$ T cells from G286 mice increases the level of CTLA-4 compared to those from non-transgenic mice. The increase in negative costimulatory signal or upregulation of CTLA-4 could be one possible mechanism for diabetes protection observed in G286 mice (Tarbell et al., 2002).

4.2 Design of novel GAD-BPI for suppressing T1D

In T1D, recruitment of leukocytes into the islet of pancreas involves a multistep process, which includes rolling, firm adhesion, and migration across the endothelium (Huang et al., 2005). β_2-integrins on leukocytes such as $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (Mac-1), $\alpha_x\beta_2$ (p150/95), and $\alpha_D\beta_2$ have a major role in adhesion and transmigration of leukocytes (Yusuf-Makagiansar et al., 2000). As indicated previously, both Th1 and Th2 can increase the expression of adhesion molecules, including ligands for β_2-integrins (i.e., ICAM-1, -2, and -3), on the vascular endothelium in the pancreas to enhance islet infiltration (Bradley et al., 1999). Blocking of ICAM-1/LFA-1-mediated T-cell adhesion can suppress diabetes progression in NOD mice (Huang et al., 2005; Moriyama et al., 1996). Linear and cyclic LABL peptides derived from the I-domain of the α_L-subunit of LFA-1 bind to D1 of ICAM-1 and inhibit ICAM-1/LFA-1-mediated (a) homotypic T-cell adhesion, (b) heterotypic T-cell adhesion to epithelial and endothelial cell monolayers, and (c) mixed lymphocyte reaction (MLR) (Tibbetts et al., 1999; Tibbetts et al., 2000; Yusuf-Makagiansar et al., 2001a; Yusuf-Makagiansar et al., 2001b; Yusuf-Makagiansar et al., 2001c). cLABL peptide can block T-cell adhesion but not monocyte adhesion to islet microvascular endothelium (Huang et al., 2005). This is because T-cell adhesion is via $\alpha_L\beta_2$ while monocyte adhesion is via $\alpha_M\beta_2$ (Mac-1); thus, cLABL peptide can selectively differentiate between α_L and α_M binding to ICAM-1.

Siahaan *et al.* developed bifunctional peptide inhibitor (BPI) molecules, which were assembled by conjugating an antigenic peptide to a cell adhesion peptide (i.e., LABL) via a linker (Table 1). The estimated length of the linker was determined by measuring the distance between the N-terminal of LABL peptide and the C-terminal of the antigenic peptide when they were docked to domain-1 (D1) of ICAM-1 (Bella et al., 1998; Casasnovas et al., 1998; Xu et al., 2002) and MHC-II (Corper et al., 2000), respectively. In this case, both ICAM-1 and MHC-II receptors were modeled as if they were protruding from the cell membranes into the extracellular space. In the case of GAD-BPI, it is a conjugate between $P_{208\text{-}217}$ peptide and LABL peptide (Table 1) (Tibbetts et al., 1999; Tibbetts et al., 2000). Using the distance estimate, a combination of glycine (G) and amino caproic acid (Acp) was used to connect LABL to GAD peptides. In general, BPI molecules are effective in suppressing different autoimmune diseases; for example, GAD-BPI, PLP-BPI, and CII-BPI molecules (Table 1) have excellent efficacy to suppress T1D (Murray et al., 2007), EAE (Kobayashi et al., 2008; Kobayashi et al., 2007; Ridwan et al., 2010; Zhao et al., 2010), and RA, respectively.

The central hypothesis is that binding of BPI molecules simultaneously to MHC-II and ICAM-1 on the surface of APC blocks the immunological synapse formation at the interface of APC-T cells. In other words, BPI molecules inhibit the translocation of Signal-1 and Signal-2 to prevent the segregation of each signal. As a result, the naïve T cells differentiate to T-reg or Th2 cells, and this process suppresses the differentiation and proliferation of Th1 and Th17 cells. This process alters the immune cell balance from inflammatory to regulatory phenotypes in an antigenic-specific manner without affecting the general immune response.

This is in contrast to the mechanism of Signal-2 or cell adhesion inhibitors that normally suppress the general immune response.

GAD-BPI can effectively suppress T1D in NOD and NOD.Scid mice. Subcutaneous (s.c.) administrations of GAD-BPI (80 nmol/injection/day) to NOD mice on days 0 and 7 after T1D stimulation with GAD peptide in CFA on day 0 effectively suppressed insulitis in NOD mice compared to PBS-treated mice (Murray et al., 2007). The majority of the islets (about 83%) in GAD-BPI-treated mice were normal or without insulitis. In contrast, only a low population of islets (about 35%) in PBS-treated mice was normal and the remaining islets (65%) had moderate to severe insulitis. The splenocytes of GAD-BPI-treated NOD mice had high levels of IL-4 compared to those of PBS-treated animals, indicating that GAD-BPI treatment increased Th2 cell differentiation and proliferation (Murray et al., 2007).

T cells isolated from splenocytes of GAD-BPI- and PBS-treated NOD mice were adoptively transferred into NOD.Scid mice that had received diabetogenic cells from NOD mice (Gonzales et al., 2001). Only 28% of NOD.Scid mice treated with T cells from GAD-BPI-treated mice had hyperglycemia (*i.e.*, blood glucose ≥250 mg/dl) at weeks 7 or 12 while 83% of NOD.Scid mice treated with T cells from PBS-treated NOD mice had hyperglycemia (Murray et al., 2007). In parallel, lower insulitis was observed in NOD.Scid mice receiving T cells of GAD-BPI-treated NOD mice than in NOD.Scid mice receiving T cells from PBS-treated NOD mice (Murray et al., 2007). This result indicates that GAD-BPI alters the composition of CD4+ T cells from effector to regulatory or suppressor cells, which have protective effects in limiting the invasion and destruction of the islets.

To evaluate whether GAD-BPI could simultaneously bind to MHC-II and ICAM-1 on the surface of APC, a colocalization study was carried out using B cells isolated from the spleens of NOD female mice (6 weeks of age). GAD-BPI, unlinked GAD + LABL peptide, and PBS were added to the B cell suspension. After washing, the cells were incubated with antibodies to MHC-II (I-A^{g7}) and ICAM-1 with two different fluorescence labels. The decoration of B cell surface by each antibody that corresponds to the fluorescence label was observed with confocal microscopy. The effect of each molecule to colocalize MHC-II and ICAM-1 on the surface of B cells was determined by merging the two different fluorescence signals from anti-MHC-II and anti-ICAM-1 mAbs. The result showed that B cells treated with GAD-BPI had about 60% of colocalization signals from both antibodies compared to about 12% of colocalization signals from B cells treated with unlinked GAD and LABL peptides (Murray et al., 2007). To further evaluate the binding properties of GAD-BPI, antibody inhibition studies were carried out. GAD-BPI inhibited binding of anti-I-A^{g7} and anti-ICAM-1 mAbs to MHC-II and ICAM-1, respectively. In addition, GAD-BPI inhibited binding of both antibodies in a cooperative manner. Therefore, colocalization and antibody inhibition studies both suggest that GAD-BPI could bind simultaneously to MHC-II and ICAM-1 on the surface of B cells.

As mentioned above, the BPI type of molecule has been used to suppress RA and EAE in animal models. EAE is an animal model for multiple sclerosis (MS). PLP-BPI derivatives are able to suppress EAE better than the parent PLP peptide ($PLP_{139-151}$), indicating that the presence of LABL peptide alters the mechanisms of action of PLP-BPI compared to PLP peptide alone (Kobayashi et al., 2008; Kobayashi et al., 2007; Ridwan et al., 2010; Zhao et al., 2010). Cytokine studies indicated that PLP-BPI treatment induced the differentiation and proliferation of T-reg and Th2 cells and suppressed the proliferation of Th17 cells. Using the same concept, CII-BPI-1 and CII-BPI-2 molecules could effectively suppress rheumatoid arthritis better than the respective antigenic peptides (CII-1 and CII-2) in the collagen-

induced arthritis (CIA) mouse. The CII-BPI molecules could also lower the production of inflammatory cells. The results indicate the BPI molecules with appropriate antigenic peptides can effectively suppress autoimmune diseases.

4.3 Blocking ICAM-1/LFA-1 interactions

Interactions between adhesion molecules such as LFA-1 (CD11a/CD18) and ICAM-1 have an important role in insulitis in NOD mice and humans (Mysliwiec et al., 1999). ICAM-1/LFA-1 interactions are involved in leukocyte adhesion to vascular endothelium prior to infiltration of the islet. Individuals with overt diabetes have high levels of CD11a on their monocytes and lymphocytes (Mysliwiec et al., 1999). Administration of anti-LFA-1 and anti-ICAM-1 mAbs to female NOD mice at 2 weeks of age completely prevented the development of spontaneous diabetes and formation of insulitis until 30 weeks of age (Moriyama et al., 1996). In contrast, T1D was observed in 60% of mice treated with control mAbs, indicating that LFA-1/ICAM-1 interactions are critical for T-cell-mediated cellular responses as well as T-cell differentiation and extravasation. However, administration of these mAbs to NOD mice at 5 weeks of age did not induce this long-lasting protection, suggesting that the mechanism of action of these mAbs is in the early onset of the disease (Moriyama et al., 1996). Anti-ICAM-1 alone could significantly inhibit T1D development; however, it could not prevent significant infiltration of mononuclear cells into the islets. The conclusion is that blocking the ICAM-1/LFA-1 pathway can induce tolerance against pancreatic β-cells.

Adoptive transfer of spelenocytes from acutely diabetic mice into NOD.Scid mice followed by treatment with anti-LFA-1/anti-ICAM-1 and anti-CD8 mAbs prevented the development of T1D. In this case, 40% of the mAb-treated recipients became diabetic and exhibited moderate-to-severe insulitis around 12 to 35 weeks after transfer (Chowdhury et al., 2002). It is suggested that the mechanism of action of anti-ICAM-1 and anti-LFA-1 mAbs to induce tolerance is due to suppression of T-cell activation and not to clonal deletion or anergy. However, other mechanisms are plausible, such as the mAbs inhibiting the infiltration of autoreactive T cells that destroy β-cells.

4.4 Modulation of B7 and CD28 functions

Modulating costimulatory signals can induce immunotolerance and influence T-cell immunodominance; B7/CD28 is one of the costimulatory signals involved in the development of T1D in NOD mice (Lenschow et al., 1996; Salomon et al., 2001; Salomon et al., 2000). CD28-deficient and B7-1/B7-2-deficient NOD mice develop a more severe diabetes than control NOD mice. In CD28-deficient mice, the diabetes is due to a decrease in or absence of T-regs; transfer of T-regs from the control NOD mice to the CD28-deficient mice prevents the development of T1D (Salomon et al., 2000). B7-1, working together with self-antigen presentation by MHC-II on B cells, can induce T-cell activation during T1D development (Bour-Jordan et al., 2007). B7-1-mediated costimulatory signal promotes epitope-spreading in T1D in NOD mice. In contrast to the blocking of ICAM-1/LFA-1 interactions, inhibition of CD28/B7 interaction suppresses the production of Th2 cytokines (IL-4 and IL-5) (Labuda et al., 1998; Salomon et al., 1998). The homeostasis of T-reg is influenced by B7/CD28 signal. Although administration of CTLA4-Ig or anti-B7-2 mAbs to NOD mice did not prevent insulitis, it prevented the onset of diabetes in animals 2-4 weeks of age (Lenschow et al., 1995). In contrast, after the animals were past 10 weeks of age,

CTLA-4Ig and anti-B7-1 mAbs could not prevent T1D development. The results suggest that these molecules block the disease between insulitis and full-blown diabetes. Administration of anti-B7-1 mAb at the onset of insulitis resulted in a more severe infiltration and rapid onset of disease in both male and female NOD mice (Lenschow et al., 1995). T cells from anti-B7-1-treated mice were highly activated as reflected in the high levels of CD69 expression. It has been suggested that B7-1 costimulatory signal regulates the development of insulitis while B7-2 costimulatory signal is necessary for full development of T1D.

4.5 Modulation of CD3 function

T1D patients treated with anti-CD3 mAb called Otelixixumab have shown improvement in clinical trials (Keymeulen et al., 2010). This monoclonal antibody suppress the function of effector T cells and upregulate the regulatory T cells. In NOD mice, administration of anti-CD3 mAb prevented the onset of T1D by downregulating Th1 response and increasing T-reg response (Masteller et al., 2002). However, treatment of T1D with the anti-CD3 mAb, Otelixixiumab, can affect other non-self-reactive T cells; therefore along with another mAb known as Teplizumab, both antibodies were pulled from clinical trials due to their severe side effects caused by their general immuno-suppression. Thus, using antigen-specific therapeutics becomes a more attractive option for treating T1D. Because T cells recognize antigens presented on MHC molecules, treating with soluble TCR ligands is also an attractive option (Masteller et al., 2002). Treatment with a soluble peptide-MHC complex is similar to anti-TCR-CD3 mAb treatment, except that it target only those T cells that are self-reactive.

4.6 Modulation of cytokines

TNF-α can negatively or positively regulate the peripheral tolerance of T cells to β-islet antigens, depending on the age of NOD mice (Lee et al., 2005). Administration of TNF-α at a non-toxic dose to female NOD mice for 21 days after birth produced an early onset T1D (Lee et al., 2005). In this case, TNF-α activates macrophages and increases the expression of maturation markers such as CD86, CD40, CD54, MHC class II, and CD119 on $CD11c^{+}CD11b^{+}$ subpopulations. In contrast, administration of anti-TNF-α mAb for 21 days after birth prevented the development of T1D for a 1-year period. However, administration of anti-TNF-α to CD28-deficient and B7-1/B7-2-deficient NOD mice lead to more severe disease than the control NOD mice (Salomon et al., 2000). B7-deficient mice lack both CD28 and CTLA-4; CTLA-4 plays a critical role in regulating autoimmune disease. TNF-α mAb could not prevent T1D development when delivered at 3-4 weeks of age (Lee et al., 2005). Anti-TNF-α increases in $CD8\alpha^{+}CD11c^{+}$ dendritic cells (DC) compared to TNF-α. Because hyperglycemia has a direct effect on the expression of TNF-α, it is thought that TNF-α induces the maturation of DC and accelerates the migration of $CD\alpha^{+}CD11c^{+}$ DCs into the lymph nodes from the pancreas.

In response to GAD recognition as antigen, NOD mice increase the production of IFN-γ specifying the involvement Th1 cells, which are predominantly found in the islets regardless of mouse age. IFN-γ-producing cells are present even after the transfer of suppressor T cells (Hartemann et al., 1999). The number of IFN-γ-secreting cells does not correlate with NOD disease progression, which suggests that there is no absolute shift in the immune response leading to β-cell destruction. Although IL-4 secreting T cells are not detected, Th2-type cells are still present in the islet, but they are in a resting state. Therefore, an increase in the

number of IL-4 secreting cells does not indicate the presence of Th2 suppressive cells in the islets. It is suggested that the destruction of β-cells may depend on either an IFN-γ-independent triggering event or the number of IFN-γ-secreting cells that reach a certain threshold in islets (Hartemann et al., 1999).

4.7 Adjuvant treatment to suppress T1D

Several studies have shown that adjuvant-containing mycobacterial preparations such as CFA or BCG effectively prevent onset and recurrence of T1D in NOD mice (Qin et al., 2004b; Qin et al., 1997). Adoptive transfer studies showed that BCG immunization of diabetic NOD mice impaired the ability of splenocytes to transfer diabetes. Histological examination indicated that splenocytes from BCG-immunized diabetic mice induced less insulitis in recipient NOD.Scid mice than did splenocytes from the saline-treated group. BCG immunization significantly decreased the proportions of CD4, CD8, and $CD45RB^{low}$ T cells by inducing apoptosis and increased CD11b positive macrophages in time-course studies (Qin et al., 2004b). In comparison to control, the total number of TNF-α- or IFN-γ-positive splenocytes and IL-4 expression were significantly increased in BCG-immunized mice. Mechanistically, *in vivo* administration of anti-IFN-γ mAb reversed the immune regulatory effect of BCG to down-regulate $CD45RB^{low}$ CD4 T cells and increase apoptosis of CD4 T cells (Qin et al., 2004b). BCG immunization also significantly increased Fas^{high}, FasL, and TNFR expression on CD4 and CD8 T cells. Furthermore, administration of anti-FasL or anti-TNFR1 mAb resulted in a significant decreased in T-cell apoptosis and increased in T-cell proliferative response. This suggests that BCG immunization down-regulates destructive autoimmunity by TNF-α-/IFN-γ-induced apoptosis of diabetogenic T cells through both Fas-FasL- and TNFR-TNF-α-mediated signaling pathways (Qin et al., 2004b).

5. Conclusions

The use of peptides and proteins for treating autoimmune diseases, including T1D, has been increasing steadily. Other than insulin, many monoclonal antibodies are being investigated as disease-modifying agents to treat T1D by tipping the balance of immune cells from effector to suppressor or regulatory cells and preventing further damage to β-cells. Recently, antigenic peptides and bifunctional peptide inhibitors (BPI) have been explored for altering the differentiation and proliferation of T cells to regulatory cells to prevent the development of diabetes. These molecules are developed to affect immune cells in an antigenic-specific manner; thus, they do not suppress the general immune response for fighting infections. However, further studies on the mechanisms of action of antigenic peptides and BPI molecules are still needed for improving the therapeutic index.

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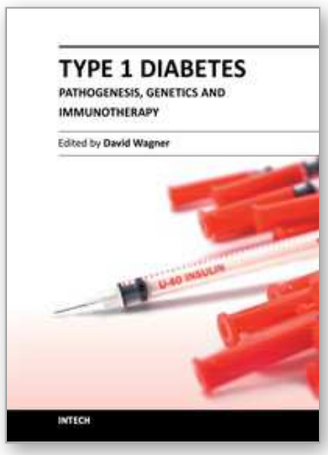

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This book is a compilation of reviews about the pathogenesis of Type 1 Diabetes. T1D is a classic autoimmune disease. Genetic factors are clearly determinant but cannot explain the rapid, even overwhelming expanse of this disease. Understanding etiology 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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