Clinical and Developmental Immunology

Type 1 Diabetes Immunological Tolerance and Immunotherapy

Guest Editors: Aziz Alami Chentoufi, Vincent Geenen, Nick Giannokakis, and Abdelaziz Amrani

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Editorial Board

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Editorial

Type 1 Diabetes Immunological Tolerance and Immunotherapy

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Type 1 diabetes (T1D) is a debilitating chronic autoimmune disease (AID) for which there is currently no preventive treatment or therapeutic strategies. Patients with T1D are also at higher risk for other autoimmune disease such as celiac disease. In genetically susceptible individuals, T1D is associated with the generation and activation of autoreactive $CD4^+$ and $CD8^+$ T cells that infiltrate the pancreas and selectively destroy the insulin-producing β-cells in the islets. The impairment of T-cell tolerance in T1D has been reported at many levels including abnormal self-antigen presentation in the thymus and periphery, autoreactive T-cell resistance to apoptosis, unbalanced immunoregulatory T-cell function, and deregulation of Th1/Th2/Th17 axes. Despite the identification of T1D-associated autoantigens and their derived $CD4^+$ and $CD8^+$ T-cell epitopes, numerous antigen-specific immunoregulatory therapies have failed when evaluated for their efficiency in the prevention and treatment of T1D. The development of antigen-specific tolerization approaches to treat or to prevent T1D would bring exceptionally high economic and health benefits. In this special issue, we accepted original research and review articles highlighting the recent advances in T1D-associated immunological tolerance mechanisms and potential immunotherapeutic strategies.

In the first paper of this special issue, A. A. Chentoufi and V. Geenen have addressed the role of thymic self-antigens expression in the control of self-reactive and regulatory T-cells generation. Also, the authors proposed the concept of negative/tolerogenic self-vaccination to modulate diabetogenic autoimmune responses. In the second paper, G. F. Hoyne described the molecular mechanisms that regulate peripheral immune responses that control organ-specific autoimmunity and highlighted the role of a range of E3 ubiquitin ligases and signaling pathways that influence the development of effector T-cell responses and T1D development. The third paper by A. A. Chentoufi et al. highlighted the recent findings and controversies regarding the tolerogenic properties of interleukin-2 (IL-2) mediated through naturally occurring regulatory T cells and discussed the link between the immunomodulatory role of IL-2 and the pathogenesis of T1D.

In the fourth paper of this special issue, the group of A. Amrani eloquently investigated the abnormal functionality of dendritic cells (DCs) in nonobese diabetic (NOD) mice through the expression of IRF4 and IRF8 genes. The results showed an upregulation of IRF4, but not IRF8, expression in $CD11c^+$ splenic DCs of NOD as compared to BALB/c mice and correlated with the increased levels of $CD4^+CD8\alpha^-$ DCs suggesting that IRF4 may be involved in abnormal DC functions in diabetes in NOD mice. In the fifth paper, the group of G. A. Passos investigated the transcriptional modulation of immune reactivity genes occurring through thymocytes maturation into peripheral autoreactive T lymphocytes. The transcriptome of thymocytes and peripheral $CD3^+$ T lymphocytes from prediabetic or diabetic mice analyzed through microarray hybridizations identified 2,771 differentially expressed genes. The analysis of the transcriptional activity of thymocytes developing into peripheral T cells revealed sequential participation of genes involved in $CD4^+/CD8^+$ T cell differentiation, tolerance

induction by regulatory T cells, and apoptosis soon after T-cell activation, while the emergence of T1D coincided with the expression of cytotoxicity and inflammatory response genes by peripheral T lymphocytes. The sixth paper by M. Delmastro and J. Piganelli described the importance of reactive oxygen species/oxidative stress as well as potential for redox modulation in the context of T1D.

In the following selected research article papers, P. Alard and Y. Zhang groups described novel therapeutic approaches that prevent and/or treat T1D in mouse model. Indeed, P. Alard group have proposed to utilize the microorganism ability to induce tolerogenic DCs to abrogate the proinflammatory process and prevent diabetes development. They have shown that LTA-treated DCs produced much more IL-12 than IL-10 and accelerated diabetes development when transferred into NOD mice. In contrast, stimulation of NOD DCs with L. Casei favored the production of IL-10 over IL-12, and their transfer decreased disease incidence in an IL-10-dependent manner. Similarly, Y. Zhang group have shown that oral administration of the CTB-Ins-GFP protein induced tolerance, delayed the development of diabetic symptoms, and suppressed diabetes onset in NOD mice. Furthermore, CTB-Ins-GFP protein increased the numbers of $CD4^+CD25^+Foxp3^+$ regulatory T cells in peripheral lymph tissues and affected the biological activity of spleen cells.

In the last series of papers, we have selected review papers describing the antigen-based therapeutic approaches. S. Culina et al. have discussed the different antigen formulations that have been considered for T1D treatment/prevention, such as proteins or peptides, either in their native form or modified ad hoc, DNA plasmids, and cell-based agents. Also, they highlighted the differences between mice model and human data that should be taken into account. Critical parameters such as administration route, dosing, and interval remain largely empirical and need to be further dissected. R. Mallone et al. described T-cell recognition of autoantigens in human T1D and their relevance to the clinical perspective. Finally, B. Philips et al. highlighted the preclinical successes and the excitement generated by phase I trials while offering alternative possibilities and new translational avenues that can be explored given the very recent disappointment in leading agents in more advanced clinical trials.

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Review Article

Thymic Self-Antigen Expression for the Design of a Negative/Tolerogenic Self-Vaccine against Type 1 Diabetes

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Before being able to react against infectious non-self-antigens, the immune system has to be educated in the recognition and tolerance of neuroendocrine proteins, and this critical process essentially takes place in the thymus. The development of the autoimmune diabetogenic response results from a thymus dysfunction in programming central self-tolerance to pancreatic insulin-secreting islet β cells, leading to the breakdown of immune homeostasis with an enrichment of islet β cell reactive effector T cells and a deficiency of β cell-specific natural regulatory T cells (nTreg) in the peripheral T-lymphocyte repertoire. Insulin-like growth factor 2 (IGF-2) is the dominant member of the insulin family expressed during fetal life by the thymic epithelium under the control of the autoimmune regulator (*AIRE*) gene/protein. Based on the close homology and cross-tolerance between insulin, the primary T1D autoantigen, and IGF-2, the dominant self-antigen of the insulin family, a novel type of vaccination, so-called "negative/tolerogenic self-vaccination", is currently developed for prevention and cure of T1D. If this approach were found to be effective for reprogramming immunological tolerance in T1D, it could pave the way for the design of negative self-vaccines against autoimmune endocrine diseases, as well as other organ-specific autoimmune diseases.

1. Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder associated, in genetically susceptible individuals, with the generation and activation of autoreactive T cells recognizing pancreatic β cell autoantigens. Autoreactive CD4+ and CD8+ T cells infiltrate pancreatic Langerhans' islets (insulitis) and selectively destroy the insulin-producing β cells in these structures. This destruction occurs silently and progressively and, in most cases, remains undetected for many years. By the time the first clinical T1D symptoms become apparent, nearly 80% of the patients' β cells have been destroyed and there is little hope for curing the disease. In humans, T1D incidence peaks around 10–14 years of age. It is estimated that T1D affects about 30 million people worldwide (approximately 10–15% of all patients with diabetes mellitus).

Major T1D autoantigens include (pro)insulin, the 65-kDa isoform of glutamic acid decarboxylase (GAD65), the islet tyrosine phosphatase IA-2, and the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). These pancreatic β-target autoantigens have been identified in both humans and nonobese diabetic (NOD) mice [1]. The generation of autoreactivity to islet β cells is the consequence of multiple various genetic defects that have an impact on fundamental immunological processes such as central and peripheral tolerance. In general, potentially autoreactive lymphocytes must encounter their cognate autoantigen during differentiation in order for the host to develop tolerance to self-antigens. This encounter may occur within the thymus or upon release into the periphery [2, 3]. Central self-tolerance occurs during T-cell differentiation in the thymus and involves deletion (negative selection) of self-reactive T cells during T-cell development in the thymus [4]. This process occurs to eliminate T cells that recognize ubiquitous or blood-borne self-antigens. T cells which recognize tissue-specific antigens (TSAs), however, were long-thought to be

tolerized in the periphery [3]. Until recently, it was widely assumed that such TSAs are excluded from the thymus, precluding central self-tolerance. To explain tolerance to TSAs, numerous peripheral tolerance mechanisms have been suggested, including ignorance and immunoregulation. At the same time it has also been argued that TSAs may reach the thymus in significant quantities and thereby induce negative selection *in situ*, especially of high-affinity T cells. This idea became more plausible when others and we found that many TSAs are expressed by rare medullary thymic epithelial cells (mTEC) [5–7]. Findings such as these challenged historical assumptions about tolerance to TSAs and intense investigations, in both mice and humans, have elucidated the identity of TSA-expressing cells in the thymus, as well as their role in the process of central self-tolerance. Others and we showed that genetically determined low levels of TSAs (i.e., insulin) expression in the thymus is associated with the generation of high numbers of autoreactive T cells in the periphery and predisposition to autoimmune disease [5–7]. The ectopic expression of many tissue-restricted antigens in the thymus is controlled by the autoimmune regulator (*AIRE*). *AIRE* is a 54.5-kDa protein with a nuclear localization and several potential DNA-binding and protein interaction domains [8]. $AIRE^{-/-}$ mice exhibit a defined profile of autoimmune diseases including T1D. It is notable that all these diseases arise due to a lack of *AIRE* expression in stromal cells of the thymus. Furthermore, *AIRE*-deficient mTEC showed a specific reduction in promiscuous transcription of genes encoding peripheral antigens, demonstrating the importance of thymus-dependent tolerance in controlling autoimmunity [9].

Although these data are appealing, they do not prove that thymic expression of TSAs automatically induces central self-tolerance to peripheral tissues. Other potential mechanisms for developing tolerance to TSAs also include thymic generation of self-antigen-specific Foxp3+CD4+CD25+ regulatory T cells or Treg ("dominant" central self-tolerance). In the last 25 years, Foxp3+CD4+CD25+ Treg have been identified as key effectors in the maintenance of peripheral tolerance. It has also been shown that the promiscuous expression of a neo-self-antigen in TEC may be involved in the selective induction and/or expansion of self-antigen-specific Foxp3+CD4+CD25+ Treg thymic precursors as early as the double-positive stage [10]. Importantly, this selection of self-antigen-specific Foxp3+ Treg is mediated by *AIRE*+ mTEC [11]. In this paper, we highlight the recent progress in the field of central and peripheral self-tolerance exemplified by T1D pathogenesis as consequence of a deleterious process in T-cell tolerance installment.

2. T1D-Associated Autoantigens and Autoimmunity

An important step to demonstrate the autoimmune nature of T1D was the discovery of autoantibodies directed against Langerhans' islet cells [12]. Major autoantigens include (pro)insulin itself, GAD65 and IGRP. The islet-specific cation efflux transporter ZnT8 (Slc30A8) and chromogranin A have been also recently reported as important autoantigens in T1D [13, 14]. However, among these autoantigens, only antigenic epitopes derived from (pro)insulin are specific of pancreatic islet β cells. Furthermore, there is now ample evidence that autoimmunity to (pro)insulin is central to autoimmune diabetes pathogenesis both in non-obese diabetic (NOD) mice and in humans [15, 16]. Clinically, detection of anti-insulin, anti-GAD65, and/or anti-IA-2 autoantibodies are very reliable markers of the autoimmune response targeting β cells. The majority of the children with recent-onset of T1D (more than 90%) have antibodies against one or several of these autoantigens in their serum. Their high predictive value is also established since they can be detected several years before the clinical signs of insulin deficiency. The predictive value of autoantibodies against several autoantigens is higher than elevated titers of one single autoantibody. The combination of autoantibodies with susceptible genetic alleles of the major histocompatibility (MHC) class II locus further increases this predictive value. Such prediction is very useful for clinical studies targeted at T1D prevention given the relatively low incidence of this disease [17, 18]. However, the pathogenic significance of T1D-related autoantibodies is rather low, if not absent [19], and the principal effectors of β cell autoimmune destruction are CD4+ and CD8+ T lymphocytes [20]. Investigation of specific T-cell responses in T1D patients is however challenging because of the very low frequency in the peripheral T-cell pool of autoreactive T cells specific for epitopes derived from (pro)insulin, GAD65, or IA-2. However, the development of sensitive and specific techniques, such as enzyme-linked immunosorbant spot assays (ELISpot) and multimers of class I/II HLA molecules complexed with T1D-related epitopes, has already provided very significant data that further document the importance of T-cell-mediated mechanisms in T1D pathogenesis [21, 22].

3. The Central Role of the Thymus in Central Self-Tolerance of Neuroendocrine Proteins and the Nature of "Neuroendocrine Self"

A major question when addressing the pathogenesis of organ-specific autoimmunity such as T1D is the origin of the self-reactive T cells that are directed against target antigens of endocrine cells. Among all lymphoid structures, the thymus is an organ that emerged some 500 million years ago, concomitantly or very shortly after recombinase-dependent adaptive immunity, with a specific function in orchestrating central immunological self-tolerance. The thymus is not an endocrine gland, but it crucially stands at the intersection between the immune and neuroendocrine systems. In this organ that is responsible for thymopoiesis, that is, generation of naïve and competent T lymphocytes, the neuroendocrine system regulates the process of T-cell differentiation from very early stages, while in parallel naïve T lymphocytes are educated to recognize and tolerate neuroendocrine gene/protein families [7, 23, 24]. Contrary to the popular opinion, the thymus continues to function throughout life and plays a fundamental role in the recovery of a competent T-cell

repertoire after intensive chemotherapy or during highly active antiretroviral chemotherapy in human immunodeficiency virus infection [25, 26]. The integrity of the somatotrope growth hormone/IGF-1 axis is known to be important for the maintenance of thymus function in adult life [27].

The thymus is the central lymphoid organ responsible for the maturation and differentiation of bone-marrow-derived thymocytes. The random generation of the T-cell repertoire, including autoreactive T cells, is regulated in the thymus by mechanisms of central self-tolerance. Anatomically, the thymus is divided into subcapsular, cortical, and medullary compartments. The stromal cells include a variety of bone-marrow-derived professional antigen-presenting cells (dendritic cells [DC], macrophages, and B cells) and endoderm-derived cortical (c) TEC and mTEC [28]. A striking morphologic feature of the medulla is the presence of Hassall's corpuscles, which consist of concentric whorls of stratified keratinizing epithelium and share antigenic properties with ectodermic epithelium [29]. The thymus constitutes the central arm of immunological self-tolerance by two essential mechanisms that are intimately associated and paradoxically mediated by the same thymic self-antigens: (a) negative selection of self-reactive T cells issued from the random recombination of TCR genes ("recessive" self-tolerance) and (b) generation of self-antigen-specific nTreg that are able to inactivate in periphery self-reactive T cells having escaped intrathymic negative selection ("dominant" self-tolerance) [30, 31].

Several groups and ourselves have demonstrated that TEC from different species constitute a site for the promiscuous transcription of a great number of genes encoding tissue-restricted antigens or belonging to neuroendocrine families, such as the neurohypophysial family, tachykinins, neurotensins, somatostatins, atrial natriuretic peptides, and the insulin family. This demonstration has radically changed our common understanding of the pathogenesis of organ-specific autoimmune endocrine diseases such as T1D. From the investigation of intrathymic expression of neuroendocrine-related self-peptide precursor genes, a series of properties can be derived that define the nature of the "neuroendocrine self". First, thymic neuroendocrine self-antigens usually correspond to peptide sequences that have been highly conserved throughout the evolution of their related family. Second, a hierarchy characterizes their expression pattern in the thymus. In the neurohypophysial family, oxytocin (OT) is the dominant peptide synthesized by TEC from different species. The binding of OT to its cognate receptor expressed by pre-T cells induces a very rapid phosphorylation of focal adhesion-related kinases. This event could play a major role in promoting establishment of synapses between immature T lymphocytes and TEC, as well as with macrophages and DC. All the genes of the insulin family are expressed in the thymus according to a precise hierarchy during fetal life: *IGF2* (cTEC and mTEC) >*IGF1*>*INS* (a few subsets of mTEC). This hierarchical pattern is meaningful because the strength of self-tolerance to a protein is proportional to its intrathymic concentration [32]. Third, neuroendocrine precursors are not processed according to the classic model of neurosecretion, but they undergo an antigenic processing for presentation by—or in association with—MHC proteins [33]. Fourth, most of neuroendocrine self-antigens are transcribed in the thymic epithelium under the control of the autoimmune regulator gene *AIRE* (see below). Fifth, intrathymic *OT* transcription precedes OT and vasopressin (VP) expression in hypothalamic magnocellular neurons. Finally, epigenetic regulation of intrathymic gene expression is strongly suggested by the loss of *IGF2* parental imprinting in human mTEC [34, 35].

This hierarchy in the organization of the thymic repertoire of neuroendocrine self-antigens is also significant from an evolutionary point of view. Since a series of essential and physiological functions had been established before the appearance of adaptive immunity in cartilaginous fishes, they had to be protected from the risk of autotoxicity inherent to this type of immunity. For example, OT is a "bonding" peptide that has been implicated at different steps of the reproductive process and thus, for species preservation, OT possibly had to be protected to a greater degree than VP, which controls water metabolism and vascular tone. Along the same line of reasoning, IGF-2 as a major factor in fetal development possibly had to be more protected than insulin, which "only" regulates glucose homeostasis. Nevertheless, because of their close homology, thymic neuroendocrine self-antigens may promote cross-tolerance to other members of their respective families. This is supported by the weaker tolerance to insulin of $Igf2^{-/-}$ mice when compared to wild-type mice [36]. Further insight into the relative influence of the central arm of immunological self-tolerance will be gained through the generation of mice with TEC-specific *Igf2* deletion, currently under development in our laboratory.

4. The Central Role of a Thymus Dysfunction in T1D Pathogenesis (Figure 1)

As hypothesized by Burnet in 1973, the pathogenesis of autoimmune diseases may first depend on the appearance of "forbidden" self-reactive clones in the peripheral T-cell repertoire [37]. In 1992, a defect in the process of intrathymic T-cell education to recognize and to tolerate OT was hypothesized to play a pivotal role in the development of hypothalamus-specific autoimmunity leading to "idiopathic" central diabetes insipidus [38]. The progressive increase in the degree of immune diversity and complexity may explain why failures in self-tolerance are increasingly detected during evolution with most such failures occurring in the human species. Since the thymus is the primary site for induction of self-tolerance, a thorough investigation of the mechanisms responsible for a breakdown of thymus-dependent tolerance should provide the scientific community with important keys to understand the mechanisms underlying the development of autoimmune responses.

A number of abnormalities of thymic morphology and cytoarchitecture have been described in T1D. Central tolerance and apoptosis of self-reactive T cells are defective in the thymus of NOD mouse [39, 40]. Transcription of insulin-related genes (*Ins*, *Igf1*, and *Igf2*) has been analyzed in

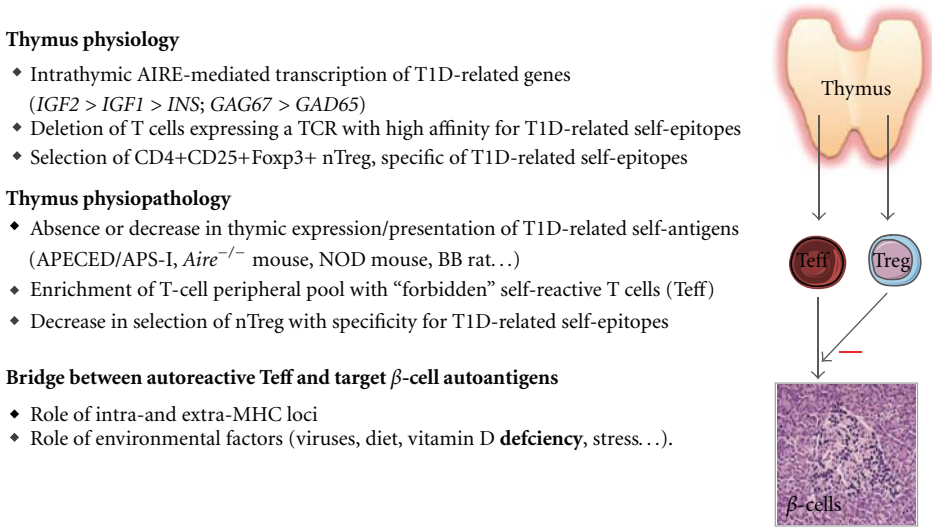

Figure 1: Thymus physiopathology and T1D development. Throughout life, the thymus selects self-tolerant and competent T cells against non-self-antigens and generates self-specific nTreg. Under control by *AIRE* for most of them, thymic epithelium transcribes genes encoding T1D-related antigens, as well as other neuroendocrine-related and tissue-restricted antigens. Absence or decrease in presentation of thymic T1D-related antigens (as observed in different animal models of autoimmune diabetes) conducts to the enrichment of the peripheral T-cell pool with "forbidden" self-reactive T cells (Teff) bearing a TCR directed against T1D-related epitopes, while thymic generation of specific nTreg is severely impaired. Combination of these two events is responsible for the breakdown of central self-tolerance to islet β cells. Both genetic and environmental factors are involved in the establishment of a molecular bridge between anti-β cell self-reactive Teff and islet β cell autoantigens. Once this bridge is formed, the autoimmune pathogenic response is triggered and leads to a progressive destruction of the β cell mass.

the thymus of diabetes-resistant (BBDR) and diabetes-prone (BBDP) rats, another model of T1D. *Ins* and *Igf1* transcripts were detected in all thymi from BBDP and BBDR rats. *Igf2* transcripts were also present in the thymus from all BBDR rats, but were not detected in the thymus from more than 80% of BBDP rats, in close concordance with the incidence (86%) of autoimmune diabetes in those rats. This defect in *Igf2* transcription in BBDP thymus could also explain both their lymphopenia (including CD8+ T cells and RT6+ Treg) and the absence of central self-tolerance to insulin-secreting islet β cells [41, 42]. As already mentioned, we have shown that susceptibility to autoimmune diabetes is correlated with the level of *Ins2* transcription in the mouse thymus [6]. Breeding of $Ins2^{-/-}$ mice onto the NOD background markedly accelerated insulitis and onset of diabetes [43]. In contrast, insulitis and diabetes were considerably reduced in $Ins1^{-/-}$ congenic NOD mice [44]. These observations are explained by the dominance of *Ins2* encoding proinsulin in the murine thymus, while *Ins1* encodes proinsulin in islet β cells. In the human species, *INS* transcripts were measured at lower levels in the fetal thymus with short class I VNTR (variable number of tandem repeats) alleles, a genetic trait of T1D susceptibility as discussed above [45, 46]. The contribution of thymic insulin in mediating central self-tolerance to islet β cells was definitively demonstrated by the rapid onset of autoimmune diabetes following thymus-specific deletion of *Ins1* and *Ins2* through an elegant transgenic construction in mice [47].

The identification of *AIRE* led to further demonstration that a thymus dysfunction plays a crucial role in the pathogenesis of organ-specific autoimmune diseases [48, 49]. Loss-of-function *AIRE* single mutations are responsible for a very rare autosomal recessive disease named autoimmune polyendocrinopathy, candidiasis, and ectodermal dystrophy (APECED), or autoimmune polyendocrine syndrome type 1 (APS-1). This syndrome develops in early childhood and is characterized by multiorgan autoimmunity and insufficiency of several endocrine glands such as parathyroids, adrenal cortex, and gonads. *AIRE* expression is maximal in the thymus, mainly in mTEC, but is absent in TEC of NOD mice [50]. Depending on their genetic background, $AIRE^{-/-}$ mice exhibit several signs of peripheral autoimmunity, which are associated with a significant decrease in thymic transcription of neuroendocrine genes (including *Ot, Npy, Igf2,* and *Ins2*), as well as other TSAs [9, 51, 52]. However, as shown for GAD67, AIRE does not control the intrathymic expression of all TSAs. By different aspects, *AIRE*-regulated transcription in the thymus differs from expression of these antigens in eutopic tissues. For example, loss of *Igf2* imprinting with biallelic transcription has been observed in mTEC [53]. The same study has shown that many TSAs in mTEC are clustered in their chromosomal location, including *AIRE*-dependent and *AIRE*-independent gene targets [53]. Extrathymic *AIRE* expression has been evidenced in secondary lymphoid organs where *AIRE* also controls the expression of TSA genes that are different from those regulated by *AIRE* in mTEC [54]. The precise molecular mechanisms by which *AIRE*

controls transcription of TSA are not completely elucidated [55, 56]. However, it more and more appears that *AIRE* uses one of its two zinc finger plant homeodomains for binding to nonmethylated histone H3K4 and activating gene expression, which establishes a relationship between chromatin regulation and tissue-specific central tolerance [57, 58]. Finally, there is mounting evidence that *AIRE* is closely implicated in mTEC differentiation (reviewed in [59]).

In collaboration with Didier Hober (Laboratory of Virology EA3610, CHRU Lille, France), we have shown that coxsackievirus B4 (CVB4) is capable to directly infect the epithelial and lymphoid compartments of the human and murine thymus and to induce a severe thymus dysfunction with massive pre-T-cell depletion and marked upregulation of MHC class I expression by TEC and by CD4+ CD8+ immature thymic T cells [60, 61]. Interestingly, outbred mice can be infected with CVB4 following an oral inoculation, which results in systemic spreading of viral RNA and a prolonged detection of CVB4 RNA in thymus, spleen, and blood up to 70 days postinoculation [62]. These findings suggest that, in addition to a role for CVB4 in breaking peripheral tolerance to islet β cells, the severe infection of the thymus by CVB4 could enhance its virulence through induction of central tolerance to the virus, and a still putative breakdown in central self-tolerance to islet β cells.

5. Thymic Control of Naturally Occurring CD4+CD25+ Treg Generation in T1D

Naturally occurring CD4+CD25+ Treg have emerged as a dominant T-cell population mediating peripheral tolerance. They are potent suppressors of organ-specific autoimmunity (T1D, inflammatory bowel disease, and gastritis), allograft rejection, graft versus host disease and control immunity to asthma and infectious agents such as parasites and viruses [63–68]. Depletion of CD4+CD25+ Treg cells leads to the development of various autoimmune diseases in genetically susceptible animals. In contrast, the adoptive transfer of CD4+CD25+ Treg in many animal models has been shown efficient in controlling organ-specific autoimmunity [69].

Multiple reports have implicated Treg in T1D prevention. Treg depletion or interference with B7/CD28 pathway in NOD mice has been shown to accelerate T1D onset [70, 71]. In aged NOD mice, autoimmune diabetes resistance has been correlated with the expansion of CD25+ CD4+ T cells with regulatory activity within inflamed pancreatic lymph nodes [72]. Recently, Chen et al. reported that Foxp3-deficient NOD mice, which are deficient in Treg, display an increased incidence and earlier onset of T1D compared with normal NOD mice, strongly implying a role for Treg in the control of T1D pathogenesis [73]. Obviously, alterations in the frequency and/or function of Treg are associated with various autoimmune diseases, including T1D [74, 75]. The quantification of naturally occurring Treg is hampered by a lack of unique surface markers. Perhaps not surprisingly, there is some controversy in the literature regarding the frequency of Treg in NOD mice, as determined by the identification of CD4+CD25 high cells in the spleen and lymphoid organs. More importantly, however, it appears that NOD mice harbor Treg that prevent disease development at an early age, but lose their functional capacity later on in life, thereby allowing pathogenic effector T cells to attack pancreatic islets [76, 77]. Similarly, the numbers of Foxp3+ Treg in the peripheral blood of T1D patients do not appear to be significantly different from healthy control subjects but there is evidence for functional defects in their suppressive capacity [78–80]. Therefore, there has been much interest in the potential use of adoptive transfer of *in vitro* expanded islet-specific Treg as a way of suppressing autoimmunity in T1D patients, perhaps preserving islet-cell function in newly diagnosed T1D.

The role of thymic self-antigens expression in the negative selection of self-reactive T cells has been well described by others and us. Many reports have shown that thymic mTEC and subsets of DC also play a critical role in the generation of antigen-specific CD4+CD25+Foxp3+ Treg [10]. Moreover, mTEC forming Hassall's corpuscles in the human thymus express thymic stromal lymphopoietin (TSLP), a molecule that can instruct thymic and peripheral DC to drive thymocyte CD4+CD8−CD25− differentiation into naturally occurring regulatory CD4+CD25+ T-cells [81, 82]. Importantly, Aschenbrenner et al. have shown that this selection of Foxp3+ Treg specific for self-antigen is mediated by *AIRE*+ mTEC, and the "routing" of mTEC-derived self-antigens (i.e., direct presentation by mTEC or via transfer to DC) may determine whether specific thymocytes are deleted or enter the Treg lineage. Indeed, this study proposed that mTEC-thymocyte interactions shape the Treg compartment and dominant tolerance, whereas hematopoietic antigen-presenting cells (mainly thymic DC) mediate recessive deletional tolerance [11]. Taken together, these results show that mTEC forming Hassall's corpuscles play an important role in central and peripheral T-cell tolerance.

6. Genetic Factors in T1D Pathogenesis

T1D is the polygenic autoimmune disease that has been most intensively investigated at the genetic level. Knowledge of genetic loci that determine susceptibility to T1D is important for identifying pathogenic pathways, for improved prediction of the disease and for selection of potential pharmacological targets. The balance between susceptibility and resistance alleles determines individual predisposition to T1D. The most significant part (±50%) of genetic susceptibility to T1D resides in the HLA class II region on chromosome 6p21, as recognized by pioneering studies [83, 84]. The major susceptibility in this region is conferred by the specific HLA class II haplotypes DR4-*DQA1*0301-DQB1*0302* (DQ8 molecule) and DR3-*DQA1*0501-DQB1*0201* (DQ2 molecule). In contrast, the allele *DQB1*0602* (DQ6 molecule) confers dominant protection against T1D. Theoretically, HLA class I proteins present antigens that are processed from endogenous proteins to CD8+ T cells, while HLA class II proteins present antigens issued from exogenous proteins to CD4+ T cells. Consequently, it has long been difficult to explain

the relationship between insulin and T1D genetic susceptibility located in the HLA class II region. This problem was solved when very elegant crystallographic studies showed that a dominant insulin epitope (InsB9-23) is presented in the binding pocket of DQ8 and DQ2 proteins [85]. Since then, a comprehensive scan of the whole HLA region, combined with potent statistical methods, has linked T1D susceptibility to HLA class I genes *HLA-B* and *HLA-A* [86].

Other genetic linkage and association studies have identified a second locus for T1D susceptibility that corresponds to a high polymorphic minisatellite constituted by a variable number of tandem repeats (VNTR) [87, 88]. This VNTR is embedded on chromosome 11p15 and controls the transcription of the insulin (*INS*) and insulin-like growth factor 2 (*IGF2*) genes downstream. Short VNTR class I alleles contain 20–63 repeats of 14-15 base pairs, while intermediate class II and long class III alleles include 64–139 and 140–210 repeats, respectively. VNTR class I alleles are associated with TID susceptibility, whereas class III alleles confer protection.

The *CTLA4* gene region on chromosome 2q33 is also associated with susceptibility to T1D [89]. The signaling between B7, expressed by professional antigen-presenting cells such as DC and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed by T cells, plays a pivotal role in peripheral T-cell tolerance. CTLA-4 is expressed neither by thymocytes nor resting T cells, but it is detectable after antigen-mediated T-cell activation and downregulates responses of activated T cells. *Ctla4* deletion in mice results in an extremely severe lymphoproliferative and an autoimmune phenotype with lethal multiorgan tissue destruction [90].

Another mutation in a non-HLA gene conferring significant susceptibility to T1D is a variant of the lymphoid tyrosine phosphatase Lyp gene (*PTPN22*), a suppressor of T-cell activation [91]. Lyp normally interacts with a C-terminal Src kinase (Csk) complex to dephosphorylate positive regulatory tyrosines and downregulate signaling from the TCR pathway. The minor allele derived from the single nucleotide polymorphism (SNP) differs in a single but crucial amino acid residue (R620W) involved in interaction of Lyp with Csk. Interestingly, the same variant R620W also increases risk to other common autoimmune diseases, such as rheumatoid arthritis, Graves' disease, and systemic lupus erythematosus [92]. However, the variant *PTPN22* 620W is a gain-of-function mutant, since it is associated with a higher catalytic activity of the encoded Lyp, a marked decrease of T-cell response to antigen stimulation, CD25 expression and IL-10 secretion from TCR stimulation, and an increase in peripheral memory CD4+ T cells [93, 94]. The role of this mutation in the pathogenesis of T1D and other autoimmune diseases remains to be further elucidated.

Different studies, including a genome-wide association analysis, have identified association of T1D with noncoding SNPs on the chromosome 10p15 region containing CD25, which encodes the high-affinity α chain of the IL2R complex [95]. Further mapping of the association between the IL2RA locus and T1D supported a role of IL2Rα in the pathogenesis of the disease, most possibly through modulation of Treg activity [96].

An association has also been found between T1D and a polymorphism of the *IGF2* receptor gene (*IGF2R*), which seems to be subject to parental imprinting since only maternal alleles at this polymorphism are associated with the disease [97]. Human T1D differs from other common autoimmune disorders, which preferentially affect females (like autoimmune diabetes in the NOD mouse). Evidence was also recently provided for an association between T1D and polymorphisms in *CYP27B1*, which encodes 1α-hydroxylase, the enzyme that transforms 25(OH) vitamin D into bioactive $1,25(OH)_2$ vitamin D3 [98].

The association between T1D and viral infections has been recently reinforced by genetic studies that have evidenced a linkage between T1D susceptibility and host genetic determinants of the antiviral responses such as the antiviral oligoadenylate synthetase (*OAS1*) and the interferon-induced helicase (*IFIH1* or *MDA5*), which intervenes in innate immunity by recognition of RNA genomes of picornaviruses (such as coxsackie viruses) [99–101]. Therefore, the question of a higher incidence of enterovirus infection during childhood in countries with a high-risk of T1D deserves to be further investigated, particularly if one seriously considers the possibility of anticoxsackie virus vaccination as a potential method for T1D prevention.

7. Prospective: The Concept of Negative/Tolerogenic Self-Vaccination

Because of its antigen-specificity, the most attractive immunomodulating approach against the development of the diabetogenic autoimmune response is the design of peptide-based therapeutic vaccines. According to the novel knowledge gained in T1D pathogenesis and the central role of a thymus dysfunction in its development, the control of the autoimmune process could be obtained by reprogramming β cell tolerance through the potent tolerogenic properties of the thymus, in particular the repertoire of thymic T1D-related self-antigens. Contrary to insulin, the "altered self-IGF-2," IGF-2, and derived epitopes might be an appropriate choice for a novel type of a negative self-vaccination that associates competition for MHC presentation and regulatory responses downstream, as well as potential bystander suppression of autoimmune responses to other T1D-related autoantigens. This hypothesis is currently being investigated by vaccination of NOD mice with recombinant human IGF-2 alone or in combination with tolerogenic adjuvants.

8. Conclusion

The thymus plays a central role in the establishment of central immunological self-tolerance towards Langerhans' insulin-secreting islet β cells, and there is now evidence that T1D development results from a breakdown of thymus-dependent tolerance to insulin family-derived epitopes. This knowledge should translate in the very near future to the design of novel tolerogenic/regulatory approaches aimed at restoring the immunological tolerance specific of islet β cells, which represents an appealing strategy for both

prevention and cure of T1D, one of the heaviest prices paid by the human species for having evolved the advantage of the extreme diversity and efficiency of adaptive immune responses against new biological threats.

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References

[1] T. P. Di Lorenzo, M. Peakman, and B. O. Roep, "Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes," *Clinical and Experimental Immunology*, vol. 148, no. 1, pp. 1–16, 2007.

[2] T. K. Starr, S. C. Jameson, and K. A. Hogquist, "Positive and negative selection of T cells," *Annual Review of Immunology*, vol. 21, pp. 139–176, 2003.

[3] L. S. K. Walker and A. K. Abbas, "The enemy within: keeping self-reactive T cells at bay in the periphery," *Nature Reviews Immunology*, vol. 2, no. 1, pp. 11–19, 2002.

[4] P. S. Ohashi, "Negative selection and autoimmunity," *Current Opinion in Immunology*, vol. 15, no. 6, pp. 668–676, 2003.

[5] A. A. Chentoufi, M. Palumbo, and C. Polychronakos, "Proinsulin expression by Hassall's corpuscles in the mouse thymus," *Diabetes*, vol. 53, no. 2, pp. 354–359, 2004.

[6] A. A. Chentoufi and C. Polychronakos, "Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes," *Diabetes*, vol. 51, no. 5, pp. 1383–1390, 2002.

[7] B. Kyewski and L. Klein, "A central role for central tolerance," *Annual Review of Immunology*, vol. 24, pp. 571–606, 2006.

[8] J. Pitkänen and P. Peterson, "Autoimmune regulator: from loss of function to autoimmunity," *Genes and Immunity*, vol. 4, no. 1, pp. 12–21, 2003.

[9] M. S. Anderson, E. S. Venanzi, L. Klein et al., "Projection of an immunological self shadow within the thymus by the aire protein," *Science*, vol. 298, no. 5597, pp. 1395–1401, 2002.

[10] J. Cabarrocas, C. Cassan, F. Magnusson et al., "Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 22, pp. 8453–8458, 2006.

[11] K. Aschenbrenner, L. M. D'Cruz, E. H. Vollmann et al., "Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire medullary thymic epithelial cells," *Nature Immunology*, vol. 8, no. 4, pp. 351–358, 2007.

[12] G. F. Bottazzo, A. Florin Christensen, and D. Doniach, "Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies," *The Lancet*, vol. 2, no. 7892, pp. 1279–1283, 1974.

[13] J. M. Wenzlau, K. Juhl, L. Yu et al., "The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 43, pp. 17040–17045, 2007.

[14] B. D. Stadinski, T. Delong, N. Reisdorph et al., "Chromogranin A is an autoantigen in type 1 diabetes," *Nature Immunology*, vol. 11, no. 3, pp. 225–231, 2010.

[15] M. Nakayama, N. Abiru, H. Moriyama et al., "Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice," *Nature*, vol. 435, no. 7039, pp. 220–223, 2005.

[16] S. C. Kent, Y. Chen, L. Bregoli et al., "Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope," *Nature*, vol. 435, no. 7039, pp. 224–228, 2005.

[17] E. A. M. Gale and P. J. Bingley, "Autoimmune type 1 diabetes," in *Immunoendocrinology in Health and Disease*, V. Geenen and G. P. Chrousos, Eds., pp. 417–438, Marcel Dekker, New York, NY, USA, 2004.

[18] A. L. Notkins and A. Lernmark, "Autoimmune type 1 diabetes: resolved and unresolved issues," *Journal of Clinical Investigation*, vol. 108, no. 9, pp. 1247–1252, 2001.

[19] S. Martin, D. Wolf-Eichbaum, G. Duinkerken et al., "Development of type 1 diabetes despite severe hereditary B-cell deficiency," *The New England Journal of Medicine*, vol. 345, no. 14, pp. 1036–1040, 2001.

[20] B. O. Roep, "The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure," *Diabetologia*, vol. 46, no. 3, pp. 305–321, 2003.

[21] S. Arif, T. I. Tree, T. P. Astill et al., "Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health," *Journal of Clinical Investigation*, vol. 113, no. 3, pp. 451–463, 2004.

[22] K. C. Herold, B. Brooks-Worrell, J. Palmer et al., "Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes," *Diabetes*, vol. 58, no. 11, pp. 2588–2595, 2009.

[23] V. Geenen and G. Kroemer, "Multiple ways to cellular immune tolerance," *Immunology Today*, vol. 14, no. 12, pp. 573–575, 1993.

[24] H. Martens, B. Goxe, and V. Geenen, "The thymic repertoire of neuroendocrine self-antigens: physiological implications in T-cell life and death," *Immunology Today*, vol. 17, no. 7, pp. 312–317, 1996.

[25] M. L. Dion, R. Bordi, J. Zeidan et al., "Slow disease progression and robust therapy-mediated CD4+ T-cell recovery are associated with efficient thymopoiesis during HIV-1 infection," *Blood*, vol. 109, no. 7, pp. 2912–2920, 2007.

[26] E. Castermans, F. Baron, E. Willems et al., "Evidence for neogeneration of T cells by the thymus after non-myeloablative conditioning," *Haematologica*, vol. 93, no. 2, pp. 240–247, 2008.

[27] G. Morrhaye, H. Kermani, J. J. Legros et al., "Impact of growth hormone (GH) deficiency and GH replacement upon thymus function in adult patients," *PLoS ONE*, vol. 4, no. 5, Article ID e5668, 2009.

[28] R. L. Boyd, C. L. Tucek, D. I. Godfrey et al., "The thymic microenvironment," *Immunology Today*, vol. 14, no. 9, pp. 445–459, 1993.

[29] D. D. Patel, L. P. Whichard, G. Radcliff, S. M. Denning, and B. F. Haynes, "Characterization of human thymic epithelial cell surface antigens: phenotypic similarity of thymic epithelial cells to epidermal keratinocytes," *Journal of Clinical Immunology*, vol. 15, no. 2, pp. 80–92, 1995.

[30] L. Klein, M. Hinterberger, G. Wirnsberger, and B. Kyewski, "Antigen presentation in the thymus for positive selection and central tolerance induction," *Nature Reviews Immunology*, vol. 9, no. 12, pp. 833–844, 2009.

[31] H. Von Boehmer, "Central tolerance: essential for preventing autoimmune disease?" *European Journal of Immunology*, vol. 39, no. 9, pp. 2313–2316, 2009.

[32] P. G. Ashton-Rickardt, A. Bandeira, J. R. Delaney et al., "Evidence for a differential avidity model of T cell selection in the thymus," *Cell*, vol. 76, no. 4, pp. 651–663, 1994.

[33] Y. Vanneste, A. Ntodou Thome, E. Vandersmissen et al., "Identification of neurotensin-related peptides in human thymic epithelial cell membranes and relationship with major histocompatibility complex class I molecules," *Journal of Neuroimmunology*, vol. 76, no. 1-2, pp. 161–166, 1997.

[34] V. Geenen, F. Brilot, I. Hansenne, and H. Martens, "Thymus and T cells," in *Encyclopedia of Neuroscience on CD-ROM*, G. Adelman and B. H. Smith, Eds., Elsevier, New York, NY, USA, 3rd edition, 2003.

[35] J. Derbinski, J. Gäbler, B. Brors et al., "Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels," *Journal of Experimental Medicine*, vol. 202, no. 1, pp. 33–45, 2005.

[36] I. Hansenne, C. Renard-Charlet, R. Greimers, and V. Geenen, "Dendritic cell differentiation and immune tolerance to insulin-related peptides in *Igf2*-deficient mice," *Journal of Immunology*, vol. 176, no. 8, pp. 4651–4657, 2006.

[37] F. M. Burnet, "A reassessment of the forbidden clone hypothesis of autoimmune disease," *The Australian journal of experimental biology and medical science*, vol. 50, no. 1, pp. 1–9, 1972.

[38] F. R. Robert, H. Martens, N. Cormann, A. Benhida, J. Schoenen, and V. Geenen, "The recognition of hypothalamo-neurohypophysial functions by developing T cells," *Developmental immunology*, vol. 2, no. 2, pp. 131–140, 1992.

[39] H. Kishimoto and J. Sprent, "A defect in central tolerance in NOD mice," *Nature Immunology*, vol. 2, no. 11, pp. 1025–1031, 2001.

[40] S. Zucchelli, P. Holler, T. Yamagata, M. Roy, C. Benoist, and D. Mathis, "Defective central tolerance induction in NOD mice: genomics and genetics," *Immunity*, vol. 22, no. 3, pp. 385–396, 2005.

[41] O. Kecha-Kamoun, I. Achour, H. Martens et al., "Thymic expression of insulin-related genes in an animal model of autoimmune type 1 diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 17, no. 2, pp. 146–152, 2001.

[42] V. Geenen and P. J. Lefèbvre, "The intrathymic expression of insulin-related genes: implications for pathophysiology and prevention of Type 1 diabetes," *Diabetes/Metabolism Reviews*, vol. 14, no. 1, pp. 95–103, 1998.

[43] K. Thébault-Baumont, D. Dubois-Laforgue, P. Krief et al., "Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice," *Journal of Clinical Investigation*, vol. 111, no. 6, pp. 851–857, 2003.

[44] H. Moriyama, N. Abiru, J. Paronen et al., "Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10376–10381, 2003.

[45] P. Vafiadis, S. T. Bennett, J. A. Todd et al., "Insulin expression in human thymus is modulated by *INS* VNTR alleles at the *IDDM2* locus," *Nature Genetics*, vol. 15, no. 3, pp. 289–292, 1997.

[46] A. Pugliese, M. Zeller, A. Fernandez et al., "The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the *INS* VNTR-*IDDM2* susceptibility locus for type 1 diabetes," *Nature Genetics*, vol. 15, no. 3, pp. 293–297, 1997.

[47] Y. Fan, W. A. Rudert, M. Grupillo, J. He, G. Sisino, and M. Trucco, "Thymus-specific deletion of insulin induces autoimmune diabetes," *EMBO Journal*, vol. 28, no. 18, pp. 2812–2824, 2009.

[48] K. Nagamine, P. Peterson, H. S. Scott et al., "Positional cloning of the APECED gene," *Nature Genetics*, vol. 17, no. 4, pp. 393–398, 1997.

[49] Finnish-German APECED Consortium, "An autoimmune disease, APECED, caused bymutations in a novel gene featuring two PHD-type zinc-finger domains," *Nature Genetics*, vol. 17, pp. 399–403, 1997.

[50] M. Heino, P. Peterson, N. Sillanpää et al., "RNA and protein expression of the murine autoimmune regulator gene (*Aire*) in normal, RelB-deficient and in NOD mouse," *European Journal of Immunology*, vol. 30, no. 7, pp. 1884–1893, 2000.

[51] C. Ramsey, O. Winqvist, L. Puhakka et al., "Aire deficient mice develop multiple features of APECED phenotype and show altered immune response," *Human Molecular Genetics*, vol. 11, no. 4, pp. 397–409, 2002.

[52] F. X. Hubert, S. A. Kinkel, P. E. Crewther et al., "Aire-deficient C57BL/6 mice mimicking the common human 13-base pair deletion mutation present with only a mild autoimmune phenotype," *Journal of Immunology*, vol. 182, no. 6, pp. 3902–3918, 2009.

[53] J. Derbinski, J. Gäbler, B. Brors et al., "Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels," *Journal of Experimental Medicine*, vol. 202, no. 1, pp. 33–45, 2005.

[54] J. M. Gardner, J. J. DeVoss, R. S. Friedman et al., "Deletional tolerance mediated by extrathymic aire-expressing cells," *Science*, vol. 321, no. 5890, pp. 843–847, 2008.

[55] D. Mathis and C. Benoist, "Aire," *Annual Review of Immunology*, vol. 27, pp. 287–312, 2009.

[56] P. Peterson, T. Org, and A. Rebane, "Transcriptional regulation by AIRE: molecular mechanisms of central tolerance," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 948–957, 2008.

[57] T. Org, F. Chignola, C. Hetényi et al., "The autoimmune regulator PHD finger binds to non-methylated histone H3K4 to activate gene expression," *EMBO Reports*, vol. 9, no. 4, pp. 370–376, 2008.

[58] A. S. Koh, A. J. Kuo, Y. P. Sang et al., "Aire employs a histone-binding module to mediate immunological tolerance, linking chromatin regulation with organ-specific autoimmunity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 41, pp. 15878–15883, 2008.

[59] M. Matsumoto, "Contrasting models for the roles of Aire in the differentiation program of epithelial cells in the thymic medulla," *European Journal of Immunology*, vol. 41, no. 1, pp. 12–17, 2011.

[60] F. Brilot, W. Chehadeh, C. Charlet-Renard, H. Martens, V. Geenen, and D. Hober, "Persistent infection of human

thymic epithelial cells by coxsackievirus B4," *Journal of Virology*, vol. 76, no. 10, pp. 5260–5265, 2002.

[61] F. Brilot, V. Geenen, D. Hober, and C. A. Stoddart, "Coxsackievirus B4 infection of human fetal thymus cells," *Journal of Virology*, vol. 78, no. 18, pp. 9854–9861, 2004.

[62] H. Jaïdane, J. Gharbi, P. E. Lobert et al., "Prolonged viral RNA detection in blood and lymphoid tissues from Coxsackievirus B4 E2 orally-inoculated Swiss mice," *Microbiology and Immunology*, vol. 50, no. 12, pp. 971–974, 2006.

[63] P. A. Taylor, R. J. Noelle, and B. R. Blazar, "CD4+CD25+ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade," *Journal of Experimental Medicine*, vol. 193, no. 11, pp. 1311–1317, 2001.

[64] B. Singh, S. Read, C. Asseman et al., "Control of intestinal inflammation by regulatory T cells," *Immunological Reviews*, vol. 182, pp. 190–200, 2001.

[65] Y. Belkaid, R. B. Blank, and I. Suffia, "Natural regulatory T cells and parasites: a common quest for host homeostasis," *Immunological Reviews*, vol. 212, pp. 287–300, 2006.

[66] I. P. Lewkowich, N. S. Herman, K. W. Schleifer et al., "CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function," *Journal of Experimental Medicine*, vol. 202, no. 11, pp. 1549–1561, 2005.

[67] U. Dittmer, H. He, R. J. Messer et al., "Functional impairment of CD8+ T cells by regulatory T cells during persistent retroviral infection," *Immunity*, vol. 20, no. 3, pp. 293–303, 2004.

[68] M. Iwashiro, R. J. Messer, K. E. Peterson, I. M. Stromnes, T. Sugie, and K. J. Hasenkrug, "Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 16, pp. 9226–9230, 2001.

[69] E. M. Shevach, R. A. DiPaolo, J. Andersson, D. M. Zhao, G. L. Stephens, and A. M. Thornton, "The lifestyle of naturally occurring CD4+CD25+ Foxp3+ regulatory T cells," *Immunological Reviews*, vol. 212, pp. 60–73, 2006.

[70] B. Salomon, D. J. Lenschow, L. Rhee et al., "B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes," *Immunity*, vol. 12, no. 4, pp. 431–440, 2000.

[71] Q. Tang, K. J. Henriksen, E. K. Boden et al., "Cutting edge: CD28 controls peripheral homeostasis of CD4+ CD25+ regulatory T cells," *Journal of Immunology*, vol. 171, no. 7, pp. 3348–3352, 2003.

[72] A. E. Herman, G. J. Freeman, D. Mathis, and C. Benoist, "CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1479–1489, 2004.

[73] Z. Chen, A. E. Herman, M. Matos, D. Mathis, and C. Benoist, "Where CD4+CD25+ T reg cells impinge on autoimmune diabetes," *Journal of Experimental Medicine*, vol. 202, no. 10, pp. 1387–1397, 2005.

[74] C. Baecher-Allan and D. A. Hafler, "Human regulatory T cells and their role in autoimmune disease," *Immunological Reviews*, vol. 212, pp. 203–216, 2006.

[75] D. Homann and M. Von Herrath, "Regulatory T cells and type 1 diabetes," *Clinical Immunology*, vol. 112, no. 3, pp. 202–209, 2004.

[76] S. Gregori, N. Giarratana, S. Smiroldo, and L. Adorini, "Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development," *Journal of Immunology*, vol. 171, no. 8, pp. 4040–4047, 2003.

[77] S. You, M. Belghith, S. Cobbold et al., "Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells," *Diabetes*, vol. 54, no. 5, pp. 1415–1422, 2005.

[78] T. Brusko and M. Atkinson, "Treg in type 1 diabetes," *Cell Biochemistry and Biophysics*, vol. 48, no. 2-3, pp. 165–175, 2007.

[79] S. Lindley, C. M. Dayan, A. Bishop, B. O. Roep, M. Peatman, and T. I. M. Tree, "Defective suppressor function in CD4+CD25+ T-cells from patients with type 1 diabetes," *Diabetes*, vol. 54, no. 1, pp. 92–99, 2005.

[80] J. H. Buckner, "Mechanisms of impaired regulation by CD4++ CD25++ Foxp3++ regulatory T cells in human autoimmune diseases," *Nature Reviews Immunology*, vol. 10, no. 12, pp. 849–859, 2010.

[81] Y. J. Liu, V. Soumelis, N. Watanabe et al., "TSLP: an epithelial cell cytokine that regulates t cell differentiation by conditioning dendritic cell maturation," *Annual Review of Immunology*, vol. 25, pp. 193–219, 2007.

[82] N. Watanabe, YI. H. Wang, H. K. Lee et al., "Hassall's corpuscles instruct dendritic cells to induce CD4+ CD25+ regulatory T cells in human thymus," *Nature*, vol. 436, no. 7054, pp. 1181–1185, 2005.

[83] J. Nerup, P. Platz, O. O. Andersen et al., "HLA antigens and diabetes mellitus," *The Lancet*, vol. 2, pp. 864–866, 1974.

[84] J. A. Noble, A. M. Valdes, M. Cook, W. Klitz, G. Thomson, and H. A. Erlich, "The role of HLA class II genes in insulin-dependent diabetes mellitus: molecular analysis of 180 Caucasian, multiplex families," *American Journal of Human Genetics*, vol. 59, no. 5, pp. 1134–1148, 1996.

[85] K. H. Lee, K. W. Wucherpfennig, and D. C. Wiley, "Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type I diabetes," *Nature Immunology*, vol. 2, no. 6, pp. 501–507, 2001.

[86] S. Nejentsev, J. M. M. Howson, N. M. Walker et al., "Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A," *Nature*, vol. 450, no. 7171, pp. 887–892, 2007.

[87] G. I. Bell, S. Horita, and J. H. Karam, "A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus," *Diabetes*, vol. 33, no. 2, pp. 176–183, 1984.

[88] S. T. Bennett, A. M. Lucassen, S. C. L. Gough et al., "Susceptibility to humantype 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulingene minisatellite locus," *Nature Genetics*, vol. 9, pp. 284–292, 1995.

[89] L. Nisticò, R. Buzzetti, L. E. Pritchard et al., "The *CTLA-4* gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes," *Human Molecular Genetics*, vol. 5, no. 7, pp. 1075–1080, 1996.

[90] P. Waterhouse, J. M. Penninger, E. Timms et al., "Lymphoproliferative disorders with early lethality in mice deficient in *CTLA-4*," *Science*, vol. 270, no. 5238, pp. 985–988, 1995.

[91] N. Bottini, L. Musumeci, A. Alonso et al., "A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes," *Nature Genetics*, vol. 36, no. 4, pp. 337–338, 2004.

[92] L. A. Criswell, K. A. Pfeiffer, R. F. Lum et al., "Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes," *American Journal of Human Genetics*, vol. 76, no. 4, pp. 561–571, 2005.

[93] T. Vang, M. Congia, M. D. Macis et al., "Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant," *Nature Genetics*, vol. 37, no. 12, pp. 1317–1319, 2005.

[94] M. Rieck, A. Arechiga, S. Onengut-Gumuscu, C. Greenbaum, P. Concannon, and J. H. Buckner, "Genetic variation in *PTPN22* corresponds to altered function of T and B lymphocytes," *Journal of Immunology*, vol. 179, no. 7, pp. 4704–4710, 2007.

[95] P. R. Burton, D. G. Clayton, L. R. Cardon et al., "Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls," *Nature*, vol. 447, no. 7145, pp. 661–678, 2007.

[96] H. Q. Qu, A. Montpetit, B. Ge, T. J. Hudson, and C. Polychronakos, "Toward further mapping of the association between the *IL2RA* locus and type 1 diabetes," *Diabetes*, vol. 56, no. 4, pp. 1174–1176, 2007.

[97] J. A. McCann, Q. X. Yong, R. Frechette, L. Guazzarotti, and C. Polychronakos, "The *insulin-like growth facfor-II* receptor gene is associated with type 1 diabetes: evidence of a maternal effect," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 11, pp. 5700–5706, 2004.

[98] R. Bailey, J. D. Cooper, L. Zeitels et al., "Association of the vitamin D metabolism gene *CYP27B1* with type 1 diabetes," *Diabetes*, vol. 56, no. 10, pp. 2616–2621, 2007.

[99] L. L. Field, V. Bonnevie-Nielsen, F. Pociot, S. Lu, T. B. Nielsen, and H. Beck-Nielsen, "*OAS1* splice site polymorphism controlling antiviral enzyme activity influences susceptibility to type 1 diabetes," *Diabetes*, vol. 54, no. 5, pp. 1588–1591, 2005.

[100] D. J. Smyth, J. D. Cooper, R. Bailey et al., "A genome-wide association study of nonsynonymous SNPs identifies a type 1 diabetes locus in the interferon-induced helicase (IFIH1) region," *Nature Genetics*, vol. 38, no. 6, pp. 617–619, 2006.

[101] H. Kato, O. Takeuchi, S. Sato et al., "Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses," *Nature*, vol. 441, no. 1, pp. 101–105, 2006.

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Review Article

Mechanisms That Regulate Peripheral Immune Responses to Control Organ-Specific Autoimmunity

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The immune system must balance the need to maintain a diverse repertoire of lymphocytes to be able to fight infection with the need to maintain tolerance to self-proteins. The immune system places strict regulation over the ability of T cells to produce the major T cell growth factor interleukin 2 as this cytokine can influence a variety of immune outcomes. T cells require the delivery of two signals, one through the antigen receptor and a second through the costimulatory receptor CD28. The immune system uses a variety of E3 ubiquitin ligases to target signaling proteins that function downstream of the TCR and CD28 receptors. Mutations in these E3 ligases can lead to a breakdown in immune tolerance and development of autoimmunity. This paper will examine the role of a range of E3 ubiquitin ligases and signaling pathways that influence the development of T-cell effector responses and the development of organ-specific autoimmune diseases such as type 1 diabetes.

1. Introduction

The immune system has evolved to protect the body from infectious pathogens through both innate and adaptive immune responses. The adaptive immune response is built upon a diverse repertoire of antigen-specific T and B lymphocytes that learn to distinguish between self- from non-self- antigens during their differentiation in the thymus and bone marrow, respectively. Central tolerance is established in the thymus by the elimination of autoreactive thymocytes that display a TCR with high affinity for self-peptide/MHC complexes [1]. Despite the relative efficiency of clonal deletion not all tissue-specific antigens are expressed in the thymus and thus a small proportion of autoreactive T cells can escape thymic deletion, complete their maturation and enter the peripheral circulation. The immune system has multiple checkpoints in place to limit the activation and expansion of these autoreactive cells in the periphery [2]. The last decade has led to an improved understanding of some of these checkpoints involved in peripheral regulation of the immune response.

Autoimmunity arises following a failure in either central or peripheral tolerance mechanisms. In the periphery the immune system has a range of mechanisms available that control the fate of autoreactive T cells, including immune privilege, immune ignorance, activation-induced cell death, clonal anergy, and immune suppression-mediated by regulatory T (Treg) cells [3–5]. Autoimmune diseases are classified as organ-specific or systemic depending on the source of the autoantigens. Type 1 diabetes (T1D) is an example of an organ-specific autoimmune disease caused by the breakdown in tolerance in both CD4+ and CD8+ T cells and B cells that express antigen receptors specific for proteins derived from the islets of Langerhans in the pancreas. Some of the key target autoantigens in T1D include insulin, GAD65, and insulin adenoma 2 (IA2) protein. Systemic autoimmune diseases are typified by systemic lupus erythematosus (SLE) or lupus and are due to the generation of high-affinity anti-self-antibodies specific for ubiquitous cellular proteins or nucleic acids.

Type 1 diabetes arises as a result of a breakdown of tolerance in islet reactive T cells. This leads to immunological destruction of the pancreatic beta cells and loss of insulin secretion that is mediated by CD4+ and CD8+ T cells [6]. Patients can produce anti-insulin antibodies indicating that T1D reflects a generalized breakdown in immunological tolerance that allows islet-reactive CD4+ Th cells to provide help to autoreactive B cells.

It was originally thought that clonal anergy and regulatory T cells were entirely distinct mechanisms that are used to control peripheral immune responses. However, studies in recent years have uncovered a remarkable overlap with the mechanisms of clonal anergy and also the generation of regulatory T cells in the periphery. In particular the discussion will examine the group of proteins known as ubiquitin ligases and how these proteins have emerged as an important class of negative regulators of the immune response not only in animals but also in humans. In addition I will examine how signaling through the AKT/mammalian target of rapamycin (mTOR) pathway has important roles in balancing the choice between immunity and suppression.

2. T-Cell Activation and Generation of Effector Responses

Naive T cells remain in a quiescent state as they circulate through secondary lymphoid tissues, the blood and lymph, and in the absence of an antigenic signal rely on survival signals transmitted via growth factor receptors (e.g., IL-7R, CD127) [7]. Importantly, quiescent cells are unable to secrete IL-2 because they recruit a number of different nuclear repressor proteins to the *Il2* gene locus (e.g., Ikaros, p50 NF-κB dimers, Blimp1, Tob, and Smad proteins) that mediate epigenetic modification to repress the *Il2* gene transcription [8–12].

T-cell activation is dependent on the delivery of two separate signals. Signal one is mediated through the TCR and signal two through the costimulatory receptor CD28 in response to binding of its ligands CD80/CD86 which leads to phosphorylation of a number of intracellular signaling pathways including phospholipase γ-1, protein kinase-θ, MAPK, JNK, PI3K, and Iκ-B kinase (IKK) that leads to the recruitment of transcription factors (e.g., NFAT, AP-1, and NF-κB) critical to the transcription of the *Il2* gene [13]. IL-2 is a multifunctional cytokine that plays a role in T-cell mitogenesis stimulating growth of activated T cells via paracrine and autocrine signalling through the IL-2 receptor (IL-2R) [14]. It can promote Th1 and Th2 cell differentiation; it is required for the generation of CD8+ T cell memory responses and plays a crucial role in the maintenance and homeostasis of Treg cells in the periphery. The delivery of a costimulatory signal during T cell activation leads to derepression of the *Il2* gene, and *Il2* mRNA transcripts become stabilized and translated into protein to drive T cell mitogenesis. Given the role that IL-2 has in coordinating the proliferative response of effector T cells, it is not surprising that the immune system has placed *Il2* gene transcription under tight regulation in an effort to restrict the inappropriate activation of this gene in naïve T cells *in vivo* and to limit the potential for a breakdown in immune tolerance and for autoimmunity. Therefore the secretion of IL-2 following T-cell activation represents an important checkpoint that can determine the outcome between immunity and tolerance [15].

3. Clonal Anergy

Ligation of TCR on T cells in the absence of CD28 costimulation leads to the development of clonal anergy which is characterized by the failure to activate the MAPK, PI3K/AKT, and the IKK pathways and results in reduced activity of the nuclear factors AP-1 and NF-κB and deficient IL-2 gene transcription, but there is elevated NFAT signalling in anergic cells [5]. The original studies performed on mouse Th1 cell clones *in vitro* showed that ligation of TCR in the absence of CD28 costimulation leads to a hyporesponsive state and a failure to secrete IL-2 leading to an abortive proliferative response [5]. It is also possible to induce anergy in naïve CD4+ and CD8+ T cells *in vivo* that is identified by a failure of these cells to secrete IL-2 and inflammatory cytokines, for example, TNF-α and IFN-γ upon restimulation with antigen. Around the same time, human CD4+ T cells were also shown to be subject to clonal anergy *in vitro* through cognate presentation of peptide between MHCII expressing T cells, (i.e., T-T presentation in the absence of APCs) [16]. The induction of anergy can also be induced by artificially elevating calcium levels which leads to activation of calcineurin and NFAT activity in the cells [17, 18]. Finally delivery of soluble antigens via the intravenous or mucosal routes is particularly conducive to tolerance induction that reflects many aspects of anergy [19–23].

Clonal anergy is an active process that requires new protein synthesis and is associated with an anergic gene expression profile that is characterized by increased expression of a number of E3 ubiquitin ligases including Cbl-b, Itch, and Grail [18]. Recent studies have also identified that the induction of anergy is associated with induction of numerous negative regulators of TCR signaling including *diacylglycerol kinase*, *caspase3*, *Traf6*, *Ikaros*, *Egr2*, *Egr3*, and *CREM* (cyclic AMP response element modulator) [18, 24–29]. Early studies showed that anergy was associated with defective Ras/MAPK activation and AP1 transactivation, but the causal relationship of Ras activation to anergy induction was difficult to prove [30, 31]. More recently gene expression profiling of anergic cells revealed that inhibitors of RasGRP1 were increased [18, 32] and diacylglycerol kinase (DGK) is required for the induction of T-cell anergy and is induced in response to NFAT. DGK functions by inhibiting Ras activation by diminishing the second messenger diacylglycerol that would activate RasGRP1 [32, 33].

4. Regulation of T-Cell Activation by E3 Ubiquitin Ligases

Multiple E3 ubiquitin ligases are required for regulation of lymphocyte development and activation and immune tolerance [34, 35]. Ubiquitination is a highly conserved process where proteins become tagged with ubiquitin, and this can target them for degradation by the proteasome. Ubiquitin is added by the sequential activity of three enzymes: E1 is an activating enzyme, E2 is a conjugating enzyme, and E3 is a ligase that attaches the ubiquitin moiety to the target protein. Proteins can either be subject to monoubiquitination or polyubiquitination (Table 1) [36, 37].

Table 1: E3 Ligases that play an important role in preventing autoimmunity.

Name	References	Type of E3 ligase	Function
Cbl-b	[40–45]	RING type	Ubiquitinates PLC-γ1 and PKC-θ downstream of TCR signalling. Also binds to and ubiquitinates P85 subunit of PI3K to inhibit CD28-mediated triggering of PI3K. Critical role in T-cell anergy.
Itch	[46–50]	HECT type	Important role in Th cell differentiation. Binds to a number of substrates including Notch, c-Flip, Smad2, p63, and p73. Ubiquitinates Bcl-10, JunB, Cbl-b, and PKC-θ and targets them for degradation. Binds to and is regulated by the E3 ligase Ndfip1.
Nedd4	[51]	HECT type	Binds to and ubiquitinates a number of targets that targets them for degradation including JunB, Cbl-b, c-Cbl, Notch, PTEN, PLC-γ1, and Bcl-10.
Ndfip1	[52–54]	HECT type	Associates with Nedd4 and is a negative regulator of Itch. Other targets include p63, p73, and c-Flip.
Grail	[55–57]	RING type	Selectively inhibits RhoGTPase activity but does not affect the Ras/MAPK kinase pathway. Inhibits IL-2 production and T-cell proliferation.
Traf6	[26, 58]	RING type	Binds to and ubiquitinates NEMO targeting it for degradation. Leads to upregulation of NF-κB signalling in response to TCR signaling.
Roquin	[59–61]	RING type	Binds to RNA-protein complexes and targets themfor degradation. Important role in posttranscriptional regulation of target gene expression. Targets include ICOS, CXCR5, PDCD1, CCL5, Il21, and CD100.

Cbl-b and its close paralogue, c-Cbl, are RING (really interesting new gene) type E3 ligases that regulate cell surface expression of activated receptors with receptor tyrosine kinase activity (e.g., the EGF receptor) through endocytosis and targeting them for ubiquitin-mediated degradation. C-Cbl is required for TCR modulation in thymocytes, while the two proteins appear to function redundantly for promoting TCR downregulation in peripheral T cells. *Cblb* is induced during the induction of anergy [17, 18, 38], and its expression is regulated by the *early growth response genes* (*Egr*) *2* and *Egr3* [28]. However *Cblb* has a nonredundant role in the induction and maintenance of peripheral T-cell anergy. *Cblb*-deficient mice are susceptible to autoimmune diseases, and T cells display hyperproliferation in response to TCR signaling and produce IL-2 in the absence of a costimulatory signal. The Komeda diabetes prone (KDP) rat strain develops spontaneous islet-specific autoimmunity that is caused by a loss of function mutation in *Cblb,* but the emergence of spontaneous disease requires additional susceptibility factors, notably a diabetes-susceptible MHC haplotype [39]. The autoimmune regulator (Aire) gene is a transcription factor that controls the expression of tissue-specific self-antigens within the thymus. In particular it is crucial for clonal deletion of self-reactive lymphocytes [40–42]. Aire deficiency in both mice and humans can trigger spontaneous autoimmune disease such as type 1 diabetes with variable latency [40, 43], and it was recently revealed that *Cblb* acts as critical failsafe mechanism to help restrain autoreactive T cells in the periphery as a result of an AIRE deficiency [44]. Combined mutations in *Aire* and *Cblb* lead to a lethal autoimmunity with destruction of the exocrine pancreas and multiorgan inflammation within 5 weeks of life, whereas on their own neither gene mutation could trigger the lethal autoimmunity [44].

Similarly, we find that islet-specific autoimmunity is only caused by *Cblb* deficiency in mice when combined with a TCR transgene that makes high numbers of islet-reactive CD4 cells in their thymus and expresses the neoself-antigen hen egg lysozyme on pancreatic beta cells [45]. The *Cblb* deficiency does not affect negative selection of autoreactive T cells or the differentiation of natural Tregs that develop in the thymus [45]. However, the islet reactive T cells from *Clbb*$^{-/-}$ *TCR x insHel* double transgenic mice proliferated strongly and produced cytokines following restimulation with self-antigen *in vitro* indicating a breakdown in T cell anergy compared to wild-type cells from *TCR x insHel* which were unresponsive to the Hel stimulation. We also observed that the Cblb deficiency reduced the formation of inducible Tregs in response to TGF-β signaling, and these cells were less suppressive compared to wild-type iTregs [45]. This will be discussed further below.

Itch is a HECT (homologous to the E6-associated protein carboxy terminus) type E3 ubiquitin ligase and is upregulated following induction of anergy [17, 46]. Itch has an N-terminal protein kinase C-related domain, four WW domains, and a C-terminal HECT domain. The Itchy mouse strain was identified as the naturally occurring non-agouti-lethal 18H mutation that leads to a loss of function mutation in the Itch gene [47]. Itch can promote the ubiquitination of multiple proteins including Bcl-10, JunB, Cbl-b, and PKC-θ [17, 48, 49]. Itch-deficient mice develop a spontaneous and lethal systemic proinflammatory disease consistent with a failure of peripheral tolerance. The disease is associated with an expansion of Th2-type T cells that trigger a chronic pulmonary interstitial inflammation with elevated levels of IgE antibodies [48]. PLC-γ1 and PKC-θ are induced by calcium/calcineurin signaling, and Itch targets both proteins for endosomal sorting and lysosomal degradation to attenuate TCR signaling [17]. Reduced levels of PLC-γ1 and PKC-θ shorten the longevity of the immunological synapse and thus reduce the interactions between T cells and APCs. In addition, Itch also targets Jun B for degradation which is required for the formation of the AP1 transcription factor [50].

Neural precursor cell-expressed developmentally down-regulated 4 (Nedd4) is a HECT E3 ligase that is also expressed in T cells and can target multiple proteins for ubiquitination and degradation including JunB, Cbl-b, c-Cbl, Notch, PTEN, PLC-γ1, and Bcl10 which indicates that there may be a functional overlap between Itch and Nedd4 in T cells. Using fetal liver (FL) chimeras, Yang et al. [51] showed that reconstitution of irradiated mice with Nedd4-deficient FL cells led to normal T-cell development but these cells proliferated poorly following stimulation *in vitro* and produced less cytokines and the recipient animals did not develop any sign of overt autoimmunity. The defective T-cell activation was independent of JunB but instead could be attributed to a failure to ubiquitinate and degrade Cblb. Therefore, Nedd4 is required to degrade Cbl-b in order for T-cell activation to proceed [51].

Ndfip1 was originally identified through its association with the HECT E3 ligase Nedd4 and was later found to be a negative regulator of Itch [52, 53]. *Ndfip1*$^{-/-}$ develops a severe inflammatory disease that manifests clinically similar to that observed in *Itch*$^{-/-}$ mice. The Ndfip1-deficient mice develop severe skin inflammation with weight loss, splenomegaly, hepatomegaly, and premature death. The similarity between the disease phenotypes of *Itch*$^{-/-}$ and *Ndfip1*$^{-/-}$ mice may relate to the target proteins regulated by Itch, for example, Jun B which is upregulated and can promote Th2 differentiation [48, 54]. However, other targets of Ndfip1 include p63, p73, and c-Flip, but it is not known if dysregulation of any of these targets was responsible for the disease phenotype.

Grail is a RING-type E3 ubiquitin ligase that was found to be induced following the induction of T-cell anergy in CD4+ T cells [55]. Ectopic expression of Grail in CD4+ T cells inhibits IL-2 production and proliferation following stimulation antigen pulsed APCs [55]. In addition over-expression of Grail in T cells can selectively inhibit RhoGTPase activity but does not affect Ras activation and MAPK signalling [56]. This suggests that Grail has a separate regulatory function in controlling TCR signaling that is independent of the *Cblb* E3 ligase. Grail-deficient mice were resistant to immune tolerance induction, and they were more susceptible to autoimmune diseases [57]. Similar to the *Cblb*$^{-/-}$ mice, naïve T cells from Grail-deficient animals display a hyperproliferative response and increased cytokine production in the absence of a costimulation. In addition, loss of Grail function Tregs displays reduced suppressive function.

Traf6 is an E3 ligase with an N-terminal RING finger domain, and one of its targets includes the NF-κB essential modifier (NEMO) which is a member of the IκB kinase complex that functions downstream of TCR signalling. T-cell-specific deletion of Traf6 leads to a multiorgan inflammatory disease with splenomegaly and lymphadenopathy. The Traf6 deficient cells display Th2 polarization and the mice produce increased levels of IgG1, IgE, and IgM. In addition, the TraF6-deficient T cells hyperproliferate in response to anti-CD3 only in the absence of CD28 costimulation, and this was related to increased phosphorylation of PI3K p85 and AKT [26, 58].

Roquin (*Rc3h1*) is a newly described RING finger protein with E3 ligase activity. The Sanroque mouse strain was identified through an ENU mutagenesis screen for regulators of systemic autoimmunity. The *Rch*31$^{san/san}$ mice carry a point mutation in the conserved Roq domain generating a hypomorphic allele that gives rise to a lethal spontaneous systemic lupus-like disease [59]. The *Rch*31$^{san/san}$ mice have spontaneous germinal centre formation, increased numbers of T follicular helper cells (Tfh), increased serum levels of double stranded DNA antibodies, and elevated serum Ig levels. Tfh cells of the *Rch*31$^{san/san}$ mice constitutively express high levels of inducible costimulator molecule (ICOS) which can promote spontaneous germinal centre responses [60]. Roquin regulates ICOS expression levels by binding to the 3′ untranslated region of the *Icos* mRNA, promoting its degradation [61]. In the sensitized *TCR x insHel* model whereby the HEL neoself-antigen is expressed on the pancreatic islets [62], the expression of a high frequency of islet reactive T cells expressing the HEL-specific TCR, these *Rch*31$^{san/san}$ *TCR x insHel* mice have rapidly develop type 1 diabetes and generate anti-Hel islet-specific autoantibodies [59]. This is an unusual feature of autoimmunity in this animal model as *Cblb*$^{-/-}$ *TCR x insHel* mice do not make anti-Hel islet autoantibodies despite developing type 1 diabetes at high frequency [45].

When the Roquin mutation was combined with the Aire deficiency in mice, it was predicted that this combination would lead to rapid autoimmune disease given the severity of systemic autoimmunity observed in the *Rch*31$^{san/san}$ mice. However, the *Aire*$^{-/-}$ *Rch*31$^{san/san}$ mice displayed normal survival rates and remained overtly healthy with no clinical signs of autoimmunity for over 140 days. In contrast, *Aire*$^{-/-}$ *Cblb*$^{-/-}$ mice died with a mean survival age of 25 d with severe weight loss, and surprisingly the immunological destruction was restricted to the exocrine tissue of the pancreas [44]. As a result, the mice did not become diabetic as the islet tissue was unaffected by the autoimmune response. These studies highlight that *Cblb* appears to have a unique role in restricting the activation of autoreactive T cells in the periphery that is especially crucial when Aire-mediated clonal deletion is compromised in the thymus.

There has been some significant progress made in the mechanisms that control TCR signalling and activation in the peripheral immune system. These checkpoints are crucial to prevent *Il2* gene transcription and inappropriate activation of autoreactive T cells. The immune system has multiple ways of dampening the TCR signal that is controlled by multiple E3 ligases. The specificity of each of these ligases is crucial to control immune responses under different environmental conditions. The outcome of responses may be influenced by the range of cytokines or even different subsets of APCs present within different tissues.

5. *Cblb* and *Itch* in Regulatory T Cell Development

Regulatory T cells that express the forkhead/winged helix transcription factor Foxp3 play a critical role *in vivo* in

suppressing immune responses. Mutations in Foxp3 in mice (scurfy) and humans with immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) both lead to overwhelming multiorgan autoimmunity, diabetes, and early death [63–65]. Numerous studies have shown that Foxp3 is a master regulator of Treg development and function [66–68]. Overexpression of Foxp3 in naïve T cells can convert them to a Treg phenotype that enables them to suppress the response of other T cells either *in vitro* or *in vivo* [66–68]. Foxp3 is a transcriptional repressor that is recruited to the *Il2* and *Ifnγ* loci in T cells and modulates histone acetylation [69]. It is now apparent that there are two types of Tregs in the immune system. The natural Tregs (nTregs) are selected in the thymus during CD4+ T-cell differentiation; these cells represent ~5–10% of the peripheral CD4+ T cell pool and play crucial roles in regulating T-cell responses to self-antigens [4]. The second subset is known as inducible Tregs whereby naïve CD4+ CD25− T cells cultured in the presence of TGF-β (IL-10 and IL-2) can switch on Foxp3 and differentiate as a Treg cell [70–73]. These iTregs function in an equivalent manner in being able to suppress responses of other T cells whether *in vitro* or *in vivo*. The mechanism of suppression by Tregs is mediated by secretion of inhibitory cytokines (e.g., IL10, IL-35, or TGF-β) and/or cell-cell contact [4].

TGF-β has long been known to exert anti-inflammatory effects on the immune system. Deletion of TGF-β leads to abnormal T cell responses and rapid autoimmunity [72, 74–76]. It also plays an important role in Treg generation and maintenance [72, 73] and together with IL-2 is important for the thymic development of nTregs [77]. TGF-β is also a potent inhibitor of T-cell proliferative response. However, in recent years there has been a significant advance made in understanding the plasticity of CD4+ Th cell lineage commitment. The Th1-Th2 paradigm stood unchallenged for nearly 20 years but in the last 5 years several new Th cell subsets have been defined. Activation of naïve T cells in the presence of TGF-β + IL-6 can induce ROR$\gamma\tau$ expression, and this promotes the differentiation of Th17 cells which play critical roles in bacterial immunity as well as being implicated in autoimmune diseases [78]. Investigating how TGF-β influences the differentiation of Th cell subsets with completely distinct effector functions (i.e., Treg (suppression) and Th17 cell autoimmunity) has been a major focus for immunologists. It is now clear that when T cells encounter TGF-β alone this can be sufficient to induce *Foxp3* expression and allow iTreg development to proceed [70–73]. In contrast, the presence of IL-6 antagonizes Foxp3 expression by a direct effect on AKT activity [79] (discussed further below).

TGF-β signalling by the TGF-βIIR leads to the phosphorylation of Smad2/3 which forms a complex with Smad4 and this complex is translocated to the nucleus to regulate transcription of target genes [80, 81]. One of the targets is Smad7 which is an inhibitory Smad used to attenuate TGF-β signalling by competing with Smad 2/3 for binding to the receptor [80, 81]. The intron 1 of the *Foxp3* gene in T cells contains two NFATs and a Smad3 binding sites that are located upstream of the Stat5 and CREB binding sites [82]. Naïve CD4+ CD25− T cells from *Cblb*$^{-/-}$ and *Itch*$^{-/-}$ mice show poor induction of *Foxp3* expression, and the iTregs induced are functionally less suppressive in coculture experiments with wild-type naïve T cells [45, 46, 83, 84].

To understand the molecular basis of Itch regulation of Foxp3 expression in iTreg cells studies by Liu and colleagues identified that TGF-β-induced early gene product (TIEG1 or KLF10) is induced in response to TGF-β stimulation [84]. Itch can bind to and ubiquitinate TIEG1 leading to both mono- and polyubiquitinated forms. Itch and TIEG1 can bind to the of *Foxp3* promoter leading to its transactivation. Forced expression of TIEG1 in wild-type T cells can induce robust *Foxp3* expression, and cell proliferation was inhibited in the presence of TGF-β [84]. In contrast, overexpression of TIEG1 in *Itch*-deficient T cells led to poor induction of *Foxp3,* and these cells were resistant to TGF-β inhibition *in vitro*. Furthermore, analysis of TIEG1$^{-/-}$ T cells demonstrated that they were also resistant to the inhibitory effects of TGF-β *in vitro,* they failed to induce *Foxp3* expression in the presence of TGF-β *in vitro,* and they were less effective in mediating suppression of growth of wild-type naïve T effector cells. Finally, replacing TIEG1 in TIEG1$^{-/-}$ T cells through retroviral transduction could restore *Foxp3* expression, and these cells could mediate immune suppression [84]. Taken together these results have identified an important pathway by which Itch and TIEG1 controls iTreg cell development by regulating Foxp3 expression. This is likely to have important implications in the control of autoimmune responses to tissue-specific antigens in the periphery.

6. Regulation of T-Cell Activation by Sensing the Environment

The AKT-mTOR pathway has emerged as a central checkpoint that controls the fate of multiple cell types in the immune system [85–87]. Signaling downstream of the TCR and growth factor receptors (e.g., IL-2R) leads to activation of PI3 kinase (PI3K), the serine threonine kinase AKT, and mTOR which in turn leads to increased activity of the cyclin-dependent kinase (e.g., CDK2) that allows cells to traverse the G1 cell cycle restriction check point to stimulate proliferation and avoid the induction of anergy. The AKT-mTOR pathway is highly conserved and is used by eukaryotic cells to integrate environmental cues such as nutrient and amino acid availability, senses energy stores, and directs cellular proliferation [88]. The AKT-mTOR pathway stimulates a switch in cellular metabolism from catabolic to anabolic pathways in activated T cells leading to an increase in glycolysis and the expression of nutrient transporters [88]. Recent studies have revealed that AKT/mTOR signaling is crucial for the survival and homeostasis of naïve and memory T cells, the differentiation of CD4+ Th cell lineages (e.g., Th1, Th2 Th17) [89], CD8+ T cell memory [90, 91], as well as the differentiation of Treg cells in the periphery [79] and lymphocyte trafficking [92]. AKT can in turn activate mTOR and induce the phosphorylation of the Foxo transcription factors (e.g., Foxo 1 and Foxo 3a) which leads to their

nuclear export and subsequent degradation [87]. Treatment of T cells with the mTOR inhibitor rapamycin could block mTOR activity and induce T cell anergy even when T cells received a TCR and costimulatory signal [93]. The induction of anergy requires the complete inhibition of mTOR activity, and there is heightened activation of the calcineurin/NFAT pathway [15]. The AKT-mTOR pathway acts downstream of several energy sensing pathways in eukaryotic cells, that can modulate expression of genes that are involved in the maintenance of anergy, or indirectly mediates degradation of the gene products [85].

7. mTOR in Treg Development and Function

As discussed above, activation of naïve T cells in the presence of TGF-β readily induces expression of *Foxp3*. This induction requires proteins that normally block PI3K activity such as Cbl-b and PTEN [94, 95]. Likewise, constitutive AKT activity in T cells can inhibit Foxp3 expression [79]. Delgoffe et al. [89] recently showed that deletion of mTOR leads to increased expression of *Foxp3* in T cells and that mTOR deficient CD4+ T cells are hypersensitive to TGF-β treatment and switch on *Foxp3*. The effect on *Foxp3* expression appears to be related to mTORC2 activity and not mTORC1. The Foxo transcription factors, Foxo1 and Foxo3a, are nuclear proteins that function downstream of AKT/mTOR signaling, and their activity is regulated by AKT-mediated phosphorylation that inactivates them and they are exported from the nucleus. One of the targets of the E3 ligase Cbl-b is the p85 subunit of PI3K. Active PI3K is required for activation of AKT and so in anergic cells, which express higher levels of Cbl-b, can inactivate PI3K signaling leading to inhibition of mTOR/AKT signalling. Two recent studies have identified that regulation of the Foxo1 and Foxo3a transcription factors is essential for TGF-β-mediated iTreg cell differentiation [83, 96].

In the absence of *Cblb* the Foxo1 and Foxo3 proteins become phosphorylated indicating heightened levels of PI3K-AKT signaling, and this leads to impaired expression of Foxp3 in $Cblb^{-/-}$ T cells when cultured in the presence of TGF-β [83, 96]. The loss of *Cblb* does not impact on nTreg differentiation in the thymus and does not influence cell numbers in the periphery. Ouyang et al. found that in the absence of both Foxo1 and Foxo3a T cells were unable to induce Foxp3 expression and when cultured in the presence of TGF-β did not affect nTreg differentiation. The iTregs from double knockout mice also showed increased expression of proinflammatory genes (e.g., IL-17 and IFN-γ) which are normally absent from iTreg cells. In addition, many of the Treg signature genes were also modulated in these cells. What is surprising is that the PI3K/AKT/mTOR/Foxo pathway appears to be more active in the iTreg cells and not the nTreg cells that develop in the thymus. From the recent studies it appears that AKT/mTOR signaling only affects the induction of Foxp3 expression and it does not have any influence on the maintenance of Foxp3 expression in iTreg cells.

8. Concluding Remarks

The development of T1D is caused by the breakdown of immune tolerance to islet-specific antigens in the pancreas. At present we do not understand how the breakdown in immune tolerance to islet-specific antigen occurs in most individuals. The last decade has seen some important developments in the identification of key genes that play an essential role in the regulation of peripheral immune responses as well as the further clarification of autoimmune checkpoints that try to limit organ-specific autoimmune diseases such as T1D. The recent discovery regarding the plasticity of CD4+ Th cell development *in vivo* has led to a greater insight into the process of disease pathology. Understanding the molecular regulation between inflammatory T cell subsets and Treg cells offers the potential for therapeutic intervention in diseases such as T1D.

Clearly the immune system places an important effort in controlling the activation of naïve T cells as it strives to prevent inappropriate T effector cell activation and differentiation. As highlighted in this paper the family of E3 ubiquitin ligases play a critical role in the immune system and dysregulation of any of one of these genes can have important implications in the development of autoimmunity by disrupting processes such as clonal anergy, Treg differentiation, and generation of autoantibody responses. The next decade should hopefully see even more exciting discoveries as the fruits of various genetic screens in both mice and humans should begin to uncover immune checkpoints that have not been previously identified and should help to provide a better understanding of the disease process of T1D in humans that may eventually lead to the "holy grail" where we can cure people of this debilitating and life-threatening disease.

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References

[1] C. C. Goodnow, J. Sprent, ST. G. B. F. De, and C. G. Vinuesa, "Cellular and genetic mechanisms of self tolerance and autoimmunity," *Nature*, vol. 435, no. 7042, pp. 590–597, 2005.

[2] H. Von Boehmer and F. Melchers, "Checkpoints in lymphocyte development and autoimmune disease," *Nature Immunology*, vol. 11, no. 1, pp. 14–20, 2010.

[3] D. Mathis and C. Benoist, "Back to central tolerance," *Immunity*, vol. 20, no. 5, pp. 509–516, 2004.

[4] S. Sakaguchi, "Naturally arising Foxp3-expressing CD25+ CD4+ regulatory T cells in immunological tolerance to self and non-self," *Nature Immunology*, vol. 6, no. 4, pp. 345–352, 2005.

[5] R. H. Schwartz, "T cell anergy," *Annual Review of Immunology*, vol. 21, pp. 305–334, 2003.

[6] D. Mathis, L. Vence, and C. Benoist, "β-cell death during progression to diabetes," *Nature*, vol. 414, no. 6865, pp. 792–798, 2001.

[7] J. Sprent, J. H. Cho, O. Boyman, and C. D. Surh, "T cell homeostasis," *Immunology and Cell Biology*, vol. 86, no. 4, pp. 312–319, 2008.

[8] S. M. Kang, A. C. Tran, M. Grilli, and M. J. Lenardo, "NF-(κ)B subunit regulation in nontransformed CD4 T lymphocytes," *Science*, vol. 256, no. 5062, pp. 1452–1455, 1992.

[9] D. Tzachanis, G. J. Freeman, N. Hirano et al., "Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells," *Nature Immunology*, vol. 2, no. 12, pp. 1174–1182, 2001.

[10] J. Wang, S. Lee, C. E. Y. Teh, K. Bunting, L. Ma, and M. F. Shannon, "The transcription repressor, ZEB1, cooperates with CtBP2 and HDAC1 to suppress IL-2 gene activation in T cells," *International Immunology*, vol. 21, no. 3, pp. 227–235, 2009.

[11] S. Grundström, P. Anderson, P. Scheipers, and A. Sundstedt, "Bcl-3 and NFkappaB p50-p50 homodimers act as transcriptional repressors in tolerant CD4+ T cells," *Journal of Biological Chemistry*, vol. 279, no. 9, pp. 8460–8468, 2004.

[12] G. A. Martins, L. Cimmino, J. Liao, E. Magnusdottir, and K. Calame, "Blimp-1 directly represses Il2 and the Il2 activator Fos, attenuating T cell proliferation and survival," *Journal of Experimental Medicine*, vol. 205, no. 9, pp. 1959–1965, 2008.

[13] A. H. Sharpe, "Mechanisms of costimulation," *Immunological Reviews*, vol. 229, no. 1, pp. 5–11, 2009.

[14] T. R. Malek and I. Castro, "Interleukin-2 receptor signaling: at the interface between tolerance and immunity," *Immunity*, vol. 33, no. 2, pp. 153–165, 2010.

[15] A. D. Wells, "New insights into the molecular basis of T cell anergy: anergy factors, avoidance sensors, and epigenetic imprinting," *Journal of Immunology*, vol. 182, no. 12, pp. 7331–7341, 2009.

[16] J. R. Lamb and M. Feldmann, "Essential requirement for major histocompatibility complex recognition in T-cell tolerance induction," *Nature*, vol. 308, no. 5954, pp. 72–74, 1984.

[17] V. Heissmeyer, F. Macián, S. H. Im et al., "Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins," *Nature Immunology*, vol. 5, no. 3, pp. 255–265, 2004.

[18] F. Macián, F. García-Cózar, S. H. Im, H. F. Horton, M. C. Byrne, and A. Rao, "Transcriptional mechanisms underlying lymphocyte tolerance," *Cell*, vol. 109, no. 6, pp. 719–731, 2002.

[19] E. R. Kearney, K. A. Pape, D. Y. Loh, and M. K. Jenkins, "Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo," *Immunity*, vol. 1, no. 4, pp. 327–339, 1994.

[20] G. F. Hoyne, R. E. O'Hehir, D. C. Wraith, W. R. Thomas, and J. R. Lamb, "Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice," *Journal of Experimental Medicine*, vol. 178, no. 5, pp. 1783–1788, 1993.

[21] B. Metzler and D. C. Wraith, "Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity," *International Immunology*, vol. 5, no. 9, pp. 1159–1165, 1993.

[22] Y. Chen, V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner, "Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis," *Science*, vol. 265, no. 5176, pp. 1237–1240, 1994.

[23] P. Garside, M. Steel, F. Y. Liew, and A. M. Mowat, "CD4 but not CD8 T cells are required for the induction of oral tolerance," *International Immunology*, vol. 7, no. 3, pp. 501–504, 1995.

[24] S. Bandyopadhyay, M. Duré, M. Paroder, N. Soto-Nieves, I. Puga, and F. Macián, "Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells," *Blood*, vol. 109, no. 7, pp. 2878–2886, 2007.

[25] J. E. Harris, K. D. Bishop, N. E. Phillips et al., "Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4+ T cells," *Journal of Immunology*, vol. 173, no. 12, pp. 7331–7338, 2004.

[26] C. G. King, J. L. Buckler, T. Kobayashi et al., "Cutting edge: requirement for TRAF6 in the induction of T cell anergy," *Journal of Immunology*, vol. 180, no. 1, pp. 34–38, 2008.

[27] I. Puga, A. Rao, and F. Macian, "Targeted cleavage of signaling proteins by caspase 3 inhibits T cell receptor signaling in anergic T cells," *Immunity*, vol. 29, no. 2, pp. 193–204, 2008.

[28] M. Safford, S. Collins, M. A. Lutz et al., "Egr-2 and Egr-3 are negative regulators of T cell activation," *Nature Immunology*, vol. 6, no. 5, pp. 472–480, 2005.

[29] R. M. Thomas, N. Chunder, C. Chen, S. E. Umetsu, S. Winandy, and A. D. Wells, "Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4+ T lymphocytes," *Journal of Immunology*, vol. 179, no. 11, pp. 7305–7315, 2007.

[30] P. E. Fields, T. F. Gajewski, and F. W. Fitch, "Blocked Ras activation in anergic CD4+ T cells," *Science*, vol. 271, no. 5253, pp. 1276–1278, 1996.

[31] S. M. Kang, B. Beverly, A. C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo, "Transactivation by AP-1 is a molecular target of T cell clonal anergy," *Science*, vol. 257, no. 5073, pp. 1134–1138, 1992.

[32] Y. Zha, R. Marks, A. W. Ho et al., "T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α," *Nature Immunology*, vol. 7, no. 11, pp. 1166–1173, 2006.

[33] B. A. Olenchock, R. Guo, J. H. Carpenter et al., "Disruption of diacylglycerol metabolism impairs the induction of T cell anergy," *Nature Immunology*, vol. 7, no. 11, pp. 1174–1181, 2006.

[34] A. E. Lin and T. W. Mak, "The role of E3 ligases in autoimmunity and the regulation of autoreactive T cells," *Current Opinion in Immunology*, vol. 19, no. 6, pp. 665–673, 2007.

[35] D. L. Mueller, "E3 ubiquitin ligases as T cell anergy factors," *Nature Immunology*, vol. 5, no. 9, pp. 883–890, 2004.

[36] C. A. Ross and C. M. Pickart, "The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases," *Trends in Cell Biology*, vol. 14, no. 12, pp. 703–711, 2004.

[37] Y. A. Lam, C. M. Pickart, A. Alban et al., "Inhibition of the ubiquitin-proteasome system in Alzheimer's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 18, pp. 9902–9906, 2000.

[38] M. S. Jeon, A. Atfield, K. Venuprasad et al., "Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction," *Immunity*, vol. 21, no. 2, pp. 167–177, 2004.

[39] N. Yokoi, K. Komeda, HE. Y. Wang et al., "Cblb is a major susceptibility gene for rat type 1 diabetes mellitus," *Nature Genetics*, vol. 31, no. 4, pp. 391–394, 2002.

[40] M. S. Anderson, E. S. Venanzi, Z. Chen, S. P. Berzins, C. Benoist, and D. Mathis, "The cellular mechanism of Aire control of T cell tolerance," *Immunity*, vol. 23, no. 2, pp. 227–239, 2005.

[41] A. Liston, D. H. D. Gray, S. Lesage et al., "Gene dosage-limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity," *Journal of Experimental Medicine*, vol. 200, no. 8, pp. 1015–1026, 2004.

[42] A. Liston, S. Lesage, J. Wilson, L. Peltonen, and C. C. Goodnow, "Aire regulates negative selection of organ-specific T cells," *Nature Immunology*, vol. 4, no. 4, pp. 350–354, 2003.

[43] J. Aaltonen, P. Björses, J. Perheentupa et al., "An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains," *Nature Genetics*, vol. 17, no. 4, pp. 399–403, 1997.

[44] C. E. Teh, S. R. Daley, A. Enders, and C. C. Goodnow, "T-cell regulation by casitas B-lineage lymphoma (Cblb) is a critical failsafe against autoimmune disease due to autoimmune regulator (Aire) deficiency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 33, pp. 14709–14714, 2010.

[45] G. F. Hoyne, E. Flening, M. Yabas et al., "Visualizing the role of Cbl-b in control of islet-reactive CD4 T cells and susceptibility to type 1 diabetes," *Journal of Immunology*, vol. 186, no. 4, pp. 2024–2032, 2011.

[46] K. Venuprasad, C. Elly, M. Gao et al., "Convergence of itch-induced ubiquitination with MEKK1-JNK signaling in Th2 tolerance and airway inflammation," *Journal of Clinical Investigation*, vol. 116, no. 4, pp. 1117–1126, 2006.

[47] W. L. Perry, C. M. Hustad, D. A. Swing, T. Norene O'Sullivan, N. A. Jenkins, and N. G. Copeland, "The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a(18H) mice," *Nature Genetics*, vol. 18, no. 2, pp. 143–146, 1998.

[48] D. Fang, C. Elly, B. Gao et al., "Dysregulation of T lymphocyte function in itchy mice: a role for Itch in T2 differentiation," *Nature Immunology*, vol. 3, no. 3, pp. 281–287, 2002.

[49] E. Scharschmidt, E. Wegener, V. Heissmeyer, A. Rao, and D. Krappmann, "Degradation of Bcl10 induced by T-cell activation negatively regulates NF-kappa B signaling," *Molecular and Cellular Biology*, vol. 24, no. 9, pp. 3860–3873, 2004.

[50] M. Gao, T. Labuda, Y. Xia et al., "Jun turnover is controlled through JNK-dependent phosphorylation of the E3 ligase itch," *Science*, vol. 306, no. 5694, pp. 271–275, 2004.

[51] B. Yang, D. L. Gay, M. K. L. MacLeod et al., "Nedd4 augments the adaptive immune response by promoting ubiquitin-mediated degradation of Cbl-b in activated T cells," *Nature Immunology*, vol. 9, no. 12, pp. 1356–1363, 2008.

[52] K. F. Harvey, L. M. Shearwin-Whyatt, A. Fotia, R. G. Parton, and S. Kumar, "N4WBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein," *Journal of Biological Chemistry*, vol. 277, no. 11, pp. 9307–9317, 2002.

[53] C. N. Jolliffe, K. F. Harvey, B. P. Haines, G. Parasivam, and S. Kumar, "Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4," *Biochemical Journal*, vol. 351, no. 3, pp. 557–565, 2000.

[54] P. M. Oliver, X. Cao, G. S. Worthen et al., "Ndfip1 protein promotes the function of itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation," *Immunity*, vol. 25, no. 6, pp. 929–940, 2006.

[55] N. Anandasabapathy, G. S. Ford, D. Bloom et al., "GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells," *Immunity*, vol. 18, no. 4, pp. 535–547, 2003.

[56] L. Su, N. Lineberry, Y. Huh, L. Soares, and C. G. Fathman, "A novel E3 ubiquitin ligase substrate screen identifies Rho guanine dissociation inhibitor as a substrate of gene related to anergy in lymphocytes," *Journal of Immunology*, vol. 177, no. 11, pp. 7559–7566, 2006.

[57] R. I. Nurieva, S. Zheng, W. Jin et al., "The E3 ubiquitin ligase GRAIL regulates T cell tolerance and regulatory T cell function by mediating T cell receptor-CD3 degradation," *Immunity*, vol. 32, no. 5, pp. 670–680, 2010.

[58] E. Chiffoleau, T. Kobayashi, M. C. Walsh et al., "TNF receptor-associated factor 6 deficiency during hemopoiesis induces Th2-polarized inflammatory disease," *Journal of Immunology*, vol. 171, no. 11, pp. 5751–5759, 2003.

[59] C. G. Vinuesa, M. C. Cook, C. Angelucci et al., "A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity," *Nature*, vol. 435, no. 7041, pp. 452–458, 2005.

[60] M. A. Linterman, R. J. Rigby, R. Wong et al., "Roquin differentiates the specialized functions of duplicated T cell costimulatory receptor genes CD28 and ICOS," *Immunity*, vol. 30, no. 2, pp. 228–241, 2009.

[61] D. Yu, A. H. M. Tan, X. Hu et al., "Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA," *Nature*, vol. 450, no. 7167, pp. 299–303, 2007.

[62] S. Akkaraju, W. Y. Ho, D. Leong, K. Canaan, M. M. Davis, and C. C. Goodnow, "A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis," *Immunity*, vol. 7, no. 2, pp. 255–271, 1997.

[63] C. L. Bennett, M. E. Brunkow, F. Ramsdell et al., "A rare polyadenylation signal mutation of the FOXP3 gene (AAUAAA → AAUGAA) leads to the IPEX syndrome," *Immunogenetics*, vol. 53, no. 6, pp. 435–439, 2001.

[64] C. L. Bennett, J. Christie, F. Ramsdell et al., "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3," *Nature Genetics*, vol. 27, no. 1, pp. 20–21, 2001.

[65] M. E. Brunkow, E. W. Jeffery, K. A. Hjerrild et al., "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse," *Nature Genetics*, vol. 27, no. 1, pp. 68–73, 2001.

[66] Y. J. Chiang, H. K. Kole, K. Brown et al., "Cbl-b regulates the CD28 dependence of T-cell activation," *Nature*, vol. 403, no. 6766, pp. 216–220, 2000.

[67] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[68] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor Foxp3," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[69] C. Chen, E. A. Rowell, R. M. Thomas, W. W. Hancock, and A. D. Wells, "Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation," *Journal of Biological Chemistry*, vol. 281, no. 48, pp. 36828–36834, 2006.

[70] W. Chen, W. Jin, N. Hardegen et al., "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3," *Journal of Experimental Medicine*, vol. 198, no. 12, pp. 1875–1886, 2003.

[71] M. C. Fantini, C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath, "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7," *Journal of Immunology*, vol. 172, no. 9, pp. 5149–5153, 2004.

[72] M. O. Li, S. Sanjabi, and R. Flavell, "Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms," *Immunity*, vol. 25, no. 3, pp. 455–471, 2006.

[73] Y. Y. Wan and R. A. Flavell, "Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 14, pp. 5126–5131, 2005.

[74] A. B. Kulkarni, C. G. Huh, D. Becker et al., "Transforming growth factor β null mutation in mice causes excessive inflammatory response and early death," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 2, pp. 770–774, 1993.

[75] J. C. Marie, J. J. Letterio, M. Gavin, and A. Y. Rudensky, "TGF-β1 maintains suppressor function and Foxp3 expression in CD4+ CD25+ regulatory T cells," *Journal of Experimental Medicine*, vol. 201, no. 7, pp. 1061–1067, 2005.

[76] M. M. Shull, I. Ormsby, A. B. Kier et al., "Targeted disruption of the mouse transforming growth factor-β1 gene results in multifocal inflammatory disease," *Nature*, vol. 359, no. 6397, pp. 693–699, 1992.

[77] Y. Liu, P. Zhang, J. Li, A. B. Kulkarni, S. Perruche, and W. Chen, "A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells," *Nature Immunology*, vol. 9, no. 6, pp. 632–640, 2008.

[78] D. R. Littman and A. Y. Rudensky, "Th17 and regulatory T cells in mediating and restraining inflammation," *Cell*, vol. 140, no. 6, pp. 845–858, 2010.

[79] S. Haxhinasto, D. Mathis, and C. Benoist, "The AKT-mTOR axis regulates de novo differentiation of CD4+ Foxp3+ cells," *Journal of Experimental Medicine*, vol. 205, no. 3, pp. 565–574, 2008.

[80] J. Massague, "How cells read TGF-β signals," *Nature Reviews Molecular Cell Biology*, vol. 1, no. 3, pp. 169–178, 2000.

[81] J. Massague, J. Seoane, and D. Wotton, "Smad transcription factors," *Genes and Development*, vol. 19, no. 23, pp. 2783–2810, 2005.

[82] Y. Tone, K. Furuuchi, Y. Kojima, M. L. Tykocinski, M. I. Greene, and M. Tone, "Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer," *Nature Immunology*, vol. 9, no. 2, pp. 194–202, 2008.

[83] Y. Harada, Y. Harada, C. Elly et al., "Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells," *Journal of Experimental Medicine*, vol. 207, no. 7, pp. 1381–1391, 2010.

[84] K. Venuprasad, H. Huang, Y. Harada et al., "The E3 ubiquitin ligase Itch regulates expression of transcription factor Foxp3 and airway inflammation by enhancing the function of transcription factor TIEG1," *Nature Immunology*, vol. 9, no. 3, pp. 245–253, 2008.

[85] G. M. Delgoffe and J. D. Powell, "MTOR: taking cues from the immune microenvironment," *Immunology*, vol. 127, no. 4, pp. 459–465, 2009.

[86] R. G. Jones and C. B. Thompson, "Revving the engine: signal transduction fuels T cell activation," *Immunity*, vol. 27, no. 2, pp. 173–178, 2007.

[87] J. D. Powell and G. M. Delgoffe, "The mammalian target of rapamycin: linking T cell differentiation, function, and metabolism," *Immunity*, vol. 33, no. 3, pp. 301–311, 2010.

[88] D. M. Sabatini, "mTOR and cancer: insights into a complex relationship," *Nature Reviews Cancer*, vol. 6, no. 9, pp. 729–734, 2006.

[89] G. M. Delgoffe, T. P. Kole, Y. Zheng et al., "The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment," *Immunity*, vol. 30, no. 6, pp. 832–844, 2009.

[90] K. Araki, A. P. Turner, V. O. Shaffer et al., "mTOR regulates memory CD8 T-cell differentiation," *Nature*, vol. 460, no. 7251, pp. 108–112, 2009.

[91] R. R. Rao, Q. Li, K. Odunsi, and P. A. Shrikant, "The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin," *Immunity*, vol. 32, no. 1, pp. 67–78, 2010.

[92] L. V. Sinclair, D. Finlay, C. Feijoo et al., "Phosphatidylinositol-3-OH kinase and nutrient-sensing mTOR pathways control T lymphocyte trafficking," *Nature Immunology*, vol. 9, no. 5, pp. 513–521, 2008.

[93] J. D. Powell, C. G. Lerner, and R. H. Schwartz, "Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation," *Journal of Immunology*, vol. 162, no. 5, pp. 2775–2784, 1999.

[94] S. Sauer, L. Bruno, A. Hertweck et al., "T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 22, pp. 7797–7802, 2008.

[95] E. A. Wohlfert, L. Gorelik, R. Mittler, R. A. Flavell, and R. B. Clark, "Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-β sensitivity in vitro and in vivo," *Journal of Immunology*, vol. 176, no. 3, pp. 1316–1320, 2006.

[96] W. Ouyang, O. Beckett, Q. Ma, J. H. Paik, R. A. Depinho, and M. O. Li, "Foxo proteins cooperatively control the differentiation of Foxp3 regulatory T cells," *Nature Immunology*, vol. 11, no. 7, pp. 618–627, 2010.

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Review Article

Type I Diabetes-Associated Tolerogenic Properties of Interleukin-2

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Type 1 Diabetes (T1D) results from insulin-producing beta cells destruction by diabetogenic T lymphocytes in humans and nonobese diabetic (NOD) mice. The breakdown of tolerance has been associated with a defect in the number and the function of naturally occurring regulatory T cells (nTreg) that are the master player in peripheral tolerance. Gene knockout experiments in mouse models have shown a nonredundant activity of IL-2 related to its critical role in inducing nTreg and controlling peripheral T cell tolerance. Whereas strong evidence has suggested that IL-2 is critically required for nTreg-mediated T1D control, several fundamental questions remain to be addressed. In this paper, we highlight the recent findings and controversies regarding the tolerogenic properties of IL-2 mediated through nTreg. We further discuss a potential link between the immunomodulatory role of interleukin-2 and the pathogenesis of type 1 diabetes.

1. Introduction

The induction of tolerance is critical for the maintenance of immune homeostasis and the prevention of autoimmune diseases, including type 1 diabetes (T1D). Tregs are crucial for suppressing autoimmune responses and maintaining peripheral immunological tolerance [1]. Defects in the number and function of immunoregulatory $CD4^+$ T cells (nTregs) play a critical role in the breakdown of immune tolerance in the experimental model of spontaneous autoimmune diabetes nonobese diabetic (NOD) mouse [2, 3] and in humans with genetic susceptibility to T1D. Tregs arise during the normal process of T cell maturation in the thymus, and their differentiation can be induced (iTreg) in the periphery by conversion of naive $CD4^+CD25^-Foxp3^-$ Tregs into $CD4^+CD25^+Foxp3^+$ Tregs [4, 5]. The influence of nTregs in maintaining T cell tolerance is strongly supported by the observations of the development of autoimmune syndromes in mice lacking nTregs and by the findings that defects in Foxp3 gene expression in humans and mice lead to autoimmune syndromes in early life [6, 7]. In agreement with these observations, the prevention of other autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and type 1 diabetes (T1D) has been achieved by reconstitution of autoimmune-prone mice with nTregs [8]. Emerging evidence has revealed the involvement of IL-2 as a major regulator of the survival and suppressive function of nTreg [9, 10]. Work from Santamaria's group [11] has revealed that IL-2 production was reduced in NOD mice and correlated with impairment in nTreg function. Furthermore, treatment with IL-2 has been shown to induce Treg expansion and activation in humans and mice [9] and protection against diabetes in NOD mice [12]. In the last decade, much progress has been made in

understanding the role of the IL-2/IL-2 receptor (IL-2R) axis in promoting nTreg differentiation and its importance in the interface between tolerance and autoimmunity. This paper primarily focuses on our current understanding of the role of IL-2/nTreg in regulating autoimmune diabetes and its potential therapeutic application in patients with T1D. Recently, it has been shown that the administration of low doses of IL-2 at the onset of diabetes can induce a long-lasting remission in NOD mice. Interestingly, IL-2 did not stimulate autoreactive effector T cells but rather specifically stimulated $CD4^+Foxp3^+$ Tregs in the pancreas, resulting in dampening the influence of the inflammatory environment [12]. Here, we further highlight the role of IL-2/IL-2R in autoimmune T1D, specifically through the modulation of nTregs development and function.

2. Biological Importance of IL-2/IL-2R Signaling

IL-2 is a 15 kDa 4-bundled α-helical protein mainly produced by activated $CD4^+$ T lymphocytes. However, the expression of IL-2 by naïve $CD8^+$ T cells, dendritic cells, and thymic cells has also been reported [13, 14]. The magnitude and duration of the T cell immune response is dependent on the interaction of IL-2 with its high-affinity IL-2 receptor (IL-2R) which is composed of α, β and γ subunits. The intermediate affinity IL-2R is composed of IL-2Rβ (CD122) and IL-2Rγ (CD132) and is constitutively expressed on resting T lymphocytes. However, IL-2Rα (CD25) is only induced after T-cell activation, which allows the formation of the high-affinity IL-2R [15]. The biological activities resulting from the binding of IL-2 to its receptor on T-cells have not yet been fully defined. Evidence has shown that in conventional T cells, the effects of occupation of the high affinity IL-2R are mediated by at least two major signaling pathways, the JAK-STAT and the PI3K pathways. The activation of the JAK-STAT pathway is initiated by the activation of the Janus Kinases (JAK) JAK1 and JAK3. JAK1 is primarily associated with the serine-rich region of the IL-2R, whereas JAK3 seems to be associated with both the proximal and distal regions of the cytoplasmic domain of the common γ-chain. The stimulation of JAK molecules initiates a cascade of activation involving the signal transducers and activators of transcription (STAT5a/b) factors as well as the phosphatidylinositol 3-kinse (PI3K) and ras-mitogen-activated protein kinase (MAPK) signaling pathways [15, 16]. The activation of these signaling molecules results in modulation of target gene expression involved in cell cycle progression (gene encoding cycling family proteins A, B, C, D2, D3, and E), anti-apoptosis (Cmyc, C-fos, C-jun, Bcl2, and bclx), and in the suppression of cytokine (SOCS and CIS) signaling (Figure 1) [17, 18].

The JAK/STAT5 pathway is important for many cellular responses, including differentiation, proliferation, and oncogenesis. For example, $STAT5^{-/-}$ mice have a profound defect in mammary gland development and in prolactin response, whereas $STAT5B^{-/-}$ mice display a defect in growth hormone response [19]. Simultaneous inactivation of STAT5A/B genes has revealed a requirement for both proteins in myeloid and lymphoid cell proliferation. Indeed, myeloid cells, mast cells, peripheral T cells, and NK and B cells display impaired proliferation and/or survival in $STAT5^{-/-}$ mice [20–22]. STAT5 has also been shown to be involved in maintaining $CD4^+CD25^+$ regulatory T cell homeostasis and self-tolerance [23], Th2 differentiation, and $CD8^+$ T cell homeostasis [24]. $STAT5^{-/-}$ mice have a decreased number of $CD8^+$ T cells, whereas STAT5 transgenic mice have an increased number of these cells, which correlates with anti-apoptotic protein Bcl-x_L expression [25]. Ectopic expression of Bcl-x rescues $STAT5^{-/-}$ BM cells from apoptosis indicating that STAT5 promotes survival of myeloid progenitor cells through the ability to induce the transcription of the Bcl-x gene [26]. Finally, $STAT5^{-/-}$ mice exhibit autoimmune pathology in a manner very similar to IL-2-deficient mice. This disease correlates with decreased numbers of nTregs, which undergo apoptosis at increased rates in the absence of STAT5 [26]. Recently, it has been reported that a patient, with a missense mutation in the STAT5B gene, had no detectable expression of STAT5B and had decreased numbers and impaired function of nTregs [27]. Similarly, JAK3−/− mice have low frequency of nTregs, increased amount of autoreactive T cells, and develop autoimmunity as is the case for IL-2-deficient and IL-2R-deficient mice [23]. These reports underlie the importance of IL-2/IL-2R signaling in the maintenance of immune tolerance.

3. Naturally Occurring $CD4^+CD25^+$ Tregs and Autoimmunity

Naturally occurring $CD4^+CD25^+$ regulatory T cells represent 5%–10% of the $CD4^+$ T cell subset that critically contributes to maintenance of peripheral immune tolerance. These cells arise in the thymus and, after migrating to the periphery, exert their immunoregulatory functions. They are potent suppressor of organ-specific autoimmune diseases such as T1D, inflammatory bowel disease and gastritis, and they control allograft rejection and immunity to infectious agents such as parasites and viruses [28–30]. In mice, a day-3 neonatal thymectomy (d3Tx) leads to the development of multiorgan autoimmune disease [31]. Subsequently, the depletion of $CD4^+CD25^+$ Tregs leads to spontaneous development of various autoimmune diseases in genetically susceptible animals as well. The adoptive transfer of $CD4^+CD25^+$ nTreg prevents the development of such organ-specific autoimmunity [32]. In the T1D NOD mouse model, it has been shown that the pool of $CD4^+CD25^+$ regulatory T cells decreases with the progression of diabetes [33, 34] and that administration of these cells affords protection against the development of diabetes [35, 36]. In the mouse, nTreg are considered as resting antigen-experienced cells and display heterogeneous combination of cell surface markers associated with naive and activated T cells. nTregs constitutively express high-affinity IL-2Rα (CD25) and Foxp3, a forkhead winged helix transcriptional regulator that controls their development and functions [37]. The mutation of Foxp3 resulted in drastic loss of nTregs and fatal lymphoproliferative process that leads to multiorgan-autoimmune diseases of nTregs in mice and humans. The transfer of nTreg cells from wild-type mice

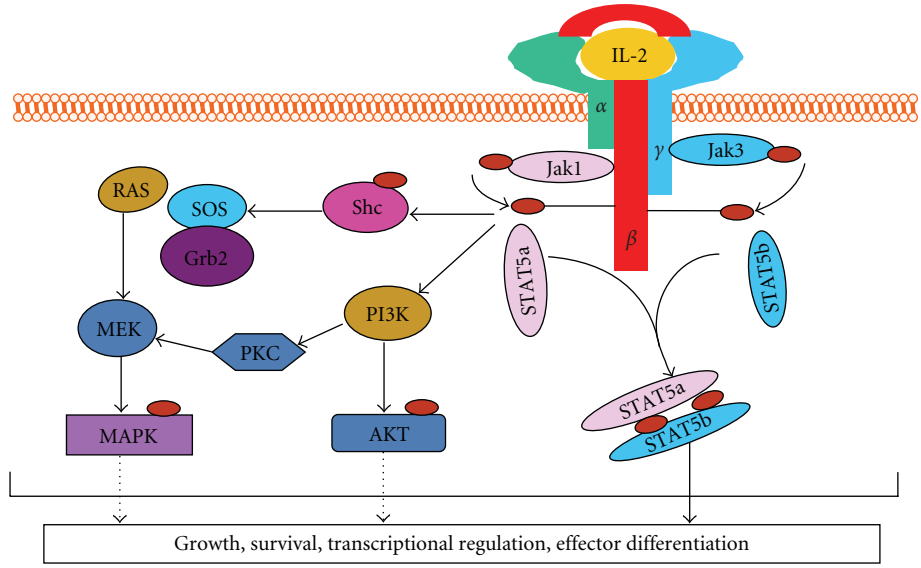

Figure 1: IL-2 receptor signaling. The binding of IL-2 to IL-2R leads to the initiation of signal transduction. Janus-activated kinase 3 (JAK3) molecules that are associated with the γc, and JAK1 molecules that are associated with IL-2Rβ, phosphorylate (●) tyrosine residues in the cytoplasmic tail of IL-2Rβ, the γc and the JAK molecules themselves. The JAKs activation induced a cascade of activation of signal transducer and activator of transcription (STAT5a/b) factors, leading to their dimerization translocation to the nucleus, as well as phosphatidylinositol 3-kinse (PI3K) and ras-mitogen-activated protein kinase (MAPK) signaling pathways. These signaling molecules activation results in, modulation of target genes expression involved in cell cycles progression, antiapoptosis, and in the suppression of cytokine signaling.

to scurfy mice rescues the animals from the fatal disease [7, 38]. Other cell surface markers have been shown to be expressed by nTregs, including high levels of CD5, CD62L, and CD69. Beside the expression of high-affinity IL-2Rα (CD25), at resting state, nTreg constitutively express high levels of other feature markers such as the glucocorticoid-induced TNFR (GITR) family of related proteins [39], OX40 (CD134) [40, 41], and CTLA-4 [42, 43] that also contribute to their suppressive function. Importantly, mice deficient in Foxp3 gene (scurfy mice) rapidly develop a fatal lymphoproliferative disease similar to that seen in mice lacking CTLA-4 or TGF-β [44]. Recently, it has been shown that OX40 is a key factor in shaping nTregs sensitivty to IL-2 and promoting their proliferation and survival toward accurate immune regulation [45].

We have previously reported that tolerogenic DCs are critical in maintaining nTregs pool in NOD mice. Using adoptive transfer experiments, we have shown that depletion of tolerogenic DCs before transferring splenocytes from diabetes-free NOD mice restored their diabetogenic potential, thereby underlying the importance of tolerogenic DC in nTregs maintenance [46]. Naïve $CD4^+CD25^-$ T cells can be also converted into regulatory-like T cells after transduction with a retroviral vector coding for the FoxP3 gene. The transfer of these converted Tregs has been shown to prevent the development of autoimmune IBD [5, 38, 47]. Collectively, these data clearly indicate that Foxp3 plays a critical role for both development and function of nTreg in mice.

4. Cytokines Profile and Cytokines Receptor Signaling in nTreg

The mechanisms by which IL-2 or other cytokines exert their effects via the cytokine receptors on nTreg have not been fully investigated yet. In conventional T cells, the effects of IL-2 are mediated by at least two major signaling pathways, the JAK-STAT and PI3K pathways. Several different studies have examined the role of JAK-STAT molecules in $CD4^+CD25^+$ nTreg development and function. The importance of JAK3 in IL-2 signaling has been further substantiated by the recent findings that homozygous point mutations or deletions of the Jak3 gene are found in autosomal recessive T-B+ severe combined immunodeficiency patients [48] as well as immunocompromised mice [49]. Indeed, mice deficient in JAK3 or STAT5A/B develop a severe immunodeficiency that is followed by multiple organ autoimmune diseases, a decreased number or the absence of $CD4^+CD25^+$ nTreg in their peripheral lymphoid organs, and early death. However, these mice have normal numbers of Tregs in the thymus, suggesting the role of these molecules in regulatory T cell development and homeostasis [23, 26]. Interestingly, the overexpression of STAT5b in transgenic mice leads to an increased number of $CD4^+CD25^+$ nTreg [50].

The role of JAK-STAT and PI3K signaling pathways in Treg proliferation and immunoregulatory function is not yet fully defined. Recently, Bensinger et al. [51] have shown that freshly isolated $CD4^+CD25^+$ nTreg maintain an intact JAK-STAT signaling pathway, whereas signaling downstream PI3K

(particularly activation of AKT and p70S6K) is negatively regulated as a result of increased expression of phosphatase and a tensin homolog deleted on chromosome 10 (PTEN). Conversely, we found that *in vitro* stimulated Treg displayed a normal PI3K pathway while inhibiting JAK-STAT pathways, which results from a downregulation of JAK-STAT molecules expression as opposed to kinase activity (Chentoufi et al. Unpublished data). The inhibition of the JAK-STAT pathway could result from the high expression of SOCS-2 and CIS molecules observed 24–48 h following nTreg stimulation with anti-CD3 and IL-2. These results may explain the hypoproliferative activity of regulatory T cells even after activation through TCR in the presence of IL-2. Moreover, IL-2 generates signaling pathways through a cytokine receptor that contains a γ-chain common to several other cytokine receptors including IL-4, IL-7, IL-9, IL-15, and IL-21. These observations add more complexity to the problem of deciphering the role of other cytokines on $CD4^+CD25^+$ nTreg.

Recently, it has been reported that IL-4 can substitute for IL-2 to induce $CD4^+CD25^+$ nTreg-mediated proliferation and suppression *in vitro*. However, nTreg obtained from IL-4−/− mice display normal suppressive activity, suggesting profound differences between *in vitro* and *in vivo* effects [52]. It has also been shown that STAT1-deficient mice are highly susceptible to autoimmune diseases resulting from a reduced number as well as a functional impairment of $CD4^+CD25^+$ nTreg, suggesting a role of IFNs on regulatory T cells [53]. Naturally occurring $CD4^+CD25^+$ T cells constitutively express mRNA coding for IL-10, IL-4, IL-17, IL-21, IFN-γ, TNF-α, and TGF-β but do not express mRNA coding for IL-2 [43].

The biological roles of cytokines production and interaction with their receptors in nTreg development and function have not yet been clearly delineated. Recently, McHugh et al. [43] have shown, through gene expression profiling experiments, that JAK-STAT regulatory proteins such as members of the SOCS family of proteins (SOCS-1, SOCS-3, and CIS) are upregulated in stimulated $CD4^+CD25^+$ nTreg as compared to conventional T cells [43]. The hypoproliferative activity of activated nTreg *in vitro* and the increased expression of SOCS molecules could suggest that these molecules play a role as an internal control mechanism for $CD4^+CD25^+$ nTreg activation and expansion. SOCS molecules function to inhibit the proliferative effects of cytokines such as IFN-γ, IL-4, and IL-2 and can antagonize JAK3 and STAT5 activities [18].

5. Critical Balance of IL-2 for Naturally Occurring $CD4^+CD25^+$ Treg-Mediated T1D Control

IL-2 was originally defined as a growth factor that stimulated the differentiation and proliferation of T lymphocytes [54]. The critical role of IL-2 on the survival and suppressive function of nTreg cells has been well documented [55]. Consistent with this, IL-2 administration has been shown to expand and activate nTreg cells in humans and mice [9, 10]. Thus, although IL-2 has pleiotropic functions, its major impact is to favor nTreg cell activity [56]. Surprisingly, in IL-2 or IL-2R knockout mice, T cells develop, and these mice acquire a lymphoproliferative syndrome and spontaneous autoimmune disease [57]. From these studies, two important roles for IL-2 in immune tolerance can be ascribed. First, the data showed that IL-2 played a critical role in programming T cells for activation-induced cell death (AICD). Second, increased evidence showed that IL-2 played an additional role in immune regulation aside from AICD. For instance, several groups have reported that in IL-2 and IL-2R knockout mice, the lymphoproliferative syndrome could be prevented by rIL-2 injection and bone marrow transplantation from wild-type mice [58].

Of importance, it was observed that IL-2 had to be provided at very early stages. For instance, delivery of IL-2 a few days after birth resulted in low efficiency of disease improvement, suggesting that it was not the lack of IL-2 during later T cell activation in the periphery that was at fault in these mice. The dependence of nTreg cells on IL-2 was first suggested by transfer experiments performed by Klebb et al. These authors transferred spleen cells or thymocytes from IL-2−/− mice treated with rIL-2 for 25–35 days into untreated 6–8-day-old IL-2−/− BALB/C mice. The IL-2 defect on the BALB/C background is particularly severe and leads to death within 3 weeks. Depending on the number of transferred cells and the organ from which they were derived, some of the recipients lived for 7–10 months. Since no more IL-2 was available during that time, the authors suggested that "IL-2 induces a postnatal differentiation/maturation of regulatory cells necessary for self and non-self-discrimination". Kramer et al. have shown that IL-2−/− and IL-2R−/− bone marrow transfer into RAG−/− mice leads to the development of IL-2 deficiency syndrome, and early death of RAG−/− recipient mice [59].

Again, the disease could be prevented by the complementation with bone marrow from IL-2-sufficient mice. Transfer of $CD4^+CD25^+$ nTreg from wild-type mice induced the development of a lymphoproliferative syndrome in IL-2Rα−/− mice [60]. The most striking observation in IL-2−/− mice models is the lack of $CD4^+$ $CD25^+$ nTreg, suggesting a critical role for IL-2/IL-2R signaling in the generation and homeostasis of these cells. Indeed, mice deficient in IL-2, IL-2R [61], JAK-3 [23], or STAT5A/B [26] have an absent or reduced number of thymic and peripheral $CD4^+$ $CD25^+$ nTreg and consequently develop multiple organ autoimmune diseases. To evaluate the role of IL-2 expression on thymic nTreg development, Malek et al. rescued IL-2Rβ knockout mice with an IL-2Rβ transgene that was predominantly expressed in the thymus with negligible expression in the periphery [60, 61]. In marked contrast to IL-2Rβ−/− mice, the IL-2Rβ transgene restored the normal levels of $CD4^+CD25^+$ nTreg and prevented the onset of a fatal autoimmune disease, further reinforcing the notion that IL-2 plays a critical role in the thymic development of nTreg.

The molecular mechanism by which IL-2 exerts its effect on the nTreg cell precursors in the thymus is as of yet not completely understood. In addition, the origin of IL-2 in the thymus does not seem to be from $CD4^+CD25^+$ nTreg themselves or hematopoietic cells, but probably derives

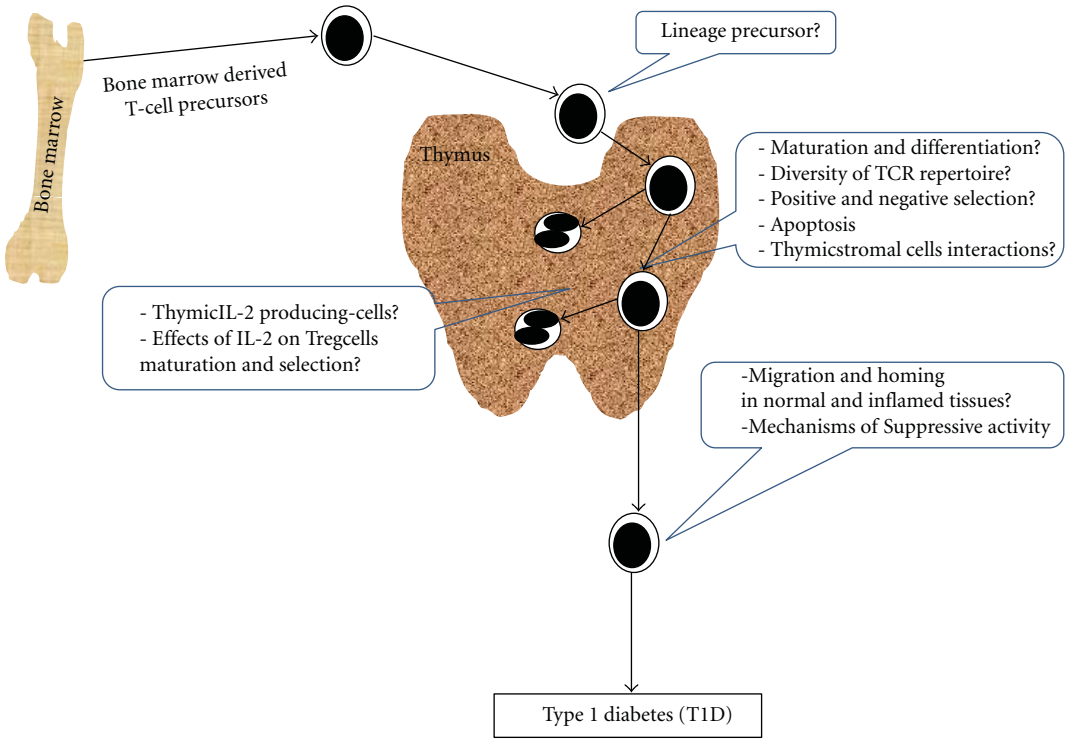

Figure 2: Fundamental questions about naturally occurring $CD4^+$ $CD25^+$ regulatory T cells development and function.

from radio-resistant thymic stromal cells. Indeed, chimeric bone marrow from IL-2−/− and IL-2Rα−/− mice develops functional $CD4^+CD25^+$ nTreg. Furthermore, lethally irradiated RAG2−/− mice that have been reconstituted with IL-2−/− bone morrow develop normal $CD4^+CD25^+$ nTreg [62]. The IL- 2 signaling requirement for peripheral expansion/homeostasis of $CD4^+CD25^+$ nTreg has been shown by Malek et al. when $CD4^+CD25^+$ nTreg from wild-type mice were adoptively transferred to IL-2R deficient mice (which normally produce IL-2). The recipient mice did not develop autoimmune diseases. In contrast, the adoptive transfer of $CD4^+CD25^+$ nTreg from wild-type mice failed to confer the same benefits in IL-2−/− mice [61].

The requirement of IL-2 for nTreg suppression activity is still very controversial. When nTreg are adoptively transferred, they lose CD25 expression and hence are no longer responsive to IL-2. Nevertheless, these cells retain their suppressive activity *in vitro* [63]. However, the addition of IL-2 to nTreg eliminates this suppressive activity *in vitro* [64]. Conversely, $CD4^+CD25^+$ nTreg from wild-type mice transferred to IL-2-deficient mice fail to prevent autoimmunity and *in vitro* suppression activity is completely abrogated by selective blocking of the IL-2 receptor on nTreg during a coculture with responder T cells [65]. Furthermore, treatment of mice with anti-IL-2 or with CTLA-4 immunoglobulin to inhibit costimulatory signals also leads to a rapid decline in the number of $CD4^+CD25^+$ T cells [36].

Besides, NOD mice present a qualitative diminution of IL-2 production [11], and a genetic predisposing factor to T1D development in humans and NOD mice is linked to IL-2/IL-2R gene polymorphisms [66]. It has been recently reported that insufficient IL-2 amounts in the pancreas are responsible for poor nTreg cell survival in this tissue, which could lead to progressive breakdown of self-tolerance and the development of diabetes in NOD mice [67]. It has been recently shown that young prediabetic NOD mice treated with low-dose IL-2 alone, or together with rapamycin, can be protected from the development of disease [67–69]. Recently, it has been shown that low-dose IL-2 administration at diabetes onset can induce long-lasting remission in the treated mice. Interestingly, IL-2 seems to specifically stimulate the $CD4^+CD25^+$ Treg cells in the pancreas rather diabetogenic effector T cells [12]. The mechanisms by which IL-2 selectively stimulate Tregs and reverse the disease in NOD diabetic mice is not yet understood. One mechanism of action of low-dose IL-2 would be the limitation of IFN-γ production by the islet-infiltrating effector T cells. In addition, by expressing high levels of the high affinity IL-2R, Tregs have an advantage in competing for low levels of IL-2 against effector T-cells in the microenvironment.

A number of contradictory observations regarding the number and function of nTreg in diabetic individuals and in mice have been reported. Regulatory T cell numbers have been reported to be decreased or normal in T1D [70–79], and functional assays have similarly described low, slightly decreased, or normal regulatory activity [80–86]. Nevertheless, a consensus regarding a few key points is developing. (i) There is clear evidence for the existence of natural regulatory T cells in both pre-T1D and postdiagnosis

T1D subjects and mice. (ii) Some of the induced Treg cells display similar antigen specificity for β-cell autoantigens of non-T1D subject's Tregs [82]. (iii) In vitro and in vivo expansion of nTreg cells have been successful both in mice and human, particularly aided by the use of rapamycin [87, 88].

6. Conclusion and Perspectives

It has now been strongly established that T cell-mediated dominant immunoregulation is essential for maintaining immunologic self-tolerance and controlling immune responses to non-self-antigens. Among the various kinds of Tregs (iTreg, Tr1, and Th3), naturally occurring $CD4^+CD25^+$ nTreg play a key role in peripheral self-tolerance. A proteomic approach might contribute better to the identification of novel molecules involved in IL-2R signaling pathways for further therapeutic methods and specific cell surface markers for better isolation and characterization purposes. *In vivo*, if IL-2 seems to play a critical role in the thymic development of nTreg, which cells produce IL-2 in the thymus, at which stage of T-cell development IL-2 exert its effects, and what is the importance of IL-2 for the nTreg precursor cells differentiation are critical questions that need to be investigated. A possible explanation is that some thymocytes are confined within special thymic microenvironments containing high doses of IL-2, which could, in principle, transform them into anergic Treg cells. Such an anergic state could be obtained *in vitro* following Th1 clones incubation with IL-2 for 24 to 48 h. These cells are rendered unresponsive to subsequent restimulation with antigen [89] although they remain fully responsive to IL-2 *in vitro* as long as IL-2 is provided. However, when restimulated with appropriate antigen and APC, they do not secrete cytokines, including IL-2 [90]. With respect to T cell maturation and central tolerance, not much is known about nTreg cells thymic maturation/differentiation. Whereas it is thought that nTreg cell generation depends on self-antigen expression in the thymus [91], a recent report from the Mathis' group [92] has shown that nTreg does not necessarily require the thymic expression of agonistic self-antigen. These questions and more need to be further investigated. Certainly, the development of technical tools and animal models will be invaluable to shed light into naturally occurring Treg biology and to provide novel immunomodulatory approaches that target (i) the downregulation of the immune system to prevent/treat autoimmune disease and allograft rejection and (ii) the control of immunity to infectious agents such as parasites and viruses (Figure 2).

Abbreviations

T1D: Type 1 diabetes
nTreg: Naturally occurring regulatory T-cells
IL-2: Interleukin-2.

Conflict of Interests

The authors have declared no conflict of interests.

References

[1] S. Sakaguchi, "Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses," *Annual Review of Immunology*, vol. 22, pp. 531–562, 2004.

[2] S. P. Berzins, E. S. Venanzi, C. Benoist, and D. Mathis, "T-cell compartments of prediabetic NOD mice," *Diabetes*, vol. 52, no. 2, pp. 327–334, 2003.

[3] S. You, M. Belghith, S. Cobbold et al., "Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells," *Diabetes*, vol. 54, no. 5, pp. 1415–1422, 2005.

[4] D. J. Campbell and S. F. Ziegler, "Opinion: FOXP3 modifies the phenotypic and functional properties of regulatory T cells," *Nature Reviews Immunology*, vol. 7, no. 4, pp. 305–310, 2007.

[5] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor Foxp3," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[6] C. L. Bennett, J. Christie, F. Ramsdell et al., "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3," *Nature Genetics*, vol. 27, no. 1, pp. 20–21, 2001.

[7] R. S. Wildin, F. Ramsdell, J. Peake et al., "X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy," *Nature Genetics*, vol. 27, no. 1, pp. 18–20, 2001.

[8] H. Liu and B. P. Leung, "$CD4^+CD25^+$ regulatory T cells in health and disease," *Clinical and Experimental Pharmacology and Physiology*, vol. 33, no. 5-6, pp. 519–524, 2006.

[9] M. Ahmadzadeh and S. A. Rosenberg, "IL-2 administration increases $CD4^+CD25^{hi}$ $Foxp3^+$ regulatory T cells in cancer patients," *Blood*, vol. 107, no. 6, pp. 2409–2414, 2006.

[10] H. Zhang, K. S. Chua, M. Guimond et al., "Lymphopenia and interleukin-2 therapy alter homeostasis of $CD4^+CD25^+$ regulatory T cells," *Nature Medicine*, vol. 11, no. 11, pp. 1238–1243, 2005.

[11] J. Yamanouchi, D. Rainbow, P. Serra et al., "Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity," *Nature Genetics*, vol. 39, no. 3, pp. 329–337, 2007.

[12] Y. Grinberg-Bleyer, A. Baeyens, S. You et al., "IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells," *Journal of Experimental Medicine*, vol. 207, no. 9, pp. 1871–1878, 2010.

[13] H. Bassiri and S. R. Carding, "A requirement for IL-2/IL-2 receptor signaling in intrathymic negative selection," *Journal of Immunology*, vol. 166, no. 10, pp. 5945–5954, 2001.

[14] F. Granucci, C. Vizzardelli, N. Pavelka et al., "Inducible IL-2 production by dendritic cells revealed by global gene expression analysis," *Nature Immunology*, vol. 2, no. 9, pp. 882–888, 2001.

[15] J. X. Lin and W. J. Leonard, "Signaling from the IL-2 receptor to the nucleus," *Cytokine and Growth Factor Reviews*, vol. 8, no. 4, pp. 313–332, 1997.

[16] K. Sugamura, H. Asao, M. Kondo et al., "The interleukin-2 receptor γ chain: its role in the multiple cytokine receptor complexes and T cell development in XSCID," *Annual Review of Immunology*, vol. 14, pp. 179–205, 1996.

[17] M. Kubo, T. Hanada, and A. Yoshimura, "Suppressors of cytokine signaling and immunity," *Nature Immunology*, vol. 4, no. 12, pp. 1169–1176, 2003.

[18] K. Shuai and B. Liu, "Regulation of JAK-STAT signalling in the immune system," *Nature Reviews Immunology*, vol. 3, no. 11, pp. 900–911, 2003.

[19] K. Paukku and O. Silvennoinen, "STATs as critical mediators of signal transduction and transcription: lessons learned from STAT5," *Cytokine and Growth Factor Reviews*, vol. 15, no. 6, pp. 435–455, 2004.

[20] M. Kieslinger, I. Woldman, R. Moriggl et al., "Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation," *Genes and Development*, vol. 14, no. 2, pp. 232–244, 2000.

[21] V. Sexl, R. Piekorz, R. Moriggl et al., "Stat5a/b contribute to interleukin 7-induced B-cell precursor expansion, but abl- and bcr/abl-induced transformation are independent of Stat5," *Blood*, vol. 96, no. 6, pp. 2277–2283, 2000.

[22] M. Socolovsky, H. S. Nam, M. D. Fleming, V. H. Haase, C. Brugnara, and H. F. Lodish, "Ineffective erythropoiesis in Stat5a5b mice due to decreased survival of early erythroblasts," *Blood*, vol. 98, no. 12, pp. 3261–3273, 2001.

[23] A. Antov, L. Yang, M. Vig, D. Baltimore, and L. Van Parijs, "Essential role for STAT5 signaling in $CD25^+CD4^+$ regulatory T cell homeostasis and the maintenance of self-tolerance," *Journal of Immunology*, vol. 171, no. 7, pp. 3435–3441, 2003.

[24] J. Zhu, J. Cote-Sierra, L. Guo, and W. E. Paul, "Stat5 activation plays a critical role in Th2 differentiation," *Immunity*, vol. 19, no. 5, pp. 739–748, 2003.

[25] J. Kelly, R. Spolski, K. Imada, J. Bollenbacher, S. Lee, and W. J. Leonard, "A role for Stat5 in $CD8^+$ T cell homeostasis," *Journal of Immunology*, vol. 170, no. 1, pp. 210–217, 2003.

[26] J. W. Snow, N. Abraham, M. C. Ma, B. G. Herndier, A. W. Pastuszak, and M. A. Goldsmith, "Loss of tolerance and autoimmunity affecting multiple organs in STAT5A/5B-deficient mice," *Journal of Immunology*, vol. 171, no. 10, pp. 5042–5050, 2003.

[27] A. C. Cohen, K. C. Nadeau, W. Tu et al., "Cutting edge: decreased accumulation and regulatory function of $CD4^+CD25^{high}$ T cells in human STAT5b deficiency," *Journal of Immunology*, vol. 177, no. 5, pp. 2770–2774, 2006.

[28] Y. Belkaid, C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks, "$CD4^+CD25^+$ regulatory T cells control Leishmania major persistence and immunity," *Nature*, vol. 420, no. 6915, pp. 502–507, 2002.

[29] M. Iwashiro, R. J. Messer, K. E. Peterson, I. M. Stromnes, T. Sugie, and K. J. Hasenkrug, "Immunosuppression by CD4 regulatory T cells induced by chronic retroviral infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 16, pp. 9226–9230, 2001.

[30] B. Singh, S. Read, C. Asseman et al., "Control of intestinal inflammation by regulatory T cells," *Immunological Reviews*, vol. 182, pp. 190–200, 2001.

[31] S. Sakaguchi, K. Fukuma, K. Kuribayashi, and T. Masuda, "Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participiation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease," *Journal of Experimental Medicine*, vol. 161, no. 1, pp. 72–87, 1985.

[32] R. S. McHugh and E. M. Shevach, "Cutting edge: depletion of $CD4^+CD25^+$ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease," *Journal of Immunology*, vol. 168, no. 12, pp. 5979–5983, 2002.

[33] T. M. Brusko, C. H. Wasserfall, M. J. Clare-Salzler, D. A. Schatz, and M. A. Atkinson, "Functional defects and the influence of age on the frequency of $CD4^+CD25^+$ T-cells in type 1 diabetes," *Diabetes*, vol. 54, no. 5, pp. 1407–1414, 2005.

[34] S. M. Pop, C. P. Wong, D. A. Culton, S. H. Clarke, and R. Tisch, "Single cell analysis shows decreasing FoxP3 and TGFβ1 coexpressing $CD4^+CD25^+$ regulatory T cells during autoimmune diabetes," *Journal of Experimental Medicine*, vol. 201, no. 8, pp. 1333–1346, 2005.

[35] Z. Chen, A. E. Herman, M. Matos, D. Mathis, and C. Benoist, "Where $CD4^+CD25^+$ T reg cells impinge on autoimmune diabetes," *Journal of Experimental Medicine*, vol. 202, no. 10, pp. 1387–1397, 2005.

[36] B. Salomon, D. J. Lenschow, L. Rhee et al., "B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes," *Immunity*, vol. 12, no. 4, pp. 431–440, 2000.

[37] S. Sakaguchi, T. Yamaguchi, T. Nomura, and M. Ono, "Regulatory T cells and immune tolerance," *Cell*, vol. 133, no. 5, pp. 775–787, 2008.

[38] R. Khattri, T. Cox, S. A. Yasayko, and F. Ramsdell, "An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells," *Nature Immunology*, vol. 4, no. 4, pp. 337–342, 2003.

[39] J. Shimizu, S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi, "Stimulation of $CD25^+CD4^+$ regulatory T cells through GITR breaks immunological self-tolerance," *Nature Immunology*, vol. 3, no. 2, pp. 135–142, 2002.

[40] C. E. Ruby, M. A. Yates, D. Hirschhorn-Cymerman et al., "Cutting edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right," *Journal of Immunology*, vol. 183, no. 8, pp. 4853–4857, 2009.

[41] I. Takeda, S. Ine, N. Killeen et al., "Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells," *Journal of Immunology*, vol. 172, no. 6, pp. 3580–3589, 2004.

[42] T. Takahashi, Y. Kuniyasu, M. Toda et al., "Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state," *International Immunology*, vol. 10, no. 12, pp. 1969–1980, 1998.

[43] R. S. McHugh, M. J. Whitters, C. A. Piccirillo et al., "$CD4^+CD25^+$ Immunoregulatory T Cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor," *Immunity*, vol. 16, no. 2, pp. 311–323, 2002.

[44] M. E. Brunkow, E. W. Jeffery, K. A. Hjerrild et al., "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse," *Nature Genetics*, vol. 27, no. 1, pp. 68–73, 2001.

[45] S. Piconese, P. Pittoni, A. Burocchi et al., "A non-redundant role for OX40 in the competitive fitness of Treg in response to IL-2," *European Journal of Immunology*, vol. 40, no. 10, pp. 2902–2913, 2010.

[46] S. Gaudreau, C. Guindi, M. Ménard, G. Besin, G. Dupuis, and A. Amrani, "Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of $CD4^+CD25^+$ regulatory T cells," *Journal of Immunology*, vol. 179, no. 6, pp. 3638–3647, 2007.

[47] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[48] M. J. Lenardo, "Interleukin-2 programs mouse $\alpha\beta$ T lymphocytes for apoptosis," *Nature*, vol. 353, no. 6347, pp. 858–861, 1991.

[49] D. C. Thomis, C. B. Gurniak, E. Tivol, A. H. Sharpe, and L. J. Berg, "Defects in B lymphocyte maturation and T lymphocyte

activation in mice lacking Jak3," *Science*, vol. 270, no. 5237, pp. 794–797, 1995.

[50] M. A. Burchill, C. A. Goetz, M. Prlic et al., "Distinct effects of STAT5 activation on $CD4^+$ and $CD8^+$ T Cell homeostasis: development of $CD4^+CD25^+$ regulatory T cells versus $CD8^+$ memory T cells," *Journal of Immunology*, vol. 171, no. 11, pp. 5853–5864, 2003.

[51] S. J. Bensinger, P. T. Walsh, J. Zhang et al., "Distinct IL-2 receptor signaling pattern in $CD4^+CD25^+$ regulatory T cells," *Journal of Immunology*, vol. 172, no. 9, pp. 5287–5296, 2004.

[52] A. M. Thornton, C. A. Piccirillo, and E. M. Shevach, "Activation requirements for the induction of $CD4^+CD25^+$ T cell suppressor function," *European Journal of Immunology*, vol. 34, no. 2, pp. 366–376, 2004.

[53] T. Nishibori, Y. Tanabe, L. Su, and M. David, "Impaired development of $CD4^+CD25^+$ regulatory T cells in the absence of STAT1: increased susceptibility to autoimmune disease," *Journal of Experimental Medicine*, vol. 199, no. 1, pp. 25–34, 2004.

[54] K. A. Smith, "Interleukin-2," *Current Opinion in Immunology*, vol. 4, no. 3, pp. 271–276, 1992.

[55] M. Papiernik, M. L. de Moraes, C. Pontoux, F. Vasseur, and C. Pénit, "Regulatory $CD4^+$T cells: expression of IL-2Rα chain, resistance to clonal deletion and IL-2 dependency," *International Immunology*, vol. 10, no. 4, pp. 371–378, 1998.

[56] T. R. Malek, A. Yu, L. Zhu, T. Matsutani, D. Adeegbe, and A. L. Bayer, "IL-2 family of cytokines in T regulatory cell development and homeostasis," *Journal of Clinical Immunology*, vol. 28, no. 6, pp. 635–639, 2008.

[57] A. Schimpl, I. Berberich, B. Kneitz et al., "IL-2 and autoimmune disease," *Cytokine and Growth Factor Reviews*, vol. 13, no. 4-5, pp. 369–378, 2002.

[58] G. Klebb, I. B. Autenrieth, H. Haber et al., "Interleukin-2 is indispensable for development of immunological self-tolerance," *Clinical Immunology and Immunopathology*, vol. 81, no. 3, pp. 282–286, 1996.

[59] S. Krämer, C. Mamalaki, I. Horak, A. Schimpl, D. Kioussis, and T. Hünig, "Thymic selection and peptide-induced activation of T cell receptor-transgenic CD8 T cells in interleukin-2-deficient mice," *European Journal of Immunology*, vol. 24, no. 10, pp. 2317–2322, 1994.

[60] T. R. Malek, B. O. Porter, E. K. Codias, P. Scibelli, and A. Yu, "Normal lymphoid homeostasis and lack of lethal autoimmunity in mice containing mature T cells with severely impaired IL-2 receptors," *Journal of Immunology*, vol. 164, no. 6, pp. 2905–2914, 2000.

[61] T. R. Malek, A. Yu, V. Vincek, P. Scibelli, and L. Kong, "CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rβ-deficient mice: implications for the nonredundant function of IL-2," *Immunity*, vol. 17, no. 2, pp. 167–178, 2002.

[62] A. R. M. Almeida, N. Legrand, M. Papiernik, and A. A. Freitas, "Homeostasis of peripheral $CD4^+$ T cells: IL-2Rα and IL-2 shape a population of regulatory cells that controls $CD4^+$ T cell numbers," *Journal of Immunology*, vol. 169, no. 9, pp. 4850–4860, 2002.

[63] M. A. Gavin, S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky, "Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells in vivo," *Nature Immunology*, vol. 3, no. 1, pp. 33–41, 2002.

[64] J. F. Bach, "Regulatory T cells under scrutiny," *Nature Reviews Immunology*, vol. 3, no. 3, pp. 189–198, 2003.

[65] M. de la Rosa, S. Rutz, H. Dorninger, and A. Scheffold, "Interleukin-2 is essential for $CD4^+CD25^+$ regulatory T cell function," *European Journal of Immunology*, vol. 34, no. 9, pp. 2480–2488, 2004.

[66] A. Vella, J. D. Cooper, C. E. Lowe et al., "Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms," *American Journal of Human Genetics*, vol. 76, no. 5, pp. 773–779, 2005.

[67] Q. Tang, J. Y. Adams, C. Penaranda et al., "Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction," *Immunity*, vol. 28, no. 5, pp. 687–697, 2008.

[68] A. Rabinovitch, W. L. Suarez-Pinzon, A. M. James Shapiro, R. V. Rajotte, and R. Power, "Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice," *Diabetes*, vol. 51, no. 3, pp. 638–645, 2002.

[69] D. V. Serreze, K. Hamaguchi, and E. H. Leiter, "Immunostimulation circumvents diabetes in NOD Lt mice," *Journal of Autoimmunity*, vol. 2, no. 6, pp. 759–776, 1989.

[70] T. Brusko, C. Wasserfall, K. McGrail et al., "No alterations in the frequency of FOXP3 regulatory T-cells in type 1 diabetes," *Diabetes*, vol. 56, no. 3, pp. 604–612, 2007.

[71] J. Grant, K. Bourcier, S. Wallace et al., "Validated protocol for FoxP3 reveals increased expression in type 1 diabetes patients," *Cytometry Part B*, vol. 76, no. 2, pp. 69–78, 2009.

[72] S. Lindley, C. M. Dayan, A. Bishop, B. O. Roep, M. Peatman, and T. I. M. Tree, "Defective suppressor function in $CD4^+CD25^+$ T-cells from patients with type 1 diabetes," *Diabetes*, vol. 54, no. 1, pp. 92–99, 2005.

[73] M. Link, L. Salur, K. Kisand, T. Rajasalu, V. Tillmann, and R. Uibo, "Higher FoxP3 mRNA expression in peripheral blood mononuclear cells of GAD65 or IA-2 autoantibody-positive compared with autoantibody-negative persons," *APMIS*, vol. 116, no. 10, pp. 896–902, 2008.

[74] W. Łuczyński, A. Stasiak-Barmuta, R. Urban, M. Urban, B. Florys, and M. Hryszko, "Lower percentages of T regulatory cells in children with type 1 diabetes—preliminary report," *Pediatric Endocrinology, Diabetes, and Metabolism*, vol. 15, no. 1, pp. 34–38, 2009.

[75] V. Öling, J. Marttila, M. Knip, O. Simell, and J. Ilonen, "Circulating $CD4^+CD25$ regulatory T cells and natural killer T cells in children with newly diagnosed type 1 diabetes or with diabetes-associated autoantibodies," *Annals of the New York Academy of Sciences*, vol. 1107, pp. 363–372, 2007.

[76] A. L. Putnam, F. Vendrame, F. Dotta, and P. A. Gottlieb, "$CD4^+CD25^+$ regulatory T cells in human autoimmune diabetes," *Journal of Autoimmunity*, vol. 24, no. 1, pp. 55–62, 2005.

[77] T. I. M. Tree, B. O. Roep, and M. Peakman, "A mini meta-analysis of studies on $CD4^+CD25^+$ T cells in human type 1 diabetes: report of the immunology of Diabetes Society T cell workshop," *Annals of the New York Academy of Sciences*, vol. 1079, pp. 9–18, 2006.

[78] Z. Vrabelova, Z. Hrotekova, Z. Hladikova, K. Bohmova, K. Stechova, and J. Michalek, "CD 127^- and $FoxP3^+$ expression on $CD25^+CD4^+$ T regulatory cells upon specific diabetogeneic stimulation in high-risk relatives of type 1 diabetes mellitus patients," *Scandinavian Journal of Immunology*, vol. 67, no. 4, pp. 404–410, 2008.

[79] Z. Yang, Z. Zhou, G. Huang et al., "The $CD4^+$ regulatory T-cells is decreased in adults with latent autoimmune diabetes," *Diabetes Research and Clinical Practice*, vol. 76, no. 1, pp. 126–131, 2007.

[80] Y. Jin, X. Chen, R. Podolsky et al., "APC dysfunction is correlated with defective suppression of T cell proliferation in

human type 1 diabetes," *Clinical Immunology*, vol. 130, no. 3, pp. 272–279, 2009.

[81] J. M. Lawson, J. Tremble, C. Dayan et al., "Increased resistance to $CD4^+CD25^{hi}$ regulatory T cell-mediated suppression in patients with type 1 diabetes," *Clinical and Experimental Immunology*, vol. 154, no. 3, pp. 353–359, 2008.

[82] S. A. Long, K. Cerosaletti, P. L. Bollyky et al., "Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in $CD4^+CD25^+$ regulatory T-cells of type 1 diabetic subjects," *Diabetes*, vol. 59, no. 2, pp. 407–415, 2010.

[83] S. A. Long, M. R. Walker, M. Rieck et al., "Functional islet-specific Treg can be generated from $CD4^+CD25^-$ T cells of healthy and type 1 diabetic subjects," *European Journal of Immunology*, vol. 39, no. 2, pp. 612–620, 2009.

[84] A. L. Putnam, T. M. Brusko, M. R. Lee et al., "Expansion of human regulatory T-cells from patients with type 1 diabetes," *Diabetes*, vol. 58, no. 3, pp. 652–662, 2009.

[85] A. Ryden, K. Stechova, M. Durilova, and M. Faresjö, "Switch from a dominant Th1-associated immune profile during the pre-diabetic phase in favour of a temporary increase of a Th3-associated and inflammatory immune profile at the onset of type 1 diabetes," *Diabetes/Metabolism Research and Reviews*, vol. 25, no. 4, pp. 335–343, 2009.

[86] A. Schneider, M. Rieck, S. Sanda, C. Pihoker, C. Greenbaum, and J. H. Buckner, "The effector T cells of diabetic subjects are resistant to regulation via $CD4^+FOXP3^+$ regulatory T cells," *Journal of Immunology*, vol. 181, no. 10, pp. 7350–7355, 2008.

[87] M. Battaglia, A. Stabilini, B. Migliavacca, J. Horejs-Hoeck, T. Kaupper, and M. G. Roncarolo, "Rapamycin promotes expansion of functional $CD4^+CD25^+$ $FOXP3^+$ regulatory T cells of both healthy subjects and type 1 diabetic patients," *Journal of Immunology*, vol. 177, no. 12, pp. 8338–8347, 2006.

[88] P. Monti, M. Scirpoli, P. Maffi et al., "Rapamycin monotherapy in patients with type 1 diabetes modifies $CD4^+CD25^+FOXP3^+$ Regulatory T-Cells," *Diabetes*, vol. 57, no. 9, pp. 2341–2347, 2008.

[89] D. B. Wilde, M. B. Prystowsky, J. M. Ely, S. N. Vogel, D. P. Dialynas, and F. W. Fitch, "Antigen-reactive cloned helper T cells. II. Exposure of murine cloned helper T cells to IL 2-containing supernatant induces unresponsiveness to antigenic restimulation and inhibits lymphokine production after antigenic stimulation," *Journal of Immunology*, vol. 133, no. 2, pp. 636–641, 1984.

[90] G. Otten, D. B. Wilde, M. B. Prystowsky et al., "Cloned helper T lymphocytes exposed to interleukin 2 become unresponsive to antigen and concanavalin A but not to calcium ionophore and phorbol ester," *European Journal of Immunology*, vol. 16, no. 3, pp. 217–225, 1986.

[91] M. S. Jordan, A. Boesteanu, A. J. Reed et al., "Thymic selection of $CD4^+CD25^+$ regulatory T cells induced by an agonist self-peptide," *Nature Immunology*, vol. 2, no. 4, pp. 301–306, 2001.

[92] H. M. van Santen, C. Benoist, and D. Mathis, "Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells," *Journal of Experimental Medicine*, vol. 200, no. 10, pp. 1221–1230, 2004.

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Research Article

IFN Regulatory Factors 4 and 8 Expression in the NOD Mouse

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Dendritic cells (DCs) contribute to islet inflammation and its progression to diabetes in NOD mouse model and human. DCs play a crucial role in the presentation of autoantigen and activation of diabetogenic T cells, and IRF4 and IRF8 are crucial genes involved in the development of DCs. We have therefore investigated the expression of these genes in splenic DCs during diabetes progression in NOD mice. We found that IRF4 expression was upregulated in splenocytes and in splenic $CD11c^+$ DCs of NOD mice as compared to BALB/c mice. In contrast, IRF8 gene expression was higher in splenocytes of NOD mice whereas its expression was similar in splenic $CD11c^+$ DCs of NOD and BALB/c mice. Importantly, levels of IRF4 and IRF8 expression were lower in tolerogenic bone marrow derived DCs (BMDCs) generated with GM-CSF as compared to immunogenic BMDCs generated with GM-CSF and IL-4. Analysis of splenic DCs subsets indicated that high expression of IRF4 was associated with increased levels of $CD4^+CD8\alpha^-IRF4^+CD11c^+$ DCs but not $CD4^-CD8\alpha^+IRF8^+CD11c^+$ DCs in NOD mice. Our results showed that IRF4 expression was up-regulated in NOD mice and correlated with the increased levels of $CD4^+CD8\alpha^-$ DCs, suggesting that IRF4 may be involved in abnormal DC functions in type 1 diabetes in NOD mice.

1. Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that play a key role in the induction of innate and adaptive immunity [1]. DCs recognize various pathogens and their components through pattern-recognition receptors, such as TLRs. Captured pathogens are processed and presented as antigenic peptides associated with MHC molecules to T cells. Consequently, DCs/T cell interaction leads to Th1 or Th2 responses and also to tolerance by inducing Treg differentiation. The great diversity of immune responses following DCs antigen presentation is attributed to the presence of multiple DCs subsets [2, 3]. At least six DCs subsets have been identified in the mouse spleen including conventional $CD11c^{high}$ DCs and $CD11c^{int}B220^+CD11b^-$ plasmacytoid DCs (pDCs) [4]. Conventional $CD11c^{high}$ are divided into three distinct subtypes, $CD4^+CD8\alpha^-CD11b^{high}$, $CD4^-CD8\alpha^+CD11b^{low}$, and $CD4^-CD8\alpha^-CD11b^{high}$ [4–7]. Conventional lymphoid $CD8\alpha^+$ and myeloid $CD8\alpha^-$ DCs are the major producers of IL-12 whereas pDCs are the producers of type I IFN [5–7]. Despite much progress in understanding the biology of DCs, molecular events that specify DC development and functions are not fully understood. Interferon regulatory factors 4 and 8 (IRF4 and IRF8), two members of the IRF transcription factor family involved in the regulation of both innate and adaptive immunity [8], have been shown to contribute to DCs development and function [9–13]. Notably, IRF4 has been shown to be required for the development of $CD11b^{high}CD8\alpha^-$ DCs subset [13], whereas IRF-8 is required for differentiation of $CD11c^+CD11b^{low}CD8\alpha^+$ DCs and pDCs subsets [14]. Indeed, mice lacking the IRF4 gene have selective defect in splenic $CD11b^{high}CD8\alpha^-$ conventional DCs [13] whereas $IRF8^{-/-}$ mice have a defect in both lymphoid and plasmacytoid DC subsets [14]. Furthermore, $CD8\alpha^+$ DCs and pDCs express high levels of IRF8, but low level of IRF4. Conversely, IRF4 expression is high in $CD4^+$ DCs and $CD4^-CD8\alpha^-CD11b^{high}$ DCs whereas IRF8 expression is low.

The Nonobese Diabetic (NOD) mouse spontaneously develops diabetes between 15 to 20 weeks of age. The onset of the disease is preceded by a period of insulitis during which the islets of Langerhans are infiltrated by autoreactive T cells and APCs [15]. Interestingly, DCs are the first cells to infiltrate the islets [16], preceding T cell infiltration [17]. Infiltrating DCs produce cytokines such as IL-12 [17], suggesting their implication in the early pathogenesis of diabetes. Furthermore, the administration of IL-12 to NOD mice results in enhanced DCs accumulation in pancreatic islets and accelerated diabetes onset [18]. Although the precise mechanism leading to the breakdown of tolerance to islets antigen is not fully understood, defects in function and maturation of DCs in NOD mice have been suggested [19]. Several studies have shown several abnormalities in BM-derived and splenic DCs of NOD mice as compared to DCs of diabetes-resistant mice [20, 21]. For example, it has been reported that the number of splenic CD8α^+ DCs and CD8α^- DCs in NOD mice is low as compared to those of the diabetes-resistant B10.BR and C57BL6J mice [22]. This decreased population of DCs could result in their reduced ability to take up and to clear dead cells [14] and to maintain self-tolerance [23].

In the present study, we have investigated the expression of IRF4 and IRF8 genes in splenocytes and DCs of diabetes-prone NOD mice and compared the results to diabetes-resistant NOR and BALB/c mice. We found an upregulated expression of IRF4 in the total splenic cell population of NOD mice as compared to BALB/c mice. Enhanced IRF4 expression was found in CD11c$^+$ splenic DCs of NOD as compared to BALB/c mice. However, IRF4 and IRF8 expression was only increased in BMDCs generated with a combination of IL-4 and GM-CSF whereas their expression was low in BMDCs generated with GM-CSF alone.

2. Materials and Methods

2.1. Mice. Male and female NOD, NOR, C57BL/6J, CD1, and BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice were housed and bred in-house under specific pathogen-free conditions and were used according to guidelines of the Institutional Animal Care Committee of the University of Sherbrooke.

2.2. Cell Lines and Antibodies. Anti-CD8α-PE (clone 53-6.7), anti-CD4-FITC/biotin/APC (clone GK1.5), anti-CD11c-FITC/biotin (clone HL3) antibodies, and streptavidin-PerCP (used for all biotin conjugated antibodies) were from Becton-Dickinson (San Jose, CA). The anti-IRF4-biotin (clone M17) and anti-IRF8-biotin (clone C19) were from Santa Cruz Biotechnology (Santa Cruz, CA). The antigoat-HRP antibody was from R and D Systems (Minneapolis, MN).

2.3. Splenic DCs Isolation. DCs purification was performed using antibody-coated magnetic beads from Miltenyi Biotec (Bergisch Gladbach, Germany) as before [24]. Briefly, spleens were digested with collagenase D (2 or 3 organs), stained with anti-CD11c-coated beads and sorted by MACS (Miltenyi Biotec). Purity of CD11c$^+$ DCs was >85% as determined by FACS.

2.4. Generation of Bone Marrow-Derived DCs. BMDCs were generated with GM-CSF (5 ng/mL) alone or in combination with IL-4 (4.5 ng/mL) (Cederlane, Burlington, ON) as described [25, 26]. On Day 7, DCs were collected for further analysis.

2.5. Real-Time PCR. Total RNA was extracted from DCs using the TRIzol reagent (Invitrogen, Carlsbad, CA). Two micrograms of RNA were reversely transcribed to cDNA using Superscript II (Invitrogen Burlington, ON). Quantitative PCR reactions were performed using Rotor-Gene 3000 (Corbett Life Science, Mortlake, New South Wales, Australia) with a 25 μL mixture composed of 2 μL of cDNA template, 2 μL PCR buffer, 2 μL of dNTP (10 mmol/L), 0.3 μL of each primer (20 pmol/mL), 0.5 μL SYBR green Quantitect SYBR Green qPCR kit (Qiagen), and 0.1 μL of Taq polymerase. The reactions were carried out with an initial denaturation at 95°C for 5 min, followed by 40 cycles of 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Amplification plots were generated using the Rotor-Gene Amplification software v6.0 (Corbett Research). HPRT was used as a reference to obtain the relative fold change for target samples using the comparative C_T method. The primers used were 5′ AATGGGAAACTCCGACAGTG3′ (IRF4 sense), 5′ TAGGAGGATCTGGCTTGTCG3′ (IRF4 antisense), 5′ GATCGAACAGATCGACAGCA3′ (IRF8 sense), 5′ AGAGCACAGCGTAACCTCGT3′ (IRF8 antisense), 5′ GTTGGATACAGGCCAGACTTTGTTG3′ (HPRT sense), and 5′ GATTCAACTTGCTCTCATCTTAGGC3′ (HPRT antisense).

2.6. Western Blots. BM-derived and splenic DCs were harvested, washed in cold PBS, and resuspended in lysis buffer containing Tris 50 mM, NaCl 0.15 M, DTT 1 mM, Triton X-100 1% (v/v), and a cocktail of protease and phosphatase inhibitors. Cell lysates were fractionated on 10% SDS-PAGE gels, transferred to a nitrocellulose membrane (Hybond-ECL Amersham Biosciences, Baie d'Urfé, QC) and incubated overnight with primary antibodies, followed by the appropriate secondary antibodies and revealed by enhanced chemiluminescence (GE Health Care, Baie d'Urfé, QC). Quantification of Western band intensities was performed by densitometry analysis of X-ray films using the NIH Image software (http://rsb.info.nih.gov/nih-image/).

2.7. Flow Cytometry. Cells were washed once with PBS supplemented with 2.5% bovine serum albumin (PBS-BSA) and incubated with the indicated mAbs for 20 min at 4°C. In the case of IRF4 and IRF8 intracellular staining, the cells were fixed with 4% paraformaldehyde for 1 h at 4°C, permeabilized with FACS buffer containing 0.1% saponin and stained with anti-IRF4 or IRF8 Abs or antirat IgG2a for 30 min at 4°C. The cells were then washed twice with PBS-BSA and analyzed by FACS using the CellQuest software (BD Biosciences) or the FCS express V3 software (De Novo Software, Los Angeles, CA).

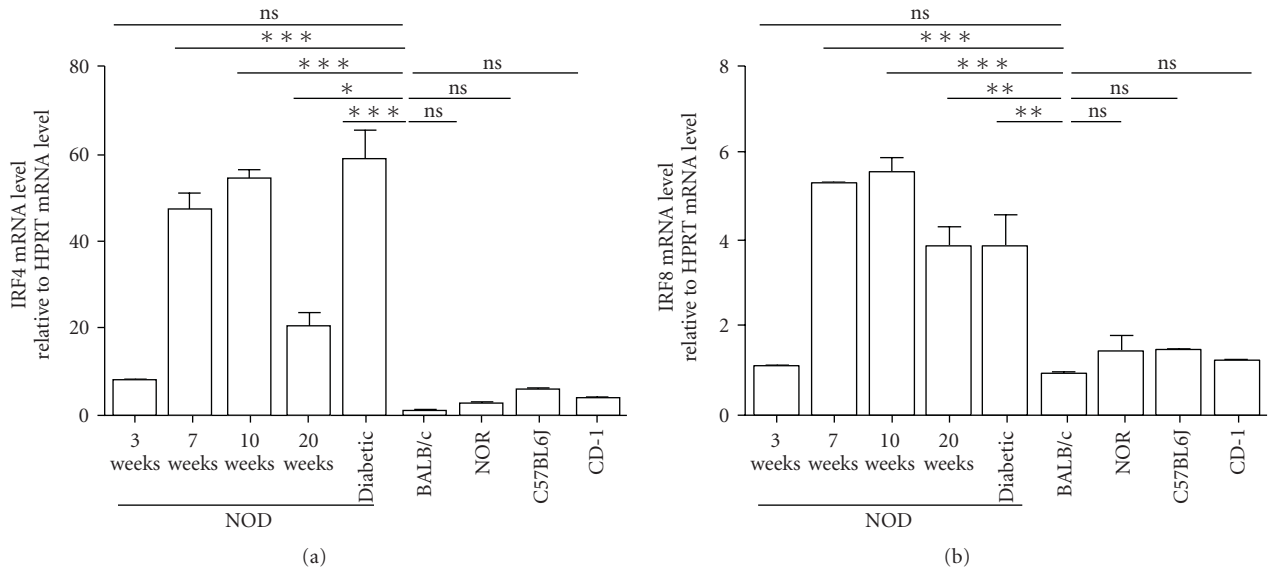

Figure 1: Age-related IRF4 and IRF8 mRNA expression levels in splenocytes of diabetes-prone and diabetes-resistant mice. Total splenic RNA was obtained from NOD, NOR, C57BL/6J, CD-1, and BALB/c mice, purified and gene expression profile of IRF4 (a) and IRF8 (b) were analyzed by real time RT-PCR. Each RNA sample was analyzed in triplicates and normalized to HPRT expression (ΔCt). Each NOD sample was normalized to a BALB/c sample (ΔΔCt = ΔCt sample −ΔCt BALB/c) and, the expression level was calculated as follows: $2^{-\Delta\Delta Ct}$. Data are shown as the average ± SD of 4 independent experiments (*$P < .05$, **$P < .01$ and ***$P < .001$).

2.8. Statistical Analysis. Two groups were compared using two-tailed unpaired Student's t test. When more that two groups were compared, one-way ANOVA Dunnett's test for multiple comparisons was used. Differences were considered to be statistically significant when a $P < .05$. Data reported here are representative of 2-3 independent experiments. Histograms and bar graph results are shown as the mean ± SEM.

3. Results

3.1. IRF4 But Not IRF8 Gene Expression Is Increased in Splenocytes of NOD Mice. In a first series of experiments, we used quantitative PCR (qPCR) to assess gene expression of IRF4 and IRF8 in splenocytes isolated from prediabetic and diabetic NOD mice as well as control diabetes-resistant congenic NOR and BALB/c mice. We found that the levels of mRNA expression of IRF4 were slightly but not significantly higher ($P > .05$) in splenocytes of 3-week old NOD (7.7 ± 0.8) mice as compared to splenocytes of diabetes-resistant BALB/c (1.04 ± 0.1), NOR (2.6 ± 0.6), C57BL/6 (5.8 ± 0.9), or CD1 (3.7 ± 0.8) mice (Figure 1(a)). The levels of IRF4 mRNA expression were significantly ($P < .001$) increased in the splenocytes of 7-, 10-, and 20-week old diabetic NOD mice (54.8 ± 1.9 and 58.9 ± 16.1, resp.) as compared with 3 weeks old NOD mice and diabetes-resistant mice (Figure 1(a)). However, the expression of IRF4 remained unchanged in the splenocytes of 3-, 6-, 10-, or 20-week old NOR and BALB/c mice (data not shown). Interestingly, IRF4 gene expression in splenocytes of nondiabetic 20-week old NOD mice was significantly lower ($P < .05$) than in 20-week old diabetic NOD mice (20 ± 5.3 compared to 53.9 ± 16.1) (Figure 1(a)).

Analysis of IRF8 gene expression revealed similar levels of IRF8 mRNA in splenocytes of BALB/c and 3-week old NOD mice (0.9 ± 0.1 as opposed to 1.1 ± 0.1) (Figure 1(b)). In addition, IRF8 gene expression was significantly ($P < .05$) increased in the splenocytes of 7-, 10-, and 20-week old NOD mice (Figure 1(b)). In contrast, the levels of IRF8 gene expression were similar in the splenocytes of 20-week old nondiabetic and diabetic NOD mice (3.8 ± 0.6 as opposed to 3.8 ± 1.1) (Figure 1(b)).

We next determined whether changes observed in the expression of the IRF4 and IRF8 genes were matched by changes at the protein levels. Total proteins extracts were prepared from the splenocytes of NOD and BALB/c mice and the expression of IRF4 and IRF8 was analyzed by Western blots (Figure 2). Results showed that IRF4 expression was significantly higher ($P < .05$) in splenocytes of 3-week old NOD mice than in BALB/c mice (1.38 ± 0.08 as opposed to 1.00 ± 0) (Figures 2(a) and 2(b)). IRF4 expression was slightly but not significantly ($P > .05$) higher in 7-, 10-, and 20-week old nondiabetic and diabetic NOD mice (1.58 ± 0.02, 1.60 ± 0.1, 1.52 ± 0.03, and 1.81 ± 0.10, resp.) as compared to IRF4 expression in 3-week old NOD mice (Figures 2(a) and 2(b)). In contrast, the protein levels of IRF8 in splenocytes of 3- and 7-week-old NOD mice were similar to those of BALB/c mice (1.09 ± 0.1, 0.98 ± 0.10 as compared to 1.00 ± 0, $P > .05$). Protein levels of IRF8 were significantly higher ($P < .05$) in 10- and 20-week-old non diabetic and diabetic NOD mice (1.16 ± 0.1, 1.22 ± 0.03, and 1.25 ± 0.10, resp.) as compared to BALB/c (1.00 ± 0) mice (Figures 2(c) and 2(d)). Together, these results suggested that gene and protein expression levels of IRF4 were upregulated in the spleen of diabetes-prone NOD mice whereas levels of IRF8 remained unchanged at

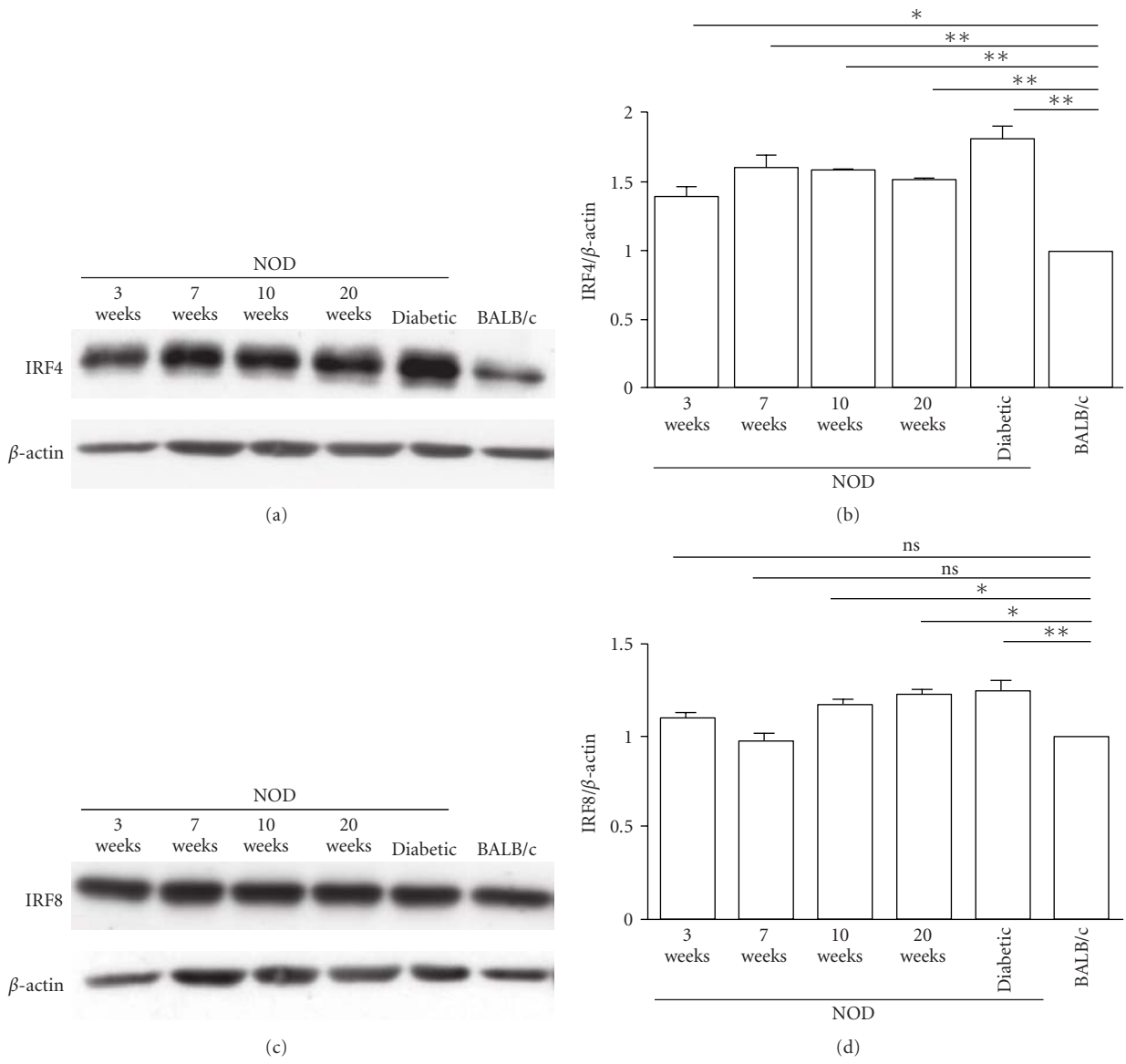

Figure 2: Age-related IRF4 and IRF8 expression levels in splenocytes of diabetes-prone NOD mice and diabetes-resistant BALB/c mice. Proteins were extracted from NOD or BALB/c mouse splenocytes and analyzed by Western blotting for IRF4 (a) or IRF8 (c) expression. The relative intensities of the bands were determined using the NIH Image software and normalized to reference actin bands to establish a ratio of (b) IRF4/actin and (d) IRF8/actin. The levels of expression in BALB/c mice were arbitrarily set at a unitary value. Data are representative of 3 independent experiments.

3 and 7 weeks of age, and were slightly increased in 10 and 20 weeks old mice when compared to BALB/c mice.

3.2. Increased Expression of IRF4 Correlated with Augmented $CD4^+CD8\alpha^-CD11c^+$ Splenic DCs in NOD Mice. To determine whether differential expression of IRF4 and IRF8 in splenocytes of NOD mice resided in splenic DCs, we examined the levels of protein expression of IRF4 and IRF8 by Western blots in $CD11c^+$-purified splenic DCs of 8-week-old NOD and BALB/c mice. Results showed (Figure 3(a)) that the expression of IRF4 was higher in splenic DCs of NOD mice as compared to BALB/c mice ($P < .05$). In contrast, IRF8 expression was similar in splenic DCs of both strains of mice (Figure 3(b)). Since IRF4 and IRF8 have been shown to be essential for the development of $CD4^+CD8\alpha^-CD11c^+$ and $CD4^-CD8\alpha^+CD11c^+$ subsets, respectively [9], we determined whether the changes observed in IRF4 and IRF8 expression in splenic DCs of NOD and BALB/c mice affected the proportions of these two DCs subsets. Results of FACS analysis (Figure 3(c)) showed no difference in the percentage of $CD4^+CD8\alpha^-IRF4^+CD11c^+$ DCs subset in splenocytes of 3-week-old NOD and BALB/c mice ($0.42\% \pm 0.03$ as opposed to $0.27\% \pm 0.03$, $P > .05$). The percentages of splenic $CD4^+CD8\alpha^-IRF4^+CD11c^+$ DCs subset were significantly ($P < .05$) increased in 7-, 10-, and 20-week-old NOD mice ($0.60\% \pm 0.030$ at 7 weeks, $0.63\% \pm 0.03$ at 10

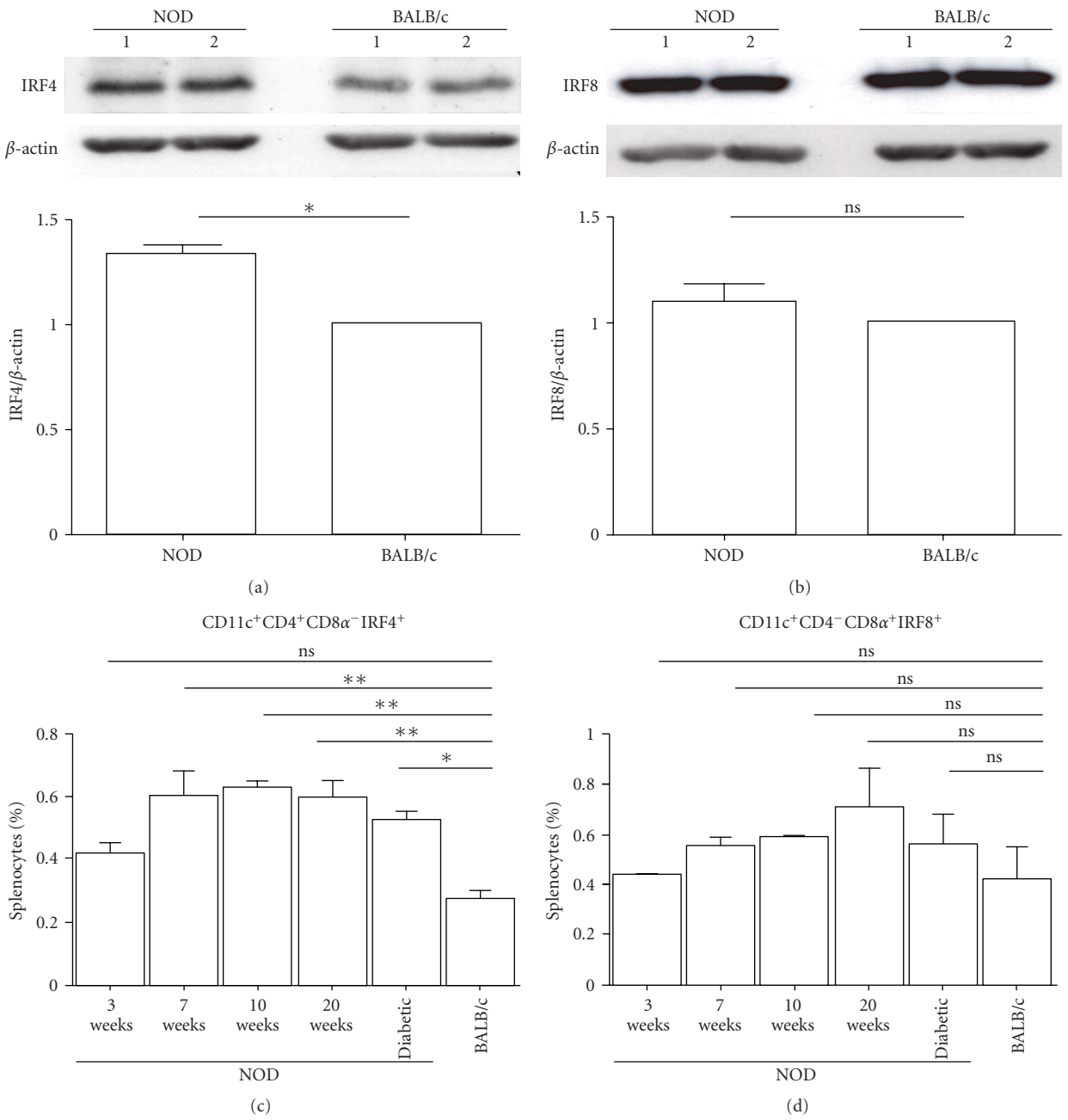

Figure 3: Expression of IRF4 in splenic DCs of diabetes-prone NOD mice and diabetes-resistant BALB/c mice. Proteins were extracted from $CD11c^+$-purified splenic DCs from NOD and BALB/c mice and analyzed by Western blotting for (a) IRF4 or (b) IRF8 expression. The relative intensities of the bands were assessed using the NIH Image software and normalized to reference actin bands to establish a ratio of IRF4/actin (a), lower panel) and (b), lower panel) IRF8/actin. The expression levels observed in BALB/c were arbitrarily set as a unitary value. Data are representative of 2 independent experiments (Exp1 and Exp2). (c) and (d) splenocytes from NOD and BALB/c mice were stained for the CD11c, CD4, and CD8α surface markers in combination with intracellular staining with an anti-IRF8 or anti-IRF4 mAb and analyzed by flow cytometry. Data represent the average percentage of $CD11c^+CD4^+CD8\alpha^-IRF4^+$-positive (c) and $CD11c^+CD4^-CD8\alpha^+IRF8^+$-positive (d) cells of two independent experiments. Error bars correspond to the averages ±S.D ($^*P < .05$, $^{**}P < .01$ and $^{***}P < .001$).

weeks, and 0.60% ± 0.07 at 20 weeks) as compared to 3 weeks old (NOD 0.42% ± 0.03) and BALB/c (0.27% ± 0.0) mice. In addition, there were no significant difference in splenic $CD4^+CD8\alpha^-IRF4^+CD11c^+$ DCs subset in 7-, 10-, and 20-week-old nondiabetic and diabetic NOD mice. In contrast, an absence of differences was noted in the percentage of $CD4^-CD8\alpha^+IRF8^+CD11c^+$ DCs subset in the splenocytes of BALB/c mice and diabetic or nondiabetic NOD mice (Figure 3(d)). Together, these data suggested that high expression of IRF4 in NOD mice was associated with enhanced splenic $CD4^+CD8\alpha^-IRF4^+CD11c^+$ DCs subset but not with splenic $CD4^-CD8\alpha^+IRF8^+CD11c^+$ DCs subset.

3.3. Increased Expression of IRF8 and IRF4 in BMDCs of NOD Mice. Defects in phenotype and tolerogenic function of BMDCs of NOD mice have also been associated with diabetes [20, 21]. We have reported that treatment of NOD mice with GM-CSF restored the tolerogenic function of myeloid DCs [24]. In addition, BMDCs generated with low dose of GM-CSF possessed tolerogenic functions when compared to immunogenic BMDCs generated with GM-CSF and IL-4 [27] (Guindi et al., unpublished data). Therefore, we determined the expression IRF4 and IRF8 in BMDCs of NOD mice generated with GM-CSF or with a combination of GM-CSF and IL-4. BMDCs generated from BALB/c mice were used as controls. Under both sets of experimental conditions, more than 95% of BMDCs were $CD8\alpha^{-}CD11c^{+}$ cells. Results showed that BMDCs generated with GM-CSF from both strains of mice expressed similar levels of IRF4 as determined by qPCR (Figure 4(a)) and by Western blot (Figures 4(b) and 4(c)). IRF4 expression was increased in BMDCs generated with a combination of GM-CSF and IL-4 and was significantly higher ($P < .05$) in NOD as compared to BALB/c mice (Figures 4(a), 4(b) and 4(c)). The expression of IRF8 was similar in BMDCs of both strain of mice generated with GM-CSF (Figures 4(d), 4(e) and 4(f)). However, BMDCs generated with a combination of GM-CSF and IL-4 expressed significantly ($P < .05$) higher levels of IRF8 as compared to BMDCs generated with GM-CSF. Importantly, the levels of expression of IRF8 were higher in the case of immunogenic BMDCs generated with GM-CSF and IL-4 from NOD than in the case of BALB/c mice (Figures 4(d), 4(e) and 4(f)). Together, these results showed that IRF4 and IRF8 were highly expressed in immunogenic BMDCs generated with a combination of GM-CSF and IL-4 in the case of both strains of mice, whereas their expression was significantly lower in the case of tolerogenic BMDCs generated with GM-CSF alone.

4. Discussion

In this study, RNA gene expression and protein analysis were used to investigate the expression profile of the two transcription factors IRF4 and IRF8 that are known to play an important role in DCs differentiation [9–13]. We found that the expression of IRF4 was enhanced in splenic DCs and in immunogenic BMDCs but not in tolerogenic BMDCs of NOD mice. The increased IRF4 expression was associated with a greater percentage of $CD4^{+}CD8\alpha^{-}IRF4^{+}CD11c^{+}$ DCs but not $CD4^{-}CD8\alpha^{+}IRF8^{-}CD11c^{+}$ DCs. In contrast, IRF8 expression remained unchanged in splenic DCs of NOD and diabetes-resistant BALB/c mice although it was significantly increased in immunogenic BMDCs but not in tolerogenic BMDCs of NOD mice.

Development of type 1 diabetes consists in a succession of events during which variation in gene expression plays a critical role in progression from islet inflammation to clinical diabetes [28, 29]. The development of microarray technology has provided a new approach to understand diabetes pathogenesis and to identify genes deregulated in type 1 diabetes including NOD mice [29, 30]. IRF4 is one candidate gene located in the *idd14* susceptibility region suspected to play a role in the development of type 1 diabetes in NOD mice [29]. IRF8, another candidate gene important in regulating Th2 immune response, has been suggested to play an important role in diabetes development [29]. Here, we found high levels of mRNA and protein expression of IRF4 in splenic cells of NOD mice when compared to BABL/c mice. IRF4 expression was particularly found to increase in 7 weeks and older NOD mice in which islet inflammation has already occurred and in diabetic NOD mice. These results suggested that up-regulation of IRF4 expression may play an important role in diabetes development in diabetes-prone NOD mice. Our results could not be explained by triggering immune response toward Th1 response. In this connection, several studies have reported that diabetes in NOD mice is a Th1-mediated disease and that immunodeviation toward a Th2 response contributes to prevention of diabetes development [31–34]. Of note, IRF4 has been shown to be essential for the Th2 response, and naïve T cells of mice deficient for IRF4 have a compromised production of IL-4 and Th2 cytokines [35]. Alternatively, the increased IRF4 expression during islet inflammation and diabetes development in NOD mice could be also explained by the requirement of IRF4 for production and responsiveness to IL-21 and for stabilization of the Th17 phenotype [36–38] that has been shown to be critical for the development of type 1 diabetes [39]. This observation may also explain the reduced expression of IRF4 in 20-week-old nondiabetic NOD mice as opposed to 20-week-old diabetic NOD mice. In contrast, the increased IRF8 expression in the spleen of diabetes-prone NOD mice may be the result of a high production of IFNγ which is known to induce IRF8 expression in macrophages and T cells [40].

IRF4 and IRF8 play a key role in molecular programs regulating DCs development and function [9–13]. Abnormal DCs development and function have been reported to be associated with diabetes in human and in NOD mice [20, 21, 41]. Here, we found that splenic DCs of NOD mice expressed high levels of IRF4 when compared to DCs of nondiabetic NOD mice and BALB/c mice, whereas the expression of IRF8 was similar in splenic DCs of NOD and BALB/c mice. The expression of IRF4 in NOD mice was associated with an increase in the number of the $CD4^{+}CD8\alpha^{-}IRF4^{+}CD11c^{+}$ DCs subset, thereby confirming that IRF4 contributed to the development of $CD4^{+}CD11c^{+}$ DCs [9, 42]. Furthermore, our data suggested that increases in IRF4 expression and $CD4^{+}CD8\alpha^{-}CD11c^{+}IRF4^{+}$ DCs population in NOD mice were associated with the abnormal function of DCs in diabetes-prone NOD mice. Several studies have also reported an abnormal function of bone marrow-derived DCs of NOD mice generated with a combination of GM-CSF and IL-4. For instance, it was found that the enhanced capacity to activate autoreactive T cells and the increased production of IL-12p70 contributed to the development of diabetes in NOD mice [43, 44]. We also observed that BMDCs of NOD mice, generated with GM-CSF were less immunogenic than BMDCs generated with a combination of GM-CSF and IL-4 (Guindi et al. submitted). This observation prompted us to compare the expression of IRF4 and IRF8 in BMDCs

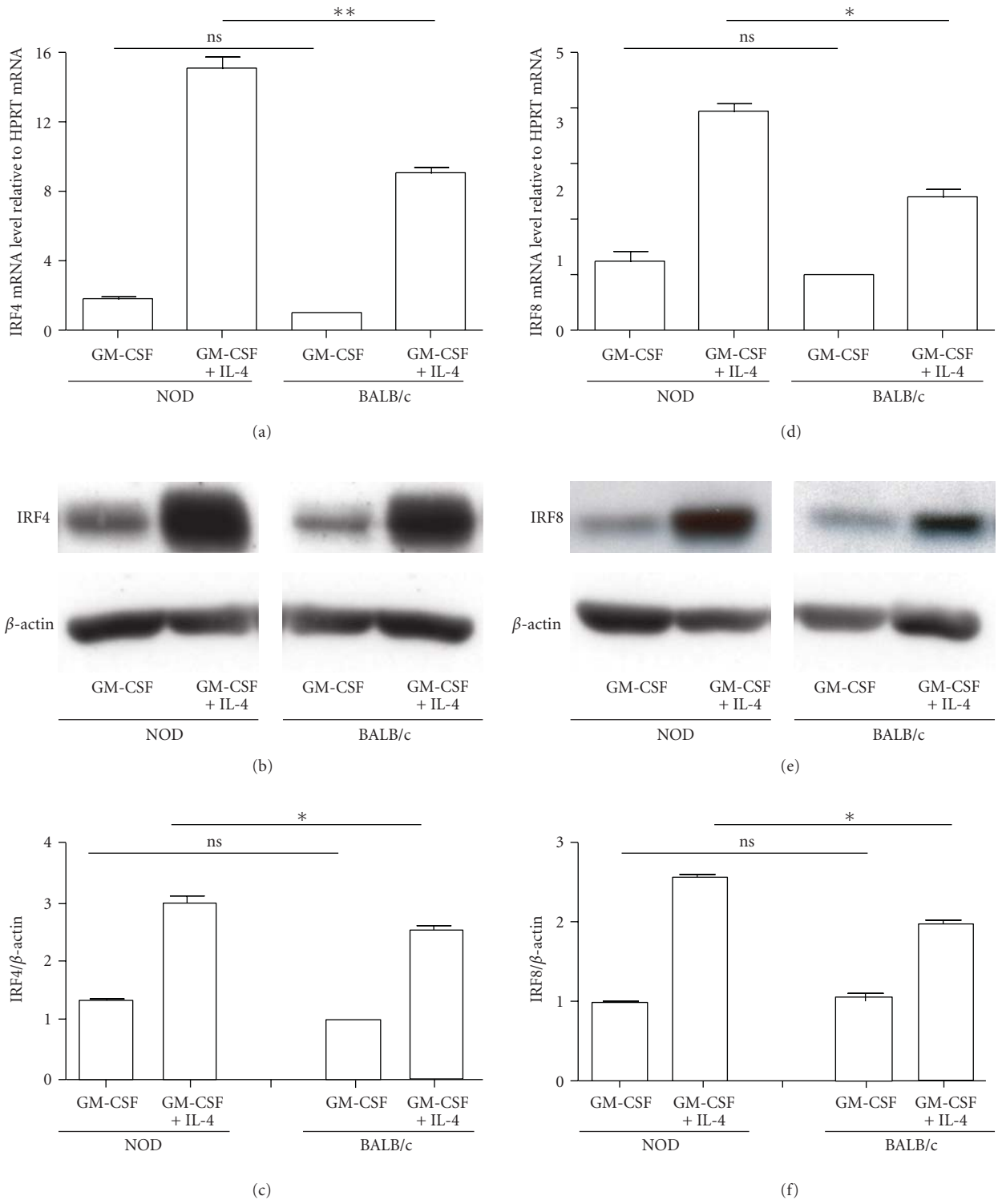

Figure 4: Reduced expression of IRF4 and IRF8 in tolerogenic BMDCs derived from NOD mice. Total RNA was extracted from BM-DCs generated with GM-CSF or a combination of GM-CSF and IL-4, purified and gene expression profiles of IRF4 (a) and IRF8 (d) were analyzed by RT-PCR. Each sample was analyzed in triplicates and normalized to HPRT expression (ΔCt). Each NOD sample was normalized to BALB/c sample (ΔΔCt = ΔCt sample −ΔCt BALB/c), and the expression levels were calculated as follows: $2^{-\Delta\Delta Ct}$. Data are shown as the average ±SD of 3-4 independent experiments (*$P < .05$ and, **$P < .01$). (b) and (e) total proteins were extracted from BM-DCs generated with GM-CSF or a combination of GM-CSF and IL-4 and analyzed by Western blotting for IRF4 (b) or IRF8 (f) expression. The relative intensities of bands were assessed using the NIH Image software and normalized to reference actin bands to establish a ratio of (c) IRF4/actin and (f) IRF8/actin. The expression levels observed in BALB/c mice were arbitrarily set as a unitary value. A representative of 2 independent experiments is shown. Error bars correspond to S.D.

generated with GM-CSF and with a combination of GM-CSF and IL-4. IRF4 and IRF8 were less expressed in BMDCs generated with GM-CSF whereas they were more expressed in BMDCs generated with GM-CSF and IL-4, suggesting that downregulation of IRF4 and IRF8 may attenuate abnormal immunogenic function of BMDCs in NOD mice. In support of this interpretation, several studies have reported that IRF8 contributes to the production of IL-12p70 by acting as transcriptional activator of the IL-12p35 and IL-12p40 genes [45–48]. Therefore, our findings of high expression of IRF8 in DCs of NOD mice may explain their abnormal high production of IL-12p70. Our data also suggested that increased levels of IRF4 in DCs contribute to their abnormal function in NOD mice. In support of this interpretation, the transcription of IRF4 has been shown to be regulated by NF-κB elements located in the IRF4 promoter region that bind Rel/NF-κB complexes [49]. In this context, increased NF-κB activation has been reported to display essential functions of BMDCs for the development of type 1 diabetes [50]. Therefore, enhanced NF-κB activation in DCs of NOD mice leading to their hyperactivation may result from upregulated IRF4 expression.

Our data reported an enhanced involvement of the IRF4 and IRF8 in DCs function in type 1 diabetes-susceptible NOD mice as opposed to control, nondiabetic mice. Further investigation on the role of IRF4 and IRF8 in diabetes development may help to determine whether IRF4 and/or IRF8 could be potential targets for therapeutic interventions in type 1 diabetes.

Abbreviations

DCs: Dendritic cells
BMDCs: Bone marrow-derived dendritic cells
GM-CSF: Granulocyte macrophage-colony stimulating factor
IRF: Interferon regulating factor.

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References

[1] J. Banchereau and R. M. Steinman, "Dendritic cells and the control of immunity," *Nature*, vol. 392, no. 6673, pp. 245–252, 1998.

[2] C. Ardavín, "Origin, precursors and differentiation of mouse dendritic cells," *Nature Reviews Immunology*, vol. 3, no. 7, pp. 582–590, 2003.

[3] F. R. Carbone and W. R. Heath, "The role of dendritic cell subsets in immunity to viruses," *Current Opinion in Immunology*, vol. 15, no. 4, pp. 416–420, 2003.

[4] K. Shortman and Y. J. Liu, "Mouse and human dendritic cell subtypes," *Nature Reviews Immunology*, vol. 2, no. 3, pp. 151–161, 2002.

[5] C. Asselin-Paturel, A. Boonstra, M. Dalod et al., "Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology," *Nature Immunology*, vol. 2, no. 12, pp. 1144–1150, 2001.

[6] H. Nakano, M. Yanagita, and M. D. Gunn, "CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells," *Journal of Experimental Medicine*, vol. 194, no. 8, pp. 1171–1178, 2001.

[7] F. P. Siegal, N. Kadowaki, M. Shodell et al., "The nature of the principal Type 1 interferon-producing cells in human blood," *Science*, vol. 284, no. 5421, pp. 1835–1837, 1999.

[8] P. Tailor, T. Tamura, and K. Ozato, "IRF family proteins and type I interferon induction in dendritic cells," *Cell Research*, vol. 16, no. 2, pp. 134–140, 2006.

[9] T. Tamura, P. Tailor, K. Yamaoka et al., "IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity," *Journal of Immunology*, vol. 174, no. 5, pp. 2573–2581, 2005.

[10] H. Tsujimura, T. Tamura, C. Gongora et al., "ICSBP/IRF-8 retrovirus transduction rescues dendritic cell development in vitro," *Blood*, vol. 101, no. 3, pp. 961–969, 2003.

[11] G. Schiavoni, F. Mattei, P. Sestili et al., "ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8α+ dendritic cells," *Journal of Experimental Medicine*, vol. 196, no. 11, pp. 1415–1425, 2002.

[12] J. Aliberti, O. Schulz, D. J. Pennington et al., "Essential role for ICSBP in the in vivo development of murine CD8α+ dendritic cells," *Blood*, vol. 101, no. 1, pp. 305–310, 2003.

[13] S. Suzuki, K. Honma, T. Matsuyama et al., "Critical roles of interferon regulatory factor 4 in CD11b CD8α+ dendritic cell development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 24, pp. 8981–8986, 2004.

[14] T. Iyoda, S. Shimoyama, K. Liu et al., "The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo," *Journal of Experimental Medicine*, vol. 195, no. 10, pp. 1289–1302, 2002.

[15] L. Castano and G. S. Eisenbarth, "Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat," *Annual Review of Immunology*, vol. 8, pp. 647–679, 1990.

[16] A. Jansen, F. Homo-Delarche, H. Hooijkaas, P. J. Leenen, M. Dardenne, and H. A. Drexhage, "Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice," *Diabetes*, vol. 43, no. 5, pp. 667–675, 1994.

[17] E. Dahlén, K. Dawe, L. Ohlsson, and G. Hedlund, "Dendritic cells and macrophages are the first and major producers of TNF-α in pancreatic islets in the nonobese diabetic mouse," *Journal of Immunology*, vol. 160, no. 7, pp. 3585–3593, 1998.

[18] S. Trembleau, T. Germann, M. K. Gately, and L. Adorini, "The role of IL-12 in the induction of organ-specific autoimmune diseases," *Immunology Today*, vol. 16, no. 8, pp. 383–386, 1995.

[19] D. V. Serreze, "Autoimmune diabetes results from genetic defects manifest by antigen presenting cells," *FASEB Journal*, vol. 7, no. 11, pp. 1092–1096, 1993.

[20] S. Boudaly, J. Morin, R. Berthier, P. Marche, and C. Boitard, "Altered dendritic cells (DC) might be responsible for regulatory T cell imbalance and autoimmunity in nonobese diabetic (NOD) mice," *European Cytokine Network*, vol. 13, no. 1, pp. 29–37, 2002.

[21] M. Feili-Hariri and P. A. Morel, "Phenotypic and functional characteristics of BM-derived DC from NOD and non-diabetes-prone strains," *Clinical Immunology*, vol. 98, no. 1, pp. 133–142, 2001.

[22] A. C. Vasquez, M. Feili-Hariri, R. J. Tan, and P. A. Morel, "Qualitative and quantitative abnormalities in splenic dendritic cell populations in NOD mice," *Clinical and Experimental Immunology*, vol. 135, no. 2, pp. 209–218, 2004.

[23] R. M. Steinman and M. C. Nussenzweig, "Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 1, pp. 351–358, 2002.

[24] S. Gaudreau, C. Guindi, M. Ménard, G. Besin, G. Dupuis, and A. Amrani, "Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of CD4+CD25+ regulatory T cells," *Journal of Immunology*, vol. 179, no. 6, pp. 3638–3647, 2007.

[25] M. B. Lutz, N. Kukutsch, A. L. J. Ogilvie et al., "An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow," *Journal of Immunological Methods*, vol. 223, no. 1, pp. 77–92, 1999.

[26] K. Inaba, M. Inaba, M. Naito, and R. M. Steinman, "Dendritic cell progenitors phagocytose particulates, including Bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo," *Journal of Experimental Medicine*, vol. 178, no. 2, pp. 479–488, 1993.

[27] M. B. Lutz, R. M. Suri, M. Niimi et al., "Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo," *European Journal of Immunology*, vol. 30, no. 7, pp. 1813–1822, 2000.

[28] S. Makino, K. Kunimoto, Y. Muraoka, Y. Mizushima, K. Katagiri, and Y. Tochino, "Breeding of a non-obese, diabetic strain of mice," *Jikken Dobutsu*, vol. 29, pp. 1–13, 1980.

[29] S. E. Eckenrode, Q. Ruan, P. Yang, W. Zheng, R. A. McIndoe, and J. X. She, "Gene expression profiles define a key checkpoint for Type 1 diabetes in NOD mice," *Diabetes*, vol. 53, no. 2, pp. 366–375, 2004.

[30] K. H. S. Wilson, S. E. Eckenrode, Q. Z. Li et al., "Microarray analysis of gene expression in the kidneys of new- and post-onset diabetic NOD mice," *Diabetes*, vol. 52, no. 8, pp. 2151–2159, 2003.

[31] C. Forestier, T. Takaki, A. Molano et al., "Improved outcomes in NOD mice treated with a novel Th2 cytokine-biasing NKT cell activator," *Journal of Immunology*, vol. 178, no. 3, pp. 1415–1425, 2007.

[32] M. Mizuno, M. Masumura, C. Tomi et al., "Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice," *Journal of Autoimmunity*, vol. 23, no. 4, pp. 293–300, 2004.

[33] L. Weiss, V. Barak, M. Zeira et al., "Cytokine production in linomide-treated NOD mice and the potential role of a Th (1)/Th(2) shift on autoimmune and anti-inflammatory processes," *Cytokine*, vol. 19, no. 2, pp. 85–93, 2002.

[34] M. Feili-Hariri, D. H. Falkner, and P. A. Morel, "Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice," *European Journal of Immunology*, vol. 32, no. 7, pp. 2021–2030, 2002.

[35] M. Lohoff, H. W. Mittrücker, S. Prechtl et al., "Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 18, pp. 11808–11812, 2002.

[36] M. Huber, A. Brüstle, K. Reinhard et al., "IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 52, pp. 20846–20851, 2008.

[37] R. Spolski, M. Kashyap, C. Robinson, Z. Yu, and W. J. Leonard, "IL-21 signaling is critical for the development of type I diabetes in the NOD mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 14028–14033, 2008.

[38] A. P. R. Sutherland, T. V. Belle, A. L. Wurster et al., "Interleukin-21 is required for the development of type 1 diabetes in nod mice," *Diabetes*, vol. 58, no. 5, pp. 1144–1155, 2009.

[39] J. A. Emamaullee, J. Davis, S. Merani et al., "Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice," *Diabetes*, vol. 58, no. 6, pp. 1302–1311, 2009.

[40] H. Wang and H. C. Morse, "IRF8 regulates myeloid and B lymphoid lineage diversification," *Immunologic Research*, vol. 43, no. 1–3, pp. 109–117, 2009.

[41] A. M. Marleau and B. Singh, "Myeloid dendritic cells in non-obese diabetic mice have elevated costimulatory and T helper-1-inducing abilities," *Journal of Autoimmunity*, vol. 19, no. 1-2, pp. 23–35, 2002.

[42] V. Saxena, J. K. Ondr, A. F. Magnusen, D. H. Munn, and J. D. Katz, "The countervailing actions of myeloid and plasmacytoid dendritic cells control autoimmune diabetes in the nonobese diabetic mouse," *Journal of Immunology*, vol. 179, no. 8, pp. 5041–5053, 2007.

[43] M. Lee, AE. Y. Kim, and Y. Kang, "Defects in the differentiation and function of bone marrow-derived dendritic cells in non-obese diabetic mice," *Journal of Korean Medical Science*, vol. 15, no. 2, pp. 217–223, 2000.

[44] J. Strid, L. Lopes, J. Marcinkiewicz et al., "A defect in bone marrow derived dendritic cell maturation in the nonobese diabetic mouse," *Clinical and Experimental Immunology*, vol. 123, no. 3, pp. 375–381, 2001.

[45] I. M. Wang, C. Contursi, A. Masumi, X. Ma, G. Trinchieri, and K. Ozato, "An IFN-γ-inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages," *Journal of Immunology*, vol. 165, no. 1, pp. 271–279, 2000.

[46] A. Masumi, S. Tamaoki, I. M. Wang, K. Ozato, and K. Komuro, "IRF-8/ICSBP and IRF-1 cooperatively stimulate mouse IL-12 promoter activity in macrophages," *FEBS Letters*, vol. 531, no. 2, pp. 348–353, 2002.

[47] C. Zhu, K. Rao, H. Xiong et al., "Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP," *Journal of Biological Chemistry*, vol. 278, no. 41, pp. 39372–39382, 2003.

[48] J. Liu, X. Guan, T. Tamura, K. Ozato, and X. Ma, "Synergistic activation of interleukin-12 p35 gene transcription by interferon regulatory factor-1 and interferon consensus sequence-binding protein," *Journal of Biological Chemistry*, vol. 279, no. 53, pp. 55609–55617, 2004.

[49] R. J. Grumont and S. Gerondakis, "Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: modulation of interferon-regulated gene expression by Rel/nuclear factor κB," *Journal of Experimental Medicine*, vol. 191, no. 8, pp. 1281–1291, 2000.

[50] S. E. Lamhamedi-Cherradi, S. Zheng, B. A. Hilliard et al., "Transcriptional regulation of Type I diabetes by NF-κB," *Journal of Immunology*, vol. 171, no. 9, pp. 4886–4892, 2003.

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Research Article

Development of Type 1 Diabetes Mellitus in Nonobese Diabetic Mice Follows Changes in Thymocyte and Peripheral T Lymphocyte Transcriptional Activity

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As early as one month of age, nonobese diabetic (NOD) mice feature pancreatic infiltration of autoreactive T lymphocytes, which destruct insulin-producing beta cells, producing autoimmune diabetes mellitus (T1D) within eight months. Thus, we hypothesized that during the development of T1D, the transcriptional modulation of immune reactivity genes may occur as thymocytes mature into peripheral T lymphocytes. The transcriptome of thymocytes and peripheral $CD3^+$ T lymphocytes from prediabetic or diabetic mice analyzed through microarray hybridizations identified 2,771 differentially expressed genes. Hierarchical clustering grouped mice according to age/T1D onset and genes according to their transcription profiling. The transcriptional activity of thymocytes developing into peripheral T lymphocytes revealed sequential participation of genes involved with $CD4^+$/$CD8^+$ T-cell differentiation (Themis), tolerance induction by Tregs (Foxp3), and apoptosis (Fasl) soon after T-cell activation (IL4), while the emergence of T1D coincided with the expression of cytotoxicity (Crtam) and inflammatory response genes (Tlr) by peripheral T lymphocytes.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease that results in the destruction of pancreatic insulin-producing beta cells [1, 2]. This destruction is a progressive process that occurs over five to eight months in the nonobese diabetic (NOD) mouse or several years in human patients [3]. The early stages of T1D pathogenesis are characterized by insulitis, an inflammation of the beta cells of the pancreas caused by lymphocyte infiltration. Nevertheless, the molecular genetics regulating the progress of beta cell failure and factors determining time of presentation of clinical diabetes are still poorly understood.

The NOD mouse is an autoimmune mouse strain and is a primary animal model used to dissect the mechanisms of lack of immune tolerance and autoimmune T1D, which reflects at least a part of human T1D [4–6]. The most important genetic determinants in susceptibility to diabetes lie in the major histocompatibility complex (MHC). Within the MHC locus, the class II molecules DQ8 and DQ2 in humans and the mouse homologue $I\text{-}Ag^7$ in the NOD mouse are thought to be particularly crucial [7]. In addition, many other genes have been identified that contribute to the development of diabetes in the NOD mouse [8].

In this murine strain, it is now clear that both the $CD4^+$ and $CD8^+$ subsets of T-cells play a role in the development of

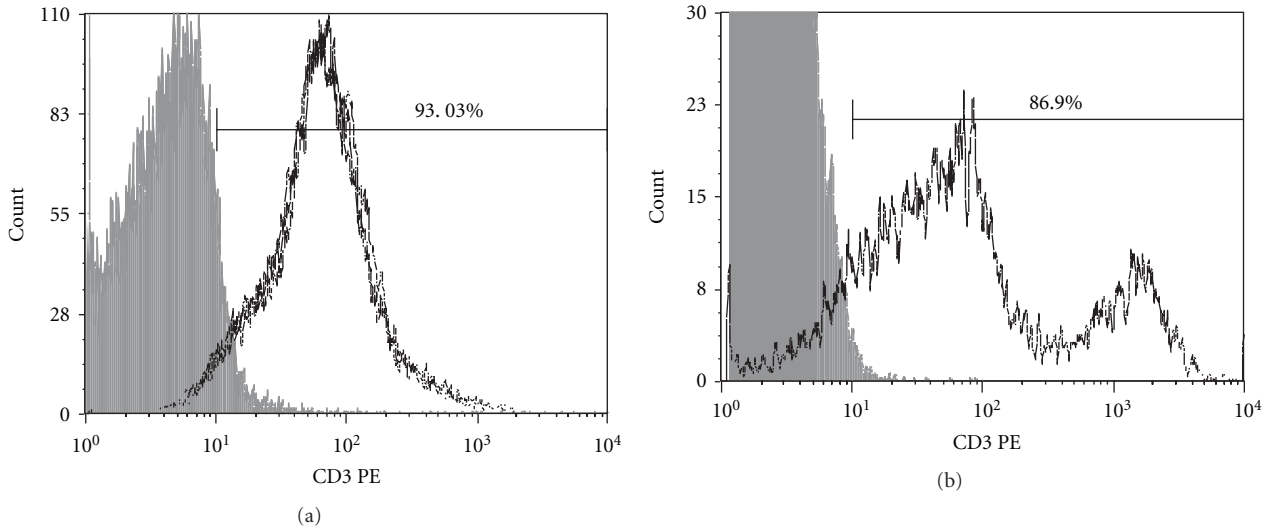

Figure 1: Fluorescent-activated cell sorting (FACS) analysis of thymocytes (approx. 93% purity) (a) and of peripheral $CD3^+$ T lymphocytes (approx. 87% purity).

disease. Diabetes does not occur in the absence of $CD4^+$ cells, as shown by studies using anti-CD4 antibodies [9] as well as in mice that lack $CD4^+$ T-cells [10], mice that are deficient in $CD8^+$ cells, either by anti-CD8 antibody injection into young mice [11], or mice in which few $CD8^+$ T-cells develop because of a genetic lack of Beta-2 microglobulin [12–14]. These findings support the idea that T1D is a function of the action of autoreactive $CD3^+$ T-cells that feature either a $CD4^+$ or $CD8^+$ phenotype.

The BDC2.5 line, which derives from a $CD4^+$ T-cell clone that is restricted by the NOD MHC class II A^{g7} molecule and specific for an unknown beta cell protein [15, 16], has been instrumental in the elucidation of several features of the immunoregulatory genes or cells that control the aggressively autoreactive T-cells in the periphery [17–20].

The differentiation into cytotoxic effector cells is the major function of $CD8^+$ T-cells, which are able to recognize antigenic peptides in the context of MHC class I molecules. These peptides are produced through the endogenous antigen presenting pathways, though evidence suggests that exogenous antigens are also presented by MHC class I molecules [21, 22].

The thymus exerts an important role in controlling autoreactive T-cells. An extremely diverse repertoire of T-cells is generated through the random rearrangement of T-cell receptor (TCR) gene segments. This random process generates autoreactive T-cells that are eventually eliminated through negative selection, which occurs in the medullar compartment of the thymic stroma in close association with the medullary thymic epithelial cells (mTECs). The negative selection plays an essential role in preventing pathogenic autoimmune reactions and/or autoimmune diseases.

The mTECs are essentially self-antigen-presenting cells. These cells express most of the parenchymal organs' self-antigens, a phenomenon that has been termed promiscuous gene expression (PGE) [23, 24]. Thymocytes are in close interaction with mTECs, establishing the thymic cross-talk. In fact, the self-antigens are coded from peripheral tissue antigen (PTA) genes. The translated PTAs are trimmed into peptides that are presented to thymocytes by means of the MHC. Dendritic cells also participate in the negative selection process after they have acquired PTA peptides from mTECs [23, 25–31].

Thymocyte clones that recognize self-peptide antigens during the cross-talk phase trigger a death gene expression cascade and die by apoptosis. Accordingly, the escaping autoreactive thymocytes from negative selection may cause severe aggressive reactions in the peripheral tissues and/or organs, provoking aggressive autoimmunity/autoimmune diseases. Thus, an imbalance in the central tolerance may have important consequences in the pathogenesis of autoimmune diseases, including T1D.

The central tolerance imbalance may explain, at least in part, the results of early studies using anti-CD3 antibodies; the results indicated that T1D in the NOD mouse is a T-cell-mediated disease [32].

We considered the following factors in our experiment: (1) peripheral T-cells represent the primary effectors in T1D autoreactivity in NOD mice; (2) the role played by autoreactive T-cells in the periphery may be a consequence of failure of the negative selection in the thymus; (3) the autoreactive phenotype of these cells may be a direct consequence of their transcriptional activity. Thus, our interest in this study was to analyze the transcriptome profile of thymocytes and peripheral $CD3^+$ lymphocytes in the course of T1D in NOD mice to elucidate the sequential participation of genes associated with autoreactivity.

2. Materials and Methods

2.1. Animals, Thymocytes and Peripheral T $CD3^+$ Lymphocyte Isolation. Female NOD mice were born in specific pathogen-free (SPF) conditions at the CEMIB-UNICAMP animal facility of the University of Campinas, SP, Brazil and maintained

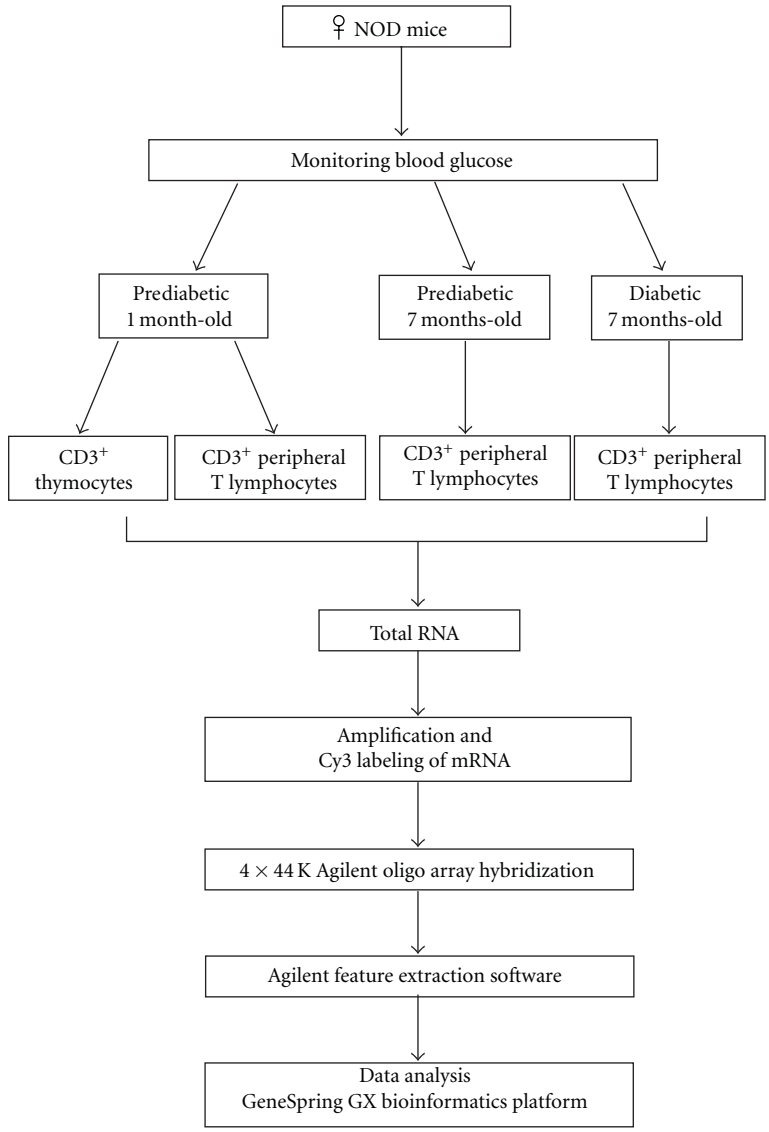

Figure 2: Experimental design of the work discriminating the biological samples used (animal groups and cell types), total RNA extraction, hybridizations, and microarray data analysis.

in SPF mini-isolators in our laboratory at the University of São Paulo, Campus of Ribeirão Preto, SP, Brazil during the experiment. We studied prediabetic 1-month-old and diabetic 7-month-old animals. Diabetes was confirmed by blood glucose levels (≥250 mg glucose/dL) using the Accu-Chek Active kit (Roche Diagnostics Brazil, São Paulo, Brazil).

The thymi from 1-month-old animals were dissected and trimmed of fat and connective tissue in DMEM/F10 medium, and thymocytes were obtained by 2-3 passages of the thymic fragments throughout a 10-μm mesh nylon membrane (Sefar Inc. Depew, NY, USA). Pelleted thymocytes were resuspended in phosphate-buffered saline (PBS). Fluorescent-activated cell sorting (FACS) analysis in a BD-FACScalibur flow cytometer with phycoerythrin-(PE-) labeled anti-CD3 antibody indicated that this procedure yielded approximately 93% purity of the thymocyte population (Figure 1(a)). These cells were then used for total RNA preparation.

The peripheral T CD3$^+$ lymphocytes from 1-month-old prediabetic, 7-month-old prediabetic, or 7-month-old diabetic animals were isolated from spleens using magnetic beads for negative selection (Pan T-cell isolation kit, mouse, Miltenyi Biotec) according to the manufacturer's instructions. FACS analysis with PE-labeled anti-CD3 antibody indicated that this procedure yielded approximately 87% purity of the CD3$^+$ T lymphocyte population (Figure 1(b)). These cells were then used for total RNA preparation. The animal experimental protocol was previously approved by the Commission for Ethics in Animal Research, Faculty of Medicine of Ribeirão Preto, USP, Brazil (Protocol no. 120/2008).

Table 1: Clusters of the differentially expressed genes and their ontology.

Cluster	Biological process	Genes
1	Activation of immune system	Cd55 Daf2 Fcer1g Klre1 Klrk1 Lax1 Lyn Malt1 Masp2 Plcg2 Tlr3 Tlr4 Tlr6 Unc93b1
	Adaptive immune response	Bcl3 Cd55 Cd74 Daf2 Fcer1g Fcgr3 Icam1 Icosl Lilrb3 Masp2 Pou2f2 Slc11a1 Tlr6
	Cell activation	Bank1 Bcl11a Bcl3 Btk Casp1 Cd74 Clcf1 Cplx2 Cxcr5 Elf4 Entpd1 Fcer1g Fcgr3 Fyb Gapt Gpr183 H2-M3 Hdac9 Hhex Icosl Il4 Irf1 Irf4 Itgax Klre1 Klrk1 Lax1 Lbp Lilrb3 Lyn Malt1 Plcg2 Pou2f2 Slc11a1 Tlr3 Tlr4 Tlr6 Vwf
	Defense response	Alox5 Bcl3 Btk Ccl19 Ccl5 Ccr2 Ccr5 Cd163 Cd180 Cd36 Cd55 Cd74 Chst2 Ciita Clec4a2 Clec4d Cnr2 Daf2 Ddx58 Fcer1g Fcgr3 H2-K1 H2-M3 Hdac9 Il18rap Il1b Irf8 Lbp Lta Ly86 Lyn Malt1 Masp2 Mefv Ncf1 Neurod2 Pglyrp1 Prg2 Samhd1 Slc11a1 Tirap Tlr3 Tlr4 Tlr6
	I-kappa8 kinase/NF-kappa8 cascade	Btk Irak2 Malt1 Rel Tirap Tlr4
	Immune effector process	Bcl3 Btk Cd55 Cd74 Cplx2 Daf2 Fcer1g Fcgr3 Icam1 Icosl Lax1 Lbp Lilrb3 Lyn Masp2 Ncf1 Pou2f2 Slc11a1
	Immune response	Bcl3 Btk Ccl19 Ccl5 Ccl9 Ccr2 Cd180 Cd55 Cd74 Ciita Clec4a2 Clec4d Cplx2 Daf2 Ddx58 Enpp1 Fasl Fcer1g Fcgr3 Fcgrt Gpr183 H2-D1 H2-K1 H2-M3 H2-Q10 H2-Q2 H2-Q7 H2-Q8 H2-T23 Hfe Icam1 Icosl Igj Il18rap Il1b Il4 Irf8 Irf8 Lax1 Lbp Lilrb3 Lta Ltb Ly86 Lyn Malt1 Masp2 Ncf1 Oas1b Pglyrp1 Plcg2 Pou2f2 Prg2 Samhd1 Slc11a1 Tirap Tlr3 Tlr4 Tlr6
	Immune response-activating signal transduction	Fcer1g Klre1 Klrk1 Lax1 Lyn Malt1 Plcg2 Tlr3 Tlr4 Tlr6 Unc93b1
	Immune system process	Bank1 Bcl11a Bcl3 Btk Casp1 Ccl19 Ccl5 Ccl9 Ccr2 Cd180 Cd300lf Cd55 Cd74 Ciita Clcf1 Clec4a2 Clec4d Cplx2 Crkl Csf1 Csf3r Cxcr5 Daf2 Ddx58 Dnase2a Elf4 Enpp1 Fasl Fcer1g Fcgr3 Fcgrt Fyb Gapt Gpr183 H2-D1 H2-K1 H2-M3 H2-Q10 H2-Q2 H2-Q7 H2-Q8 H2-T23 Hdac9 Hfe Hhex Icam1 Icosl Igj Il18rap Il1b Il4 Irf1 Irf4 Irf8 Itgax Klre1 Klrk1 Lax1 Lbp Lilrb3 Lta Ltb Ly86 Lyn Malt1 Masp2 Myo1e Ncf1 Oas1b Pglyrp1 Plcg2 Pou2f2 Prg2 Samhd1 Slc11a1 Terc Tirap Tlr3 Tlr4 Tlr6 Tnfrsf13c Unc93b1
	Inflammatory response	Alox5 Btk Ccl19 Ccl5 Ccr2 Cd163 Cd180 Cd55 Chst2 Cnr2 Daf2 Fcgr3 Hdac9 Il1b Lbp Lta Ly86 Lyn Masp2 Mefv Ncf1 Slc11a1 Tirap Tlr3 Tlr4 Tlr6
	Lymphocyte activation	Bank1 Bcl11a Bcl3 Cd74 Clcf1 Cxcr5 Elf4 Gapt Gpr183 H2-M3 Hdac9 Hhex Icosl Il4 Irf1 Itgax Klre1 Klrk1 Lax1 Malt1 Plcg2 Pou2f2 Slc11a1
	Lymphocyte activation during immune response	Bcl3 Gpr183 H2-M3 Plcg2 Slc11a1
	Lymphocyte mediated immunity	Bcl3 Cd55 Cd74 Daf2 Fcer1g Fcgr3 Icam1 Icosl Lilrb3 Masp2 Pou2f2 Slc11a1
	Positive regulation of interferon-gamma production	Bcl3 H2-M3 Irf8 Klre1 Klrk1 Lta Slc11a1 Tlr4
	Regulation of defense response	Adrb2 Anxa1 Cadm1 Ccl5 Ccr5 Cnr2 Crtam Fcer1g Fcgr3 H2-Bl H2-M3 Klrb1b Klre1 Klrk1 Lta Nt5e Tgm2 Tlr3 Tlr4 Tlr6 Unc93b1
	Regulation of immune effector process	Cadm1 Crtam Fcer1g Fcgr3 H2-Bl H2-K1 H2-M3 Hmox1 Klrb1b Klre1 Klrk1 Lta
	Regulation of immune response	Btla Cadm1 Cd55 Crtam Daf2 Fcer1g Fcgr3 H2-Bl H2-K1 H2-M3 Il4 Klrb1b Klre1 Klrk1 Lax1 Lta Lyn Malt1 Masp2 Plcg2 Slc11a1 Tlr3 Tlr4 Tlr6 Tnfrsf13c Unc93b1
	Regulation of inflammatory response	Adrb2 Anxa1 Ccl5 Cnr2 Fcer1g Fcgr3 Lta Nt5e Tgm2 Tlr4
	Regulation of leucocyte mediated cytotoxicity	Cadm1 Crtam H2-Bl H2-K1 H2-M3 Klrb1b Klre1 Klrk1
	Apoptosis	Actc1 Bag3 Dapl1 Dedd2 Fasl Gramd4 Hipk2 Nfkb1 Nod1 Pea15a Pim2 Pmaip1 Psen2 Ripk1 Sgk1 Sgms1 Shisa5 Tmem173 Traf1 Traf3ip2 Traf5

Table 1: Continued.

Cluster	Biological process	Genes
	Immune response	B2m Ccl3 Ccl5 Cxcl9 Eomes Fasl Foxp3 H2-Q10 Il18r1 Il1rl1 Il7r Irgm1 Myo1f Pf4 Psen2 Tgtp1 Tlr1 Tmem173 Tnfaip8l2 Tnfsf8 Traf3ip2
2	Immune system process	B2m Ccl3 Ccl5 Cxcl9 Eomes Fasl Flt3l Foxp3 Gimap5 H2-Q10 Il18r1 Il1rl1 Il2rb Il7r Irf1 Irgm1 Jak3 Myo1f Nfkb1 P2rx7 Pf4 Pik3cd Psen2 Slamf1 Tgfbr2 Tgtp1 Tlr1 Tmem173 Tnfaip8l2 Tnfsf8 Traf3ip2
	Regulation of I-kappaB kinase/NF-kappaB cascade	Card6 Il1rl1 Nod1 Pim2 Tgm2
	Regulation of signal transduction	Arhgef12 Arhgef3 Arrb1 Arrb2 Axin2 Card6 Cd44 Fasl Furin Il1rl1 Nod1 P2rx7 Pim2 Psen2 Rasa3 Rasgrp2 Rgs11 Runx2 S1pr1 Smad7 Socs3 Spry2 Tgm2 Zeb2
3	Immune system process	Add2 Ahsp Ank1 Ccl4 Ccr2 Cd48 Cebpa Ctla4 Ctse Elane Epas1 Epb4.2 Gimap5 Gm5077 Id2 Ifng Il1r1 Il1rl1 Il1rl2 Itgam Junb Klf1 Klf11 Mpo Plscr1 Polr3c Samhd1 Spna1 Tal1 Tgtp1 Trim10 Txnrd2 Zbtb32
4	Cell communication	Atg16l1 Bmp8a Cacna1c Cblc Chat Cldn5 Cxcr7 Drd5 Erbb3 Gad1 Gad2 Gast Gja5 Gnal Gpr12 Gpr173 Gpr82 Grp Grpr Hnf1b Itgax Kcnk2 Lin7a Mrgprb2 Ngfr Olfr1010 Olfr1022 Olfr1048 Olfr107 Olfr1090 Olfr1115 Olfr1161 Olfr128 Olfr1377 Olfr1384 Olfr1388 Olfr1459 Olfr1462 Olfr1469 Olfr1495 Olfr304 Olfr33 Olfr350 Olfr365 Olfr516 Olfr523 Olfr556 Olfr606 Olfr62 Olfr651 Olfr68 Olfr684 Olfr724 Olfr768 Olfr770 Olfr784 Olfr790 Olfr796 Olfr845 Olfr889 Olfr904 Olfr924 Olfr974 Park2 Pdx1 Pik3c2g Plat Pth Rab3b Rab3c Slc1a2 Slc6a4 Syn2 Taar4 Tacr1 Upk1a Vmn2r26 Vmn2r81 Xcr1
	G-protein coupled receptor protein signaling pathway	Cxcr7 Drd5 Gast Gnal Gpr12 Gpr173 Gpr82 Grp Grpr Kcnk2 Mrgprb2 Olfr1010 Olfr1022 Olfr1048 Olfr107 Olfr1090 Olfr1115 Olfr1161 Olfr128 Olfr1377 Olfr1384 Olfr1388 Olfr1459 Olfr1462 Olfr1469 Olfr1495 Olfr304 Olfr33 Olfr350 Olfr365 Olfr516 Olfr523 Olfr556 Olfr606 Olfr62 Olfr651 Olfr68 Olfr684 Olfr724 Olfr768 Olfr770 Olfr784 Olfr790 Olfr796 Olfr845 Olfr889 Olfr904 Olfr924 Olfr974 Pth Taar4 Tacr1 Vmn2r26 Vmn2r81 Xcr1
	Signal transduction	Bmp8a Cblc Cxcr7 Drd5 Erbb3 Gast Gnal Gpr12 Gpr173 Gpr82 Grp Grpr Itgax Kcnk2 Mrgprb2 Ngfr Olfr1010 Olfr1022 Olfr1048 Olfr107 Olfr1090 Olfr1115 Olfr1161 Olfr128 Olfr1377 Olfr1384 Olfr1388 Olfr1459 Olfr1462 Olfr1469 Olfr1495 Olfr304 Olfr33 Olfr350 Olfr365 Olfr516 Olfr523 Olfr556 Olfr606 Olfr62 Olfr651 Olfr68 Olfr684 Olfr724 Olfr768 Olfr770 Olfr784 Olfr790 Olfr796 Olfr845 Olfr889 Olfr904 Olfr924 Olfr974 Pdx1 Pik3c2g Plat Pth Rab3b Rab3c Taar4 Tacr1 Upk1a Vmn2r26 Vmn2r81 Xcr1
	Apoptosis, apoptosis regulation	Alms1 Bcl2l1 Birc5 Bub1 Bub1b Casp6 Ckap2 Cul7 E2f1 E2f2 Egln3 Epha2 Fas Ift57 Krt18 Krt8 Lig4 Phlda1 Rad21 Rtn3 Stk3 Tfdp1 Tia1 Tpx2 Traf4 Trp53inp1 Vdac1
	Cell activation	Ada Bcl11b Ccnd3 Cd4 Cd8a Cxcl12 Fas Hdac7 Hells Lig4 Ly6d Msh6 Patz1 Rag1 Rorc Satb1 Sox4 Themis
5	Cell cycle	1190002H23Rik Anapc5 Anln Aspm Aurka B230120H23Rik Birc5 Bub1 Bub1b C79407 Casc5 Ccdc99 Ccna2 Ccnb1 Ccnb2 Ccnd3 Ccne2 Ccnf Ccng2 Cdc20 Cdc25a Cdc25c Cdc45 Cdca2 Cdca3 Cdca5 Cdk1 Cdkn1a Cdkn2c Cdkn3 Cenpe Cenpj Cep55 Chek1 Ckap2 Ckap5 Cul7 E2f1 E2f2 E2f3 E2f7 Ercc6l Esco2 Espl1 F630043A04Rik Fam33a Fbxo5 Gas2l3 Gsg2 H2afx Haus2 Hells Kif11 Kif2c Kifc1 Lig4 Mad1l1 Mki67 Myb Ncapd2 Ncaph Ndc80 Nde1 Nek2 Nsl1 Nuf2 Nusap1 Pard6g Phgdh Prc1 Psrc1 Pttg1 Rad21 Rad51c Rbbp4 Rcc1 Sgol1 Sgol2 Skp2 Spag5 Spc25 Stmn1 Tacc2 Tacc3 Tfdp1 Tpx2 Trp53inp1 Tubb5 Tubg1 Ube2c Uhrf1 Wee1

Table 1: Continued.

Cluster	Biological process	Genes
	Cell proliferation	Alms1 Aspm Bcl2l1 Ccnd3 Cxcl12 Gins1 Hells Hmgb1 Lig4 Lipa Mki67 Ncapg2 Nde1 Satb1 Tacc2 Tacc3 Uhrf1 Vegfa
	Lymphocyte activation	Ada Bcl11b Ccnd3 Cd4 Cd8a Cxcl12 Faz Hdac7 Hells Lig4 Ly6d Msh6 Patz1 Rag1 Rorc Satb1 Sox4 Themis
	Lymphocyte differentiation	Ada Bcl11b Cd4 Cd8a Fas Hdac7 Hells Lig4 Ly6d Patz1 Rag1 Rorc Satb1 Sox4 Themis
	T-cell activation	Bcl11b Ccnd3 Cd4 Cd8a Cxcl12 Fas Lig4 Patz1 Rag1 Rorc Satb1 Sox4 Themis
	T-cell differentiation	Bcl11b Cd4 Cd8a Fas Lig4 Patz1 Rag1 Rorc Satb1 Sox4 Themis
	V(D)J recombination	Bcl11b Lig4 Rag1 Xrcc6
	CD8-positive, alpha-beta T-cell differentiation	Pax1 Satb1
	Cell differentiation	Acan Bcl11b Bcl2l1 Bcl6 Bmp7 Cby1 Cdkn1c Cux1 Cxcl12 Dyrk1b Ephb2 Gjc1 Gpc2 Hdac2 Id3 Ift81 Igfbp3 Lhx2 Lig4 Morc1 Msi2 Myh10 Notch1 Notch3 Ntn1 Paqr5 Pax1 Pias2 Pitx2 Ptprf Rag1 Rag2 Runx1 Satb1 Sox11 Spata6 Spo11 Stra8 Tbata Thy1 Whrn
6	Cell-cell adhesion	Acan Arvcf Jup Lmo4 Mcam Ncam1 Ntn1 Ptprf Pvrl3 Vangl2
	Lymphocyte differentiation	Bcl11b Bcl6 Lig4 Pax1 Rag1 Rag2 Satb1
	Somatic diversification and recombination of T-cell receptor genes	Bcl11b Lig4
	T-cell activation	Bcl11b Cxcl12 Lig4 Pax1 Rag1 Rag2 Satb1 Sla2
	T-cell differentiation	Bcl11b Lig4 Pax1 Rag1 Rag2 Satb1
	T-cell receptor V(D)J recombination	Bcl11b Lig4
	V(D)J recombination	Bcl11b Lig4 Rag1 Rag2

2.2. Total RNA Preparation. The total RNA was extracted from 1×10^7 thymocytes or peripheral T $CD3^+$ lymphocytes using a mirVana total RNA isolation kit (Ambion) according to the manufacturer's instructions. RNA preparations were confirmed to be free of proteins and phenol by UV spectrophotometry. The state of degradation was assessed by microfluidic electrophoresis using Agilent RNA Nano 6000 chips and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples that were free of proteins and phenol and featured an RNA Integrity Number (RIN) $\geq$ 9.0 were used.

2.3. RNA Amplification, Labeling, Microarray Hybridization and, Data Analysis. Changes in gene expression were evaluated using the Agilent one-color (Cy3 fluorochrome) microarray-based gene expression platform according to the manufacturer's instructions. For hybridization onto whole mouse genome 4 $\times$ 44 K 60-mer oligonucleotide arrays (G4122F, Agilent Technologies, Palo Alto, CA, USA), 500 ng total RNA was used in the one-color Quick Amp labeling kit (Agilent Technologies, Santa Clara, CA, USA). Samples of complementary RNA (cRNA) were hybridized for 18 h at 42°C in a rotator oven and were then washed. The array slides were scanned using a DNA microarray scanner (Agilent Technologies), and the hybridization signals were extracted using the Agilent Feature Extraction software version 10.5.

Gene expression profiles from independent preparations of thymocytes (from 1-month-old prediabetic mice) or $CD3^+$ peripheral lymphocytes (from 1-month-old prediabetic, 7-month-old prediabetic or 7-month-old diabetic mice) were analyzed through comparisons of the microarray hybridizations of the respective samples. Figure 2 depicts the experimental design for further comparison of the gene profiling.

A complete file that provides all of the genes present in the microarray used in this study, as well as the experimental conditions, is available online at the MIAME public database [33], ArrayExpress accession E-MEXP 3047.

The microarray numerical quantitative data were normalized to the 75th percentile and were analyzed using the GeneSpring GX bioinformatics platform [34] according to the default instructions allowing hierarchical clustering of samples of mice or genes based on ANOVA statistical analysis ($P < .01$) with a fold change >2.0 and an uncentered Pearson correlation metrics [35]. The similarities and dissimilarities in gene expression are presented as dendrograms, in which the pattern and length of the branches reflect the relatedness of the samples or genes, and heat maps.

2.4. Gene Ontology. Microarray data analysis was used to identify gene expression based on combined information from the public databases DAVID [36] and SOURCE [37]. These databases show gene annotation enrichment analysis, functional annotation clustering, BioCarta and KEGG pathway mapping (DAVID), or microarray data and sequencing

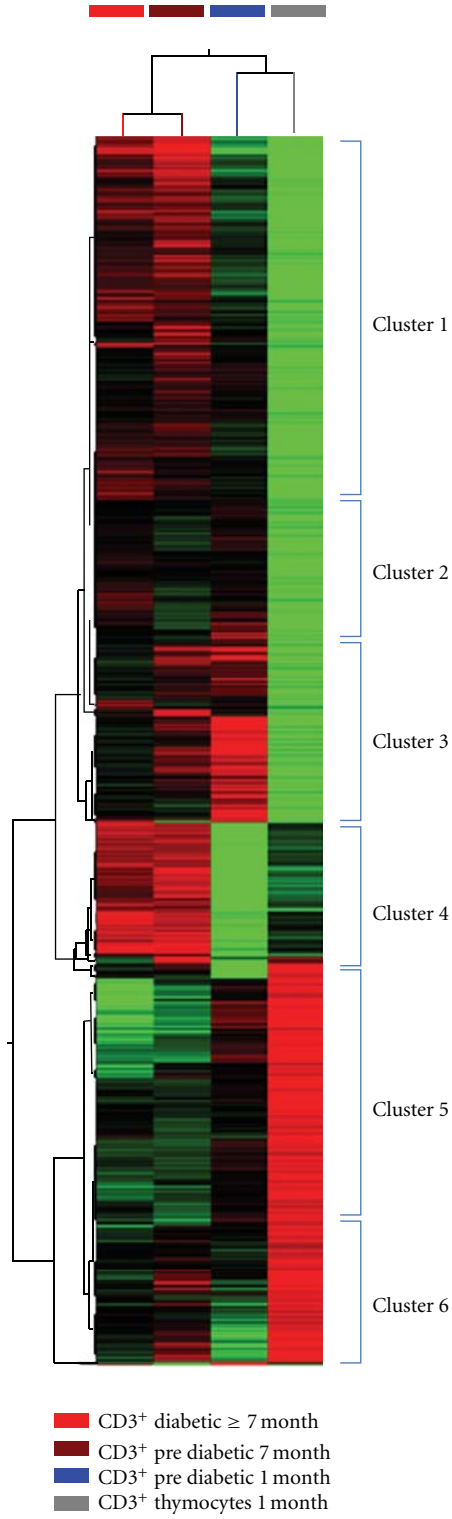

Figure 3: Hierarchical clustering of thymocytes and peripheral CD3+ lymphocytes of nonobese diabetic (NOD) mice based on microarray gene expression profiling. Dendrograms and heat maps were obtained using the Cluster-Tree View program within the GeneSpring GX (Agilent) platform. Red = upregulation, green = downregulation, and black = unmodulated (Pearson correlation metrics, 75 percentile). The 2,771 differentially expressed genes were divided in six clusters (clusters 1 to 6) according to their relative expression levels and ontology.

of cDNA clones from different organ/tissues (SOURCE), including GenBank accession number, chromosomal location and the molecular/biological function of each gene analyzed.

2.5. Oligonucleotide Primer Design and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Microarray data were confirmed using qRT-PCR for the genes listed below that were differentially expressed between thymocytes and peripheral CD3+ T lymphocytes. The cDNA sequences of these genes were retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/GenbankSearch.html), and the Primer3 web tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was used to select pairs of oligonucleotide primers spanning an intron/exon junction and with consideration of the alternative transcripts. An optimal melting temperature of 60°C was standardized for all genes. The following forward and reverse sequences are given in the 5′ to 3′ orientation: Hypoxanthine phosphoribosyltransferase 1 (HPRT1, accession number NM_000194.2, GACCAGTCAA CAGGGGACAT′ and CTGCATTGTTTTGCCAGTGT); Fas ligand (Fasl, accession number NM_000639, ACTCCGTG AGTTCACCAACC and GTGGGGGTTCCCTGTTAAAT); Toll-like receptor 3 (Tlr3, accession number BC068487, TTGTCTTCTGCACGAACCTG and CCCGTTCCCAACTT TGTAGA); Toll-like receptor 4 (Tlr4, accession number NM_138554, TCAGAACTTCAGTGGCTGGA CCTGGGGA AAAACTCTGGAT); Forkhead box P3 (Foxp3, accession number NM_014009, TCTTCGAGGAGCCAGAAGAG and GCTCCAGAGACTGCACCACT); Thymocyte selection associated (Themis, accession number NM_001164685, AAATG AAGCTCACCTTGCTCA and ATCCTGGCCACTTTCATC TG). HPRT1 was used as the constitutively expressed gene. Transcriptional expression levels were determined using a StepOne Real-Time PCR System (Applied Biosystems, USA). The ΔΔCT relative normalization method was used as described previously. We used the GraphPad Prism 5.00 tool (http://www.graphpad.com/prism/Prism.html) to run one-way or two-way ANOVAs with Bonferroni's correction statistics.

3. Results

Although the expression pattern remained unchanged between thymocytes and peripheral T lymphocytes from prediabetic and diabetic animals for the majority of the 44,000 sequences tested, which presented a control/test ratio ≈ 1.0 (Pearson correlation), 2,771 genes were found to be differentially expressed. Hierarchical clustering of the data allowed for the identification of clusters of upregulated (induced) and downregulated (repressed) genes. Changes in gene expression profile could be observed when comparing thymocytes from 1-month-old prediabetic mice with peripheral CD3+ lymphocytes from 1-month-old prediabetic, 7-month-old prediabetic, or 7-month-old diabetic mice to observe the modulated genes that coincided with the development of T1D.

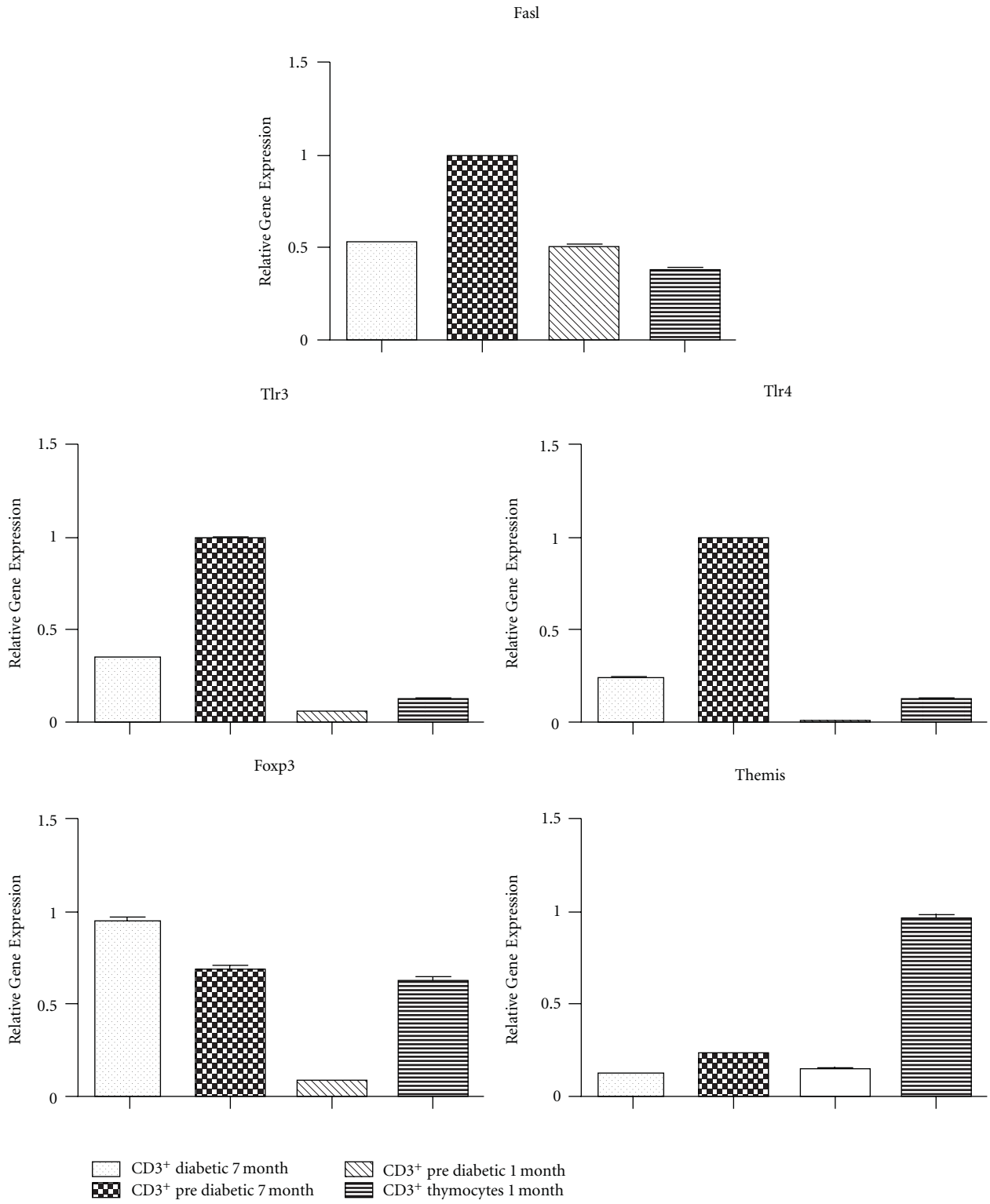

Figure 4: Confirmation of microarray data by qRT-PCR. The Fasl, Tlr3, and Tlr4 genes were downregulated in thymocytes from prediabetic animals, Foxp3 was upregulated in peripheral $CD3^+$ lymphocytes from diabetic animals, and Themis was upregulated in thymocytes from prediabetic animals.

Hierarchical clustering analysis depicted in Figure 3 shows variability in the hybridization signatures between cell types. The upper horizontal dendrogram (cell samples) demonstrates that this variability could distinguish cells according to their developmental phase, namely, thymocytes (prediabetic animals) from peripheral $CD3^+$ lymphocytes (prediabetic and diabetic animals). The left vertical dendrogram shows the genes that were differentially expressed (up- or downregulated) according to their respective biological function, and the genes were divided into six clusters (Figure 3 and Table 1). Clusters 1 to 4 contain genes downregulated in thymocytes and peripheral $CD3^+$ lymphocytes from 1-month-old prediabetic animals and progressively upregulated in peripheral $CD3^+$ lymphocytes from 7-month-old prediabetic and diabetic animals.

Cluster 1 comprises 767 differentially expressed genes, and the following genes were highlighted because they participate in immune processes potentially implicated in the pathogenesis of T1D: IL4, involved with T-cell activation, Crtam, involved in cytotoxicity and Tlr3, Tlr4, and Tlr6, involved in inflammatory response.

Cluster 2 comprises 340 differentially expressed genes, and the following genes were highlighted: Fasl, involved in the induction of apoptosis by extracellular signals and FoxP3, involved in $CD25^+$ alpha-beta T-regulatory (Treg) cell differentiation and positive regulation of T-cell tolerance induction.

Cluster 3 comprises 435 differentially expressed genes, and the following genes were highlighted: Chemokine (C–C motif) ligand 4 gene (Ccl4) and Chemokine (C–C motif) receptor 2 gene (Ccr2), both involved in inflammatory response; CD48 antigen gene, involved in T-cell activation; Cytotoxic T-lymphocyte-associated protein 4 gene (Ctla4), involved in negative regulation of Treg cell differentiation; Interferon gamma gene (Ifng), involved in positive regulation of T-cell proliferation; Integrin alpha M gene (Itgam), involved in cell-cell adhesion.

Cluster 4 comprises 292 differentially expressed genes, and the following genes were highlighted: Integrin alpha X (Itgax) and Claudin 5 (Cldn5) gene, involved in cell-cell adhesion; Gap junction protein, alpha 5 (Gja5) gene, involved in cell-cell signaling; HNF1 homeobox 1 transcription factor, involved in maturity onset diabetes of the young and circadian regulation of transcription; and Gastrin (Gast) and Guanine nucleotide-binding protein 1 (Gna1) gene, both involved in protein signaling pathway. Interestingly, this cluster comprises various members of the olfactory receptor (Olfr) gene family, which is involved with cell signaling pathways.

Finally, clusters 5 and 6 comprise 610 and 327 genes, respectively, which feature a different expression pattern than those included in the previous clusters, that is, they were mainly upregulated in thymocytes from prediabetic mice and progressively downregulated in peripheral $CD3^+$ lymphocytes from prediabetic and diabetic animals. These two clusters contain genes involved in CD4/CD8 cell differentiation (Themis) and V(D)J recombination (Rag1 and Rag2).

Using qRT-PCR, we assayed the expression levels of five differentially expressed genes (Fasl, Tlr3, Tlr4, Foxp3 and Themis) that are associated with thymocyte selection, T-cell differentiation, cell activation, or inflammation. The expression levels obtained with qRT-PCR method were comparable with microarrays, for example; the Fasl, Tlr3 and Tlr4 genes were downregulated in thymocytes from prediabetic animals, Foxp3 was upregulated in peripheral $CD3^+$ lymphocytes from diabetic animals, and Themis was upregulated in thymocytes from prediabetic animals (Figure 4).

4. Discussion

In this study, we assessed the hypothesis that the transcriptional modulation of immune reactivity genes may occur during the development of T1D as thymocytes mature into peripheral T lymphocytes. Consequently, the emergence of T1D might follow a pattern of the transcriptional activity of these cells, sequentially featuring genes associated with a negative selection of thymocytes, T-cell maturation, differentiation, and autoreactivity.

As discussed in a previous study [38], diabetes in NOD mice is similar to T1D patients, and the progress of diabetes in these animals occurs in two stages. In the first stage, autoreactive $CD8^+$ T lymphocytes infiltrate the pancreatic islet by 1 month after the birth. However, most pancreatic islets are preserved at this phase, and the animals are clinically healthy. Stage one can persist for months because the autoimmune attack is under control and is relatively nondestructive. In the second stage, most of pancreatic islets are destructed and animals are often diabetic.

Because these animals spontaneously develop autoimmune diabetes mellitus that is similar to human T1D, including the presence of pancreas-specific autoantibodies and autoreactive $CD4^+$ or $CD8^+$ T-cells and synteny to human chromosomal linkage groups associated with T1D, they are a classical model system for investigating autoimmune T1D and/or failure in the tolerance mechanisms [4, 6, 28]. Thus, we chose to employ the NOD mouse as a model system given the difficulty of easily testing this theory in humans.

To exclude the influence of genetic backgrounds of nonautoimmune mouse strains, we compared only groups of NOD mice in two distinct phases of autoimmunity, namely, prediabetic (1- or 7-month-old) and diabetic ($\geq$7-month-old).

Moreover, by establishing transcriptome comparisons between cells from prediabetic and diabetic animals or between thymocytes and peripheral T lymphocytes, it was possible to find genes temporally regulated (because T1D emerges according to age) and regulated according to T-cell development.

The procedures for isolation of thymocytes and peripheral $CD3^+$ T lymphocytes used in this work yielded purities (approximately 93% for thymocytes and 85% for peripheral $CD3^+$ T lymphocytes) that are comparable to automated cell sorting of T-cells. Moreover, the peripheral $CD3^+$ T lymphocytes were isolated by negative separation, minimizing eventual cell activation by artifacts.

Figure 3 (clusters 1 to 4) and Table 1 show the gene expression pattern observed in thymocytes and peripheral $CD3^+$ lymphocytes from prediabetic 1-month-old animals. The gene expression in these cells could be considered to occur in the first stage of T1D, and this expression features the down-regulation of genes involved with the activation of the immune system/adaptive response, cell activation, NF-KappaB cascade, immune effector/immune response processes, inflammatory response, lymphocyte activation, the regulation of interferon-gamma production, the regulation of leukocyte-mediated cytotoxicity, apoptosis, cell communication, and signal transduction. Altogether, these biological processes are necessary for the development of lymphocyte differentiation and autoreactivity.

The thymocytes from prediabetic 1-month-old animals featured down-regulation of genes associated with several biological processes that were gradually upregulated in the peripheral $CD3^+$ lymphocytes from prediabetic 1-month-old, prediabetic 7-month-old, and diabetic 7-month-old animals (Figure 3 and Table 1). Among these processes and their respective genes, we selected some of the most important to discuss because of their direct or indirect association with aggressive autoimmunity.

The apoptosis process featured genes including Fas ligand (Fasl), TNF receptor-associated factor (Traf1 and Traf5), Traf3 interacting protein 2 (Traf3ip2), and Tumor necrosis factor (ligand) superfamily, member 8 (Tnfsf8 or TRAIL).

Because apoptosis is the final stage of the negative selection of thymocytes in the thymus [29], the down-regulation of these genes may favor the survival of autoreactive thymocyte clones, including those that recognize pancreatic beta cell autoantigens.

A role for TRAIL in T1D in NOD mice has been evidenced by its blockade with consequent exacerbation of the disease [39] and by its systemic delivery indicating that T1D can be prevented by TRAIL overexpression through an enhancement of the tissue inhibitor of the metalloproteinase-1 (TIMP-1) function [40]. The authors concluded that elevated TIMP-1 production inhibits the activity of matrix metalloproteinases, which may contribute to the suppression of the transmigration of diabetogenic T-cells into the pancreatic islets and protects pancreatic beta cells from cytokine-induced apoptosis.

The tolerance induction process featured Forkhead box P3 (Foxp3) gene, which was also downregulated in thymocytes from 1-month-old prediabetic animals. This gene is involved in tolerance induction via $CD4^+CD25^+$ T-regulatory cells (Tregs) [41–43], a process that directly activates any of the steps required for tolerance, a physiologic state in which the immune system does not react destructively against self-components. Thus, their deregulation may impair the Treg-mediated suppression of aggressive autoimmunity, favoring the survival of autoreactive thymocytes in the thymus.

The T-cell activation process featured several downregulated genes in thymocytes from 1-month-old prediabetic animals, from which we highlight Chemokine (C–C ligand motif) ligand 4 (Ccl4), Chemokine (C–C motif) receptor 2 (Ccr2), Cd48 antigen and Interferon, gamma (Ifng), and Interleukin 4 (IL4) genes. These genes were gradually upregulated in peripheral CD3+ lymphocytes from prediabetic to diabetic animals and may increase the activation of autoreactive T-cell clones in the periphery.

Interestingly, the G-protein-coupled receptor protein signaling pathway and signal transduction processes shared several members of the olfactory gene family (Olfr), which is involved in sensory perception of smell through receptor and signal transducer activity. The two pathways also shared bone morphogenetic protein 8a (Bmp8a) gene, which is involved in growth and ossification. These genes were downregulated in thymocytes from 1-month-old prediabetic animals and were gradually upregulated in peripheral $CD3^+$ lymphocytes from prediabetic to diabetic animals. Despite their disparate biological processes in the context of T1D, these genes may play a role in the receptor-mediated signal transduction activity of peripheral T-cells in diabetic animals.

Dissimilar from the pattern of genes related to the processes discussed above, the T-cell receptor V(D)J recombination and $CD4^+/CD8^+$ T-cell differentiation featured genes were upregulated in thymocytes and $CD3^+$ peripheral T lymphocytes from prediabetic 1-month-old animals and were gradually downregulated in peripheral CD3+ lymphocytes from prediabetic and diabetic 7-month-old animals.

Among these genes, we highlight the recombination activating genes (Rag-1 and Rag-2) that code the recombinase catalytic complex involved in the recognition of recombination signal sequences (RSS) within the T-cell receptor loci (TCR alpha, beta, gamma or delta), cutting and recombining DNA during aleatory generation of TCR diversity. The generation of TCR diversity implies the production of T-cell clones directed to foreign antigens and also autoreactive clones. Autoreactive clones are normally eliminated by apoptosis throughout the negative selection process.

Also, Adenosine deaminase (Ada) gene, which is involved in a process that increases the frequency of T-cell differentiation in the thymus, and Cytotoxic T-lymphocyte-associated protein 4 (Ctla4) gene, which in contrast to Ada gene, is involved in the negative regulation of T-cell proliferation, were both identified.

Finally, we found the Thymocyte selection associated (Themis) gene, which is expressed in the thymus and to a lesser extent in the spleen but is not detectable in nonlymphoid tissues. This gene is highly expressed in thymocytes between the pre-T-cell antigen receptor (pre-TCR) and positive-selection checkpoints and is expressed at a low level in mature T-cells (at the protein level). Themis is also implicated in the control of T-helper CD4+/cytotoxic CD8+ cell fate. Moreover, the CD4 as well as CD8a antigen genes, which define the respective T-helper $CD4^+$ or T cytotoxic CD8+ phenotypes, were also found.

During their permanence within the thymus, thymocytes activate a developmentally complex mechanism because of close contact with thymic stroma. The medullary thymic epithelial cells (mTECs), which form the stromal medullar compartment, present peripheral tissue antigens (PTAs) to thymocytes to eliminate autoreactive clones by inducing

apoptosis (negative selection), leading to tolerance induction [23, 24].

Failure in the expression of specific PTA genes in the thymic stroma is strongly associated with aggressive autoimmunity as recently observed during the development of T1D in NOD mice [38] or collagen-induced arthritis in DBA-1/J mice [44]. This indeed led to survival of autoreactive thymocyte clones that once in the periphery, mediate autoimmune attack of target structures such as pancreatic beta cells.

To better understand the control of transcriptional activity of thymocytes specifically associated with the induction of tolerance and negative selection within the thymus or cytotoxicity and inflammatory response in peripheral T lymphocytes, we propose further experiments to evaluate the participation of microRNAs (miRNAs) that once dysregulated might be associated with aggressive autoimmunity of T1D.

5. Conclusion

In this study, we were able to construct a transcriptional profile of T-cell development comparing thymocytes with peripheral CD3+ lymphocytes in the context of the emergence of T1D. The sequential participation of genes involved with the main steps of T-cell development such as the generation of TCR diversity, $CD4^+/CD8^+$ cell fate, apoptosis, and negative selection demonstrated that the T1D autoimmune phenotype in NOD mice runs in parallel with transcriptome changes of T-cells. The results obtained confirm our initial hypothesis.

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References

[1] A. L. Notkins and Å. Lernmark, "Autoimmune type 1 diabetes: resolved and unresolved issues," *Journal of Clinical Investigation*, vol. 108, no. 9, pp. 1247–1252, 2001.

[2] B. Regnault, J. Osorio Y Fortea, D. Miao, G. Eisenbarth, and E. Melanitou, "Early over expression of messenger RNA for multiple genes, including insulin, in the Pancreatic Lymph Nodes of NOD mice is associated with Islet Autoimmunity," *BMC Medical Genomics*, vol. 2, article no. 63, 2009.

[3] E. H. Leiter, M. Prochazka, and D. L. Coleman, "The non-obese diabetic (NOD) mouse," *American Journal of Pathology*, vol. 128, no. 2, pp. 380–383, 1987.

[4] M. A. Atkinson and E. H. Leiter, "The NOD mouse model of type 1 diabetes: as good as it gets?" *Nature Medicine*, vol. 5, no. 6, pp. 601–604, 1999.

[5] M. S. Anderson and J. A. Bluestone, "The NOD mouse: a model of immune dysregulation," *Annual Review of Immunology*, vol. 23, pp. 447–485, 2005.

[6] J. P. Driver, D. V. Serreze, and Y. -G. Chen, "Mouse models for the study of autoimmune type 1 diabetes: a NOD to similarities and differences to human disease," *Seminars in Immunopathology*, vol. 33, no. 1, pp. 67–87, 2011.

[7] H. Ikegami, S. Makino, E. Yamato et al., "Identification of a new susceptibility locus for insulin-dependent diabetes mellitus by ancestral haplotype congenic mapping," *Journal of Clinical Investigation*, vol. 96, no. 4, pp. 1936–1942, 1995.

[8] L. S. Wicker, J. A. Todd, and L. B. Peterson, "Genetic control of autoimmune diabetes in the NOD mouse," *Annual Review of Immunology*, vol. 13, pp. 179–200, 1995.

[9] J. A. Shizuru, C. Taylor-Edwards, B. A. Banks, A. K. Gregory, and C. G. Fathman, "Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes," *Science*, vol. 240, no. 4852, pp. 659–662, 1988.

[10] F. S. Wong, I. Visintin, LI. Wen, J. Granata, R. Flavell, and C. A. Janeway, "The role of lymphocyte subsets in accelerated diabetes in nonobese diabetic-rat insulin promoter-B7-1 (NOD-RIP-B7-1) mice," *Journal of Experimental Medicine*, vol. 187, no. 12, pp. 1985–1993, 1998.

[11] BO. Wang, A. Gonzalez, C. Benoist, and D. Mathis, "The role of CD8 T cells in the initiation of insulin-dependent diabetes mellitus," *European Journal of Immunology*, vol. 26, no. 8, pp. 1762–1769, 1996.

[12] J. Katz, C. Benoist, and D. Mathis, "Major histocompatibility complex class I molecules are required for the development of insulitis in non-obese diabetic mice," *European Journal of Immunology*, vol. 23, no. 12, pp. 3358–3360, 1993.

[13] D. V. Serreze, E. H. Leiter, G. J. Christianson, D. Greiner, and D. C. Roopenian, "Major histocompatibility complex class I-deficient NOD-B2m(null) mice are diabetes and insulitis resistant," *Diabetes*, vol. 43, no. 3, pp. 505–509, 1994.

[14] T. Sumida, M. Furukawa, A. Sakamoto et al., "Prevention of insulitis and diabetes in β-microglobulin-deficient non-obese diabetic mice," *International Immunology*, vol. 6, no. 9, pp. 1445–1449, 1994.

[15] J. D. Katz, B. Wang, K. Haskins, C. Benoist, and D. Mathis, "Following a diabetogenic T cell from genesis through pathogenesis," *Cell*, vol. 74, no. 6, pp. 1089–1100, 1993.

[16] B. Bergman and K. Haskins, "Islet-specific T-cell clones from the NOD mouse respond to β-granule antigen," *Diabetes*, vol. 43, no. 2, pp. 197–203, 1994.

[17] A. Gonzalez, J. D. Katz, M. G. Mattei, H. Kikutani, C. Benoist, and D. Mathis, "Genetic control of diabetes progression," *Immunity*, vol. 7, no. 6, pp. 873–883, 1997.

[18] O. Kanagawa, A. Militech, and B. A. Vaupel, "Regulation of diabetes development by regulatory T cells in pancreatic islet antigen-specific TCR transgenic nonobese diabetic mice," *Journal of Immunology*, vol. 168, no. 12, pp. 6159–6164, 2002.

[19] L. Poirot, C. Benoist, and D. Mathis, "Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 21, pp. 8102–8107, 2004.

[20] S. Zucchelli, P. Holler, T. Yamagata, M. Roy, C. Benoist, and D. Mathis, "Defective central tolerance induction in NOD mice: genomics and genetics," *Immunity*, vol. 22, no. 3, pp. 385–396, 2005.

[21] K. L. Rock, S. Gamble, and L. Rothstein, "Presentation of exogenous antigen with class I major histocompatibility complex molecules," *Science*, vol. 249, no. 4971, pp. 918–921, 1990.

[22] M. Kovacsovics-Bankowski and K. L. Rock, "Presentation of exogenous antigens by macrophages: analysis of major

histocompatibility complex class I and II presentation and regulation by cytokines," *European Journal of Immunology*, vol. 24, no. 10, pp. 2421–2428, 1994.

[23] B. Kyewski and J. Derbinski, "Self-representation in the thymus: an extended view," *Nature Reviews Immunology*, vol. 4, no. 9, pp. 688–698, 2004.

[24] D. A. R. Magalhães, E. L. V. Silveira, C. M. Junta et al., "Promiscuous gene expression in the thymus: the root of central tolerance," *Clinical and Developmental Immunology*, vol. 13, no. 2–4, pp. 81–99, 2006.

[25] L. Klein and B. Kyewski, "Self-antigen presentation by thymic stromal cells: a subtle division of labor," *Current Opinion in Immunology*, vol. 12, no. 2, pp. 179–186, 2000.

[26] J. Derbinski, A. Schulte, B. Kyewski, and L. Klein, "Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self," *Nature Immunology*, vol. 2, no. 11, pp. 1032–1039, 2001.

[27] B. Kyewski and L. Klein, "A central role for central tolerance," *Annual Review of Immunology*, vol. 24, pp. 571–606, 2006.

[28] Y. Takahama, "Journey through the thymus: stromal guides for T-cell development and selection," *Nature Reviews Immunology*, vol. 6, no. 2, pp. 127–135, 2006.

[29] G. A. Hollönder, "Claudins provide a breath of fresh Aire," *Nature Immunology*, vol. 8, no. 3, pp. 234–236, 2007.

[30] M. Irla, S. Hugues, J. Gill et al., "Autoantigen-specific interactions with $CD4^+$ thymocytes control mature medullary thymic epithelial cell cellularity," *Immunity*, vol. 29, no. 3, pp. 451–463, 2008.

[31] J. Villaseñor, W. Besse, C. Benoist, and D. Mathis, "Ectopic expression of peripheral-tissue antigens in the thymic epithelium: probabilistic, monoallelic, misinitiated," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 41, pp. 15854–15859, 2008.

[32] A. R. Hayward and M. Shreiber, "Neonatal injection of CD3 antibody into nonobese diabetic mice reduces the incidence of insulitis and diabetes," *Journal of Immunology*, vol. 143, no. 5, pp. 1555–1559, 1989.

[33] http://www.ebi.ac.uk/miamexpress.

[34] http://www.agilent.com/chem/genespring.

[35] M. B. Eisen, P. T. Spellman, P. O. Brown, and D. Botstein, "Cluster analysis and display of genome-wide expression patterns," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 25, pp. 14863–14868, 1998.

[36] http://david.abcc.ncifcrf.gov.

[37] http://smd.stanford.edu/cgi-bin/source/sourceSearch.

[38] T. A. Fornari, P. B. Donate, C. Macedo, M. M. C. Marques, D. A. Magalhães, and G. A. S. Passos, "Age-related deregulation of Aire and peripheral tissue antigen genes in the thymic stroma of non-obese diabetic (NOD) mice is associated with autoimmune type 1 diabetes mellitus (DM-1)," *Molecular and Cellular Biochemistry*, vol. 342, no. 1-2, pp. 21–28, 2010.

[39] Q. S. Mi, D. Ly, S. E. Lamhamedi-Cherradi et al., "Blockade of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice," *Diabetes*, vol. 52, no. 8, pp. 1967–1975, 2003.

[40] S. Kang, E.-J. Park, Y. Joe et al., "Systemic delivery of TNF-Related Apoptosis-Inducing Ligand (TRAIL) elevates levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) and prevents type 1 diabetes in nonobese diabetic mice," *Endocrinology*, vol. 151, no. 12, pp. 5638–5646, 2010.

[41] J. D. Fontenot, M. A. Gavin, and A. Y. Rudensky, "Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells," *Nature Immunology*, vol. 4, no. 4, pp. 330–336, 2003.

[42] S. Hori, T. Nomura, and S. Sakaguchi, "Control of regulatory T cell development by the transcription factor Foxp3," *Science*, vol. 299, no. 5609, pp. 1057–1061, 2003.

[43] J. H. Buckner, "Mechanisms of impaired regulation by $CD4^+$ $CD25^+$ $FOXP3^+$ regulatory T cells in human autoimmune diseases," *Nature Reviews Immunology*, vol. 10, no. 12, pp. 849–859, 2010.

[44] P. B. Donate, T. A. Fornari, C. M. Junta et al., "Collagen induced arthritis (CIA) in mice features regulatory transcriptional network connecting major histocompatibility complex (MHC H2) with autoantigen genes in the thymus," *Immunobiology*, vol. 216, no. 5, pp. 591–603, 2011.

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Review Article

Oxidative Stress and Redox Modulation Potential in Type 1 Diabetes

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Redox reactions are imperative to preserving cellular metabolism yet must be strictly regulated. Imbalances between reactive oxygen species (ROS) and antioxidants can initiate oxidative stress, which without proper resolve, can manifest into disease. In type 1 diabetes (T1D), T-cell-mediated autoimmune destruction of pancreatic β-cells is secondary to the primary invasion of macrophages and dendritic cells (DCs) into the islets. Macrophages/DCs, however, are activated by intercellular ROS from resident pancreatic phagocytes and intracellular ROS formed after receptor-ligand interactions via redox-dependent transcription factors such as NF-κB. Activated macrophages/DCs ferry β-cell antigens specifically to pancreatic lymph nodes, where they trigger reactive T cells through synapse formation and secretion of proinflammatory cytokines and more ROS. ROS generation, therefore, is pivotal in formulating both innate and adaptive immune responses accountable for islet cell autoimmunity. The importance of ROS/oxidative stress as well as potential for redox modulation in the context of T1D will be discussed.

1. Introduction

Oxidation-reduction or redox reactions are pivotal to maintaining life through respiration, metabolism, and energy supply. Mitochondria, which are known to be the powerhouses of the cell, possess the ability to utilize nutrients to generate energy (redox potential) via the electron transport chain, which donates electrons to oxygen to yield ATP and H_2O [1, 2]. Consequently, oxygen free radicals, known as superoxide (O_2^-), are nonenzymatically leaked from the mitochondria and react with other molecules to create reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hydroxyl radical (OH^-), all of which can alter DNA, proteins, carbohydrates, and nucleic acids [3–5] and may eventually lead to irreversible damage. The inability of a cell's antioxidant defenses to overcome oxidative injury and accretion of severe ROS-mediated damage over time will eventually lead to cell death [5–7]. In order to maintain a reduced environment, several cellular antioxidant defenses are in place, including glutathione, glutathione peroxidase, catalase, and three different superoxide dismutase (SOD) enzymes: SOD1, 2, and 3, located in different subcellular and extracellular locations. A basal level of "accidental" superoxide is accumulated in healthy individuals [1, 8], which has been widely hypothesized to be responsible for aging and the associated pathologies [9–11]. However, oxidative stress occurs from an imbalance between ROS and antioxidant actions. During chronic oxidative stress caused by environmental factors (i.e., UV light, ionizing radiation, toxic substances), infections, or lack of dietary antioxidants, an inequity of cellular reducing equivalents capable of detoxifying the increased burden of ROS has marked effects on normal cellular processes. However, in times of oxidative stress, normal cellular respiration is also still functioning, resulting in dysregulated mitochondrial free radical production and disparity between ROS generation and antioxidant

defenses [6, 12]. The combination of stress-induced and conventional mitochondrial dysfunction can manifest into disease states, including cancer [13–15], rheumatoid arthritis [16, 17], neurological disorders [18–21], pulmonary diseases [22], and type 1 diabetes [23–26].

2. Redox and Inflammation

What once was thought to be solely derived from the mitochondria, reactive oxygen species have now been shown to be produced by an important family of primarily immune system-associated enzymes [27–29]. The NADPH oxidase (NOX) family of enzymes is designed to combine NADPH and oxygen to actively generate superoxide. Activated phagocytes, such as macrophages, monocytes, and dendritic cells (DCs), as well as neutrophils, form ROS within the phagosomal membrane for efficient killing of a wide array of invading pathogens [30]. The protection afforded by the phagocytes is crucial, but not without side effects. Production of highly permeable reactive oxygen species (i.e., H_2O_2) causes leakage of these molecules from phagocytes and therefore, unwanted effects on bystander cells [31, 32]. In an environment high in oxidative stress, these bystander reactions drive increased activation of the immune system, cell damage, and progression to disease. For example, NOX-derived ROS have been shown to stimulate mitogenic signaling and proliferation [33, 34], which can have potential deleterious consequences on the promotion of tumorigenesis [35, 36] and in the context of autoimmunity, can lead to T cell expansion [37]. Additionally, H_2O_2 can augment monocyte chemokine receptor surface expression critical for migration to sites of infection and inducing inflammation [38] as well as can promote VEGF signaling to trigger angiogenesis, with implications in cancer and tumor progression [39]. Furthermore, ROS generated from both mitochondria and NADPH oxidase complexes can also act intra-cellularly as well as inter-cellularly as signal transduction molecules. Hydrogen peroxide has been suggested to inactivate protein phosphatases [40], as well as to activate protein tyrosine kinases [41, 42] and metalloproteases through the oxidation of critical cysteine residues [43, 44]. Phosphatases such as SHP-1 serve to decrease inflammation by inhibiting tyrosine kinase activity, yet this type of regulation is lost upon cysteine oxidation [45–48]. Similarly, latent metalloproteases require oxidation for activation and, in the presence of hypochlorous acid (HOCL) and H_2O_2, secretion of chemotactic mediators (L-selectin and proinflammatory TNFα) is highly increased [49], thus enhancing inflammation. In addition, H_2O_2 has been demonstrated to freely cross the plasma membrane and activate NF-κB, a redox-dependent transcription factor [50, 51]. NF-κB plays a major role in immunity by promoting proinflammatory cytokine production, cell proliferation, and inflammation. In general, receptor-ligand interactions are known to generate ROS [52, 53]. In the immune system specifically, LPS interaction with Toll-like receptor 4 (TLR4) has been shown to facilitate the binding of TLR4 to NADPH oxidase 4 (Nox4) and subsequently release ROS [54], resulting in the activation of NF-κB and generation of proinflammatory cytokines IL-1β and TNFα [53]. In a highly oxidized environment, the binding of pathogens to innate cell receptors can lead to hyperresponsiveness [55], suggesting inflammation is secondary to oxidative stress [25, 56]. Not only are phagocytic cells critical for early pathogen recognition through receptor-ligand interaction, they are also necessary for activation of the adaptive immune response. Following antigen recognition by phagocytic antigen-presenting cells (APC), an adaptive immune response is acquired in secondary lymphoid organs through synapse formation of APCs with lymphocytes, as well as from critical innate-derived ROS and third signal proinflammatory cytokines (TNFα, IL-1β) enhancing T-cell activation, proliferation, and effector function [37, 57]. Within this interaction, the H_2O_2 made by the phagocytes is able to traverse the synapse and act upon the T cells, at concentrations ranging from 10–100 μM [58, 59], resulting in a feed-forward mechanism stimulating T-cell-specific NF-κB activity and subsequent proinflammatory cytokine production. Similar effects of ROS are also seen on B cells [60]. Moreover, antigen stimulation of the TCR also drives endogenous production of H_2O_2 through the T cell's own NOX enzyme [28, 61]. Intracellular H_2O_2 can then signal and lead to T-cell proliferation, apoptosis [61, 62], and in conjunction with proinflammatory cytokines, promote T-cell effector function [37, 52, 63]. Therefore, in the presence of oxidative stress, an inability to balance the oxidation with antioxidant enzymes can drive chronic inflammation from both the innate and adaptive arms of the immune response [64], manifesting into many clinically relevant diseases, particularly type 1 diabetes.

3. Oxidative Stress and Type 1 Diabetes

Type 1 diabetes or insulin-dependent diabetes mellitus (T1D) is an autoimmune disorder involving immune-mediated recognition of islet β-cells by autoreactive T cells, which leads to the liberation of ROS and proinflammatory cytokines, resulting in the destruction of pancreatic β-cells in the islets of Langerhans and loss of insulin secretion. Patients with T1D must constantly prevent hyperglycemia by administering exogenous insulin or in the situation of severe hyperglycemic unawareness, by undergoing islet transplantation. Despite a multitude of efforts in trying to specify the exact etiology, the cause of T1D is still under debate. The combinatorial effects of genetic susceptibility, environmental factors, and dietary deficiencies are known to contribute to disease origin; however, the impact of oxidative stress in a genetically susceptible individual is of particular interest. Oxidative stress, as stated above, occurs when the generation of ROS overcomes the scavenging abilities of antioxidants. Such instances may be mediated by genetic lack of antioxidant enzymes as well as environmental triggers like viral infections. Overall, oxidative stress has been linked to β-cell cytotoxicity [65–67] and has been suggested to play a role in T1D pathology [68–71]. Several studies show that the total serum antioxidant status, as measured by urate, Vitamin C, and total plasma antioxidant levels, of prediabetic and T1D patients is lower in comparison to age-matched controls [72, 73], which inevitably leads to greater oxidative modification

of proteins and lipids [74]. Other literature illustrates a connection between viruses, ROS production, and type 1 diabetes onset. Gamble et al. demonstrated a positive correlation between type 1 diabetes onset and Coxsackie B4 virus infection through antibody titer measurements [75, 76]. Furthermore, such infections have been shown to cause indirect [77] and direct β-cell damage [78] and to stimulate the β-cells into secreting inflammatory mediators themselves [79]. ROS are made following viral infection from activated phagocytes [80, 81], as mentioned previously, and work to not only cause cellular injury but also can activate inflammatory, redox-dependent transcription factors, such as NF-κB, perpetuating inflammation. Viral-mediated ROS production or a reduction in antioxidants can have severe consequences as β-cells are more prone to oxidative damage than most other tissues. The β-cell mitochondria have exceptionally low levels of glutathione peroxidase, superoxide dismutase, and catalase activity [24, 82–84]. Because of this low antioxidant defense, β-cells can be clearly disrupted by oxidative stress and, in genetically predisposed individuals, results in easy targets for a subsequent cytokine-mediated autoimmune attack.

Mitochondrial and NOX-derived ROS both have implications in β-cell destruction and T1D. Increased glucose causes rapid induction of the tricarboxylic acid (TCA) cycle within the β-cell mitochondria, which can lead to augmented ROS production [85]. The superoxide leaked from mitochondria can then form H_2O_2 and work to uncouple glucose metabolism from insulin secretion [86]. Ultimately, high levels of mitochondrial ROS can cause β-cell death [87, 88]. Intriguingly, models of T1D induce disease by generating toxic amounts of ROS within the islets (i.e., streptozotocin and alloxan) [89]. Alloxan is easily taken up by β-cells [90], where it is reduced into dialuric acid and subsequently reoxidized to establish a redox cycle [91]. ROS generated by alloxan treatment have been shown to promote islet β-cell DNA fragmentation, culminating in cell death [92]. In contrast, an alloxan-resistant strain of mice, the ALR mouse, shows increased ROS dissipation and resistance to islet destruction [23, 93, 94], further implicating the importance of oxidative stress and T1D. Streptozotocin (STZ), on the other hand, causes β-cell DNA alkylation and eventually drains the cellular NAD^+ and ATP source in an effort to repair the DNA [95]. Xanthine oxidase is then able to utilize dephosphorylated ATP as a substrate for superoxide production [96]. Additionally, STZ metabolism increases the levels of islet cell nitric oxide (NO) [97], which together with superoxide can generate peroxynitrite ($ONOO^-$). Detection of peroxynitrite in prediabetic nonobese diabetic mouse (NOD) islets suggests importance of this ROS in β-cell death [71]. Similarly, NOX enzymes have been detected within the pancreatic β-cells [98, 99]. Hyperglycemia can increase the assembly of NOX enzymes through its p47phox subunit, and therefore, enhance superoxide generation [100] and facilitate β-cell death.

4. Immunology of T1D

Autoimmune diabetes onset is preceded by infiltration of immune cells into the pancreatic islets. Ultimately, a breach in tolerance to self-antigens allows for autoreactive T cells to become activated and attack the β-cells, resulting in the loss of insulin secretion. However, innate immune cells, such as macrophages and DCs, are of the first cells to enter the islets during insulitis [101, 102]. Although resident macrophages are present in the pancreas at all times, acquisition of antigen is required for macrophage activation and the production of cytokines. As described above, genetic and environmental factors can lead to cell destruction, releasing β-cell-specific antigens as well as ROS [103]. Macrophages will phagocytose dying β-cells and present antigen in the context of their MHC molecules. In humans, specific HLA molecules HLA-DR3 and DR4 are correlated with a susceptibility to T1D [103, 104]. Moreover, the ROS created by the initial insult to the islets are able to stimulate the activation of redox-dependent NF-κB and other transcription factors within the macrophages [105]. Activated macrophages secrete a mixture of proinflammatory cytokines such as TNFα, IL-6, IL-1β, and ROS, which can start to damage the pancreatic β-cells [106–108]. IL-1β can cause extensive cytolysis in β-cells [109] through the upregulation of iNOS and subsequent generation of nitric oxide (NO) [110, 111], whereas TNFα enhances IL-1β-mediated islet destruction and helps activate APCs and T cells [112–114], but does not cause direct β-cell apoptosis *in vivo* [114].

ROS and cytokines released by APCs not only promote β-cell damage, but also help to generate an adaptive immune response, which in T1D, is the crucial step in autoimmune destruction. It is well established that chronic elicitation of antigens to innate immune cells in a highly oxidized environment will lead to MHC-peptide presentation, perpetuating an adaptive immune response [115, 116] (Figure 1).

In the context of continuous β-cell ablation, macrophages can phagocytose dying cells and migrate to the pancreatic lymph node where they interact with naïve T-cells. It is this aforementioned synapse that enables T-cell proliferation and effector function to occur. In the presence of all three necessary signals: (1) MHC-peptide, (2) costimulation, and (3) soluble third signal, in this case consisting of ROS, IL-1β, and TNFα, T cells become activated via NFAT and NF-κB [117–121]. Furthermore, IL-12 released from macrophages can differentiate $CD4^+$ T cells into the TH1 lineage via signaling through STAT4 [122–125]. $CD4^+$ TH1 cells then home to the site of antigen production, the β-cells, and call in other T cells and more APCs through the secretion of IFNγ. IFNγ has some indirect effects on β-cells, including potentiating the maturation of pancreatic APCs, which can then elicit an even greater T-cell response [126]. Additionally, neutralization of IFNγ in NOD mice has been shown to reduce both diabetes and insulitis [127], whereas IFNγR-deficient NOD mice demonstrate delayed insulitis, but do not develop T1D [128]. Proinflammatory cytokines TNFα, IL-1β, and IFNγ all play a role in β-cell death primarily through activation of redox-regulated transcription factors NF-κB and STAT1 [129–131]. Combinations of TNFα with IFNγ or IL-1β are necessary for primary murine β-cell death [132], and TNFα/IFNγ act synergistically to activate the stress-activated proapoptotic JNK/SAPK pathway, which promotes β-cell apoptosis via p53 and intracellular ROS [133]. The activation of NF-κB can

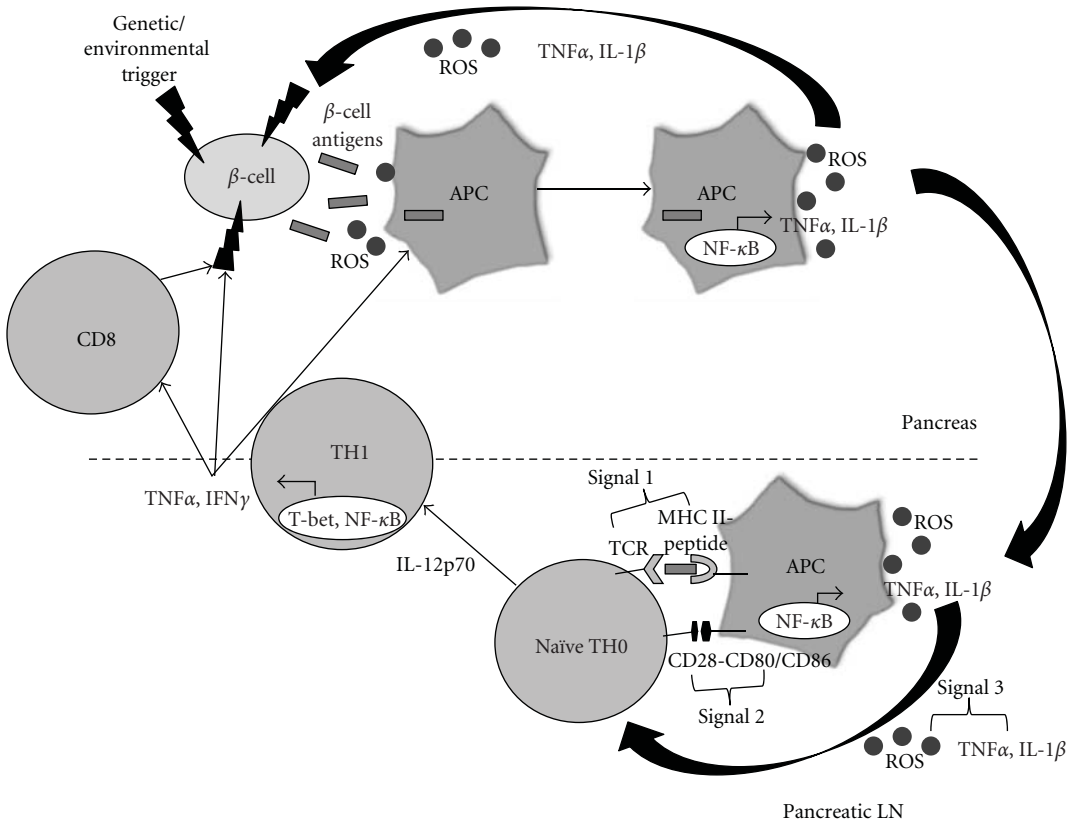

Figure 1: *Role of redox in the immunopathology of type 1 diabetes.* An initial genetic or environmental insult to the beta cell triggers the release of beta cell antigens as well as the production of ROS. Beta cell antigens are phagocytosed, and ROS are able to stimulate redox-dependent transcription factors such as NF-κB, which leads to APC activation and cytokine secretion. ROS and proinflammatory cytokines secreted by APCs act as the third signal within the T-cell-APC immunological synapse, which occurs in the pancreatic lymph node. ROS play a critical role in the progression of naïve TH0 cells to cytokine-secreting TH1 cells. Release of IFNγ by TH1 cells then works directly on the beta cells as well as activates more APCs and CD8 cells, all of which can impart deleterious effects on the islets.

also increase iNOS and Fas expression, potential inducers of cell death, while downregulating the antiapoptotic Bcl-2 gene [134]. Apoptosis of β-cells is also mediated partially by T-cell expression of Fas ligand, TNFα, and perforin/granzyme [114, 134]. Specifically, $CD4^+$ T cells are thought to be sufficient for T1D onset [135, 136], whereas $CD8^+$ T cells seem to play a lesser role in the final stage of autoimmune destruction [137]. It is known, however, that synergy between both CD4 and CD8 T cells results in absolute transfer of diabetes in rodent models [138, 139]. Although specific to the model of autoimmune diabetes, TNFα secretion from $CD4^+$ T cells can activate TNFR1 on β-cells and cause apoptosis [140], while $CD8^+$ T cells can kill NOD β-cells by a Fas-dependent mechanism [141] or by perforin release [142]. Ultimately, T-cell exacerbation of β-cell death comes from endogenous generation of ROS and cytokines following APC activation [143] that can perpetuate islet destruction through a feed forward mechanism. Overall, ROS are crucial in not only activating the initial infiltrating macrophages and DCs [144] via the common denominator NF-κB, but also for subsequently driving an adaptive TH1 immune response that is necessary for total ablation of β-cells and progression to T1D [134, 136]. Therefore, therapies would be most beneficial if there was not only protection of the β-cells from ROS, but also inhibition of the ROS-mediated autoimmune attack, possibly by preventing NF-κB activation, ensuing inflammation, and the initiation of the adaptive immune response.

5. Controlling Redox in T1D

Glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase are categorized as the most crucial antioxidant enzymes; however, islets inherently contain only a fraction of the enzymatic activities in comparison to liver, which possesses the highest abundance [145]. Because of the low antioxidant defenses present in pancreatic islets, therapeutic strategies to enhance antioxidants and reducing capabilities are of utmost importance. Studies utilizing overexpression of GPX1, SOD1 (Cu/Zn SOD), SOD2 (MnSOD), or SOD mimetic administration in insulinoma cell lines such as NIT-1 and INS-1 afforded protection from ROS and reactive nitrogen species (RNS) *in vitro* [25, 146, 147]. Usage of SOD mimetics in other inflammatory models has also demonstrated diminutions in proinflammatory cytokines [148, 149]. Furthermore, stable transfection of

insulin-producing RINm5F cells with GPX, catalase, and Cu/Zn SOD resulted in defenses against cytokine toxicity imparted by the combination of IL-1β, TNFα, and IFNγ [150]. Antioxidant overexpression has been linked to not only protection against ROS and cytokines, but also to enhanced cell proliferation and decreased death. PDX1, a transcription factor necessary for β-cell differentiation, survival, and insulin synthesis [151], is also very responsive to ROS [152], where high oxidation causes a cytoplasmic relocation of PDX1 out of the nucleus, increased degradation of the protein, and subsequent dysfunction of β-cells [153, 154]. By alleviating ROS within the islets, PDX1 protein has exhibited stability and enhanced function in type 2 diabetes models [155], which can also have implications in T1D for stabilizing β-cell function and survival. Other experiments utilize transgene or adenoviral technology to overexpress antioxidant genes within the β-cells to specifically show islet-mediated versus autoimmune protection from T1D. These studies have elicited conflicting results. For example, overexpression of metallothionein and catalase in β-cells was unable to delay or inhibit spontaneous diabetes onset within NOD mice and promoted reduced activation of the PDX1 survival pathway [156]. Metallothionein proteins are intracellular, cysteine-rich molecules with high redox potential [157]. Similarly, transgenic expression of extracellular SOD in β-cells does not confer any difference in T1D incidence in comparison to control NOD mice [158]. These results suggest that basal levels of ROS production are necessary for β-cell function, possibly by triggering appropriate insulin signaling and regulating cell survival [159]. In contrast, overexpression of thioredoxin, a redox-regulated protein which helps repair ROS-damaged proteins and DNA, constitutes protection of β-cells from autoimmune and STZ-induced diabetes [160]. β-cell-specific transgenic expression of catalase and metallothionein is also able to shield isolated islets from hydrogen peroxide and reduce the effects of STZ treatment [161–163]. Transgenic expression of heme oxygenase-1, which has crucial cytoprotective functions against oxidative stress and inflammation, can improve insulitis and spontaneous diabetes in NOD mice [164], and alloxan-induced diabetes is also reduced following overexpression of Cu/Zn SOD in β-cells [165]. Moreso, precedence for the importance of enhancing islet-associated antioxidant levels has been demonstrated at the genetic level, in which mice resistant to alloxan treatment (ALR mice) exhibit protection from diabetes [94, 166]. This finding particularly helps further justify the need for therapeutic discovery and necessary experiments to determine druggable targets based upon modulation of antioxidant function.

Systemic administration of antioxidants, in comparison to overexpression studies, shows more consistency in ameliorating T1D. Administration of 16 mg/kg/day of a potent antioxidant to young NOD mice resulted in a reduction of diabetes incidence from 89% in controls to 44% in the treated animals [167]. Furthermore, after a multiple low dose administration of STZ, addition of zinc sulphate to the drinking water of animals was able to increase metallothionein levels, inhibiting the onset of T1D [168], whereas intraperitoneal injections of butylated hydroxyanisole (BHA) antioxidant were able to attenuate the production of proinflammatory cytokines by islets and macrophages, thereby lowering insulitis and hyperglycemia [169]. Such uniformity in these results versus the transgenic expression of multiple antioxidants, as discussed above, may relate to the ability of systemic therapies to not only protect the β-cells but to also inhibit immune system activation and inflammation. Adenoviral delivery of systemic heme oxygenase to NOD mice decreased insulitis and T1D incidence; however, this alleviation was associated with a decrease in mature DCs and TH1 effector function [170]. Additionally, ALR mice resistant to alloxan-induced diabetes contain specific genetic modifications conferring systemic elevation of antioxidants, resulting in neutrophils with reduced superoxide bursts [171]. In an *in vitro* system using the antioxidant probucol, which can delay alloxan-induced [172] and spontaneous diabetes in rats [173], macrophages exhibit decreased H_2O_2 production, thus maintaining islet viability [174]. Further reports on the effects of systemic antioxidants on innate immunity include studies from our lab utilizing metalloporphyrin-based catalytic antioxidants (CA) with bone marrow-derived macrophages. The CA houses a metal center that catalyzes superoxide dismutation, mimicking SOD activity [175, 176], and is able to scavenge a broad range of ROS including O_2^-, H_2O_2, $ONOO^-$, and lipid peroxyl radicals [53, 177, 178]. Following treatment with CA and LPS stimulation of macrophages, the production of nitrite (NO_2^-), O_2^- TNFα, and IL-1β was significantly reduced in comparison to control [25, 53]. This effect was mediated by the ability of CA to oxidize the p50 subunit of NF-κB within the nucleus, inhibiting its binding to DNA and subsequent transcription of proinflammatory cytokines [53]. Redox modulation of transcription factor DNA binding has previously been demonstrated for NF-κB as well as other eukaryotic molecules [179, 180]. Inhibition of NF-κB has been well established as an effective method of thwarting the immune response and resolving inflammation to maintain β-cell integrity [181, 182]; however, we are the first to illustrate a link between metalloporphyrin catalytic antioxidants, blockade of NF-κB activation, and delayed autoimmune diabetes, as described below.

The activation of macrophages and T cells relies on oxidative stress, which ultimately leads to the progression of T1D. Based upon this fact, CA was also investigated in the context of CD4 and CD8 T cells. The BDC-2.5 TCR-Tg TH1 cell clone, which has recently been described as specific for the protein ChgA, a member of the granin family of neuroendocrine secretory proteins [183], causes rapid transfer of diabetes into NOD.*scid* recipients [184]. By utilizing this method, pretreatment of NOD.*scid* mice with CA prior to adoptive transfer of the BDC-2.5 clone inhibits the infiltration of T cells into the pancreas, significantly delaying T1D onset. Moreover, APC-dependent BDC-2.5 T cell proliferation and IFNγ production are also reduced after *in vitro* CA treatment [25]. To further delineate the mechanism of diminished T-cell effector function, *in vivo* treatment of NOD and BDC-2.5 TCR-Tg mice with CA was able to decrease innate-derived third signal synthesis, primarily consisting of TNFα, resulting in antigen-specific T cell hyporesponsiveness [37]. Similar results were

found upon CA treatment in the context of CD8 T cells, reducing proliferation, cytokine production, and cytolytic effector molecules of CTLs [185]. Interestingly, by inhibiting NADPH oxidase in NOD animals (NOD.*Ncf1*^{m1J}) in an effort to genetically mimic systemic CA administration, not only is NOX-derived superoxide production eliminated, but T cells show reduced TH1 responses, granting protection from T1D onset [120]. Earlier studies by Chaudhri et al. supported our experimentation by demonstrating attenuation of T-cell proliferation and IL-2R expression following antioxidant treatment [186, 187]. Such findings point to the possibility and importance of redox modulation in not only regulating the innate immune cells, but also impacting the T cells which formulate an adaptive immune response crucial for the autoimmune attack in T1D (Figure 2).

In addition to decreasing oxidative stress imposed on the islets, which can directly damage β-cells or indirectly stimulate the autoreactive immune response to become activated, redox modulation may also be useful for decreasing the unyielding ER stress within the β-cells. Because the β-cells are a constant source of insulin and insulin must be folded properly for secretion, the importance of balancing a high protein-folding load with survival of the cells increases substantially in comparison to other nonsecretory cells [188]. An overload of misfolded proteins may eventually result in cell death, if not properly resolved. An early study by Lo et al. highlighted the susceptibility of β-cells to ER stress by overexpressing MHC class II proteins in islets, essentially overwhelming the protein folding machinery and leading to apoptosis [189]. Other more recent studies show biochemical connections between ER stress-induced apoptosis and β-cell death, through both calcium-dependent and independent molecules [190–192]. To reconcile protein misfolding within the ER, the unfolded protein response, or UPR, is consequently triggered [193, 194]. The UPR acts as a backup mechanism to protect cells from accumulating unfolded proteins and to restore the balance between the protein folding machinery and the secretory pathway [195]. However, an accumulation of unfolded proteins during severe ER stress is sometimes unable to be resolved by the UPR, as characterized in the Akita mouse which contains a mutation in the proinsulin 2 gene that disrupts insulin folding, retains it within the ER, activates UPR, yet still eventually leads to β-cell death [196, 197]. Moreover, ROS have been suggested in supporting the UPR towards a more proapoptotic than proadaptive level [198], further illustrating the importance of regulating oxidative stress to maintain β-cell survival. Although the UPR paradoxically utilizes an oxidative environment within the ER to correctly fold proteins (i.e., disulfide bond formation), sustained oxidative stress can perpetuate the UPR to a level that promotes apoptosis [198, 199]. Additionally, the abundance of ROS present during continued unadapted ER stress can trigger apoptosis in neighboring cells as well. This is especially critical in islet β-cells, where their ability to handle oxidative stress is already reduced because of low levels of antioxidants [24, 82–84]. More pertinent is when unresolved ER stress leads to dying β-cells containing the misfolded proteins. These cells can be taken up by resident pancreatic APCs and presented to autoreactive T cells within the pancreatic lymph nodes. This type of event may stimulate the reactivity of T cells to formerly tolerated "neo-autoantigens," which can ultimately promote more β-cell destruction and eventual development of autoimmune diabetes [200, 201]. A study conducted by Malhotra, et al. shows that antioxidant treatment of CHO cells results in not only decreased oxidative stress, but also decreased misfolded proteins, reduced activation of the UPR, and enhanced secretion of proteins [188]. Thus, it appears that a temporal or redox balance is essential for optimal β-cell function. In situations where the β-cell may experience environmental stressors that lead to disruption of the ER-machinery, the results may set in motion both ER-stress-induced UPR and the expression of misfolded proteins in an oxidative environment, further providing an optimal milieu for driving autoreactive T cells to become activated. Therefore, redox modulation may serve yet another purpose: to help reduce ER stress and subsequently maintain β-cell viability.

Although the ability to predict susceptibility to type 1 diabetes is becoming increasingly accurate [202], and therefore, prophylactic treatment of patients with antioxidant therapeutics is not out of the realm of possibilities, currently a more feasible option for individuals with chronic hyperglycemia is to undergo islet transplantation. Islets, like any other transplantable organ, are in short supply; however, maintaining function and viability of transplanted islets is the major drawback of the procedure [161]. Not only are islets susceptible to immune rejection, but hypoxia during isolation and transplantation is the primary cause of β-cell death [203]. Because of their low resistance to ROS [24, 82–84], β-cells are especially vulnerable to oxidative damage and ischemia-reperfusion injury [204, 205]. In order to combat this weakness, the application of antioxidants seems a suitable alternative, as they have shown promise in liver and kidney transplantations [206, 207]. Longer allograft survival times have been demonstrated with mouse islets soaked with hydroxyl-radical inhibitors prior to transplantation [208] and with multiple *in vivo* administrations of SOD and catalase prior to and after islet transplantation [209]. Likewise, transduction of islets with heme oxygenase-1 or SOD2 genes was able to improve viability and insulin secretion *in vitro* [210] and elicit greater functionality upon transplantation in comparison to controls [211], respectively. Furthermore, we have also demonstrated benefit using the catalytic antioxidant approach, whereby adding CA during and after human islet isolation enhanced cell survival and function, allowing for normalization of STZ-induced diabetic NOD.*scid* mice [212]. Additionally, CA is not only able to protect human islets from STZ cell damage, but can also protect murine islets from both antigen-independent innate-mediated inflammation and antigen-dependent T-cell-mediated allograft rejection [204]. Overall, unlike common antirejection drugs, which are outstanding at protecting against the adaptive immune response but fail to shield islets from ROS/inflammation [213, 214], our CA treatment is nontoxic to islets and can alleviate

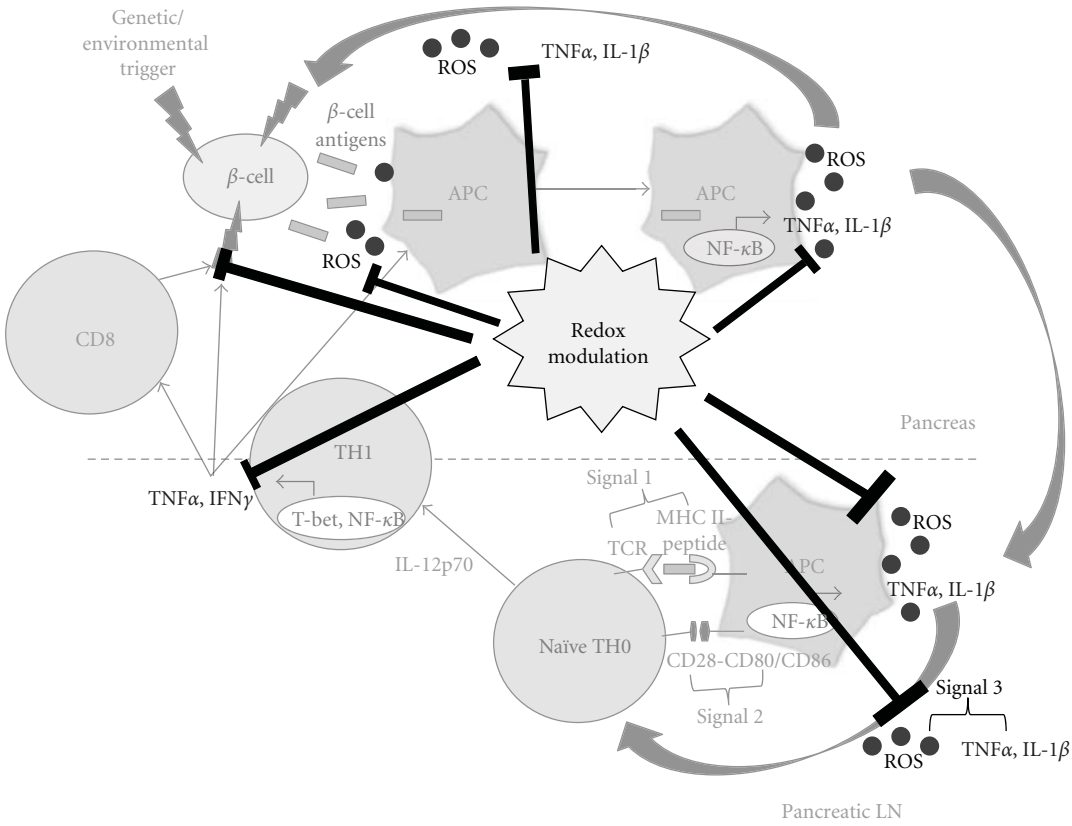

Figure 2: *Role of redox modulation in controlling ROS-mediated beta cell destruction*. Redox modulation has shown promise in blocking the production of ROS and its ability to activate APCs, resulting in diminished TH1 cell activation and effector function, which ultimately may help regulate beta-cell destruction.

both the alloimmune [204] and autoimmune responses [25, 37, 53, 185].

6. Conclusion

Although redox has been extensively studied in the context of both T1D and type 2 diabetes [85, 215], the plethora of literature discussed above shows the implications of ROS in all stages of autoimmune T1D, including the primary "trigger", the initiation of insulitis by the innate immune system, and the acquisition of T-cell-mediated autoreactivity. These studies open the door to novel ideas of redox modulation, such as targeting ROS-dependent immunological metalloproteases [43, 44, 49] or disrupting the autoreactive T-cell pool, as described [37, 120, 185]. Moreover, a study evaluating self-antigen-primed T cells demonstrates how NO is able to reduce FOXP3 expression and subsequently decrease Tregs in autoimmune disorders [216], illustrating how intricate and vast the role of redox is in the immune response and where future studies may focus. In addition to effects on the target organ(s) and the immune system, autoimmunity also gives rise to systemic problems, and in the context of diabetes, ROS have been characterized as crucial elements promoting hyperglycemia-induced diabetic complications, especially those involving the vasculature [6, 217]. One important study conducted by Ling et al. provided evidence of oxidative stress-mediated vascular complications in prediabetic NOD mice [218], which exemplifies the importance of ROS in not only exacerbation of disease, but also on initiation of T1D and nonhyperglycemic associated pathologies. Furthermore, utilizing antioxidants, such as Vitamin E, cannot only assuage vascular activation [219], but can also grant protection from the loss of secondary target organ function, such as the kidneys [220]. Therefore, oxidative stress affects every aspect of T1D and the benefit of redox modulation may be more important than once thought. Optimal treatments may have to incorporate antioxidants with anti-inflammatory agents, such as inhibitors of NF-κB activation, and must also take into consideration the limitations associated with utilization of intact enzyme/protein therapies, including bioavailability, immunogenicity-limited cellular accessibility, and cost of production. However, with the advent of newer nonpeptidyl small compounds, alleviating oxidative stress through antioxidant therapy appears to be a plausible druggable target. This therapy should restore balance between oxidation and reduction, leading to resolution of inflammation, thus reducing the autoimmune destruction of the islet β-cells.

References

[1] J. M. McCord, "The evolution of free radicals and oxidative stress," *American Journal of Medicine*, vol. 108, no. 8, pp. 652–659, 2000.

[2] P. Rich, "Chemiosmotic coupling: the cost of living," *Nature*, vol. 421, no. 6923, p. 583, 2003.

[3] A. Boveris, N. Oshino, and B. Chance, "The cellular production of hydrogen peroxide," *Biochemical Journal*, vol. 128, no. 3, pp. 617–630, 1972.

[4] J. F. Turrens, "Superoxide production by the mitochondrial respiratory chain," *Bioscience Reports*, vol. 17, no. 1, pp. 3–8, 1997.

[5] F. B. Johnson, D. A. Sinclair, and L. Guarente, "Molecular biology of aging," *Cell*, vol. 96, no. 2, pp. 291–302, 1999.

[6] J. Limón-Pacheco and M. E. Gonsebatt, "The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress," *Mutation Research*, vol. 674, no. 1-2, pp. 137–147, 2009.

[7] J. Chandra, A. Samali, and S. Orrenius, "Triggering and modulation of apoptosis by oxidative stress," *Free Radical Biology and Medicine*, vol. 29, no. 3-4, pp. 323–333, 2000.

[8] D. Harman, "The aging process," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 11, pp. 7124–7128, 1981.

[9] T. Finkel and N. J. Holbrook, "Oxidants, oxidative stress and the biology of ageing," *Nature*, vol. 408, no. 6809, pp. 239–247, 2000.

[10] A. Mitsui, J. Hamuro, H. Nakamura et al., "Overexpression of human thioredoxin in transgenic mice controls oxidative stress and life span," *Antioxidants and Redox Signaling*, vol. 4, no. 4, pp. 693–696, 2002.

[11] S. E. Schriner, N. J. Linford, G. M. Martin et al., "Medecine: extension of murine life span by overexpression of catalase targeted to mitochondria," *Science*, vol. 308, no. 5730, pp. 1909–1911, 2005.

[12] B. Halliwell, "Antioxidants in human health and disease," *Annual Review of Nutrition*, vol. 16, pp. 33–50, 1996.

[13] J. E. Klaunig, Y. Xu, J. S. Isenberg et al., "The role of oxidative stress in chemical carcinogenesis," *Environmental Health Perspectives*, vol. 106, no. 1, pp. 289–295, 1998.

[14] L. W. Oberley and G. R. Buettner, "Role of superoxide dismutase in cancer: a review," *Cancer Research*, vol. 39, no. 4, pp. 1141–1149, 1979.

[15] S. Toyokuni, "Persistent oxidative stress in cancer," *FEBS Letters*, vol. 358, no. 1, pp. 1–3, 1995.

[16] S. Bashir, G. Harris, M. A. Denman, D. R. Blake, and P. G. Winyard, "Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases," *Annals of the Rheumatic Diseases*, vol. 52, no. 9, pp. 659–666, 1993.

[17] S. Jaswal, H. C. Mehta, A. K. Sood, and J. Kaur, "Antioxidant status in rheumatoid arthritis and role of antioxidant therapy," *Clinica Chimica Acta*, vol. 338, no. 1-2, pp. 123–129, 2003.

[18] D. A. Butterfield and C. M. Lauderback, "Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid β-peptide-associated free radical oxidative stress," *Free Radical Biology and Medicine*, vol. 32, no. 11, pp. 1050–1060, 2002.

[19] P. Jenner and C. W. Olanow, "Oxidative stress and the pathogenesis of Parkinson's disease," *Neurology*, vol. 47, no. 6, pp. S161–S170, 1996.

[20] I. I. Kruman, W. A. Pedersen, J. E. Springer, and M. P. Mattson, "ALS-linked Cu/Zn-SOD mutation increases vulnerability of motor neurons to excitotoxicity by a mechanism involving increased oxidative stress and perturbed calcium homeostasis," *Experimental Neurology*, vol. 160, no. 1, pp. 28–39, 1999.

[21] M. A. Smith, P. L. Richey Harris, L. M. Sayre, J. S. Beckman, and G. Perry, "Widespread peroxynitrite-mediated damage in Alzheimer's disease," *Journal of Neuroscience*, vol. 17, no. 8, pp. 2653–2657, 1997.

[22] I. Rahman, S. K. Biswas, and A. Kode, "Oxidant and antioxidant balance in the airways and airway diseases," *European Journal of Pharmacology*, vol. 533, no. 1–3, pp. 222–239, 2006.

[23] J. Chen, A. M. Gusdon, T. C. Thayer, and C. E. Mathews, "Role of increased ROS dissipation in prevention of T1D: lessons from the ALR mouse," *Annals of the New York Academy of Sciences*, vol. 1150, pp. 157–166, 2008.

[24] S. Lenzen, "Oxidative stress: the vulnerable β-cell," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 343–347, 2008.

[25] J. D. Piganelli, S. C. Flores, C. Cruz et al., "A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone," *Diabetes*, vol. 51, no. 2, pp. 347–355, 2002.

[26] W. I. Sivitz and M. A. Yorek, "Mitochondrial dysfunction in diabetes: from molecular mechanisms to functional significance and therapeutic opportunities," *Antioxidants and Redox Signaling*, vol. 12, no. 4, pp. 537–577, 2010.

[27] B. M. Babior, R. S. Kipnes, and J. T. Curnutte, "Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent," *Journal of Clinical Investigation*, vol. 52, no. 3, pp. 741–744, 1973.

[28] S. H. Jackson, S. Devadas, J. Kwon, L. A. Pinto, and M. S. Williams, "T cells express a phagocyte-type NADPH oxidase that is activated after T cell receptor stimulation," *Nature Immunology*, vol. 5, no. 8, pp. 818–827, 2004.

[29] Y. A. Suh, R. S. Arnold, B. Lassegue et al., "Cell transformation by the superoxide-generating oxidase Mox1," *Nature*, vol. 401, no. 6748, pp. 79–82, 1999.

[30] J. D. Lambeth, "NOX enzymes and the biology of reactive oxygen," *Nature Reviews Immunology*, vol. 4, no. 3, pp. 181–189, 2004.

[31] J. M. McCord, "Oxygen-derived radicals: a link between reperfusion injury and inflammation," *Federation Proceedings*, vol. 46, no. 7, pp. 2402–2406, 1987.

[32] W. F. Petrone, D. K. English, K. Wong, and J. M. McCord, "Free radicals and inflammation: superoxide-dependent activation of a neutrophil chemotactic factor in plasma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 77, no. 2, pp. 1159–1163, 1980.

[33] R. S. Arnold, J. Shi, E. Murad et al., "Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 10, pp. 5550–5555, 2001.

[34] G. A. C. Murrell, M. J. O. Francis, and L. Bromley, "Modulation of fibroblast proliferation by oxygen free radicals," *Biochemical Journal*, vol. 265, no. 3, pp. 659–665, 1990.

[35] J. L. Arbiser, J. Petros, R. Klafter et al., "Reactive oxygen generated by Nox1 triggers the angiogenic switch," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 2, pp. 715–720, 2002.

[36] S. S. Brar, T. P. Kennedy, A. B. Sturrock et al., "An NAD(P)H oxidase regulates growth and transcription in melanoma cells," *American Journal of Physiology*, vol. 282, no. 6, pp. C1212–C1224, 2002.

[37] H. M. Tse, M. J. Milton, S. Schreiner, J. L. Profozich, M. Trucco, and J. D. Piganelli, "Disruption of innate-mediated proinflammatory cytokine and reactive oxygen species third signal leads to antigen-specific hyporesponsiveness," *Journal of Immunology*, vol. 178, no. 2, pp. 908–917, 2007.

[38] A. Saccani, S. Saccani, S. Orlando et al., "Redox regulation of chemokine receptor expression," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 6, pp. 2761–2766, 2000.

[39] M. Ushio-Fukai, "VEGF signaling through NADPH oxidase-derived ROS," *Antioxidants and Redox Signaling*, vol. 9, no. 6, pp. 731–739, 2007.

[40] S. G. Rhee, S. W. Kang, W. Jeong, T. S. Chang, K. S. Yang, and H. A. Woo, "Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins," *Current Opinion in Cell Biology*, vol. 17, no. 2, pp. 183–189, 2005.

[41] S. G. Rhee, "H_2O_2, a necessary evil for cell signaling," *Science*, vol. 312, no. 5782, pp. 1882–1883, 2006.

[42] R. L. Whisler, M. A. Goyette, I. S. Grants, and Y. G. Newhouse, "Sublethal levels of oxidant stress stimulate multiple serine/threonine kinases and suppress protein phosphatases in Jurkat T cells," *Archives of Biochemistry and Biophysics*, vol. 319, no. 1, pp. 23–35, 1995.

[43] K. K. Nelson and J. A. Melendez, "Mitochondrial redox control of matrix metalloproteinases," *Free Radical Biology and Medicine*, vol. 37, no. 6, pp. 768–784, 2004.

[44] Y. Wang, A. H. Herrera, Y. Li, K. K. Belani, and B. Walcheck, "Regulation of mature ADAM17 by redox agents for L-selectin shedding," *Journal of Immunology*, vol. 182, no. 4, pp. 2449–2457, 2009.

[45] S. C. You, Y. O. Sun, and Z. Zhu, "Tyrosine phosphatase SHP-1 in oxidative stress and development of allergic airway inflammation," *American Journal of Respiratory Cell and Molecular Biology*, vol. 39, no. 4, pp. 412–419, 2008.

[46] J. M. Cunnick, J. F. Dorsey, L. Mei, and J. Wu, "Reversible regulation of SHP-1 tyrosine phosphatase activity by oxidation," *Biochemistry and Molecular Biology International*, vol. 45, no. 5, pp. 887–894, 1998.

[47] K. Lee and W. J. Esselman, "Inhibition of PTPs by H_2O_2 regulates the activation of distinct MAPK pathways," *Free Radical Biology and Medicine*, vol. 33, no. 8, pp. 1121–1132, 2002.

[48] M. Reth, "Hydrogen peroxide as second messenger in lymphocyte activation," *Nature Immunology*, vol. 3, no. 12, pp. 1129–1134, 2002.

[49] M. Pietri, B. Schneider, S. Mouillet-Richard et al., "Reactive oxygen species-dependent TNF-α converting enzyme activation through stimulation of 5-HT and α autoreceptors in neuronal cells," *FASEB Journal*, vol. 19, no. 9, pp. 1078–1087, 2005.

[50] M. Meyer, H. L. Pahl, and P. A. Baeuerle, "Regulation of the transcription factors NF-κB and AP-1 by redox changes," *Chemico-Biological Interactions*, vol. 91, no. 2-3, pp. 91–100, 1994.

[51] R. Schreck, P. Rieber, and P. A. Baeuerle, "Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1," *EMBO Journal*, vol. 10, no. 8, pp. 2247–2258, 1991.

[52] Y. J. Suzuki, H. J. Forman, and A. Sevanian, "Oxidants as stimulators of signal transduction," *Free Radical Biology and Medicine*, vol. 22, no. 1-2, pp. 269–285, 1996.

[53] H. M. Tse, M. J. Milton, and J. D. Piganelli, "Mechanistic analysis of the immunomodulatory effects of a catalytic antioxidant on antigen-presenting cells: implication for their use in targeting oxidation-reduction reactions in innate immunity," *Free Radical Biology and Medicine*, vol. 36, no. 2, pp. 233–247, 2004.

[54] H. S. Park, H. Y. Jung, E. Y. Park, J. Kim, W. J. Lee, and Y. S. Bae, "Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF-κB," *Journal of Immunology*, vol. 173, no. 6, pp. 3589–3593, 2004.

[55] J. D. Crapo, "Oxidative stress as an initiator of cytokine release and cell damage," *European Respiratory Journal, Supplement*, vol. 44, pp. 4s–6s, 2003.

[56] E. Ho and T. M. Bray, "Antioxidants, NFκB activation, and diabetogenesis," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 222, no. 3, pp. 205–213, 1999.

[57] J. M. Curtsinger, C. S. Schmidt, A. Mondino et al., "Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells," *Journal of Immunology*, vol. 162, no. 6, pp. 3256–3262, 1999.

[58] H. M. Lander, "An essential role for free radicals and derived species in signal transduction," *FASEB Journal*, vol. 11, no. 2, pp. 118–124, 1997.

[59] C. F. Nathan and R. K. Root, "Hydrogen peroxide release from mouse peritoneal macrophages. Dependence on sequential activation and triggering," *Journal of Experimental Medicine*, vol. 146, no. 6, pp. 1648–1662, 1977.

[60] Y. Zhang, J. Wienands, C. Zürn, and M. Reth, "Induction of the antigen receptor expression on B lymphocytes results in rapid competence for signaling of SLP-65 and Syk," *EMBO Journal*, vol. 17, no. 24, pp. 7304–7310, 1998.

[61] S. Devadas, L. Zaritskaya, S. G. Rhee, L. Oberley, and M. S. Williams, "Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and Fas ligand expression," *Journal of Experimental Medicine*, vol. 195, no. 1, pp. 59–70, 2002.

[62] M. Los, W. Dröge, K. Stricker, P. A. Baeuerle, and K. Schulze-Osthoff, "Hydrogen peroxide as a potent activator of T lymphocyte functions," *European Journal of Immunology*, vol. 25, no. 1, pp. 159–165, 1995.

[63] M. Karin, "The NF-κB activation pathway: its regulation and role in inflammation and cell survival," *Cancer Journal from Scientific American*, vol. 4, no. 1, pp. S92–S99, 1998.

[64] G. Gloire, S. Legrand-Poels, and J. Piette, "NF-κB activation by reactive oxygen species: fifteen years later," *Biochemical Pharmacology*, vol. 72, no. 11, pp. 1493–1505, 2006.

[65] R. Bottino, A. N. Balamurugan, H. Tse et al., "Response of human islets to isolation stress and the effect of antioxidant treatment," *Diabetes*, vol. 53, no. 10, pp. 2559–2568, 2004.

[66] A. Rabinovitch, "Free radicals as mediators of pancreatic islet β-cell injury in autoimmune diabetes," *Journal of Laboratory and Clinical Medicine*, vol. 119, no. 5, pp. 455–456, 1992.

[67] P. O. T. Tran, S. M. Parker, E. LeRoy et al., "Adenoviral overexpression of the glutamylcysteine ligase catalytic subunit protects pancreatic islets against oxidative stress," *Journal of Biological Chemistry*, vol. 279, no. 52, pp. 53988–53993, 2004.

[68] I. C. West, "Radicals and oxidative stress in diabetes," *Diabetic Medicine*, vol. 17, no. 3, pp. 171–180, 2000.

[69] E. Horio, M. Fukuda, H. Katoh et al., "Reactive oxygen intermediates in autoimmune islet cell destruction of the NOD mouse induced by peritoneal exudate cells (rich in macrophages) but not T cells," *Diabetologia*, vol. 37, no. 1, pp. 22–31, 1994.

[70] J. Nerup, T. Mandrup-Poulsen, J. Molvig, S. Helqvist, L. Wogensen, and J. Egeberg, "Mechanisms of pancreatic β-cell destruction in type I diabetes," *Diabetes Care*, vol. 11, supplement 1, pp. 16–23, 1988.

[71] W. L. Suarez-Pinzon, C. Szabó, and A. Rabinovitch, "Development of autoimmune diabetes in NOD mice is associated with the formation of peroxynitrite in pancreatic islet β-cells," *Diabetes*, vol. 46, no. 5, pp. 907–911, 1997.

[72] S. R. J. Maxwell, H. Thomason, D. Sandler et al., "Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus," *European Journal of Clinical Investigation*, vol. 27, no. 6, pp. 484–490, 1997.

[73] B. Ročić, M. Vučić, J. Knežević-Ćuća et al., "Total plasma antioxidants in first-degree relatives of patients with insulin-dependent diabetes," *Experimental and Clinical Endocrinology and Diabetes*, vol. 105, no. 4, pp. 213–217, 1997.

[74] S. A. Santini, G. Marra, B. Giardina et al., "Defective plasma antioxidant defenses and enhanced susceptibility to lipid peroxidation in uncomplicated IDDM," *Diabetes*, vol. 46, no. 11, pp. 1853–1858, 1997.

[75] D. R. Gamble and K. W. Taylor, "Seasonal incidence of diabetes mellitus," *British medical journal*, vol. 3, no. 671, pp. 631–633, 1969.

[76] D. R. Gamble, K. W. Taylor, and H. Cumming, "Coxsackie viruses and diabetes mellitus," *British Medical Journal*, vol. 4, no. 5887, pp. 260–262, 1973.

[77] T. J. Coleman, D. R. Gamble, and K. W. Taylor, "Diabetes in mice after Coxsackie B 4 virus infection," *British medical journal*, vol. 3, no. 5870, pp. 25–27, 1973.

[78] J. W. Yoon, T. Onodera, and A. L. Notkins, "Virus-induced diabetes mellitus. XV. Beta cell damage and insulin-dependent hyperglycemia in mice infected with Coxsackie virus B4," *Journal of Experimental Medicine*, vol. 148, no. 4, pp. 1068–1080, 1978.

[79] A. K. Berg, O. Korsgren, and G. Frisk, "Induction of the chemokine interferon-gamma-inducible protein-10 in human pancreatic islets during enterovirus infection," *Diabetologia*, vol. 49, no. 11, pp. 2697–2703, 2006.

[80] B. Xie, J. F. Zhou, Q. Lu, C. J. Li, and P. Chen, "Oxidative stress in patients with acute coxsackie virus myocarditis," *Biomedical and Environmental Sciences*, vol. 15, no. 1, pp. 48–57, 2002.

[81] E. Peterhans, M. Grob, T. Bürge, and R. Zanoni, "Virus-induced formation of reactive oxygen intermediates in phagocytic cells," *Free Radical Research Communications*, vol. 3, no. 1-5, pp. 39–46, 1987.

[82] S. Lenzen, J. Drinkgern, and M. Tiedge, "Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues," *Free Radical Biology and Medicine*, vol. 20, no. 3, pp. 463–466, 1996.

[83] M. Tiedge, S. Lortz, J. Drinkgern, and S. Lenzen, "Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells," *Diabetes*, vol. 46, no. 11, pp. 1733–1742, 1997.

[84] K. Grankvist, S. L. Marklund, and I. B. Taljedal, "CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse," *Biochemical Journal*, vol. 199, no. 2, pp. 393–398, 1981.

[85] P. Newsholme, E. P. Haber, S. M. Hirabara et al., "Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity," *Journal of Physiology*, vol. 583, no. 1, pp. 9–24, 2007.

[86] P. Maechler, L. Jornot, and C. B. Wollheim, "Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells," *Journal of Biological Chemistry*, vol. 274, no. 39, pp. 27905–27913, 1999.

[87] T. Nishikawa, D. Edelstein, X. L. Du et al., "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage," *Nature*, vol. 404, no. 6779, pp. 787–790, 2000.

[88] N. Welsh and C. Hellerstrom, "In vitro restoration of insulin production in islets from adult rats treated neonatally with streptozotocin," *Endocrinology*, vol. 126, no. 4, pp. 1842–1848, 1990.

[89] T. Szkudelski, "The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas," *Physiological Research*, vol. 50, no. 6, pp. 537–546, 2001.

[90] D. C. Weaver, M. L. McDaniel, and P. E. Lacy, "Alloxan uptake by isolated rat islets of langerhans," *Endocrinology*, vol. 102, no. 6, pp. 1847–1855, 1978.

[91] R. Munday, "Dialuric acid autoxidation: effects of transition metals on the reaction rate and on the generation of "active oxygen" species," *Biochemical Pharmacology*, vol. 37, no. 3, pp. 409–413, 1988.

[92] N. Takasu, I. Komiya, T. Asawa, Y. Nagasawa, and T. Yamada, "Streptozocin- and alloxan-induced H_2O_2 generation and DNA fragmentation in pancreatic islets: HO as mediator for DNA fragmentation," *Diabetes*, vol. 40, no. 9, pp. 1141–1145, 1991.

[93] T. Ino, Y. Kawamoto, K. Sato et al., "Selection of mouse strains showing high and low incidences of alloxan-induced diabetes," *Jikken dobutsu. Experimental animals*, vol. 40, no. 1, pp. 61–67, 1991.

[94] C. E. Mathews, R. T. Graser, A. Savinov, D. V. Serreze, and E. H. Leiter, "Unusual resistance of ALR/Lt mouse β cells to autoimmune destruction: role for β cell-expressed resistance determinants," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 1, pp. 235–240, 2001.

[95] S. Sandler and I. Swenne, "Streptozotocin, but not alloxan, induces DNA repair synthesis in mouse pancreatic islets in vitro," *Diabetologia*, vol. 25, no. 5, pp. 444–447, 1983.

[96] M. Nukatsuka, H. Sakurai, Y. Yoshimura, M. Nishida, and J. Kawada, "Enhancement by streptozotocin of O_2 radical generation by the xanthine oxidase system of pancreatic β-cells," *FEBS Letters*, vol. 239, no. 2, pp. 295–298, 1988.

[97] K. D. Kroncke, K. Fehsel, A. Sommer, M. L. Rodriguez, and V. Kolb-Bachofen, "Nitric oxide generation during cellular metabolization of the diabetogenic N-methyl-N-nitrosourea streptozotozin contributes to islet cell DNA damage," *Biological Chemistry Hoppe-Seyler*, vol. 376, no. 3, pp. 179–185, 1995.

[98] D. Morgan, H. R. Oliveira-Emilio, D. Keane et al., "Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line," *Diabetologia*, vol. 50, no. 2, pp. 359–369, 2007.

[99] H. R. Oliveira, R. Verlengia, C. R. O. Carvalho, L. R. G. Britto, R. Curi, and A. R. Carpinelli, "Pancreatic β-cells express phagocyte-like NAD(P)H oxidase," *Diabetes*, vol. 52, no. 6, pp. 1457–1463, 2003.

[100] R. Gyurko, C. C. Siqueira, N. Caldon, LI. Gao, A. Kantarci, and T. E. Van Dyke, "Chronic hyperglycemia predisposes to exaggerated inflammatory response and leukocyte dysfunction in Akita mice," *Journal of Immunology*, vol. 177, no. 10, pp. 7250–7256, 2006.

[101] L. A. O'Reilly, P. R. Hutchings, P. R. Crocker et al., "Characterization of pancreatic islet cell infiltrates in NOD mice: effect of cell transfer and transgene expression," *European Journal of Immunology*, vol. 21, no. 5, pp. 1171–1180, 1991.

[102] K. Lee, K. Amano, and J. Yoon, "Evidence for initial involvement of macrophage in development of insulitis in NOD mice," *Diabetes*, vol. 37, no. 7, pp. 989–991, 1988.

[103] J. Nerup, T. Mandrup-Poulsen, S. Helqvist et al., "On the pathogenesis of IDDM," *Diabetologia*, vol. 37, supplement 2, pp. S82–S89, 1994.

[104] J. L. Tiwari and P. I. Terasaki, "HLA-DR and disease associations," *Progress in Clinical and Biological Research*, vol. 58, pp. 151–163, 1981.

[105] F. J.T. Staal, M. Roederer, L. A. Herzenberg, and L. A. Herzenberg, "Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 24, pp. 9943–9947, 1990.

[106] W. Held, H. R. MacDonald, I. L. Weissman, M. W. Hess, and C. Mueller, "Genes encoding tumor necrosis factor alpha and granzyme A are expressed during development of autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, pp. 2239–2243, 1990.

[107] Z. Jiang and B. A. Woda, "Cytokine gene expression in the islets of the diabetic Biobreeding/Worcester rat," *Journal of Immunology*, vol. 146, no. 9, pp. 2990–2994, 1991.

[108] T. Mandrup-Poulsen, G. A. Spinas, S. J. Prowse et al., "Islet cytotoxicity of interleukin 1. Influence of culture conditions and islet donor characteristics," *Diabetes*, vol. 36, no. 5, pp. 641–647, 1987.

[109] T. Mandrup-Poulsen, J. Egeberg, and J. Nerup, "Ultrastructural studies of time-course and cellular specificity of interleukin-1 mediated islet cytotoxicity," *Acta Pathologica Microbiologica et Immunologica Scandinavica C*, vol. 95, no. 2, pp. 55–63, 1987.

[110] C. Southern, D. Schulster, and I. C. Green, "Inhibition of insulin secretion by interleukin-1β and tumour necrosis factor-α via an L-arginine-dependent nitric oxide generating mechanism," *FEBS Letters*, vol. 276, no. 1-2, pp. 42–44, 1990.

[111] M. Arnush, M. R. Heitmeier, A. L. Scarim, M. H. Marino, P. T. Manning, and J. A. Corbett, "IL-1 produced and released endogenously within human islets inhibits β cell function," *Journal of Clinical Investigation*, vol. 102, no. 3, pp. 516–526, 1998.

[112] T. Mandrup-Poulsen, K. Bendtzen, C. A. Dinarello, and J. Nerup, "Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic β-cell cytotoxicity," *Journal of Immunology*, vol. 139, no. 12, pp. 4077–4082, 1987.

[113] I. L. Campbell, L. Oxbrow, J. West, and L. C. Harrison, "Regulation of MHC protein expression in pancreatic beta-cells by interferon-gamma and tumor necrosis factor-alpha," *Molecular Endocrinology*, vol. 2, pp. 101–107, 1988.

[114] H. E. Thomas and T. W. H. Kay, "Beta cell destruction in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse," *Diabetes/Metabolism Research and Reviews*, vol. 16, no. 4, pp. 251–261, 2000.

[115] M. Torres and H. J. Forman, "Redox signaling and the MAP kinase pathways," *BioFactors*, vol. 17, no. 1-4, pp. 287–296, 2003.

[116] Y. Murata, T. Shimamura, and J. Hamuro, "The polarization of Th1/Th2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production," *International Immunology*, vol. 14, no. 2, pp. 201–212, 2002.

[117] C. A. Dinarello, J. G. Cannon, and J. W. Mier, "Multiple biological activities of human recombinant interleukin 1," *Journal of Clinical Investigation*, vol. 77, no. 6, pp. 1734–1739, 1986.

[118] E. A. Green, E. E. Eynon, and R. A. Flaveirti, "Local expression of TNFα in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens," *Immunity*, vol. 9, no. 5, pp. 733–743, 1998.

[119] C. Huang, J. Li, M. Costa et al., "Hydrogen peroxide mediates activation of nuclear factor of activated T cells (NFAT) by nickel subsulfide," *Cancer Research*, vol. 61, no. 22, pp. 8051–8057, 2001.

[120] H. M. Tse, T. C. Thayer, C. Steele et al., "NADPH oxidase deficiency regulates Th lineage commitment and modulates autoimmunity," *Journal of Immunology*, vol. 185, no. 9, pp. 5247–5258, 2010.

[121] M. L. Schmitz, S. Bacher, and O. Dienz, "NF-κB activation pathways induced by T cell costimulation," *FASEB Journal*, vol. 17, no. 15, pp. 2187–2193, 2003.

[122] Y.-H. Chung, H.-S. Jun, Y. Kang et al., "Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes in diabetes-resistant BioBreeding rats," *Journal of Immunology*, vol. 159, no. 1, pp. 466–471, 1997.

[123] A. Holz, B. Adrian, B. Coon, T. Wolfe, M. J. Grusby, and M. G. Von Herrath, "Disruption of the STAT4 signaling pathway protects from autoimmune diabetes while retaining antiviral immune competence," *Journal of Immunology*, vol. 163, no. 10, pp. 5374–5382, 1999.

[124] N. G. Jacobson, S. J. Szabo, R. M. Weber-Nordt et al., "Interleukin 12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat)3 and Stat4," *Journal of Experimental Medicine*, vol. 181, no. 5, pp. 1755–1762, 1995.

[125] S. E. Macatonia, C.-S. Hsieh, K. M. Murphy, and A. O'Garra, "Dendritic cells and macrophages are required for Th1 development of CD4+ t cells from $\alpha\beta$ TCR transgenic mice: IL-12 substitution for macrophages to stimulate IFN-γ production is IFN-γ-dependent," *International Immunology*, vol. 5, no. 9, pp. 1119–1128, 1993.

[126] N. Sarvetnick, J. Shizuru, D. Liggitt et al., "Loss of pancreatic islet tolerance induced by β-cell expression of interferon-γ," *Nature*, vol. 346, no. 6287, pp. 844–847, 1990.

[127] M. Debray-Sachs, C. Carnaud, C. Boitard et al., "Prevention of diabetes in NOD mice treated with antibody to murine IFNγ," *Journal of Autoimmunity*, vol. 4, no. 2, pp. 237–248, 1991.

[128] BO. Wang, I. André, A. Gonzalez et al., "Interferon-γ impacts at multiple points during the progression of autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 25, pp. 13844–13849, 1997.

[129] M. Cnop, N. Welsh, J.-C. Jonas, A. Jörns, S. Lenzen, and D. L. Eizirik, "Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes: many differences, few similarities," *Diabetes*, vol. 54, supplement 2, pp. S97–S107, 2005.

[130] D. L. Eizirik, F. Moore, D. Flamez, and F. Ortis, "Use of a systems biology approach to understand pancreatic β-cell death in Type 1 diabetes," *Biochemical Society Transactions*, vol. 36, no. 3, pp. 321–327, 2008.

[131] F. Moore, N. Naamane, M. L. Colli et al., "STAT1 is a master regulator of pancreatic β-cell apoptosis and islet inflammation," *Journal of Biological Chemistry*, vol. 286, no. 2, pp. 929–941, 2011.

[132] L. A. Stephens, H. E. Thomas, L. Ming, M. G.R. Darwiche, L. Volodin, and T. W.H. Kay, "Tumor necrosis factor-α-activated cell death pathways in NIT-1 insulinoma cells and primary pancreatic β cells," *Endocrinology*, vol. 140, no. 7, pp. 3219–3227, 1999.

[133] W. H. Kim, J. W. Lee, B. Gao, and M. H. Jung, "Synergistic activation of JNK/SAPK induced by TNF-α and IFN-γ: apoptosis of pancreatic β-cells via the p53 and ROS pathway," *Cellular Signalling*, vol. 17, no. 12, pp. 1516–1532, 2005.

[134] D. L. Eizirik and T. Mandrup-Poulsen, "A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis," *Diabetologia*, vol. 44, no. 12, pp. 2115–2133, 2001.

[135] M. O. Kurrer, S. V. Pakala, H. L. Hanson, and J. D. Katz, "β cell apoptosis in T cell-mediated autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 1, pp. 213–218, 1997.

[136] A. Suri and J. D. Katz, "Dissecting the role of CD4+ T cells autoimmune diabetes through the use of TCR transgenic mice," *Immunological Reviews*, vol. 169, pp. 55–65, 1999.

[137] T. P. DiLorenzo, R. T. Graser, T. Ono et al., "Major histocompatibility complex class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and use a prevalent T cell receptor α chain gene rearrangement," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 21, pp. 12538–12543, 1998.

[138] A. Bendelac, C. Carnaud, C. Boitard, and J. F. Bach, "Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ cells," *Journal of Experimental Medicine*, vol. 166, no. 4, pp. 823–832, 1987.

[139] S. W. Christianson, L. D. Shultz, and E. H. Leiter, "Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice: relative contributions of CD4+ and CD8+ T-cells from diabetic versus prediabetic NOD.NON-Thy-1 donors," *Diabetes*, vol. 42, no. 1, pp. 44–55, 1993.

[140] S. V. Pakala, M. Chivetta, C. B. Kelly, and J. D. Katz, "In autoimmune diabetes the transition from benign to pernicious insulitis requires an islet cell response to tumor necrosis factor α," *Journal of Experimental Medicine*, vol. 189, no. 7, pp. 1053–1062, 1999.

[141] A. Amrani, J. Verdaguer, B. Anderson, T. Utsugi, S. Bou, and P. Santamaria, "Perforin-independent β-cell destruction by diabetogenic CD8+ T lymphocytes in transgenic nonobese diabetic mice," *Journal of Clinical Investigation*, vol. 103, no. 8, pp. 1201–1209, 1999.

[142] D. Kägi, B. Odermatt, P. Seiler, R. M. Zinkernagel, T. W. Mak, and H. Hengartner, "Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice," *Journal of Experimental Medicine*, vol. 186, no. 7, pp. 989–997, 1997.

[143] W. L. Suarez-Pinzon and A. Rabinovitch, "Approaches to type 1 diabetes prevention by intervention in cytokine immunoregulatory circuits," *International Journal of Experimental Diabetes Research*, vol. 2, no. 1, pp. 3–17, 2001.

[144] T. Mandrup-Poulsen, "The role of interleukin-1 in the pathogenesis of IDDM," *Diabetologia*, vol. 39, no. 9, pp. 1005–1029, 1996.

[145] X. G. Lei and M. Z. Vatamaniuk, "Two tales of antioxidant enzymes on beta cells and diabetes," *Antioxid Redox Signal*. In press.

[146] D. A. Lepore, T. A. Shinkel, N. Fisicaro et al., "Enhanced expression of glutathione peroxidase protects islet β cells from hypoxia-reoxygenation," *Xenotransplantation*, vol. 11, no. 1, pp. 53–59, 2004.

[147] C. Moriscot, M. J. Richard, M. C. Favrot, and P. Y. Benhamou, "Protection of insulin-secreting INS-1 cells against oxidative stress through adenoviral-mediated glutathione peroxidase overexpression," *Diabetes and Metabolism*, vol. 29, pp. 145–151, 2003.

[148] S. Cuzzocrea, E. Mazzon, L. Dugo et al., "Protective effects of a new stable, highly active SOD mimetic, M40401 in splanchnic artery occlusion and reperfusion," *British Journal of Pharmacology*, vol. 132, no. 1, pp. 19–29, 2001.

[149] D. Salvemini, E. Mazzon, L. Dugo et al., "Pharmacological manipulation of the inflammatory cascade by the superoxide dismutase mimetic, M40403," *British Journal of Pharmacology*, vol. 132, no. 4, pp. 815–827, 2001.

[150] S. Lortz, M. Tiedge, T. Nachtwey, A. E. Karlsen, J. Nerup, and S. Lenzen, "Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes," *Diabetes*, vol. 49, no. 7, pp. 1123–1130, 2000.

[151] M. Sander and M. S. German, "The β cell transcription factors and development of the pancreas," *Journal of Molecular Medicine*, vol. 75, no. 5, pp. 327–340, 1997.

[152] H. Kaneto, Y. Kajimoto, J. Miyagawa et al., "Beneficial effects of antioxidants in diabetes: possible protection of pancreatic β-cells against glucose toxicity," *Diabetes*, vol. 48, no. 12, pp. 2398–2406, 1999.

[153] D. Kawamori, Y. Kajimoto, H. Kaneto et al., "Oxidative stress induces nucleo-cytoplasmic translocation of pancreatic transcription factor Pdx-1 through activation of c-jun NH2-terminal kinase," *Diabetes*, vol. 52, no. 12, pp. 2896–2904, 2003.

[154] M. J. Boucher, L. Selander, L. Carlsson, and H. Edlund, "Phosphorylation marks IPF1/PDX1 protein for degradation by glycogen synthase kinase 3-dependent mechanisms," *Journal of Biological Chemistry*, vol. 281, no. 10, pp. 6395–6403, 2006.

[155] X. D. Wang, M. Z. Vatamaniuk, S. K. Wang, C. A. Roneker, R. A. Simmons, and X. G. Lei, "Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice," *Diabetologia*, vol. 51, no. 8, pp. 1515–1524, 2008.

[156] X. Li, H. Chen, and P. N. Epstein, "Metallothionein and catalase sensitize to diabetes in nonobese diabetic mice: reactive oxygen species may have a protective role in pancreatic β-cells," *Diabetes*, vol. 55, no. 6, pp. 1592–1604, 2006.

[157] P. Coyle, J. C. Philcox, L. C. Carey, and A. M. Rofe, "Metallothionein: the multipurpose protein," *Cellular and Molecular Life Sciences*, vol. 59, no. 4, pp. 627–647, 2002.

[158] J. Sandström, L. M. Jonsson, H. Edlund, D. Holmberg, and S. L. Marklund, "Overexpression of extracellular-SOD in islets of nonobese diabetic mice and development of diabetes," *Free Radical Biology and Medicine*, vol. 33, no. 1, pp. 71–75, 2002.

[159] B. J. Goldstein, M. Kalyankar, and X. Wu, "Redox paradox: insulin action is facilitated by insulin-stimulated reactive oxygen species with multiple potential signaling targets," *Diabetes*, vol. 54, no. 2, pp. 311–321, 2005.

[160] M. Hotta, F. Tashiro, H. Ikegami et al., "Pancreatic β cell-specific expression of thioredoxin, an antioxidative and anti-apoptotic protein, prevents autoimmune and streptozotocin-induced diabetes," *Journal of Experimental Medicine*, vol. 188, no. 8, pp. 1445–1451, 1998.

[161] B. O. Xu, J. T. Moritz, and P. N. Epstein, "Overexpression of catalase provides partial protection to transgenic mouse beta cells," *Free Radical Biology and Medicine*, vol. 27, no. 7-8, pp. 830–837, 1999.

[162] P. Y. Benhamou, C. Moriscot, M. J. Richard et al., "Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets," *Diabetologia*, vol. 41, no. 9, pp. 1093–1100, 1998.

[163] H. Chen, E. C. Carlson, L. Pellet, J. T. Moritz, and P. N. Epstein, "Overexpression of metallothionein in pancreatic β-cells reduces streptozotocin-induced DNA damage and diabetes," *Diabetes*, vol. 50, no. 9, pp. 2040–2046, 2001.

[164] S. H. Huang, C. H. Chu, J. C. Yu et al., "Transgenic expression of haem oxygenase-1 in pancreatic beta cells protects non-obese mice used as a model of diabetes from autoimmune destruction and prolongs graft survival following islet transplantation," *Diabetologia*, vol. 53, no. 11, pp. 2389–2400, 2010.

[165] H. M. Kubisch, J. Wang, T. M. Bray, and J. P. Phillips, "Targeted overexpression of Cu/Zn superoxide dismutase protects pancreatic β-cells against oxidative stress," *Diabetes*, vol. 46, no. 10, pp. 1563–1566, 1997.

[166] C. E. Mathews and E. H. Leiter, "Constitutive differences in antioxidant defense status distinguish alloxan-resistant and alloxan-susceptible mice," *Free Radical Biology and Medicine*, vol. 27, no. 3-4, pp. 449–455, 1999.

[167] A. Rabinovitch, W. L. Suarez, and R. F. Power, "Lazaroid antioxidant reduces incidence of diabetes and insulitis in nonobese diabetic mice," *Journal of Laboratory and Clinical Medicine*, vol. 121, no. 4, pp. 603–607, 1993.

[168] P. Ohly, C. Dohle, J. Abel, J. Seissler, and H. Gleichmann, "Zinc sulphate induces metallothionein in pancreatic islets of mice and protects against diabetes induced by multiple low doses of streptozotocin," *Diabetologia*, vol. 43, no. 8, pp. 1020–1030, 2000.

[169] S. D. Stosic-Grujicic, D. M. Miljkovic, I. D. Cvetkovic, D. D. Maksimovic-Ivanic, and V. Trajkovic, "Immunosuppressive and anti-inflammatory action of antioxidants in rat autoimmune diabetes," *Journal of Autoimmunity*, vol. 22, no. 4, pp. 267–276, 2004.

[170] C. M. Hu, H. H. Lin, M. T. Chiang, PI. F. Chang, and L. Y. Chau, "Systemic expression of heme oxygenase-1 ameliorates type 1 diabetes in NOD mice," *Diabetes*, vol. 56, no. 5, pp. 1240–1247, 2007.

[171] C. E. Mathews, B. D. Dunn, M. O. Hannigan, C. K. Huang, and E. H. Leiter, "Genetic control of neutrophil superoxide production in diabetes-resistant ALR/Lt mice," *Free Radical Biology and Medicine*, vol. 32, no. 8, pp. 744–751, 2002.

[172] M. Matsushita, G. Yoshino, M. Iwai et al., "Protective effect of probucol on alloxan diabetes in rats," *Diabetes Research and Clinical Practice*, vol. 7, no. 4, pp. 313–316, 1989.

[173] A. L. Drash, W. A. Rudert, S. Borquaye, R. Wang, and I. Lieberman, "Effect of probucol on development of diabetes mellitus in BB rats," *American Journal of Cardiology*, vol. 62, no. 3, pp. 27B–30B, 1988.

[174] M. Fukuda, H. Ikegami, Y. Kawaguchi, T. Sano, and T. Ogihara, "Antioxidant, probucol, can inhibit the generation of hydrogen peroxide in islet cells induced by macrophages and prevent islet cell destruction in NOD mice," *Biochemical and Biophysical Research Communications*, vol. 209, no. 3, pp. 953–958, 1995.

[175] I. Batinić-Haberle, L. Benov, I. Spasojević, and I. Fridovich, "The ortho effect makes manganese(III) meso-tetrakis(N-methylpyridinium- 2-yl)porphyrin a powerful and potentially useful superoxide dismutase mimic," *Journal of Biological Chemistry*, vol. 273, no. 38, pp. 24521–24528, 1998.

[176] R. F. Pasternack, A. Banth, J. M. Pasternack, and C. S. Johnson, "Catalysis of the disproportionation of superoxide by metalloporphyrins. III," *Journal of Inorganic Biochemistry*, vol. 15, no. 3, pp. 261–267, 1981.

[177] B. J. Day, I. Batinic-Haberle, and J. D. Crapo, "Metalloporphyrins are potent inhibitors of lipid peroxidation," *Free Radical Biology and Medicine*, vol. 26, no. 5-6, pp. 730–736, 1999.

[178] G. Ferrer-Sueta, D. Vitturi, I. Batinić-Haberle et al., "Reactions of manganese porphyrins with peroxynitrite and carbonate radical anion," *Journal of Biological Chemistry*, vol. 278, no. 30, pp. 27432–27438, 2003.

[179] Y. Huang and F. E. Domann, "Redox modulation of AP-2 DNA binding activity in vitro," *Biochemical and Biophysical Research Communications*, vol. 249, no. 2, pp. 307–312, 1998.

[180] YI. Sun and L. W. Oberley, "Redox regulation of transcriptional activators," *Free Radical Biology and Medicine*, vol. 21, no. 3, pp. 335–348, 1996.

[181] N. Giannoukakis, W. A. Rudert, M. Trucco, and P. D. Robbins, "Protection of human islets from the effects of interleukin-1β by adenoviral gene transfer of an IκB repressor," *Journal of Biological Chemistry*, vol. 275, no. 47, pp. 36509–36513, 2000.

[182] H. Heimberg, Y. Heremans, C. Jobin et al., "Inhibition of cytokine-induced NF-κB activation by adenovirus-mediated expression of a NF-κB super-repressor prevents β-cell apoptosis," *Diabetes*, vol. 50, no. 10, pp. 2219–2224, 2001.

[183] B. D. Stadinski, T. Delong, N. Reisdorph et al., "Chromogranin A is an autoantigen in type 1 diabetes," *Nature Immunology*, vol. 11, no. 3, pp. 225–231, 2010.

[184] C. M. Dobbs and K. Haskins, "Comparison of a T cell clone and of T cells from a TCR transgenic mouse: TCR transgenic T cells specific for self-antigen are atypical," *Journal of Immunology*, vol. 166, no. 4, pp. 2495–2504, 2001.

[185] M. M. Sklavos, H. M. Tse, and J. D. Piganelli, "Redox modulation inhibits CD8+ T cell effector function," *Free Radical Biology and Medicine*, vol. 45, no. 10, pp. 1477–1486, 2008.

[186] G. Chaudhri, I. A. Clark, and N. H. Hunt, "Effect of antioxidants on primary alloantigen-induced T cell activation and proliferation," *Journal of Immunology*, vol. 137, no. 8, pp. 2646–2652, 1986.

[187] G. Chaudhri, N. H. Hunt, I. A. Clark, and R. Ceredig, "Antioxidants inhibit proliferation and cell surface expression of receptors for interleukin-2 and transferrin in T lymphocytes stimulated with phorbol myristate acetate and ionomycin," *Cellular Immunology*, vol. 115, no. 1, pp. 204–213, 1988.

[188] J. D. Malhotra, H. Miao, K. Zhang et al., "Antioxidants reduce endoplasmic reticulum stress and improve protein secretion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 47, pp. 18525–18530, 2008.

[189] D. Lo, L. C. Burkly, G. Widera et al., "Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells," *Cell*, vol. 53, no. 1, pp. 159–168, 1988.

[190] L. Jiang, F. Allagnat, E. Nguidjoe et al., "Plasma membrane Ca2+-ATPase overexpression depletes both mitochondrial and endoplasmic reticulum Ca2+ stores and triggers apoptosis in insulin-secreting BRIN-BD11 cells," *Journal of Biological Chemistry*, vol. 285, no. 40, pp. 30634–30643, 2010.

[191] X. Lei, S. Zhang, S. E. Barbour et al., "Spontaneous development of endoplasmic reticulum stress that can lead to diabetes mellitus is associated with higher calcium-independent phospholipase A expression: a role for regulation by SREBP-1," *Journal of Biological Chemistry*, vol. 285, no. 9, pp. 6693–6705, 2010.

[192] S. Ramanadham, F.-F. Hsu, S. Zhang et al., "Apoptosis of insulin-secreting cells induced by endoplasmic reticulum stress is amplified by overexpression of group VIA calcium-independent phospholipase A2 (iPLA2 beta) and suppressed by inhibition of iPLA2 beta," *Biochemistry*, vol. 43, no. 4, pp. 918–930, 2004.

[193] F. R. Papa, C. Zhang, K. Shokat, and P. Waiter, "Bypassing a kinase activity with an ATP-competitive drug," *Science*, vol. 302, no. 5650, pp. 1533–1537, 2003.

[194] D. Ron, "Stressed cells cope with protein overload," *Science*, vol. 313, no. 5783, pp. 52–53, 2006.

[195] C. Patil and P. Walter, "Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals," *Current Opinion in Cell Biology*, vol. 13, no. 3, pp. 349–355, 2001.

[196] T. Izumi, H. Yokota-Hashimoto, S. Zhao, J. Wang, P. A. Halban, and T. Takeuchi, "Dominant negative pathogenesis by mutant proinsulin in the Akita diabetic mouse," *Diabetes*, vol. 52, no. 2, pp. 409–416, 2003.

[197] J. Wang, T. Takeuchi, S. Tanaka et al., "A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse," *Journal of Clinical Investigation*, vol. 103, no. 1, pp. 27–37, 1999.

[198] C. X. Santos, L. Y. Tanaka, J. Wosniak, and F. R. Laurindo, "Mechanisms and implications of reactive oxygen species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase," *Antioxid Redox Signal*, vol. 11, pp. 2409–2427, 2009.

[199] R. J. Kaufman, "Orchestrating the unfolded protein response in health and disease," *Journal of Clinical Investigation*, vol. 110, no. 10, pp. 1389–1398, 2002.

[200] L. A. Casciola-Rosen, G. J. Anhalt, and A. Rosen, "DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis," *Journal of Experimental Medicine*, vol. 182, no. 6, pp. 1625–1634, 1995.

[201] S. G. Fonseca, M. Burcin, J. Gromada, and F. Urano, "Endoplasmic reticulum stress in β-cells and development of diabetes," *Current Opinion in Pharmacology*, vol. 9, no. 6, pp. 763–770, 2009.

[202] E. Bonifacio and A. G. Ziegler, "Advances in the prediction and natural history of type 1 diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 39, no. 3, pp. 513–525, 2010.

[203] A. M. Davalli, L. Scaglia, D. H. Zangen, J. Hollister, S. Bonner-Weir, and G. C. Weir, "Vulnerability of islets in the immediate posttransplantation period: dynamic changes in structure and function," *Diabetes*, vol. 45, no. 9, pp. 1161–1167, 1996.

[204] M. M. Sklavos, S. Bertera, H. M. Tse et al., "Redox modulation protects islets from transplant-related injury," *Diabetes*, vol. 59, no. 7, pp. 1731–1738, 2010.

[205] M. D. Menger, P. Vajkoczy, R. Leiderer, S. Jager, and K. Messmer, "Influence of experimental hyperglycemia on microvascular blood perfusion of pancreatic islet isografts," *Journal of Clinical Investigation*, vol. 90, no. 4, pp. 1361–1369, 1992.

[206] W. Land, H. Schneeberger, S. Schleibner et al., "The beneficial effect of human recombinant superoxide dismutase on acute and chronic rejection events in recipients of cadaveric renal transplants," *Transplantation*, vol. 57, no. 2, pp. 211–217, 1994.

[207] H. Petrowsky, B. Dippe, P. Geck et al., "Do oxygen radicals play a role in primary dysfunction of transplanted livers following preservation in University of Wisconsin solution?" *Transplantation Proceedings*, vol. 27, no. 1, pp. 729–731, 1995.

[208] J. Mendola, J. R. Wright, and P. E. Lacy, "Oxygen free-radical scavengers and immune destruction of murine islets in allograft rejection and multiple low-dose streptozocin-induced insulitis," *Diabetes*, vol. 38, no. 3, pp. 379–385, 1989.

[209] I. N. Nomikos, Y. Wang, and K. J. Lafferty, "Involvement of O_2 radicals in 'autoimmune' diabetes," *Immunology and Cell Biology*, vol. 67, no. 1, pp. 85–87, 1989.

[210] Y. X. Li, GE. Li, W. P. Dong, DA. R. Lu, and J. M. Tan, "Protection of human islets from induction of apoptosis and improved islet function with HO-1 gene transduction," *Chinese Medical Journal*, vol. 119, no. 19, pp. 1639–1645, 2006.

[211] S. Bertera, M. L. Crawford, A. M. Alexander et al., "Gene transfer of manganese superoxide dismutase extends islet graft function in a mouse model of autoimmune diabetes," *Diabetes*, vol. 52, no. 2, pp. 387–393, 2003.

[212] R. Bottino, A. N. Balamurugan, S. Bertera, M. Pietropaolo, M. Trucco, and J. D. Piganelli, "Preservation of human islet cell functional mass by anti-oxidative action of a novel SOD mimic compound," *Diabetes*, vol. 51, no. 8, pp. 2561–2567, 2002.

[213] A. N. Balamurugan, R. Bottino, N. Giannoukakis, and C. Smetanka, "Prospective and challenges of islet transplantation for the therapy of autoimmune diabetes," *Pancreas*, vol. 32, no. 3, pp. 231–243, 2006.

[214] N. Zhang, D. Su, S. Qu et al., "Sirolimus is associated with reduced islet engraftment and impaired β-cell function," *Diabetes*, vol. 55, no. 9, pp. 2429–2436, 2006.

[215] H. Kaneto, N. Katakami, M. Matsuhisa, and T. A. Matsuoka, "Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis," *Mediators of Inflammation*, vol. 2010, Article ID 453892, 2010.

[216] S. Brahmachari and K. Pahan, "Myelin basic protein priming reduces the expression of Foxp3 in T cells via nitric oxide," *Journal of Immunology*, vol. 184, no. 4, pp. 1799–1809, 2010.

[217] A. Stirban, P. Rösen, and D. Tschoepe, "Complications of type 1 diabetes: new molecular findings," *Mount Sinai Journal of Medicine*, vol. 75, no. 4, pp. 328–351, 2008.

[218] X. Ling, A. Cota-Gomez, N. C. Flores et al., "Alterations in redox homeostasis and prostaglandins impair endothelial-dependent vasodilation in euglycemic autoimmune nonobese diabetic mice," *Free Radical Biology and Medicine*, vol. 39, no. 8, pp. 1089–1098, 2005.

[219] T. Yoshikawa and N. Yoshida, "Vitamin E and leukocyte-endothelial cell interactions," *Antioxidants and Redox Signaling*, vol. 2, no. 4, pp. 821–825, 2000.

[220] M. A. Haidara, D. P. Mikhailidis, M. A. Rateb et al., "Evaluation of the effect of oxidative stress and vitamin E supplementation on renal function in rats with streptozotocin-induced Type 1 diabetes," *Journal of Diabetes and its Complications*, vol. 23, no. 2, pp. 130–136, 2009.

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Research Article

NOD Dendritic Cells Stimulated with *Lactobacilli* Preferentially Produce IL-10 versus IL-12 and Decrease Diabetes Incidence

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Dendritic cells (DCs) from NOD mice produced high levels of IL-12 that induce IFNγ-producing T cells involved in diabetes development. We propose to utilize the microorganism ability to induce tolerogenic DCs to abrogate the proinflammatory process and prevent diabetes development. NOD DCs were stimulated with *Lactobacilli* (nonpathogenic bacteria targeting TLR2) or lipoteichoic acid (LTA) from *Staphylococcus aureus* (TLR2 agonist). LTA-treated DCs produced much more IL-12 than IL-10 and accelerated diabetes development when transferred into NOD mice. In contrast, stimulation of NOD DCs with *L. casei* favored the production of IL-10 over IL-12, and their transfer decreased disease incidence which anti-IL-10R antibodies restored. These data indicated that *L. casei* can induce NOD DCs to develop a more tolerogenic phenotype via production of the anti-inflammatory cytokine, IL-10. Evaluation of the relative production of IL-10 and IL-12 by DCs may be a very useful means of identifying agents that have therapeutic potential.

1. Introduction

Proinflammatory cytokines are increased during the active stages of type 1 diabetes and appear to be involved in disease development in NOD mice [1, 2]. DCs and macrophages from NOD mice have been shown to produce higher levels of proinflammatory cytokines, including IL-12 (p70) and TNF-α [3–5], as a result of NFκB hyperactivity [6–9] by comparison to diabetes-resistant strains of mice, that is, C57BL/6, BALB/c, and NOR [6]. Moreover, myeloid DCs cultured from NOD bone marrow or spleen exhibit hyperactivation of NFκB in response to several stimuli [8]. Taken together, these data strongly suggest that a defect in regulation of NFκB activation exists in NOD DCs and macrophages and may play a key role in the modulation of APC function in NOD mice. Similarly, elevated IL-12 production as well as polymorphisms in the region of the IL-12 gene has also been associated with individuals at high risk for type 1 diabetes [10, 11]. The increase in IL-12 in NOD mice is particularly significant since it leads to activation of IFNγ-producing T cells which can mediate disease development [1, 2, 12]. Furthermore, suppression of IL-12 in NOD mice has been shown to reduce cellular infiltration in islets and prevent the development of diabetes [13]. Therefore, switching the cytokine profile of NOD DCs from proinflammatory to anti-inflammatory may be an effective strategy to abrogate activation of pathogenic Th1 cells and prevent diabetes development, since DCs have been shown to orchestrate the delicate balance between T-cell immunity and regulation in NOD mice [14].

Bacteria isolated from the normal gut flora are nonpathogenic and include the Lactobacillus species. Various strains of *Lactobacilli*, including *L. casei*, *L. reuteri,* and *L. plantarum*, have been shown to prevent diabetes [15, 16], collagen-induced arthritis [17], and colitis [18, 19] in animal models. In addition, *Lactobacilli* have been used to manage allergic diseases and are associated with improvement of various gastrointestinal diseases in humans [20, 21]. Recent studies have shown that various types of microorganisms,

including bacteria, fungi, and parasites, can evade the immune system by inducing tolerogenic APC [22–24] and/or regulatory T cells [23, 25–28]. Some of these microorganisms, including *Lactobacilli*, elicit an anti-inflammatory response, at least in part, via Toll-like Receptor 2 (TLR2) ligation [26, 29, 30]. Components of microbial cell walls that target TLR2 have also been found to induce DCs from nonautoimmune-prone mice to produce anti-inflammatory cytokines and induce regulatory cells [29, 31–33]. There is a possibility, therefore, that stimulation of NOD DCs with *Lactobacilli* or TLR2 ligands could polarize them toward an anti-inflammatory phenotype that could protect against disease development. We propose that cytokine profiles, particularly IL-12 and IL-10, could be useful predictors of the ability of stimuli to induce DCs that may be used as a treatment for the prevention of diabetes. In the current study, we tested a TLR2 agonist, lipoteichoic acid (LTA), derived from *S. aureus,* and three different strains of *Lactobacilli* for their ability to switch the NOD DCs phenotype to an anti-inflammatory tolerogenic phenotype. We report in the present paper that transfer of BM-DCs induced to produce IL-10 into NOD mice delayed onset and decreased incidence of diabetes, whereas transfer of BM-DCs induced to produce IL-12 has the opposite effect, that is, an acceleration in diabetes onset and increase in incidence of diabetes.

2. Materials and Methods

2.1. Mice. Female C57BL/6 and NOD mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained at the University of Louisville facility according to institutional animal care and use committee (IACUC) guidelines. Mice were anaesthetized with bromoethanol before euthanasia in order to harvest organs.

2.2. Antibodies and Flow Cytometry. FITC-anti-B7-1, FITC-anti-B7-2, PEcy7-anti-CD11b, and PE-anti-CD11c antibodies were purchased (BD Pharmingen, San Diego, CA). Cells were incubated with Fc block then labeled with antibodies for 20 min in DPBS 1% FCS, 0.1% $NaNO_3$ and washed twice. Cells were analyzed by FACS using a FACScalibur (Becton Dickinson, Palo Alto, CA).

2.3. Generation of Bone-Marrow-Derived Dendritic Cells. Bone-marrow-derived dendritic cells (BM-DCs) were generated by culturing bone marrow cells for 12 days with Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF) (PeproTech, Rocky Hill, NJ) in super complete medium containing RPMI (Mediatech, Herndon, VA) supplemented with 1% Hepes buffer (MP Biomedicals, LLC, Solon, OH), 1% sodium pyruvate, 1% L-glutamine, 1% penicillin-streptomycin, 1% nonessential amino acids (Mediatech, Herndon, VA), 0.1% β2-mercaptoethanol (Sigma-Aldrich, Saint-Louis, MO), and 10% fetal calf serum (FCS) (Hyclone, Logan, UT). Briefly, 5×10^6 bone marrow cells were cultured in large petri dishes (Nunc, Roskilde, Denmark) in 10 mL of culture medium containing 5 ng/mL of GM-CSF. New culture medium (10 mL) was added to the petri dishes at day 4. At days 6 and 9, half of the old cell culture medium (10 mL) was removed from each petri dish and replaced by new culture medium containing GM-CSF (5 ng/mL), and at day 12, cells were harvested.

2.4. Lactobacilli Cultures. The following *Lactobacilli* strains were used: human *Lactobacillus reuteri* DSM 2016 (DSMZ, Braunschweig, Germany), human *Lactobacillus plantarum* LP299v (PROBI, Lund, Sweden), and human *Lactobacillus casei* B255 (NIZO, Ede, The Netherlands). *Lactobacilli* were grown on deMan-Rogosa-Sharp (MRS) media (Difco Laboratories, Detroit, MI) under anaerobic conditions, and a single colony was expanded in MRS broth (Difco Laboratories, Detroit, MI) under anaerobic conditions and frozen in glycerol. *Lactobacilli* were thawed twice a week and grown for 20 hrs in MRS broth under anaerobic conditions, then expanded in large quantities until they reached the postexponential phase, that is, 12–16 hrs, depending on the strain.

2.5. Dendritic Cell Stimulation and Transfer. BM-DCs were stimulated overnight with different stimuli including 1, 10, or 100 μg/mL of LPS-free Lipoteichoic Acid (LTA) from *Staphylococcus aureus* (Invivogen, San Diego, CA), 100 ng/mL of LPS (Invivogen, San Diego, CA), or 10×10^6 CFU/mL, 1×10^6 CFU/mL (low dose) or 20×10^6 CFU/mL (high dose) *Lactobacilli*. The supernatants were harvested and stored at -20°C until assayed using quantitative enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. For the *in vivo* experiments, gentamycin 10 μg/mL was included in the culture containing *Lactobacilli* to eliminate any living bacteria, and the BM-DCs harvested after 24 hrs, washed twice with HBSS. 1×10^6 treated BM-DCs were transferred into 6–8-week-old NOD mice (n = 4–11 mice/group). In some experiments, 0.5 mg/mouse of anti-IL-10R antibodies or isotype control (BioXCell, West Lebanon, NH) was injected once a week for a period of four weeks. Sera were collected 28 hrs following injection and stored at -20°C until assayed using ELISA kits (eBioscience, San Diego, CA) according to the manufacturer's instructions. Blood glucose was monitored weekly until 30 weeks of age using blood glucose strips and a blood glucose meter (Home Diagnostics, Inc., Ft Lauderdale, FL). Mice were considered diabetic when glucose levels were >300 mg/dl for two consecutive weeks.

2.6. Statistical Analysis. Data were analyzed using either the Student's t-test, Wilcoxon test, or nonparametric log-rank test. Each experiment was repeated with reproducible results 2–4 times.

3. Results

3.1. Effect of a TLR-2 Ligand on NOD BM-DCs Activation and Phenotype. Antigen presenting cells (APCs) from adult NOD mice have been shown to produce high levels of the proinflammatory cytokine, IL-12 [3–5] in response to LPS and express lower levels of costimulatory molecules such as B7-1 and B7-2 [34, 35]. The overproduction of IL-12 by NOD APC is thought to contribute significantly to disease development, since it leads to activation of IFNγ-producing T cells which mediate diabetes [1, 2, 12]. Because

TLR2 agonists have been shown to induce production of anti-inflammatory cytokines in nonautoimmune mice and healthy human [29, 31–33], we examined whether a TLR2 ligand, lipoteichoic acid (LTA) can induce an anti-inflammatory (tolerogenic) phenotype in NOD DCs. We compared cytokine production and costimulatory molecule expression by bone-marrow-derived dendritic cells (BM-DCs) from NOD and B6 mice after stimulation with LTA isolated from *Staphylococcus aureus (S. aureus)*. DCs were cultured either in media (none), or in the presence of 10 μg/mL of LPS-free LTA or 100 ng/mL of LPS. After 24 hrs, supernatants were collected and measured for the presence of the anti-inflammatory cytokine, IL-10, or the proinflammatory cytokine, IL-12, by ELISA. As reported previously, upon stimulation with LPS, NOD DCs produced considerably more IL-12 than B6 DCs (Figure 1(b)) and about the same amount of IL-10 (Figure 1(a)). Surprisingly, stimulation with LTA from *S. aureus* also induced NOD DCs to produce more IL-12 compared to B6 DCs (0.5 ng/mL versus 0.1 ng/mL) as shown in Figures 1(a) and 1(b). Although IL-10 production upon LTA stimulation was about the same or even a bit higher in NOD DCs compared to B6 DCs (0.18 ng/mL versus 0.12 ng/mL), the production of IL-12 relative to IL-10 was much greater in NOD DCs compared to B6 DCs (Figures 1(a) and 1(b)). Moreover, fewer NOD DCs expressed B7-1 and B7-2 in response to LTA and LPS compared to B6 DCs, and the level of expression of B7-2 was also lower in NOD DCs. In summary, the TLR2 ligand, LTA, had a differential effect on the cytokine profile produced by NOD and B6 DCs: LTA induced more IL-12 than IL-10 in NOD DCs while inducing the same quantity of IL-12 and IL-10 in B6 DCs.

3.2. Effect of LTA-Treated BM-DCs on Diabetes Development in NOD Mice. Since the data described above suggested that LTA from *S. aureus* promotes a more proinflammatory than tolerogenic phenotype in NOD BM-DCs, we examined the relationship between cytokine production by DCs and disease development. BM-DCs from NOD mice were cultured in the absence (none or no treatment) or in the presence of LTA from *S. aureus* (LTA-treated NOD DCs) for 24 hrs. Supernatants and cells were harvested, and supernatants tested for the presence of IL-12 and IL-10. DCs from these cultures were injected into 6-week-old NOD mice and disease incidence was assessed. Similar to the results displayed in Figure 1(a), LTA from *S. aureus* induced much more IL-12 than IL-10 production (IL-12 : IL-10 ratio of 6.6) by NOD BM-DCs (data not shown). Interestingly, 100% of NOD mice injected with LTA-treated NOD DCs developed diabetes by 15 weeks of age (Figure 2(a), open circles) indicating that a single injection of LTA-treated NOD DCs could significantly accelerate disease onset. Moreover, we found that the level of IL-12 was higher in the serum of mice injected with LTA-treated NOD DCs (Figure 2(b), left panel), whereas the level of IL-10 was not significantly different (Figure 2(b)). Because various concentrations of LTA could affect DCs differentially, we tested a lower as well as a higher dose of LTA. As shown in Figure 2(c), DCs treated with 1 μg/mL, 10 μg/mL or 100 μg/mL of LTA and injected into young NOD mice accelerated diabetes development in a similar manner. Altogether, these data suggest that injection of LTA-treated NOD DCs may contribute to diabetes pathogenesis by producing high levels of the proinflammatory cytokine, IL-12.

3.3. Treatment with Different Strains of Lactobacilli Induces NOD DCs to Produce Different Cytokine Profiles. Dendritic cells play a pivotal role in the differentiation of Th1, Th2, Th3, and regulatory T cells throughout the gastrointestinal tract [36]. *Lactobacilli* are bacteria that compose the normal flora, and feeding *Lactobacilli* has been shown to be protective in various models of autoimmune diseases, including type 1 diabetes [15, 16], collagen-induced arthritis [17], and colitis [18, 19]. It is thought that *Lactobacilli* mediate their protective effect by modulating DCs cytokine production and surface molecule expression [37]. We tested the effects of stimulating NOD and B6 DCs with three different strains of *Lactobacilli* on cytokine production and costimulatory molecule expression in order to assess whether there were any differences in the ability of these strains to induce anti-inflammatory phenotypes in NOD DCs. These three strains included *Lactobacillus casei* (*L. casei*), *Lactobacillus plantarum* (*L. plantarum*), and *Lactobacillus reuteri* (*L. reuteri*) and were selected based on their ability to induce tolerance [38] or prevent disease in animal models, including diabetes [16], arthritis [17, 39], and colitis [18, 19]. We examined the production of the proinflammatory cytokine, IL-12, and anti-inflammatory cytokine, IL-10, by NOD and B6 BM-DCs stimulated with each of the strains of *Lactobacilli* described above for 24 hrs. Interestingly, cytokine production varied dramatically depending on the strain of *Lactobacilli* used and the source of DCs, that is, NOD versus B6 mice (Figures 3(a) and 3(b)). *L. reuteri* induced NOD BM-DCs to produce the highest level of IL-12 (Figure 3(a), fourth black column) and among the lowest levels of IL-10 (Figure 3(a), fourth white column), with IL-12 produced about an 11-fold higher level than IL-10. *L. plantarum* induced a low to intermediate level of IL-12 and a very low level of IL-10 in NOD BM-DCs, and IL-12 production (Figure 3(a), third black column) was about 3-fold higher than IL-10 production (Figure 3(a), third white column). In contrast, *L. casei* not only induced NOD BM-DCs to produce the highest level of IL-10 (Figure 3(a), second white column), but also the lowest level of IL-12 (Figure 3(a), second black column), and four times more IL-10 than IL-12 was produced. Interestingly, IL-10 production was much higher, in general, in B6 DCs stimulated with all three strains of *Lactobacilli*, with *L. casei* inducing the highest levels of IL-10 and the lowest levels of IL-12 (Figure 3(b)). As shown in Figures 3(c) and 3(d), all three strains of *Lactobacilli* induced approximately the same percentages of NOD BM-DCs to express B7-1 and B7-2, but to a lower extent than B6 BM-DCs. These data suggest that the ability of *Lactobacilli* to induce an anti-inflammatory versus proinflammatory phenotype in NOD DCs varies according to the strain, and *L. casei* appears to be the only strain of the three tested that induces an anti-inflammatory phenotype. Furthermore, NOD DCs appeared to be much less predisposed to produce IL-10 in response to *Lactobacilli* compared to B6 DCs.

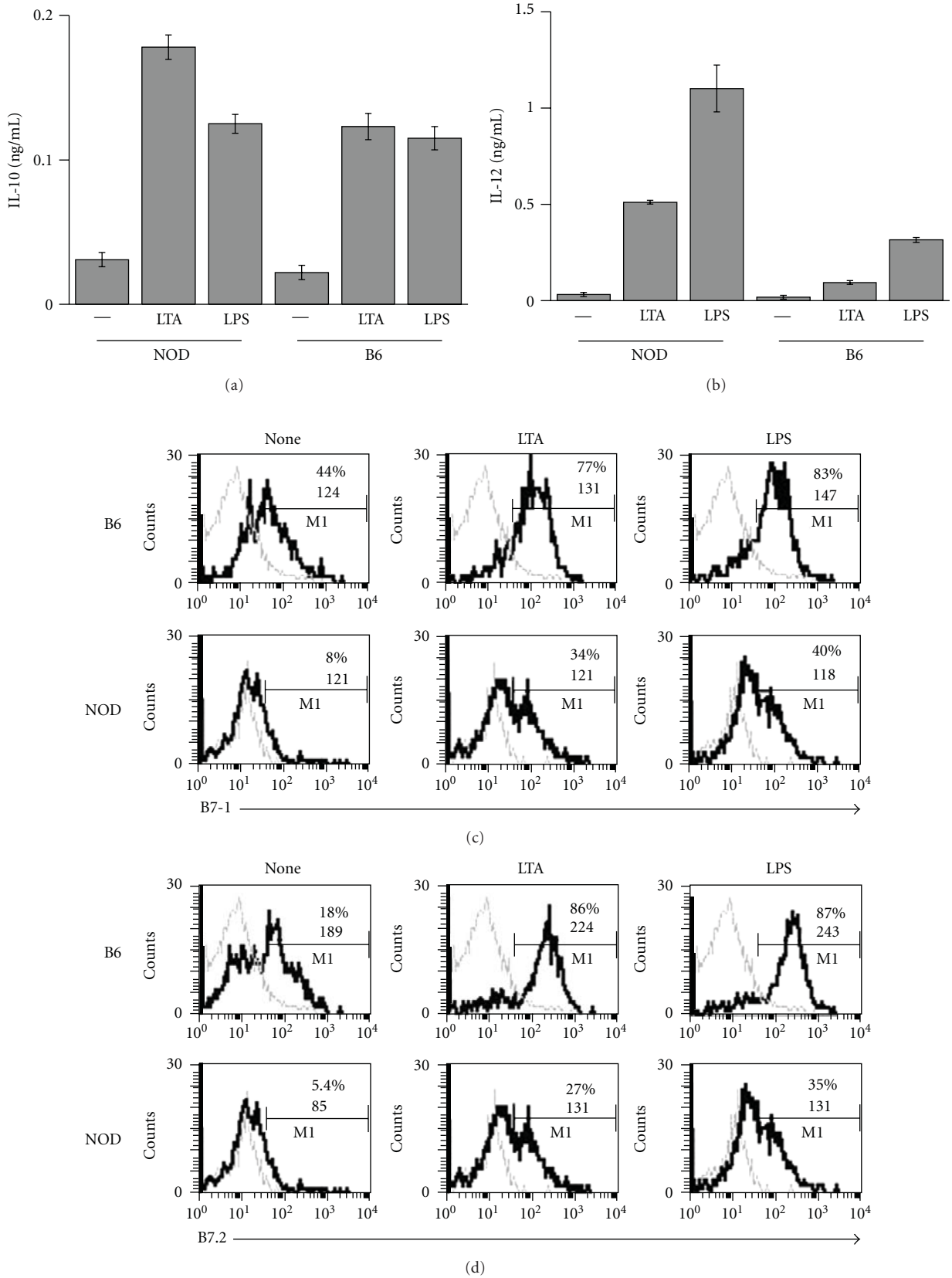

Figure 1: Effect of LTA on NOD and B6 DCs cytokine production and surface marker expression. BM-DCs from NOD mice were stimulated *in vitro* with 10 μg/mL of LTA from *S. aureus* (LTA), 100 ng/mL of LPS, or medium alone (—or none). After 24 hrs, supernatants were collected and measured by ELISA for the presence of IL-10 (a) or IL-12 (b), or cells were collected and labeled with anti-CD11c, anti-CD11b, and anti-B7-1 (c) or anti-B7-2 (d) antibodies, and analyzed by FACS after gating on $CD11b^{+}CD11c^{int}$ cells.

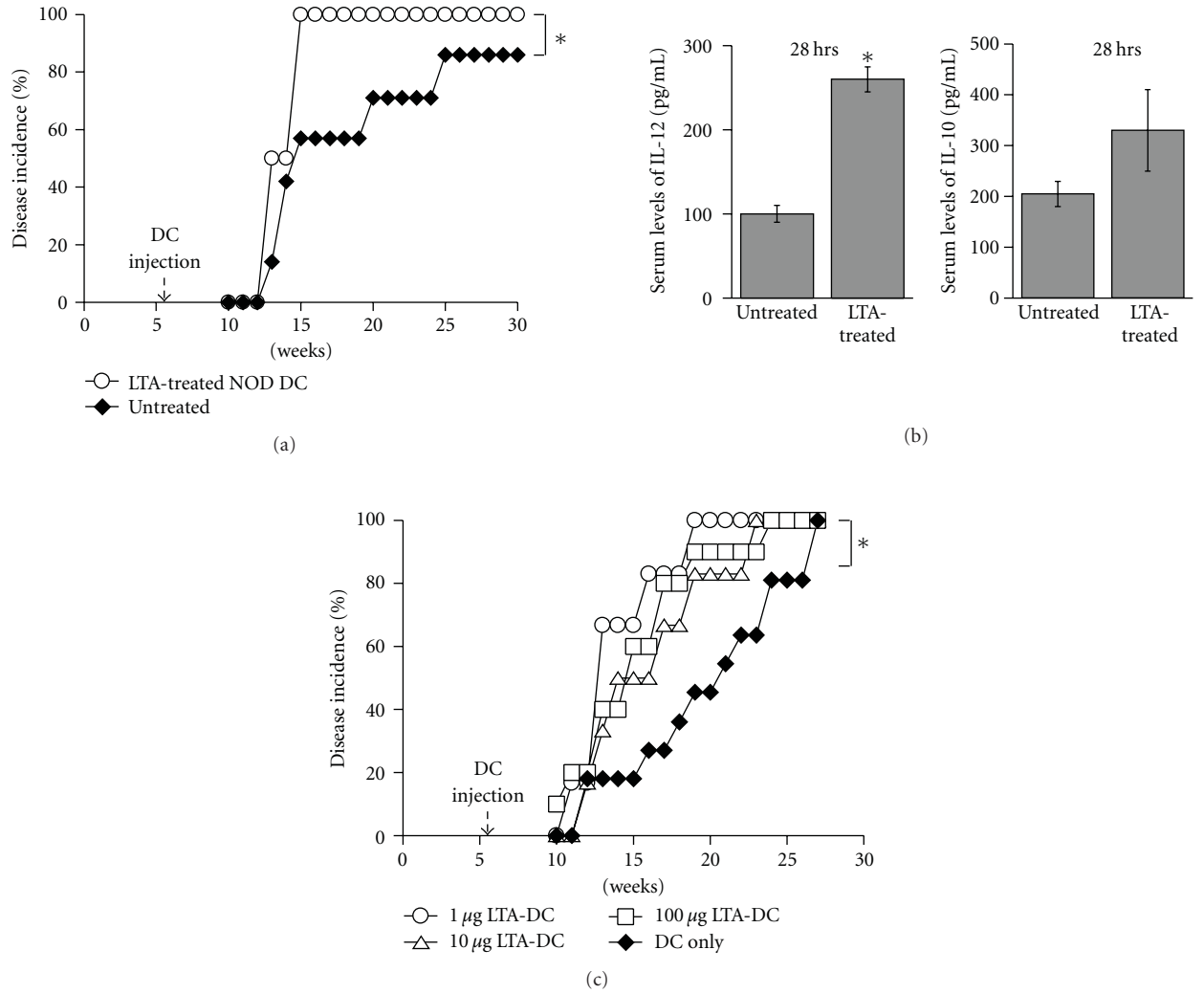

Figure 2: Incidence of diabetes in NOD mice injected with LTA-treated DCs. BM-DCs from NOD mice were stimulated *in vitro* overnight with 10 μg/mL of LTA from *S. aureus* (LTA) or medium alone (none), washed, and one million DCs were injected into six-week-old NOD mice (n = 5-6), and blood glucose was monitored weekly (a). Sera from pooled NOD mice untreated or injected with DCs treated with LTA were collected at 28 hrs after injection and tested for the presence of IL-12 and IL-10 by ELISA (b). One million DCs untreated or treated with 1, 10 or 100 μg/mL of LTA were injected into six-week-old NOD mice (n = 6–11), and blood glucose was monitored weekly (c). Mice were considered diabetic when their blood glucose levels were >300 dL/mL for 2 consecutive weeks. *indicates a significant difference from the "DC only" or "untreated" group at $P < .01$.

3.4. NOD DCs Treated with Different Doses of L. casei Produce Different Cytokine Profiles and Induce a Different Disease Outcome When Injected into NOD Mice. The dose of *Lactobacilli* has been shown to strongly influence the type of cytokine produced by DCs from B6 mice *in vitro* [37]. To determine the effect of dose of *L. casei* on cytokine production by NOD DCs, BM-DCs from NOD mice were cultured either in media (none) or in the presence of 1×10^6 CFU/mL (Low-dose) or 20×10^6 CFU/mL (High dose) of *L. casei*. After 24 hrs, supernatants were collected and measured for the presence of IL-10 or IL-12 by ELISA. The high dose of *L. casei* (LC) induced much more IL-10 (Figure 4(a), third white column) than the low dose of LC (Figure 4(a), second white column), and four times more IL-10 than IL-12 (Figure 4(a), third black column). These data indicate that high-dose LC is optimal for inducing NOD DCs to produce a more anti-inflammatory and tolerogenic phenotype.

We next examined whether injection of NOD DCs treated with either a high or low dose of LC into NOD mice translates into different disease outcomes. BM-DCs from NOD mice were cultured in the presence of 1×10^6 CFU/mL (low LC) or 20×10^6 CFU/mL of *L. casei* (high LC). Gentamycin was included in the culture medium, and no live bacteria were detected at the end of the culture (i.e., after 24 hrs). After 24 hrs, cells were harvested and injected into 6-week-old NOD mice and disease onset was monitored. In mice injected with NOD BM-DCs treated with low-dose LC, 100% of the mice develop diabetes by 18 weeks

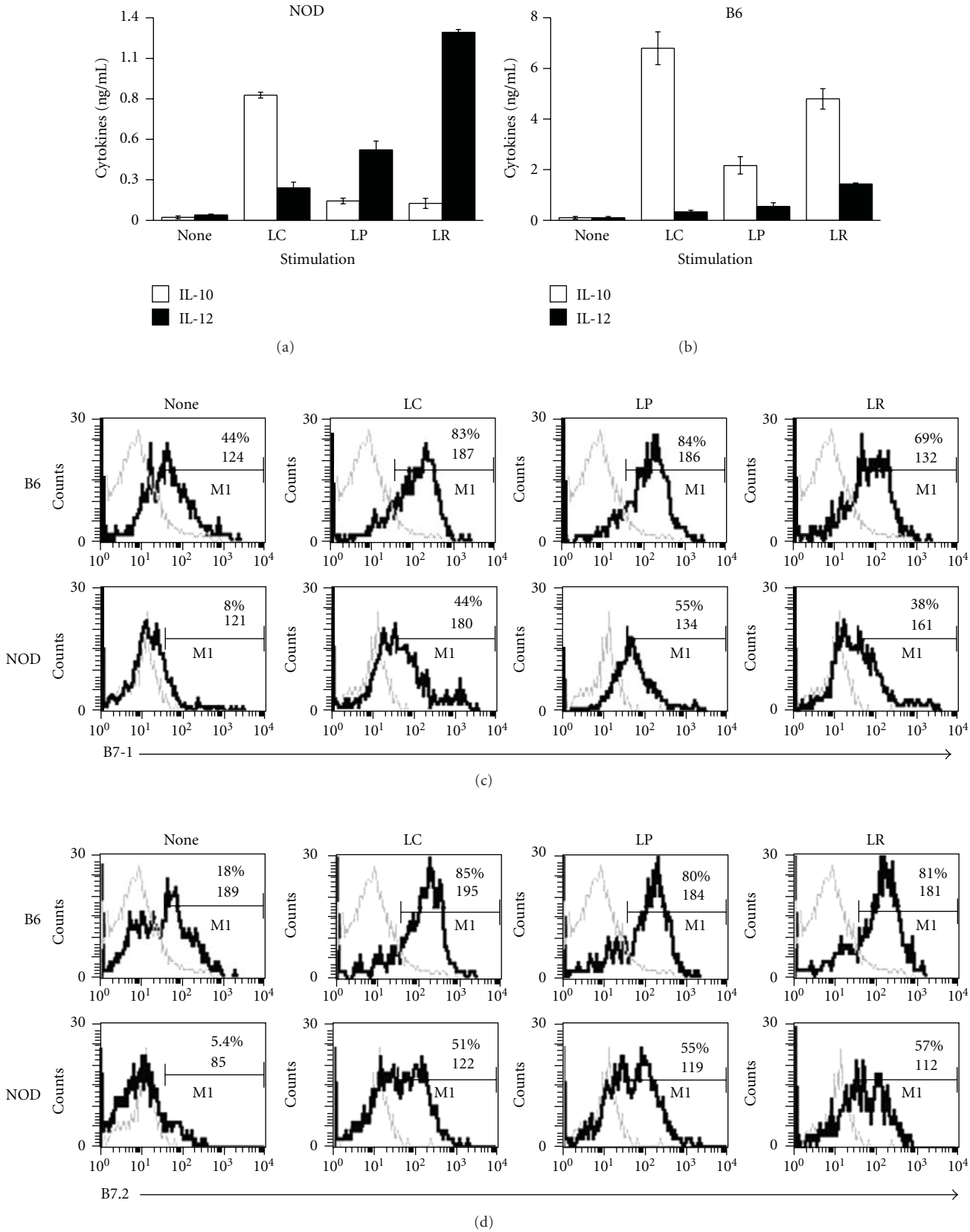

Figure 3: Activation of NOD and B6 DCs with different strains of *Lactobacilli in vitro*. BM-DCs from NOD or control B6 mice were stimulated for 24 hrs with 10×10^6 CFU/mL *L. casei* (LC), *L. plantarum* (LP), *L. reuteri* (LR), or medium alone (none). After 24 hrs, supernatants were collected and measured by ELISA for the presence of IL-10 or IL-12 (a and b), or cells were collected and labeled with anti-CD11c, anti-CD11b, and anti-B7-1 (c) or anti-B7-2 (d) antibodies, and analyzed by FACS after gating on $CD11b^+CD11c^{int}$ cells.

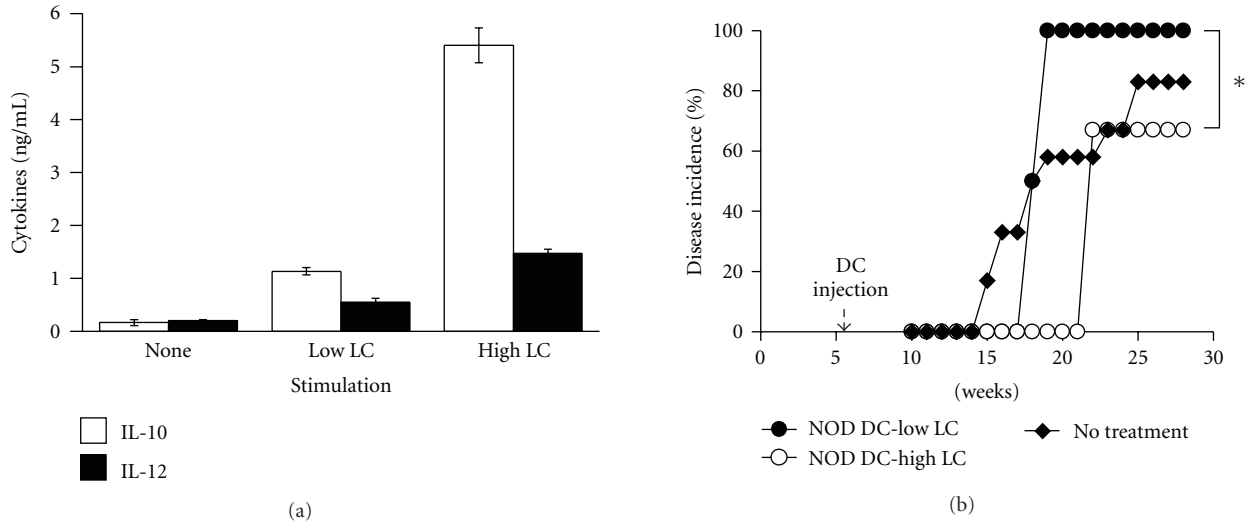

Figure 4: Effect of different doses of *L. casei* on the profiles of IL-10 and IL-12 production by NOD DCs *in vitro* and disease incidence. BM-DCs from NOD mice were stimulated with either 1×10^6 CFU/mL (Low LC), 20×10^6 CFU/mL (High LC) of the *L. casei* or medium alone (none). After 24 hrs, supernatants were collected and measured by ELISA for the presence of IL-10 (white bar) or IL-12 (black bar) (a). Six-week-old NOD mice were left untreated or received a single i.v. injection of 1×10^6 DCs treated with either low (1×10^6 CFU/mL-low LC) or high (20×10^6 CFU/mL-high LC) dose of *L. casei* (LC) ($n = 4$–6) (b). Blood glucose was measured weekly and diabetes incidence determined. *indicates a significant difference at $P < .05$.

of age (Figure 4(b), closed circle). In contrast, NOD mice injected with NOD DCs treated with high-dose LC exhibited a delay in disease onset by about 4 weeks (from 17 to 21 weeks of age), as well as a significant decrease in diabetes incidence from 100% to 60% by 30 weeks (Figure 4(b), open circle). These data suggest that the dose of *Lactobacilli* used to stimulate DCs determines the cytokine profile and, consequently, disease outcome.

3.5. Injection of IL-10-Producing NOD DCs into NOD Mice Decreases Diabetes Incidence. We next tested whether injections of LC-treated NOD DCs in NOD mice could actually prevent diabetes development in an IL-10 dependent manner. We administered one or two injections of NOD BM-DCs treated with high-dose LC starting at 6 weeks of age. As shown in Figures 5(a) and 5(c), injections of LC-treated DCs significantly decreased diabetes incidence by about 40% by 30 weeks in comparison to untreated NOD mice. No statistical differences were found between the untreated and untreated- DCs-injected groups, and one injection was enough to decrease disease incidence. We next examined the serum levels of IL-10 in NOD mice 28 hrs after injection of BM-DCs treated with high doses of *L. casei*. As shown in Figure 5(b), NOD mice injected with LC-treated DCs produced higher levels of IL-10 in serum 28 hrs following injection compared to noninjected NOD mice, suggesting LC-treated BM-DCs continue to produce IL-10 after injection and IL-10 may modulate the immune response *in vivo*. At 6 hrs after injection, very low levels of cytokines were detected with no differences among the different groups (data not shown), suggesting that cytokine production/accumulation requires additional time in order to allow differences to be detected. We finally tested whether the production of IL-10 by LC-treated DCs was responsible for the decrease in diabetes incidence. NOD mice were injected with anti-IL-10R or isotype control antibodies for a four-week period and LC-treated DCs. The treatment with anti-IL-10R antibodies completely abrogated production of IL-10 detected in the serum of mice injected with LC-treated DCs (data not shown). The incidence of disease was not altered in NOD mice treated with anti-IL-10R antibody only (Figure 5(c), closed diamond) compared to NOD mice injected with untreated DCs and treated with isotype control (Figure 5(c), open triangle) or anti-IL-10R antibody (data not shown). More importantly, mice injected with LC-treated DCs and treated with anti-IL-10R antibodies (Figure 5(c), closed circle) exhibited a higher diabetes incidence than mice that were injected with LC-treated DCs and treated with isotype control (Figure 5(c), open circle), indicating that IL-10 production plays an important role in LC-treated DCs-mediated protection. Altogether, these data suggest that injections of LC-treated DCs early in the disease process may be effective in preventing full-blown disease in a significant proportion of NOD mice, and IL-10 production by DCs may be useful to predict the effectiveness of the treatment.

4. Discussion

Previous reports have shown that feeding *Lactobacilli* has a protective effect in NOD mice [15, 16]. In the current study, we examined whether stimulation of NOD DCs with *Lactobacilli* and subsequent transfer into young NOD mice could have a similar protective effect, and whether cytokine profiles produced by DCs after stimulation could be a useful

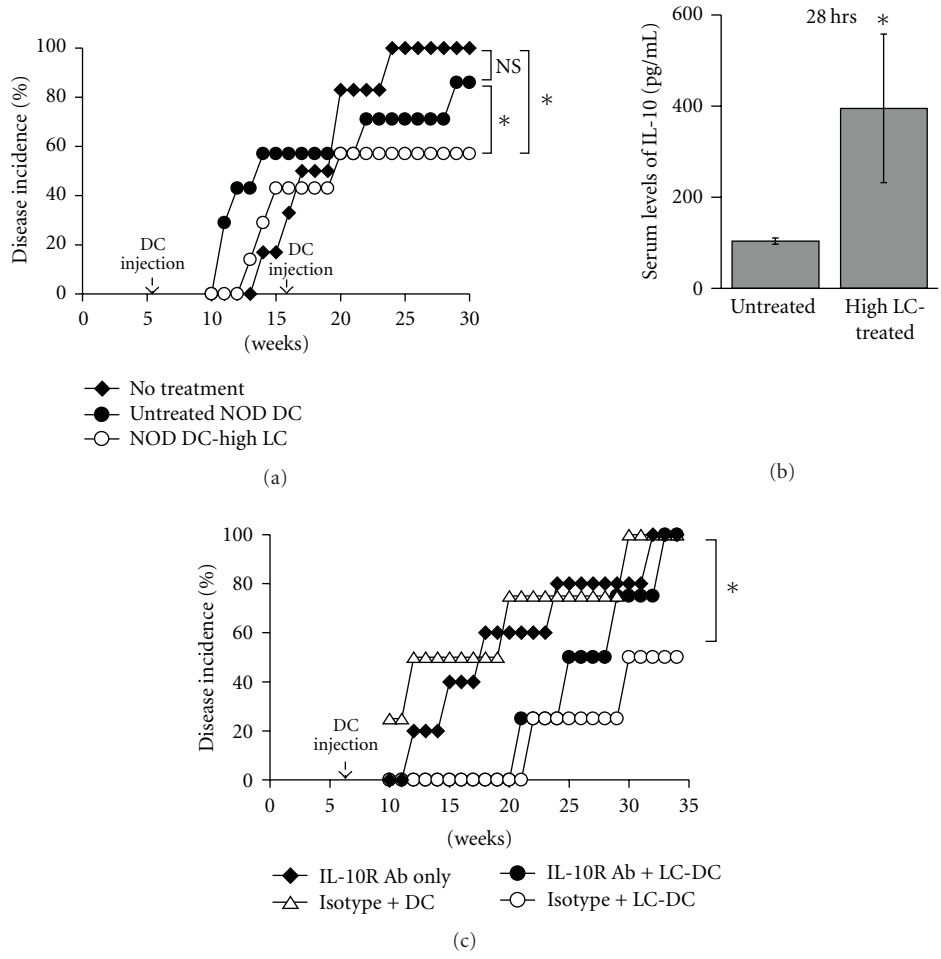

Figure 5: Injection of *Lactobacilli*-treated DCs into NOD mice confers some protection. Six-week-old NOD mice received two injections of 1×10^6 DCs treated with high dose (20×10^6 CFU/mL; NOD-high LC) of LC at 6 and 16 weeks of age ($n = 4$–6) and blood glucose was measured weekly and diabetes incidence determined (a). Sera from NOD mice uninjected or injected with DCs treated with high-dose LC (high LC) were collected at 28 hrs after injection, and tested for the presence of IL-10 by ELISA (b). Six-week-old NOD mice left uninjected or injected with 1×10^6 untreated DCs ($n = 4$-5) or DCs treated with high dose of LC ($n = 4$) received anti-IL-10R or isotype control antibodies, and blood glucose was measured weekly and diabetes incidence determined (c). *indicates a significant difference at $P < .03$.

predictor of treatment efficacy. We found that stimulation of NOD DCs with three different strains of *Lactobacilli* induced upregulation of costimulatory molecules, but had differential effects on cytokine production. *L. casei* induced production of higher levels of IL-10 while *L. reuteri* and *L. plantarum* induced higher levels of IL-12, corroborating previous findings showing that different strains of *Lactobacilli* differentially modulate expression of cytokines in DCs from B6 (diabetes-resistant) mice [37]. Injection of IL-10-producing NOD DCs stimulated with *L. casei* significantly decreased disease incidence. Surprisingly, NOD DCs treated with the TLR2 agonist, lipoteichoic acid (LTA) produced large amounts of IL-12 and accelerated diabetes onset and increased disease incidence upon transfer into NOD mice. These data indicate that NOD DCs can be manipulated to become tolerogenic, and that the cytokine profile elicited can predict the ability of these cells to prevent diabetes upon injection into NOD mice.

NOD DCs have been shown to express lower levels of costimulatory molecules, such as B7 [35], and in the current study, we found that stimulation with *Lactobacilli* restored normal levels of B7-1 and B7-2 expression on the surface of NOD DCs, suggesting that *Lactobacilli* can induce NOD DCs maturation. The upregulation of B7 on NOD DCs following *Lactobacilli* treatment is particularly significant, since B7 has been found to be important for regulation of diabetes [40], and regulatory cell development and homeostasis [41, 42]. On one hand, treatment of NOD mice with anti-B7-1 antibodies accelerated diabetes development, suggesting that B7-1 may be associated with induction of regulatory cells that control diabetes development [40]. On the other hand, treatment with anti-B7-2 antibodies prevented diabetes,

implicating B7-2 in disease pathogenesis [40]. There is a possibility that expression of B7-1 and B7-2 by different antigen-presenting cells play different and opposing roles. Expression of B7-1 on DCs may play a unique role in regulatory cell induction, since DCs are very potent activators of regulatory cells that can control diabetes [43, 44]. In contrast, expression of B7-2 on B cells may be very important for diabetes induction, since B cells have been shown to be crucial for diabetes development and are excellent inducers of IFNγ-producing T cells that respond to pancreatic antigens [45–47]. Expression of B7-2 on DCs may, therefore, not necessarily be associated with pathogenicity in NOD mice.

Furthermore, the cytokines produced by DCs may be more crucial in determining the status of the DCs in terms of their immunogenicity versus tolerogenicity despite the expression of costimulatory molecules. We have found that a single injection of LC-treated DCs is sufficient to affect diabetes development. Several studies have reported decrease in diabetes incidence following one injection of DCs whose cytokine profile was modulated to produce IL-10 and not IL-12 [48–50]. Since the lifespan of DCs, once injected *in vivo*, is around 12–15 days, it is safe to assume that these DCs may be able to promote long-term tolerance or immunity according to their cytokine production profiles. Indeed, IL-10-producing mature DCs expressing high levels of B7-1 and B7-2 have been shown to induce the development of Tr1 cells that can inhibit inflammation in a model of asthma [51]. A recent study has also shown that injection of GM-CSF into prediabetic NOD mice prevents diabetes development by inducing IL-10-producing tolerogenic DCs that sustain the suppressive function of $CD4^+CD25^+$ regulatory T cells [52]. Similarly, DCs harvested from the pancreatic LN of NOD mice do not produce IL-12 and are tolerogenic as reflected by the protection mediated by their transfer [48]. There is also a possibility that a regulatory Th2 response has been induced after transfer of *L. casei*-treated DCs as found previously after transfer of BM-DCs generated in the presence of GM-CSF/IL-4 [53] or splenic Flt-3L-derived DCs [50]. Conversely, we found that LTA-treated DCs produced much more IL-12 than IL-10, and without upregulating high expression of B7-1 or B7-2 accelerated diabetes when injected into NOD mice. Therefore, our data appear to show that the profile of cytokines produced by DCs may play a determinant role in their ability to induce a pathogenic versus a tolerogenic response.

The role that IL-10 plays in diabetes development in NOD mice is complex, since IL-10 appears to be involved in diabetes pathogenesis as well as diabetes prevention. On the one hand, there is evidence that IL-10 is involved at some level in diabetes pathogenesis. First, neutralization of IL-10 with anti-IL-10 antibodies effectively blocks insulitis development in NOD mice [54]. In addition, the incidence of diabetes is enhanced in transgenic IL-10-NOD mice expressing IL-10 in the glucagon-producing α-cells of the pancreas [55], and adoptive transfers of prediabetic or diabetic wild-type NOD splenocytes into these transgenic IL-10-NOD mice accelerates diabetes [56]. Interestingly, injection of IL-10-deficient NOD splenocytes into transgenic IL-10-NOD. SCID mice accelerates diabetes, demonstrating that pancreatic IL-10, not peripheral IL-10, appears to play a role in diabetes pathogenesis [56]. On the other hand, IL-10 appears to also play an important role in diabetes prevention. Intraperitoneal injection of a long-lived noncytolytic murine IL-10/Fc fusion protein into young NOD mice prevents insulitis and diabetes by blocking the production of proinflammatory cytokines and IFNγ [57], and IL-10-transduced islet-specific $CD4^+$ T-cells prevent diabetes transfer in NOD mice [58]. Furthermore, systemic delivery of IL-10 by intramuscular injection of expression plasmid DNA can prevent diabetes in NOD mice [59]. Finally, prevention of diabetes by injection of IL-10 expressing vector into NOD mice is associated with an increase in $CD4^+CD25^+$ regulatory T cells [60]. Taken together, these data, and our data suggest that timing, location, and concentration of IL-10 may determine whether IL-10 is protective or disease exacerbating. Our data suggest that IL-10 produced by *Lactobacilli*-treated NOD BM-DCs is involved in the protection observed in our model system and could be having a direct impact or acts via induction of regulatory T cells as shown previously [60, 61]. We attempted to block the protective effect induced by *Lactobacilli*-treated NOD DCs injected into NOD mice using anti-IL10R antibodies *in vivo*. There was a decrease in protection in mice treated with the anti-IL-10R antibodies when compared to mice treated with isotype control, confirming a role for IL-10 in diabetes protection mediated by injection of *Lactobacilli*-treated DCs. Although IL10-deficient NOD mice are not readily available, it would be interesting to test whether BM-DCs from IL-10-deficient NOD mice are capable of preventing diabetes, or whether feeding *Lactobacilli* to IL-10-deficient NOD mice can still affect diabetes development.

Lactobacilli have been shown to mediate their effects via TLR-2 [30, 62], possibly via Lipoteichoic acid (LTA), a TLR-2 agonist and one of the main immunostimulatory components of *Lactobacilli* bacteria. However, in contrast to the whole *Lactobacillus* organism, we have found that LTA isolated from *S. aureus* induced NOD BM-DCs to produce much more IL-12 than IL-10 and upregulated the expression of B7-1 and B7-2 on the surface of NOD BM-DCs. Consequently, NOD BM-DCs treated with LTA from *S. aureus* failed to protect NOD mice from diabetes and, in fact, appeared to exacerbate disease. This is not surprising since IL-12 have been reported to mediate disease development in NOD mice via activation of T cells producing IFNγ [2, 12, 13]. Analysis of mutant *L. plantarum* that exhibit altered teichoic acid biosynthesis indicates that alteration in LTA structure correlates with a change in its ability to induce proinflammatory versus anti-inflammatory responses [63]. Although we cannot rule out that components other than LTA may be involved in the induction of IL-10 by *Lactobacilli*, the predominant production of IL-12 versus IL-10 by NOD BM-DCs stimulated with LTA isolated from *S. aureus* could be due to differences in the structure of LTA by comparison to *Lactobacilli*, in general, and could also contribute to the differences in cytokine production between the different strains of *Lactobacilli* [10, 11]. It would be interesting to determine whether LTA isolated from *Lactobacilli* behave differently than LTA isolated from *S. aureus*.

5. Conclusions

Our data indicate that transfer of differentially stimulated DCs into NOD mice can accelerate or prevent diabetes development depending on the cytokine profile of the DCs. Depending on the strain of *Lactobacilli* and the dose, DCs can be induced to secrete anti-inflammatory cytokines that appear to mediate some diabetes protection. The specific molecular component from *Lactobacilli* that affords protection in NOD mice remains unknown. It will, therefore, be very important to determine the nature of the putative protective component(s) that preferentially induces tolerance versus inflammation, as well as the signalling pathways involved in this process. With this knowledge in hand, it should be possible to design more efficacious DCs-based therapies capable of completely preventing or treating disease. Furthermore, by using pancreatic antigens along with this strategy, it may be possible to generate tolerogenic DCs that are capable of inducing potent antigen-specific regulatory cells and, therefore, are more efficacious in preventing or treating disease.

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References

[1] L. Adorini, S. Gregori, J. Magram, and S. Trembleau, "The role of IL-12 in the pathogenesis of Th1 cell-mediated autoimmune diseases," *Annals of the New York Academy of Sciences*, vol. 795, pp. 208–215, 1996.

[2] H. S. Jun, C. S. Yoon, L. Zbytnuik, N. Van Rooijen, and J. I. W. Yoon, "The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice," *Journal of Experimental Medicine*, vol. 189, no. 2, pp. 347–358, 1999.

[3] D. G. Alleva, E. B. Johnson, J. Wilson, D. I. Beller, and P. J. Conlon, "SJL and NOD macrophages are uniquely characterized by genetically programmed, elevated expression of the IL-12(p40) gene, suggesting a conserved pathway for the induction of organ-specific autoimmunity," *Journal of Leukocyte Biology*, vol. 69, no. 3, pp. 440–448, 2001.

[4] D. G. Alleva, R. P. Pavlovich, C. Grant, S. B. Kaser, and D. I. Beller, "Aberrant macrophage cytokine production is a conserved feature among autoimmune-prone mouse strains: elevated interleukin (IL)-12 and an imbalance in tumor necrosis factor-α and IL-10 define a unique cytokine profile in macrophages from young nonobese diabetic mice," *Diabetes*, vol. 49, no. 7, pp. 1106–1115, 2000.

[5] J. Liu and D. Beller, "Aberrant production of IL-12 by macrophages from several autoimmune-prone mouse strains is characterized by intrinsic and unique patterns of NF-κB expression and binding to the IL-12 p40 promoter," *Journal of Immunology*, vol. 169, no. 1, pp. 581–586, 2002.

[6] B. Poligone, D. J. Weaver, P. Sen, A. S. Baldwin, and R. Tisch, "Elevated NF-κB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function," *Journal of Immunology*, vol. 168, no. 1, pp. 188–196, 2002.

[7] P. Sen, S. Bhattacharyya, M. Wallet et al., "NF-κB hyperactivation has differential effects on the APC function of nonobese diabetic mouse macrophages," *Journal of Immunology*, vol. 170, no. 4, pp. 1770–1780, 2003.

[8] D. J. Weaver, B. Poligone, T. Bui, U. M. Abdel-Motal, A. S. Baldwin, and R. Tisch, "Dendritic cells from nonobese diabetic mice exhibit a defect in NF-κB regulation due to a hyperactive IκB kinase," *Journal of Immunology*, vol. 167, no. 3, pp. 1461–1468, 2001.

[9] W. Wheat, R. Kupfer, D. Gutches et al., "Increased NF-κB activity in B cells and bone marrow-derived dentritic cells from NOD mice," *European Journal of Immunology*, vol. 34, no. 5, pp. 1395–1404, 2004.

[10] G. Morahan, D. Huang, S. I. Ymer et al., "Linkage disequilibrium of a type 1 diabetes susceptibility locus with a regulatory IL12B allele," *Nature Genetics*, vol. 27, no. 2, pp. 218–221, 2001.

[11] M. Szelachowska, A. Kretowski, and I. Kinalska, "Increased in vitro interleukin-12 production by peripheral blood in high-risk IDDM first degree relatives," *Hormone and Metabolic Research*, vol. 29, no. 4, pp. 168–171, 1997.

[12] S. Trembleau, G. Ferma, E. Bosi, A. Mortara, M. K. Gately, and L. Adorini, "Interleukin 12 administration induces T helper type 1 cells and accelerates autoimmune diabetes in NOD mice," *Journal of Experimental Medicine*, vol. 181, no. 2, pp. 817–821, 1995.

[13] S. Trembleau, G. Penna, S. Gregori, M. K. Gately, and L. Adorini, "Deviation of pancreas-infiltrating cells to Th2 by interleukin-12 antagonist administration inhibits autoimmune diabetes," *European Journal of Immunology*, vol. 27, no. 9, pp. 2330–2339, 1997.

[14] J. Lo and M. J. Clare-Salzler, "Dendritic cell subsets and type I diabetes: focus upon DC-based therapy," *Autoimmunity Reviews*, vol. 5, no. 6, pp. 419–423, 2006.

[15] F. Calcinaro, S. Dionisi, M. Marinaro et al., "Oral probiotic administration induces interleukin-10 production and prevents spontaneous autoimmune diabetes in the non-obese diabetic mouse," *Diabetologia*, vol. 48, no. 8, pp. 1565–1575, 2005.

[16] T. Matsuzaki, Y. Nagata, S. Kado et al., "Prevention of onset in an insulin-dependent diabetes mellitus model, NOD mice, by oral feeding of Lactobacillus casei," *APMIS*, vol. 105, no. 8, pp. 643–649, 1997.

[17] I. Kato, K. Endo-Tanaka, and T. Yokokura, "Suppressive effects of the oral administration of Lactobacillus casei on type ii collagen-induced arthritis in DBA/1 mice," *Life Sciences*, vol. 63, no. 8, pp. 635–644, 1998.

[18] K. L. Madsen, J. S. Doyle, L. D. Jewell, M. M. Tavernini, and R. N. Fedorak, "Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice," *Gastroenterology*, vol. 116, no. 5, pp. 1107–1114, 1999.

[19] M. Schultz, C. Veltkamp, L. A. Dieleman et al., "Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice," *Inflammatory Bowel Diseases*, vol. 8, no. 2, pp. 71–80, 2002.

[20] N. Borruel, F. Casellas, and F. Guarner, "Probiotics and inflammatory bowel diseaseProbióticos y enfermedad inflamatoria intestinal," *Gastroenterologia y Hepatologia*, vol. 25, no. 9, pp. 552–559, 2002.

[21] M. Kalliomäki, S. Salminen, H. Arvilommi, P. Kero, P. Koskinen, and E. Isolauri, "Probiotics in primary prevention

of atopic disease: a randomised placebo-controlled trial," *The Lancet*, vol. 357, no. 9262, pp. 1076–1079, 2001.

[22] T. B. H. Geijtenbeek, S. J. Van Vliet, E. A. Koppel et al., "Mycobacteria target DC-SIGN to suppress dendritic cell function," *Journal of Experimental Medicine*, vol. 197, no. 1, pp. 7–17, 2003.

[23] P. J. Ross, E. D. C. Lavelle, K. H. G. Mills, and A. P. Boyd, "Adenylate cyclase toxin from bordetella pertussis synergizes with lipopolysaccharide to promote Innate Interleukin-10 production and enhances the induction of Th2 and regulatory T cells," *Infection and Immunity*, vol. 72, no. 3, pp. 1568–1579, 2004.

[24] A. Sing, A. Roggenkamp, A. M. Geiger, and J. Heesemann, "Yersinia enterocolitica evasion of the host innate immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice," *Journal of Immunology*, vol. 168, no. 3, pp. 1315–1321, 2002.

[25] Y. Belkaid, K. F. Hoffmann, S. Mendez et al., "The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure," *Journal of Experimental Medicine*, vol. 194, no. 10, pp. 1497–1506, 2001.

[26] L. E. Layland, R. Rad, H. Wagner, and C. U. Prazeres da Costa, "Immunopathology in schistosomiasis is controlled by antigen-specific regulatory T cells primed in the presence of TLR2," *European Journal of Immunology*, vol. 37, no. 8, pp. 2174–2184, 2007.

[27] J. Madura Larsen, C. Stabell Benn, Y. Fillie, D. van der Kleij, P. Aaby, and M. Yazdanbakhsh, "BCG stimulated dendritic cells induce an interleukin-10 producing T-cell population with no T helper 1 or T helper 2 bias in vitro," *Immunology*, vol. 121, no. 2, pp. 276–282, 2007.

[28] A. S. McKee and E. J. Pearce, "$CD25^+CD4^+$ cells contribute to Th2 polarization during helminth infection by suppressing Th1 response development," *Journal of Immunology*, vol. 173, no. 2, pp. 1224–1231, 2004.

[29] S. Dillon, S. Agrawal, K. Banerjee et al., "Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance," *The Journal of Clinical Investigation*, vol. 116, no. 4, pp. 916–928, 2006.

[30] L. H. Zeuthen, L. N. Fink, and H. Frøkiær, "Toll-like receptor 2 and nucleotide-binding oligomerization domain-2 play divergent roles in the recognition of gut-derived lactobacilli and bifidobacteria in dendritic cells," *Immunology*, vol. 124, no. 4, pp. 489–502, 2008.

[31] S. Dillon, A. Agrawal, T. Van Dyke et al., "A Toll-Like receptor 2 ligand stimulates Th2 responses in vivo, via induction of extracellular signal-regulated Kinase mitogen-activated protein Kinase and c-Fos in dendritic cells," *Journal of Immunology*, vol. 172, no. 8, pp. 4733–4743, 2004.

[32] M. G. Netea, R. Sutmuller, C. Hermann et al., "Toll-Like Receptor 2 Suppresses Immunity against Candida albicans through Induction of IL-10 and regulatory T Cells," *Journal of Immunology*, vol. 172, no. 6, pp. 3712–3718, 2004.

[33] D. van der Kleij, E. Latz, J. F. H. M. Brouwers et al., "A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization," *The Journal of Biological Chemistry*, vol. 277, no. 50, pp. 48122–48129, 2002.

[34] S. Boudaly, J. Morin, R. Berthier, P. Marche, and C. Boitard, "Altered dendritic cells (DC) might be responsible for regulatory T cell imbalance and autoimmunity in nonobese diabetic (NOD) mice," *European Cytokine Network*, vol. 13, no. 1, pp. 29–37, 2002.

[35] E. Dahlén, G. Hedlund, and K. Dawe, "Low CD86 expression in the nonobese diabetic mouse results in the impairment of both T cell activation and CTLA-4 up-regulation," *Journal of Immunology*, vol. 164, no. 5, pp. 2444–2456, 2000.

[36] P. Marteau, P. Seksik, and R. Jian, "Probiotics and health: new facts and ideas," *Current Opinion in Biotechnology*, vol. 13, no. 5, pp. 486–489, 2002.

[37] H. R. Christensen, H. Frokiaer, and J. J. Pestka, "Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells," *Journal of Immunology*, vol. 168, no. 1, pp. 171–178, 2002.

[38] H. H. Smits, A. Engering, D. van der Kleij et al., "Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 6, pp. 1260–1267, 2005.

[39] E. Baharav, F. Mor, M. Halpern, and A. Weinberger, "Lactobacillus GG bacteria ameliorate arthritis in Lewis rats," *Journal of Nutrition*, vol. 134, no. 8, pp. 1964–1969, 2004.

[40] D. J. Lenschow, S. C. Ho, H. Sattar et al., "Differential effects of anti-B7-1 and anti-B7-2 monoclonal antibody treatment on the development of diabetes in the nonobese diabetic mouse," *Journal of Experimental Medicine*, vol. 181, no. 3, pp. 1145–1155, 1995.

[41] J. Lohr, B. Knoechel, S. Jiang, A. H. Sharpe, and A. K. Abbas, "The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens," *Nature Immunology*, vol. 4, no. 7, pp. 664–669, 2003.

[42] Q. Tang, K. J. Henriksen, E. K. Boden et al., "Cutting edge: CD28 controls peripheral homeostasis of $CD4^+CD25^+$ regulatory T cells," *Journal of Immunology*, vol. 171, no. 7, pp. 3348–3352, 2003.

[43] S. Yamazaki, T. Iyoda, K. Tarbell et al., "Direct expansion of functional $CD25^+CD4^+$ regulatory T cells by antigen-processing dendritic cells," *Journal of Experimental Medicine*, vol. 198, no. 2, pp. 235–247, 2003.

[44] K. V. Tarbell, S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman, "$CD25^+CD4^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1467–1477, 2004.

[45] M. Falcone, J. Lee, G. Patstone, B. Yeung, and N. Sarvetnick, "B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice," *Journal of Immunology*, vol. 161, no. 3, pp. 1163–1168, 1998.

[46] A. M. Marleau, K. L. Summers, and B. Singh, "Differential contributions of APC subsets to t cell activation in nonobese diabetic mice," *Journal of Immunology*, vol. 180, no. 8, pp. 5235–5249, 2008.

[47] P. A. Silveira, E. Johnson, H. D. Chapman, T. Bui, R. M. Tisch, and D. V. Serreze, "The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors," *European Journal of Immunology*, vol. 32, no. 12, pp. 3657–3666, 2002.

[48] M. J. Clare-Salzler, J. Brooks, A. Chai, K. Van Herle, and C. Anderson, "Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer," *The Journal of Clinical Investigation*, vol. 90, no. 3, pp. 741–748, 1992.

[49] L. Ma, S. Qian, X. Liang et al., "Prevention of diabetes in NOD mice by administration of dendritic cells deficient in nuclear transcription factor-κB activity," *Diabetes*, vol. 52, no. 8, pp. 1976–1985, 2003.

[50] J. Morin, B. Faideau, M. C. Gagnerault, F. Lepault, C. Boitard, and S. Boudaly, "Passive transfer of flt-3L-derived dendritic cells delays diabetes development in NOD mice and associates with early production of interleukin (IL)-4 and IL-10 in the spleen of recipient mice," *Clinical and Experimental Immunology*, vol. 134, no. 3, pp. 388–395, 2003.

[51] O. Akbari, R. H. DeKruyff, and D. T. Umetsu, "Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen," *Nature Immunology*, vol. 2, no. 8, pp. 725–731, 2001.

[52] S. Gaudreau, C. Guindi, M. Ménard, G. Besin, G. Dupuis, and A. Amrani, "Granulocyte-macrophage colony-stimulating factor prevents diabetes development in NOD mice by inducing tolerogenic dendritic cells that sustain the suppressive function of $CD4^+CD25^+$ regulatory T cells," *Journal of Immunology*, vol. 179, no. 6, pp. 3638–3647, 2007.

[53] M. Feili-Hariri, D. H. Falkner, and P. A. Morel, "Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice," *European Journal of Immunology*, vol. 32, no. 7, pp. 2021–2030, 2002.

[54] M. S. Lee, R. Mueller, L. S. Wicker, L. B. Peterson, and N. Sarvetnick, "IL-10 is necessary and sufficient for autoimmune diabetes in conjunction with NOD MHC homozygosity," *Journal of Experimental Medicine*, vol. 183, no. 6, pp. 2663–2668, 1996.

[55] M. Moritani, K. Yoshimoto, F. Tashiro et al., "Transgenic expression of IL-10 in pancreatic islet A cells accelerates autoimmune insulitis and diabetes in non-obese diabetic mice," *International Immunology*, vol. 6, no. 12, pp. 1927–1936, 1994.

[56] G. Rajagopalan, Y. C. Kudva, M. M. Sen et al., "IL-10-deficiency unmasks unique immune system defects and reveals differential regulation of organ-specific autoimmunity in non-obese diabetic mice," *Cytokine*, vol. 34, no. 1-2, pp. 85–95, 2006.

[57] X. X. Zheng, A. W. Steele, W. W. Hancock et al., "A noncytolytic IL-10/Fc fusion protein prevents diabetes, blocks autoimmunity, and promotes suppressor phenomena in NOD mice," *Journal of Immunology*, vol. 158, no. 9, pp. 4507–4513, 1997.

[58] M. Moritani, K. Yoshimoto, S. Ii et al., "Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes: a gene therapy model for autoimmune diabetes," *The Journal of Clinical Investigation*, vol. 98, no. 8, pp. 1851–1859, 1996.

[59] Y. Nitta, F. Tashiro, M. Tokui et al., "Systemic delivery of interleukin 10 by intramuscular injection of expression plasmid DNA prevents autoimmune diabetes in nonobese diabetic mice," *Human Gene Therapy*, vol. 9, no. 12, pp. 1701–1707, 1998.

[60] K. S. Goudy, B. R. Burkhardt, C. Wasserfall et al., "Systemic overexpression of IL-10 induces $CD4^+CD25^+$ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion," *Journal of Immunology*, vol. 171, no. 5, pp. 2270–2278, 2003.

[61] Y. C. Zhang, A. Pileggi, A. Agarwal et al., "Adeno-associated virus-mediated IL-10 gene therapy inhibits diabetes recurrence in syngeneic islet cell transplantation of NOD mice," *Diabetes*, vol. 52, no. 3, pp. 708–716, 2003.

[62] C. Maldonado Galdeano and G. Perdigón, "The probiotic bacterium Lactobacillus casei induces activation of the gut mucosal immune system through innate immunity," *Clinical and Vaccine Immunology*, vol. 13, no. 2, pp. 219–226, 2006.

[63] C. Grangette, S. Nutten, E. Palumbo et al., "Enhanced antiinflammatory capacity of a Lactobacillus plantarum mutant synthesizing modified teichoic acids," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 29, pp. 10321–10326, 2005.

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Research Article

Protection against Autoimmune Diabetes by Silkworm-Produced GFP-Tagged CTB-Insulin Fusion Protein

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In animals, oral administration of the cholera toxin B (CTB) subunit conjugated to the autoantigen insulin enhances the specific immune-unresponsive state. This is called oral tolerance and is capable of suppressing autoimmune type 1 diabetes (T1D). However, the process by which the CTB-insulin (CTB-INS) protein works as a therapy for T1D *in vivo* remains unclear. Here, we successfully expressed a green fluorescent protein- (GFP-) tagged CTB-Ins (CTB-Ins-GFP) fusion protein in silkworms in a pentameric form that retained the native ability to activate the mechanism. Oral administration of the CTB-Ins-GFP protein induced special tolerance, delayed the development of diabetic symptoms, and suppressed T1D onset in nonobese diabetic (NOD) mice. Moreover, it increased the numbers of $CD4^+CD25^+Foxp3^+$ T regulatory (Treg) cells in peripheral lymph tissues and affected the biological activity of spleen cells. This study demonstrated that the CTB-Ins-GFP protein produced in silkworms acted as an oral protein vaccine, inducing immunological tolerance involving $CD4^+CD25^+Foxp3^+$ Treg cells in treating T1D.

1. Introduction

Oral tolerance refers to the physiological response of an organism remaining in a state of specific immunological unresponsiveness to some food antigens that is orally administered [1]. Data indicate that this can be used to treat autoimmune diseases, like type 1 diabetes (T1D), which has specific postulated autoantigens, such as insulin and glutamic acid decarboxylase 65 [2–5]. Indeed, in animal models, oral administration of tissue-specific antigen insulin was able to prevent T1D [6]. However, a major problem remains to be solved if oral tolerance is employed to treat human autoimmune diseases: how to obtain sufficient amounts of autoantigens for repeated oral administration because humans have an enormous intestinal absorptive surface area. Thus, the effective use of oral tolerance to treat autoimmune diseases may depend on the use of mucosal adjuvants to enhance efficacy.

As a mucosal carrier, the nontoxic cholera toxin B subunit (CTB) is conjugated to autoantigens for the induction of oral tolerance [7]. It has also been demonstrated in similar systems [8–10] that conjugating antigen to CTB can increase efficiency and thus reduce effective antigen doses. The therapeutic applications of CTB-mediated oral tolerance, as demonstrated in animal models, include the prevention and treatment of T-cell-mediated autoimmune diabetes [11].

The intestinal mucosa is the central location for the induction of oral tolerance [12]. Orally administrated, cholera toxin (CT), a potent mucosal modulator, induces the appearance of cells that share some common traits with Peyer's patch (PP) M-cells in mice [13]. Glycoprotein 2 on M-cells serves as a transcytotic receptor for mucosal antigens [14]. Additionally, it has been shown that dendritic cells (DCs) in the gut-associated lymphoid tissue (GALT) take up antigen and transfer it to T cells to generate Treg cells in oral tolerance-treated arthritic mice [15]. Sublingual administration of CTB-conjugated antigen to B-cell-deficient mice sharply reduced $CD4^+CD25^+Foxp3^+$ Treg cells compared with the wild type [16, 17]. Together,

Table 1: Five primers synthesized for the construction of the fusion proteins.

No.	Sequence	Length
P1	5′-CG*GGATCC*ATGATTAAATTAAAATTTGG-3′ (BamHI)	28 bp
P2	5′-**GGGGCCGGGGCC**GTTGCAGTAGTTCTCCA-3′ (GPGP)	29 bp
P3	5′-**GGGGCCGGGGCC**ATTTGCCATACTAAT-3′ (GPGP)	27 bp
P4	5′-**GGCCCCCGGCCCC**GTGAGCAAGGGCGAGGA-3′ (GPGP)	29 bp
P5	5′-TG*CTCGAG*TTACTTGTACAGCTCGTCC-3′ (XhoI)	27 bp

The italics in P1 and P5 indicate BamHI and XhoI restriction endonuclease sites, respectively. Bold type indicates the linker peptide (GPGP).

these findings suggest that the regulatory T cells elicited by mucosal immunization with CTB conjugated peptides are unique and distinct from those that arise from spontaneous endogenous priming. This protein vaccine therapy may be useful in treating autoimmune diseases.

T1D is a spontaneous autoimmune disease associated with the pancreas in which damage to the insulin-producing β-cells disturbs glucose metabolism and leads to a series of complications [18]. The autoantigen insulin conjugated to CTB produced in silkworms can induce oral tolerance, protecting against T1D in NOD mice [19]. Using the silkworm as a "bioreactor" to obtain large amounts of proteins through the insect baculovirus expression system (BES) has many merits [20, 21]. As the green fluorescent protein (GFP) is widely used to reveal intestinal epithelial immunoreaction by tagging antibodies or microorganisms [13, 14], we expressed a GFP-tagged CTB-Ins (CTB-Ins-GFP) protein by a Bac-to-Bac silkworm BES in this study. We found that the CTB-Ins-GFP fusion protein was efficiently produced in both BmN cells and silkworms and possessed the functional characteristics of native CTB. Oral administration of the protein was capable of inducing specific immune tolerance, delaying diabetes symptoms, and suppressing T1D onset in NOD mice. The specific tolerance could increase the $Foxp3^+$ regulatory T cell proportions in peripheral lymph tissues and suppress the biological functions of spleen lymphocytes in mice. This silkworm-produced active CTB-Ins-GFP protein, administered as a vaccine protein, was able to induce insulin specific oral tolerance, which is related to increased Treg cells in the treatment of T1D.

2. Material and Methods

2.1. Reagents, Cell Lines, Silkworms, and Mice. DNA manipulation and PCR amplification kits were purchased from TaKaRa Biomedicals (Japan). The rabbit anticholera toxin primary serum, bacterial CTB peptides, and monosialoganglioside-GM1 were from Sigma-Aldrich (USA). A mouse regulatory T-cell staining kit was obtained from eBioscience (USA). Transwell chambers ($\varnothing = 8\,\mu$m) were purchased from Corning Incorporated (USA). The silkworm BmN cells were cultured in TC-100 medium (Gibco-BRL, USA) containing 10% fetal calf serum (Gibco-BRL, USA) and 50 μg/mL gentamycin at 27°C. Fifth-instar silkworm *B. mori* larvae (Jingsong × Haoyue, Showa) were fed fresh mulberry leaves and reared under a photoperiod schedule of 12-h light and 12-h darkness at 25 ± 1°C. Female nonobese diabetic (NOD) mice and NOD severe combined immunodeficient (NOD/SCID) mice were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (SLAC, CAS, China), and housed at the central animal facility, where they were screened for bacterial and viral pathogens.

2.2. Construction of Recombinant Bacmid Vectors. Five primers were designed prior to the construction of the CTB-GFP and CTB-Ins-GFP fusion genes (Table 1). Using the recombinant PAK-CTB-INS plasmid and the pEGFP plasmid [22] as templates, the CTB-GFP and CTB-Ins-GFP fusion genes were amplified by splice overlap extension PCR (SOE-PCR), identified, and subcloned into the donor pFastBac1 plasmid. Then, the recombinant bacmid vectors were constructed and verified by PCR identification and fragment sequencing.

2.3. Acquisition of Recombinant Baculovirus in BmN Cells. A subconfluent monolayer of BmN cells was transfected with the recombinant bacmid vectors using Lipofectamine 2000 (Invitrogen, USA). The recombinant virus was generated in the transfected BmN cells after 3–5 days and verified by detection of green fluorescent light under a fluorescent microscope (Nikon, Japan). Viral genomic DNA was then extracted using the Wizard genomic DNA purification kit (Promega, USA) and identified by PCR amplification and fragment sequencing. Prior to the next viral infection for fusion protein expression, the dilution of the recombinant virus was calculated using the Reed-Muench method.

2.4. Expression and Collection of the Fusion Proteins. BmN cells (4×10^6) were infected by BmNPV CTB-GFP and BmNPV CTB-Ins-GFP at MOI = 10 and collected at 2–7 days after infection. Harvested BmN cells were suspended in 0.5 mL phosphate-buffered Saline (PBS) and lysed by gentle sonication several times on ice. They were centrifuged and the supernatant removed and stored at −20°C. The fifth-instar silkworm larvae were needle inoculated with the viral solutions (1×10^7 pfu/mL) into their body cavities. The hemolymph of the larvae were collected at 2–7 days after inoculation, centrifuged to remove insoluble impurities, and stored at −20°C.

2.5. Western Blot and ELISA Assay. To detect the expression of monomeric or pentameric fusion proteins, the cell-lysed supernatants or hemolymph samples were diluted and separated by 12% SDS-PAGE. Samples were either boiled or loaded directly on the gel. The separated protein bands were

then transferred to a nitrocellulose (NC) filter membrane. Detection of the immunoreaction was performed with an enhanced chemiluminescence (ECL) Western blotting kit (Biologicacl Industries, Israel). Rabbit anticholera toxin antiserum (Sigma, USA) and rabbit anti-GFP primary antibody (Epitomics, USA) were used for the immunoreactions.

A semiquantitative ELISA was used to investigate the expression level of the fusion protein. A 96-well microtiter plate was loaded with dilutions of the cell-lysed supernatant (1 : 20–1 : 100) or hemolymph (1 : 500–1 : 2000) in bicarbonate buffer, pH 9.6 (15 mM Na_2CO_3, 35 mM $NaHCO_3$) at 4°C overnight. The plate was blocked with 1% BSA at 37°C for 1 h and then washed with PBS containing 0.05% Tween-20 (PBST). A 1 : 5000 dilution of anticholera toxin antiserum in 1% BSA was added at 37°C for 2 h. It was then washed with PBST and incubated with a 1 : 10000 dilution of antirabbit IgG conjugated with horseradish peroxidase at 37°C for 40 min. Finally, the chromogenic substrate O-phenylenediamine was added at 37°C for 30 min to develop color and 2 M H_2SO_4 (50 μL/well) was added to stop the reaction. The absorbance at 492 nm was measured in a Labsystems Multiscan MS ELISA plate reader (Labsystems, Finland). Serial dilutions of bacterial CTB (Sigma, USA) were used to generate the standard curve to calculate the results.

2.6. GM1 Ganglioside-Binding Assay. A GM1-ELISA was performed to detect the affinity of silkworm-derived fusion proteins for GM1 ganglioside. The microtiter plates were coated with monosialoganglioside-GM1 (Sigma, USA) by incubating the plates with 50 μL/well of GM1 in methanol at 4°C overnight. The wells were then blocked with BSA solution, and the dilutions of hemolymph were added. The remainder of the procedure was identical to the semiquantitative ELISA assay described above. The same dilutions of normal hemolymph and serial dilutions of bacterial CTB were used as a negative control and to generate the standard curve, respectively.

2.7. Induction of Oral Tolerance. Five-week-old female NOD mice were divided into four groups and fed with Saline, hemolymph synthesized CTB-GFP protein, CTB-Ins-GFP protein, or CTB-INS protein. Beginning at 5 weeks of age, the mice were fed an equal amount of hemolymph (about 100–300 μL) every other day until 10 weeks of age. Each feeding of hemolymph delivered approximately 50 μg of the corresponding fusion proteins. The animals were sacrificed at 10 weeks of age for antibody titer assays and pancreatic islet histopathological analysis.

2.8. Pancreatic Islet Histopathological Analysis. To evaluate insulitis in experimental NOD mice, the extent of lymphocyte infiltration in the islets was measured. At 10 weeks of age, six mice in each group were sacrificed and the pancreas removed. Each pancreas was fixed in PBS-buffered paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin. The degree of insulitis was evaluated using a standardized scoring system with two independent observers using a semiquantitative scale ranging from 0 to 4: 0, normal islet with no sign of T-cell infiltration, 1, focal peri-islet T-cell infiltration, 2, more extensive peri-islet infiltration but with lymphocytes less than one-third of the islet area, 3, intraislet T-cell infiltration in one-third to one-half of the islet area, and 4, extensive intraislet inflammation involving more than half of the islet area. At least 20 islets were scored for each animal.

2.9. Assessment of Diabetes. Incidences of diabetes were compared among mice fed the CTB-INS, CTB-Ins-GFP, or CTB-GFP fusion proteins or Saline. The feeding schedule was the same as mentioned above and continued for 30 weeks. Starting at 10 weeks of age, the development of diabetes was monitored and confirmed by measuring blood glucose using the ACCU-CHEK III system (Roche Diagnostics Ltd., Shanghai, China). A mouse with a blood glucose level above 16.7 mM for 2 consecutive weeks was considered diabetic.

2.10. Gut Mucosal Binding Assay. Mice were anesthetized with ether and kept warm on a 37°C warming pad during the assay. Silkworm-derived CTB-GFP or CTB-Ins-GFP fusion proteins were injected into the ligated intestinal loops. After incubation for 30–60 min, the mice were sacrificed and part of the intestinal loops (about 5 mm) was excised [14]. After washing, 8 μm frozen sections of the specimens were prepared. The locations of the proteins were verified by fluorescent microscopy.

2.11. Quantification of Serum Antibody Subtypes. Treated NOD mouse serum was quantified for anti-CTB and anti-insulin antibody subtype using ELISA. Human insulin (Novo Nordisk) or bacterial CTB were used as the well-coating antigens and after blocking, serial dilutions of pooled sera were added to the coated microtiter plate wells. Horseradish peroxidase-conjugated antimouse IgG1, IgG2a, IgA, or IgE antibody (SABC, China) was used as the secondary antibody. The absorption value was measured as described above. The titer was defined as the reciprocal of the highest dilution of the sample giving an absorption signal above background, and was individually determined for each sample.

2.12. Cytometric Analysis of Treg Cell Flow. Lymphocytes in the spleen, intestinal lymph nodes, and blood of 10-week-old treated NOD mice were obtained with the help of mouse lymphocyte separation medium (Dakewe Biotech Company, Limited, China). Then, the assay was performed with a mouse regulatory T-cell staining kit, which contains FITC-antiCD4 antibody, PE-antiCD25 antibody, and PE-cy5-antiFoxp3 antibody, according to the manufacturer's protocol. Proportions of Treg cells in the lymph tissues were analyzed by flow cytometric analysis of lymphocyte phenotypic markers with a FACScan flow cytometer (Beckman Coulter, USA).

2.13. Spleen Lymphocyte BrdU Label Analysis. Spleen lymphocytes were cultured with 10 μM 5-bromo-2-deoxyuridine for 1 day to label the proliferated cells as in a previous study [23]. They were then fixed and treated with Triton X-100 and H_2O_2, denatured with HCl, and neutralized with

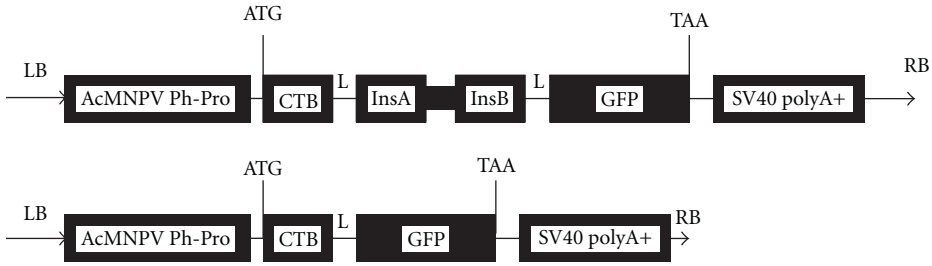

Figure 1: Schematic structure of the CTB-Ins-GFP fusion gene. AcMNPV Ph-Pro: *Autographa californica* multiple nuclear polyhedrosis virus polyhedrin promoter, CTB: cholera toxin B subunit, L: linker peptide (GPGP), InsA: human insulin subunit A, InsB: human insulin subunit B (InsA and InsB are linked by mini-C peptide (RRGSKR)), GFP: green fluorescent protein, and LB and RB: left and right border, respectively, of the donor vector pFastBac1.

boric acid, all at room temperature. Following blocking, the cells were treated with mouse anti-BrdU antibody (Boster, China) then incubated with biotin-labeled antimouse IgG antibody. Next, ABC compound was added to the cells. DAB was used to develop the corresponding brown color with hematoxylin used as a counterstain. Samples were analyzed using a microscope at 400x.

2.14. Spleen Lymphocyte Transwell Assay. Spleen lymphocytes (1×10^5) were isolated from NOD mice, and cultured in the upper chamber of 24-well Transwell plates with RPMI 1640 serum-free medium. IL2 was added in the normal culture medium (150 IU/mL) and placed in the bottom chamber and the cells were cultured for about 14 h at 37°C with 5% CO_2. Next, the cells were fixed and stained with crystal violet solution after the cells upon the chamber were clear. Samples were analyzed using a microscope at 400x.

2.15. Adoptive Transfer of Diabetes. Splenocytes from 10-week-old NOD mice fed CTB-Ins-GFP fusion proteins were assessed for the capacity to suppress the diabetogenic activity of splenocytes taken from an acutely diabetic donor. Briefly, splenocytes (1×10^7 cells) from the three treated groups, but not Saline-fed NOD mice, were mixed with splenocytes (1×10^7 cells) from newly diabetic NOD mice and given by intravenous injection (i.v.) into the tail veins of 6–8-week-old NOD/SCID mice. Mice receiving only 1×10^7 diabetic splenocytes were used as a control. Recipient mice were monitored for the development of diabetes for 15 weeks.

2.16. Statistical Analysis. Survival analyses with Kaplan-Meier estimates were used to evaluate the difference in diabetes incidence among the NOD mice groups. Results were otherwise analyzed by using *t*-tests or ANOVA for multiple comparisons. A P value of less than .05 was considered to indicate statistical significance in all cases.

3. Results

3.1. Construction and Identification of the Recombinant BmNPV CTB-Ins-GFP. Using pEGFP, PAK-CTB-INS, and five primers, we obtained the GFP, CTB, and CTB-Ins genes by traditional PCR. Using the SOE-PCR method, the GFP gene was anchored separately at the 3′-end of the CTB and CTB-Ins genes to form the CTB-GFP and CTB-Ins-GFP fusion genes, respectively. A flexible hinge tetrapeptide (GPGP) was introduced between the two peptides to maintain the natural conformation of CTB/CTB-Ins and GFP (Figure 1). The segments were confirmed by autosequencing and then subcloned into the pFastBac1 vector. Results of the enzyme digestion, PCR amplification, full-length nucleotide sequencing, and deduced amino acid sequences showed the fusion genes were exactly inserted into the pFastBac1 vector (data not shown). Then, they were transformed into the *E. coli* host strain DH10α (competent cells) to generate recombinant bacmid vectors. PCR results showed that the recombinant bacmid vectors were successfully constructed (data not shown). The BmN cells were transfected by the recombinant bacmid vectors to obtain recombinant viruses. Viral genomic DNA was extracted and recombined by PCR amplification (data not shown). According to the Reed-Muench formula, the dilutions of the recombinant viruses were 1.17×10^9 for BmNPV CTB-Ins-GFP and 5.62×10^9 for BmNPV CTB-GFP (data not shown).

3.2. High Expression of the CTB-Ins-GFP Recombinant Protein. When infected with the recombinant virus, BmN cells and silkworm larvae showed virus infection symptoms and started to produce CTB-GFP and CTB-Ins-GFP fusion proteins in the cytoplasm and body cavity, respectively (Figure 2(a)). The presence of the proteins was determined by Western blot analysis with anti-CTB or -GFP antibodies (Figure 2(b)). We found that the oligomeric fusion proteins (CTB-GFP protein ~195 kDa; CTB-Ins-GFP protein ~240 kDa) were dissociated into monomers by boiling and subsequently migrated as a specific band with an apparent molecular weight (CTB-GFP protein ~39 kDa; CTB-Ins-GFP protein ~48 kDa), with normal samples as negative controls. No immunospecific signal corresponded in molecular mass to the CTB-GFP or CTB-Ins-GFP fusion proteins in normal cells or normal hemolymph samples. Moreover, the ELISA results showed that the highest detectable level of the CTB-GFP and CTB-Ins-GFP fusion proteins yielded by BmN cells was 0.037 mg/2×10^6 cells on the 6th day after infection (Figure 2(c)). In the infected silkworm larvae, the recombinant fusion proteins were efficiently expressed

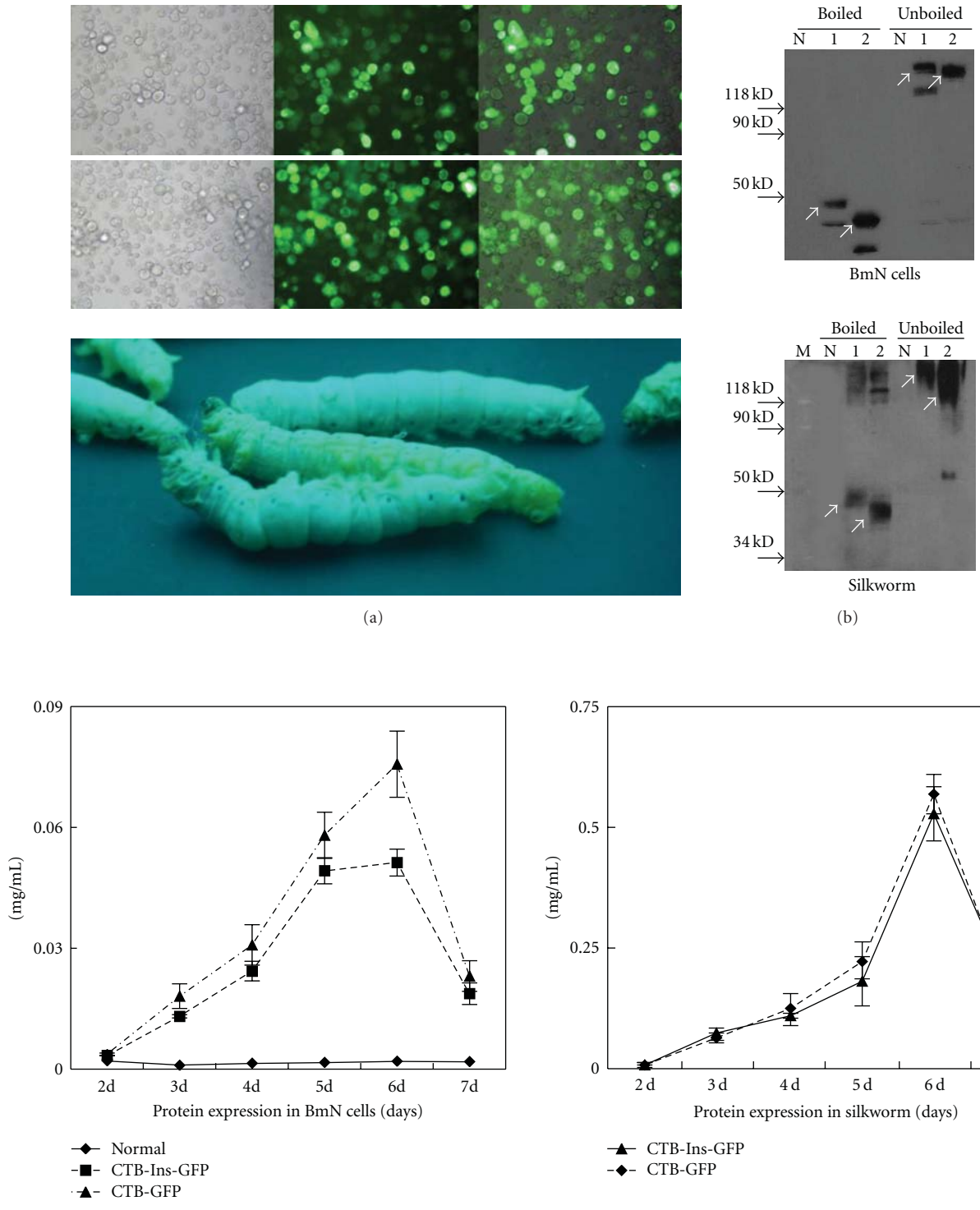

Figure 2: Identification of the expressed fusion proteins. (a) Fusion protein expression in BmN cells and silkworm *B. mori* fifth-instar larvae. Left to right: BmNPV CTB-GFP or BmNPV CTB-Ins-GFP 5 days after infection under normal light, blue light, and overlapped. Magnification, 200x. Lower: *B. mori* fifth-instar normal larvae and larvae infected with recombinant viruses 5 days after infection under ultraviolet radiation. (b) Western blot analysis of fusion protein expression. Lane N: control, lane 1: CTB-Ins-GFP protein, lane 2: CTB-GFP protein. GFP antibody was used as the primary antibody. Silkworm: lane M: molecular weight standard, lane N: normal sample, lane 1: CTB-Ins-GFP protein, lane 2: CTB-GFP protein. Anti-CTB serum was used as the primary antibody. The arrows denote the pentamers (~240, 195 kDa) and monomers (~48, 39 kDa) of the CTB-Ins-GFP and CTB-GFP proteins. (c) ELISA semiquantification analysis of protein expression. Concentrations on days after infection were calculated according to the standard curve of bacterial CTB. Data are presented as the mean concentration ± SD on each day. The experiment was repeated twice.

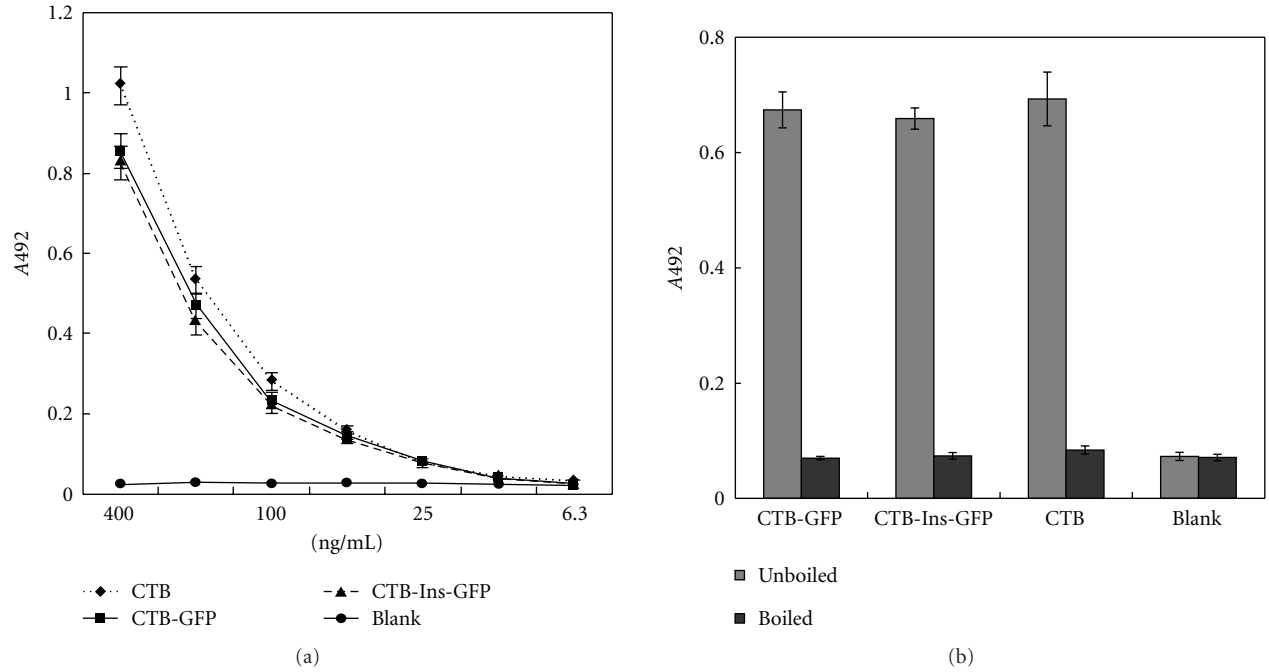

Figure 3: GM1 affinity assay. (a) Reactivity of the CTB-Ins-GFP protein with the GM1 ganglioside and a native bacterial CTB control. (b) Boiling induced pentamer dissociation into monomers. Approximately equal amounts of the three different samples indicated were used to measure A492 signal levels. Data represent the mean A492 values ± SD of each sample. The experiment was repeated twice.

and released into the larval hemolymph. On the 6th day after infection, the maximum amount of fusion protein in the hemolymph reached 0.58 mg/mL with an average of 0.4 mL hemolymph per larva (Figure 2(c)). The results demonstrated that the silkworm is a powerful "bioreactor" for protein production.

3.3. Strong Affinity of the Recombinant Protein for the GM1 Ganglioside. The CTB-based protein in its natural conformation forms a pentameric structure and possesses an affinity for the GM1 ganglioside, so we used the GM1-ELISA method with GM1 ganglioside as the capture molecule to confirm the specific affinity of the fusion protein. An increase in the concentration-specific absorption signal was observed in contrast to the bacterial pentameric CTB standard curve, indicating that the recombinant protein existed as pentamers, because only pentameric protein binds to GM1 ganglioside (Figure 3(a)). However, the heat-treated protein lost its affinity for GM1 ganglioside (Figure 3(b)). The silkworm-derived recombinant protein exhibited the biochemical and antigenic properties of CTB necessary for the next study. The GM1-binding ability also suggested proper folding of the CTB-GFP and CTB-Ins-GFP molecules, resulting in a functional pentameric structure.

3.4. Feeding of CTB-Ins-GFP Protein Reduces Insulitis and Suppresses Diabetes. To investigate the impact on insulitis as a result of feeding CTB-Ins-GFP protein to NOD mice, 5-week-old female NOD mice were given larval hemolymph containing recombinant CTB-GFP or CTB-Ins-GFP proteins or Saline, with a group fed hemolymph containing CTB-INS as a positive control, until 10 weeks. Then, their pancreatic tissues were collected and the islets individually scored for insulitis. Representative pancreatic islets from animals fed CTB-Ins-GFP or CTB-INS fusion proteins had insulitis scores of 1 and 2 (Figure 4(a)(2), (3)), whereas animals fed CTB-GFP or Saline had insulitis scores of 3 and 4 (Figure 4(a)(4)) with normal mice as a negative control (Figure 4(a)(1)). The ANOVA test revealed a significant reduction of insulitis in mice fed CTB-Ins-GFP or CTB-INS in contrast to the CTB-GFP or Saline groups (1.1 ± 0.3 and 1.4 ± 0.3 versus 2.9 ± 0.5 and 3.1 ± 0.6, resp.; $P < .05$; Figure 4(b)). No significant difference in insulitis scores was found in mice fed CTB-GFP or Saline. These results showed the CTB-Ins-GFP protein synthesized by the silkworm effectively suppressed insulitis.

We then investigated whether long-term oral administration of the CTB-Ins-GFP fusion protein could prevent diabetes. Prediabetic female NOD mice were orally administered the CTB-Ins-GFP fusion protein from 5 to 35 weeks of age with a CTB-INS protein-fed group as a control. In the early stages, mice in all groups had normal appetites and weight gains, and no apparent difference was observed (data not shown). Then, the mice were found to have a decrease in body weight and an increase in appetite (data not shown). At 35 weeks of age, only 39% and 33% (7/18 and 6/18) of the NOD mice that received CTB-INS (group 1) or CTB-Ins-GFP (group 2) fusion protein developed diabetes compared with 67% and 70% (12/18 and 7/10) of those fed the CTB-GFP peptide (group 3) or Saline (group 4), respectively (groups 1 and 2 versus groups 3 and 4, $P = .0276$; group 1 versus group 2, $P = .9683$; Figure 5). Although this result

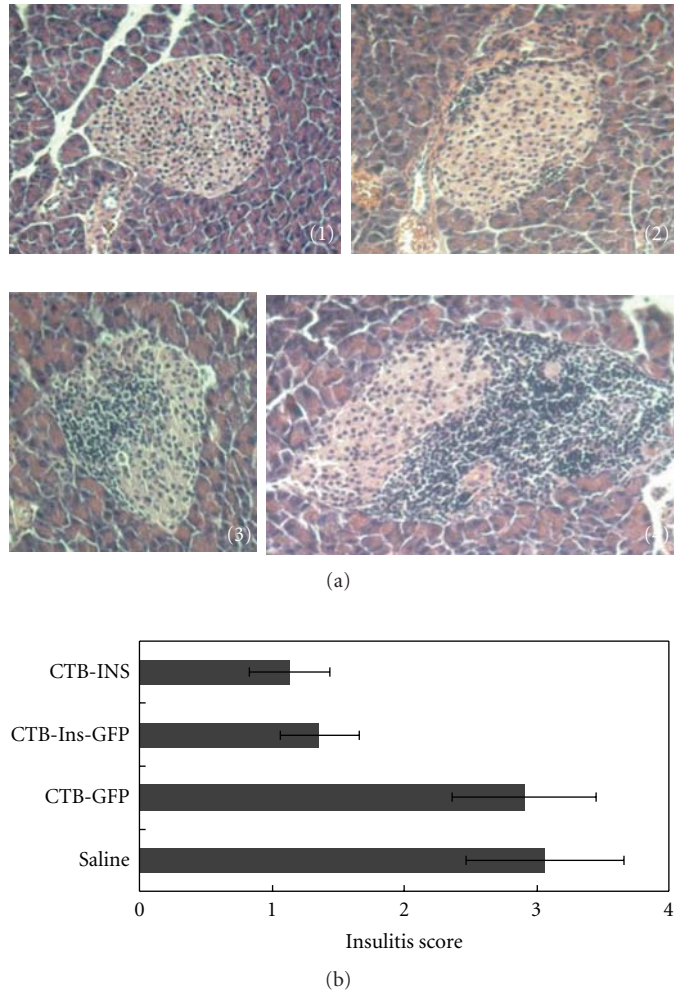

Figure 4: Insulitis score analysis. (a) Representative histopathological pancreatic islets from normal mice or experimental NOD mice. (b) Semiquantitative analysis of pancreatic islet insulitis score. Data are expressed as the mean score for each group ± SD. The ANOVA test revealed a significant reduction in insulitis in mice fed CTB-INS or CTB-Ins-GFP in contrast to the CTB-GFP or Saline group (1.1 ± 0.3 and 1.4 ± 0.3 versus 2.9 ± 0.5 and 3.1 ± 0.6, resp., $P < .05$). No significant difference in insulitis score was found in mice fed either CTB-GFP or Saline. Six mice per group were tested in two separate experiments.

showed that feeding CTB-Ins-GFP fusion protein might not totally suppress diabetes development in NOD mice, the incidence of diabetes decreased markedly in the CTB-Ins-GFP- and CTB-INS protein-fed groups, compared with the CTB-GFP and Saline groups. The percentage of mice fed CTB-GFP alone did not differ significantly from the Saline control group. These results suggest that the CTB-Ins-GFP fusion protein, orally administered, may offer an alternative approach to effectively prevent or even treat diabetes.

3.5. Interaction between the Recombinant Protein and Gut Mucosa. To verify the interaction of the fusion protein with the intestine, we obtained 8 μm of frozen gut sections of NOD mice from the protein binding assay. Photomicrographs showed that the GFP tag was effectively located on the fusion proteins similarly to other tagged probes or antibodies, as expected (Figure 6). We concluded that the fusion GFP-tagged proteins had complete GFP ability and could be used to trace the process of protein transportation in the intestinal mucosa by direct visualization of the GFP tag.

3.6. Feeding with CTB-Ins-GFP Fusion Protein Induced a Humoral Immune Response. Generally, oral administration of certain protein antigens can induce humoral immunoreactions to the antigen, so we assayed 10-week-old female NOD mouse anti-CTB and anti-insulin antibody serum titers to investigate whether feeding Saline (group 1), CTB-GFP (group 2), CTB-Ins-GFP (group 3), or CTB-INS (group 4) hemolymph would induce specific humoral immune responses. We found that NOD mice fed CTB-Ins-GFP or CTB-INS hemolymph had both anti-CTB and anti-insulin antibodies in their sera (data not shown). Serum anti-CTB IgG1 levels in animals fed CTB-GFP, CTB-Ins-GFP, or CTB-INS hemolymph were significantly higher than in the Saline group (antibody titer: 758 ± 140, 734 ± 133, and 812 ± 150 versus 79 ± 18, resp.; $n = 9$ per

Table 2: Treg cell proportion in the peripheral lymph tissues of NOD mice.

Type of mice	Material oral administrated	$CD4^+CD25^+Foxp3^+$ T cell's proportion (%)					
		Nondiabetes			Diabetes		
		Spleen	Lymph node	Blood	Spleen	Lymph node	Blood
NOD mice	Saline	1.45 ± 0.38	1.82 ± 0.36	2.22 ± 0.14	5.42 ± 0.83	8.11 ± 1.6	20.63 ± 5.79
	CTB-GFP	1.54 ± 0.42	1.93 ± 0.29	2.19 ± 0.2			
	CTB-Ins-GFP	2.75 ± 0.34*	2.9 ± 0.42*	3.61 ± 0.70*			
	CTB-INS	2.87 ± 0.35****	3.16 ± 0.53*	3.71 ± 0.78*			
Normal mice		0.89 ± 0.11	± 0.28	1.5 ± 0.36			

Splenocyte (1×10^5 cells) isolated from CTB-INS, CTB-Ins-GFP, CTB-GFP, Saline-fed NOD mice at 10 weeks of age were treated with the mouse regulatory T cell staining kit by the protocol. Splenocytes of the normal mice and the diabetic NOD mice (from the diabetes onset experiment) was used as contrasts. These treated splenocytes were examined by FACScan flow cytometer for $CD4^+CD25^+Foxp3^+$ Treg cell proportions. Results are presented as the mean titer values ± S.D. Four mice per group were tested in two separate experiments.
*Values $P < .05$ is for statistical differences among mice groups treated with CTB-INS, CTB-Ins-GFP, CTB-GFP, and Saline.

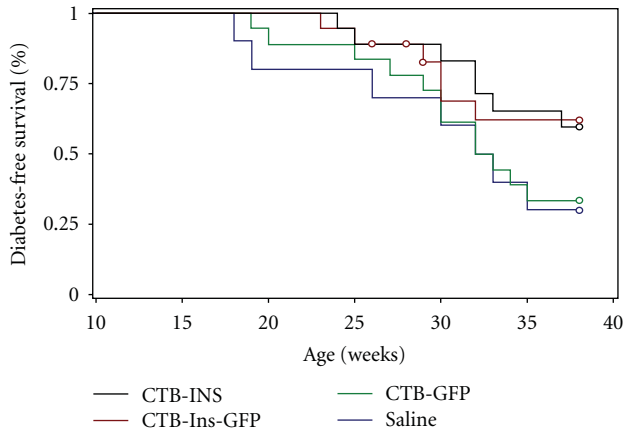

Figure 5: Effect of fusion protein feeding on diabetes development. Five-week-old female NOD mice were fed CTB-INS (group 1, $n = 18$, black), CTB-Ins-GFP (group 2, $n = 18$, red), CTB-GFP (group 3, $n = 18$, green), or Saline (group 4, $n = 10$, blue) four times per week until 38 weeks of age. Diabetes was confirmed by hyperglycemia (>16.7 mM glucose) for 2 consecutive weeks. The ANOVA test revealed a significant reduction in T1D onset in groups 1 and 2 compared with groups 3 and 4 ($P = .0276$). No significant difference was found between group 1 and group 2 ($P = .9683$).

group; $P < .05$). A significant increase in serum IgG1 anti-insulin antibody titers in NOD mice fed CTB-Ins-GFP or CTB-INS hemolymph was observed compared with the CTB-GFP and Saline groups (antibody titer: 523 ± 109 and 581 ± 96 versus 73 ± 34 and 88 ± 39, resp.; $n = 9$ per group; $P < .05$), with no significant difference in serum IgG2a antibodies (Figure 7(a)). Additionally, IgA antibodies, rather than IgE, in the sera of groups 2–4 were elevated in contrast to group 1 (antibody titer: 126 ± 28, 133 ± 29, and 154 ± 39 versus 20 ± 6, resp.; $n = 9$ per group; $P < .05$; Figure 7(b)). These findings indicate that oral insulin-induced Th2 lymphocyte-mediated oral tolerance resulted in predominantly IgG1 rather than other antibody isotypes.

3.7. Upregulation of Treg Cells in the Spleen, Intestinal Lymph Nodes, and Blood. We assayed the $CD4^+CD25^+Foxp3^+$ T cells in the peripheral lymph tissues of 10-week-old female NOD mice by FACScan flow cytometry. The results showed that the proportions in CTB-INS and CTB-Ins-GFP fed NOD mice were significantly elevated in contrast to the CTB-GFP and Saline groups (2.87 ± 0.35% and 2.75 ± 0.34% versus 1.54 ± 0.42% and 1.45 ± 0.38%, resp., in the spleen, 3.16 ± 0.53% and 2.9 ± 0.42% versus 1.93 ± 0.29% and 1.82 ± 0.36%, resp., in intestinal lymph nodes, and 3.71 ± 0.78% and 3.61 ± 0.7% versus 2.19 ± 0.27% and 2.22 ± 0.14%, resp., in blood, $P < .05$; Table 2), which were correlated with the suppression of T1D onset, and all were higher than in normal mice (0.89 ± 0.11%, 1.01 ± 0.28%, and 1.5 ± 0.36% in the spleen, intestinal lymph nodes, and blood, resp.). However, in diabetic NOD mice, the $CD4^+CD25^+Foxp3^+$ T cell proportions were much higher than that in any other group (5.42 ± 0.83%, 8.11 ± 1.6%, 20.63 ± 5.79% in the spleen, intestinal lymph nodes, and blood, resp., $P < .05$), and we believe that this was a result of a T1D autoimmune feedback mechanism. The results indicated that the induced oral tolerance specifically increased the proportions of $CD4^+CD25^+Foxp3^+$ T cells, which might suppress T1D progression in the peripheral lymph system.

3.8. Effect on Spleen Lymphocytes. To demonstrate the putative suppressive regulatory effect of induced Treg (iTreg) cells, we determined the proliferative and migratory abilities of spleen cells in treated NOD mice *in vitro* by BrdU label analysis and a Transwell assay. The performances indicated that specific immune suppression existed in the CTB-INS- and CTB-Ins-GFP- treated NOD mice, manifested by the suppression of the ability of the cells to proliferate and migrate (proliferative ability: CTB-INS- and CTB-Ins-GFP-treated groups: 60.19 ± 6.51% and 59.1 ± 5.38%, resp., $P = .00074$, versus CTB-GFP and Saline treated groups or normal mice 67.87 ± 4.13%, 69.11 ± 3.6%, and 71.67 ± 6.73%, resp., $P = .00009$; migratory ability: CTB-INS and CTB-Ins-GFP- treated groups: 128 ± 8 and 115 ± 9, resp., $P = .025$, versus CTB-GFP- and Saline-treated groups or normal mice: 200 ± 17, 191 ± 11, and 182 ± 28, resp., $P = .0068$; Figure 8). This suggests that oral tolerance might

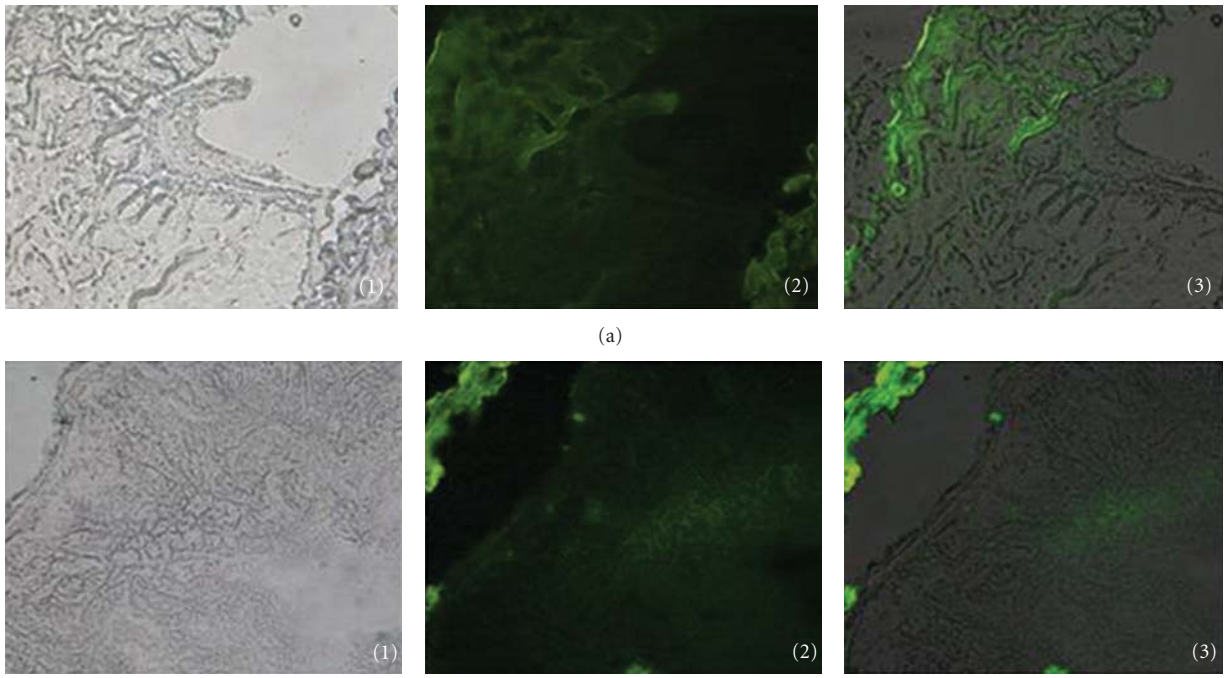

Figure 6: Intestinal mucosal binding assay. Images of the NOD mice frozen gut sections of (a)NOD mice fed CTB-GFP and (b) NOD mice fed CTB-Ins-GFP. Gut sections under (1) normal light, (2) blue light, and (3) overlapped. Magnification is 40x.

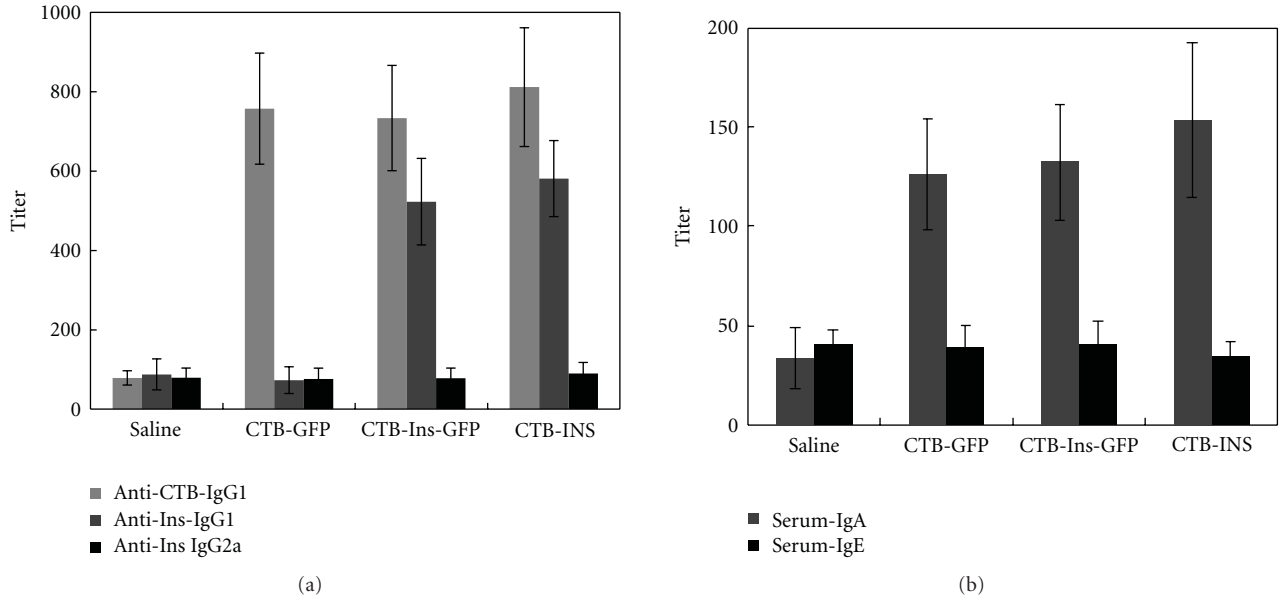

Figure 7: Serum antibody assay of treated NOD mice. (a) Anti-CTB IgG1, anti-insulin IgG1, and IgG2a serum antibody titers and (b) IgA and IgE serum titers in mice fed CTB-INS, CTB-Ins-GFP, CTB-GFP, or Saline. Results are presented as the mean titer values ± SD. Four or five mice per group were individually tested in two separate experiments.

suppress spleen lymphocyte activity via the iTreg cells in some way.

3.9. Adoptive Transfer of Treatment Regulatory Cells. To determine whether administration of the CTB-Ins-GFP protein protected NOD animals from the development of diabetes via some regulatory cells, we used an adoptive transfer model. Splenocytes from 10-week-old CTB-INS- (group 1), CTB-Ins-GFP- (group 2), and CTB-GFP (group 3)-fed NOD mice were mixed with diabetogenic splenocytes and given i.v. to 6–8-week-old syngeneic NOD/SCID recipient mice. Mice receiving only diabetogenic splenocytes

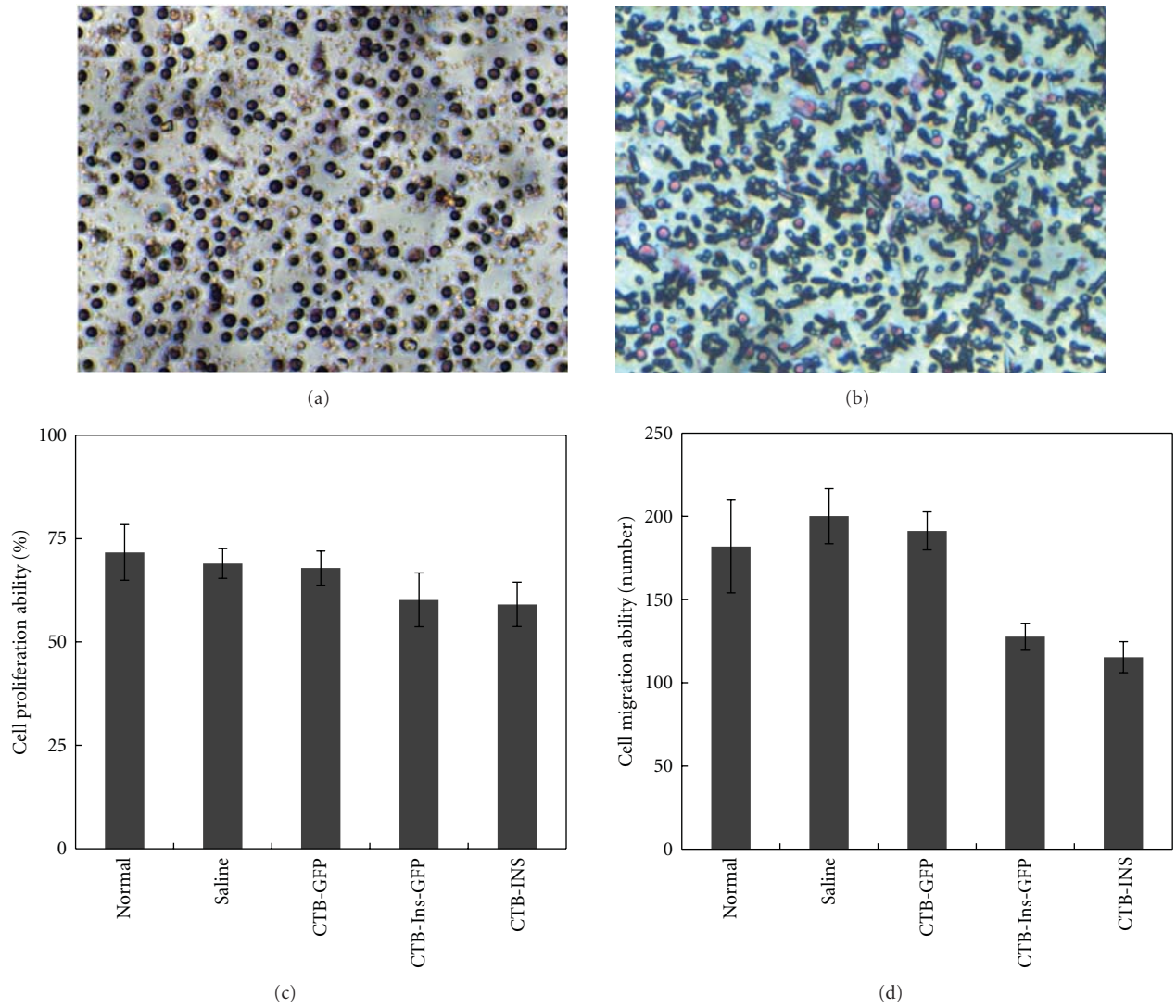

Figure 8: Proliferation and migration abilities of splenocytes in treated NOD mice. Splenocytes (1×10^7 cells) isolated from CTB-INS-, CTB-Ins-GFP-, CTB-GFP-, Saline-fed and normal mice at 10 weeks of age were cultured in 24-well plates with 10 μM BrdU for 24 h. Cells were immunohistochemically stained and examined for stained cell proportions (representative of proliferative ability) according to the protocol. Or splenocytes were cultured in the upper chamber of Transwell plates with 0.6 mL of normal culture medium (IL2 150 IU/mL) placed in the bottom chamber at 37°C with 5% CO_2 for 14 h. The migrated cells were fixed, stained, and numbered under the microscope. A. Splenocyte BrdU assay. Magnification is 400x. (b) Splenocyte Transwell assay. The magnification is 400x. (c) Statistical analysis of proliferative ability. (d) Statistical analysis of migratory ability. All results are presented as the mean titer values ± SD. Five mice per group were tested in two separate experiments.

(group 4) were used as a negative control. We found 83% and 100% (5/6 and 6/6) of the NOD/SCID recipient mice receiving splenocytes from group 3 and group 4 NOD mice, respectively, developed diabetes, whereas splenocytes from group 1 and group 2 NOD mice were able to prevent diabetes in 67% and 50% (4/6 and 3/6) of NOD/SCID mice, respectively (groups 1 and 2 versus groups 3 and 4, $P = .0001$; group 3 versus group 4, $P = .6064$; Figure 9). These results demonstrated that oral administration of CTB-INS or CTB-Ins-GFP fusion proteins generated some regulatory cells, including increased $CD4^+CD25^+Foxp3^+$ Treg cells, which could transfer suppressive ability in NOD mice.

4. Discussion

Oral tolerance is a potential therapeutic strategy, because it can induce "inappropriate" immune responses to specific "self" antigen proteins that precede overt autoimmune disease, such as T1D [1]. Human insulin is the major autoantigen orally administered in treating T1D via induction of special mucosal tolerance [6, 11, 19]. However, the obvious effect and application of this approach depends not only on the availability of autoantigens produced in quantity, but also on the detailed mechanisms of tolerance to make it amenable to clinical testing.

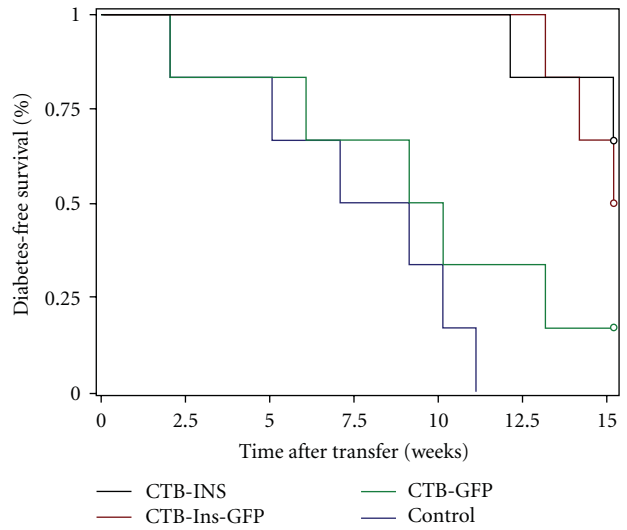

Figure 9: Splenocytes from insulin-fed NOD mice prevented diabetes after adoptive transfer. Splenocytes (1×10^7 cells) isolated from CTB-INS- (group 1, black), CTB-Ins-GFP- (group 2, red), and CTB-GFP- (group 3, green) fed mice at 10 weeks of age were mixed with diabetogenic splenocytes (1×10^7 cells) from diabetic NOD mice, and then cotransferred to 8-week-old NOD/SCID mice ($n = 6$ for all four groups) with a diabetogenic splenocyte-only injection (group 4, blue) as a control. The development of diabetes in the recipients was monitored for 15 weeks. The ANOVA test revealed a significant reduction in T1D onset in groups 1 and 2 compared with groups 3 and 4 ($P = .0001$). No significant difference was found between groups 3 and 4 ($P = .6064$).

Repeated oral administration of large doses of insulin is essential in maintaining specific unresponsiveness for long periods because of the large absorptive intestinal mucosal area. The CTB peptide as a mucosal carrier was applied successfully to induce peripheral immunological tolerance [7, 11]. In previous studies, it was shown that specific tolerance could be enhanced by conjugating insulin to the CTB peptide [19]. The CTB-conjugated protein functions in a pentameric structure, interacting with GM1 ganglioside expressed on the intestinal epithelial cells, facilitating the mucosal immune reaction and increasing the autoantigen concentration. Others have reported that CTB may enhance the immune reaction by affecting the intestinal tight junction [24]. Moreover, Terahara et al. [13] found that orally administered CT induced villous M-like cells in mice. Comprehensive gene expression revealed that they share traits with both intestinal epithelial cells and PP M-cells. The latter play significant roles in mucosal immunity. In a previous study of T1D treatment in NOD mice by oral insulin, Zhang et al. [6] found that administration of 1 mg of porcine insulin was an effective oral dose. However, we were able to decrease the effective dose of the autoantigen in the active CTB pentameric form to about 50 μg. Together, our data and the results from other researchers support the theory that low doses administrated for oral tolerance favor active suppression, whereas high doses favor clonal anergy/deletion [25, 26].

So far, CTB-based fusion proteins have been successfully achieved in *E. coli* [8], potatoes [27], tomatoes [28], tobacco [9], and even rice [10]. In this study, we used silkworm to express the fusion proteins by insect BES. The insect BES was first used in 1993 to obtain the cytokine interferon-α and has been broadly applied to produce proteins due to its numerous virtues [20]. In recent years, the improved BES, the so-called Bac-to-Bac BES, has been used more, because it is quicker, more efficient, and allows easier identification with lower workloads [29]. In the present study, we produced protein at levels up to 0.58 mg/mL. Additionally, the proteinase inhibitors [30] and biocapsule-like fat of silkworms may keep the protein of interest stable and protect it against gastrointestinal degradation, making the hemolymph especially appropriate for oral administration. The results indicate that Bac-to-Bac BES is suitable for producing the large quantities of proteins or polypeptides of interest.

The precise mechanism by which the CTB-Ins-GFP fusion protein enhances oral tolerance remains unclear. As a popular fluorescent protein first discovered in 1962, GFP was expressed in *E. coli* to reveal that FimH+ bacteria combine glycoprotein 2 of the intestinal M-cells to initiate a mucosal immune response [14]. It was also useful in work on the localization, structure, and dynamic processes of molecules, cells, and organisms, such as viruses, and even in interaction studies among DNA, RNA, and proteins [31, 32]. In the present study, the fusion protein was shown to interact with the gut mucosa, but appeared in neither the spleen nor the intestinal lymph nodes (data not shown). The orally administered protein has a low bioavailability, less than 3.1% [33]. It was previously reported that low amounts of insulin induced regulatory $CD4^+$ T-cells [34–36]. In other diseases, like the arthritis mouse model, orally administered type II collagen was shown to induce $CD4^+CD25^+Foxp3^+$ regulatory cells by IDO expressing $CD11c^+$ DCs in intestinal Peyer's patches in the treatment of arthritis [37–39]. Moreover, oral tolerance induced by OVA coupled to CTB promoting OVA specific regulatory $CD4^+CD25^+Foxp3^+$ T-cells was dependent on B lymphocytes not only in the mucosal inductive draining lymph node but also at the inflamed effector sites to suppress effector T-cells [16, 40, 41]. It was also observed that the expression of CD86 in B-cells decreased, whereas IL-10 producing $CD19^+$ B-cells simultaneously increased. These Treg cells were reported to induce other $CD4^+$ cells into becoming suppressors in addition to inducing the apoptosis or depletion of effector T-cells by contact-dependent or cytokine-dependent action mechanisms. According with this, our results indicate that CTB enhances immunomodulation of insulin by the humoral and Th2-like immune responses and induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. The adoptive cotransfer experiment also showed that the regulatory cells were functionally active. This is probably because the autoantigen insulin plays an important role in T cell differentiation into Treg cell lineages. Administration of exogenous CTB-Ins-GFP fusion protein may create a special microenvironment in the gut that can promote beneficial humoral Th2-like immune responses, instead of harmful Th1-like immune reactions [42–44], and also insulin-specific regulatory T cells by some immature DCs or special B

cells in the intestine, which play an essential role in the development of spleen cells [45]. Although the exact process by which the protein treats T1D remains to be discovered, these findings validate the use of the autoantigen insulin as an ideal immunotherapy strategy in the treatment of diseases involving $CD4^+CD25^+Foxp3^+$ Treg cells.

Treg cells are attracting great interest because of their potential therapeutic role in autoimmune diseases [46]. To some extent, T1D can be "cured" by pancreatic transplantation [47], gene therapy [48, 49], or injection of specific autoantibodies, such as anti-CD3 antibodies [50]. However, none of these meet the requirements of an ideal cure [51]. Our work offers support for the use of oral tolerance in treating T1D. Generally, our data and other studies suggest that the antigen amount, delivery mode, and/or coadministered adjuvant molecules may have profound effects on directing the mechanism of tolerance. Successful application of oral tolerance in the treatment of human diseases will depend not only on the detailed functional mechanisms but also on strategies that can improve antigen presentation, alter the administered dose and formulations, use potent mucosal adjuvants, target the correct cells in the gut-liver axis, develop immune biomarkers for detecting effects, use combination therapies with other immunomodulatory agents, and target the right patient populations [52–55].

5. Conclusions

In summary, this study demonstrates that low doses of orally administered silkworm-produced CTB-Ins-GFP protein can effectively suppress the development of diabetes in NOD mice, where the treatment function involves inducing $CD4^+CD25^+Foxp3^+$ Treg cells. Our experimental evidence lends further support to the hypothesis that orally administering the CTB-Ins protein may be a feasible way to treat T1D patients, and oral tolerance may be a powerful therapeutic strategy for treating autoimmune diseases.

Conflict of Interests

The authors indicate no potential conflict of interests.

Acknowledgments

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References

[1] P. Garside and A. M. Mowat, "Oral tolerance," *Seminars in Immunology*, vol. 13, no. 3, pp. 177–185, 2001.

[2] J. F. Elliott, H. Y. Qin, S. Bhatti et al., "Immunization with the larger isoform of mouse glutamic acid decarboxylase GAD) prevents autoimmune diabetes in NOD mice," *Diabetes*, vol. 43, no. 12, pp. 1494–1499, 1994.

[3] V. K. Ramiya, X. Z. Shang, P. G. Pharis et al., "Antigen based therapies to prevent diabetes in NOD mice," *Journal of Autoimmunity*, vol. 9, no. 3, pp. 349–356, 1996.

[4] V. K. Ramiya and N. K. Maclaren, "Insulin in diabetes prevention," *Hormone Research*, vol. 48, no. 4, pp. 67–70, 1997.

[5] Z. Gong, L. Pan, Y. Le et al., "Glutamic acid decarboxylase epitope protects against autoimmune diabetes through activation of Th2 immune response and induction of possible regulatory mechanism," *Vaccine*, vol. 28, no. 24, pp. 4052–4058, 2010.

[6] Z. J. Zhang, L. Davidson, G. Eisenbarth, and H. L. Weiner, "Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 22, pp. 10252–10256, 1991.

[7] J. B. Sun, J. Holmgren, and C. Czerkinsky, "Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 23, pp. 10795–10799, 1994.

[8] Z. Gong, X. Long, L. Pan et al., "Cloning, expression, purification and characterization of the cholera toxin B subunit and triple glutamic acid decarboxylase epitopes fusion protein in Escherichia coli," *Protein Expression and Purification*, vol. 66, no. 2, pp. 191–197, 2009.

[9] S. Rosales-Mendoza, A. G. Alpuche-Solis, R. E. Soria-Guerra et al., "Expression of an Escherichia coli antigenic fusion protein comprising the heat labile toxin B subunit and the heat stable toxin, and its assembly as a functional oligomer in transplastomic tobacco plants," *Plant Journal*, vol. 57, no. 1, pp. 45–54, 2009.

[10] T. Nochi, H. Takagi, Y. Yuki et al., "Rice-based mucosal vaccine as a global strategy for cold-chain- and needle-free vaccination," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 26, pp. 10986–10991, 2007.

[11] I. Bergerot, C. Ploix, J. Petersen et al., "A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 9, pp. 4610–4614, 1997.

[12] M. R. Neutra, A. Frey, and J. P. Kraehenbuhl, "Epithelial M cells: gateways for mucosal infection and immunization," *Cell*, vol. 86, no. 3, pp. 345–348, 1996.

[13] K. Terahara, M. Yoshida, O. Igarashi et al., "Comprehensive gene expression profiling of peyer's patch m cells, villous m-like cells, and intestinal epithelial cells," *Journal of Immunology*, vol. 180, no. 12, pp. 7840–7846, 2008.

[14] K. Hase, K. Kawano, T. Nochi et al., "Uptake through glycoprotein 2 of FimH + bacteria by M cells initiates mucosal immune response," *Nature*, vol. 462, no. 7270, pp. 226–230, 2009.

[15] S. Y. Min, K. S. Park, M. L. Cho et al., "Antigen-induced, tolerogenic CD11c+,CD11b+ dendritic cells are abundant in Peyer's patches during the induction of oral tolerance to type II collagen and suppress experimental collagen-induced arthritis," *Arthritis and Rheumatism*, vol. 54, no. 3, pp. 887–898, 2006.

[16] J. B. Sun, C. F. Flach, C. Czerkinsky, and J. Holmgren, "B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit," *Journal of Immunology*, vol. 181, no. 12, pp. 8278–8287, 2008.

[17] L. A. De-Gennaro, A. F. Popi, S. R. D. Almeida, J. D. Lopes, and M. Mariano, "B-1 cells modulate oral tolerance in mice," *Immunology Letters*, vol. 124, no. 2, pp. 63–69, 2009.

[18] M. A. Atkinson and N. K. Maclaren, "The pathogenesis of insulin-dependent diabetes mellitus," *New England Journal of Medicine*, vol. 331, no. 21, pp. 1428–1436, 1994.

[19] Z. Gong, Y. Jin, and Y. Zhang, "Oral administration of a cholera toxin B subunit-insulin fusion protein produced in silkworm protects against autoimmune diabetes," *Journal of Biotechnology*, vol. 119, no. 1, pp. 93–105, 2005.

[20] T. A. Kost, J. P. Condreay, and D. L. Jarvis, "Baculovirus as versatile vectors for protein expression in insect and mammalian cells," *Nature Biotechnology*, vol. 23, no. 5, pp. 567–575, 2005.

[21] L. K. Miller, "Baculoviruses for foreign gene expression in insect cells," *Biotechnology*, vol. 10, pp. 457–465, 1988.

[22] G. Zhang, V. Gurtu, and S. R. Kain, "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells," *Biochemical and Biophysical Research Communications*, vol. 227, no. 3, pp. 707–711, 1996.

[23] L. Langroudi, Z. M. Hassan, M. Ebtekar, M. Mahdavi, N. Pakravan, and S. Noori, "A comparison of low-dose cyclophosphamide treatment with artemisinin treatment in reducing the number of regulatory T cells in murine breast cancer model," *International Immunopharmacology*, vol. 10, no. 9, pp. 1055–1061, 2010.

[24] A. Fasano, B. Baudry, D. W. Pumplin et al., "Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 12, pp. 5242–5246, 1991.

[25] Y. H. Chen and H. L. Weiner, "Dose-dependent activation and deletion of antigen-specific T cells following oral tolerance," *Annals of the New York Academy of Sciences*, vol. 778, pp. 111–121, 1996.

[26] N. Van Houten and S. F. Blake, "Direct measurement of anergy of antigen-specific T cells following oral tolerance induction," *Journal of Immunology*, vol. 157, no. 4, pp. 1337–1341, 1996.

[27] T. Arakawa, D. K. X. Chong, J. Lawrence Merritt, and W. H. R. Langridge, "Expression of cholera toxin B subunit oligomers in transgenic potato plants," *Transgenic Research*, vol. 6, no. 6, pp. 403–413, 1997.

[28] M. K. Sharma, N. K. Singh, D. Jani et al., "Expression of toxin co-regulated pilus subunit a (TCPA) of Vibrio cholerae and its immunogenic epitopes fused to cholera toxin B subunit in transgenic tomato (Solanum lycopersicum)," *Plant Cell Reports*, vol. 27, no. 2, pp. 307–318, 2008.

[29] S. Li, D. T. M. Ip, H. Q. Lin et al., "High-level expression of functional recombinant human butyrylcholinesterase in silkworm larvae by Bac-to-Bac system," *Chemico-Biological Interactions*, vol. 187, no. 1–3, pp. 101–105, 2010.

[30] H. Fujii, H. Doira, and H. Iwamoto, "Synthesis and resorption of a humoral chymotrypsin inhibitor, CI-8, by fat body of the silkworm, Bombyx mori," *Insect Biochemistry and Molecular Biology*, vol. 30, no. 5, pp. 363–368, 2000.

[31] H. H. Gerdes and C. Kaether, "Green fluorescent protein: applications in cell biology," *FEBS Letters*, vol. 389, no. 1, pp. 44–47, 1996.

[32] B. L. Epel, H. S. Padgett, M. Heinlein, and R. N. Beachy, "Plant virus movement protein dynamics probed with a GFP-protein fusion," *Gene*, vol. 173, no. 1, pp. 75–79, 1996.

[33] W. Zhang, Z. Lv, Z. Nie et al., "Bioavailability of orally administered rhGM-CSF: a single-dose, randomized, open-label, two-period crossover trial," *PLoS ONE*, vol. 4, no. 5, Article ID e5353, 2009.

[34] I. Bergerot, N. Fabien, V. Maguer, and C. Thivolet, "Oral administration of human insulin to NOD mice generates CD4+ T cells that suppress adoptive transfer of diabetes," *Journal of Autoimmunity*, vol. 7, no. 5, pp. 655–663, 1994.

[35] C. Aspord and C. Thivolet, "Nasal administration of CTB-insulin induces active tolerance against autoimmune diabetes in non-obese diabetic (NOD) mice," *Clinical and Experimental Immunology*, vol. 130, no. 2, pp. 204–211, 2002.

[36] R. Mukherjee, P. Chaturvedi, H. Y. Qin, and B. Singh, "CD4+CD25+ regulatory T cells generated in response to insulin B:9-23 peptide prevent adoptive transfer of diabetes by diabetogenic T cells," *Journal of Autoimmunity*, vol. 21, no. 3, pp. 221–237, 2003.

[37] M. J. Park, S. Y. Min, K. S. Park et al., "Indoleamine 2,3-dioxygenase-expressing dendritic cells are involved in the generation of CD4+CD25+ regulatory T cells in Peyer's patches in an orally tolerized, collagen-induced arthritis mouse model," *Arthritis Research and Therapy*, vol. 10, no. 1, article R11, 2008.

[38] K. S. Park, M. J. Park, M. L. Cho et al., "Type II collagen oral tolerance; mechanism and role in collagen-induced arthritis and rheumatoid arthritis," *Modern Rheumatology*, vol. 19, no. 6, pp. 581–589, 2009.

[39] O. Jaen, S. Rullé, N. Bessis, A. Zago, M. C. Boissier, and G. Falgarone, "Dendritic cells modulated by innate immunity improve collagen-induced arthritis and induce regulatory T cells in vivo," *Immunology*, vol. 126, no. 1, pp. 35–44, 2009.

[40] J. B. Sun, N. Cuburu, M. Blomquist, B. L. Li, C. Czerkinsky, and J. Holmgren, "Sublingual tolerance induction with antigen conjugated to cholera toxin B subunit induces foxp3CD25+CD4+ regulatory T cells and suppresses delayed-type hypersensitivity reactions," *Scandinavian Journal of Immunology*, vol. 64, no. 3, pp. 251–259, 2006.

[41] J. B. Sun, C. Czerkinsky, and J. Holmgren, "Sublingual "oral tolerance" induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells," *Scandinavian Journal of Immunology*, vol. 66, no. 2-3, pp. 278–286, 2007.

[42] M. Polanski, N. S. Melican, J. Zhang, and H. L. Weiner, "Oral administration of the immunodominant B-chain of insulin reduces diabetes in a Co-transfer model of diabetes in the NOD mouse and is associated with a switch from Th1 to Th2 cytokines," *Journal of Autoimmunity*, vol. 10, no. 4, pp. 339–346, 1997.

[43] B. Hartmann, K. Bellmann, I. Ghiea, R. Kleemann, and H. Kolb, "Oral insulin for diabetes prevention in NOD mice: potentiation by enhancing Th2 cytokine expression in the gut through bacterial adjuvant," *Diabetologia*, vol. 40, no. 8, pp. 902–909, 1997.

[44] S. T. Azar, H. Tamim, H. N. Beyhum, M. Zouhair Habbal, and W. Y. Almawi, "Type I (insulin-dependent) diabetes is a Th1- and Th2-mediated autoimmune disease," *Clinical and Diagnostic Laboratory Immunology*, vol. 6, no. 3, pp. 306–310, 1999.

[45] E. D. W. Suh, B. P. Vistica, C. C. Chan, J. M. Raber, I. Gery, and R. B. Nussenblatt, "Splenectomy abrogates the induction of oral tolerance in experimental autoimmune uveoretinitis," *Current Eye Research*, vol. 12, no. 9, pp. 833–839, 1993.

[46] J. A. Dromey, B. H. Lee, H. Yu et al., "Generation and expansion of regulatory human CD4+ T-cell clones specific for pancreatic islet autoantigens," *Journal of Autoimmunity*, vol. 36, no. 1, pp. 47–55, 2011.

[47] A. N. Balamurugan, R. Bottino, N. Giannoukakis, and C. Smetanka, "Prospective and challenges of islet transplantation

for the therapy of autoimmune diabetes," *Pancreas*, vol. 32, no. 3, pp. 231–243, 2006.

[48] N. Giannoukakis and M. Trucco, "Gene therapy for type 1 diabetes: a proposal to move to the next level," *Current Opinion in Molecular Therapeutics*, vol. 7, no. 5, pp. 467–475, 2005.

[49] A. D'Anneo, P. Rood, R. Bottino, A. N. Balamurugan, J. He, and N. Giannoukakis, "Gene therapy for type 1 diabetes: is it ready for the clinic?" *Immunologic Research*, vol. 36, no. 1-3, pp. 83–89, 2006.

[50] J. Smith-Palmer, B. H. Curtis, K. S. Boye, G. Goodall, and S. R. Pillemer, "Anti-CD3 monoclonal antibody treatment in newly diagnosed Type 1 diabetes patients: a hypothetical modelling analysis," *Diabetic Medicine*, vol. 27, no. 2, pp. 189–196, 2010.

[51] N. Giannoukakis, B. Phillips, and M. Trucco, "Toward a cure for type 1 diabetes mellitus: diabetes-suppressive dendritic cells and beyond," *Pediatric Diabetes*, vol. 9, no. 3, pp. 4–13, 2008.

[52] M. Peakman and C. M. Dayan, "Antigen-specific immunotherapy for autoimmune disease: fighting fire with fire?" *Immunology*, vol. 104, no. 4, pp. 361–366, 2001.

[53] A. Wakabayashi, Y. Kumagai, E. Watari et al., "Importance of gastrointestinal ingestion and macromolecular antigens in the vein for oral tolerance induction," *Immunology*, vol. 119, no. 2, pp. 167–177, 2006.

[54] A. M. C. Faria and H. L. Weiner, "Oral tolerance: therapeutic implications for autoimmune diseases," *Clinical and Developmental Immunology*, vol. 13, no. 2-4, pp. 143–157, 2006.

[55] H. Waldmann, "Tolerance: an overview and perspectives," *Nature Reviews Nephrology*, vol. 6, no. 10, pp. 569–576, 2010.

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Review Article

Antigen-Based Immune Therapeutics for Type 1 Diabetes: Magic Bullets or Ordinary Blanks?

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The ideal drug of modern medicine is the one that achieves its therapeutic target with minimal adverse effects. Immune therapy of Type 1 diabetes (T1D) is no exception, and knowledge of the antigens targeted by pathogenic T cells offers a unique opportunity towards this goal. Different antigen formulations are being considered, such as proteins or peptides, either in their native form or modified *ad hoc*, DNA plasmids, and cell-based agents. Translation from mouse to human should take into account important differences, particularly in the time scale of autoimmune progression, and intervention. Critical parameters such as administration route, dosing and interval remain largely empirical and need to be further dissected. T1D staging through immune surrogate markers before and after treatment will be key in understanding therapeutic actions and to finally turn ordinary blanks into magic bullets.

1. Introduction

Type 1 diabetes (T1D), one of the most common autoimmune diseases, stems from defects in central and peripheral tolerance that lead to progressive T-cell-mediated destruction of insulin-producing β cells in pancreatic islets. Clinically, this destruction results in the inability of affected individuals to produce the insulin required to properly regulate glucose metabolism, causing substantial morbidity and mortality. Although metabolic derangements are only the consequences of the underlying autoimmune pathogenesis, they remain the only targets of mainstream insulin therapy. Immune-targeted interventions which could correct autoimmune mechanisms are, therefore, intensively sought as a more rational approach. Preclinical studies largely take advantage of the nonobese diabetic (NOD) mouse, which is one rare instance of autoimmune model, where disease develops spontaneously rather than being induced by experimental manipulation.

2. Translating the NOD Mouse into Human

There is a long list of immune biologics that have spectacular effects in preventing T1D in the NOD mouse. However, only a handful of them has shown some effect once translated into clinical trials. There are a number of reasons explaining these discrepancies which should be kept in mind. First is the phylogenetic difference between the murine and human immune system, which is not surprising considering that the two species diverged ~70 million years ago [1]. Second, although the NOD is a spontaneous model of T1D which is linked to MHC susceptibility haplotypes and involves a complex immune interplay like in human, there are important discrepancies. These include characteristics of the insulitis infiltrate, autoantibody (aAb) specificities, and association with other autoimmune manifestations (e.g., sialitis). Third, the NOD is an inbred strain composed of genetically identical animals. As such, it can be assimilated to one single T1D patient, and indeed a very peculiar one.

Further underrepresenting human disease, these mice are kept under identical environmental conditions, protected from most infectious threats. Fourth, disease and treatment kinetics are quite different, as NOD mice are most commonly treated for preventing diabetes at an early stage, before the appearance of any circulating autoimmune marker such as anti-insulin aAbs (IAA). This is rarely possible in humans, as further discussed. Indeed, when we look at immune therapies which work in the NOD mouse once the disease has become clinically overt, the list of effective treatments falls much shorter. The challenge that intervention trials face in humans is thus a formidable one. For this reason, we will focus our discussion on antigen- (Ag-) specific agents that have already been tested or are soon to enter clinical trials, reasoning that our goal is to cure men rather than mice.

3. Immune Therapy for Type 1 Diabetes: Whom for?

Studies in the NOD mouse suggest that the great majority of the β-cell mass (~75%) has already been destroyed by the time of diabetes onset [2]. A recent meta-analysis and mathematical modeling of three landmark histopathological studies of human T1D pancreata [3–5] suggests that this is also the case in humans [6]. However, the extent of β-cell destruction varies with age, ranging from 85% in children to 40% in adults [6]. This is probably due to a combination of factors, which include a physiological age-related decline in β-cell mass, differences in insulin needs according to body weight, growth and insulin sensitivity, and the degree of β-cell autoimmunity, which may be more aggressive in younger patients. One limitation of most histopathological studies is that measurement of residual β-cell mass was based on enumeration of insulin-positive cells within the islets. It is, thus, possible that viable β cells not producing insulin because of functional impairment are missed, leading to an overestimation. It is also difficult to relate these histopathological estimates of residual β-cell mass with those of residual β-cell function, as determined by stimulated C-peptide secretion. However, recent studies suggest that insulin production may be more substantial at diagnosis than had been previously appreciated and that residual insulin secretion may persist in a subgroup of T1D patients [7]. Further complicating the picture, the question of whether significant β-cell renewal occurs in T1D patients remains unsettled [8].

Whichever the real extent of β-cell destruction at the time of T1D onset, it is clear that interventions aimed at correcting immune mechanisms should be implemented as early as possible, ideally at a preclinical stage in as yet healthy subjects at risk of developing disease (Figure 1). A related problem is, therefore, to reliably identify these subjects. Although genetic markers (particularly HLA Class II susceptibility alleles) and serum aAbs against the β-cell Ags insulin, glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), and zinc transporter 8 (ZnT8) greatly help to stratify T1D risk, they fall short in accuracy, as they can predict the risk but not the time course of disease development (i.e., the "if", but not the "when"), and much less when only one aAb marker is present. The accuracy of these predictions needs to be very stringent if used to decide enrollment into a clinical trial, because the risk-benefit balance is a delicate one for T1D. We are not facing a rapidly lethal disease but rather one that, despite difficult daily management and long-term complications, carries a life expectancy which is getting closer and closer to normal. This benign prognosis needs to be weighed against the risks of experimental immune therapeutics, whose long-term adverse events are frequently unknown. It is, therefore, ethically acceptable to trial only those subjects in whom T1D will eventually develop. Given the efforts and difficulties needed to recruit an adequate number of at-risk subjects, T1D prevention studies have been quite limited and performed mostly in subjects at very high risk. Trials in newly diagnosed T1D patients are more common, with the inner constraint that, even if the autoimmune process is effectively halted, there is limited clinical benefit to be expected from rescuing the residual β-cell mass. This is particularly daunting in T1D children, in whom the remaining β cells are fewer than in adults and may be as few as 15% [6]. In this respect, combination of immune biologics with strategies aimed at replacing or regenerating lost β cells may expand the optimal time window for intervention.

4. Immune Suppression versus Tolerance Restoration

Two broad strategies could theoretically be followed to intervene on β-cell autoimmunity. The first one would consist of correcting the environmental causes triggering autoimmunity. However, this is certainly the most frustrating failure of half a century of T1D research, which has elucidated a wealth of immune mechanisms without identifying the environmental *primi moventes*. The only emerging exception may be the role of the gut microbiome, as data obtained in the NOD mouse suggests that altering composition of intestinal flora may offer new treatment paths in the future [9]. Factors responsible for insulin resistance are also emerging contributors to T1D development, but they probably play a precipitating rather than causative role. Nonetheless, insulin resistance has recently been recognized as a powerful T1D risk factor [10], and clinical trials with drugs such as metformin acting on metabolic pathways keep being investigated [11–13].

The second, currently more viable, strategy is to correct immune mechanisms. In doing this, an important difference needs to be made between immune suppression and tolerance restoration. With immune suppression, a generalized status of lessened reactivity is induced so that the body has decreased responses to Ag challenges of any kind. This exposes to increased risks of infection and secondary tumor growth. Moreover, treatment needs to be lifelong, as its effects are lost after discontinuation. Immune suppression is typically induced by drugs that act on common signaling pathways used by key immune cells. Examples of such drugs used in the past are cyclosporin A [14] and, in more

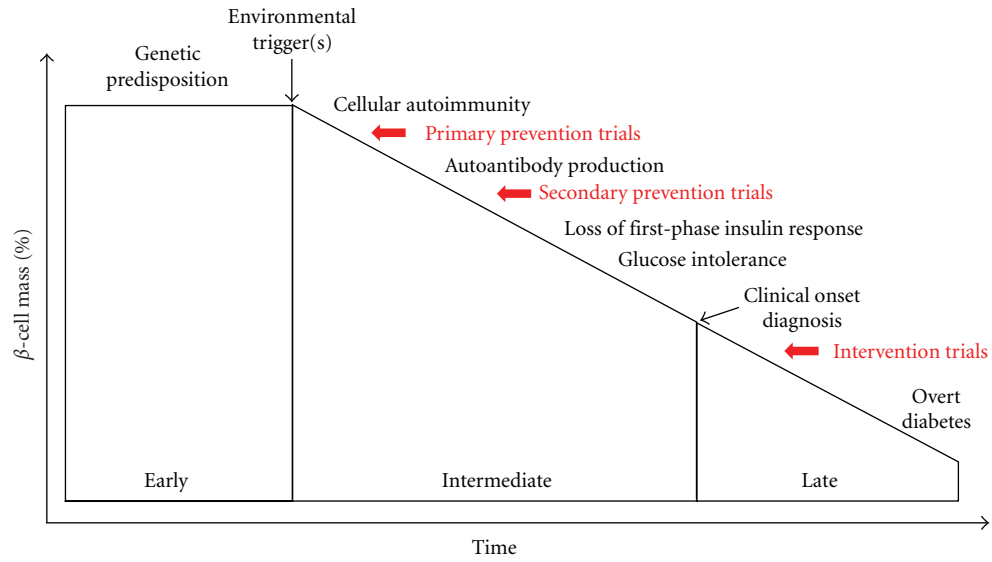

Figure 1: Stages of disease progression and intervention in T1D. Progression over time (X-axis) from simple genetic susceptibility to β-cell autoimmunity and T1D is plotted against residual β-cell mass (Y-axis). The time points at which immune therapies are administered are shown in red.

recent years, anti-CD25 monoclonal antibodies (mAbs) and mycofenolate mofetil, used either alone or in combination [15].

With immune tolerance restoration, the induced effect is not generalized but limited to specific types of responses, ideally only to responses specific to β-cell Ags. Thus, the immune system remains capable of responding to infectious and tumoral threats. Treatment should be limited in time, while the effects should persist after discontinuation. This is because an "active" effect is induced by boosting natural immune tolerance mechanisms. With this frame of reference, it is evident why immune restoration strategies are preferable. Given their more selective action and limited duration of treatment, their risk-benefit balance is more attractive, especially if they have to be applied in prevention trials. Although several non-Ag-specific therapeutic mAbs are also under scrutiny [16], agents that exploit the target β-cell Ags themselves may offer the best warranties of Ag-specific immune tolerance.

5. Which β-Cell Antigens?

The list of β-cell Ags relevant to T1D autoimmunity is quite long [17], but few of them have resisted the proof of time and proved to be key targets of aAb and/or T-cell responses. These Ags are insulin and its precursors proinsulin (PI) and preproinsulin (PPI), GAD, IA-2, ZnT8, and islet glucose-6-phosphatase catalytic subunit-related protein (IGRP). The latter is the only one to be exclusively described as a target of T cells [18, 19], as aAbs have been sought after but not yet found.

A popular tenet in autoimmunity is that there could be one primary self Ag which initiates pathogenesis. Tissue destruction through targeting of this Ag could further release other ones, thus amplifying the autoimmune cascade through a phenomenon known as epitope spreading. In the NOD mouse, insulin has been identified as the initiating β-cell Ag. The importance of insulin is supported by data on insulin knockout NOD mice. Different from humans, rodents express two isoforms, referred to as insulin 1 and 2. NOD mice defective for the insulin 2 gene, the prevalent isoform in the thymus, display accelerated T1D [20], likely related to defective deletion of insulin-reactive T cells [21]. Conversely, NOD mice defective for the insulin 1 gene, one of the two isoforms expressed in the islets, are less susceptible to T1D [22]. However, insulin 1 knockout islets transplanted into recently diabetic wild-type NOD mice become infiltrated and only transiently reverse T1D, suggesting that insulin is an early but not exclusive target [22]. More recent evidence from Nakayama et al. further suggests that insulin may be the initiating β-cell Ag in T1D [23]. These authors produced NOD mice, where the endogenous insulin 1 and 2 genes have been deleted and replaced by a hormonally active insulin transgene carrying a single amino acid mutation at position B16. These mice are completely protected from T1D and insulitis [23]. Intriguingly, the introduced substitution affects insulin recognition by both CD4+ and CD8+ T cells, as two overlapping immunodominant epitopes have been described in this region: insulin $B_{9–23}$ [24] and insulin $B_{15–23}$ [25], respectively. These data suggest that recognition of these immunodominant epitopes by CD4+ and/or CD8+ T cells may be a mandatory early event in T1D pathogenesis. Studies by Krishnamurthy et al. further corroborated the hypothesis that insulin is the initiating Ag in the T1D of the NOD mouse, because mice rendered tolerant to insulin by transgenic overexpression of insulin 2 in Ag-presenting cells do not develop the immunodominant $IGRP_{206–214}$-specific responses and are protected from T1D [26]. Conversely, mice made tolerant to IGRP by the same means are not protected

from T1D [26], suggesting that IGRP-specific responses lay downstream of insulin-specific ones in the pathogenic cascade. The prerequisite of insulin-specific responses for T1D to develop is even found in NOD8.3 mice, which are transgenic for a T-cell receptor recognizing the $IGRP_{206–214}$ epitope [27].

Despite strong evidence pointing to insulin's triggering role, identifying and targeting more β-cell Ags remain a high-priority goal. Several considerations justify these efforts. First, the evidence supporting insulin's critical role has been obtained in the NOD model, but similar evidence in human T1D is confined to the early appearance of IAA. Second, once autoimmune T-cell (and B-cell) responses to β cells are initiated, the specificity of these responses rapidly enlarges to include more Ags (epitope spreading). As a result, responses to the presumable triggering Ag can be rapidly overgrown by secondary responses, as exemplified by IGRP-specific CD8+ T cells, which rapidly outnumber insulin-specific cells in the NOD model [28]. This consideration is particularly relevant in human, as even prevention trials enroll at-risk subjects at a relatively late stage, once the first signs of β-cell autoimmunity (i.e., aAbs) are already detectable, and Ag targeting has probably already diversified. Third, even among inbred NOD mice kept under identical environmental exposure, variations exist in the specificity of CD8+ T-cell responses [29]. Such variations are certainly more extensive in the outbred human population. Fourth, drawing a straight correlation between NOD mice and humans is a gross approximation even in the case of target Ags. Indeed, data in the NOD mouse suggest that GAD and IA-2 are dispensable Ags [30, 31], while they are major targets of aAbs and T-cell responses in humans, and even promising therapeutics in the case of GAD [32]. Of the β-cell Ags described, only insulin and GAD have reached the stage of clinical trials and will be discussed, as summarized in Table 1.

Ag-based biologics can be divided into the following categories (Figure 2):

(i) whole proteins,

(ii) peptides,

(iii) modified protein and peptide Ags,

(iv) DNA plasmids,

(v) Ag-specific cell therapies.

6. Whole Proteins

The main advantage of using whole proteins is that they cover the complete amino acid sequence potentially available for epitope processing and presentation by Ag-presenting cells. Contrary to peptide epitopes, one single agent can be used in all patients, independently of their HLA haplotypes. However, production of the protein in recombinant form at sufficient purity and clinical grade can be challenging, as these preparations are frequently spiked with small contaminants carried over from the host bacteria, yeasts, or baculoviral systems that need to be removed. Only insulin and GAD have so far been employed as whole protein agents for T1D.

6.1. Insulin. The rationale of insulin-based T1D clinical trials is twofold. First, to restore insulin-specific immune tolerance. Second, to put the β cell "at rest", by providing hormonally active exogenous insulin, thus avoiding to overload the endogenous secretory capacity. Indeed, metabolic stress could not only precipitate β-cell apoptosis [33], but also make β cells more immunogenic and thus susceptible to destruction by autoreactive T cells [34].

Despite this appealing rationale and solid (pre)clinical grounds obtained in the NOD mouse [35–37] and in small-scale pilot human studies [38–40], a number of insulin-based trials, both preventative and interventional, have been disappointingly unsuccessful. Insulin of human origin was used in all of these studies. In the diabetes prevention trial-1 (DPT-1) [41], subjects at high risk of developing T1D were enrolled. These were defined as being positive for islet cell aAbs and already displaying early alterations of insulin secretion, documented by loss of the first-phase insulin response. For such subjects, the projected 5-year risk is >50%. Participants were treated with parenteral insulin—subcutaneous injections twice daily, plus annual intravenous infusions—for a median followup of 3.7 years. Subcutaneous ultralente insulin was administered at a dose of 0.125 U/kg twice daily and intravenous regular insulin given every 12 months for 4 days at a basal rate of 0.015 U/kg/h, which was increased for meals. Despite this intensive treatment, there was no protection on subsequent T1D development. Similar results were obtained in a smaller European trial employing subcutaneous insulin [42]. This outcome is not as surprising when comparing the DPT-1 strategy with its founding preclinical studies in the NOD mice [35]. Indeed, NOD mice were treated at doses of 0.5 U per animal, that is, ~25 U/kg, a dose which is 200-fold higher of what was used in the DPT-1. Regimens like those used in mouse studies would correspond to 1,750 U for an average adult human, which is far above the maximal tolerable dose. Moreover, in most studies, NOD mice were treated continuously for up to 6 months. It is, thus, possible that insulin treated ongoing diabetes rather than preventing incipient disease in some animals. The risk of hypoglycemia is a major concern for translation into human, while mice are quite resistant to insulin-induced hypoglycemia, possibly due to stronger counter-regulatory hormone responses. Longer-acting insulins such as glargine are now available which display a lower risk of hypoglycemia and constant levels throughout most of the day. This pharmacokinetic profile could be interesting not only in widening the therapeutic window of safe dosage, but also by providing low level Ag persistence without significant pulsatility, which may be more effective for tolerance induction [43]. Surprisingly, a mutated insulin (B25Asp) devoid of hypoglycemic activity (due to very low binding affinity for the insulin receptor) and preserving immunogenicity [44] has never been considered for clinical trials, despite encouraging studies in the NOD mouse [45]. B25Asp insulin could be administered at much higher doses, similar to those used in mice, while minimizing the risk of hypoglycemia.

The DPT-1 trial also comprised an oral arm, in which at-risk subjects were treated by oral insulin (7.5 mg/day) for a

Table 1: Clinical trials in T1D using antigen-specific strategies.

Antigen type	Antigen	Formulation	Route	Trial (Phase)	Subjects	Outcome and/or immune biomarkers	Reference
Protein	Insulin	Short-acting insulin	Intravenous	Intravenous insulin (Phase I)	Recent-onset T1D	Higher stimulated C peptide and lower HbA1c versus s.c. NPH insulin	[38]
Protein	Insulin	(Ultra)lente/regular insulin (s.c.) + short-acting insulin (i.v.)	Subcutaneous + Intravenous	Joslin Insulin Prophylaxis Trial (Phase I)	At risk	Suggestive of efficacy	[39]
Protein	Insulin	Lente/short-acting insulin (s.c.) + short-acting insulin (i.v.)	Subcutaneous + Intravenous	Schwabing Insulin Prophylaxis Trial (Phase I)	At risk	Suggestive of efficacy	[40]
Protein	Insulin	Ultralente insulin (s.c.) + short-acting insulin (i.v.)	Subcutaneous + Intravenous	DPT-1 Parenteral arm (Phase III)	At risk	No effect	[41]
Protein	Insulin	Ultralente insulin	Subcutaneous	EPPSCIT (Phase II)	At risk	No effect	[42]
Protein	Insulin	Short-acting insulin	Oral	ORALE (Phase II)	Recent-onset T1D	No effect	[46]
Protein	Insulin	Short-acting insulin	Oral	IMDIAB VII (Phase II)	Recent-onset T1D	No effect	[47]
Protein	Insulin	Short-acting insulin	Oral	DPT-1 Oral arm (Phase III)	At risk	Some efficacy in IAA^+ subjects	[49]
Protein	Insulin	Short-acting insulin	Oral	Oral insulin tolerance (Phase III)	Recent-onset T1D	1 mg improved C-peptide responses in older patients;10 mg accelerated C-peptide decline in younger patients	[48]
Protein	Insulin	Short-acting insulin	Oral	NIH/ADA/JDRF oral insulin (Phase III)	At-risk IAA^+	Ongoing	NCT 00419562
Protein	Insulin	Short-acting insulin	Intranasal	INIT-I (Phase I)	At-risk IAA^+	Increase in aAb and decrease in T-cell proliferative responses to insulin	[50]
Protein	Insulin	Short-acting insulin	Intranasal	DIPP (Phase III)	At risk	No effect	[51]
Protein	Insulin	Short-acting insulin	Intranasal	Intranasal insulin in T1D patients (Phase II)	Recent-onset, non-insulin-dependent T1D	No effect; decrease in IFN-γ T-cell responses to PI; decrease in Ab responses to exogenous insulin	[52]
Protein	Insulin	Short-acting insulin	Intranasal	INIT-II (Phase II)	At risk with preserved 1st phase insulin response	Ongoing	NCT 00336674
Protein	Insulin	Short-acting insulin	Oral or Intranasal	Pre-POINT	IAA^- chidren at high genetic risk for T1D	Planned	[123]
Protein	GAD	Recombinant GAD in alum (Diamyd)	Subcutaneous	Swedish Diamyd (Phase II)	Recent-onset T1D	Slower decline in fasting and stimulated C-peptide secretion; increase in anti-GAD aAbs and in FowP3 and TGF-β mRNA	[32]

Table 1: Continued.

Antigen type	Antigen	Formulation	Route	Trial (Phase)	Subjects	Outcome and/or immune biomarkers	Reference
Protein	GAD	Recombinant GAD in alum (Diamyd)	Subcutaneous	EU Diamyd (Phase III)	Recent-onset T1D	Ongoing	NCT 00723411
Protein	GAD	Recombinant GAD in alum (Diamyd)	Subcutaneous	US Diamyd (DIAPREVENT) (Phase III)	Recent-onset T1D	Ongoing	NCT 00751842
Protein	GAD	Recombinant GAD in alum	Subcutaneous	NIDDK/ADA/JDRF GAD-alum (Phase II)	Recent-onset T1D	Ongoing	NCT 00529399
Protein	GAD	Recombinant GAD in alum (Diamyd)	Subcutaneous	DIAPREV-IT (Phase II)	At risk, GAD $aAb^+ + \geq 1$ other aAb	Ongoing	NCT 01122446
Peptide	Insulin	Insulin B chain in incomplete Freund's adjuvant	Intramuscular	IBC-VS01 (Phase I)	Recent-onset IAA^+ T1D	Increased TGF-β production	[66]
Peptide	Proinsulin	PI_{C19-A3}	Intradermal	PI peptide immunotherapy (Phase I)	Long-standing T1D	Transient PI-specific IL-10 secretion in 3/18 patients at 30 μg	[67]
Modified peptide	Insulin	NBI-6024 (B_{9-23} APL)	Subcutaneous	NBI-6024-0003 (Phase I)	Recent-onset T1D	Shift from Th1 to Th2 responses	[76]
Modified peptide	Insulin	NBI-6024 (B_{9-23} APL)	Subcutaneous	Neurocrine NBI-6024 (Phase II)	Recent-onset T1D	No effect	[77]
Modified protein	Insulin	Insulin-coupled ECDI-fixed autologous leukocytes	?	ITN insulin-coupled leukocytes	At risk	Planned	[86]
DNA plasmid	Proinsulin	BHT-3021 (PI plasmid)	Intramuscular	Bayhill BHT-3021 (Phase I)	Recent-onset T1D	Ongoing	NCT 00453375
Ag-specific cell therapy	None	Autologous monocyte-derived DCs treated with CD40/CD80/CD86 antisense oligonucleotides	Intradermal	Pittsburgh DC vaccine (Phase I)	Long-standing T1D	Ongoing	NCT 00445913

median of 4.3 years. Also, in this case, no significant protection was induced, in line with the negative results of smaller trials [46–48]. However, analysis of a subgroup positive for IAA suggested a potential benefit, as the annualized T1D rate was 6.2% with oral insulin and 10.4% with placebo [49]. It is possible that the intervention may be more effective in these individuals due to a more active autoimmunity against insulin. This observation has prompted a new oral insulin trial focused on IAA+ at-risk subjects (ClinicalTrials.gov NCT00419562). It also underlines the importance of a through autoimmunity profiling to optimize enrollment (see below).

Similar prevention and intervention trials using intranasal insulin administration proved safe but did not yield significant T1D protection [50–52]. One further prevention trial is ongoing (ClinicalTrial.gov NCT00336674). Interestingly, no study has so far tried to administer PI rather than insulin. This could be attractive for a number of reasons. First, PI has a much lower affinity for the insulin receptor. Although it could be degraded into insulin once administered, the window of safe doses not engendering hypoglycemia could be wider. Second, a number of critical epitopes have been described which are specific of PI, as they reside either in the C peptide or at its junction with the B chain [19, 53]. Important epitopes have also been described which are specific of PPI, as they lie in the leader sequence [19, 34, 54]. Although PPI is more difficult to produce due to its lower solubility in water, this characteristic could be even advantageous to obtain a depot effect once injected subcutaneously.

6.2. GAD. In a recent intervention trial, Ludvigsson et al. treated new-onset (<18 mo) T1D children positive for anti-GAD aAbs with two subcutaneous injections of GAD (20 μg) or placebo in alum adjuvant and followed this children for 30 months. Although there was no change in insulin

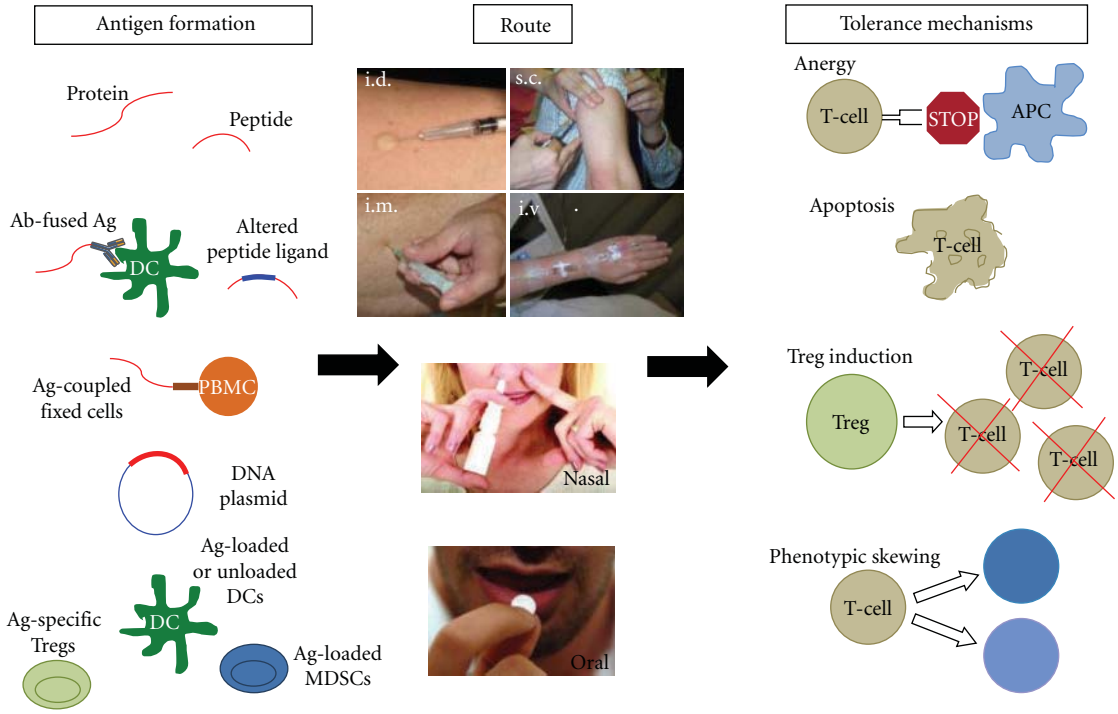

Figure 2: Ag-specific immune therapies. Different Ag formulations can be administered via different routes, triggering various tolerance mechanisms. APC: Ag-presenting cell; DC: dendritic cell; i.d.: intradermal; i.m., intramuscular; i.v.: intravenous; MDSCs: myeloid-derived suppressor cells; PBMC: peripheral blood mononuclear cell; s.c.: subcutaneous; Tregs: regulatory T cells.

requirements, there was a slower decline in the fasting and stimulated C-peptide secretion (a common measure of residual β-cell function). Importantly, this effect was observed only in those patients treated within 6 months of diagnosis. Similar observations have been made in other clinical trials, such as the European anti-CD3 trial, where lower insulin requirements were observed only in those patients who had higher residual β-cell function at the time of enrollment [55]. Taken together, they suggest that "the earlier the better," and that interventions at a preclinical stage would be much more beneficial.

In the GAD trial, the effect on C-peptide secretion was accompanied by an increase in anti-GAD aAb titers and in mRNA expression of FoxP3 and transforming growth factor (TGF)-β in peripheral blood mononuclear cells, which could suggest a regulatory switch. It may be counter-intuitive that the GAD vaccine is administered along with an alum adjuvant, which is known to favor immunogenic rather than tolerogenic responses, especially with the "prime-boost" strategy used in this trial. This peculiarity may raise the possibility that the therapeutic effect may be due, at least in part, to the high titers of anti-GAD Abs induced after vaccination [56]. Indeed, a protective effect of anti-islet aAbs has been evoked to explain the lower T1D risk observed in the offspring of aAb+ T1D mothers compared to aAb-negative ones [57]. Alternative strategies using GAD vaccination in the absence of adjuvant would be equally worth testing. Larger intervention trials using the GAD alum preparation are in progress in Europe and US and will allow to validate the effect on C-peptide secretion and to further explore its immune correlates (ClinicalTrials.gov NCT00723411, NCT00751842, and NCT00529399). A prevention trial is also in progress in Sweden (NCT01122446).

7. Peptides

Peptides have the significant edge of being easier to synthesize at high purity than recombinant proteins. Long peptides may have the advantage of covering multiple epitopes, thus targeting different T-cell specificity. Moreover, the use of long peptides requires processing before loading on HLA molecules. This requirement may allow some selectivity in the Ag-presenting cells targeted, as only professional Ag-presenting cells—most notably immature, tolerogenic dendritic cells—can efficiently process and present exogenous peptides. However, there are also multiple drawbacks compared to protein Ags. First, although peptides are smaller molecules and thus deliver up to 50 times more agent on a weight-for-weight basis when compared with protein Ags, the life span of peptides in blood—and likely in other tissues—is very short, in the order of minutes [58]. Second, peptides (but also epitopes processed from protein Ags) may be presented in alternative ways, thus triggering unwanted T-cell activation [59, 60]. Third, the same peptide dose can trigger diverse effects on different T cells, depending on their relative Ag avidity (e.g., simple activation versus activation-induced apoptosis) [61, 62]. Fourth, activation-induced

apoptosis, which is one of the mechanisms through which peptides may work, develops through a phase of deleterious activation before driving T cells to death. The drawbacks of this type of peptide-based approaches are exemplified by the disappointing results obtained on multiple sclerosis patients. Two clinical trials were prematurely terminated because of disease flare-ups rather than remissions [63–65]. Although these trials employed altered peptide ligands (see below), the same caveats may apply to native peptides.

There is no sufficient hindsight from T1D clinical trials to judge whether these theoretical hurdles are encountered. In a Phase I study on 12 new-onset T1D patients, a single intramuscular administration of insulin B chain (i.e., a 30-amino-acid polypeptide) or placebo in incomplete Freund's adjuvant was safe and induced robust insulin-specific humoral and T-cell responses but no difference in stimulated C-peptide responses. Given the small sample size and patients' recruitment irrespective of residual C-peptide secretion, it is not possible to draw conclusions concerning clinical efficacy. However, B-chain-stimulated CD4+ T cells from B-chain-treated patients exhibited higher TGF-β secretion in the first 3 months after treatment as compared to T cells from placebo-treated patients, yielding clones with profiles suggestive of a regulatory phenotype [66].

A Phase I clinical trial with a DR*04:01-restricted PI peptide C19-A3 in long-standing (>5 years) T1D patients has also been reported [67]. This trial employed intradermal administration at a dose of 30 or 300 μg repeated 3 times within 2 months, enrolling 18 patients in each arm (peptide versus no treatment). The therapy was well tolerated and did not induce proinflammatory reactivation of PI-specific T cells, but rather transient PI-specific IL-10 secretion in 3 of the 18 patients treated at the lower dose. As is the case for Phase I trials, the aim of this study was to assess safety and not clinical efficacy, and the inclusion criteria reflect this rationale (long-standing T1D patients with glucagon-stimulated C peptide $\leq$0.2 nmol/L; control group left untreated, i.e., no placebo). Although the observed safety profile is reassuring, it should be noted that PI-specific T-cell responses were not detectable at baseline in these long-standing patients, contrary to what previously observed in new-onset ones [68]. Thus, the effect of peptide administration may be different in the presence of recently *in vivo* primed PI-specific T cells and of a higher residual β-cell mass.

Clinical trials with a peptide derived from the heat-shock protein (hsp60) immunodominant epitope 437–460 (DiaPep277) showed marginally preserved endogenous insulin production in some newly diagnosed T1D patients [69, 70]. The mechanism by which the DiaPep277 may function is not completely understood. Qualifying hsp60 as a target Ag in T1D autoimmunity is debatable, and alternative, non-Ag-specific mechanisms are possible. Indeed, hsp60 has been shown to activate T cells via the Toll-like receptor 2 and to inhibit T-cell chemotaxis [71]. This pathway could also induce the shift from T helper (Th)1 to Th2 cytokines observed in humans. Thus, this mode of action would categorize DiaPep277 as a systemic rather than an Ag-specific immune modulator.

8. Modified Protein and Peptide Ags

Disarming pathogenic T cells by modifying the epitopes they recognize at key amino acid positions is an option that has been sought in several autoimmune diseases. TCR interaction with these modified epitopes, called altered peptide ligands (APLs), can result in only partial activation and dramatically different T-cell phenotypes, ranging from inducing selective stimulatory functions to completely turning off their functional capacity [72]. The central role of the insulin $B_{9–23}$ epitope in the NOD mouse [23, 73] and its immunodominance in T1D patients [74] led to develop an APL version called NBI-6024. This $B_{9–23}$ APL showed promising results in NOD mice [75]. Results of a Phase I clinical study on recent-onset T1D patients suggested that NBI-6024 treatment shifted Th1 responses to a Th2 phenotype [76]. However, a subsequent Phase II, dose-ranging trial testing repeated NBI-6024 subcutaneous treatment at 0.1, 0.5, or 1 mg did not preserve β-cell function [77]. Clinical trials with APLs in multiple sclerosis further chilled down enthusiasm for this approach. Two Phase II trials with a myelin basic protein APL were prematurely terminated, as treatment led to disease exacerbations and hypersensitivity reactions [63, 64]. This dramatic experience underlines the difficulty of developing one single APL which would induce the same effect on all cognate T cells. It is possible that the APL effect may be dependent on parameters such as effect-ive dose delivered and T-cell avidity, activation status and phenotype, and thus trigger different polarizing signals [65].

As DCs present Ags in a tolerogenic manner in the steady state, for example, in the absence of infection or inflammation, selective Ag delivery to DCs promotes tolerance in the absence of maturation stimuli [78–80]. To this end, a number of mAbs against endocytic receptors expressed by DCs have been developed, with the aim of using them to target Ag delivery. DEC-205 (CD205) is one of such receptors, which is expressed on the CD8+ DC subset capable to efficiently cross-present [81]. Besides selective targeting, this *in vivo* Ag delivery approach would obviate the need for leukapheresis and *ex vivo* DC manipulation. On these grounds, the group of T. DiLorenzo delivered the IGRP mimotope NRP-V7 *in vivo* to murine DCs by fusing it with an anti-DEC-205 mAb. Proliferation of transferred NRP-V7-specific T cells was initially observed, but this was followed by deletion. Tolerance was achieved because rechallenge of mice with NRP-V7 in adjuvant did not induce an immune response. NRP-V7 delivery through DEC-205 was tolerogenic only when performed in the steady state. As expected, co-administration of anti-CD40 mAb and polyI:C to mature DCs led instead to enhanced expansion of transferred T cells [82]. Although evidence of T1D protection is still missing, this approach may prove of great interest when combined with suitable epitopes. Moving towards this direction, the feasibility of DEC-205 delivery of the entire PPI molecule is

under investigation [83]. This would facilitate translation to patients expressing diverse MHC molecules.

Another original system of tolerogenic Ag delivery has been developed by Miller and coworkers [84–86]. This consists in administration of self peptides covalently cross-linked to cells via ethylene carbodiimide (ECDI). In preclinical models of various autoimmune diseases, this approach involves ECDI cross-linking of self proteins or peptides to syngeneic splenocytes. Intravenous injection of these Ag-coupled splenocytes is highly effective at inducing tolerance for both prevention and treatment of various autoimmune diseases in mouse models, including experimental autoimmune encephalomyelitis (a mouse model of multiple sclerosis) [87] and T1D in the NOD mouse [88]. Ag-coupled splenocytes behave like apoptotic bodies and are rapidly uptaken by Ag-presenting cells, much more efficiently than soluble protein or peptide Ags [89]. Ag presentation leads to tolerance induction by inducing Ag-specific T-cell unresponsiveness *via* two synergistic mechanisms: programmed death (PD)-1/PD ligand 1-mediated anergy and T regulatory cell (Treg) activation [88]. Processing and presentation of Ags coupled to apoptotic bodies gives the advantage of inducing tolerance by cellular carriers fixed with peptides, intact proteins, or even crude homogenates of the target organ [87]. This tolerogenic system is currently being tested in multiple sclerosis, using ECDI-fixed autologous peripheral blood leukocytes coupled with a cocktail of seven myelin peptides. A similar trial using insulin-coupled autologous leukocytes for prevention of T1D is under development by the Immune Tolerance Network.

9. DNA Plasmids

DNA vaccines are the simplest embodiment of vaccines that, rather than consisting of the Ag itself, provide genes for endogenous synthesis of the protein Ag. The idea stemmed from the need to efficiently deliver protein and polypeptide Ags to the MHC Class I pathway for presentation to and stimulation of CD8+ T-cell responses. Inducing such responses with exogenous protein Ags is not efficient, as it requires cross-presentation, a requirement which can be fulfilled mostly if not exclusively by specialized DC subsets. As DNA-encoded Ags are endogenously translated by transfected cells, DNA immunization has the potential to result in conventional priming as well as cross-priming.

There are many ways to efficiently deliver DNA vaccines [90], and this strategy has been exploited for many research applications such as T-cell epitope identification [91, 92]. One strategy consists of bombarding the epidermis with gold microbeads coated with plasmid, which can directly transfect Langerhans cells causing their rapid migration to draining lymph nodes. Alternatively, plasmids can be intramuscularly injected, leading to predominant transfection of myocytes and less efficient Ag presentation. This latter approach is currently been explored for tolerance induction, using a plasmid encoding for proinsulin (BHT-3021) in T1D patients with less than 5 years of disease (ClinicalTrials.gov NCT00453375). The relatively long disease duration used for patient recruitment and the low potency of DNA vaccines documented in other clinical trials may be important factors in the final outcome.

10. Ag-Specific Cell Therapies

Ag-specific tolerance restoration could be induced not only by using suitable Ag proteins or peptides, but also by adoptively transferring cells armed to be selective for the targets of choice. These approaches are being explored for Tregs and DCs [93].

Expansion protocols to obtain high numbers of CD4+ Tregs suitable for *in vivo* cell therapies have been developed for both mouse and human [94–97]. However, these cells remain polyclonal in nature although a shortcut has been obtained in murine models by expanding T cells from mice transgenic for a T-cell receptor which recognizes a specific β-cell epitope such as BDC2.5. Although some pioneering reports of expanded human β-cell Ag-specific Tregs have been published [98, 99], it is not clear whether these cells would have suitable characteristics to exert therapeutic effects once transferred *in vivo*. Further incertitude is added by a recent paper describing that polyclonal murine CD4+ Tregs can revert their phenotype after *in vivo* transfer, losing FoxP3 expression and acquiring pathogenic potential. Indeed, these "ex-Tregs" were found to accumulate spontaneously in the insulitis of NOD mice, suggesting that this phenomenon may not be peculiar of transfer settings. Even more worryingly, these ex-Tregs were capable of efficiently transferring disease, similar to standard T effector cells [100].

Another cell-based approach consists of loading immature DCs with the Ag of interest. This type of approach has previously been shown to be efficient at promoting Ag-specific immune tolerance [101, 102]. This strategy is now being tested for T1D intervention in a Phase I safety study led by M. Trucco. Tolerogenic autologous monocyte-derived DCs are being generated by treating them *ex vivo* with antisense oligonucleotides targeting the CD40, CD80, and CD86 costimulatory molecules [103]. These modified DCs are being compared with control autologous monocyte-derived DCs left untreated. The fact that intravenously administered bone-marrow-derived DCs accumulate predominantly in the spleen and the pancreatic and tracheal lymph nodes [104] justifies the choice of not loading these DCs with exogenous Ag. These DCs may acquire islet Ags *in vivo* in pancreatic lymph nodes and could thus modulate effector and regulatory T-cell responses to T1D-relevant Ags even without deliberate prior Ag loading. The other peculiarity of this trial is that DCs (which are not used fresh but cryopreserved) will be administered intradermally closest to the physical location of the pancreas, as both sites may drain to the same lymph nodes (ClinicalTrials.gov NCT00445913). Importantly, preclinical studies suggest that the therapeutic effect of these unloaded tolerogenic DCs remains Ag-specific, as splenic T cells from treated mice proliferated to allo-Ags *ex vivo* [103].

Another cell population which may soon be tested for cell-based immunotherapy is myeloid-derived suppressor

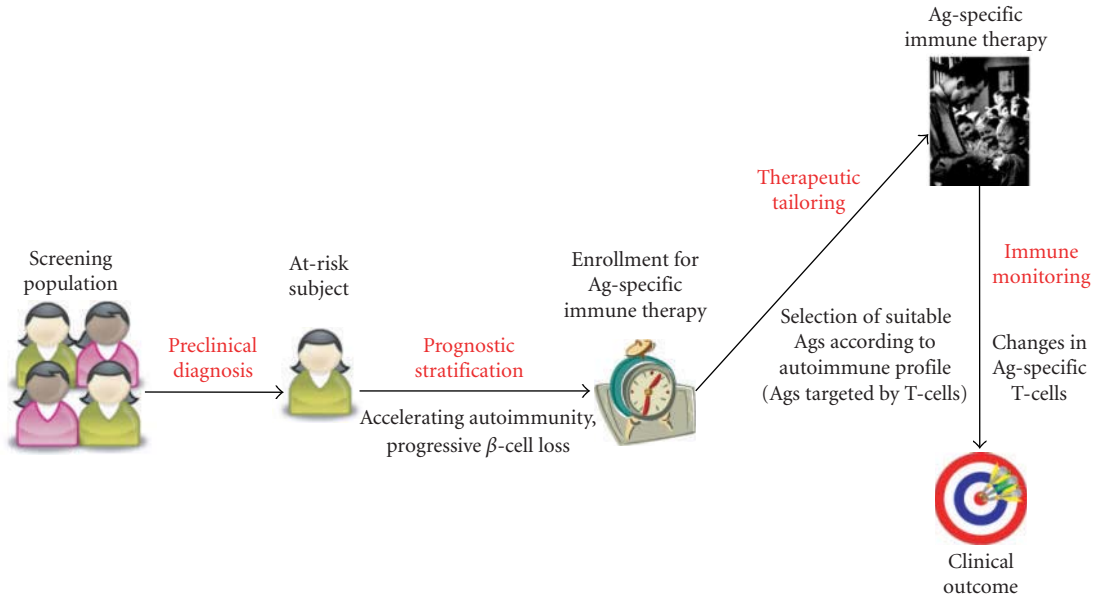

Figure 3: "Immune staging" of T1D. Biomarkers of β-cell autoimmunity such as aAbs and T cells could help to identify at-risk subjects at an early stage (preclinical diagnosis) and to follow them up over time to decide the need for immune therapy and the best timing for treatment (prognostic stratification). Ag-specific immune therapy could be personalized for each subject by administering therapeutic formulations of those Ags targeted by aAb and/or T-cell responses. Modifications induced on such responses could be followed in real time during treatment, thus allowing to assess immune efficacy prior to and independent of clinical outcome.

cells (MDSCs). These are cells of myeloid origin with immunoregulatory activity that can suppress Ag-specific and nonspecific T-cell responses *via* different mechanisms in cancer [105–107], transplantation [108], and T1D [109]. Pre-clinical study testing the protective role of these cells in T1D were performed in RIP-HA mice transferred with HA-specific CD4+ T cells, where it was shown that HA-loaded MDSCs could act as APCs in an Ag-specific fashion to induce anergy of effector T cells, development of Tregs, and T1D prevention [109].

11. The Importance of Immune Surrogate Markers

In light of the formidable challenge of rescuing a significant β-cell mass in already diabetic patients, it is important to evaluate not only clinical (lower HbA1c values and reduced insulin requirements) and metabolic (C-peptide secretion) endpoints, but also immune surrogate endpoints. This is even more important in trials not showing clinical benefit, or not poised to detect such benefits, as is commonly the case for phase I studies. Indeed, the lack of clinical effects could be open to disparate interpretation, including late treatment in front of full-blown disease, the need to target multiple Ags in advanced T1D once epitope spreading has occurred, or failure to restore immune tolerance. These possibilities can be sorted out by immune monitoring analyses performed before, during, and after treatment, particularly by scrutinizing changes induced in T-cell responses specific to the administered Ag (Figure 3). Validation and standardization of blood sample processing and T-cell assay procedures is an important goal to this end [110–112].

We recently reported a proof-of-concept study on aAb+ diabetic patients not requiring insulin at the time of diagnosis. These patients were treated with intranasal insulin in an attempt to save residual β cells. Although nasal insulin treated patients eventually progressed towards insulin dependency at a rate similar to placebo-treated ones, we could document successful induction of insulin-specific immune tolerance both at the T-cell and antibody level [52]. Contrary to what observed in the placebo arm, patients treated with intranasal insulin displayed significant reductions in frequencies of interferon-γ-secreting PI-specific T-cell responses, using high sensitivity T-cell assays that amplify responses by means of an accelerated cocultured DC (acDC) stimulation (Martinuzzi et al., under revision). This effect was Ag-specific, as it was not observed for responses towards the tetanus toxoid recall Ag. The initiation of insulin therapy further documented that this PI-specific tolerance was operational *in vivo*, as intranasal insulin treated subjects failed to develop anti-insulin antibodies [52]. These results suggest that this intervention is immunological effective, but not sufficient to rescue β cells, probably because administered too late, at an advanced stage where most islets have already disappeared and β-cell autoimmunity has already spread too far on additional Ag specificities. Knowing whether Ag-specific T-cell responses are modified and in which individuals provides key mechanistic information to plan further trials and modify therapeutic strategies accordingly. This information may also be critical to optimize enrollment strategies. Indeed, pretreatment testing for T-cell reactivities

could allow to focus enrollment on those patients displaying active insulin-specific autoimmune responses, as there might be little benefit in treating those who do not harbor such responses (Figure 3). Imaging techniques allowing to visualize β-cell mass and infiltration will provide additional tools for immune-based pretreatment staging and post-treatment monitoring [113].

12. Combination Therapies

Despite the mentioned evidence in the NOD mouse for an initiating role of insulin as the primary β-cell Ag target, the same principle may not apply to outbred humans, due to the variable genetic and environmental background and the later timing of intervention and even prevention trials, at a stage where epitope spreading is likely to have occurred. Therefore, strategies where multiple β-cell Ags (e.g., PI and GAD) are combined may yield synergistic effects and more substantial clinical benefit. This possibility will certainly be explored in upcoming clinical trials. Another option under consideration consists of combining Ags with immune modulatory mAbs such as anti-CD3 and anti-CD20, which have already been trialed alone, sometimes with encouraging results [114]. Besides the potential for synergy, this approach may allow to reduce mAb dosing, thus limiting unwanted side effects and facilitating repeated treatment [115–117]. Combination of tolerogenic strategies with approaches aimed at replacing [118, 119] or regenerating [120] β cells are also attractive, as they may allow to intervene at later stages. Although a first clinical trial testing exenatide alone or in combination with daclizumab did not show any effect [121], other agents and combinations thereof need to be tested before drawing definite conclusions.

13. Conclusions

We are moving towards a new era of clinical trials in T1D, where the long-sought goal of Ag-specific immune tolerance is becoming at reach. To turn these promises into reality, it will be important to comparatively evaluate several aspects that remain poorly defined. (1) Administration routes: intravenous, subcutaneous, intradermal, and mucosal. Regarding the latter, it is surprising that only oral and intranasal routes have been investigated for T1D, leaving out the sublingual approach despite its successful track record for allergy desensitization [122]. (2) Ag dose, identifying suitable therapeutic ranges, which will critically depend on the nature of the Ag and the route of administration. We have discussed the hurdles of translating the insulin doses used in NOD mouse studies into clinical trials. Importantly, difference insulin formulations may widen the therapeutic window. Another key difference between preclinical and human studies is the timing of intervention. A proposed primary prevention trial (Pre-POINT) [123] aims to address this issue of disease stage by intervening with oral or nasal insulin in children genetically predisposed to T1D, before the appearance of the first signs of β-cell autoimmunity (i.e., in children who have not yet developed IAA). (3) Frequency and interval of administration, keeping in mind that approaches promoting longer persistence of low Ag loads may be beneficial over high-load spikes [43]. Ancillary mechanistic studies should accompany these trials to clarify modes of action and help optimizing the risk-to-benefit ratio. Autoimmune T-cell profiling may be particularly useful for tailoring and monitoring treatment in each individual patient.

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References

[1] J. Mestas and C. C. W. Hughes, "Of mice and not men: differences between mouse and human immunology," *Journal of Immunology*, vol. 172, no. 5, pp. 2731–2738, 2004.

[2] S. Sreenan, A. J. Pick, M. Levisetti, A. C. Baldwin, W. Pugh, and K. S. Polonsky, "Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse," *Diabetes*, vol. 48, no. 5, pp. 989–996, 1999.

[3] W. Gepts, "Pathologic anatomy of the pancreas in juvenile diabetes mellitus," *Diabetes*, vol. 14, no. 10, pp. 619–633, 1965.

[4] A. K. Foulis, M. A. Farquharson, and R. Hardman, "Aberrant expression of Class II major histocompatibility complex molecules by B cells and hyperexpression of Class I major histocompatibility complex molecules by insulin containing islets in Type 1 (insulin-dependent) diabetes mellitus," *Diabetologia*, vol. 30, no. 5, pp. 333–343, 1987.

[5] A. K. Foulis, C. N. Liddle, and M. A. Farquharson, "The histopathology of the pancreas in Type I (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom," *Diabetologia*, vol. 29, no. 5, pp. 267–274, 1986.

[6] D. J. Klinke, "Extent of beta cell destruction is important but insufficient to predict the onset of type 1 diabetes mellitus," *PLoS ONE*, vol. 3, no. 1, Article ID e1374, 2008.

[7] N. A. Sherry, E. B. Tsai, and K. C. Herold, "Natural history of β-cell function in type 1 diabetes," *Diabetes*, vol. 54, supplement 2, pp. S32–S39, 2005.

[8] E. Akirav, J. A. Kushner, and K. C. Herold, "β-cell mass and type 1 diabetes: going, going, gone?" *Diabetes*, vol. 57, no. 11, pp. 2883–2888, 2008.

[9] L. I. Wen, R. E. Ley, P. Y. U. Volchkov et al., "Innate immunity and intestinal microbiota in the development of Type 1 diabetes," *Nature*, vol. 455, no. 7216, pp. 1109–1113, 2008.

[10] S. Fourlanos, P. Narendran, G. B. Byrnes, P. G. Colman, and L. C. Harrison, "Insulin resistance is a risk factor for progression to Type 1 diabetes," *Diabetologia*, vol. 47, no. 10, pp. 1661–1667, 2004.

[11] G. Pagano, V. Tagliaferro, and Q. Carta, "Metformin reduces insulin requirement in Type 1 (insulin-dependent) diabetes," *Diabetologia*, vol. 24, no. 5, pp. 351–354, 1983.

[12] J. Hamilton, E. Cummings, V. Zdravkovic, D. Finegood, and D. Daneman, "Metformin as an adjunct therapy in adolescents with type 1 diabetes and insulin resistance: a randomized controlled trial," *Diabetes Care*, vol. 26, no. 1, pp. 138–143, 2003.

[13] S. S. Lund, L. Tarnow, A. S. Astrup et al., "Effect of adjunct metformin treatment in patients with type-1 diabetes and persistent inadequate glycaemic control. A randomized study," *PLoS ONE*, vol. 3, no. 10, Article ID e3363, 2008.

[14] C. R. Stiller, J. Dupre, and M. Gent, "Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset," *Science*, vol. 223, no. 4643, pp. 1362–1367, 1984.

[15] P. A. Gottlieb, S. Quinlan, H. Krause-Steinrauf et al., "Failure to preserve β-cell function with mycophenolate mofetil and daclizumab combined therapy in patients with new-onset type 1 diabetes," *Diabetes Care*, vol. 33, no. 4, pp. 826–832, 2010.

[16] J. A. Bluestone, K. Herold, and G. Eisenbarth, "Genetics, pathogenesis and clinical interventions in type 1 diabetes," *Nature*, vol. 464, no. 7293, pp. 1293–1300, 2010.

[17] S. M. Lieberman and T. P. DiLorenzo, "A comprehensive guide to antibody and T-cell responses in type 1 diabetes," *Tissue Antigens*, vol. 62, no. 5, pp. 359–377, 2003.

[18] S. M. Lieberman, A. M. Evans, B. Han et al., "Identification of the β cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8384–8388, 2003.

[19] R. Mallone, E. Martinuzzi, P. Blancou et al., "CD8+ T-cell responses identify β-cell autoimmunity in human type 1 diabetes," *Diabetes*, vol. 56, no. 3, pp. 613–621, 2007.

[20] K. Thébault-Baumont, D. Dubois-Laforgue, P. Krief et al., "Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice," *Journal of Clinical Investigation*, vol. 111, no. 6, pp. 851–857, 2003.

[21] B. Faideau, C. Lotton, B. Lucas et al., "Tolerance to proinsulin-2 is due to radioresistant thymic cells," *Journal of Immunology*, vol. 177, no. 1, pp. 53–60, 2006.

[22] H. Moriyama, N. Abiru, J. Paronen et al., "Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10376–10381, 2003.

[23] M. Nakayama, N. Abiru, H. Moriyama et al., "Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice," *Nature*, vol. 435, no. 7039, pp. 220–223, 2005.

[24] D. Daniel, R. G. Gill, N. Schloot, and D. Wegmann, "Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice," *European Journal of Immunology*, vol. 25, no. 4, pp. 1056–1062, 1995.

[25] F. S. Wong, J. Karttunen, C. Dumont et al., "Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library," *Nature Medicine*, vol. 5, no. 9, pp. 1026–1031, 1999.

[26] B. Krishnamurthy, N. L. Dudek, M. D. McKenzie et al., "Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP," *Journal of Clinical Investigation*, vol. 116, no. 12, pp. 3258–3265, 2006.

[27] B. Krishnamurthy, L. Mariana, S. A. Gellert et al., "Autoimmunity to both proinsulin and IGRP is required for diabetes in nonobese diabetic 8.3 TCR transgenic mice," *Journal of Immunology*, vol. 180, no. 7, pp. 4458–4464, 2008.

[28] A. Amranl, J. Verdaguer, P. Serra, S. Tafuro, R. Tan, and P. Santamaria, "Progression of autoimmune diabetes driven by avidity maturation of a T- cell population," *Nature*, vol. 406, no. 6797, pp. 739–742, 2000.

[29] S. M. Lieberman, T. Takaki, B. Han, P. Santamaria, D. V. Serreze, and T. P. DiLorenzo, "Individual nonobese diabetic mice exhibit unique patterns of CD8+ T cell reactivity to three islet antigens, including the newly identified widely expressed dystrophia myotonica kinase," *Journal of Immunology*, vol. 173, no. 11, pp. 6727–6734, 2004.

[30] A. Kubosaki, S. Gross, J. Miura et al., "Targeted disruption of the IA-2β gene causes glucose intolerance and impairs insulin secretion but does not prevent the development of diabetes in NOD mice," *Diabetes*, vol. 53, no. 7, pp. 1684–1691, 2004.

[31] E. Jaeckel, L. Klein, N. Martin-Orozco, and H. Von Boehmer, "Normal incidence of diabetes in NOD mice tolerant to glutamic acid decarboxylase," *Journal of Experimental Medicine*, vol. 197, no. 12, pp. 1635–1644, 2003.

[32] J. Ludvigsson, M. Faresjö, M. Hjorth et al., "GAD treatment and insulin secretion in recent-onset type 1 diabetes," *The New England Journal of Medicine*, vol. 359, no. 18, pp. 1909–1920, 2008.

[33] J. Chandra, B. Zhivotovsky, S. Zaitsev, L. Juntti-Berggren, P. O. Berggren, and S. Orrenius, "Role of apoptosis in pancreatic β-cell death in diabetes," *Diabetes*, vol. 50, supplement 1, pp. S44–S47, 2001.

[34] A. Skowera, R. J. Ellis, R. Varela-Calviño et al., "CTLs are targeted to kill β cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope," *Journal of Clinical Investigation*, vol. 118, no. 10, pp. 3390–3402, 2008.

[35] M. A. Atkinson, N. K. Maclaren, and R. Luchetta, "Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy," *Diabetes*, vol. 39, no. 8, pp. 933–937, 1990.

[36] P. Gladstone and G. T. Nepom, "The prevention of IDDM: injecting insulin into the cytokine network," *Diabetes*, vol. 44, no. 7, pp. 859–862, 1995.

[37] N. Schloot and G. S. Eisenbarth, "Isohormonal therapy of endocrine autoimmunity," *Immunology Today*, vol. 16, no. 6, pp. 289–294, 1995.

[38] S. C. Shah, J. I. Malone, and N. E. Simpson, "A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 320, no. 9, pp. 550–554, 1989.

[39] R. J. Keller, G. S. Eisenbarth, and R. A. Jackson, "Insulin prophylaxis in individuals at high risk of type I diabetes," *The Lancet*, vol. 341, no. 8850, pp. 927–928, 1993.

[40] M. Füchtenbusch, W. Rabl, B. Grassl, W. Bachmann, E. Standl, and A. G. Ziegler, "Delay of Type I diabetes in high risk, first degree relatives by parenteral antigen administration: the Schwabing insulin Prophylaxis Pilot Trial," *Diabetologia*, vol. 41, no. 5, pp. 536–541, 1998.

[41] Diabetes Prevention Trial-Type 1 Diabetes Study G, "Effects of insulin in relatives of patients with type 1 diabetes mellitus," *The New England Journal of Medicine*, vol. 346, pp. 1685–1691, 2002.

[42] J. C. Carel, P. Landais, P. Bougnères, C. Rodriguez-Villar, R. Casamitjana, and I. Conget, "Therapy to prevent type 1 diabetes mellitus," *The New England Journal of Medicine*, vol. 347, no. 14, pp. 1115–1116, 2002.

[43] J. C. Guéry, F. Galbiati, S. Smiroldo, and L. Adorini, "Selective development of T helper (Th)2 cells induced by continuous administration of low dose soluble proteins to normal and β2-microglobulin-deficient BALB/c mice," *Journal of Experimental Medicine*, vol. 183, no. 2, pp. 485–497, 1996.

[44] A. Volund, J. Brange, K. Drejer et al., "In vitro and in vivo potency of insulin analogues designed for clinical use," *Diabetic Medicine*, vol. 8, no. 9, pp. 839–847, 1991.

[45] D. G. Karounos, J. S. Bryson, and D. A. Cohen, "Metabolically inactive insulin analog prevents type I diabetes in prediabetic NOD mice," *Journal of Clinical Investigation*, vol. 100, no. 6, pp. 1344–1348, 1997.

[46] L. Chaillous, H. Lefevre, C. Thivolet et al., "Oral insulin administration and residual beta-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale group," *The Lancet*, vol. 356, pp. 545–549, 2000.

[47] P. Pozzilli, D. Pitocco, N. Visalli et al., "No effect of oral insulin on residual beta-cell function in recent-onset Type I diabetes (the IMDIAB VII)," *Diabetologia*, vol. 43, no. 8, pp. 1000–1004, 2000.

[48] B. Ergun-Longmire, J. Marker, A. Zeidler et al., "Oral insulin therapy to prevent progression of immune-mediated (Type 1) diabetes," *Annals of the New York Academy of Sciences*, vol. 1029, pp. 260–277, 2004.

[49] J. S. Skyler, "Effects of oral insulin in relatives of patients with type 1 diabetes: the diabetes prevention trial-type 1," *Diabetes Care*, vol. 28, no. 5, pp. 1068–1076, 2005.

[50] L. C. Harrison, M. C. Honeyman, C. E. Steele et al., "Pancreatic β-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes," *Diabetes Care*, vol. 27, no. 10, pp. 2348–2355, 2004.

[51] K. Näntö-Salonen, A. Kupila, S. Simell et al., "Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial," *The Lancet*, vol. 372, no. 9651, pp. 1746–1755, 2008.

[52] S. Fourlanos, C. Perry, S. A. Gellert et al., "Evidence that nasal insulin induces immune tolerance to insulin in adults with autoimmune diabetes," *Diabetes*, vol. 60, no. 4, pp. 1237–1245, 2011.

[53] A. Toma, S. Haddouk, J. P. Briand et al., "Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 30, pp. 10581–10586, 2005.

[54] A. Toma, T. Laïka, S. Haddouk et al., "Recognition of human proinsulin leader sequence by class I-restricted T-cells in HLA-A$*$0201 transgenic mice and in human type 1 diabetes," *Diabetes*, vol. 58, no. 2, pp. 394–402, 2009.

[55] B. Keymeulen, E. Vandemeulebroucke, A. G. Ziegler et al., "Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes," *The New England Journal of Medicine*, vol. 352, no. 25, pp. 2598–2608, 2005.

[56] M. Chéramy, C. Skoglund, I. Johansson, J. Ludvigsson, C. S. Hampe, and R. Casas, "GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD enzyme activity and humoral response," *Clinical Immunology*, vol. 137, pp. 31–40, 2010.

[57] K. Koczwara, E. Bonifacio, and A. G. Ziegler, "Transmission of maternal islet antibodies and risk of autoimmune diabetes in offspring of mothers with type 1 diabetes," *Diabetes*, vol. 53, no. 1, pp. 1–4, 2004.

[58] S. Casares, C. A. Bona, and T. D. Brumeanu, "Enzymatically mediated engineering of multivalent MHC class II-peptide chimeras," *Protein Engineering*, vol. 14, no. 3, pp. 195–200, 2001.

[59] N. R. Martinez, P. Augstein, A. K. Moustakas et al., "Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1365–1371, 2003.

[60] A. Pugliese, "Peptide-based treatment for autoimmune diseases: learning how to handle a double-edged sword," *Journal of Clinical Investigation*, vol. 111, no. 9, pp. 1280–1282, 2003.

[61] R. Mallone, S. A. Kochik, E. M. Laughlin et al., "Differential recognition and activation thresholds in human autoreactive GAD-specific T-cells," *Diabetes*, vol. 53, no. 4, pp. 971–977, 2004.

[62] R. Mallone, S. A. Kochik, H. Reijonen et al., "Functional avidity directs T-cell fate in autoreactive CD4+ T cells," *Blood*, vol. 106, no. 8, pp. 2798–2805, 2005.

[63] B. Bielekova, B. Goodwin, N. Richert et al., "Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand," *Nature Medicine*, vol. 6, no. 10, pp. 1167–1175, 2000.

[64] L. Kappos, G. Comi, H. Panitch et al., "Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial," *Nature Medicine*, vol. 6, no. 10, pp. 1176–1182, 2000.

[65] C. P. Genain and S. S. Zamvil, "Specific immunotherapy: one size does not fit all," *Nature Medicine*, vol. 6, no. 10, pp. 1098–1100, 2000.

[66] T. Orban, K. Farkas, H. Jalahej et al., "Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy," *Journal of Autoimmunity*, vol. 34, no. 4, pp. 408–415, 2010.

[67] S. L. Thrower, L. James, W. Hall et al., "Proinsulin peptide immunotherapy in type 1 diabetes: report of a first-in-man Phase I safety study," *Clinical and Experimental Immunology*, vol. 155, no. 2, pp. 156–165, 2009.

[68] S. Arif, T. I. Tree, T. P. Astill et al., "Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health," *Journal of Clinical Investigation*, vol. 113, no. 3, pp. 451–463, 2004.

[69] I. Raz, D. Elias, A. Avron, M. Tamir, M. Metzger, and I. R. Cohen, "β-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial," *The Lancet*, vol. 358, no. 9295, pp. 1749–1753, 2001.

[70] I. Raz, A. Avron, M. Tamir et al., "Treatment of new-onset type 1 diabetes with peptide DiaPep277® is safe and associated with preserved beta-cell function: extension of a randomized, double-blind, phase II trial," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 4, pp. 292–298, 2007.

[71] A. Zanin-Zhorov, G. Nussbaum, S. Franitza, I. R. Cohen, and O. Lider, "T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors," *The FASEB Journal*, vol. 17, no. 11, pp. 1567–1569, 2003.

[72] J. Sloan-Lancaster and P. M. Allen, "Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology," *Annual Review of Immunology*, vol. 14, pp. 1–27, 1996.

[73] D. Daniel and D. R. Wegmann, "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 2, pp. 956–960, 1996.

[74] D. G. Alleva, P. D. Crowe, L. Jin et al., "A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin," *Journal of Clinical Investigation*, vol. 107, no. 2, pp. 173–180, 2001.

[75] D. G. Alleva, A. Gaur, L. Jin et al., "Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9-23) peptide," *Diabetes*, vol. 51, no. 7, pp. 2126–2134, 2002.

[76] D. G. Alleva, R. A. Maki, A. L. Putnam et al., "Immunomodulation in type 1 diabetes by NBI-6024, an altered peptide ligand of the insulin B epitope," *Scandinavian Journal of Immunology*, vol. 63, no. 1, pp. 59–69, 2006.

[77] M. Walter, A. Philotheou, F. Bonnici, A. G. Ziegler, and R. Jimenez, "No effect of the altered peptide ligand NBI-6024 on β-cell residual function and insulin needs in new-onset type 1 diabetes," *Diabetes Care*, vol. 32, no. 11, pp. 2036–2040, 2009.

[78] D. Hawiger, K. Inaba, Y. Dorsett et al., "Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo," *Journal of Experimental Medicine*, vol. 194, no. 6, pp. 769–779, 2001.

[79] L. Bonifaz, D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman, "Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance," *Journal of Experimental Medicine*, vol. 196, no. 12, pp. 1627–1638, 2002.

[80] L. C. Bonifaz, D. P. Bonnyay, A. Charalambous et al., "In vivo targeting of antigens to maturing dendritic cells via the DEC-205 receptor improves T cell vaccination," *Journal of Experimental Medicine*, vol. 199, no. 6, pp. 815–824, 2004.

[81] D. Dudziak, A. O. Kamphorst, G. F. Heidkamp et al., "Differential antigen processing by dendritic cell subsets in vivo," *Science*, vol. 315, no. 5808, pp. 107–111, 2007.

[82] A. Mukhopadhaya, T. Hanafusa, I. Jarchum et al., "Selective delivery of β cell antigen to dendritic cells in vivo leads to deletion and tolerance of autoreactive CD8+ T cells in NOD mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 17, pp. 6374–6379, 2008.

[83] G. Mukherjee and T. P. Dilorenzo, "The immunotherapeutic potential of dendritic cells in type 1 diabetes," *Clinical and Experimental Immunology*, vol. 161, no. 2, pp. 197–207, 2010.

[84] S. D. Miller, R. P. Wetzig, and H. N. Claman, "The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells," *Journal of Experimental Medicine*, vol. 149, no. 3, pp. 758–773, 1979.

[85] S. D. Miller, D. M. Turley, and J. R. Podojil, "Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease," *Nature Reviews Immunology*, vol. 7, no. 9, pp. 665–677, 2007.

[86] X. Luo, K. C. Herold, and S. D. Miller, "Immunotherapy of type 1 diabetes: where are we and where should we be going?" *Immunity*, vol. 32, no. 4, pp. 488–499, 2010.

[87] M. K. Kennedy, L. J. Tan, M. C. Dal Canto et al., "Inhibition of murine relapsing experimental autoimmune encephalomyelitis by immune tolerance to proteolipid protein and its encephalitogenic peptides," *Journal of Immunology*, vol. 144, no. 3, pp. 909–915, 1990.

[88] B. T. Fife, I. Guleria, M. G. Bupp et al., "Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway," *Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2737–2747, 2006.

[89] K. Inaba, S. Turley, F. Yamaide et al., "Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells," *Journal of Experimental Medicine*, vol. 188, no. 11, pp. 2163–2173, 1998.

[90] J. J. Donnelly, B. Wahren, and M. A. Liu, "DNA vaccines: progress and challenges," *Journal of Immunology*, vol. 175, no. 2, pp. 633–639, 2005.

[91] P. Blancou, R. Mallone, E. Martinuzzi et al., "Immunization of HLA class I transgenic mice identifies autoantigenic epitopes eliciting dominant responses in type 1 diabetes patients," *Journal of Immunology*, vol. 178, no. 11, pp. 7458–7466, 2007.

[92] E. Martinuzzi, F. A. Lemonnier, C. Boitard, and R. Mallone, "Measurement of CD8+ T cell responses in human type 1 diabetes," *Annals of the New York Academy of Sciences*, vol. 1150, pp. 61–67, 2008.

[93] J. A. Bluestone, A. W. Thomson, E. M. Shevach, and H. L. Weiner, "What does the future hold for cell-based tolerogenic therapy?" *Nature Reviews Immunology*, vol. 7, no. 8, pp. 650–654, 2007.

[94] Q. Tang, K. J. Henriksen, M. Bi et al., "In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1455–1465, 2004.

[95] K. V. Tarbell, S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman, "CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1467–1477, 2004.

[96] M. Battaglia, A. Stabilini, B. Migliavacca, J. Horejs-Hoeck, T. Kaupper, and M. G. Roncarolo, "Rapamycin promotes expansion of functional CD4+CD25+FOXP3+ regulatory T cells of both healthy subjects and type 1 diabetic patients," *Journal of Immunology*, vol. 177, no. 12, pp. 8338–8347, 2006.

[97] A. L. Putnam, T. M. Brusko, M. R. Lee et al., "Expansion of human regulatory T-cells from patients with type 1 diabetes," *Diabetes*, vol. 58, no. 3, pp. 652–662, 2009.

[98] S. A. Long, M. R. Walker, M. Rieck et al., "Functional islet-specific Treg can be generated from CD4+CD25 T cells of healthy and type 1 diabetic subjects," *European Journal of Immunology*, vol. 39, no. 2, pp. 612–620, 2009.

[99] J. A. Dromey, B. H. Lee, H. Yu et al., "Generation and expansion of regulatory human CD4(+) T-cell clones specific for pancreatic islet autoantigens," *Journal of Autoimmunity*, vol. 36, pp. 47–55, 2010.

[100] X. Zhou, S. L. Bailey-Bucktrout, L. T. Jeker et al., "Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo," *Nature Immunology*, vol. 10, no. 9, pp. 1000–1007, 2009.

[101] M. V. Dhodapkar and R. M. Steinman, "Antigen-bearing immature dendritic cells induce peptide-specific CD8(+) regulatory T cells in vivo in humans," *Blood*, vol. 100, no. 1, pp. 174–177, 2002.

[102] H. Hackstein, A. E. Morelli, and A. W. Thomson, "Designer dendritic cells for tolerance induction: guided not misguided missiles," *Trends in Immunology*, vol. 22, no. 8, pp. 437–442, 2001.

[103] J. Machen, JO. Harnaha, R. Lakomy, A. Styche, M. Trucco, and N. Giannoukakis, "Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells," *Journal of Immunology*, vol. 173, no. 7, pp. 4331–4341, 2004.

[104] R. J. Creusot, S. S. Yaghoubi, P. Chang et al., "Lymphoid tissue-specific homing of bone marrow-derived dendritic cells," *Blood*, vol. 113, no. 26, pp. 6638–6647, 2009.

[105] S. Kusmartsev, Y. Nefedova, D. Yoder, and D. I. Gabrilovich, "Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive

oxygen species," *Journal of Immunology*, vol. 172, no. 2, pp. 989–999, 2004.

[106] A. H. Zea, P. C. Rodriguez, M. B. Atkins et al., "Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion," *Cancer Research*, vol. 65, no. 8, pp. 3044–3048, 2005.

[107] BO. Huang, P. Y. Pan, Q. Li et al., "Gr-1CD115 immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host," *Cancer Research*, vol. 66, no. 2, pp. 1123–1131, 2006.

[108] M. R. Garcia, L. Ledgerwood, Y. Yang et al., "Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice," *Journal of Clinical Investigation*, vol. 120, no. 7, pp. 2486–2496, 2010.

[109] B. Yin, G. Ma, C.-Y. Yen et al., "Myeloid-derived suppressor cells prevent type 1 diabetes in murine models," *Journal of Immunology*, vol. 185, no. 10, pp. 5828–5834, 2010.

[110] K. C. Herold, B. Brooks-Worrell, J. Palmer et al., "Validity and reproducibility of measurement of islet autoreactivity by T-cell assays in subjects with early type 1 diabetes," *Diabetes*, vol. 58, no. 11, pp. 2588–2595, 2009.

[111] S. I. Mannering, F. S. Wong, I. Durinovic-Belló et al., "Current approaches to measuring human islet-antigen specific T cell function in type 1 diabetes," *Clinical and Experimental Immunology*, vol. 162, no. 2, pp. 197–209, 2010.

[112] R. Mallone, S. I. Mannering, B. M. Brooks-Worrell et al., "Isolation and preservation of peripheral blood mononuclear cells for analysis of islet antigen-reactive T cell responses: position statement of the T-Cell Workshop Committee of the Immunology of Diabetes Society," *Clinical and Experimental Immunology*, vol. 163, no. 1, pp. 33–49, 2011.

[113] J. L. Gaglia, A. R. Guimaraes, M. Harisinghani et al., "Noninvasive imaging of pancreatic islet inflammation in type 1A diabetes patients," *Journal of Clinical Investigation*, vol. 121, no. 1, pp. 442–445, 2011.

[114] M. D. Pescovitz, C. J. Greenbaum, H. Krause-Steinrauf et al., "Rituximab, B-lymphocyte depletion, and preservation of beta-cell function," *The New England Journal of Medicine*, vol. 361, no. 22, pp. 2143–2152, 2009.

[115] D. Bresson, L. Togher, E. Rodrigo et al., "Anti-CD3 and nasal proinsulin combination therapy enhances remission from recentonset autoimmune diabetes by inducing Tregs," *Journal of Clinical Investigation*, vol. 116, no. 5, pp. 1371–1381, 2006.

[116] D. Bresson and M. von Herrath, "Moving towards efficient therapies in type 1 diabetes: to combine or not to combine?" *Autoimmunity Reviews*, vol. 6, no. 5, pp. 315–322, 2007.

[117] J. B. Matthews, T. P. Staeva, P. L. Bernstein, M. Peakman, and M. Von Herrath, "Developing combination immunotherapies for type 1 diabetes: recommendations from the ITN-JDRF Type 1 Diabetes Combination Therapy Assessment Group," *Clinical and Experimental Immunology*, vol. 160, no. 2, pp. 176–184, 2010.

[118] A. M. J. Shapiro, J. R. T. Lakey, E. A. Ryan et al., "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen," *The New England Journal of Medicine*, vol. 343, no. 4, pp. 230–238, 2000.

[119] A. M. J. Shapiro, C. Ricordi, B. J. Hering et al., "International trial of the Edmonton protocol for islet transplantation," *The New England Journal of Medicine*, vol. 355, no. 13, pp. 1318–1330, 2006.

[120] P. Collombat, X. Xu, P. Ravassard et al., "The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into α and subsequently β cells," *Cell*, vol. 138, no. 3, pp. 449–462, 2009.

[121] K. I. Rother, L. M. Spain, R. A. Wesley et al., "Effects of exenatide alone and in combination with daclizumab on β-cell function in long-standing type 1 diabetes," *Diabetes Care*, vol. 32, no. 12, pp. 2251–2257, 2009.

[122] M. Larché, "Update on the current status of peptide immunotherapy," *Journal of Allergy and Clinical Immunology*, vol. 119, no. 4, pp. 906–909, 2007.

[123] P. Achenbach, J. Barker, and E. Bonifacio, "Modulating the natural history of type 1 diabetes in children at high genetic risk by mucosal insulin immunization," *Current Diabetes Reports*, vol. 8, no. 2, pp. 87–93, 2008.

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Review Article

T Cell Recognition of Autoantigens in Human Type 1 Diabetes: Clinical Perspectives

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Type 1 diabetes (T1D) is an autoimmune disease driven by the activation of lymphocytes against pancreatic β-cells. Among β-cell autoantigens, preproinsulin has been ascribed a key role in the T1D process. The successive steps that control the activation of autoreactive lymphocytes have been extensively studied in animal models of T1D, but remains ill defined in man. In man, T lymphocytes, especially $CD8^+$ T cells, are predominant within insulitis. Developing T-cell assays in diabetes autoimmunity is, thus, a major challenge. It is expected to help defining autoantigens and epitopes that drive the disease process, to pinpoint key functional features of epitope-specific T lymphocytes along the natural history of diabetes and to pave the way towards therapeutic strategies to induce immune tolerance to β-cells. New T-cell technologies will allow defining autoreactive T-cell differentiation programs and characterizing autoimmune responses in comparison with physiologically appropriate immune responses. This may prove instrumental in the discovery of immune correlates of efficacy in clinical trials.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease driven by the activation of lymphocytes against pancreatic β-cells. While the successive steps that control the activation of autoreactive lymphocytes have been extensively studied in animal models, the disease process remains ill defined in the human [1]. However, the predominant role of T lymphocytes is characteristic of both mouse and human T1D. In the mouse, T1D is transferred into naive recipients by T cells, is prevented by antibodies that target T lymphocyte activation, and fails to develop when key genes in T lymphocyte differentiation or activation are non functional [2]. T1D is a highly multigenic disease both in the mouse [3] and in the human [4]. In man, T lymphocytes, especially $CD8^+$ T cells, are predominant within insulitis in most [5–11], although not all [12], observations. Occurrence of T1D in a patient deprived of B lymphocytes further underscores the role of T lymphocytes [13].

Remarkable features in human T1D are the long preclinical phase that precedes the development of full-blown hyperglycemia [14] and the high recurrence level of autoimmunity in long-standing patients who have been treated with exogenous insulin for years [15]. The first detection of autoantibodies can occur at any time during life. However, it peaks at one to three years of age in a large subset of children who develop early T1D. A second incidence peak is seen around puberty and show more heterogeneous autoantibody profiles than in early forms of T1D [14]. Rapid diabetes recurrence is seen in T1D recipients of an isograft from a discordant, nondiabetic twin and is accompanied by an almost exclusive $CD8^+$ T-cell islet infiltration [15]. It, thus, seems that β-cell-specific T lymphocytes maintain immune memory for years after T1D onset. However, differentiation patterns of autoreactive T lymphocytes, once diabetes diagnosed, remain largely unknown.

Among β-cell autoantigens (Table 1), proinsulin has been ascribed a key role in the T1D process [1, 16]. In the NOD

Table 1: Autoantigens defined as recognized by T cells in human T1D. Listing has been limited to autoantigens for which evidence of recognition has been obtained in the human or, if only in the mouse, data are expected in the human.

autoantigen	expression	Subcellular location	Involvement in the NOD mouse	Human T1D		
				autoantibodies	$CD4^+$ T cells	$CD8^+$ T cells
Insulin	β-cell, thymus	secretory granule	+	+	+	+
*GAD 65	neuroendocrine	synaptic-like microvesicles	+	+	+	+
GAD 67	neuroendocrine	cytosol	+	+	+	+
IA-2 (ICA512)	neuroendocrine	secretory granule		+	+	+
IA-2 β/phogrin	neuroendocrine	secretory granule		+	+	+
IGRP	β-cell	endoplasmic reticulum	+	?	+	+
Chromogranin	neuroendocrine	Secretory granule	+	?	?	?
ZnT8	β-cell	secretory granule	?	+	?	?
HSP-60 HSP-70	ubiquitous	mitochondria	+	+	+	?
Glima-38		secretory granule	?	+	?	?
Amylin/IAPP		secretory granule	?	?	?	+
CD38	ubiquitous	?	?	$\pm$	?	?

GAD: glutamate decarboxylase; IA-2: islet antigen 2; ZnT8; HSP: heat shock protein; IAPP; IGRP; ICA: islet cell antibody.
?: no positive results reported.

mouse, injection of insulin-specific T-cell clones accelerates diabetes and protection is obtained by injecting insulin in incomplete Freund's adjuvant in prediabetic mice [2, 17]. Altered diabetes development in proinsulin $1^{-/-}$ or $2^{-/-}$ NOD mice makes a strong case for the primary role of insulin in this model [18–20]. By contrast, deficient expression of glutamate decarboxylase (GAD) or islet antigen 2 (IA-2) has no striking effect on diabetes development in this model [21, 22]. Antigen spreading may thus explain the activation of T cells against a long list of autoantigens once the autoimmune process on. T cell clones that are specific for GAD, chromogranin and islet-glucose-6-phosphatase catalytic subunit-related protein (IGRP) are indeed detected and transfer diabetes into naive NOD recipients [23–26]. In man, insulin and proinsulin are common targets of autoantibodies and T cells in (pre)diabetic individuals [27–37]. Insulin autoantibodies (IAA) are the first to be detected in children at risk for T1D and carry a high positive predictive value for diabetes in siblings of T1D patients [14, 28]. However, autoantibodies and T cells have been detected against autoantigens other than insulin in the human [38].

The immune image of the β-cell is that of native components of the β-cell membrane in their three-dimensional conformation, as seen by B lymphocytes, and, more importantly, of fragments of intracellular β-cell proteins in the form of 8–11 mer peptides loaded onto class I major histocompatibility complex (MHC) molecules, as seen on the β-cell surface by $CD8^+$ T lymphocytes. In addition, professional antigen-presenting cells (APCs) present fragments of autoantigens that are phagocytosed following the release of subcellular β-cell particles or β-cell debris in the extracellular milieu and loaded onto MHC class I and class II molecules. Given the key role of T lymphocytes in the T1D disease process, the cartography of autoantigen-derived peptides that are presented to class I-restricted $CD8^+$ T cells and class II-restricted $CD4^+$ T cells, although still incomplete, will be the main focus of this paper.

2. T Cell Recognition of Insulin

Both direct evidence in the mouse and indirect evidence in the human point at insulin as a key autoantigen in T1D autoimmunity. The search for T cell recognition of insulin and the characterization of insulin epitopes recognized by T lymphocytes along disease development is thus a major challenge.

2.1. $CD4^+$ T Cell Responses to Proinsulin. The study of T cell responses to β-cell autoantigens have long been limited to MHC class II-restricted responses but have faced major methodological caveats, precluding translation into routine laboratory procedures. $CD4^+$ T cell responses to exogenous insulin have first been studied [39]. They have been shown to be exacerbated in response to inhaled insulin in T1D patients and in patients treated with insulin analogs, in particular insulin detemir. They are beyond the scope of this paper that will focus on T cell responses to insulin as part of the autoimmune response to β-cells. Autoimmunity to insulin seen in the rare insulin autoimmune syndrome in patients with Grave's disease will not be detailed either [39]. Following studies associating the detection of antibodies to exogenous insulin to HLA-DR4, HLA-DR4-restricted T cell responses have first been prioritized. The characterization of the high susceptibility DQ8 molecule led to the characterization of DQ8-restricted responses in addition to DR4-restricted responses.

Proliferative T cell responses have been reported in the human against both insulin and proinsulin, especially in recent-onset T1D patients and prediabetic individuals [29–31, 35] although also in nondiabetic subjects in some reports [36, 40]. Despite treatment with insulin, long-standing T1D patients were often found low responders [30, 31], as also observed in case of $CD8^+$ T cells [32]. An inverse correlation has been observed between the presence of anti-insulin autoantibodies and T cell responses to proinsulin [29, 30], although not to insulin [40] and not in all studies [40, 41]. Some patients showed a response to proinsulin, although not to insulin, indicating that C-peptide residues were among the epitopes recognized [30]. Responses to insulin have been observed in 25% of T1D patients and 10% of siblings in a model in which proliferative responses were increased in siblings of T1D patients, although not T1D patients themselves, by addition of anti-DQ antibodies, implying the presence of primed suppressive HLA-DQ-restricted T cell responses to insulin in siblings [42].

Epitopes of proinsulin have been characterized in the human [43] (Table 2). Using class II knockout mice that were transgenic for the DQ8 diabetes susceptibility class II allele, two immunodominant preproinsulin regions have been defined, spanning residues 1–24 and 44–63, respectively. Immunodominant regions, that is, 14–33 and 74–93, were different in diabetes resistant transgenic mice that express the diabetes resistant DQ6 allele [44]. Epitopes spanning the C-peptide and A-chain junction have also been reported as immunodominant in DR1*0401 transgenic mice [45]. Using a preproinsulin peptide library, immunodominant epitopes located within the C peptide (C_{13-29}) and B chain (B_{11-27}) were preferentially recognized by $CD4^+$ T cells from autoantibody positive individuals at high risk for T1D development whereas $CD4^+$ T cells from insulin-treated T1D patients were responsive to native insulin and insulin B chain (B_{1-16}). Unexpectedly, an IL-4 and IL-10 response was predominant in both the naive CD45A and memory CD45RO T cell compartments [36].

The role of the insulin B chain peptide B_{9-23} in the NOD mouse led to test whether this peptide could also be recognized in human T1D. Importantly, the three dimensional structure of the DQ8 molecule complexed with insulin peptide B_{9-23} has been determined [46]. Short-term T cell lines obtained following a 7–10 day incubation of peripheral blood mononuclear cells (PMBCs) from 10/12 recent-onset T1D patients, while not from controls, have been shown highly proliferative to B_{9-23}. Insulin B_{9-23}-specific T cell lines were restricted by HLA-DQ8 which shows striking structural similarities with the NOD mouse IA^{g7} class II molecule. Substantial numbers of interferon γ-producing cells were detected in most recent-onset T1D patients and prediabetic subjects using an ELISpot assay [34]. However, the extent to which a B_{9-23} proliferative assay can apply to routine evaluation in T1D remains elusive. Presentation of peptide B_{9-23} was confirmed in class II knock out mice that were made transgenic for the human DQ8 allele [47]. The characterization of peptides eluted from HLA-DR4 class II molecules further allowed defining naturally processed proinsulin epitopes that clustered in the C peptide and C peptide-A chain junction. Significant responses were observed against C_{13-32}, C_{19}-A_3 and C_{22}-A_5. A positive interferon γ response to proinsulin peptides was detected in 56% of 25 T1D patients and in none of 14 control subjects. By contrast, an IL-10 response to proinsulin peptides was detected in one out of four control subjects and T1D patients. An inverse correlation was observed between the interferon γ and the IL-10 response to IA-2 and proinsulin peptides, although not to tetanus toxoid, in patients and control subjects. Type 1 diabetic patients who showed an IL-10 response were older at onset of diabetes than patients who showed an interferon γ response [48].

In a different set of experiments, expansion of T cells from pancreatic draining lymph nodes of subjects with T1D and controls allowed characterizing T cell clones. While T cell clones from control individuals were highly polyclonal in light of heterogeneous Vβ T cell receptor usage, around 50% of T cell clones from 2 of 3 T1D patients expressed identical Vβ chains, favoring an antigen-driven expansion of T cells. Half of clonally expanded clones from the 2 patients were specific for insulin A_{1-15}. Both patients were HLA-DR401 which is strongly associated with susceptibility to T1D, but also to insulin antibodies in insulin-treated patients. Both patients, however, were long-standing T1D subjects who had been treated with insulin for over than 10 years when lymph node were collected. No response to insulin in blood, spleen or pancreatic lymph nodes from a type 2 diabetic patient treated with insulin was observed. It is hypothesized that T cells residing in the pancreatic lymph nodes may persist in individuals in whom β cells have been eliminated for years [49]. The use of a human DR4B1*0401-restricted $CD4^+$ T cell clone that was obtained from a prediabetic, insulin autoantibody-positive child further allowed showing that posttranslational modifications of insulin epitopes impacts on recognition by autoreactive T cells. The T cell clone was specific for A_{1-13} and proliferated to human islet insulin, indicating that the epitope was derived from native insulin. The authors failed to obtain $CD4^+$ T cell clones specific for this epitope from two healthy donors. Recognition by the A_{1-13}-specific T cell clone was dependent on the formation of a disulfide bond between adjacent cysteine residues A_6 and A_7 which, however, did not alter peptide binding to HLA-DR4. The A_6 and A_7 cysteine residues were required for T cell recognition by this clone, while the A_{11} cysteine residue was not. Recognition of A_{1-13} was dependent on the presence of oxidized residues that allowed formation of a disulfide bond between residues A_6 and A_7 [50]. These data strengthen previous evidence that the oxidation state of insulin-derived peptides plays a role in recognition by insulin A_{1-14}-specific T cells. Murine I-A^b and I-A^d-restricted polyclonal T cells and T cell clones that were specific for bovine A_{1-14} were shown to require reduction of disulfide bonds for recognition [51]. Human DR1-restricted T cell lines derived from insulin-treated patients were shown to require intact disulfide bonds at A_6 and A_7 [52].

2.2. $CD8^+$ T Cell Responses to Proinsulin. In human T1D, a number of proinsulin epitopes that are presented by MHC

Table 2: Class II-restricted* $CD4^+$ T-cell epitopes on human preproinsulin.

§Epitope preproinsulin	§Epitope Insulin nomenclature	MHC restriction	responders	references
PPI_{1-24}	L_{1-24}	DQ8	Transgenic mice	[44]
PPI_{11-26}	L_{11}-B_2	DRB1*0401	Transgenic mice	[45]
PPI_{14-33}	L_{14}-B_9	DQ6	Transgenic mice	[44]
PPI_{20-36}	L_{20}-B_{12}	DRB1*0401	Transgenic mice	[45]
PPI_{21-36}	L_{21}-B_{12}	DR4	Transgenic mice	[36, 45]
PPI_{33-47}	B_{9-23}	DQ8	At risk/recent-onsetTransgenic mice	[35, 47]
PPI_{35-51}	B_{11-27}	DR16	At risk	[36]
PPI_{44-63}	B_{20}-C_7	DQ8	Transgenic mice	[44]
PPI_{59-74}	C_3-C_{18}	DR	Human T cell lines	[140]
PPI_{73-90}	C_{17}-A_1	DR4	Transgenic mice	[45]
PPI_{75-91}	C_{19}-A_3	DR4	T1D	[48]
PPI_{78-94}	C_{22}-A_5	DR4	T1D	[48]
PPI_{74-93}	C_{19}-A_4	DQ6	Transgenic mice	[44]
PPI_{70-93}	C_{13}-A_6	DRB1*0401	Transgenic mice	[45]
PPI_{85-101}	C_{29}-A_{12}	DRB1*0401	Transgenic mice	[45]
PPI_{69-88}	C_{13-32}	DR4	T1D	[48]
PPI_{75-92}	C_{19}-A_3	DR4	T1D	[48]
PPI_{78-94}	C_{22}-A_5	DR4	T1D	[48]
PPI_{27-102}	C_{29}-A_{12}	DR4	Transgenic mice	[45]
PPI_{90-104}	A_{1-15}, A_{1-13}	DRB1*0401	T cell clones	[49, 51]
PPI_{90-102}			Long-standing T1D	

§The preproinsulin nomenclature here refers to the human preproinsulin sequence (errors in some publications have been corrected here, which explains differences with cited references):
leader sequence: MALWMRLLPLLALLALWGPDPAAA;
B chain: FVNQHLCGSHLVEALYLVCGERGFFYTPKT;
C peptide: (RR)EAEDLQVGQVELGGGPGASGLQPLALEGSLQ(RR), (R) are excised during insulin processing;
A chain: GIVEQCCTSICSLYQLENYCN.
§§Epitopes for which class II-restriting alleles have not been defined are not indicated in this table: PPI_{1-16} (L_{1-16}), PPI_{5-20} (L_{5-20}), PPI_{9-24} (L_{9-24}), PPI, L_{13}-B_4, L_{17}-B_8, B_{1-16}, B_6–B_{22}, B_{16-32}, B_{25}-C_9, PPI_{67-83}, C_{13-29} [36]; B_1–B_{17}, B_{11}–B_{27}, B_{20}-C_4, B_{24}-C_4, B_{30}-C_{14}, C_8–C_{24}, C_{18}-A_1, C_{28}-A_{11}, A_6–A_{21} [141]; B_{10-25}, B_{25}-C_8, [140–142].

class I alleles have been characterized (Table 3). In a first study using peptide library-mediated *in vitro* assembly of class I molecules, proinsulin peptides have been defined on the basis of their association with HLA-B8, A2, and B15. Several epitopes harbor anchor residues that were only weakly predicted or not predicted by common algorithms or that did not contain canonical allele-specific binding motifs [53]. Preproinsulin epitopes that carry C-terminal residues that are generated by proteasome digestion *in vitro* follow SYFPEITHI and BIMAS algorithm prediction and bind *in vitro* to purified class II allele have been further characterized [54–56]. In case of the common HLA-A*0201 allele, immunogenicity in class I knock out A2.1 transgenic mice has further been evidenced [54, 56]. However, self-tolerance to mouse proinsulin epitopes is expected to interfere with immunogenicity of human proinsulin peptides in these mice. CTL that could be maintained *in vitro* after restimulation were cytotoxic to A2.1 target cells, indicating that corresponding proinsulin epitopes were naturally processed by cells expressing proinsulin. Further studies characterized selected peptides within the proinsulin B-C region for recognition by peripheral blood mononuclear cells from A1, A2, A3, A11, A24, B8, and B18 type 1 diabetic patients [55] and peptides located within the preproinsulin leader sequence [56, 57]. T cells specific for leader sequence $peptide_{15-24}$ were shown cytotoxic to human islets expressing HLA-A*0201, bringing further evidence that corresponding T cells may participate to β-cell destruction along the human disease process [57]. Leader sequence $peptide_{14-23}$ has been shown distinct from $peptide_{15-24}$ [56], but $peptide_{15-24}$ has been eluted from HLA-A*0201 molecules [57]. A majority of T1D patients shows significant responses to at least one of the peptides covering the whole preproinsulin sequence, while no response is usually observed in control individuals, including type 2 diabetic patients who are treated with exogenous insulin. There is no correlation between the prevalence of responses to proinsulin peptides and the affinity levels of peptide binding to purified HLA class I molecules. In many patients, responses are observed to several peptides. However, the long preclinical phase that precedes clinical diabetes does not preclude that a more restricted set of peptides is recognized at initiation of the autoimmune process. More surprisingly, proinsulin peptides were recognized both in recent-onset and long-standing diabetic patients [55, 56]. This may indicate that long-term memory class I-restricted T cells persist in the long term

Table 3: $CD8^+$ T-cell epitopes on human preproinsulin.

Epitope preproinsulin	Epitope Insulin nomenclature	MHC restriction	responders	references
PPI_{2-10}	L_{2-10}	HLA-A*0201	Recent-onset T1D	[96]
PPI_{2-11}	L_{2-11}	HLA-A*0201	Recent-onset T1D	[56]
PPI_{2-11}	L_{2-11}	HLA-A24	Recent-onset T1D	[56]
PPI_{2-11}	L_{2-11}	HLA-B8	Recent-onset T1D	[56]
PPI_{6-14}/PPI_{6-16}	L_{6-14}/L_{6-16}	HLA-A*0201	Recent-onset T1D	[56]
PPI_{14-23}	L_{14-23}	HLA-A*0201	Recent-onset T1D	[56]
PPI_{15-25}	L_{14}-B_1	HLA-A*0201	Recent-onset T1D	[56]
PPI_{15-24}	L_{15}–L_{24}	HLA-A*0201	Recent-onset T1D	[57]
PPI_{15-25}	L_{14}-B_1	HLA-A*0201	Transgenic mice	[54]
PPI_{23-42}	L_{23-18}	HLA-A24	Recent-onset T1D	[56]
PPI_{34-42}	B_{10-18}	HLA-A*0201	Recent-onset T1D Islet graft rejection Transgenic mice	[54, 55, 58]
PPI_{38-46}	B_{14-22}	HLA-A3	Recent-onset T1D	[55]
PPI_{38-46}	B_{14-22}	HLA-A11	Recent-onset T1D	[55]
PPI_{39-47}	B_{15-23}	HLA-A24	Recent-onset T1D	[141]
PPI_{39-48}	B_{15-24}	HLA-A24	Recent-onset T1D	[55]
PPI_{41-50}	B_{17-26}	HLA-A1	Recent-onset T1D	[55]
PPI_{41-50}	B_{17-26}	HLA-A3	Recent-onset T1D	[55]
PPI_{41-50}	B_{17-26}	HLA-A11	Recent-onset T1D	[55]
PPI_{42-51}	B_{18-27}	HLA-A1	Recent-onset T1D Transgenic mice	[54, 55]
PPI_{42-51}	B_{18-27}	HLA-A*0201	Recent-onset T1D	[55]
PPI_{42-51}	B_{18-27}	HLA-B8	Recent-onset T1D	[55]
PPI_{42-51}	B_{18-27}	HLA-B18	Recent-onset T1D	[55]
PPI_{44-51}	B_{20-27}	HLA-A1	Recent-onset T1D	[55]
PPI_{44-51}	B_{20-27}	HLA-B8	Recent-onset T1D	[55]
PPI_{45-53}	B_{21-29}	HLA-A3	Recent-onset T1D	[55]
PPI_{49-57}	B_{25}-C_1	HLA-B8	Recent-onset T1D	[55]
PPI_{51-61}	B_{27}-C_5	HLA-B8	Recent-onset T1D	[55]
PPI_{76-84}	C_{20-28}	HLA-A*0201	Transgenic mice Recent-onset T1D	[54, 96]
PPI_{83-89}	C_{27}-$C_{(34)}$	HLA-A*0201	Transgenic mice	[54]
PPI_{85-94}	C_{29}-A_5	HLA-A*0201	Transgenic mice Recent-onset T1D	[54, 96]
PPI_{90-99}	A_{1-10}	HLA-A*0201	Transgenic mice	[54]
$PPI_{101-109}$	A_{12-20}	HLA-A*0201	Transgenic mice Recent-onset T1D	[54, 96]

range in patients who have been deprived of residual β cells for years. Long term persistence of memory $CD8^+$ T cells may explain the dramatic recurrence of T1D in recipients of hemigrafts from monozygotic, diabetes-discordant twins [15]. Reactivity to one of the B chain peptides identified, $preproinsulin_{33-47}/B_{10-18}$, was shown to elicit a $CD8^+$ T cell response in long-standing T1D patients who undergo islet graft rejection using an ELISpot for granzyme, interferon γ and IL-10 production and immunostaining with A2.1-peptide tetramers [58]. In the NOD mouse in which no T cell activation is seen in mice deprived of β-cells at an early stage of the autoimmune process, full activation of T cells is seen in the absence of residual β-cells once the autoimmune process initiated [59]. The frequency of preproinsulin-specific $CD8^+$ T cells has been estimated, using interferon γ ELISpot assays, at a median frequency of 0.004% (range 0.0008–0.08%) of PMBCs [32]. In this last study, they waned within 6 months after diabetes onset. However, persistence of $CD8^+$ T cell responses have been observed in long-standing, insulin-treated T1D patients, in particular to B chain peptides, as opposed to leader sequence peptides [55, 56]. The combination of high sensitivity flow

cytometry detection and multiplex fluorescent reagents [60] is likely to allow high throughput $CD8^+$ T cell analyses complemented by functional studies in a near future.

3. T Cell Recognition of GAD, IA-2, and IGRP

While insulin can be defined as a primary target of the autoimmune response to β-cells in the NOD mouse, direct evidence is lacking in the human. T cell recognition of autoantigens other than insulin has been defined in the human. The lack of routine human T-cell assays is due to intrinsic difficulties in measuring T-cell responses, in particular in case of $CD4^+$ T cells [61]. Circulating antigen-specific T cells are present at a very low frequency. Although these cells are sometimes detectable *ex vivo*, their rarity challenges the sensitivity of technologies such as enzyme-linked immunospot (ELISpot) and flow cytometry. Peptides that bind to HLA class II molecules for presentation and recognition by T-cells have been more difficult to characterize in case of $CD4^+$ than $CD8^+$ T cells, due to looser binding constraints in case of class II than class I epitopes. Humanized mouse models expressing human class I or class II HLA molecules have largely been used to define class I-restricted and class II-restricted epitopes.

3.1. $CD4^+$ T Cells. Following the discovery of insulin as an autoantigen in T1D, GAD was discovered as a second autoantigen based on homologies of antigen precipitates with the target of autoantibody in stiff man syndrome. IA-2 was then discovered in 1994. Autoantibodies detected in recent-onset T1D patients recognize predominantly the cytoplasmic domain of IA-2, which shares 80% sequence homology with another tyrosine phosphatase, also known as phogrin [62, 63]. Several approaches have been developed to define $CD4^+$ T cells in T1D. An extensive description of epitopes recognized has been provided in a previous review [43]. Increased proliferation of $CD4^+$ T cells has been reported in the presence of GAD extracted from pig or human brain and islets [64, 65], overlapping peptides covering the GAD65 and GAD67 sequences, in particular a region covering residues 473–555, while other regions were recognized by T cells from both T1D patients and controls [66], recombinant GAD65 and GAD67 [67–69] in up to two third of patients with recent-onset or subjects at risk for T1D. However, proliferative responses have also been observed in normal individuals. An inverse relation between the detection of anti-GAD autoantibodies and proliferative responses to GAD has been documented [69]. Proliferative responses to a GAD region located at positions 247–279 has been correlated with responses to residues 32–47 of the coxsackie P2-C viral sequence [70]. It is noteworthy that this region is located outside of the human GAD65 region ($GAD_{473-543}$) that was shown to be immunodominant in T1D patients [66]. T cell proliferative responses were also searched against selected GAD peptides, such as $GAD65_{506-518}$ which shows striking homology with $proinsulin_{24-36}$ [33, 71] or $GAD65_{247-279}$, a response that was correlated to T cell proliferative responses to coxsackie viral peptide P2-C_{32-47} [70]. DQ-restricted responses to recombinant GAD have further been defined in patients [42]. Several publications have since reported proliferative responses of $CD4^+$ T cells from T1D patients or autoantibody-positive subjects at risk for T1D in the presence of IA-2 or IA-2 peptides [40, 72]. Using IFN-γ/IL-4 double-color ELISPOT, IA-2-specific, interferon γ-secreting PMBCs were detected *ex vivo* in T1D patients while not in controls [73]. A dominant IA-$2_{805-820}$ epitope in these studies was shown to share 56% identity with a VP7 rotavirus protein [72]. However, difficulties to develop reliable assays in these pioneering studies have hampered further studies and may have explained variable outcomes, low reproducibility of data, difficult discrimination of responses in T1D patients and controls and failure to establish clinically relevant assays. Standardization of conditions of antigen presentation, restriction by class II HLA molecules and profiles of cytokines produced along these assays were usually unknown in these studies.

The use of transgenic mice expressing functional MHC class II molecules have been useful in helping to characterize GAD and IA-2 class II epitopes. The first class II transgenic mice to be developed carried a DRB1*0401 susceptibility allele. They led to identification of two peptides, selected among overlapping 20 mer peptides as binding to DRB1*0401, as immunogenic and naturally processed by DRB1*0401-expressing mouse spleen cells, $GAD65_{274-286}$ and $GAD65_{115-127}$ [74]. Another study in DR4-transgenic mice found that $GAD65_{274-286}$ and $GAD65_{115-127}$ and an additional peptide, $GAD65_{551-565}$, were immunodominant [75]. $GAD65_{551-565}$ was shown to be naturally processed by using a combination of chromatography and mass spectrometry of peptides bound by HLA-DR401 molecules [76]. However, the class II susceptibility molecule that confers the highest susceptibility in T1D is the HLA-DQA1*0301/DQB1*0302 (DQ8) dimer. Humanized class II mice devoid of endogenous mouse class II genes and expressing DQ8 have been used to characterize autoantigen epitopes presented by DQ8. $GAD_{247-266}$ which shows homology with coxsackie P2-C, and $GAD_{509-528}$-specific, DQ8-restricted Th1 $CD4^+$ T cell lines were shown to induce insulitis after adoptive transfer into DQ8-expressing mice treated with a very low dose of streptozotocin [77]. Notably, there are two amino acid differences in $GAD_{247-266}$ (GAD_{252} and GAD_{256}) and one amino acid difference in $GAD_{509-528}$ (GAD_{509}) between the murine and the human sequence. A strong $CD4^+$ T cell response was observed and human GAD65 epitopes ($GAD_{497-517}$, $GAD_{527-547}$, $GAD_{537-557}$) identified using a pool of 19 20–23 mers overlapping peptides spanning two large regions of GAD 65 in transgenic mice expressing DQ8 and backcrossed onto the NOD background for two generations [78].

Following identification of $GAD65_{551-565}$ as naturally processed and as eliciting a T cell response in recent-onset T1D patients and individuals at risks [73], soluble HLA-DR401 or -DR404 TMrs complexed to $GAD65_{551-565}$ were used to analyze circulating T-cells from recent-onset T1D patients and at-risk subjects. This allowed detecting high

avidity $CD4^{high}$ tetramer-positive cells after expansion *in vitro* and activation on specific plate-bound class II-peptide monomers [79]. Seemingly, expansions were detected in the presence of $GAD65_{555-567}$ $GAD65_{274-286}$ peptides, and $proinsulin_{B24-C36}$, using TMrs. [80]. Generating a panel of GAD65-specific T cell lines from HLA-DR*0301/*0401 recent-onset T1D patients, epitopes have also been identified. Two were presented by DR4-expressing APCs, one covering amino acid residues 270–283, in close proximity, but outside the homology region shared with Coxsackie virus P2-C protein, a second covering residues 556–575, the peptide largely overlapping with a 20 mer peptide having the highest affinity to DRB1*0401 among known GAD65 peptides. Both were characterized in two T1D patients carrying the high susceptibility HLA-DR*0401/DQB1*0302 haplotype. Two epitopes ($GAD_{146-165}$ and $GAD_{174-185}$) were presented by APCs expressing the susceptibility HLA-DR*1601 allele, but one ($GAD_{206-225}$) was presented by APCs expressing the resistance allele HLA-DR*1501 [81].

Among the many GAD epitopes characterized, $GAD_{555-567}$ has led to extensive studies, both *ex vivo* and in transgenic mice. The transfer of a DR4-restricted $GAD_{555-567}$-specific $CD4^+$ T cell clone induces insulitis in $Rag^{-/-}$I-$A^{b-/-}$B6 DR4 transgenic mice [82] while $CD4^+$ T cells carrying TCR transgenes from two distinct $GAD_{555-567}$-specific CD4 T cell clones in B6 DR4-transgenic mice remained tolerant, although through different mechanisms [83]. An increase in the avidity of $CD4^+$ T cell recognition of $GAD_{555-567}$ has been reported in three prediabetic subjects along progression from autoantibody positivity to clinical T1D [84].

In addition to studies of direct interactions of $CD4^+$ T cells and class II-restricted peptides, cellular binding assays have been used for studying peptide-class II interactions for a large number of DR and DQ molecules. A clustering of peptides has been identified in the COOH-terminal region of GAD and promiscuous peptides have been identified. Most peptides were further shown to bind both diabetes-predisposing and diabetes-protective class II molecules [85–87] and DR as well as DQ molecules [88]. However, limitations in these studies rely with the likelihood that, in contrast with antiviral responses, peptides recognized along the autoimmune response to β-cells cannot be predicted on an affinity basis [55, 56], many peptides showing low to medium affinity being recognized and inducing immunogenic responses in humanized mice as recently shown in a DQ8 NOD transgenic mouse [89].

Similar strategies have been followed to study $CD4^+$ T cell recognition of IA-2 and IGRP. Using libraries of synthetic peptides overlapping the intracytoplasmic domain of IA-2, a dominant epitope recognized by DR4-restricted T cells from subjects at risk for T1D has first been identified, $IA\text{-}2_{805-820}$, which has 100% similarity with a sequence of the rotavirus VP7 protein [90]. Studying a panel of naturally processed islet epitopes by elution from APCs bearing HLA-DR4, $IA\text{-}2_{652-80}$, $IA\text{-}2_{709-35}$, $IA\text{-}2_{752-75}$, $IA\text{-}2_{793-817}$, $IA\text{-}2_{853-72}$, $IA\text{-}2_{955-76}$-specific $CD4^+$ T cells have been identified as proinflammatory T cells. Interestingly in this study, the majority of nondiabetic, HLA-matched controls also showed a response against islet peptides, but with the phenotype profile of IL-10-secreting T cells [48]. Two phogrin DQ8 epitopes ($ICA512_{640-659}$ and $ICA512_{755-776}$) previously defined as recognized by diabetogenic T cells in the NOD mouse has been further identified using transgenic mice expressing DQ8 on the NOD background [91]. The evidence for molecular mimicry between an IA-2 (and GAD) epitope and the rotavirus VP7 protein has been further detailed by showing strong binding of both autoantigen and viral peptides to HLA-DRB1*04 and cross recognition by IA-2-specific T cells [92]. The 831–860 region of IA-2 frequently recognized by autoantibodies has been shown to be recognized by IL-10-secreting T cells from T1D patients [93]. Following identification of DRA1*0101/DRB1*0401-retricted $IGRP_{23-35}$ and $IGRP_{247-259}$ and DRA1*0101/DRB1*0301-retricted $IGRP_{13-25}$ and $IGRP_{226-238}$ epitopes, IGRP-specific $CD4^+$ T cells have been detected in more than 80% of DRB1*0401 or DRB1*0301 healthy and T1D subjects [94].

Interestingly, autoantigen-specific $CD4^+$ T cells have been studied in very different clinical settings, including in autoantibody-positive individuals at risk for T1D, in recent-onset T1D patients and in patients undergoing pancreas/kidney as recently reported in 3 patients [95] or islet transplant [58]. Autoantibodies were detected either pretransplant or reappeared 5 and 6 years posttransplant in still normoglycemic patients, somewhat paralleling insulitis, whatever the immunosuppression used. GAD-specific $CD4^+$ T cells were detected using DRB1*0405 and DRB4*0101 TMrs and IGRP-specific $CD8^+$ T-cells were detected using HLA-A2/A*0201 class I pentamers along followup. Autoreactive T-cells are temporarily inhibited by immunosuppression, their reappearance is followed by further loss of insulin secretion [95].

3.2. $CD8^+$ T Cells. The first evidence for the recognition of GAD by A*0201-restricted $CD8^+$ T cells was obtained in one asymptomatic and two recent diabetic patients. $CD8^+$ T cells detected in this study were shown to target HLA-A*0201 peptide $GAD_{114-123}$ and were cytotoxic to autologous antigen-presenting cells incubated with the $GAD_{114-123}$ peptide or infected with a recombinant vaccinia virus expressing GAD65 [38]. A list of epitopes along with class I-restricted HLA molecule is provided in Table 4. The recognition of the $GAD_{114-123}$ epitope was confirmed by another study using an interferon γ Elispot assay [96]. Another peptide from IA-2 ($IA\text{-}2_{797-805}$) was reported as the target of cytotoxic T cells, but both in T1D patients and control individuals [97]. Using algorithms to predict nonameric β-cell peptides that would bind to the common HLA-A*0201 allele and an interferon γ Elispot assay, a human islet amyloid polypeptide (IAPP) precursor protein, 6 out of 9 recent-onset T1D patients, but none of longstanding T1D patients, were shown to recognize preproIAPP peptide $IAPP_{5-13}$[98]. Another IAPP peptide ($IAPP_{9-17}$) was defined using the same approach and an assay evaluating granzyme B secretion, along with IGRP peptides $IGRP_{215-223}$, $IGRP_{152-160}$, $IGRP_{228-236}$ and $IGRP_{266-273}$ glial fibrillary acidic protein (GFAP) peptides $GFAP_{143-151}$and $GFAP_{214-222}$, $IA\text{-}2_{172-180}$, and $IA\text{-}2_{482-490}$,

Table 4: $CD8^+$ T-cell epitopes of human T1D autoantigens (other than preproinsulin).

Epitope preproinsulin	MHC restriction	responders	references
$GAD_{114-123}$	HLA-A*0201	Recent-onset T1D Transgenic mice	[38, 96, 103]
$GAD_{114-122}$	HLA-A*0201	Recent-onset T1D	[101]
$GAD_{110-118}$	HLA-A*0201	Transgenic mice	[103]
$GAD_{159-167}$	HLA-A*0201	Transgenic mice	[103]
$GAD_{476-484}$	HLA-A*0201	Transgenic mice	[103]
$GAD_{536-545}$	HLA-A*0201	Transgenic mice	[103]
$IAPP_{5-13}$	HLA-A*0201	Recent-onset T1D	[98]
$IAPP_{9-17}$	HLA-A*0201	At risk Recent-onset T1D T1D	[99, 100]
$IGRP_{215-223}$	HLA-A*0201	Recent-onset T1D	[100]
$IGRP_{152-160}$	HLA-A*0201	At risk T1D	[99, 100]
$IGRP_{228-236}$	HLA-A*0201	Transgenic mice Recent-onset T1D	[96, 102]
$IGRP_{266-273}$	HLA-A*0201	Transgenic mice Recent-onset T1D	[96, 102]
$IGRP_{206-214}$	HLA-A*0201	Transgenic mice	[102]
$IGRP_{337-345}$	HLA-A*0201	Transgenic mice	[102]
$IGRP_{265-273}$	HLA-A*0201	Transgenic mice	[102]
$GFAP_{143-151}$	HLA-A*0201	At risk T1D	[99]
$GFAP_{214-222}$	HLA-A*0201	At risk T1D	[99]
IA-$2_{797-805}$		Normal subjects Recent-onset T1D	[97]
IA-$2_{172-180}$	HLA-A*0201	Recent-onset T1D	[100]
IA-$2_{482-490}$	HLA-A*0201	Recent-onset T1D	[100]
$IA2_{790-798}$	HLA-A*0201	Transgenic mice	[103]
$IA2_{805-813}$	HLA-A*0201	Transgenic mice	[103]
$IA2_{830-839}$	HLA-A*0201	Transgenic mice	[103]
$IA2_{962-970}$	HLA-A*0201	Transgenic mice	[103]

[95, 99, 100]. A strong inverse correlation between the binding affinity of β-cell peptides to HLA-A*0201 and CTL responses against those peptides was observed in recent-onset type 1 diabetic patients. These data confirmed that many β-cell epitopes are recognized by CTLs in recent-onset type 1 diabetic patients. Interestingly, IA-2 and GAD have been defined as a key autoantigen in T1D on the basis of the predictive value of anti-IA2 and anti-GAD autoantibodies in prediabetic individuals, while IGRP and IAPP have not been defined as key targets of autoantibodies in T1D in the human. $GAD_{114-122}$-specific $CD8^+$ T cells, as well as GAD-specific and insulin-specific $CD4^+$ T cells, have further been detected exclusively in T1D patients within the memory $CD45RO^+$ T cell population while naïve CD45RO T cell stained with HLA-0201*-$GAD_{114-122}$ tetramers were discriminative between control and T1D patients [101]. A combinatorial quantum dot MHC multimer technique has further allowed detecting expansions of HLA-A*0201-restricted $CD8^+$ T cells that were specific for IA-$2_{797-805}$, $GAD65_{114-123}$, $IGRP_{265-273}$, and $preproIAPP_{5-13}$ in recent onset diabetes patients, with a specificity ranging from 87% to 100% and a sensitivity ranging from 25% to 40%, and in islet transplantation recipients [60].

The use of HLA-A*0201 transgenic mice has first reported or confirmed the characterization of class I-restricted peptides that are potentially presented to $CD8^+$ T cells in the human. $IGRP_{206-214}$, $IGRP_{337-345}$ and $IGRP_{265-273}$ have been identified in HLA-A*0201 transgenic mice on the NOD genetic background and shown to be targeted by pathogenic $CD8^+$ T cells [102]. The systematic immunization of HLA-A*0201 transgenic mice using plasmids encoding GAD65 or the catalytic unit of the intracellular domain of IA-2 has allowed defining 5 GAD peptides ($GAD_{110-118}$, $GAD_{114-123}$, $GAD_{159-167}$, $GAD_{476-484}$, and $GAD_{536-545}$) and 4 IA-2 peptides ($IA2_{790-798}$, $IA2_{805-813}$, $IA2_{830-839}$, and

$IA2_{962-970}$) that were recognized by $CD8^+$ T cells from T1D patients, 3 of which ($GAD_{114-123}$, $GAD_{536-545}$, and $IA2_{805-813}$) in more than 25% patients [103].

Shifts both in frequency and in immunodominance of $CD8^+$ T-cell responses have been observed within months following T1D onset and were more rapid than changes in autoantibody titers. Positive T-cell responses to islet epitopes ($GAD65_{114-123}$, $GAD65_{536-545}$, $IGRP_{228-236}$, PPI_{2-10}, PPI_{34-42}, PPI_{42-51}, and $PI_{101-109}$) observed at diagnosis were shown to drop to non detectable levels, while newly targeted epitopes were evidenced, in particular proinsulin B_{18-27}, $IA\text{-}2_{206-214}$, and $IGRP_{265-273}$. However, of a total of positive T-cell responses to islet epitopes observed at diagnosis, 26 of 42 dropped to nondetectable levels, while new epitopes were targeted in only 5 [32].

3.3. Regulatory T Cells. The role of protective $CD4^+$ T cells in T1D has first been defined in the NOD mouse [104]. In the human, initial reports documenting decreased numbers of $CD4^+$ T regulatory (T_{reg}) cells defined as $CD25^+$ [105] were not confirmed by later works using more specific surface phenotyping [106]. Few papers addressed the issue of T_{reg} by analyzing autoantigen-specific T_{reg}. Despite the importance of this question, the characterization of islet-specific Tregs is still in its infancy, as it is rather difficult to detect them (their frequency being probably even lower than that of the corresponding effectors) and to expand them *in vitro* (most of these cells being characterized by a state of anergy that needs to be reversed).

The characterization of both proinflammatory (IFN-γ-producing) and regulatory (IL-10-producing) $CD4^+$ T-cell responses against proinsulin and IA-2 indicate that T_{reg} are possibly key players. While T1D patients harbored predominant IFN-γ responses, healthy subjects were characterized by higher frequencies of IL-10 responses specific for the same epitopes. The same was not true for responses against an irrelevant Ag such as tetanus toxoid. Moreover, T1D patients displaying higher IL-10 responses were characterized by an older age T1D onset, suggesting that these regulatory may counterbalance autoimmune effectors, at least transiently [48]. IL-10-secreting $CD4^+$ T_{reg} specific for proinsulin and IA-2 epitopes have been characterized as suppressive *in vitro* in healthy subjects. This suppressive activity is however not linked to IL-10 secretion, but rather to elimination of Ag-presenting cells [107]. Importantly, we previously showed that GAD-specific effector $CD4^+$ T cells cloned from T1D patients could also be rendered anergic and suppressive upon sustained Ag-specific *in vitro* stimulation [108]. Similar observations were made by showing that patients harboring the protective I/III and III/III insulin VNTR haplotypes displayed a threefold higher IL-10 release in proinsulin-specific memory T cells. These data are consistent with the hypothesis that VNTR-induced higher insulin levels in the thymus promote Treg generation, offering an additional explanation for the protective effect of the VNTR class III alleles [109]. Seemingly, cloned PPI_{70-90}-specific DRB1*0401-restricted human T cells have been characterized as expressing a downregulatory T helper 2 phenotype through predominant production of IL-5 and IL-10 and low interferon γ production [110]. DRB1*0401-restricted $CD4^+$ T cells that are specific for $GAD65_{555-567}$ have been identified in normal individuals using GAD65-specific class II TMrs after expansion in the presence of peptide, following removal of $CD4^+CD25^+T_{reg}$. Their expansion was reversed adding back $CD4^+CD25^+T_{reg}$ [111, 112]. Taken together, these observations suggest that detection of T-cell autoreactivity should not be considered pathological *per se*, but should rather be interpreted by functional profiling. Regulatory PI- and GAD-specific T-cell clones can be obtained *in vitro* under standard stimulating conditions in the absence of any exogenous cytokines [113]. Similarly, GAD- and IGRP-specific $CD4^+$ Treg clones can be obtained by *in vitro* stimulation of FoxP3-negative $CD4^+$ T cells [114]. Whether such clones are only generated *in vitro* or can also be isolated *ex vivo* and their mechanism(s) of suppression remain important questions for further investigation. On the same line, there have been reports indicating that GAD-specific circulating $CD4^+$ T cells show an activation phenotype that is not seen in control individuals, likely a memory phenotype and are more prone to proliferate while less dependent on CD28/B7-1 costimulation [115]. GAD65- and proinsulin-specific T-cells, including cells that were specific for $GAD_{106-125}$, $GAD_{526-545}$, $GAD_{266-285}$, $GAD_{556-575}$, $hPPI_{72-90}$ and $hPPI_{94-110}$, have been shown to coexpress CD25 and CD134 (OX40) as a distinctive feature when compared to T cells from healthy subjects [116]. Beyond, $CD4^+$ T cells, polyclonal regulatory $CD8^+$ T cells have also been characterized. $CD8^+$ $CD45RA^+$ $CD27^-$ T cells have been shown to control GAD65-specific $CD4^+$ T cell expansions through a contact dependent mechanism and the production of IL-10 [117].

4. Therapeutic Trials Using Peptides or Autoantigens in the Human

The characterization of T-cell epitopes is expected to help developing T cell assays to be used in the followup of immunotherapy trials in T1D, thus providing surrogate end point markers of tolerance induction that may prove more reliable than current autoantibody assays [118, 119] and pave the way towards antigen or peptide-specific immunotherapy.

4.1. Mechanisms of Tolerance. A major advantage of antigen or peptide-specific immunotherapy over other forms of immunotherapy in human autoimmune diseases is in focusing treatment on self-reactive T cell clones without impairing immune responses to unrelated antigens, especially tumoral or infectious antigens. In T1D in particular, this may prove of outmost importance considering that insulin therapy has gained in efficacy and safety over years, explaining the progressively decreasing mortality gap between T1D patients and the general population [120]. Although nonspecific immune suppression has been shown partially effective in preserving β-cells from autoimmune destruction in recent-onset T1D patients, immunosuppressive drugs used to suppress the immune response have shown major side effects

that preclude their use in the long-term range. Peptide and/or antigen-specific immunotherapy is thus likely to allow optimal risk/benefit ratio in T1D.

Immune tolerance is ensured in a succession of checkpoints by a variety of mechanisms affecting differentiating lymphocytes in central lymphoid organs as well as mature lymphocytes in the periphery. Tolerance in the periphery rely on deletion of autoreactive cells, on ignorance of self antigens, on active mechanisms that imprint an intrinsic status on tolerant lymphocytes in the form of anergy or immune deviation and on extrinsic mechanisms that involve regulatory cells. Molecular interactions in the presentation of autoantigen in the periphery are central to the tolerance process and in strategies aiming at restoring or inducing immune tolerance in autoimmunity. Elimination or reprogramming of deleterious autoreactive cells and activation of regulatory cells to control autoimmune effectors are the major outcomes expected from antigen or peptide-specific immunotherapy. These mechanisms have been clearly documented in preclinical models but are only starting to be implemented in human studies.

Key advantages of inducing antigen-specific immune tolerance have been underscored but depend on mechanisms of tolerance induction. First, it may not require knowing the initiating target autoantigen, nor the fine specificity of T cells involved. Induction of regulatory T cells, whatever their specificity, may induce bystander immunomodulation within inflammatory sites, for instance, through *in situ* production of protective cytokines, spreading of Th2 responses or promoting tolerizing antigen presentation. Factors affecting the efficiency of antigen-based immunotherapy include the size of the antigen used, the autoantigen expression pattern, the stage of the disease process at time of administering the tolerizing autoantigen, the crypticity of the epitopes presented, the autoantigen administration route and dose [121].

Given the molecular constraints of T cell activation, the induction of peptide-specific tolerance is expected to require presentation of specific peptides to autoreactive $CD4^+$ T cells in a noninflammatory environment. Induction of immune tolerance by injection of high doses of soluble peptide or antigen or DNA vaccination has proven efficient in experimental models of autoimmunity, but concerns have been raised by the risk of either exacerbating the autoimmune process in some experimental conditions or inducing anaphylactic reactions. The induction of tolerance by the mucosal (oral or nasal) route has seemingly been shown efficient, mostly in preventing autoimmunity, in preclinical models but has failed to apply to human diseases. Mechanisms of action of the mucosal route differ depending on the dose of antigen delivered. The dose chosen in human trials has so far been random. A promising approach to induce tolerance in autoimmune diseases is intravenous injection of antigen-coupled, ethylene carbodiimide-(ECDI-) fixed splenocytes. It has shown efficient in animal models, but is more complex to set up in the human. Among mechanisms involved, both the induction of anergy, at least in part through suboptimal costimulatory signaling, and presentation of the tolerizing epitope by plasmacytoid dendritic cells have been evidenced. As a last example, altered peptide ligands (APLs), either antagonistic or partial agonist APLs, have been successfully used in preclinical models to prevent autoimmunity through anergy, immune deviation or bystander suppression. As in case of soluble peptide or antigen, the use of APLs has raised safety issues relating with either exacerbation of autoimmunity or anaphylactic reactions in the human in multiple sclerosis [122]. Deviation of autoimmunity to different targets is another danger that should not be excluded [1, 123, 124].

4.2. Insulin Trials. As a key autoantigen in T1D, insulin has been used in several trials to downregulate the autoimmune response in recent onset patients or to prevent the full destruction of β-cells in prediabetic subjects. The first trial ever to modulate β-cell destruction used intravenous insulin delivered by an external artificial pancreas to maintain glycemic values between 3.3 and 4.4 mmol/L during 14 days at onset of T1D. This study in 12 T1D patients was compared to conventional treatment using subcutaneous NPH insulin injections ($n = 14$) and showed significantly higher C-peptide values in the experimental as compared to the conventional group at one-year. Insulin doses in the experimental group were in the order of 3 U/kg/d. Mechanisms may have involved β-cell rest as well immunomodulation through intravenous delivery of insulin [125]. It is possible that efficient metabolic control rather than high insulin doses explain the preservation of β cell function in this study. In a comparable study in recent-onset T1D, the nine patients who received high-dose intravenous insulin infusion and the ten patients under intensive-therapy group equally preserved β cell function along a one year follow-up [126]. By contrast, in the DCCT study, intensive insulin therapy allowed maintaining higher C-peptide levels than conventional treatment with one or two injections a day [127]. Fifteen years later, the first randomized, double-blind crossover study using nasal delivery of insulin in 38 prediabetic, autoantibody-positive individuals showed an increase in anti-insulin antibodies and a decrease in T cell proliferative responses to insulin, while no acceleration of diabetes development and stable first-phase insulin response to glucose in the 26 individuals who did not develop diabetes was observed at one year [128]. The Diabetes Prevention Trial-Type 1 Diabetes Study [129] screened 84 228 first and second-degree relatives of T1D patients to select 3152 autoantibody-positive individuals and assigned 339 with a projected five year risk over 50% to close observation or low-dose subcutaneous ultralente insulin, 0.25 U/kg/d plus annual 4 day courses of continuous intravenous insulin infusions with no delay in diabetes development after a median followup of 3.7 years. In a group of individuals with a five year projected risk of 26%–50%, no significant difference in the development of T1D was observed between individuals randomly assigned to oral insulin *versus* individuals assigned to placebo. However, in a subgroup of individuals with anti-insulin autoantibodies ≥ 80 nU/mL, 6.2% individuals receiving oral insulin developed T1D, as

compared to 10.4% of those receiving placebo ($P < .015$), suggesting that oral insulin should be tested specifically in this subgroup of individuals [130]. However, a more recent, double-blind, randomised, controlled study in 115 individuals receiving intranasal insulin (1 U/kg/d) *versus* 109 infants and siblings with ≥2 autoantibodies showed no difference between the two groups [131]. It is even possible in this study that nasal insulin had an accelerating effect on T1D development in individuals with ≥3 autoantibodies against different antigen specificities. These data face major caveats. First, the dose of insulin delivered has little rationale. Second, it is likely that prediabetes corresponds to fully activated autoimmunity involving an already expanded T cell repertoire. In new-onset T1D patients, oral delivery of human insulin at doses ranging from 2.5 to 7.5 mg/d failed to show any benefit on C-peptide secretion at one year [132, 133]. Again, new-onset T1D is likely a late stage hardly accessible to down regulation of the autoimmune reaction. Administration of insulin B-chain in incomplete Freund's adjuvant has been shown to elicit strong B and T cell immune responses to insulin in 12 subjects with recent-onset T1D, but no C-peptide benefit over a two-year followup [134]. Finally, beyond the use of insulin as a key autoantigen to downregulate the autoimmune response to β-cells in prediabetes and in new-onset T1D, a key issue remains whether or not immediate insulin therapy should be started in noninsulin dependent diabetes patients who are tested positive for islet cell autoantibodies, in particular anti-GAD autoantibodies. Sixty anti-GAD positive patients with a five year non-insulin dependent diabetes profile were randomized to either early insulin or sulfonylureas, tested annually for C-peptide secretion under an oral glucose tolerance test and followed up for 57 months. The progression to insulin dependence was observed in 3 out of 30 patients in the insulin group while in 13 of 30 in the sulfonylurea group [135], in contrast with other studies [136].

4.3. Glutamate Decarboxylase Trials. Following a dose-finding study in patients with latent autoimmune diabetes in adults indicating the safety of a primary injection and a booster injection of 20 μg each of recombinant human GAD in a standard vaccine formulation with alum as adjuvant [137], a double-blind randomised study was performed in 35 GAD autoantibody-positive T1D patients who had fasting C-peptide levels >0.1 nmol/L (0.3 ng/mL) within 18 months of diabetes diagnosis. Patients in the treated group received two injections of GAD at 1 and 30 days of the study. The decrease in fasting C-peptide values and stimulated secretion with time was significantly lower by 23% and 29%, respectively, in treated *versus* placebo patients, 30 months after the first GAD injection. No adverse effects were observed. An increase in anti-GAD autoantibodies was seen in patients who received GAD injections and peaked at 3 months of the study. Interleukins 5, 10, 13, and 17, interferon-γ and TNF-α production by T cells in response to GAD *in vitro* were seen in treated patients, while not in the placebo group [138].

4.4. Heat Shock Protein Trials. Heat shock protein 60 (hsp60) has been discussed following NOD studies as an autoantigen in T1D and T cell clones specific for hsp60 peptide p277 characterized in this model. Several trials have been conducted in the human using a 24-aminoacid peptide derived from the C-terminus of human hsp60 in patients with established T1D, including a trial in children with T1D. While no effect on C-peptide preservation was observed in the pediatric study, one of the randomized study performed in 35 adult T1D patients within <6 months following diagnosis reported significant preservation of C-peptide values in the treated group as opposed to significant decrease in placebo-treated patients. T-cell responses showed increased interferon γ and decreased interleukin 13 production in response to hsp60 than to p277 and less interferon γ, more interleukin 10 and 13 in the treated group than in the placebo group [139].

5. Conclusion

Developing T-cell assays that allow characterizing diabetes autoimmunity is a major challenge in human T1D. It is expected to help defining epitopes that are recognized in T1D, to pinpoint key functional features of epitope-specific T lymphocytes along the natural history of the diabetes process and to pave the way towards therapeutic strategies to induce immune tolerance to β-cells. New T-cell technologies are expected to allow defining autoreactive T-cell differentiation programs and characterizing autoimmune responses in comparison to physiologically appropriate immune responses. This may allow additional mechanistic studies and prove instrumental in the discovery of immune correlates of efficacy in clinical trials, as initially reported using autoantibody assays and now an open field for T cell assays.

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References

[1] B. Faideau, E. Larger, F. Lepault, J. C. Carel, and C. Boitard, "Role of β-cells in type 1 diabetes pathogenesis," *Diabetes*, vol. 54, no. 2, pp. S87–S96, 2005.

[2] M. S. Anderson and J. A. Bluestone, "The NOD mouse: a model of immune dysregulation," *Annual Review of Immunology*, vol. 23, pp. 447–485, 2005.

[3] U. C. Rogner, C. Boitard, J. Morin, E. Melanitou, and P. Avner, "Three loci on mouse chromosome 6 influence onset and final incidence of type I diabetes in NOD.C3H congenic strains," *Genomics*, vol. 74, no. 2, pp. 163–171, 2001.

[4] J. A. Todd, "Etiology of type 1 diabetes," *Immunity*, vol. 32, no. 4, pp. 457–467, 2010.

[5] N. Itoh, T. Hanafusa, A. Miyazaki et al., "Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients," *The Journal of Clinical Investigation*, vol. 92, no. 5, pp. 2313–2322, 1993.

[6] N. Somoza, F. Vargas, C. Roura-Mir et al., "Pancreas in recent onset insulin-dependent diabetes mellitus: changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor Vβ usage, and cytokine profile," *The Journal of Immunology*, vol. 153, no. 3, pp. 1360–1377, 1994.

[7] A. Willcox, S. J. Richardson, A. J. Bone, A. K. Foulis, and N. G. Morgan, "Analysis of islet inflammation in human type 1 diabetes," *Clinical and Experimental Immunology*, vol. 155, no. 2, pp. 173–181, 2009.

[8] A. Hanninen, S. Jalkanen, M. Salmi, S. Toikkanen, G. Nikolakaros, and O. Simell, "Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus," *The Journal of Clinical Investigation*, vol. 90, no. 5, pp. 1901–1910, 1992.

[9] G. F. Bottazzo, B. M. Dean, and J. M. McNally, "In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis," *The New England Journal of Medicine*, vol. 313, no. 6, pp. 353–360, 1985.

[10] A. K. Foulis, M. McGill, and M. A. Farquharson, "Insulitis in type 1 (insulin-dependent) diabetes mellitus in man—macrophages, lymphocytes, and interferon-γ containing cells," *Journal of Pathology*, vol. 165, no. 2, pp. 97–103, 1991.

[11] A. Willcox, S. J. Richardson, A. J. Bone, A. K. Foulis, and N. G. Morgan, "Analysis of islet inflammation in human type 1 diabetes," *Clinical and Experimental Immunology*, vol. 155, no. 2, pp. 173–181, 2009.

[12] B. Conrad, E. Weidmann, G. Trucco et al., "Evidence for superantigen involvement in insulin-dependent diabetes mellitus aetiology," *Nature*, vol. 371, no. 6495, pp. 351–355, 1994.

[13] S. Martin, D. Wolf-Eichbaum, G. Duinkerken et al., "Development of type 1 diabetes despite severe hereditary B-cell deficiency," *The New England Journal of Medicine*, vol. 345, no. 14, pp. 1036–1040, 2001.

[14] A. G. Ziegler and G. T. Nepom, "Prediction and pathogenesis in type 1 diabetes," *Immunity*, vol. 32, no. 4, pp. 468–478, 2010.

[15] R. K. Sibley, D. E. R. Sutherland, F. Goetz, and A. F. Michael, "Recurrent diabetes mellitus in the pancreas iso- and allograft. A light and electron microscopic and immunohistochemical analysis of four cases," *Laboratory Investigation*, vol. 53, no. 2, pp. 132–144, 1985.

[16] F. Homo-Delarche and C. Boitard, "Autoimmune diabetes: the role of the islets of Langerhans," *Immunology Today*, vol. 17, no. 10, pp. 456–460, 1996.

[17] D. Daniel, R. G. Gill, N. Schloot, and D. Wegmann, "Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice," *European Journal of Immunology*, vol. 25, no. 4, pp. 1056–1062, 1995.

[18] K. Thébault-Baumont, D. Dubois-Laforgue, P. Krief et al., "Acceleration of type 1 diabetes mellitus in proinsulin 2-deficient NOD mice," *The Journal of Clinical Investigation*, vol. 111, no. 6, pp. 851–857, 2003.

[19] H. Moriyama, N. Abiru, J. Paronen et al., "Evidence for a primary islet autoantigen (preproinsulin 1) for insulitis and diabetes in the nonobese diabetic mouse," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10376–10381, 2003.

[20] M. Nakayama, J. N. Beilke, J. M. Jasinski et al., "Priming and effector dependence on insulin B:9-23 peptide in NOD islet autoimmunity," *The Journal of Clinical Investigation*, vol. 117, no. 7, pp. 1835–1843, 2007.

[21] T. Yamamoto, E. Yamato, F. Tashiro et al., "Development of autoimmune diabetes in glutamic acid decarboxylase 65 (GAD65) knockout NOD mice," *Diabetologia*, vol. 47, no. 2, pp. 221–224, 2004.

[22] A. Kubosaki, J. Miura, and A. L. Notkins, "IA-2 is not required for the development of diabetes in NOD mice," *Diabetologia*, vol. 47, no. 1, pp. 149–150, 2004.

[23] D. Zekzer, F. S. Wong, O. Ayalon et al., "GAD-reactive CD4+ Th1 cells induce diabetes in NOD/SCID mice," *The Journal of Clinical Investigation*, vol. 101, no. 1, pp. 68–73, 1998.

[24] S. M. Lieberman, A. M. Evans, B. Han et al., "Identification of the β cell antigen targeted by a prevalent population of pathogenic CD8 T cells in autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 14, pp. 8384–8388, 2003.

[25] K. Haskins and M. McDuffie, "Acceleration of diabetes in young NOD mice with a CD4 islet-specific T cell clone," *Science*, vol. 249, no. 4975, pp. 1433–1436, 1990.

[26] B. D. Stadinski, T. Delong, N. Reisdorph et al., "Chromogranin A is an autoantigen in type 1 diabetes," *Nature Immunology*, vol. 11, no. 3, pp. 225–231, 2010.

[27] L. Yu, D. T. Robles, N. Abiru et al., "Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 4, pp. 1701–1706, 2000.

[28] A. G. Ziegler, M. Hummel, M. Schenker, and E. Bonifacio, "Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study," *Diabetes*, vol. 48, no. 3, pp. 460–468, 1999.

[29] R. J. Keller, "Cellular immunity to human insulin in individuals at high risk for the development of type I diabetes mellitus," *Journal of Autoimmunity*, vol. 3, no. 3, pp. 321–327, 1990.

[30] D. Dubois-LaForgue, J. C. Carel, P. F. Bougnères, J. G. Guillet, and C. Boitard, "T-cell response to proinsulin and insulin in type 1 and pretype 1 diabetes," *Journal of Clinical Immunology*, vol. 19, no. 2, pp. 127–134, 1999.

[31] N. C. Schloot, B. O. Roep, D. Wegmann et al., "Altered immune response to insulin in newly diagnosed compared to insulin-treated diabetic patients and healthy control subjects," *Diabetologia*, vol. 40, no. 5, pp. 564–572, 1997.

[32] E. Martinuzzi, G. Novelli, M. Scotto et al., "The frequency and immunodominance of islet-specific CD8 T-cell responses change after type 1 diabetes diagnosis and treatment," *Diabetes*, vol. 57, no. 5, pp. 1312–1320, 2008.

[33] G. Rudy, N. Stone, L. C. Harrison et al., "Similar peptides from two beta cell autoantigens, proinsulin and glutamic acid decarboxylase, stimulate T cells of individuals at risk for insulin-dependent diabetes," *Molecular Medicine*, vol. 1, no. 6, pp. 625–633, 1995.

[34] D. G. Alleva, P. D. Crowe, L. Jin et al., "A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin," *The Journal of Clinical Investigation*, vol. 107, no. 2, pp. 173–180, 2001.

[35] L. D. Petersen, G. Duinkerken, G. J. Bruining, R. A. W. Van Lier, R. R. P. De Vries, and B. O. Roep, "Increased numbers of

in vivo activated T cells in patients with recent onset insulin-dependent diabetes mellitus," *Journal of Autoimmunity*, vol. 9, no. 6, pp. 731–737, 1996.

[36] I. Durinovic-Belló, M. Schlosser, M. Riedl et al., "Pro- and anti-inflammatory cytokine production by autoimmune T cells against preproinsulin in HLA-DRB1*04, DQ8 Type 1 diabetes," *Diabetologia*, vol. 47, no. 3, pp. 439–450, 2004.

[37] P. Naquet, J. Ellis, D. Tibensky et al., "T cell autoreactivity to insulin in diabetic and related non-diabetic individuals," *The Journal of Immunology*, vol. 140, no. 8, pp. 2569–2578, 1988.

[38] P. Panina-Bordignon, R. Lang, P. M. Van Endert et al., "Cytotoxic T cells specific for glutamic acid decarboxylase in autoimmune diabetes," *Journal of Experimental Medicine*, vol. 181, no. 5, pp. 1923–1927, 1995.

[39] S. E. Fineberg, T. T. Kawabata, D. Finco-Kent, R. J. Fountaine, G. L. Finch, and A. S. Krasner, "Immunological responses to exogenous insulin," *Endocrine Reviews*, vol. 28, no. 6, pp. 625–652, 2007.

[40] I. Durinovic-Bellò, M. Hummel, and A. G. Ziegler, "Cellular immune response to diverse islet cell antigens in IDDM," *Diabetes*, vol. 45, no. 6, pp. 795–800, 1996.

[41] I. Durinovic-Bellò, M. Hummel, and A. G. Ziegler, "Cellular immune response to diverse islet cell antigens in IDDM," *Diabetes*, vol. 45, no. 6, pp. 795–800, 1996.

[42] T. I. M. Tree, G. Duinkerken, S. Willemen, R. R. P. De Vries, and B. O. Roep, "HLA-DQ-regulated T-cell responses to islet cell autoantigens insulin and GAD65," *Diabetes*, vol. 53, no. 7, pp. 1692–1699, 2004.

[43] T. P. Di Lorenzo, M. Peakman, and B. O. Roep, "Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes," *Clinical and Experimental Immunology*, vol. 148, no. 1, pp. 1–16, 2007.

[44] R. Raju, S. R. Munn, and C. S. David, "T cell recognition of human pre-proinsulin peptides depends on the polymorphism at HLA DQ locus: a study using HLA DQ8 and DQ6 transgenic mice," *Human Immunology*, vol. 58, no. 1, pp. 21–29, 1997.

[45] M. Congia, S. Patel, A. P. Cope, S. De Virgiliis, and G. Sønderstrup, "T cell epitopes of insulin defined in HLA-DR4 transgenic mice are derived from preproinsulin and proinsulin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3833–3838, 1998.

[46] K. H. Lee, K. W. Wucherpfennig, and D. C. Wiley, "Structure of a human insulin peptide-HLA-DQ8 complex and susceptibility to type I diabetes," *Nature Immunology*, vol. 2, no. 6, pp. 501–507, 2001.

[47] E. Havari, A. M. Lennon-Dumenil, L. Klein et al., "Expression of the B7.1 costimulatory molecule on pancreatic β cells abrogates the requirement for CD4 T cells in the development, of type 1 diabetes," *The Journal of Immunology*, vol. 173, no. 2, pp. 787–796, 2004.

[48] S. Arif, T. I. Tree, T. P. Astill et al., "Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health," *The Journal of Clinical Investigation*, vol. 113, no. 3, pp. 451–463, 2004.

[49] S. I. Mannering, L. C. Harrison, N. A. Williamson et al., "The insulin A-chain epitope recognized by human T cells is posttranslationally modified," *Journal of Experimental Medicine*, vol. 202, no. 9, pp. 1191–1197, 2005.

[50] S. C. Kent, Y. Chen, L. Bregoli et al., "Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope," *Nature*, vol. 435, no. 7039, pp. 224–228, 2005.

[51] P. E. Jensen, "Reduction of disulfide bonds during antigen processing: evidence from a thiol-dependent insulin determinant," *Journal of Experimental Medicine*, vol. 174, no. 5, pp. 1121–1130, 1991.

[52] G. G. Miller, J. F. Hoy, L. J. Nell, and J. W. Thomas, "Antigen processing and the human T cell receptor repertoire for insulin," *The Journal of Immunology*, vol. 141, no. 10, pp. 3293–3298, 1988.

[53] L. Chang, L. Kjer-Nielsen, S. Flynn et al., "Novel strategy for identification of candidate cytotoxic T-cell epitopes from human preproinsulin," *Tissue Antigens*, vol. 62, no. 5, pp. 408–417, 2003.

[54] Y. Hassainya, F. Garcia-Pons, R. Kratzer et al., "Identification of naturally processed HLA-A2—restricted proinsulin epitopes by reversed immunology," *Diabetes*, vol. 54, no. 7, pp. 2053–2059, 2005.

[55] A. Toma, S. Haddouk, J. P. Briand et al., "Recognition of a subregion of human proinsulin by class I-restricted T cells in type 1 diabetic patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 30, pp. 10581–10586, 2005.

[56] A. Toma, T. Laïka, S. Haddouk et al., "Recognition of human proinsulin leader sequence by class I-restricted T-cells in HLA-A∗0201 transgenic mice and in human type 1 diabetes," *Diabetes*, vol. 58, no. 2, pp. 394–402, 2009.

[57] A. Skowera, R. J. Ellis, R. Varela-Calviño et al., "CTLs are targeted to kill β cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope," *The Journal of Clinical Investigation*, vol. 118, no. 10, pp. 3390–3402, 2008.

[58] G. G. M. Pinkse, O. H. M. Tysma, C. A. M. Bergen et al., "Autoreactive CD8 T cells associated with β cell destruction in type 1 diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 51, pp. 18425–18430, 2005.

[59] E. Larger, C. Becourt, J. F. Bach, and C. Boitard, "Pancreatic islet β cells drive T cell-immune responses in the nonobese diabetic mouse model," *Journal of Experimental Medicine*, vol. 181, no. 5, pp. 1635–1642, 1995.

[60] J. H. Velthuis, W. W. Unger, J. R. F. Abreu et al., "Simultaneous detection of circulating autoreactive $CD8^+$ T-cells specific for different islet cell-associated epitopes using combinatorial MHC multimers," *Diabetes*, vol. 59, no. 7, pp. 1721–1730, 2010.

[61] M. Peakman, T. I. Tree, J. Endl, P. Van Endert, M. A. Atkinson, and B. O. Roep, "Characterization of preparations of GAD65, proinsulin, and the islet tyrosine phosphatase IA-2 for use in detection of autoreactive T-cells in type 1 diabetes: report of phase II of the second international immunology of diabetes society workshop for standardization of T-cell assays in type 1 diabetes," *Diabetes*, vol. 50, no. 8, pp. 1749–1754, 2001.

[62] M. S. Lan, J. Lu, Y. Goto, and A. L. Notkins, "Molecular cloning and identification of a receptor-type protein tyrosine phosphatase, IA-2, from human insulinoma," *DNA and Cell Biology*, vol. 13, no. 5, pp. 505–514, 1994.

[63] D. U. Rabin, S. M. Pleasic, J. A. Shapiro et al., "Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases," *The Journal of Immunology*, vol. 152, no. 6, pp. 3183–3188, 1994.

[64] H. J. DeAizpurua, M. C. Honeyman, L. C. Harrison et al., "Glutamic acid decarboxylase (GAD) in fetal pig proislets is recognised by antibodies and stimulates T cells from humans

with pre-clinical insulindependent diabetes mellitus," *Journal of Autoimmunity*, vol. 5, pp. 759–770, 1992.

[65] L. C. Harrison, M. C. Honeyman, H. J. DeAizpurua et al., "Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes," *The Lancet*, vol. 341, no. 8857, pp. 1365–1369, 1993.

[66] T. Lohmann, R. D. G. Leslie, M. Hawa, M. Geysen, S. Rodda, and M. Londei, "Immunodominant epitopes of glutamic acid decarboxylase 65 and 67 in insulin-dependent diabetes mellitus," *The Lancet*, vol. 343, no. 8913, pp. 1607–1608, 1994.

[67] M. C. Honeyman, D. S. Cram, and L. C. Harrison, "Glutamic acid decarboxylase 67-reactive T cells: a marker of insulin-dependent diabetes," *Journal of Experimental Medicine*, vol. 177, no. 2, pp. 535–540, 1993.

[68] M. A. Atkinson, D. L. Kaufman, L. Campbell et al., "Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes," *The Lancet*, vol. 339, no. 8791, pp. 458–459, 1992.

[69] L. C. Harrison, M. C. Honeyman, H. J. DeAizpurua et al., "Inverse relation between humoral and cellular immunity to glutamic acid decarboxylase in subjects at risk of insulin-dependent diabetes," *The Lancet*, vol. 341, no. 8857, pp. 1365–1369, 1993.

[70] M. A. Atkinson, M. A. Bowman, L. Campbell, B. L. Darrow, D. L. Kaufman, and N. K. Maclaren, "Cellular immunity to a determinant common to glutamate decarboxylase and Coxsackie virus in insulin-dependent diabetes," *The Journal of Clinical Investigation*, vol. 94, no. 5, pp. 2125–2129, 1994.

[71] L. C. Harrison, "Antigen-specific therapy for autoimmune disease: prospects for the prevention of insulin-dependent diabetes," *Molecular Medecine*, vol. 1, no. 7, pp. 722–727, 1995.

[72] M. C. Honeyman, N. L. Stone, and L. C. Harrison, "T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA- 2: potential for mimicry with rotavirus and other environmental agents," *Molecular Medicine*, vol. 4, no. 4, pp. 231–239, 1998.

[73] P. A. Ott, B. A. Herzog, S. Quast et al., "Islet-cell antigen-reactive T cells show different expansion rates and Th1/Th2 differentiation in type 1 diabetic patients and healthy controls," *Clinical Immunology*, vol. 115, no. 1, pp. 102–114, 2005.

[74] L. S. Wicker, S. L. Chen, G. T. Nepom et al., "Naturally processed T cell epitopes from human glutamic acid decarboxylase identified using mice transgenic for the type 1 diabetes-associated human MHC class II allele, DRB1*0401," *The Journal of Clinical Investigation*, vol. 98, no. 11, pp. 2597–2603, 1996.

[75] S. D. Patel, A. P. Cope, M. Congia et al., "Identification of immunodominant T cell epitopes of human glutamic acid decarboxylase 65 by using HLA-DR(α1*0101,β1*0401) transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 15, pp. 8082–8087, 1997.

[76] G. T. Nepom, J. D. Lippolis, F. M. White et al., "Identification and modulation of a naturally processed T cell epitope from the diabetes-associated autoantigen human glutamic acid decarboxylase 65 (hGAD65)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 4, pp. 1763–1768, 2001.

[77] L. I. Wen, F. S. Wong, L. Burkly et al., "Induction of insulitis by glutamic acid decarboxylase peptide-specific and HLA-DQ8-restricted CD4 T cells from human DQ transgenic mice," *The Journal of Clinical Investigation*, vol. 102, no. 5, pp. 947–957, 1998.

[78] R. S. Abraham, S. B. Wilson, N. F. De Souza, J. L. Strominger, S. R. Munn, and C. S. David, "NOD background genes influence T cell responses to GAD 65 in HLA-DQ8 transgenic mice," *Human Immunology*, vol. 60, no. 7, pp. 583–590, 1999.

[79] H. Reijonen, E. J. Novak, S. Kochik et al., "Detection of GAD65-specific T-cells by major histocompatibility complex class II tetramers in type 1 diabetic patients and at-risk subjects," *Diabetes*, vol. 51, no. 5, pp. 1375–1382, 2002.

[80] V. Öling, J. Marttila, J. Ilonen et al., "GAD65- and proinsulin-specific CD4 T-cells detected by MHC class II tetramers in peripheral blood of type 1 diabetes patients and at-risk subjects," *Journal of Autoimmunity*, vol. 25, no. 3, pp. 235–243, 2005.

[81] J. Endl, H. Otto, G. Jung et al., "Identification of naturally processed T cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T lymphocytes of recent onset IDDM patients," *The Journal of Clinical Investigation*, vol. 99, no. 10, pp. 2405–2415, 1997.

[82] J. A. Gebe, K. A. Unrath, B. B. Yue, T. Miyake, B. A. Falk, and G. T. Nepom, "Autoreactive human T-cell receptor initiates insulitis and impaired glucose tolerance in HLA DR4 transgenic mice," *Journal of Autoimmunity*, vol. 30, no. 4, pp. 197–206, 2008.

[83] J. A. Gebe, B. B. Yue, K. A. Unrath, B. A. Falk, and G. T. Nepom, "Restricted autoantigen recognition associated with deletional and adaptive regulatory mechanisms," *The Journal of Immunology*, vol. 183, no. 1, pp. 59–65, 2009.

[84] N. E. Standifer, E. A. Burwell, V. H. Gersuk, C. J. Greenbaum, and G. T. Nepom, "Changes in autoreactive T cell avidity during type 1 diabetes development," *Clinical Immunology*, vol. 132, no. 3, pp. 312–320, 2009.

[85] A. Geluk, K. E. Van Meijgaarden, N. C. Schloot, J. W. Drijfhout, T. H. M. Ottenhoff, and B. O. Roep, "HLA-DR binding analysis of peptides from islet antigens in IDDM," *Diabetes*, vol. 47, no. 10, pp. 1594–1601, 1998.

[86] E. Harfouch-Hammoud, T. Walk, H. Otto et al., "Identification of peptides from autoantigens GAD65 and IA-2 that bind to HLA class II molecules predisposing to or protecting from type 1 diabetes," *Diabetes*, vol. 48, no. 10, pp. 1937–1947, 1999.

[87] G. Fenalti, C. S. Hampe, Y. Arafat et al., "COOH-terminal clustering of autoantibody and T-cell determinants on the structure of GAD65 provide insights into the molecular basis of autoreactivity," *Diabetes*, vol. 57, no. 5, pp. 1293–1301, 2008.

[88] X. Ge, J. D. Piganelli, H. M. Tse et al., "Modulatory role of DR4- to DQ8-restricted CD4 T-cell responses and type 1 diabetes susceptibility," *Diabetes*, vol. 55, no. 12, pp. 3455–3462, 2006.

[89] K. Y. Chang and E. R. Unanue, "Prediction of HLA-DQ8 β cell peptidome using a computational program and its relationship to autoreactive T cells," *International Immunology*, vol. 21, no. 6, pp. 705–713, 2009.

[90] M. C. Honeyman, N. L. Stone, and L. C. Harrison, "T-cell epitopes in type 1 diabetes autoantigen tyrosine phosphatase IA- 2: potential for mimicry with rotavirus and other environmental agents," *Molecular Medicine*, vol. 4, no. 4, pp. 231–239, 1998.

[91] K. Kelemen, P. A. Gottlieb, A. L. Putnam, H. W. Davidson, D. R. Wegmann, and J. C. Hutton, "HLA-DQ8-associated T cell responses to the diabetes autoantigen phogrin (IA-2β) in human prediabetes," *The Journal of Immunology*, vol. 172, no. 6, pp. 3955–3962, 2004.

[92] M. C. Honeyman, N. L. Stone, B. A. Falk, G. Nepom, and L. C. Harrison, "Evidence for molecular mimicry between human T cell epitopes in rotavirus and pancreatic islet autoantigens," *The Journal of Immunology*, vol. 184, no. 4, pp. 2204–2210, 2010.

[93] S. M. Weenink, J. Lo, C. R. Stephenson et al., "Autoantibodies and associated T-cell responses to determinants within the 831-860 region of the autoantigen IA-2 in Type 1 diabetes," *Journal of Autoimmunity*, vol. 33, no. 2, pp. 147–154, 2009.

[94] J. Yang, N. A. Danke, D. Berger et al., "Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive $CD4^+$ T cells in human subjects," *The Journal of Immunology*, vol. 176, no. 5, pp. 2781–2789, 2006.

[95] F. Vendrame, A. Pileggi, E. Laughlin et al., "Recurrence of type 1 diabetes after simultaneous pancreas-kidney transplantation, despite immunosuppression, is associated with autoantibodies and pathogenic autoreactive CD4 T-cells," *Diabetes*, vol. 59, no. 4, pp. 947–957, 2010.

[96] R. Mallone, E. Martinuzzi, P. Blancou et al., "CD8+ T-cell responses identify β-cell autoimmunity in human type 1 diabetes," *Diabetes*, vol. 56, no. 3, pp. 613–621, 2007.

[97] K. Takahashi, M. C. Honeyman, and L. C. Harrison, "Cytotoxic T cells to an epitope in the islet autoantigen IA-2 are not disease-specific," *Clinical Immunology*, vol. 99, no. 3, pp. 360–364, 2001.

[98] C. Panagiotopoulos, H. Qin, R. Tan, and C. B. Verchere, "Identification of a β-cell-specific HLA class I restricted epitope in type 1 diabetes," *Diabetes*, vol. 52, no. 11, pp. 2647–2651, 2003.

[99] N. E. Standifer, Q. Ouyang, C. Panagiotopoulos et al., "Identification of novel HLA-A*0201-restricted epitopes in recent-onset type 1 diabetic subjects and antibody-positive relatives," *Diabetes*, vol. 55, no. 11, pp. 3061–3067, 2006.

[100] Q. Ouyang, N. E. Standifer, H. Qin et al., "Recognition of HLA class I-restricted β-cell epitopes in type 1 diabetes," *Diabetes*, vol. 55, no. 11, pp. 3068–3074, 2006.

[101] P. Monti, M. Scirpoli, A. Rigamonti et al., "Evidence for in vivo primed and expanded autoreactive T cells as a specific feature of patients with type 1 diabetes," *The Journal of Immunology*, vol. 179, no. 9, pp. 5785–5792, 2007.

[102] T. Takaki, M. P. Marron, C. E. Mathews et al., "HLA-A*0201-restricted T cells from humanized NOD mice recognize autoantigens of potential clinical relevance to type 1 diabetes," *The Journal of Immunology*, vol. 176, no. 5, pp. 3257–3265, 2006.

[103] P. Blancou, R. Mallone, E. Martinuzzi et al., "Immunization of HLA class I transgenic mice identifies autoantigenic epitopes eliciting dominant responses in type 1 diabetes patients," *The Journal of Immunology*, vol. 178, no. 11, pp. 7458–7466, 2007.

[104] C. Boitard, R. Yasunami, M. Dardenne et al., "T cell-mediated inhibition of the transfer of autoimmune diabetes in NOD mice," *The Journal of Experimental Medecine*, vol. 169, no. 5, pp. 1669–1680, 1989.

[105] A. Kukreja, G. Cost, J. Marker et al., "Multiple immuno-regulatory defects in type-1 diabetes," *The Journal of Clinical Investigation*, vol. 109, no. 1, pp. 131–140, 2002.

[106] W. Liu, A. L. Putnam, Z. Xu-yu et al., "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells," *Journal of Experimental Medicine*, vol. 203, no. 7, pp. 1701–1711, 2006.

[107] T. I. M. Tree, J. Lawson, H. Edwards et al., "Naturally arising human CD4+ T-cells that recognize islet autoantigens and secrete interleukin-10 regulate proinflammatory T-cell responses via linked suppression," *Diabetes*, vol. 59, no. 6, pp. 1451–1460, 2010.

[108] R. Mallone, S. A. Kochik, H. Reijonen et al., "Functional avidity directs T-cell fate in autoreactive CD4+ T cells," *Blood*, vol. 106, no. 8, pp. 2798–2805, 2005.

[109] I. Durinovic-Belló, E. Jelinek, M. Schlosser et al., "Class III alleles at the insulin VNTR polymorphism are associated with regulatory T-cell responses to proinsulin epitopes in HLA-DR4, DQ8 individuals," *Diabetes*, vol. 54, supplement 2, pp. S18–S24, 2005.

[110] I. Durinovic-Belló, S. Rosinger, J. A. Olson et al., "DRB1*0401-restricted human T cell clone specific for the major proinsulin 73-90 epitope expresses a down-regulatory T helper 2 phenotype," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 31, pp. 11683–11688, 2006.

[111] N. A. Danke, J. Yang, C. Greenbaum, and W. W. Kwok, "Comparative study of GAD65-specific CD4+ T cells in healthy and type 1 diabetic subjects," *Journal of Autoimmunity*, vol. 25, no. 4, pp. 303–311, 2005.

[112] N. A. Danke, D. M. Koelle, C. Yee, S. Beheray, and W. W. Kwok, "Autoreactive T cells in healthy individuals," *The Journal of Immunology*, vol. 172, no. 10, pp. 5967–5972, 2004.

[113] J. A. Dromey, B. H. Lee, H. Yu et al., "Generation and expansion of regulatory human CD4(+) T-cell clones specific for pancreatic islet autoantigens," *Journal of Autoimmunity*, vol. 36, no. 1, pp. 47–55, 2011.

[114] S. A. Long, K. Cerosaletti, P. L. Bollyky et al., "Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4(+)CD25(+) regulatory T-cells of type 1 diabetic subjects," *Diabetes*, vol. 59, no. 2, pp. 407–415, 2010.

[115] V. Viglietta, S. C. Kent, T. Orban, and D. A. Hafler, "GAD65-reactive T cells are activated in patients with autoimmune type 1a diabetes," *The Journal of Clinical Investigation*, vol. 109, no. 7, pp. 895–903, 2002.

[116] J. Endl, S. Rosinger, B. Schwarz et al., "Coexpression of CD25 and OX40 (CD134) receptors delineates autoreactive T-cells in type 1 diabetes," *Diabetes*, vol. 55, no. 1, pp. 50–60, 2006.

[117] E. A. James and W. W. Kwok, "CD8 suppressor-mediated regulation of human CD4+ T cell responses to glutamic acid decarboxylase 65," *European Journal of Immunology*, vol. 37, no. 1, pp. 78–86, 2007.

[118] C. Boitard, G. Feutren, L. Castano et al., "Effect of cyclosporin A treatment on the production of antibody in insulin-dependent (type I) diabetic patients," *The Journal of Clinical Investigation*, vol. 80, no. 6, pp. 1607–1612, 1987.

[119] B. O. Roep and M. Peakman, "Surrogate end points in the design of immunotherapy trials: emerging lessons from type 1 diabetes," *Nature Reviews Immunology*, vol. 10, no. 2, pp. 145–152, 2010.

[120] A. M. Secrest, D. J. Becker, S. F. Kelsey, R. E. LaPorte, and T. J. Orchard, "Cause-specific mortality trends in a large population-based cohort with long-standing childhood-onset type 1 diabetes," *Diabetes*, vol. 59, no. 12, pp. 3216–3222, 2010.

[121] J. Tian and D. L. Kaufman, "Antigen-based therapy for the treatment of type 1 diabetes," *Diabetes*, vol. 58, no. 9, pp. 1939–1946, 2009.

[122] S. D. Miller, D. M. Turley, and J. R. Podojil, "Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease," *Nature Reviews Immunology*, vol. 7, no. 9, pp. 665–677, 2007.

[123] B. Salomon, L. Rhee, H. Bour-Jordan et al., "Development of spontaneous autoimmune peripheral polyneuropathy in B7-2-deficient NOD mice," *Journal of Experimental Medicine*, vol. 194, no. 5, pp. 677–684, 2001.

[124] N. Prevot, C. Briet, and H. Lassmann, "Abrogation of ICOS/ICOSL costimulation in NOD mice results in autoimmune deviation towards the neuromuscular system," *European Journal of Immunology*, vol. 40, pp. 2267–2276, 2010.

[125] S. C. Shah, J. I. Malone, and N. E. Simpson, "A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus," *The New England Journal of Medicine*, vol. 320, no. 9, pp. 550–554, 1989.

[126] O. Schnell, B. Eisfelder, E. Standl et al., "High-dose intravenous insulin infusion versus intensive insulin treatment in newly diagnosed IDDM," *Diabetes*, vol. 46, no. 10, pp. 1607–1611, 1997.

[127] The Diabetes Control and Complications Trial, "Effect of intensive therapy on residual β-cell function in patients with type 1 diabetes in the Diabetes Control and Complications Trial," *Annals of Internal Medecine*, vol. 128, pp. 517–523, 1998.

[128] L. C. Harrison, M. C. Honeyman, C. E. Steele et al., "Pancreatic β-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes," *Diabetes Care*, vol. 27, no. 10, pp. 2348–2355, 2004.

[129] diabetes Prevention Trial–Type 1 Diabetes Study Group, "Effects of insulin in relatives of patients with Type 1 Diabetes Mellitus," *The New England Journal of Medecine*, vol. 346, pp. 1685–1691, 2002.

[130] J. S. Skyler, J. P. Krischer, and J. Wolfsdorf, "Effects of oral insulin in relatives of patients with type 1 diabetes: the diabetes prevention trial-type 1," *Diabetes Care*, vol. 28, no. 5, pp. 1068–1076, 2005.

[131] K. Näntö-Salonen, A. Kupila, S. Simell et al., "Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial," *The Lancet*, vol. 372, no. 9651, pp. 1746–1755, 2008.

[132] L. Chaillous, H. Lefèvre, C. Thivolet et al., "Oral insulin administration and residual β-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial," *The Lancet*, vol. 356, no. 9229, pp. 545–549, 2000.

[133] P. Pozzilli, D. Pitocco, N. Visalli et al., "No effect of oral insulin on residual beta-cell function in recent-onset Type I diabetes (the IMDIAB VII)," *Diabetologia*, vol. 43, no. 8, pp. 1000–1004, 2000.

[134] T. Orban, K. Farkas, H. Jalahej et al., "Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy," *Journal of Autoimmunity*, vol. 34, no. 4, pp. 408–415, 2010.

[135] T. Maruyama, S. Tanaka, A. Shimada et al., "Insulin intervention in slowly progressive insulin-dependent (type 1) diabetes mellitus," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 6, pp. 2115–2121, 2008.

[136] M. Alvarsson, G. Sundkvist, I. Lager et al., "Effects of insulin vs. glibenclamide in recently diagnosed patients with type 2 diabetes: a 4-year follow-up," *Diabetes, Obesity and Metabolism*, vol. 10, no. 5, pp. 421–429, 2008.

[137] C. D. Agardh, C. M. Cilio, A. Lethagen et al., "Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes," *Journal of Diabetes and its Complications*, vol. 19, no. 4, pp. 238–246, 2005.

[138] J. Ludvigsson, M. Faresjö, M. Hjorth et al., "GAD treatment and insulin secretion in recent-onset type 1 diabetes," *The New England Journal of Medicine*, vol. 359, no. 18, pp. 1909–1920, 2008.

[139] I. Raz, D. Elias, A. Avron, M. Tamir, M. Metzger, and I. R. Cohen, "β-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial," *The Lancet*, vol. 358, no. 9295, pp. 1749–1753, 2001.

[140] G. Semana, R. Gausling, R. A. Jackson, and D. A. Hafler, "T cell autoreactivity to proinsulin epitopes in diabetic patients and healthy subjects," *Journal of Autoimmunity*, vol. 12, no. 4, pp. 259–267, 1999.

[141] I. Durinovic-Belló, B. O. Boehm, and A. G. Ziegler, "Predominantly recognized ProInsulin T helper cell epitopes in individuals with and without islet cell autoimmunity," *Journal of Autoimmunity*, vol. 18, no. 1, pp. 55–66, 2002.

[142] K. Kimura, T. Kawamura, S. Kadotani, H. Inada, S. Niihira, and T. Yamano, "Peptide-specific cytotoxicity of T lymphocytes against glutamic acid decarboxylase and insulin in type 1 diabetes mellitus," *Diabetes Research and Clinical Practice*, vol. 51, no. 3, pp. 173–179, 2001.

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Review Article

Current State of Type 1 Diabetes Immunotherapy: Incremental Advances, Huge Leaps, or More of the Same?

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Thus far, none of the preclinically successful and promising immunomodulatory agents for type 1 diabetes mellitus (T1DM) has conferred stable, long-term insulin independence to diabetic patients. The majority of these immunomodulators are humanised antibodies that target immune cells or cytokines. These as well as fusion proteins and inhibitor proteins all share varying adverse event occurrence and severity. Other approaches have included intact putative autoantigens or autoantigen peptides. Considerable logistical outlays have been deployed to develop and to translate humanised antibodies targeting immune cells, cytokines, and cytokine receptors to the clinic. Very recent phase III trials with the leading agent, a humanised anti-CD3 antibody, call into question whether further development of these biologics represents a step forward or more of the same. Combination therapies of one or more of these humanised antibodies are also being considered, and they face identical, if not more serious, impediments and safety issues. This paper will highlight the preclinical successes and the excitement generated by phase II trials while offering alternative possibilities and new translational avenues that can be explored given the very recent disappointment in leading agents in more advanced clinical trials.

1. Introduction

Type 1 diabetes is an autoimmune disease clinically characterized by hyperglycemia underlai by a significant loss of pancreatic insulin-producing beta cell mass. Even though normoglycemia is achieved with pharmacologic insulin replacement, the underlying autoimmune response that impairs and eventually eradicates the beta cells is not treated. Insulin replacement cannot prevent the peripheral complications, a major source of patient morbidity and mortality. Strategies like beta cell replacement with cadaver donor islets still face the impediment of autoimmunity in addition to allogeneic rejection. There is therefore a need to develop methods that directly suppress or eliminate autoimmunity and allow a possible regenerative process.

Activated autoreactive T cells are the mediator of beta cell destruction and therefore a prime therapeutic target. Other T cell subpopulations help determine the responsiveness of cytotoxic T-cells. T helper (Th) cells are one of these populations and are divided into 3 groups based on their cytokine production profiles: proinflammatory Th1 and Th17 and anti-inflammatory Th2. The balance of Th cell populations is an important regulator of the immune system and is often examined after immunotherapy treatments, along with anti-inflammatory T-regulatory (Treg) cells. In addition to these cell types, antigen-presenting cells (APCs) such as dendritic cells (DCs) and B cells are responsible for the direct activation of T cells in response to specific antigens. Various techniques of immunomodulation have been employed in animal models to directly or indirectly regulate cytotoxic T-cell activation utilizing these different target cell populations. Here we will discuss their progress through clinical trials and offer some commentary on whether they represent incremental advances, huge leaps in terms of curative outcome and/or improvement of insulin requirements, or more of the same.

2. To Prevent or to Reverse?

The identification of multiple genetic susceptibility loci over the past decade, when coupled with the presence in high titers of the traditional autoantibody markers in first-degree relatives of T1DM patients, offers a preventive interventional opportunity. By initiating immunomodulation in such preclinically diabetic individuals, it is theoretically possible to mitigate clinical onset of the disease. Statistically, a variety of modeling outcomes suggest that such an approach could be beneficial, although much of the optimism rests on biological data from mouse studies which may not be mirrored in humans. Furthermore, even though genetic and humoral risk may be considerable, they do not always result in clinical disease [75]. The therapist thus faces two dilemmas: (i) are the benefits of prevention worth the risks of the adverse events of current immunomodulation approaches? and (ii) are the benefits of prevention worth the considerable logistical outlays required to screen and treat all those who meet "high-risk" status? The first is the most germane, especially since the long-term effects on the immune system of newer immunomodulation agents are unknown. Furthermore, there are the real risks that latent infections due to dormant viruses could become productive and life threatening as well as the possibility that modulation of immune cells could provoke latent or low-grade autoimmunity other than T1DM. These valid arguments form the cornerstone against which any preventive immunomodulation approach will have to push to successfully enter clinical trials other than phase I safety studies.

On the other hand, attempting immunomodulation in individuals who exhibit clinical disease is better justifiable as the autoimmunity is not speculative (unlike in prevention approaches) but a fact. This then leads to the question of what is considered the point of "too late" at which immunomodulation is ineffective and only adverse events will plague the patient without any possibility of real benefit.

The most straightforward answer is to identify a time window that defines a period between the onset of clinical disease and the last possible point inside which immunomodulation will result in the preservation and/or restoration of a beta cell mass adequate enough to reduce the concentration of, or even obviate, exogenous insulin replacement. Traditionally, this window has been termed the "honeymoon" period; however, a number of studies suggest that it can extend further on, as C-peptide can be detected in adult individuals who have the disease for many years [76, 77]. The diabetic inflammation of the islets of Langerhans need not be associated with beta cell destruction. It is now well accepted that insulitis impairs beta cell sensing of glucose with/without concomitant impairment in insulin production and secretion in the absence of significant irreversible physical elimination of beta cells [78–80]. Studies also show that beta cells can be differentiated from pancreatic progenitors when inflammation is controlled and suppressed. In fact, under some conditions, suppression of islet inflammation also can be permissive for the replication, even if limited, of existing beta cells [81–84]. The key then is to find a means of first suppressing the islet inflammation to restore the function of residual beta cells and then, once this is secured, to identify a method to ensure that beta-cell-reactive T cells are prevented from reaching the islets and/or that their function is silenced. This second requirement necessitates the establishment of some form of tolerance or regulation of autoreactive immune cells. A product that achieves both concurrently would be best.

3. Biomarkers of Efficacy

Although a number of surrogate measurements are accepted in clinical trials, thus far there are no bona fide biomarkers of therapeutic success. The presence of autoantibodies supports the autoimmune nature of T1DM and differentiates it from type 2 diabetes and other forms of metabolic glucohomeostasis impairment, but their disappearance cannot yield information on success of therapies. Cellular proliferation to islet-specific proteins and peptides can differentiate normal from disease-susceptible individuals; however, no causal relationship between disease process and specific cellular response has been established. Frequencies of cell populations in peripheral blood associated with immune regulation (i.e., Foxp3+ Tregs) have been suggested as markers of disease severity and stage and success of immunomodulation, but there is very little functional evidence in support of these propositions. Thus, in the absence of specific and therapy-related biomarkers, the field has accepted physiologic endpoints as surrogates of therapy efficacy.

In the absence of bona fide biomarkers of immune efficacy, physiologic measurements become of paramount importance. Examples, for T1DM, include maintenance or increase of pretreatment C-peptide levels, improvement of mixed meal-stimulated insulin production and consequent reduction of glucose levels in blood, improvements in glycated HbA1c and, in certain instances, reduction of exogenously administered insulin or complete cessation thereof for a period of time. As will be discussed later, the values between pretreatment and posttreatment physiological measurements in a number of recent immunomodulation clinical trials, in particular if promoted and implemented close to the clinical onset of the disease, have required stringent statistical analysis to identify differences, and in many respects, the pronouncements of success for some immunomodulation treatments require considerable faith in the outcome of statistical treatments of the data.

The frustration with many immunomodulation trials is the disconnect between preclinical data in mouse and rat models of T1DM and the physiologic outcomes in T1DM patients.

4. Preclinical Models: Is Efficacy Predictive of Outcome in Humans?

Currently, there are only two spontaneously occurring, genetically susceptible animal models of T1DM: the heavily popular nonobese diabetic/LtJ strain (NOD) and the diabetes-prone biobreeding rat (BB) [85]. The intensity with which many studies are performed in these two strains

overlooks some obvious, for the sake of clinical translation, inconvenient facts. First, the two strains are analogous to clones of two individual humans. Second, the differences between man and murine (or rat) immune systems, responses, and immunopathology of T1DM is as different as are the known and perceived similarities [85]. The lymphopenia that characterises the BB rat model is counterintuitive to how autoimmunity develops in this species, even though the target of immune dysfunction and destruction are the islets. Further confounding the issue is the presence of other autoimmunities and inflammatory conditions in the NOD mouse, calling into question just how tissue restricted is its autoimmunity. Another difference lies in the difference in the incidence and prevalence of the disease between female and male NOD mice, a difference that is not observed in humans. The predictable time-at-onset of disease in NOD mice and BB rats is not as simple to extrapolate to humans. Interindividual heterogeneity in humans lies in the different HLA susceptibility alleles, the non-HLA susceptibility loci, the primary autoantigen(s), and the actual composition of peripheral tolerogenic cell populations that wax and wane during preclinical disease progression. Even at the time of clinical onset, the actual mass of functional and potentially functional residual beta cells is unknown, and this heterogeneity can determine success or failure of a particular immunomodulator.

What is sobering is that, of more than 230 therapeutic strategies employed (to various levels of success) in NOD mice and BB rats, fewer than ten have proven to be of any clinical utility, and therefore, only a few have progressed to advanced trials [85, 86]. This should caution the investigator that the outcomes in NOD mice can be instructive for some human patients but not all. The overwhelming number of studies in NOD mice and the few viable therapeutics translated to the clinic offer the following lessons: (i) early prevention is simple in NOD mice (more than 65% of studies began some form of therapy between 4–6 weeks); (ii) treating new-onset diabetic NOD mice is difficult; (iii) dosage of the agent, especially putative autoantigen peptide formulation may make or break the success; (iv) most studies in NOD mice cease the monitoring at less than 40 weeks of age; (v) nonspecific microbes can influence the outcome in NOD mice. Considerable effort and logistics have been deployed in the past two decades to use insulin administration as a prophylactic intervention [87], and more recently, to employ an anti-CD3 monoclonal antibody in new-onset human disease [88–90]. The optimism and enthusiasm in both these high-profile multicenter trials was supported by data on the resoundingly successful prevention and "reversal of disease" in NOD mice [91–93]. Nevertheless, insulin administration has yet to demonstrate any successful preventive outcome in humans [87, 94–97], and very recent phase III trials of one embodiment of the anti-CD3 antibody failed to reach the primary end-point (http://www.rttnews.com/Content/BreakingNews.aspx?Id=1451366&SM=1&SimRec=1 and http://www.bizjournals.com/washington/www.bizjournals.com/washington/quick_news/2010/10/macrogenics-lilly-abandon-diabetes-drug.html). While "more of the same" autoantigen interventions can be justified by the apparent absence of adverse events noted in the insulin trials (and more recent GAD- and HSP-derived peptide studies), the choice of "more of the same" approaches that involve immunodepletion (chemical or biologic agent-mediated), even if supported by data from NOD mice and BB rats, has to be tempered by unsupportive data in advanced trials as well as borderline outcomes in early-phase trials where heavy statistical treatment might be used to demonstrate efficacy, even if minor. Nevertheless, in the absence of any other animal model, the NOD mouse and the BB rat will continue to be the proving grounds of preclinical efficacy.

5. Translation of Successful Preclinical Studies into the Clinic: the Outcomes and the Potential in Humans

The cyclosporin A and the steroid-azathioprine trials proved that established T1DM was reversible [98–100] and, more importantly, that a reserve of beta cell mass was able to restore normoglycemia contingent on suppression of inflammation and autoimmunity [101, 102]. Nevertheless, once cyclosporin A administration was suspended/terminated, the disease reappeared with a vigor no different than that during pretreatment. From a historical perspective, the first successful immunomodulation-based reversal of T1DM was achieved by lymphocyte-specific serum in BB rats [103]. It would be 20 years later that this approach would be attempted in humans [104], although the significant side effects precluded the justification to further explore this approach. Since then, a number of preclinical studies, mainly in NOD mice, have been clinically-translated.

In 1985, Eisenbarth and colleagues reported that steroid-supplemented antithymocyte globulin administration into new-onset T1DM patients reduced insulin requirements. However, this approach was abandoned due to the thrombocytopenia that the procedure was generating [105]. Since then, antithymocyte globulin has been reconsidered and, in new-onset patients, carefully adjusted dosing slowed C-peptide decline [106]. Antithymocyte globulin binds to the CD3 complex on T cells as one of its many targets and causes significantly greater T-cell depletion. Thus, its justification is hampered by significantly greater adverse events and immunosuppression compared to anti-CD3 antibodies.

The initial excitement generated over insulin administration was tempered by the outcome of a major clinical study which showed few, if any, beneficial outcomes [87]. More recent clinical studies using monoclonal antibodies targeting CD3 and CD20 have also been disappointing despite the convincing and robust successes in preclinical studies [65, 89] (http://www.rttnews.com/Content/BreakingNews.aspx?Id=1451366&SM=1&SimRec=1 and http://www.bizjournals.com/washington/quick_news/2010/10/macrogenics-lilly-abandon-diabetes-drug.html).

Long before confirmation in genetic models, insulin and proinsulin were strong contenders as the T1DM-initiating autoantigens [107–114]. As autoantigens, many proposed that the exposure of the T1DM immune system to exogenous insulin in a manner that could modulate immunity towards

insulin-specific tolerance could prevent disease in prediabetic states and perhaps delay the progression to overt clinical hyperglycemia in more advanced, but subclinical, states [115–118]. Persistent oral insulin treatment of NOD mice delayed T1DM onset and reduced disease incidence in NOD mice [119]. The addition of adjuvants provided a practical benefit in reducing the amount of exogenous insulin required to achieve the beneficial effects [120]. Largely based on the data supporting the view that mucosal delivery of soluble peptides promotes tolerance to them, a number of efforts targeted, in addition to oral, intranasal insulin or insulin-derived peptide , aerosolisation into prediabetic NOD mice. In these studies, diabetes was significantly delayed [71, 117, 121, 122]. These very promising studies with what essentially was a simple intervention led to the DPT-1 multicenter trial which determined the efficacy of oral insulin in first- and second-degree relatives of T1DM patients deemed to fall into high-risk status based on metabolic, immune, and genetic evidence [73, 123]. Other than a possible benefit in individuals with the highest autoantibody titers, the DPT-1 study failed to delay or prevent T1DM. A similarly disappointing outcome resulted in another prevention trial where insulin was administered intranasally Näntö-Salonen et al, [124]. However, a very small subgroup of autoantibody-positive patients was identified in which some effect was shown. The general view of nonpharmacologic insulin therapy is that, if beneficial, it probably will be restricted to very well-defined and characterised subpopulations of patients. It is unclear what can distinguish these patients from a general at-risk population.

A number of other trials were initiated since then without any significant benefits. The IMDIAB trial showed that oral insulin provided no benefit over placebo after a one-year followup in terms of mean C-peptide secretion and insulin requirements. The ORALE trial, comparing low- with high-dose oral insulin (2.5 mg/day versus 7.5 mg/day), after one year could not discern any benefit in decelerating the loss of physiologic beta cell function [125]. It was recently shown that combined intranasal insulin with CD3 antibody was able to reverse new-onset T1DM in NOD mice, although the effect was quite likely due to the CD3 antibody and the characteristics of the NOD mouse cohorts and not due to the insulin [126].

The most recent attempts at using insulin to improve functional beta cell mass involve intramuscular injection of human insulin B chain in incomplete Freund's adjuvant as well as subcutaneous injection of a DNA plasmid vector encoding proinsulin see [127] and http://www.bayhill-.bayhilltherapeutics.com/. The outcomes of these proposals rest on the publication of phase II studies which are currently at various stages of implementation.

As strong an argument for GAD can be made in the etiopathogenesis of T1DM as for insulin. The 65 KDa isoform of GAD has been demonstrated to be a target of early-insulitic T cells *in vitro* and *in vivo* [128, 129]. Administration of GAD into very young NOD mice suppresses anti-GAD T-cell reactivity as well as disease onset [129, 130]. GAD is equally effective when administered into older NOD mice [130]. The relevance of GAD to human T1DM relies on the presence of GAD autoantibodies in prediabetic humans and is one of three reliable markers of susceptibility [131].

Two clinical trials have used human GAD65 with alum as adjuvant to determine improvement of physiologic beta cell function. One was conducted in LADA patients [132] and determined that this formulation increased fasting and stimulated C-peptide at 24 weeks compared to baseline, a benefit that was associated with an increase of CD4+ CD25+ Tregs. The other study considered the effects of GAD65 in alum in recent-onset T1DM individuals (10–18 years of age) on the rate of decline of stimulated C-peptide [133]. The study revealed a slower rate of decline of stimulated C-peptide in GAD-treated diabetics compared to the placebo group.

Early studies showed that intrathymic administration of Hsp60-derived peptides was prophylactic in NOD mice even though Hsp60 was not considered a bona fide autoantigen involved in T1DM initiation [134]. More experiments in NOD mice revealed that the p277 peptide of Hsp60 was quite effective in suppressing disease progression [135]. These data compelled the development of a human equivalent of p277 (DiaPep277; a 24 amino acid synthetic peptide derived from the C terminus of human hsp60).

DiaPep277 has been evaluated in phase II studies [136], and data demonstrate some degree of preservation of stimulated C-peptide secretion.

Since then, three other studies have been conducted in new-onset T1DM patients [137–139]. In two of these studies, adult patients were the study population, and these recipients exhibited better, yet limited, C-peptide production than placebo-treated individuals. In contrast, DiaPep277 did not offer any objective benefits in young new-onset patients [137].

T-cell depletion targeting the CD3 complex with the OKT3 monoclonal antibody was successfully employed about two decades ago [91]. Administration of the antibody into diabetic NOD mice resulted in complete remission of disease [91, 92]. One caveat of this particular antibody was that, under some situations, it could activate T cells and therefore it was not considered suitable for human use. Soon thereafter, FcR nonbinding embodiments were manufactured which exhibited either IgG1 Fc chain or which eliminated glycosylation sites of the original OKT3 clone. These variants were found to be less activating than OKT3 [140, 141]. Herold and colleagues initially demonstrated that a short-term administration of the nonglycosylated variant into new-onset T1DM patients suppressed the loss of beta cell function as measured indirectly by surrogate physiologic markers [90, 142]. Some patients maintained improved physiologic markers of beta cell function for as much as two years following the treatment in the absence of systemic immunosuppression. A second round of anti-CD3 antibody injections was postulated as necessary to maintain the beneficial outcome. However, additional injections of the antibody were shown to confer more serious, previously undocumented clinical complications.

T1DM is unquestionably a T-cell-mediated disease; however, other cell populations have been implicated in the onset,

and early progression, of the disease, including B-lymphocytes. Accumulated findings in NOD mice demonstrated that B-cell depletion could be beneficial [143–145], even some studies suggested the contrary [146] where adoptive transfer of T1DM into NOD-SCID mice was possible in the absence of B cells and antibodies. B cells can also participate in disease exacerbation by promoting epitope spreading as shown by Tian and colleagues [147]. Indeed, capture of autoantigens such as GAD65 by B-cell surface immunoglobulin (Ig) is followed by processing and presentation of T-cell determinants by B cells, a step that is crucial for activation of autoreactive T cells and induction of diabetes [148–150]. B-cell-mediated processing of self-Ags may contribute to the generation of an inflammatory microenvironment in the pancreas, which is critical for overcoming the regulatory barrier(s) to initiation of diabetes in NOD mice [151]. Antigen-specific B cells and their antibodies are essential in catalyzing determinant-spreading reaction *via* (a) generation of novel previously cryptic epitopes through altered antigen processing or (b) facilitation of T-cell activation through generation of ligands with higher affinity for TCR and delivery of costimulatory signals. Ag processing through surface receptor-mediated internalization, for example, Fc receptors and soluble Ig, is different from that occurring through pinocytosis and phagocytosis [152].

Pescovitz and colleagues demonstrated a small but statistically significant improvement in physiologic markers of beta cell function in new-onset patients [65]. Nevertheless, the improvement was transient as placebo and rituximab-treated patients eventually (by two years) exhibited an identical decline in beta cell function. One possible mechanism could involve the restriction of epitope spreading by B cells after rituximab-mediated deletion, or the repopulation by B-cells that process and present epitopes that compete with diabetes-relevant epitopes.

Autoreactive T cells have been frequently associated with the production of TH1-type proinflammatory cytokines. It is reasonable, therefore, to use cytokine blockade as a unique or part of a combination therapeutic strategy. IL-1beta, TNFalpha, and type 1 interferons have been historically the first to exhibit direct beta cell cytotoxicity [153–155].

Recently, administration of etanercept, a soluble TNFalpha receptor, in an early-phase efficacy trial resulted in a reduction in the loss of C-peptide in T1DM patients [156]. It is important to note, however, that TNFalpha blockade may have unwanted outcomes; TNFalpha can prevent diabetes in older NOD mice even when splenocytes from diabetic NOD mice are adoptively transferred [157]. Furthermore, TNFalpha blockade may require careful consideration of the age of the individuals being treated since blockade in younger NOD mice prevented disease, while in older NOD mice it accelerated T1DM.

Even though IL-1beta is now known to exert early-onset impairment in beta cell function and mediates early-phase recruitment of immune cells into islets [158–161], it is unclear if IL-1beta blockade will have any beneficial outcome in prevention of disease or reversal of new-onset disease. In certain populations characterised by metabolic diabetes (LADA, type 2 diabetes), recent trials with the IL-1 receptor antagonist protein (IRAP, Anakinra) improved glucose control [162], and mechanistically it appears that this benefit is achieved by blockade of IL-1beta impairment of immune cell activity (i.e., inflammation) and blockade of IL-1beta effects on beta cell impairment. It is unclear if administration of the IL-1 receptor antagonist protein (Anakinra) will be of any benefit in new-onset cases, especially as the autoimmunity in new-onset disease is largely IL-1beta independent.

6. Unconventional Therapies That Might Be Clinically Justifiable

An unexpected recent discovery revolves around the realisation that alpha 1 antitrypsin (A1AT) may possess direct anti-inflammatory and perhaps tolerogenic effects, although they are very likely nonantigen specific. A1AT is a serpin whose administration into new onset T1DM NOD mice reversed disease [163]. In addition to its apparent anti-inflammatory effects on insulitis, it promoted regeneration of beta cells and, very likely in an indirect manner, it improved insulin sensitivity. As unclear as the mechanism of action of A1AT may be, equally unclear is the mechanism of another unconventional T1DM treatment using the antileukemia drug, Gleevec [164].

7. Combination Therapies

Recent discussions have focused on combining an immunomodulator successful in preventing/reversing T1DM in preclinical studies and human trials with autoantigen(s) and/or biologic agents that on their own improve the function and/or the mass of residual beta cells. These discussions have also proposed combining two or more immunomodulators. Examples include combining the anti-CD3 antibody with beta-cell-protective and function-enhancing GLP-1 agonists [165, 166], IL-1-neutralising antibodies with the anti-CD3 antibody (http://www.diabetestrialnet.org/studies/index.htm), and anti-CD3 antibodies with traditional pharmacologic immunosuppressives like rapamycin (http://www.diabetestrialnet.org/studies/index.htm).

The problem with these approaches is that the non-anti-CD3 antibody of the combination, on its own had either no effect on prevention or reversal or is not part of any known mechanism that can suppress autoimmunity. Additionally, combining agents where each alone confers obvious side effects could imperil the patient in an additive or multiplicative manner. Last, any benefits a T-cell-depleting antibody may confer on preferential expansion of regulatory T cells could be negated by the effect of pharmacologic inhibitors on Treg function and differentiation. It is our view that combining biologic agents in the absence of solid preclinical data is premature and potentially harmful. It is also unclear, even in the absence of additive adverse events, what improvement addition of another immunomodulator will have on the benefits of the CD3 antibody.

8. Patient-Specific Cell Therapy as a Viable Approach

In 2007, Voltarelli and colleagues first reported that autologous nonmyeloablative hematopoietic stem cell transplantation preceded by cyclophosphamide, granulocyte-colony stimulating factor (G-CSF) stem cell mobilization, and antithymocyte globulin rendered 14/15 new-onset T1DM patients insulin nonrequiring for an average of 16 months [167, 168]. It is unclear if such an approach will be approved elsewhere, indeed in children, and the significant toxicity in all patients calls into question whether this approach is justified for an achievement of 16-month insulin-free state. Nevertheless, hematopoietic stem cell transplantation is clinically feasible and offers one means of either repopulating a depleted immune system with a greater contingent of suppressive immune cells compared to pretreatment or establishing tolerance, even transiently. However, existing autoreactive immune cells will still exist and safe approaches to suppress them must still be employed.

Cell therapy, especially with dendritic cells, is a clinical reality that has also recently been accepted by health providers (http://www.provenge.com/). Overwhelming studies indicate that downregulated costimulation capacity in DC can subserve a tolerogenic therapeutic outcome [169–175]. Costimulation-impaired (or downregulated), functionally-immature DC administration achieves long term and stable allograft survival in a variety of mouse and rat models and prevents a number of autoimmune diseases [176–183]. Mechanistically, functionally immature DCs. act by inducing anergy either via direct cell contact and/or cytokines [172, 184, 185] or, as described more recently, by upregulating the number and function of immune cell subsets, especially the regulatory populations which include Foxp3+ CD25+ CD4+ T cells and a class of CD8+ immunosuppressive T cells [186–194].

Table 1 lists the models where immature DC administration has proven successful. In addition to anergy, Foxp3+ CD25+ CD4+ T-cell numbers have been observed to be increased by the exogenous supply of tolerogenic DC [195–199]. Immature or semimature DC can induce the differentiation of naturally-occurring (thymic) T regulatory cells (CD4+ CD25+) as well as IL-10-producing CD4+ T cells *in vitro* and *in vivo* [195, 198]. Overexpression of the Jagged-1 gene in DC results in the augmentation of antigen-specific TGFbeta-producing CD4+ CD25+ CD4+ T-cells [200]. Functionally immature DCs convert naive T cells into IL-10-secreting cells *in vitro,* and antigen-pulsed DCs injected subcutaneously into humans augment the number of IL-10-producing CD8+ T cells and trigger a reduction in IFNgamma+ T cells [201, 202]. Despite the abundance of data in support of immature DCs as functional inducers of regulatory immune cells, other investigators have discovered that the state of maturity may not be relevant for the induction of immunosuppressive endogenous T cells. For example, similar to the studies by Morel and colleagues [2], mature DCs were shown to expand CD4+ CD25+ CD4+ Foxp3+ T cells which were functionally suppressive and capable of preventing diabetes in the NOD mouse [203–205]. Whether this reflects a restimulation of existing CD4+ CD25+ T cells or their expansion is unknown. We have shown that DCs maintained in a functionally immature state following *in vitro* treatment with NF-kappaB decoys and antisense oligodeoxyribonucleotides (AS-ODNs) to the CD40, CD80, and CD86 costimulatory molecules are diabetes preventive in the NOD mouse [6, 8, 206] and may involve short-range IL-7 signaling, quite likely inside the pancreatic lymph node of NOD mice, to augment the number of CD4+ CD25+ Treg via suppression of apoptosis of a preexisting pool [206]. Multiple injections of AS-ODN-treated DCs in NOD mice maintained the animals as diabetes free without affecting the overall T-cell activity against alloantigens. Furthermore, repeated administrations of co-stimulation-deficient DC reverses hyperglycemia in new-onset diabetes NOD mice. The promising results from this study, together with the low risk of the procedure, helped in gaining approval by the Food and Drug Administration (FDA) office for a phase I clinical trial which has recently ended with very interesting outcomes, the most important being the complete absence of any adverse events. Adult (18 years or older) volunteers with a documented evidence of insulin-requiring T1D of at least 5 years duration were enrolled. Leukocytes of the patients were obtained by apheresis, and DCs were generated *in vitro* and engineered in GMP facilities with the addition of AS-ODN. These DCs expressed low levels of CD80, CD86, and CD40 and were then injected into the patient by intradermal/subcutaneous administration at an anatomical site proximal to the pancreas. The choice of this anatomic region for DC delivery was based on the location of the lymphatic conduits (microvessels) that drain the injection site and favor migration to the pancreatic and para-aortic lymph nodes. We conjecture, based on previous rodent studies, that once inside the pancreatic lymph nodes, the injected DCs will acquire molecules that have drained from the pancreas. These molecules can be acquired by passive drainage or can be transferred to the exogenously supplied DC by antigen-presenting cells coming into the pancreatic lymph nodes from apoptotic islets. Therefore, either by direct uptake of "naked" molecules or antigen transfer by migratory antigen-presenting cells, the exogenously administered DC will acquire the antigen specificity. Alternatively, our administered DC could be recruited from the lymph nodes draining the injection site to the apoptotic islets where they could indirectly deliver an anergizing signal to the T cells they encounter. They can also induce regulatory immune cells. The recent concept of contrasuppression, or alternatively, aggregational suppression (network of intercommunicating DC : Treg) could be operative in our system [207–211]. The vicious cycle that promotes the T cell-mediated anti-beta-cell-antigen-spreading phenomenon will be interrupted this way, enabling the recovery of the physiologic endocrine function of the gland with time. The abrogation of the autoimmune diabetogenic insult should be sufficient to promote rescue or regeneration of the insulin-producing beta cells in the host endocrine pancreas, even after the onset of the disease. Currently, there are no methods to directly test this mechanism *in vivo* in humans; however, mouse and nonhuman primate models offer a number of opportunities.

Table 1: Models of immunotherapeutic DC administration to prolong graft survival and to treat autoimmunity.

Autoimmune disease
Type 1 diabetes
(i) Pancreatic lymph node DC (untreated) [1]
(ii) Mature bone-marrow-derived DC (GM-CSF/IL-4 propagated) or transduced with IL-4 vector [2–4], or TGFbeta [5]
(iii) NF-kappaB oligonucleotide decoy propagated or NF-kappaB inhibition [6, 7]
(iv) Antisense oligonucleotide (CD40-, CD80-, CD86-) propagated [8]
(v) Vitamin D receptor ligands [9, 10]
(vi) Other DC embodiments [11–16]
Thyroiditis
(i) *In vitro* generation with TNFa and supernatant of a GM-CSF-transduced cell line [17, 18]
(ii) GM-CSF generated followed by *in vivo* administration of GM-CSF [19]
Experimental encephalomyelitis (Multiple sclerosis model)
(i) *In vitro* generation with TNFa and supernatant of a GM-CSF-transduced cell line [17, 18]
(ii) VIP and GM-CSF *in vitro* propagation [20, 21]
(iii) TGFbeta and MBP antigen [22]
Myasthenia gravis
(i) RelB knockdown [23]
Arthritis
(i) VIP and GM-CSF *in vitro* propagation [20, 21]
(ii) IL-4- or IL-10-expressing bone-marrow-derived DC and derivative exosome preparations [24–26]
(iii) CD95- (Fas ligand-) expressing bone marrow-derived DC [27, 28]
Gastritis
(i) IL-10- expressing DC [29]
Allergy/asthma
(i) IL-10- overexpressing vector [30]
(ii) Allergen overexpression [31]
Allotransplantation/xenotransplantation
Administration of bone-marrow-derived DC from transplant organ donor
(i) DC propagated in low-concentration GM-CSF [32–34]
(ii) DC propagated in GM-CSF, IL-10, TGFb and matured with LPS [35]
(iii) *In vitro* blockade of NF-kappaB by adding aspirin, vitamin D_3 metabolites/analogues, glucosamine, N-acetyl-cysteine, corticosteroids, cyclosporin A, rapamycin, deoxyspergualin, and mycophenolate mofetil [36–51]
(iv) Gene engineering *in vitro*; DC expressing IL-10, TGFbeta, CTLA-4Ig, indolamine-2,3 dioxygenase, Fas-ligand [52–60]
Administration of transplant-recipient-derived DC prior to transplantation
(i) DC pulsed with class I MHC allopeptide or other alloantigens [61, 62]
(ii) Rapamycin and donor tissue lysate [63]

Table 1 lists the many methods to generate tolerogenic DCs. Some methods converge upon identical cellular and molecular pathways (augmentation of Treg numbers, NF-kappaB inhibition, costimulation blockade). Others are not so obvious. A cautionary detail needs insertion herein: it is important to understand that transplantation immunobiology may not be identical to autoimmunity and vice versa. Indeed, there are numerous instances where a therapeutic regimen achieving long-term allograft survival has failed to abrogate autoimmunity [212–219]. Therefore, DCs that are able to suppress donor-specific antigen alloreactivity may not evoke the appropriate regulatory mechanisms capable of controlling and reversing autoimmunity. Also, although different DC products may exhibit similar cell surface phenotypes and nuclear proteome signatures, by no means can this be a predictor of mechanism of action. Cell viability has rarely been examined in these DC products, and not all potential mechanisms of action were considered. It is possible that all listed DC embodiments may intersect at immune regulatory cell levels, but this remains to be determined. The site of action of many DC products, exogenously administered, is currently unknown. While it is generally believed that the DC activity will occur inside the pancreatic lymph nodes, there are equally likely possibilities that the activity of immune tolerance may in fact occur extralymphatically and around the islets, or inside many lymph nodes, anatomically distant from the pancreas. The study by Ludewig and colleagues demonstrating a nascent

peri-islet lymphoid ultrastructure during diabetes onset is very interesting and instructive and awaits the discovery of a mechanism [220].

Table 1 highlights the studies where animals were treated with tolerogenic DCs pulsed with putative autoantigens. Although the ongoing insulitis drives migration of exogenously administered DCs into the islets, where they acquire the antigen specificity to provide beta cell antigens (from apoptotic/necrotic beta cells) to pancreatic lymph-node-resident regulatory immune cells like Foxp3+ CD25+ CD4+ T cells, it is currently unknown if *ex vivo* pulsing with putative autoantigen(s) could stabilize class I MHC to yield more specific Treg *in vivo*. Our data, however, indicate that the supply of putative autoantigens may not be necessary given the endogenous supply acquired by migratory exogenous DC inside the insulitic lesion. Fathman and colleagues have shown that exogenous DC administration results in preferential accumulation of the DC inside the pancreatic lymph nodes and the omentum [3, 221, 222]. The critical factor in any tissue-targeted approach will be to maintain the DCs in a functionally immature state during and after their accumulation inside that tissue [6, 8, 178, 180, 206].

That our CD40-, CD80-, and CD86-impaired DCs exhibited exemplary safety motivates us to proceed with phase II efficacy trials in new-onset T1DM patients with the objective of decelerating the rate of decline of residual beta cell mass, improving glucose profiles, and perhaps reducting insulin requirements. We anticipate beginning such a trial in the next 12–18 months.

T regulatory cells (Tregs) are another potential cell population that can be used for suppressing the autoimmunity of T1DM. A variety of methods have been employed to expand natural Tregs and to differentiate induced Tregs from peripheral blood progenitors. These cells have been shown to control ongoing autoimmunity and promote diabetes reversal in NOD mice [5, 223–225]. It is unclear whether antigen specificity is relevant in this approach because both antigen-specific and nonspecific expanded Tregs are equally efficacious in suppressing T1DM. Furthermore, Tregs recognising one antigen can have a broad effect on suppression effectors reactive to other antigens [5, 205]. Clinical protocols for T-cell administration into humans exist and patient-specific polyclonal Tregs can, under current conditions, be employed in at least phase I studies.

A particularly interesting discovery very recently indicating that aryl-hydrocarbon receptor-activated CD4+ T cells preferentially differentiate into stably suppressive Foxp3 Tregs, offers therapeutic opportunities that can be exploited by oral route of drug administration [226, 227]. If safe, orally stable aryl hydrocarbon receptor agonists are developed, one could envisage a situation where mucosal CD4+ T-cells could be induced to differentiate into stably suppressive Foxp3+ Tregs. This could be achieved with chemicals or small molecules alone or formulated into nanodelivery particles.

Biopolymers offer a unique platform that can carry antibodies, small molecules, chemical drugs, short sequences of DNA/RNA, and even plasmid vectors [228–231]. Nanoparticles and microspheres in particular can be exploited to deliver immunomodulators on their surface, or inside the polymer matrix. Two recent approaches demonstrate that T1DM can be reversed by administration of microparticles carrying immunomodulatory molecules. We have shown that a microsphere formulation comprised of PEG, polyvinyl pyrrolidone (PVP), and poly-L-lysine-hydrobromide and short antisense oligonucleotides (AS-ODNs) targeting the primary transcripts of the CD40, CD80, and CD86 costimulatory genes effectively prevents and reverses type 1 diabetes in the NOD mouse model [232]. Although the mechanism is not yet fully clear, we propose that the microspheres are taken up following subcutaneous injection (close to an abdominal anatomic site drained in part by the pancreatic lymph nodes) by migratory dendritic cells which then accumulate inside the pancreatic lymph nodes. These dendritic cells exhibit downregulated CD40, CD86, and CD80 costimulation (as a consequence of the effects of the antisense), effectively being turned into tolerogenic dendritic cells. In the pancreatic lymph nodes, these dendritic cells could promote increased regulatory T-cell prevalence which, in an antigen-specific manner, could suppress the activation and overall function of autoreactive T cells. Santamaria and colleagues prevented and reversed new-onset disease using nanoparticle-bound autoantigen-derived peptides complexed to MHC proteins. Mechanistically, these investigators showed that the peptide-MHC conjugate was recognised by low peptide-avid autoreactive CD8+ T cells that differentiated into regulatory CD8+ T cells specific for those autoantigen-derived peptides. Furthermore, the use of nanoparticle-conjugated human-relevant peptide-HLA conjugates achieved identical outcomes in human HLA-transgenic diabetic mice [233]. Microparticles offer a unique delivery platform that ranges from injectable to inhalable to topical.

9. Conclusion and Perspectives

Table 2 lists past and current immunomodulatory clinical trials and their status. Most have failed to meet their primary end-point. The reasons suggested for the disappointments using autoantigens or derivative peptides can be summarised as follows: (i) the administration route and dosage were not optimal; (ii) peptides may not behave identically to intact proteins and may not promote tolerogenic antigen presentation; (iii) regulatory immune cell responses to peptides are different from protein-derived peptide antigens; (iv) masking critical epitopes *in vivo*; (v) timing of administration and the stage of disease when the intact autoantigens or autoantigen-derived peptides were administered. It is well accepted that advanced-stage disease including clinically T1DM is associated with epitope spreading, so that even if tolerance was achievable to one autoantigen, a number of others that have since appeared would still aggravate autoimmunity.

Reports from recently completed phase III trials using one embodiment of the humanised CD3 antibody were not encouraging. The underlying reasons for why the primary end-points were not achieved, even previous studies were demonstrated to be successful, yet limited in time, are not at all clear at present but could involve one or more of the following: (i) variation among the T1DM patients in actual residual beta cell mass, in that the outcome

Table 2: Clinical trial history for the immunomodulatory treatment of type I diabetes and current state/outcomes.

Treatment	Clinical phase	Last update	Notes
Anti-CD3	Phase III, canceled	2010	Failed to change patient outcomes, and the phase III study was canceled early [64]
Anti-CD20 (rituximab)	Phase II, completed	2009	Beta cell mass preservation, but no change in C-peptide levels or insulin independence [65]
AS-ODN dendritic cells	Phase I, completed	2011	Treatment safety demonstrated
GAD65 protein (Diamyd)	Phase III, Ongoing	2010	Phase II displayed elevated anti-inflammatory cytokines and Treg cells. Insulin independence was not addressed [66, 67]
HSP60 (DiaPep277)	Phase III, ongoing	2008	Phase II trials display a trend of increased C-peptide levels, anti-inflammatory cytokines, and anti-inflammatory T-helper 2 cells [68]
Insulin APL (NBI-6042)	Phase II, failure	2009	Beta cell mass was unaffected [69, 70]
Insulin (intranasal)	Pilot	2004	Decreased T-cell responsiveness to insulin in patients expressing two to three diabetes-related autoantibodies [71, 72]. Additional clinical trials (phase I–III) are underway
Insulin (oral)	Phase I, failure	2005	Initial trials showed no prevention or delay of type 1 diabetes, but additional trials are underway [73, 74]

reflects the enrollment of individuals with a more aggressive autoimmunity and hence poorer residual beta cell mass and (ii) the possibility that the time of administration varied between this phase III and previous trials; it is well known that administration of immunomodulatory agents during different times of the day as well as during different points of biologic cycles can profoundly affect the immune response [234–237].

In addition to the anti-CD3 antibody, there are other monoclonal antibodies targeting cytokines as well as fusion proteins interfering with costimulation and serving the role of cytokine decoys. There again, the question is, do these agents represent a promise of a huge leap, an incremental advance, or more of the same? The question is indeed speculative as trials involving some of these agents are ongoing or have not yet started. However, the antibody-based trials are instructive. First, these biologic agents confer variable adverse events. Despite the arguments that these agents can represent antigen-specific approaches, they do not. At best, they promote the homeostatic expansion of new immune cell repertoires that, at various stages of autoimmunity, can act beneficially or can aggravate disease. Recent data indicate that antithymocyte globulin depletion of T cells promotes the homeostatic expansion of T cells with a temporally favorable expansion of naturally occurring (thymic) Foxp3+ Tregs [238]. This observation can guide a better approach to promoting homeostatic expansion of Tregs without considerable T-cell depletion and may be instructive for therapies like the anti-CD3 antibody. This phenomenon also appears possible following B-cell depletion with rituximab (i.e., homeostatic expansion of naive B-cells with favorable expansion of B cells with possible regulatory activity [239]). Thus, if consideration is given to understand the timing of repopulation of the periphery with regulatory cells following T-cell or B-cell depletion, more of the same could yield huge leaps in terms of therapeutic success in new onset T1DM.

Another consideration that has been discussed but seldom implemented experimentally is the route of administration of autoantigens and derivative peptides with or without adjuvants or immunomodulators/anti-inflammatory agents. Accumulating evidence suggests that the pancreatic lymph nodes are the major, if not the most important, site of autoimmunity triggering and progression [240–243]. Indeed, regulatory immune cells have been identified to expand and to differentiate from precursors inside draining lymph nodes of tissues that are targets of autoimmunity [207, 244–247]. These observations should be instructive in how to approach ongoing autoimmunity with the aim of inducing antigen-specific suppression. As most, if not all, of pancreatic beta cell antigens are concentrated inside the pancreatic lymph nodes by fluid dynamic flow or are brought inside the pancreatic lymph nodes by antigen-presenting cells, they would drive the acquisition of antigen specificity by immune cells modulated inside the same lymph nodes even by an antigen-nonspecific approach. For example, one could administer costimulation-blocking agents in a manner where they accumulate preferentially inside the pancreatic lymph nodes and allow the bulk flow, or the cross-presentation of beta-cell-derived antigens by antigen-presenting cells that

have traveled along islet-draining lymphatics, to direct the antigen specificity in conferring antigen-specific immune hyporesponsiveness that is known to occur in response to costimulation blockade.

With the disappointing outcomes of the DPT-1 and related insulin-based trials as well as the failure of industry-sponsored phase III trials with one embodiment of the anti-CD3 antibody, one could reasonably question the value in pursuing further immunodepletion interventions as well as autoantigen-alone trials. There is merit in the argument that the lack of biomarkers to more precisely measure residual beta cell mass that is functional imperils these studies. There is also merit in the arguments that the time of administration of the agents in the phase of the disease, even in new-onset-cases, is critical and needs a comprehensive evaluation. We argue that more of the same cannot go on without such biomarkers and a better understanding of the balance of immunoregulatory cells between the time of clinical onset and 6–12 months from this time in T1DM patients. The variability in immunoregulatory cell numbers and their function has to be securely identified in large populations. In the meantime, many of the resources of research support have been skewed heavily in these approaches with trepidation of supporting cell therapies that are otherwise safe. We propose that cell therapies be seriously considered for at least phase I studies. Furthermore, a new avenue of studies has emerged which attempts to promote accumulation of immunoregulatory cells inside the pancreatic lymph nodes. A better understanding of the pancreatic lymphatics, we believe, will be important to develop these approaches.

It is now evident that the explosion of obesity in the past ten years confounds the diagnosis of T1DM. Metabolic impairments in glucose homeostasis can mask underlying autoimmunity. Furthermore, obesity-related inflammation in individuals genetically at risk for T1DM could accelerate the onset of autoimmunity. A careful examination of the relationship between obesity-related inflammation and autoimmunity progression, we believe, challenges both the "huge leaps" and the "more of the same" approaches in immunomodulation of T1DM. This is relevant not only for obesity, but for LADA as well. In this regard, it is possible that conditioning the obese patient with anti-inflammatory agents will decelerate and permit the detection of underlying autoimmunity. The suppression of inflammation may be beneficial as a front-line conditioning prior to full immunomodulation.

Conflict of Interests

Dr. Giannoukakis, Dr. Phillips and Dr. Trucco do not have any conflict of interest related to the agents or the studies referred to in this review.

References

[1] M. J. Clare-Salzler, J. Brooks, A. Chai, K. Van Herle, and C. Anderson, "Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer," *Journal of Clinical Investigation*, vol. 90, no. 3, pp. 741–748, 1992.

[2] M. Feili-Hariri, X. Dong, S. M. Alber, S. C. Watkins, R. D. Salter, and P. A. Morel, "Immunotherapy of NOD mice with bone marrow-derived dendritic cells," *Diabetes*, vol. 48, no. 12, pp. 2300–2308, 1999.

[3] R. J. Creusot, S. S. Yaghoubi, K. Kodama et al., "Tissue-targeted therapy of autoimmune diabetes using dendritic cells transduced to express IL-4 in NOD mice," *Clinical Immunology*, vol. 127, no. 2, pp. 176–187, 2008.

[4] M. Feili-Hariri, D. H. Falkner, A. Gambotto et al., "Dendritic cells transduced to express interleukin-4 prevent diabetes in nonobese diabetic mice with advanced insulitis," *Human Gene Therapy*, vol. 14, no. 1, pp. 13–23, 2003.

[5] X. Luo, K. V. Tarbell, H. Yang et al., "Dendritic cells with TGF-beta1 differentiate naïve $CD4^+$ $CD25^-$ T cells into islet-protective Foxp3+ regulatory T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 8, pp. 2821–2826, 2007.

[6] L. Ma, S. Qian, X. Liang et al., "Prevention of diabetes in NOD mice by administration of dendritic cells deficient in nuclear transcription factor-κB activity," *Diabetes*, vol. 52, no. 8, pp. 1976–1985, 2003.

[7] A. Hernandez, M. Burger, B. B. Blomberg et al., "Inhibition of NF-κB during human dendritic cell differentiation generates anergy and regulatory T-cell activity for one but not two human leukocyte antigen DR mismatches," *Human Immunology*, vol. 68, no. 9, pp. 715–729, 2007.

[8] J. Machen, J. Harnaha, R. Lakomy, A. Styche, M. Trucco, and N. Giannoukakis, "Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells," *Journal of Immunology*, vol. 173, no. 7, pp. 4331–4341, 2004.

[9] L. Adorini and G. Penna, "Induction of tolerogenic dendritic cells by vitamin D receptor agonists," *Handbook of Experimental Pharmacology*, vol. 188, pp. 251–273, 2009.

[10] L. Adorini, "Tolerogenic dendritic cells induced by vitamin D receptor ligands enhance regulatory T cells inhibiting autoimmune diabetes," *Annals of the New York Academy of Sciences*, vol. 987, pp. 258–261, 2003.

[11] G. Besin, S. Gaudreau, M. Menard, C. Guindi, G. Dupuis, and A. Amrani, "Thymic stromal lymphopoietin and thymic stromal lymphopoietin-conditioned dendritic cells induce regulatory T-cell differentiation and protection of NOD mice against diabetes," *Diabetes*, vol. 57, no. 8, pp. 2107–2117, 2008.

[12] M. Buckland and G. Lombardi, "Aspirin and the induction of tolerance by dendritic cells," *Handbook of Experimental Pharmacology*, vol. 188, pp. 197–213, 2009.

[13] H. Kared, A. Masson, H. Adle-Biassette, J. F. Bach, L. Chatenoud, and F. Zavala, "Treatment with granulocyte colony—stimulating factor prevents diabetes in NOD mice by recruiting plasmacytoid dendritic cells and functional $CD4^+$ $CD25^+$ regulatory T-cells," *Diabetes*, vol. 54, no. 1, pp. 78–84, 2005.

[14] J. Morin, B. Faideau, M. C. Gagnerault, F. Lepault, C. Boitard, and S. Boudaly, "Passive transfer of flt-3L-derived dendritic cells delays diabetes development in NOD mice and associates with early production of interleukin (IL)-4 and IL-10 in the spleen of recipient mice," *Clinical and Experimental Immunology*, vol. 134, no. 3, pp. 388–395, 2003.

[15] G. Papaccio, F. Nicoletti, F. A. Pisanti, K. Bendtzen, and M. Galdieri, "Prevention of spontaneous autoimmune diabetes in NOD mice by transferring in vitro antigen-pulsed syngeneic dendritic cells," *Endocrinology*, vol. 141, no. 4, pp. 1500–1505, 2000.

[16] R. J. Steptoe, J. M. Ritchie, L. K. Jones, and L. C. Harrison, "Autoimmune diabetes is suppressed by transfer of pro-insulin-encoding Gr-1$^+$ myeloid progenitor cells that differentiate in vivo into resting dendritic cells," *Diabetes*, vol. 54, no. 2, pp. 434–442, 2005.

[17] M. Menges, S. Rossner, C. Voigtlander et al., "Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity," *The Journal of Experimental Medicine*, vol. 195, no. 1, pp. 15–22, 2002.

[18] P. Verginis, H. S. Li, and G. Carayanniotis, "Tolerogenic semimature dendritic cells suppress experimental autoimmune thyroiditis by activation of thyroglobulin-specific CD4$^+$CD25$^+$ T cells," *Journal of Immunology*, vol. 174, no. 11, pp. 7433–7439, 2005.

[19] E. Gangi, C. Vasu, D. Cheatem, and B. S. Prabhakar, "IL-10-producing CD4$^+$CD25$^+$ regulatory T cells play a critical role in granulocyte-macrophage colony-stimulating factor-induced suppression of experimental autoimmune thyroiditis," *Journal of Immunology*, vol. 174, no. 11, pp. 7006–7013, 2005.

[20] A. Chorny, E. Gonzalez-Rey, A. Fernandez-Martin, D. Ganea, and M. Delgado, "Vasoactive intestinal peptide induces regulatory dendritic cells that prevent acute graft-versus-host disease while maintaining the graft-versus-tumor response," *Blood*, vol. 107, no. 9, pp. 3787–3794, 2006.

[21] A. Chorny, E. Gonzalez-Rey, A. Fernandez-Martin, D. Pozo, D. Ganea, and M. Delgado, "Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 38, pp. 13562–13567, 2005.

[22] D. E. Faunce, A. Terajewicz, and J. Stein-Streilein, "Cutting edge: in vitro-generated tolerogenic APC induce CD8$^+$ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis," *Journal of Immunology*, vol. 172, no. 4, pp. 1991–1995, 2004.

[23] Y. Zhang, H. Yang, B. Xiao et al., "Dendritic cells transduced with lentiviral-mediated RelB-specific ShRNAs inhibit the development of experimental autoimmune myasthenia gravis," *Molecular Immunology*, vol. 46, no. 4, pp. 657–667, 2009.

[24] S. H. Kim, S. Kim, C. H. Evans, S. C. Ghivizzani, T. Oligino, and P. D. Robbins, "Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4," *Journal of Immunology*, vol. 166, no. 5, pp. 3499–3505, 2001.

[25] S. H. Kim, E. R. Lechman, N. Bianco et al., "Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis," *Journal of Immunology*, vol. 174, no. 10, pp. 6440–6448, 2005.

[26] J. D. Whalen, A. W. Thomson, L. Lu, P. D. Robbins, and C. H. Evans, "Viral IL-10 gene transfer inhibits DTH responses to soluble antigens: evidence for involvement of genetically modified dendritic cells and macrophages," *Molecular Therapy*, vol. 4, no. 6, pp. 543–550, 2001.

[27] S. Hoves, S. W. Krause, D. Halbritter et al., "Mature but not immature Fas Ligand (CD95L)-transduced human monocyte-derived dendritic cells are protected from Fas-mediated apoptosis and can be used as killer APC," *Journal of Immunology*, vol. 170, no. 11, pp. 5406–5413, 2003.

[28] S. Hoves, S. W. Krause, H. Herfarth et al., "Elimination of activated but not resting primary human CD4$^+$ and CD8$^+$ T cells by Fas ligand (FasL/CD95L)-expressing Killer-dendritic cells," *Immunobiology*, vol. 208, no. 5, pp. 463–475, 2004.

[29] M. Torisu, H. Murakami, F. Akbar et al., "Protective role of interleukin-10-producing regulatory dendritic cells against murine autoimmune gastritis," *Journal of Gastroenterology*, vol. 43, no. 2, pp. 100–107, 2008.

[30] E. Henry, C. J. Desmet, V. Garzé et al., "Dendritic cells genetically engineered to express IL-10 induce long-lasting antigen-specific tolerance in experimental asthma," *Journal of Immunology*, vol. 181, no. 10, pp. 7230–7242, 2008.

[31] K. Wu, Y. Bi, K. Sun, J. Xia, Y. Wang, and C. Wang, "Suppression of allergic inflammation by allergen-DNA-modified dendritic cells depends on the induction of Foxp3+ regulatory T cells," *Scandinavian Journal of Immunology*, vol. 67, no. 2, pp. 140–151, 2008.

[32] F. Fu, Y. Li, S. Qian et al., "Costimulatory molecule-deficient dendritic cell progenitors (MHC class II+, CD80(dim), CD86-) prolong cardiac allograft survival in nonimmunosuppressed recipients," *Transplantation*, vol. 62, no. 5, pp. 659–665, 1996.

[33] M. B. Lutz, R. M. Suri, M. Niimi et al., "Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo," *European Journal of Immunology*, vol. 30, no. 7, pp. 1813–1822, 2000.

[34] C. Rastellini, L. Lu, C. Ricorbi, T. E. Starzl, A. S. Rao, and A. W. Thomson, "Granulocyte/macrophage colony-stimulating factor-stimulated hepatic dendritic cell progenitors prolong pancreatic islet allograft survival," *Transplantation*, vol. 60, no. 11, pp. 1366–1370, 1995.

[35] K. Sato, N. Yamashita, N. Yamashita, M. Baba, and T. Matsuyama, "Regulatory dendritic cells protect mice from murine acute graft-versus-host disease and leukemia relapse," *Immunity*, vol. 18, no. 3, pp. 367–379, 2003.

[36] S. Gregori, M. Casorati, S. Amuchastegui, S. Smiroldo, A. M. Davalli, and L. Adorini, "Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance," *The Journal of Immunology*, vol. 167, no. 4, pp. 1945–1953, 2001.

[37] M. D. Griffin, W. Lutz, V. A. Phan, L. A. Bachman, D. J. McKean, and R. Kumar, "Dendritic cell modulation by 1alpha,25 dihydroxyvitamin D3 and its analogs: a vitamin D receptor-dependent pathway that promotes a persistent state of immaturity in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 12, pp. 6800–6805, 2001.

[38] H. Hackstein, A. E. Morelli, A. T. Larregina et al., "Aspirin inhibits in vitro maturation and in vivo immunostimulatory function of murine myeloid dendritic cells," *Journal of Immunology*, vol. 166, no. 12, pp. 7053–7062, 2001.

[39] H. Hackstein, T. Taner, A. F. Zahorchak et al., "Rapamycin inhibits IL-4-induced dendritic cell maturation in vitro and dendritic cell mobilization and function in vivo," *Blood*, vol. 101, no. 11, pp. 4457–4463, 2003.

[40] H. Hackstein and A. W. Thomson, "Dendritic cells: emerging pharmacological targets of immunosuppressive drugs," *Nature Reviews Immunology*, vol. 4, no. 1, pp. 24–34, 2004.

[41] J. I. Lee, R. W. Ganster, D. A. Geller, G. J. Burckart, A. W. Thomson, and L. Lu, "Cyclosporine A inhibits the expression of costimulatory molecules on in vitro-generated dendritic cells: association with reduced nuclear translocation of nuclear factor kappa B," *Transplantation*, vol. 68, no. 9, pp. 1255–1263, 1999.

[42] L. Ma, W. A. Rudert, J. Harnaha et al., "Immunosuppressive effects of glucosamine," *Journal of Biological Chemistry*, vol. 277, no. 42, pp. 39343–39349, 2002.

[43] R. Matasic, A. B. Dietz, and S. Vuk-Pavlovic, "Cyclo-oxygenase-independent inhibition of dendritic cell maturation by aspirin," *Immunology*, vol. 101, no. 1, pp. 53–60, 2000.

[44] M. K. Matyszak, S. Citterio, M. Rescigno, and P. Ricciardi-Castagnoli, "Differential effects of corticosteroids during different stages of dendritic cell maturation," *European Journal of Immunology*, vol. 30, no. 4, pp. 1233–1242, 2000.

[45] A. Mehling, S. Grabbe, M. Voskort, T. Schwarz, T. A. Luger, and S. Beissert, "Mycophenolate mofetil impairs the maturation and function of murine dendritic cells," *Journal of Immunology*, vol. 165, no. 5, pp. 2374–2381, 2000.

[46] M. Nouri-Shirazi and E. Guinet, "Direct and indirect cross-tolerance of alloreactive T cells by dendritic cells retained in the immature stage," *Transplantation*, vol. 74, no. 7, pp. 1035–1044, 2002.

[47] G. Penna and L. Adorini, "1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation," *The Journal of Immunology*, vol. 164, no. 5, pp. 2405–2411, 2000.

[48] L. Piemonti, P. Monti, P. Allavena et al., "Glucocorticoids affect human dendritic cell differentiation and maturation," *Journal of Immunology*, vol. 162, no. 11, pp. 6473–6481, 1999.

[49] D. L. Roelen, D. H. Schuurhuis, D. E. M. Van Den Boogaardt et al., "Prolongation of skin graft survival by modulation of the alloimmune response with alternatively activated dendritic cells," *Transplantation*, vol. 76, no. 11, pp. 1608–1615, 2003.

[50] J. M. Thomas, J. L. Contreras, X. L. Jiang et al., "Peri-transplant tolerance induction in macaques: early events reflecting the unique synergy between immunotoxin and deoxyspergualin," *Transplantation*, vol. 68, no. 11, pp. 1660–1673, 1999.

[51] O. Vosters, J. Nève, D. De Wit, F. Willems, M. Goldman, and V. Verhasselt, "Dendritic cells exposed to Nacystelyn are refractory to maturation and promote the emergence of alloreactive regulatory T cells," *Transplantation*, vol. 75, no. 3, pp. 383–389, 2003.

[52] C. A. Bonham, L. Peng, X. Liang et al., "Marked prolongation of cardiac allograft survival by dendritic cells genetically engineered with NF-κB oligodeoxyribonucleotide decoys and adenoviral vectors encoding CTLA4-Ig," *Journal of Immunology*, vol. 169, no. 6, pp. 3382–3391, 2002.

[53] P. T. H. Coates, R. Krishnan, S. Kireta, J. Johnston, and G. R. Russ, "Human myeloid dendritic cells transduced with an adenoviral interleukin-10 gene construct inhibit human skin graft rejection in humanized NOD-scid chimeric mice," *Gene Therapy*, vol. 8, no. 16, pp. 1224–1233, 2001.

[54] R. M. Gorczynski, J. Bransom, M. Cattral et al., "Synergy in induction of increased renal allograft survival after portal vein infusion of dendritic cells transduced to express TGFbeta and IL-10, along with administration of CHO cells expressing the regulatory molecule OX-2," *Clinical Immunology*, vol. 95, no. 3, pp. 182–189, 2000.

[55] L. Lu, A. Gambotto, W. C. Lee et al., "Adenoviral delivery of CTLA4lg into myeloid dendritic cells promotes their in vitro tolerogenicity and survival in allogeneic recipients," *Gene Therapy*, vol. 6, no. 4, pp. 554–563, 1999.

[56] W. P. Min, R. Gorczynski, X. Y. Huang et al., "Dendritic cells genetically engineered to express Fas ligand induce donor-specific hyporesponsiveness and prolong allograft survival," *Journal of Immunology*, vol. 164, no. 1, pp. 161–167, 2000.

[57] R. W. O'Rourke, S. M. Kang, J. A. Lower et al., "A dendritic cell line genetically modified to express CTLA4-Ig as a means to prolong islet allograft survival," *Transplantation*, vol. 69, no. 7, pp. 1440–1446, 2000.

[58] T. Takayama, A. E. Morelli, P. D. Robbins, H. Tahara, and A. W. Thomson, "Feasibility of CTLA4lg gene delivery and expression in vivo using retrovirally transduced myeloid dendritic cells that induce alloantigen-specific T cell anergy in vitro," *Gene Therapy*, vol. 7, no. 15, pp. 1265–1273, 2000.

[59] T. Takayama, Y. Nishioka, L. Lu, M. T. Lotze, H. Tahara, and A. W. Thomson, "Retroviral delivery of viral interleukin-10 into myeloid dendritic cells markedly inhibits their allostimulatory activity and promotes the induction of T-cell hyporesponsiveness," *Transplantation*, vol. 66, no. 12, pp. 1567–1574, 1998.

[60] P. Terness, T. M. Bauer, L. Röse et al., "Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites," *Journal of Experimental Medicine*, vol. 196, no. 4, pp. 447–457, 2002.

[61] M. Garrovillo, A. Ali, and S. F. Oluwole, "Indirect allorecognition in acquired thymic tolerance: induction of donor-specific tolerance to rat cardiac allografts by allopeptide-pulsed host dendritic cells," *Transplantation*, vol. 68, no. 12, pp. 1827–1834, 1999.

[62] Y. R. Kuo, C. W. Huang, S. Goto et al., "Alloantigen-pulsed host dendritic cells induce T-cell regulation and prolong allograft survival in a rat model of hindlimb allotransplantation," *Journal of Surgical Research*, vol. 153, no. 2, pp. 317–325, 2009.

[63] T. Taner, H. Hackstein, Z. Wang, A. E. Morelli, and A. W. Thomson, "Rapamycin-treated, alloantigen-pulsed host dendritic cells induce Ag-specific T cell regulation and prolong graft survival," *American Journal of Transplantation*, vol. 5, no. 2, pp. 228–236, 2005.

[64] "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 33, supplement 1, pp. S62–S69, 2010.

[65] M. D. Pescovitz, C. J. Greenbaum, H. Krause-Steinrauf et al., "Rituximab, B-lymphocyte depletion, and preservation of beta-cell function," *New England Journal of Medicine*, vol. 361, no. 22, pp. 2143–2152, 2009.

[66] M. Chéramy, C. Skoglund, I. Johansson, J. Ludvigsson, C. S. Hampe, and R. Casas, "GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD_{65} enzyme activity and humoral response," *Clinical Immunology*, vol. 137, no. 1, pp. 31–40, 2010.

[67] J. Ludvigsson, "Immune intervention in children with type 1 diabetes," *Current Diabetes Reports*, vol. 10, no. 5, pp. 370–379, 2010.

[68] V. A. L. Huurman, P. E. van der Meide, G. Duinkerken et al., "Immunological efficacy of heat shock protein 60 peptide DiaPep277^{TM} therapy in clinical type I diabetes," *Clinical and Experimental Immunology*, vol. 152, no. 3, pp. 488–497, 2008.

[69] D. G. Alleva, R. A. Maki, A. L. Putnam et al., "Immunomodulation in type 1 diabetes by NBI-6024, an altered peptide ligand of the insulin B epitope," *Scandinavian Journal of Immunology*, vol. 63, pp. 59–69, 2006.

[70] M. Walter, A. Philotheou, F. Bonnici, A. G. Ziegler, and R. Jimenez, "No effect of the altered peptide ligand NBI-6024 on β-cell residual function and insulin needs in new-onset type 1 diabetes," *Diabetes Care*, vol. 32, no. 11, pp. 2036–2040, 2009.

[71] L. C. Harrison, M. Dempsey-Collier, D. R. Kramer, and K. Takahashi, "Aerosol insulin induces regulatory CDS $\gamma\delta$ T cells that prevent murine insulin-dependent diabetes," *Journal of Experimental Medicine*, vol. 184, no. 6, pp. 2167–2174, 1996.

[72] L. C. Harrison, M. C. Honeyman, C. E. Steele et al., "Pancreatic β-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes," *Diabetes Care*, vol. 27, no. 10, pp. 2348–2355, 2004.

[73] J. S. Skyler, J. P. Krischer, J. Wolfsdorf et al., "Effects of oral insulin in relatives of patients with type 1 diabetes: the diabetes prevention trial-type 1," *Diabetes Care*, vol. 28, no. 5, pp. 1068–1076, 2005.

[74] J. L. Mahon, J. M. Sosenko, L. Rafkin-Mervis et al., "The TrialNet natural history study of the development of type 1 diabetes: objectives, design, and initial results," *Pediatric Diabetes*, vol. 10, no. 2, pp. 97–104, 2009.

[75] H. T. A. Siljander, S. Simell, A. Hekkala et al., "Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population," *Diabetes*, vol. 58, no. 12, pp. 2835–2842, 2009.

[76] M. Abdul-Rasoul, H. Habib, and M. Al-Khouly, "'The honeymoon phase' in children with type 1 diabetes mellitus: frequency, duration, and influential factors," *Pediatric Diabetes*, vol. 7, no. 2, pp. 101–107, 2006.

[77] F. Lombardo, M. Valenzise, M. Wasniewska et al., "Two-year prospective evaluation of the factors affecting honeymoon frequency and duration in children with insulin dependent diabetes mellitus: the key-role of age at diagnosis," *Diabetes, Nutrition and Metabolism*, vol. 15, no. 4, pp. 246–251, 2002.

[78] L. Papoz, F. Lenegre, J. Hors et al., "Probability of remission in individual in early adult insulin dependent diabetic patients: results from the cyclosporine diabetes French study group," *Diabete et Metabolisme*, vol. 16, no. 4, pp. 303–310, 1990.

[79] S. A. Dib and M. B. Gomes, "Etiopathogenesis of type 1 diabetes mellitus: prognostic factors for the evolution of residual beta cell function," *Diabetology & Metabolic Syndrome*, vol. 1, article 25, 2009.

[80] H. B. Mortensen, P. Hougaard, P. Swift et al., "New definition for the partial remission period in children and adolescents with type 1 diabetes," *Diabetes Care*, vol. 32, no. 8, pp. 1384–1390, 2009.

[81] J. J. Meier, A. Bhushan, A. E. Butler, R. A. Rizza, and P. C. Butler, "Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration?" *Diabetologia*, vol. 48, no. 11, pp. 2221–2228, 2005.

[82] N. A. Sherry, J. A. Kushner, M. Glandt, T. Kitamura, A. M. B. Brillantes, and K. C. Herold, "Effects of autoimmunity and immune therapy on β-cell turnover in type 1 diabetes," *Diabetes*, vol. 55, no. 12, pp. 3238–3245, 2006.

[83] E. Akirav, J. A. Kushner, and K. C. Herold, "β-cell mass and type 1 diabetes: going, going, gone?" *Diabetes*, vol. 57, no. 11, pp. 2883–2888, 2008.

[84] L. Tian, J. Gao, J. Hao et al., "Reversal of new-onset diabetes through modulating inflammation and stimulating beta-cell replication in nonobese diabetic mice by a dipeptidyl peptidase IV inhibitor," *Endocrinology*, vol. 151, no. 7, pp. 3049–3060, 2010.

[85] B. O. Roep, M. Atkinson, and M. von Herrath, "Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes," *Nature Reviews Immunology*, vol. 4, no. 12, pp. 989–997, 2004.

[86] M. A. Atkinson and E. H. Leiter, "The NOD mouse model of type 1 diabetes: as good as it gets?" *Nature Medicine*, vol. 5, no. 6, pp. 601–604, 1999.

[87] P. Pozzilli, "The DPT-1 trial: a negative result with lessons for future type 1 diabetes prevention," *Diabetes/Metabolism Research and Reviews*, vol. 18, no. 4, pp. 257–259, 2002.

[88] B. C. Bisikirska and K. C. Herold, "Use of anti-CD3 monoclonal antibody to induce immune regulation in type 1 diabetes," *Annals of the New York Academy of Sciences*, vol. 1037, pp. 1–9, 2004.

[89] K. C. Herold, S. E. Gitelman, U. Masharani et al., "A single course of anti-CD3 monoclonal antibody hOKT3γ1(ala-ala) results in improvement in C-peptide responses and clinical parameters for at least 2 years after onset of type 1 diabetes," *Diabetes*, vol. 54, no. 6, pp. 1763–1769, 2005.

[90] B. Keymeulen, E. Vandemeulebroucke, A. G. Ziegler et al., "Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes," *New England Journal of Medicine*, vol. 352, no. 25, pp. 2598–2608, 2005.

[91] L. Chatenoud, "Use of CD3 antibodies in transplantation and autoimmune diseases," *Transplantation Proceedings*, vol. 26, no. 6, pp. 3191–3193, 1994.

[92] L. Chatenoud, J. Primo, and J. F. Bach, "CD3 antibody-induced dominant self tolerance in overtly diabetic NOD mice," *Journal of Immunology*, vol. 158, no. 6, pp. 2947–2954, 1997.

[93] L. Chatenoud, "CD3-specific antibody-induced active tolerance: from bench to bedside," *Nature Reviews Immunology*, vol. 3, no. 2, pp. 123–132, 2003.

[94] J. Ludvigsson, "The role of immunomodulation therapy in autoimmune diabetes," *Journal of Diabetes Science and Technology*, vol. 3, no. 2, pp. 320–330, 2009.

[95] M. Rewers and P. Gottlieb, "Immunotherapy for the prevention and treatment of type 1 diabetes: human trials and a look into the future," *Diabetes Care*, vol. 32, no. 10, pp. 1769–1782, 2009.

[96] J. Tian and D. L. Kaufman, "Antigen-based therapy for the treatment of type 1 diabetes," *Diabetes*, vol. 58, no. 9, pp. 1939–1946, 2009.

[97] D. K. Wherrett and D. Daneman, "Prevention of type 1 diabetes," *Endocrinology and Metabolism Clinics of North America*, vol. 38, no. 4, pp. 777–790, 2009.

[98] C. R. Stiller, J. Dupre, M. Gent et al., "Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset," *Science*, vol. 223, no. 4643, pp. 1362–1367, 1984.

[99] G. Feutren, L. Papoz, R. Assan et al., "Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial," *Lancet*, vol. 2, no. 8499, pp. 119–124, 1986.

[100] J. Silverstein, N. Maclaren, W. Riley, R. Spillar, D. Radjenovic, and S. Johnson, "Immunosuppression with azathioprine and prednisone in recent-onset insulin-dependent diabetes mellitus," *New England Journal of Medicine*, vol. 319, no. 10, pp. 599–604, 1988.

[101] E. Strandell, D. L. Eizirik, and S. Sandler, "Reversal of β-cell suppression in vitro in pancreatic islets isolated from nonobese diabetic mice during the phase preceding insulin-dependent diabetes mellitus," *Journal of Clinical Investigation*, vol. 85, no. 6, pp. 1944–1950, 1990.

[102] S. Sreenan, A. J. Pick, M. Levisetti, A. C. Baldwin, W. Pugh, and K. S. Polonsky, "Increased β-cell proliferation and reduced mass before diabetes onset in the nonobese diabetic mouse," *Diabetes*, vol. 48, no. 5, pp. 989–996, 1999.

[103] A. A. Like, A. A. Rossini, D. L. Guberski, M. C. Appel, and R. M. Williams, "Spontaneous diabetes mellitus: reversal and prevention in the BB/W rat with antiserum to rat lymphocytes," *Science*, vol. 206, no. 4425, pp. 1421–1423, 1979.

[104] B. O. Roep, I. Stobbe, G. Duinkerken et al., "Auto- and alloimmune reactivity to human islet allografts transplanted into type 1 diabetic patients," *Diabetes*, vol. 48, no. 3, pp. 484–490, 1999.

[105] G. S. Eisenbarth, S. Srikanta, R. Jackson et al., "Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus," *Diabetes Research*, vol. 2, no. 6, pp. 271–276, 1985.

[106] F. Saudek, T. Havrdova, P. Boucek, L. Karasova, P. Novota, and J. Skibova, "Polyclonal anti-T-cell therapy for type 1 diabetes mellitus of recent onset," *The Review of Diabetic Studies*, vol. 1, no. 2, pp. 80–88, 2004.

[107] Y. Fan, W. A. Rudert, M. Grupillo, J. He, G. Sisino, and M. Trucco, "Thymus-specific deletion of insulin induces autoimmune diabetes," *EMBO Journal*, vol. 28, no. 18, pp. 2812–2824, 2009.

[108] P. Vafiadis, S. T. Bennett, J. A. Todd et al., "Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus," *Nature Genetics*, vol. 15, no. 3, pp. 289–292, 1997.

[109] A. A. Chentoufi and C. Polychronakos, "Insulin expression levels in the thymus modulate insulin-specific autoreactive T-cell tolerance: the mechanism by which the IDDM2 locus may predispose to diabetes," *Diabetes*, vol. 51, no. 5, pp. 1383–1390, 2002.

[110] A. A. Chentoufi, M. Palumbo, and C. Polychronakos, "Proinsulin expression by Hassall's corpuscles in the mouse thymus," *Diabetes*, vol. 53, no. 2, pp. 354–359, 2004.

[111] A. Pugliese, M. Zeller, A. Fernandez Jr. et al., "The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes," *Nature Genetics*, vol. 15, no. 3, pp. 293–297, 1997.

[112] D. R. Wegmann and G. S. Eisenbarth, "It's insulin," *Journal of Autoimmunity*, vol. 15, no. 3, pp. 286–291, 2000.

[113] W. Chen, I. Bergerot, J. F. Elliott et al., "Evidence that a peptide spanning the B-C junction of proinsulin is an early autoantigen epitope in the pathogenesis of type 1 diabetes," *Journal of Immunology*, vol. 167, no. 9, pp. 4926–4935, 2001.

[114] G. S. Eisenbarth, "Insulin autoimmunity: Iinmunogenetics/immunopathogenesis of type 1A diabetes," *Annals of the New York Academy of Sciences*, vol. 1005, pp. 109–118, 2003.

[115] P. A. Gottlieb and G. S. Eisenbarth, "Insulin-specific tolerance in diabetes," *Clinical Immunology*, vol. 102, no. 1, pp. 2–11, 2002.

[116] A. Muir, A. Peck, M. Clare-Salzler et al., "Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-γ transcription," *Journal of Clinical Investigation*, vol. 95, no. 2, pp. 628–634, 1995.

[117] D. Daniel and D. R. Wegmann, "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 2, pp. 956–960, 1996.

[118] B. Hartmann, K. Bellmann, I. Ghiea, R. Kleemann, and H. Kolb, "Oral insulin for diabetes prevention in NOD mice: potentiation by enhancing Th2 cytokine expression in the gut through bacterial adjuvant," *Diabetologia*, vol. 40, no. 8, pp. 902–909, 1997.

[119] Z. J. Zhang, L. Davidson, G. Eisenbarth, and H. L. Weiner, "Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 22, pp. 10252–10256, 1991.

[120] I. Bergerot, C. Ploix, J. Petersen et al., "A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 9, pp. 4610–4614, 1997.

[121] C. Aspord and C. Thivolet, "Nasal administration of CTB-insulin induces active tolerance against autoimmune diabetes in non-obese diabetic (NOD) mice," *Clinical and Experimental Immunology*, vol. 130, no. 2, pp. 204–211, 2002.

[122] W. Chen, I. Bergerot, J. F. Elliott et al., "Evidence that a peptide spanning the B-C junction of proinsulin is an early autoantigen epitope in the pathogenesis of type 1 diabetes," *Journal of Immunology*, vol. 167, no. 9, pp. 4926–4935, 2001.

[123] J. M. Sosenko, J. P. Palmer, C. J. Greenbaum et al., "Patterns of metabolic progression to type 1 diabetes in the diabetes prevention trial-type 1," *Diabetes Care*, vol. 29, no. 3, pp. 643–649, 2006.

[124] K. Näntö-Salonen, A. Kupila, S. Simell et al. et al., "Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial," *Lancet.*, vol. 372, no. 9651, pp. 1746–1755, 2008.

[125] L. Chaillous, H. Lefevre, C. Thivolet et al., "Oral insulin administration and residual beta-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale group," *Lancet*, vol. 356, no. 9229, pp. 545–549, 2000.

[126] D. Bresson, L. Togher, E. Rodrigo et al., "Anti-CD3 and nasal proinsulin combination therapy enhances remission from recentonset autoimmune diabetes by inducing Tregs," *Journal of Clinical Investigation*, vol. 116, no. 5, pp. 1371–1381, 2006.

[127] T. Orban, K. Farkas, H. Jalahej et al., "Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy," *Journal of Autoimmunity*, vol. 34, no. 4, pp. 408–415, 2010.

[128] D. L. Kaufman, M. Clare-Salzler, J. Tian et al., "Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes," *Nature*, vol. 366, no. 6450, pp. 69–72, 1993.

[129] R. Tisch, X. D. Yang, S. M. Singer, R. S. Liblau, L. Fugger, and H. O. McDevitt, "Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice," *Nature*, vol. 366, no. 6450, pp. 72–75, 1993.

[130] J. Tian, M. Clare-Salzler, A. Herschenfeld et al., "Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice," *Nature Medicine*, vol. 2, no. 12, pp. 1348–1353, 1996.

[131] R. D. G. Leslie, M. A. Atkinson, and A. L. Notkins, "Autoantigens IA-2 and GAD in Type I (insulin-dependent) diabetes," *Diabetologia*, vol. 42, no. 1, pp. 3–14, 1999.

[132] C. D. Agardh, C. M. Cilio, Å. Lethagen et al., "Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes," *Journal of Diabetes and its Complications*, vol. 19, no. 4, pp. 238–246, 2005.

[133] J. Ludvigsson, M. Faresjö, M. Hjorth et al., "GAD treatment and insulin secretion in recent-onset type 1 diabetes," *New England Journal of Medicine*, vol. 359, no. 18, pp. 1909–1920, 2008.

[134] M. A. Atkinson, L. A. Holmes, D. W. Scharp, P. E. Lacy, and N. K. Maclaren, "No evidence for serological autoimmunity to islet cell heat shock proteins in insulin dependent diabetes," *Journal of Clinical Investigation*, vol. 87, no. 2, pp. 721–724, 1991.

[135] D. Elias and I. R. Cohen, "Treatment of autoimmune diabetes and insulitis in NOD mice with heat shock protein 60 peptide p277," *Diabetes*, vol. 44, no. 9, pp. 1132–1138, 1995.

[136] V. A. L. Huurman, K. Decochez, C. Mathieu, I. R. Cohen, and B. O. Roep, "Therapy with the hsp50 peptide DiaPep277™ in C-peptide positive type 1 diabetes patients," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 4, pp. 269–275, 2007.

[137] N. C. Schloot, G. Meierhoff, C. Lengyel et al., "Effect of heat shock protein peptide DiaPep277 on beta-cell function in paediatric and adult patients with recent-onset diabetes mellitus type 1: two prospective, randomized, double-blind phase II trials," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 4, pp. 276–285, 2007.

[138] L. Lazar, R. Ofan, N. Weintrob et al., "Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study," *Diabetes/Metabolism Research and Reviews*, vol. 23, no. 4, pp. 286–291, 2007.

[139] I. Raz, D. Elias, A. Avron, M. Tamir, M. Metzger, and I. R. Cohen, "β-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial," *Lancet*, vol. 358, no. 9295, pp. 1749–1753, 2001.

[140] B. C. Bisikirska and K. C. Herold, "Regulatory T cells and type 1 diabetes," *Current Diabetes Reports*, vol. 5, no. 2, pp. 104–109, 2005.

[141] K. C. Herold and L. Taylor, "Treatment of Type 1 diabetes with anti-CD3 monoclonal antibody: induction of immune regulation?" *Immunologic Research*, vol. 28, no. 2, pp. 141–150, 2003.

[142] K. C. Herold, W. Hagopian, J. A. Auger et al., "Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus," *New England Journal of Medicine*, vol. 346, no. 22, pp. 1692–1698, 2002.

[143] D. V. Serreze, H. D. Chapman, D. S. Varnum et al., "B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new 'speed congenic' stock of NOD.Igμ(null) mice," *Journal of Experimental Medicine*, vol. 184, no. 5, pp. 2049–2053, 1996.

[144] C. Y. Hu, D. Rodriguez-Pinto, W. Du et al., "Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice," *Journal of Clinical Investigation*, vol. 117, no. 12, pp. 3857–3867, 2007.

[145] Y. Xiu, C. P. Wong, J. D. Bouaziz et al., "B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in FcγR effector functions," *Journal of Immunology*, vol. 180, no. 5, pp. 2863–2875, 2008.

[146] B. J. Miller, M. C. Appel, J. J. O'Neil, and L. S. Wicker, "Both the Lyt-2+ and L3T4+ T cell subsets are required for the transfer of diabetes in nonobese diabetic mice," *Journal of Immunology*, vol. 140, no. 1, pp. 52–58, 1988.

[147] J. Tian, D. Zekzer, Y. Lu, H. Dang, and D. L. Kaufman, "B cells are crucial for determinant spreading of T cell autoimmunity among β cell antigens in diabetes-prone nonobese diabetic mice," *Journal of Immunology*, vol. 176, no. 4, pp. 2654–2661, 2006.

[148] M. Falcone, J. Lee, G. Patstone, B. Yeung, and N. Sarvetnick, "B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice," *Journal of Immunology*, vol. 161, no. 3, pp. 1163–1168, 1998.

[149] D. V. Serreze, S. A. Fleming, H. D. Chapman, S. D. Richard, E. H. Leiter, and R. M. Tisch, "B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice," *Journal of Immunology*, vol. 161, no. 8, pp. 3912–3918, 1998.

[150] P. A. Silveira, E. Johnson, H. D. Chapman, T. Bui, R. M. Tisch, and D. V. Serreze, "The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors," *European Journal of Immunology*, vol. 32, no. 12, pp. 3657–3666, 2002.

[151] S. A. W. Greeley, D. J. Moore, H. Noorchashm et al., "Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice," *Journal of Immunology*, vol. 167, no. 8, pp. 4351–4357, 2001.

[152] H. Reijonen, T. L. Daniels, A. Lernmark, and G. T. Nepom, "GAD65-specific autoantibodies enhance the presentation of an immunodominant T-cell epitope from GAD65," *Diabetes*, vol. 49, no. 10, pp. 1621–1626, 2000.

[153] A. Rabinovitch, W. Sumoski, R. V. Rajotte, and G. L. Warnock, "Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture," *Journal of Clinical Endocrinology and Metabolism*, vol. 71, no. 1, pp. 152–156, 1990.

[154] A. Rabinovitch, "An update on cytokines in the pathogenesis of insulin-dependent diabetes mellitus," *Diabetes/Metabolism Reviews*, vol. 14, no. 2, pp. 129–151, 1998.

[155] A. Rabinovitch, W. L. Suarez-Pinzon, K. Strynadka et al., "Human pancreatic islet beta-cell destruction by cytokines is independent of nitric oxide production," *The Journal of Clinical Endocrinology & Metabolism*, vol. 79, no. 4, pp. 1058–1062, 1994.

[156] L. Mastrandrea, J. Yu, T. Behrens et al., "Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study," *Diabetes Care*, vol. 32, no. 7, pp. 1244–1249, 2009.

[157] C. O. Jacob, S. Aiso, S. A. Michie, H. O. McDevitt, and H. Acha-Orbea, "Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-alpha and interleukin 1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 3, pp. 968–972, 1990.

[158] C. A. Dinarello, "Interleukin-1β and the autoinflammatory diseases," *New England Journal of Medicine*, vol. 360, no. 23, pp. 2467–2470, 2009.

[159] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," *Annual Review of Immunology*, vol. 27, pp. 519–550, 2009.

[160] C. A. Dinarello, "Interleukin-1 in the pathogenesis and treatment of inflammatory diseases," *Blood*, vol. 117, no. 14, pp. 3720–3732, 2011.

[161] C. A. Dinarello, "Blocking interleukin-1beta in acute and chronic autoinflammatory diseases," *Journal of Internal Medicine*, vol. 269, pp. 16–28, 2011.

[162] M. Y. Donath and T. Mandrup-Poulsen, "The use of interleukin-1-receptor antagonists in the treatment of diabetes mellitus," *Nature Clinical Practice Endocrinology and Metabolism*, vol. 4, no. 5, pp. 240–241, 2008.

[163] M. Koulmanda, M. Bhasin, L. Hoffman et al., "Curative and beta cell regenerative effects of alpha1-antitrypsin treatment in autoimmune diabetic NOD mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 42, pp. 16242–16247, 2008.

[164] C. Louvet, G. L. Szot, J. Lang et al., "Tyrosine kinase inhibitors reverse type 1 diabetes in nonobese diabetic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 48, pp. 18895–18900, 2008.

[165] N. Ogawa, J. F. List, J. F. Habener, and T. Maki, "Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4," *Diabetes*, vol. 53, no. 7, pp. 1700–1705, 2004.

[166] N. A. Sherry, W. Chen, J. A. Kushner et al., "Exendin-4 improves reversal of diabetes in NOD mice treated with anti-CD3 monoclonal antibody by enhancing recovery of β-cells," *Endocrinology*, vol. 148, no. 11, pp. 5136–5144, 2007.

[167] C. E. Couri, M. C. Oliveira, A. B. Stracieri et al., "C-peptide levels and insulin independence following autologous non-myeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus," *Journal of the American Medical Association*, vol. 301, no. 15, pp. 1573–1579, 2009.

[168] J. C. Voltarelli, C. E. Couri, A. B. Stracieri et al., "Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus," *Journal of the American Medical Association*, vol. 297, no. 14, pp. 1568–1576, 2007.

[169] P. Toby, H. Coates, and A. W. Thomson, "Dendritic cells, tolerance induction and transplant outcome," *American Journal of Transplantation*, vol. 2, no. 4, pp. 299–307, 2002.

[170] H. Hackstein, A. E. Morelli, and A. W. Thomson, "Designer dendritic cells for tolerance induction: guided not misguided missiles," *Trends in Immunology*, vol. 22, no. 8, pp. 437–442, 2001.

[171] A. E. Morelli and A. W. Thomson, "Dendritic cells: regulators of alloimmunity and opportunities for tolerance induction," *Immunological Reviews*, vol. 196, pp. 125–146, 2003.

[172] M. Nouri-Shirazi and A. W. Thomson, "Dendritic cells as promoters of transplant tolerance," *Expert Opinion on Biological Therapy*, vol. 6, no. 4, pp. 325–339, 2006.

[173] R. M. Steinman, D. Hawiger, and M. C. Nussenzweig, "Tolerogenic dendritic cells," *Annual Review of Immunology*, vol. 21, pp. 685–711, 2003.

[174] R. M. Steinman, K. Inaba, S. Turley, P. Pierre, and I. Mellman, "Antigen capture, processing, and presentation by dendritic cells: recent cell biological studies," *Human Immunology*, vol. 60, no. 7, pp. 562–567, 1999.

[175] R. M. Steinman and M. C. Nussenzweig, "Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 1, pp. 351–358, 2002.

[176] R. Bottino, P. Lemarchand, M. Trucco, and N. Giannoukakis, "Gene- and cell-based therapeutics for type I diabetes mellitus," *Gene Therapy*, vol. 10, no. 10, pp. 875–889, 2003.

[177] D. Chen, R. Sung, and J. S. Bromberg, "Gene therapy in transplantation," *Transplant Immunology*, vol. 9, no. 2–4, pp. 301–314, 2002.

[178] N. Giannoukakis, W. A. Rudert, P. D. Robbins, and M. Trucco, "Targeting autoimmune diabetes with gene therapy," *Diabetes*, vol. 48, no. 11, pp. 2107–2121, 1999.

[179] N. Giannoukakis, A. W. Thomson, and P. D. Robbins, "Gene therapy in transplantation," *Gene Therapy*, vol. 6, no. 9, pp. 1499–1511, 1999.

[180] N. Giannoukakis and M. Trucco, "Gene therapy for type 1 diabetes," *American Journal of Therapeutics*, vol. 12, pp. 512–528, 2005.

[181] I. H. Tarner and C. G. Fathman, "The potential for gene therapy in the treatment of autoimmune disease," *Clinical Immunology*, vol. 104, no. 3, pp. 204–216, 2002.

[182] I. H. Tarner, A. J. Slavin, J. McBride et al., "Treatment of autoimmune disease by adoptive cellular gene therapy," *Annals of the New York Academy of Sciences*, vol. 998, pp. 512–519, 2003.

[183] M. Trucco, P. D. Robbins, A. W. Thomson, and N. Giannoukakis, "Gene therapy strategies to prevent autoimmune disorders," *Current Gene Therapy*, vol. 2, no. 3, pp. 341–354, 2002.

[184] W. Chen, "Dendritic cells and $CD4^+CD25^+$ T regulatory cells: crosstalk between two professionals in immunity versus tolerance," *Frontiers in Bioscience*, vol. 11, pp. 1360–1370, 2006.

[185] S. Hugues, A. Boissonnas, S. Amigorena, and L. Fetler, "The dynamics of dendritic cell-T cell interactions in priming and tolerance," *Current Opinion in Immunology*, vol. 18, no. 4, pp. 491–495, 2006.

[186] S. Beissert, A. Schwarz, and T. Schwarz, "Regulatory T cells," *Journal of Investigative Dermatology*, vol. 126, no. 1, pp. 15–24, 2006.

[187] A. H. Enk, "DCs and cytokines cooperate for the induction of tregs," *Ernst Schering Research Foundation Workshop*, no. 56, pp. 97–106, 2006.

[188] S. Huber and C. Schramm, "TGF-beta and $CD4^+CD25^+$ regulatory T cells," *Frontiers in Bioscience*, vol. 11, pp. 1014–1023, 2006.

[189] J. Lohr, B. Knoechel, and A. K. Abbas, "Regulatory T cells in the periphery," *Immunological Reviews*, vol. 212, pp. 149–162, 2006.

[190] M. G. Roncarolo, S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M. K. Levings, "Interleukin-10-secreting type 1 regulatory T cells in rodents and humans," *Immunological Reviews*, vol. 212, pp. 28–50, 2006.

[191] E. M. Shevach, R. A. DiPaolo, J. Andersson, D. M. Zhao, G. L. Stephens, and A. M. Thornton, "The lifestyle of naturally occurring CD4CD25 Foxp3 regulatory T cells," *Immunological Reviews*, vol. 212, pp. 60–73, 2006.

[192] Q. Tang and J. A. Bluestone, "Regulatory T-cell physiology and application to treat autoimmunity," *Immunological Reviews*, vol. 212, pp. 217–237, 2006.

[193] J. Verhagen, K. Blaser, C. A. Akdis, and M. Akdis, "Mechanisms of allergen-specific immunotherapy: T-regulatory cells and more," *Immunology and Allergy Clinics of North America*, vol. 26, no. 2, pp. 207–231, 2006.

[194] L. Zhang, H. Yi, X. P. Xia, and Y. Zhao, "Transforming growth factor-beta: an important role in CD4$^+$ CD25$^+$ regulatory T cells and immune tolerance," *Autoimmunity*, vol. 39, no. 4, pp. 269–276, 2006.

[195] M. Battaglia, S. Gregori, R. Bacchetta, and M.-G. Roncarolo, "Tr1 cells: from discovery to their clinical application," *Seminars in Immunology*, vol. 18, no. 2, pp. 120–127, 2006.

[196] F. J. D. Mennechet and G. Uzé, "Interferon-λ-treated dendritic cells specifically induce proliferation of FOXP3-expressing suppressor T cells," *Blood*, vol. 107, no. 11, pp. 4417–4423, 2006.

[197] S. Rutella, G. Bonanno, A. Procoli et al., "Hepatocyte growth factor favors monocyte differentiation into regulatory interleukin (IL)-10IL-12 accessory cells with dendritic-cell features," *Blood*, vol. 108, no. 1, pp. 218–227, 2006.

[198] S. Vigouroux, E. Yvon, E. Biagi, and M. K. Brenner, "Antigen-induced regulatory T cells," *Blood*, vol. 104, no. 1, pp. 26–33, 2004.

[199] N. Watanabe, Y. H. Wang, H. K. Lee et al., "Hassall's corpuscles instruct dendritic cells to induce CD4 CD25 regulatory T cells in human thymus," *Nature*, vol. 436, no. 7054, pp. 1181–1185, 2005.

[200] E. S. Yvon, S. Vigouroux, R. F. Rousseau et al., "Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells," *Blood*, vol. 102, no. 10, pp. 3815–3821, 2003.

[201] M. V. Dhodapkar, R. M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj, "Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells," *Journal of Experimental Medicine*, vol. 193, no. 2, pp. 233–238, 2001.

[202] H. Jonuleit, E. Schmitt, G. Schuler, J. Knop, and A. H. Enk, "Induction of interleukin 10-producing, nonproliferating CD4$^+$ T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells," *Journal of Experimental Medicine*, vol. 192, no. 9, pp. 1213–1222, 2000.

[203] S. Yamazaki, T. Iyoda, K. Tarbell et al., "Direct expansion of functional CD25$^+$ CD4$^+$ regulatory T cells by antigen-processing dendritic cells," *Journal of Experimental Medicine*, vol. 198, no. 2, pp. 235–247, 2003.

[204] C. Brinster and E. M. Shevach, "Bone marrow-derived dendritic cells reverse the anergic state of CD4$^+$ CD25$^+$ T cells without reversing their suppressive function," *Journal of Immunology*, vol. 175, no. 11, pp. 7332–7340, 2005.

[205] K. V. Tarbell, S. Yamazaki, K. Olson, P. Toy, and R. M. Steinman, "CD25$^+$ CD4$^+$ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1467–1477, 2004.

[206] J. Harnaha, J. Machen, M. Wright et al., "Interleukin-7 is a survival factor for CD4$^+$ CD25$^+$ T-cells and is expressed by diabetes-suppressive dendritic cells," *Diabetes*, vol. 55, no. 1, pp. 158–170, 2006.

[207] P. Chappert, M. Leboeuf, P. Rameau et al., "Antigen-driven interactions with dendritic cells and expansion of Foxp3+ regulatory T cells occur in the absence of inflammatory signals," *Journal of Immunology*, vol. 180, no. 1, pp. 327–334, 2008.

[208] R. J. DiPaolo, C. Brinster, T. S. Davidson, J. Andersson, D. Glass, and E. M. Shevach, "Autoantigen-specific TGFbeta-induced Foxp3+ regulatory T cells prevent autoimmunity by inhibiting dendritic cells from activating autoreactive T cells," *The Journal of Immunology*, vol. 179, no. 7, pp. 4685–4693, 2007.

[209] T. Lehner, "Special regulatory T cell review: the resurgence of the concept of contrasuppression in immunoregulation," *Immunology*, vol. 123, no. 1, pp. 40–44, 2008.

[210] Y. Onishi, Z. Fehervari, T. Yamaguchi, and S. Sakaguchi, "Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 29, pp. 10113–10118, 2008.

[211] G. Xia, J. He, and J. R. Leventhal, "Ex vivo-expanded natural CD4CD25 regulatory T cells synergize with host T-cell depletion to promote long-term survival of allografts," *American Journal of Transplantation*, vol. 8, no. 2, pp. 298–306, 2008.

[212] E. J. Gordon, L. S. Wicker, L. B. Peterson et al., "Autoimmune diabetes and resistance to xenograft transplantation tolerance in NOD mice," *Diabetes*, vol. 54, no. 1, pp. 107–115, 2005.

[213] T. G. Markees, D. V. Serreze, N. E. Phillips et al., "NOD mice have a generalized defect in their response to transplantation tolerance induction," *Diabetes*, vol. 48, no. 5, pp. 967–974, 1999.

[214] T. Pearson, T. G. Markees, D. V. Serreze et al., "Genetic disassociation of autoimmunity and resistance to costimulation blockade-induced transplantation tolerance in nonobese diabetic mice," *Journal of Immunology*, vol. 171, no. 1, pp. 185–195, 2003.

[215] T. Pearson, T. G. Markees, D. V. Serreze et al., "Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms?" *Annals of the New York Academy of Sciences*, vol. 1005, pp. 148–156, 2003.

[216] T. Pearson, T. G. Markees, L. S. Wicker et al., "NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction," *Diabetes*, vol. 52, no. 2, pp. 321–326, 2003.

[217] A. A. Rossini, "Autoimmune diabetes and the circle of tolerance," *Diabetes*, vol. 53, no. 2, pp. 267–275, 2004.

[218] A. A. Rossini, J. P. Mordes, D. L. Greiner, and J. S. Stoff, "Islet cell transplantation tolerance," *Transplantation*, vol. 72, no. 8, pp. S43–S46, 2001.

[219] E. Seung, J. P. Mordes, D. L. Greiner, and A. A. Rossini, "Induction of tolerance for islet transplantation for type 1 diabetes," *Current Diabetes Reports*, vol. 3, no. 4, pp. 329–335, 2003.

[220] B. Ludewig, B. Odermatt, S. Landmann, H. Hengartner, and R. M. Zinkernagel, "Dendritic cells induce autoimmune diabetes and maintain disease via de novo formation of local lymphoid tissue," *Journal of Experimental Medicine*, vol. 188, no. 8, pp. 1493–1501, 1998.

[221] R. J. Creusot, S. S. Yaghoubi, P. Chang et al., "Lymphoid tissue-specific homing of bone marrow-derived dendritic cells," *Blood*, vol. 113, no. 26, pp. 6638–6647, 2009.

[222] R. J. Creusot, P. Chang, D. G. Healey, I. Y. Tcherepanova, C. A. Nicolette, and C. G. Fathman, "A short pulse of IL-4 delivered by DCs electroporated with modified mRNA can both prevent and treat autoimmune diabetes in NOD mice," *Molecular Therapy*, vol. 18, pp. 2112–2120, 2010.

[223] E. Godebu, D. Summers-Torres, M. M. Lin, B. J. Baaten, and L. M. Bradley, "Polyclonal adaptive regulatory CD4 cells that can reverse type I diabetes become oligoclonal long-term protective memory cells," *Journal of Immunology*, vol. 181, no. 3, pp. 1798–1805, 2008.

[224] Q. Tang, K. J. Henriksen, M. Bi et al., "In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes," *Journal of Experimental Medicine*, vol. 199, no. 11, pp. 1455–1465, 2004.

[225] S. E. Weber, J. Harbertson, E. Godebu et al., "Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo," *Journal of Immunology*, vol. 176, no. 8, pp. 4730–4739, 2006.

[226] R. Gandhi, D. Kumar, E. J. Burns et al., "Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3+ regulatory T cells," *Nature Immunology*, vol. 11, no. 9, pp. 846–853, 2010.

[227] L. Apetoh, F. J. Quintana, C. Pot et al., "The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27," *Nature Immunology*, vol. 11, no. 9, pp. 854–861, 2010.

[228] V. R. Sinha and A. Trehan, "Biodegradable microspheres for parenteral delivery," *Critical Reviews in Therapeutic Drug Carrier Systems*, vol. 22, no. 6, pp. 535–602, 2005.

[229] M. E. Keegan and W. M. Saltzman, "Surface-modified biodegradable microspheres for DNA vaccine delivery," *Methods in Molecular Medicine*, vol. 127, pp. 107–113, 2006.

[230] F. H. Xu and Q. Zhang, "Recent advances in the preparation progress of protein/peptide drug loaded PLA/PLGA microspheres," *Yao Xue Xue Bao*, vol. 42, no. 1, pp. 1–7, 2007.

[231] S. Tamilvanan, R. V. Babu, K. Kannan, S. K. Basu, and B. Sa, "Manufacturing techniques and excipients used during the design of biodegradable polymer-based microspheres containing therapeutic peptide/protein for parenteral controlled drug delivery," *PDA Journal of Pharmaceutical Science and Technology*, vol. 62, no. 2, pp. 125–154, 2008.

[232] B. Phillips, K. Nylander, J. Harnaha et al., "A microsphere-based vaccine prevents and reverses new-onset autoimmune diabetes," *Diabetes*, vol. 57, no. 6, pp. 1544–1555, 2008.

[233] S. Tsai, A. Shameli, J. Yamanouchi et al., "Reversal of autoimmunity by boosting memory-like autoregulatory T cells," *Immunity*, vol. 32, no. 4, pp. 568–580, 2010.

[234] L. Glass-Marmor, T. Paperna, Y. Galboiz, and A. Miller, "Immunomodulation by chronobiologically-based glucocorticoids treatment for multiple sclerosis relapses," *Journal of Neuroimmunology*, vol. 210, no. 1-2, pp. 124–127, 2009.

[235] O. Karabay, A. Temel, A. G. Koker, M. Tokel, M. Ceyhan, and E. Kocoglu, "Influence of circadian rhythm on the efficacy of the hepatitis B vaccination," *Vaccine*, vol. 26, no. 9, pp. 1143–1144, 2008.

[236] P. Lissoni, "Modulation of anticancer cytokines IL-2 and IL-12 by melatonin and the other pineal indoles 5-methoxytryptamine and 5-methoxytryptophol in the treatment of human neoplasms," *Annals of the New York Academy of Sciences*, vol. 917, pp. 560–567, 2000.

[237] P. H. Langlois, M. H. Smolensky, W. P. Glezen, and W. A. Keitel, "Diurnal variation in responses to influenza vaccine," *Chronobiology International*, vol. 12, no. 1, pp. 28–36, 1995.

[238] S. Gurkan, Y. Luan, N. Dhillon et al., "Immune reconstitution following rabbit antithymocyte globulin," *American Journal of Transplantation*, vol. 10, no. 9, pp. 2132–2141, 2010.

[239] A. Palanichamy, J. Barnard, B. Zheng et al., "Novel human transitional B cell populations revealed by B cell depletion therapy," *Journal of Immunology*, vol. 182, no. 10, pp. 5982–5993, 2009.

[240] B. Regnault, Y. F. J. Osorio, D. Miao, G. Eisenbarth, and E. Melanitou, "Early over expression of messenger RNA for multiple genes, including insulin, in the Pancreatic Lymph Nodes of NOD mice is associated with Islet Autoimmunity," *BMC Medical Genomics*, vol. 2, article 63, 2009.

[241] L. Yip, L. Su, D. Sheng et al., "Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes," *Nature Immunology*, vol. 10, no. 9, pp. 1026–1033, 2009.

[242] S. Pang, L. Zhang, H. Wang et al., "$CD8^+$ T cells specific for β cells encounter their cognate antigens in the islets of NOD mice," *European Journal of Immunology*, vol. 39, no. 10, pp. 2716–2724, 2009.

[243] B. Calderon, A. Suri, M. J. Miller, and E. R. Unanue, "Dendritic cells in islets of Langerhans constitutively present beta cell-derived peptides bound to their class II MHC molecules," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 16, pp. 6121–6126, 2008.

[244] K. M. Wheeler, E. T. Samy, and K. S. K. Tung, "Cutting edge: normal regional lymph node enrichment of antigen-specific regulatory T cells with autoimmune disease-suppressive capacity," *Journal of Immunology*, vol. 183, no. 12, pp. 7635–7638, 2009.

[245] E. T. Samy, L. A. Parker, C. P. Sharp, and K. S. K. Tung, "Continuous control of autoimmune disease by antigen-dependent polyclonal $CD4^+CD25^+$ regulatory T cells in the regional lymph node," *Journal of Experimental Medicine*, vol. 202, no. 6, pp. 771–781, 2005.

[246] J. C. Ochando, A. C. Yopp, Y. Yang et al., "Lymph node occupancy is required for the peripheral development of alloantigen-specific $Foxp3^+$ regulatory T cells," *Journal of Immunology*, vol. 174, no. 11, pp. 6993–7005, 2005.

[247] T. Hiura, H. Kagamu, S. Miura et al., "Both regulatory T cells and antitumor effector T cells are primed in the same draining lymph nodes during tumor progression," *Journal of Immunology*, vol. 175, no. 8, pp. 5058–5066, 2005.