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Chapter I.2

Pathogenic Mechanisms in Type 1 Diabetes: The Islet is Both Target and Driver of Disease

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■ Abstract

Recent advances in our understanding of the pathogenesis of type 1 diabetes have occurred in all steps of the disease. This review outlines the pathogenic mechanisms utilized by the immune system to mediate destruction of the pancreatic beta-cells. The autoimmune response against beta-cells appears to begin in the pancreatic lymph node where T cells, which have escaped negative selection in the thymus, first meet beta-cell antigens presented by dendritic cells. Proinsulin is an important antigen in early diabetes. T cells migrate to the islets via the circulation and establish insulitis initially around the islets. T cells within insulitis are specific for islet antigens rather than bystanders. Pathogenic $CD4^+$ T cells may recognize peptides from proinsulin which are produced locally within the islet. $CD8^+$ T cells differentiate into effector T cells in islets and then kill beta-cells, primarily via the perforin-granzyme pathway. Cytokines do not appear to be important cytotoxic molecules *in vivo*. Maturation of the immune response within the islet is now understood to contribute to diabetes, and highlights the islet as both driver and target of the disease. The majority of our knowledge of these pathogenic processes is derived from the NOD mouse model, although some processes are mirrored in the human disease. However, more work is required to translate the data from the NOD mouse to our understanding of human diabetes pathogenesis. New technology, especially MHC tetramers and modern imaging, will enhance our understanding of the pathogenic mechanisms.

Keywords: type 1 diabetes · beta-cell · CTL · NOD mouse · islet · CD4+ T cell · insulitis · effector mechanism

1. Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease and represents a failure of self from non-self discrimination, the fundamental task of the immune system. How the immune system goes about this immensely complex task, based both on sequence differences and pattern recognition, is an enduring central question in immunology. While much more is known about the biological mechanisms of the immune system than ever before, our knowledge remains incomplete.

Pathogenic mechanisms are events that are part of the chain of causation of a disease. They can be observed by microscopy or other technologies and follow genetic and environmental etiological factors. In autoimmune diabetes, the expression of perforin in cytotoxic T cells is a decisive pathogenic mechanism because when perforin is deficient diabetes is prevented. However, these mechanisms are not etiological factors as polymorphisms in their genes do not play a critical role. In T1D, pathogenic mechanisms lead to the destruction of pancreatic beta-cells and the eventual presentation of hyperglycemia after months or years of

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preclinical disease activity. A detailed knowledge of the chain of causative events is critical to our ability to design and implement rational therapies.

An increasingly detailed knowledge of how diabetes progresses is being obtained in parallel with advances in other areas such as therapeutics. A chain of causation implies that causation can be tested, and this requires the use of mouse models. In T1D research, the NOD mouse and all its transgenic variants remain the most commonly used models. Their weakness is that they represent just one genotype, whereas human T1D is much more diverse. There is also the ubiquitous risk of self-congratulation about our understanding of diabetes in mice, while our knowledge of human disease is much more limited.

Abbreviations:
CCR7 – C-C motif chemokine receptor 7
CFSE – carboxyfluorescein succinimidyl ester
CTL – cytotoxic T lymphocyte
CXCL9 – C-X-C motif chemokine ligand 9
CXCR3 – C-X-C motif chemokine receptor 3
DC – dendritic cell
ELIspot – enzyme-linked immuno spot
FADD – Fas-associated death domain
GAD65 – glutamic acid decarboxylase 65
GFAP – glial fibrillary acidic protein
HLA – human leukocyte antigen
IA-2 – islet cell antigen 512
ICAM-1 – intercellular adhesion molecule 1
IFNγ – interferon gamma
IFNγR – interferon gamma receptor
IGRP – islet-specific glucose-6-phosphatase catalytic subunit-related protein
IL-1 – interleukin 1
IL-1R – interleukin 1 receptor
KLRG1 – killer cell lectin-like receptor G1
MHC – major histocompability complex
NK cell – natural killer cell
NKT cell – natural killer T cell
NO – nitric oxide
NOD – nonobese diabetic
NOD IL-R – IL-1 receptor-deficient NOD mice
NODTNFR1 – NOD mice deficient in TNF receptor 1 subunit
nPOD – Network for Pancreatic Organ Donors with Diabetes
PBMC – peripheral blood mononuclear cell
PD-1 – programmed death 1
PDL-1 – programmed death 1 ligand
PLN – pancreatic lymph node
PPI – pre-proinsulin
RIP-LCMV – rat insulin promoter lymphocytic choriomeningitis virus
SOCS1 – suppressor of cytokine signaling 1
T1D – type 1 diabetes
TCR – T cell receptor
Teff – effector T cell
TNFα – tumor necrosis factor alpha
VCAM-1 – vascular cell adhesion molecule 1

This review describes the immune-mediated pathogenic mechanisms leading to diabetes. The data described is primarily in the NOD mouse, but also in humans where available. This includes activation, the mechanisms used to induce beta-cell death, and events beyond this point. In particular, we discuss the important roles the islet environment and beta-cell play in propagating the immune response, which ultimately results in beta-cell destruction and disease.

1.1 T lymphocytes destroy beta-cells

Beta-cell destruction in T1D is principally mediated by T lymphocytes. Autoreactive cytotoxic $CD8^+$ T lymphocytes (CTLs) are the main mediators of beta-cell destruction in both the NOD mouse and humans. Effector $CD4^+$ T cells also have an important role. Several $CD8^+$ and $CD4^+$ T cell clones specific for beta-cell antigens have been isolated from mice. These isolated clones are independently capable of transferring disease to immunocompromised hosts [1, 2]. Similarly, transgenic expression of T cell receptor (TCR) from various $CD4^+$ and $CD8^+$ T cell clones results in accelerated diabetes on the NOD background [3, 4]. However, in non-transgenic mice, both $CD8^+$ and $CD4^+$ T cells are essential for the development of spontaneous diabetes as a deficiency of either prevents the development of insulitis and diabetes [4].

Furthermore, both $CD8^+$ and $CD4^+$ T cells are required for efficient adoptive transfer of disease [2]. Non-depleting anti-CD4 and/or anti-CD8 antibodies can also prevent insulitis and diabetes in young NOD mice, and they can reverse disease in recent-onset NOD mice, through alterations in cytokine production and maintenance of immuno regulation [5, 6]. Collectively, these data demonstrate the importance of both autoreactive $CD8^+$ and $CD4^+$ T cells in mediating spontaneous diabetes.

1.2 Function of other immune cells in beta-cell destruction

Other immune cell subsets that contribute to diabetes include B lymphocytes, macrophages and dendritic cells (DCs). B-cell-derived autoantibodies against various islet antigens including (pro)insulin and glutamic acid decarboxylase 65 (GAD65) are the earliest indicators of beta-cell autoimmunity. They are measurable in peripheral blood, and foreshadow the onset of disease. NOD mice, deficient in B cells via the Igμ null mutation, are completely protected from insulitis and diabe-

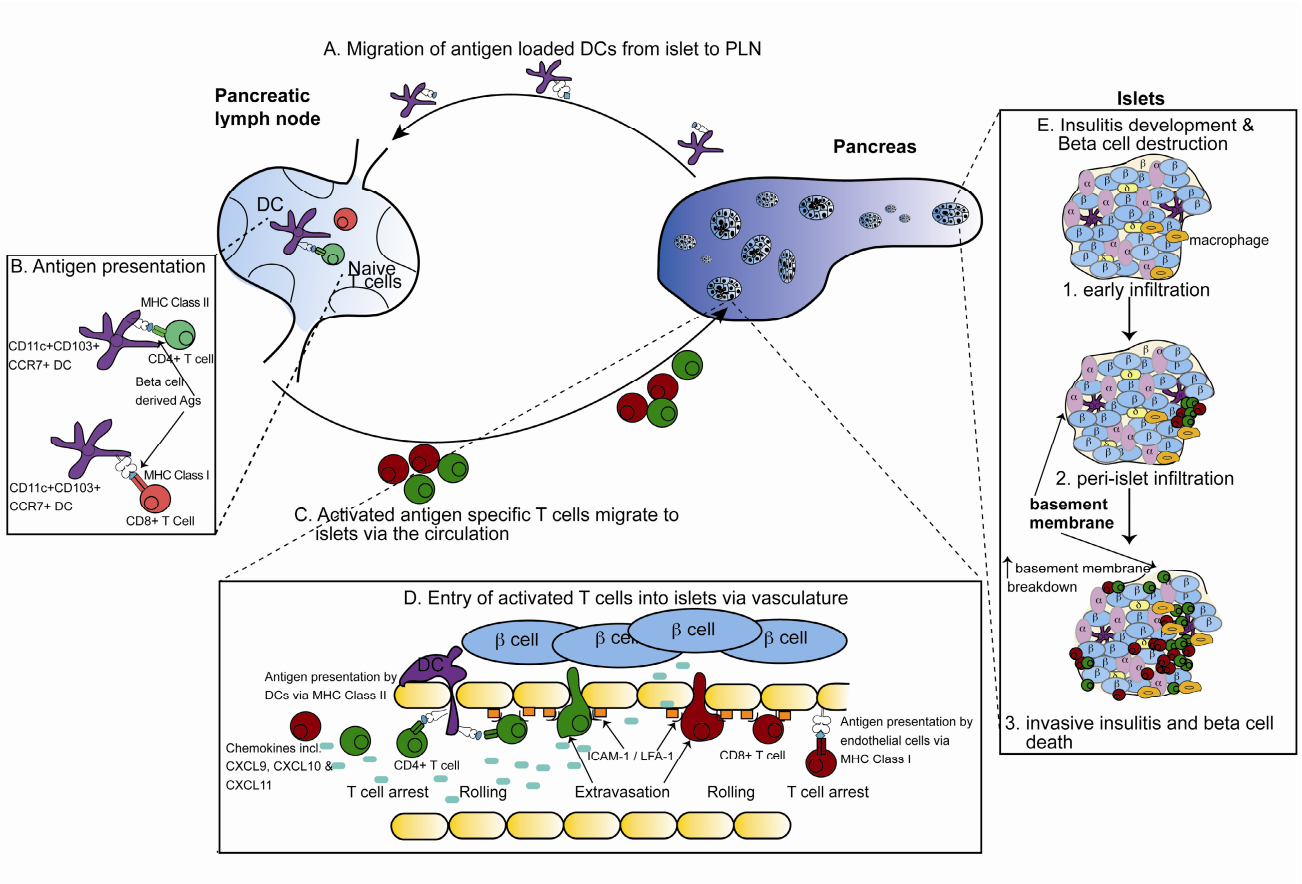

Figure 1. Activation, migration, entry, and infiltration of immune cells into islets during spontaneous diabetes. A-C: Dendritic cells loaded with beta-cell antigens migrate from islets to pancreatic lymph nodes and present antigen to naïve autoreactive $CD4^+$ and $CD8^+$ T cells. These T cells undergo initial activation and migrate towards the pancreas via the circulation. **D**: Entry for $CD4^+$ T cells and $CD8^+$ T cells into islets and presentation ($CD8^+$ T cells). Once arrested, T cells roll and extravasate via integrins and adhesion molecules. **E**: Insulitis development in islets. Initial macrophage infiltration occurs, followed by T cell infiltration, and the formation of peri-islet insulitis. The islet basement membrane breaks down and invasive insultis with concurrent beta-cell destruction occurs.

tes. Depletion of B cells using anti-CD20 also protects NOD mice, or reduces diabetes, depending on the age of administration [7, 8]. In addition, the skewing of the B cell repertoire towards islet antigens, for example through the transgenic expression of insulin-binding immunoglobulin heavy chains in B cells, promotes the development of diabetes [9].

Despite strong evidence for the requirement of B cells in diabetes, their exact function remains unclear. Secretion of antibodies with direct effects on beta-cells, or the presentation of antigens to $CD4^+$ T cells via MHC class II, are the two proposed B cell functions. Recent studies have clarified this and demonstrated that diabetes cannot be transferred by antibody or B cells alone [10]. Instead, B cells contribute primarily as antigen-presenting cells [10-12]. Using a series of transgenic and mutant mice with increased B cell activation, Silva *et al*. demonstrated that B cells act to enhance the accumulation and function of islet-reactive $CD4^+$ T cells promoting diabetes development [10]. Macrophages and natural killer (NK) cells have also been proposed as effector cells, recruited to the islet by $CD4^+$ T cells to induce beta-cell death [13, 14]. The primary function of DCs in spontaneous diabetes is antigen presentation both in the periphery and within the islet. This is critical to the initiation and progression of disease [15-18].

2. The pancreatic lymph node

2.1 Autoreactive T cells are primed in the pancreatic lymph nodes

The pancreatic lymph node (PLN) is the site where naïve T cells are activated by islet antigens (**Figure 1**). T cells that respond to islet antigens can be detected in the PLNs before the onset of insulitis [19]. Adoptively transferred TCR transgenic T cells specific for islet antigens are activated in the pancreatic lymph nodes prior to entering the circulation and infiltrating the islets [15, 19-21]. Activation of adoptively transferred T cells in the PLN are detected from about three weeks of age, suggesting that islet-derived antigens are not presented there until this time [20, 22]. Consistent with this finding, removal of the PLN at 3 weeks of age results in a dramatic decrease in the incidence of diabetes [23]. The initiation of beta-cell-specific T cell responses in the PLN infers that islet antigens are delivered there via the lymphatic circulation. Paradoxically, the islets lack lymphatic circulation, suggesting that delivery may require antigen-transit through lymphatics found at the periphery of islets [24].

Migration of antigen-loaded DCs rather than passive drainage probably provides the passage for antigen delivery from the islets to the PLNs (**Figure 1**) [25]. DCs loaded with beta-cell-derived proteins can be visualized within the PLNs [26]. Two subsets of DCs have been described within islets, the $CD11b^+$ ($CD11c^+CD11b^+CD103^-$) and the $CD103^+$ ($CD11c^+CD11b^{lo}CD103^+$) DCs. The $CD103^+$ subset, but not the $CD11b^+$ subset, expresses CCR7, a chemokine receptor associated with DC migration into draining lymph nodes [27, 28]. $CD103^+$ DCs were numerically deficient in the PLNs of CCR7 knockout mice, suggesting that $CD103^+$ DC represents the major migratory population derived from islets (**Figure 1**) [28]. DCs presenting beta-cell proteins to $CD4^+$ T cells in the PLNs have been described as $CD11c^+$CD11b variable [26]. The DCs that cross-present beta-cell proteins in the PLNs have been described as $CD11c^+CD8^+$ [15], $CD11c^+CD103^+CD11b^{+/-}$ [28], or $CD11c^+CD11b^{-/lo}$ merocytic DCs [29]. Merocytic DCs have been associated with induction of T1D [29]. Notably, CD8 expression was not detected on islets or merocytic DCs, but on cross-tolerizing DCs [15]. Given this phenotypic distinction, it is possible that different DC populations are involved in the induction of disease ($CD8^-$ DC) and cross-tolerance ($CD8^+$ DC).

The response to islet antigen is affected by the phenotype, maturity, and activation status of the DCs. Normally, presentation of beta-cell antigens in the PLNs can lead to T cell depletion after an initial period of expansion [15, 30]. An imbalance in the DC subsets within the NOD mouse may impair this process of depletion. Defective cross-tolerization in NOD mice has been associated with a genetically determined deficiency in cross-tolerizing $CD8^+$ DC [31, 32]. A key determinant of initiation of a pathogenic CD8 T cell response is the provision of CD4 T cell help and the licensing of DCs [33]. Transgenic NOD mice that express the costimulatory molecule B7-1 (CD80) on beta-cells develop accelerated autoimmunity and beta-cell destruction without a need for $CD4^+$ T cells [34]. This indicates that $CD4^+$ T cells are required in non-transgenic NOD mice (when costimulatory molecules are not expressed by beta-cells) to license DCs to facilitate the activation of $CD8^+$ T cells [34]. This is consistent with $CD4^+$ T cells licensing DCs.

2.2 Insulin is the primary antigen in NOD mice

The autoreactive T cells and antibodies that develop during the course of NOD diabetes target several proteins expressed by beta-cells, including proinsulin, islet cell antigen 512 (IA-2), GAD, islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), zinc transporter 8, and chromagranin A [35-39]. Of these proteins, proinsulin is central to the development of diabetes. However, other studies favor a role for antigens derived from neuroendocrine tissues that surround the islets, for example glial fibrillary acidic protein (GFAP) expressed by peri-islet Swann cells as initiating antigen upstream of insulin [40].

Perturbations of proinsulin expression are uniquely able to alter the course of diabetes in NOD mice. Transgenic overexpression of *Ins2,* with the aim of inducing proinsulin-specific tolerance, prevents insulitis and T1D [38, 41]. Concomitant induction of tolerance IGRP-reactive T cells of these mice provided direct evidence that the response against IGRP is downstream of that to proinsulin [36]. Conversely, despite the respective induction of tolerance in IGRP- and GAD-specific T cells by overexpression of IGRP or GAD, diabetes was not prevented [36, 37].

Gene knockout experiments similarly indicated a central role for insulin in diabetes. Insulin is encoded by two genes in mice: *Ins1* and *Ins2*, with

the latter being the dominant form expressed in the thymus. Knockouts of the *Ins1* or *Ins2* genes respectively retard or accelerate diabetes [42, 43]. Knockout of both *Ins1* and *Ins2*, with restoration of hormonal function by transgenic expression of insulin, with an inactivated CD4 and CD8 epitope, completely prevents the development of anti-insulin antibodies, insulitis, and diabetes [44]. Knockouts of GAD65 or IA-2 expression does not alter diabetes onset in NOD mice [45, 46]. These findings are consistent with the hypothesis that proinsulin is the primary antigen that initiates disease.

It is clear that $CD4^+$ T cell responses against the insulin B9-23 epitope are essential for diabetes to develop in NOD mice. Evidence for this observation comes from Unanue's lab. They identified two types of $CD4^+$ T cells recognizing insulin, "type A" T cells that respond to the intact insulin protein and the B9-23 peptide and "type B" T cells that only respond to the B9-23 peptide [17, 47]. Importantly, they showed that only type B T cells are pathogenic in NOD mice [48]. This surprising finding is largely explained by the fact that the insulin B9-23 peptide can bind to $I\text{-}A^{g7}$ in at least two overlapping registers [35, 48]. The minimal epitope B13-21 binds to $I\text{-}A^{g7}$ with high avidity. It is the predominant register presented when the epitope is derived from intact protein, and it is recognized by type A T cells. The pathogenic type B T cells recognize $I\text{-}A^{g7}$ with the B9-23-derived peptide bound in a register that is shifted by one amino acid, presenting the minimal epitope of B12-20. This peptide-MHC complex is unstable and only occurs in later endosomes, in the presence of free peptide [48]. Based on this finding, Unanue and colleagues suggest that pathogenic T cell responses against insulin arise in the NOD mouse because type A-specific $CD4^+$ T cells, but not type B-specific $CD4^+$ T cells, are deleted in the thymus where the insulin B13-21/$I\text{-}A^{g7}$ complexes are abundant [48]. However, in pancreatic islets, where the local concentration of insulin and free insulin B chain peptides is relatively high, sufficient insulin B12-20 complexes form to activate type B insulin B9-23-specific $CD4^+$ T cells, which escape thymic deletion [48].

2.3 Antigen specificity of autoreactive T cells in human type 1 diabetes

Current evidence suggests that T cell responses to proinsulin are also essential for the development of T1D in humans [49, 50]. The T1D susceptibility locus, IDDM2, maps to a variable number of tandem repeats upstream of the proinsulin gene and is believed to regulate its expression in the thymus, which in turn affects central tolerance to proinsulin [51, 52]. Peripheral blood mononuclear cells (PBMCs) from T1D patients produce interferon γ (IFNγ) when stimulated with proinsulin peptides, whereas PBMC from HLA-matched healthy subjects produce IL-10.

$CD8^+$ T cell responses to proinsulin peptides have been measured by ELIspot in PBMCs of patients with T1D, but not HLA-matched healthy control subjects [53]. Human $CD8^+$ T cell clones specific for an epitope in the leader sequence of human pre-proinsulin (PPI) have been isolated. A PPI-derived peptide presented by HLA-A2 (15-23) was identified by elution and mass spectrometry. Skowera *et al*. were then able to isolate $CD8^+$ T cell clones specific for $PPI_{15\text{-}23}$ and to identify that responses to this epitope were more readily detected in people with T1D than in HLA-matched healthy donors [54]. Recent X-ray crystallography revealed the interaction between HLA-A2/$PPI_{15\text{-}23}$ peptide/TCR [55]. Most attempts to define $CD8^+$ T cell responses have focused on HLA A2, but recently an HLA A24-restricted epitope derived from proinsulin, $PPI_{3\text{-}11}$, has been identified using a similar peptide elution approach [56].

The specificity of human autoimmune $CD4^+$ T cell responses against proinsulin is described in detail. The development of efficient CFSE-based cloning methods facilitates the isolation of human $CD4^+$ T cell clones specific for proinsulin from the peripheral blood of individuals with established T1D [57]. Interestingly, there is a second overlapping epitope, presumably presented in a different register [58]. T cell responses to several other islet antigens are also associated with immune-mediated destruction of the insulin-producing beta-cells. These mechanisms have been extensively reviewed elsewhere [59].

The possibility that T cell epitopes formed by posttranslational modification are the targets of pathogenic T-cell responses adds a new dimension to the analysis of autoimmune T cell specificity. At this point, no single epitope formed by posttranslational modification has been shown to drive beta-cell autoimmunity. The pathogenic NOD mouse T cell receptor, BDC2.5, has been shown to recognize chromogranin A [35]. More recent data suggests that the epitope recognized by BDC2.5 is formed by transglutaminase-mediated glutamine deamidation [60]. In humans, $CD4^+$ T cell responses against an epitope derived from the A chain of

human insulin depend on posttranslational modification. In this case, the modification is a vicinal disulfide bond between adjacent cysteine residues. This modification occurs spontaneously during the processing of intact proinsulin [57].

3. Migration of effector cells from lymph nodes to islets

3.1 Islet-infiltrating T cells are antigen-specific

Once activated in PLNs, T cells migrate via the circulation to the islets (**Figure 1**). Only activated, but not naïve, beta-cell-specific T cells are able to infiltrate non-inflamed islets. This is consistent with a requirement for T cell activation in PLNs for insulitis to occur [61-63]. Inflamed islets may allow the entry of some non-specific and non-activated T cells, but ultimately the T cells that accumulate within the islet lesion are predominantly islet-antigen-specific [62, 64, 65]. The selection of antigen-specific T cells is likely favored both by cognate antigen enhancement of binding and entry from the blood vessels, as well as cognate antigen-dependent expansion within the islets (see below).

3.2 Entry of immune cells into the islets

The initial infiltration of the islets requires recognition of antigen in the islets, thereby favoring entry of islet-specific T cells [61-63]. Microscopic analysis suggests that such cognate interactions may occur at the blood-islet interface [61-63]. *In vitro* analysis showed that insulin-specific CD8 T cells kill islet-endothelial cells in a cognate manner, suggesting that islet endothelial cells may cross-present insulin to CD8 T cells enabling binding and integrin activation [63]. Whilst endothelial cells are deficient in class II molecules, islet DCs (class II+) are intimately associated with the blood vessels. They frequently extend dendrites into the vessel lumen and provide a point of contact for CD4 T cells [28, 61]. The efficacy of CD4 T cell localization is further enhanced by interactions with intercellular adhesion molecule 1 (ICAM-1) (**Figure 1**) [62].

Once infiltration has commenced, the islet becomes more receptive to further infiltration and progression of disease [61, 62, 66, 67]. The role of integrins, selectins, and chemokines in this process has been comprehensively reviewed [68]. Both infiltrating immune cells and resident islet cells are involved. The islet itself is an important modifier of outcome. Infiltration by islet-specific CD4 T cells induces the upregulation of ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) in blood vessels, and ICAM-1 on beta-cells and DCs [61, 62]. Furthermore, insulitis triggers IFN-response genes. Many of these genes are expressed by the non-leukocyte component of the islet [61, 62, 66]. Chemokines are prominent among the induced genes, including *Cxcl9, Cxcl10*, and *Cxcl11* which attract T cells via the CXCR3 receptor [61, 62, 66].

4. Insulitis

4.1 NOD mouse insulitis

As diabetes progresses, the islets become infiltrated by immune cells. This process is termed insulitis (**Figure 1**). Histological analysis has shown that macrophages and DCs are dominant in the early spontaneous infiltration of NOD islets [69, 70]. The detection of cells that express CD11c or F4/80 in T cell-deficient NOD.*scid* islets has been used to assert that DCs and macrophages infiltrate islets first in the absence of T cells [69]. However, these cells are sparse, and it is now recognized that DCs resident within healthy islets express these same markers [16, 28]. Increasing islet infiltration and other changes associated with inflammation result in an increase in DC number, suggesting additional DC infiltration during disease progression [16]. Though rare, some T cells are detected in early NOD islet infiltrates, suggesting that these could be the first cells to infiltrate the islets [69, 71].

Initial insulitis is considered benign and is visualized around the periphery of individual islets. It seems probable that this peri-islet infiltrate originates from the islet-efferent blood vessels that form a network at the periphery of the islets, consisting of efferent capillaries converging at post-capillary venules [72]. The progression from benign peri-islet infiltrate to islet invasion and beta-cell destruction is poorly understood. However, it is widely believed that the destructive infiltrate originates from the peri-islet infiltrate. It has been proposed that the peri-islet basement membrane acts as a physical barrier to leukocyte migration into the islets. Histological examination in NOD mice showed that progression to destructive insulitis is associated with destruction of the peri-islet basement membrane (**Figure 1**) [73].

Recently, the islets of NOD mice have been shown to express high levels of the glycosaminoglycan, heparan sulfate, which is essential for

Table 1. Effects of genetic mutations within effector pathways on diabetes in NOD mice

Effector mechanism/ pathway	Mouse strain	Effect on diabetes	Proposed mechanism	Reference
Perforin & granzymes	Perforin -/-	Significant reduction	Prevention of granzyme-mediated beta-cell death	[92, 93]
	GranzymeB -/-	None	Redundancy in granzymes required. Possible effect on CD8+ activation	[97]
Fas - FasL	NODlpr	Complete protection	Abnormal immune cell function	[99]
	Fascg	Reduction	Minor role of Fas in beta-cell death	[102]
	dnFADD	Reduction	Minor role of Fas in beta-cell death	[101]
	Bid -/-	None	Minor role of Fas in beta-cell death	[105]
IFNγ	IFNγ	Slight delay		Reviewed in [113]
	IFNγR1	None	Promotes T cell migration	
	IFNγR2	None		
	RIP-ΔγR	None	Upregulation of beta-cell MHC class I expression	
TNF	TNFR1-/-	Complete protection	Increased Treg function	[115, 116]
IL-1	IL-1R-/-	Slight reduction & delay	CD4+ effector T cell expansion, decline in Treg number	[112, 114]
IL-21	IL-21R-/-	Significant protection	Promotes CTL, B cell, and CD4+ T cell function	[139-141]

Legend: Bid – BH3-interacting domain death agonist, CTL – cytotoxic T lymphocyte, dnFADD – dominant-negative Fas-associated death domain, Fascg – dominant-negative form of Fas. IFNγ – interferon gamma, IFNγR1/2 – interferon gamma receptor 1/2, MHC – major histocompatibility complex, NOD – nonobese diabetic, NODlpr – CD95-deficient variant (lpr) of the NOD mouse, RIP – rat insulin promoter, TNF – tumor necrosis factor, TNFR1 – tumor necrosis factor receptor 1.

beta-cell survival. Immune cells infiltrating the islets were also shown to produce heparanase, an active herparan sulfate-degrading enzyme, which could act to promote invasive insulitis [74]. Similarly, the local upregulation of hyaluronic acid could also drive the progression to destructive insulitis by its interaction with CD44 on diabetogenic cells [67]. This assertion was supported by the blockade of adoptive transfer of diabetes by *in vivo* administration of either anti-CD44 mAb or hyaluronidase [67]. Alternatively, following adoptive transfer of activated beta-cell-specific CD4 T cells, it was concluded that these T cells enter the islets directly from the circulation [61]. Whatever mechanisms are involved, it is clear that insulitis in the NOD mouse follows a distinct and well characterized pattern; it presents as an early peri-islet insulitis that progressively develops to destructive insulitis with concurrent beta-cell destruction.

4.2 Insulitis in human islets

Insulitis is also a feature of the development of diabetes in humans. Histopathological analysis of pancreata collected at or near diagnosis revealed infiltration of T cells, B cells, and macrophages into the islets [75]. $CD8^+$ T cells are the most abundant leukocytes, while $CD4^+$ T cells are less in number than both $CD8^+$ T cells and macrophages [76-78]. The infiltration of islets is often accompanied by a marked upregulation of MHC class I protein on islets under immune attack [76, 78]. This and the prevalence of $CD8^+$ T cells within the islet lesions, suggests that $CD8^+$ T cells are the dominant effector cells in human diabetes.

Insulitis in humans differs in several features from that of the NOD mouse. One of the most striking differences is that human islet infiltration and destruction is not uniform or synchronous. Frequently, it only affects a small number of islets, and lobular areas of the pancreas are affected differently [77]. A recent study of pancreata collected from autoantibody positive pre-clinical patients demonstrated the presence of insulitis in 2 out of 62 patients, and <10% of the islets studied within these two samples were affected [79]. Similarly, a study of recent onset diabetic pancreata detected small amounts of infiltrate, with a few immune cells per islet [80]. Human insulitis is qualitatively

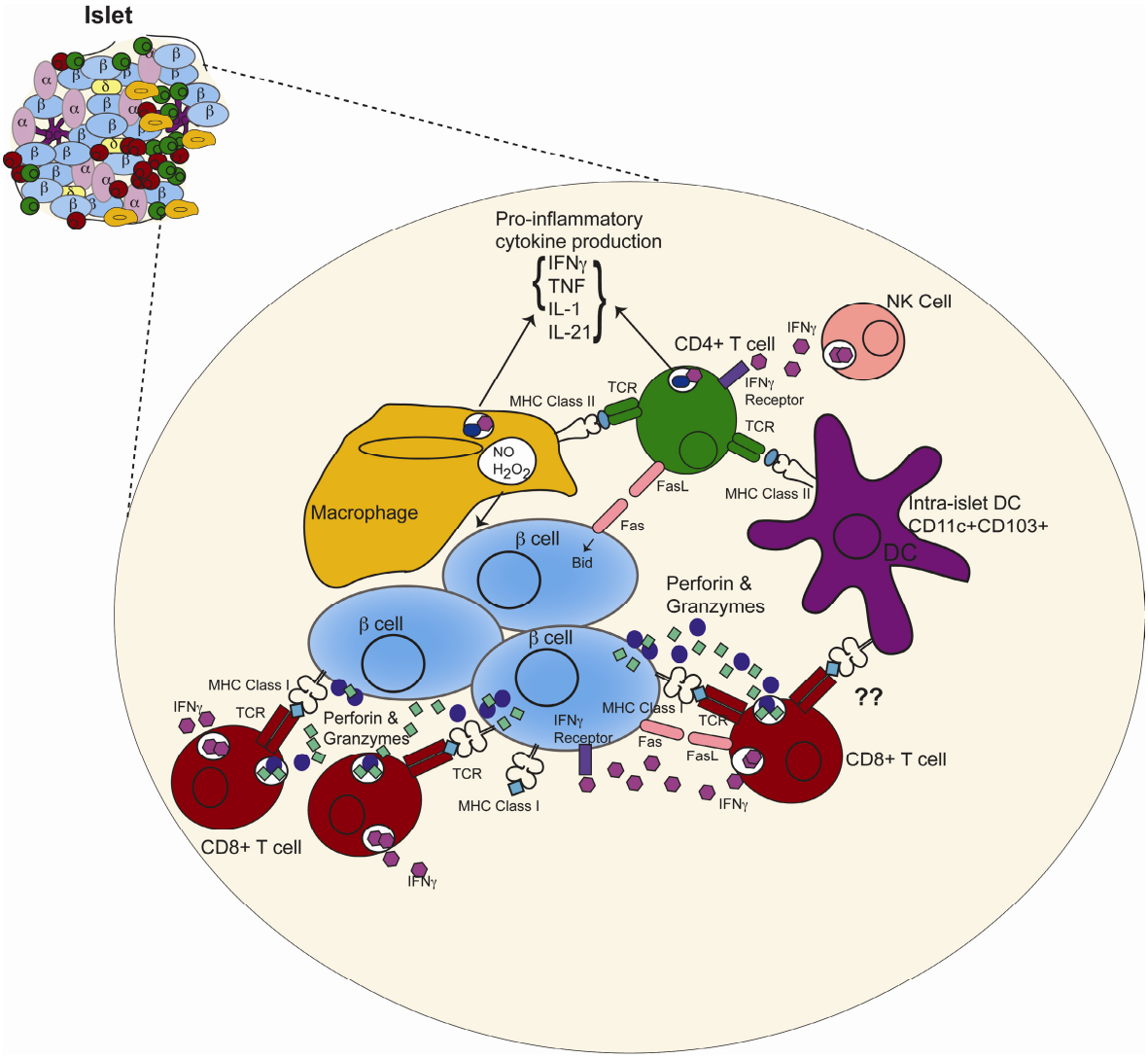

Figure 2. Effector cells and their actions in mediating beta-cell destruction. Cytotoxic T lymphocytes are the main mediators of beta-cell death through the production of perforin and granzymes. Fas-FasL interactions have a minor role in beta-cell destruction. $CD4^+$ T cells do not directly kill beta-cells, instead they are proposed to activate macrophages to kill beta-cell cells via NO and reactive oxygen species. Pro-inflammatory cytokines are produced by $CD4^+$, CTLs, and macrophages, and act to regulate the immune response. The presence of NK cells within the islets is proposed to promote effector $CD4^+$ T cell function. Intra-islet DCs present antigen to $CD4^+$ T cells and may present antigen to CTLs. B cells are also present in the islet and secrete autoantibodies and promote $CD4^+$ T cell function through antigen presentation (not shown). Figure is prepared from descriptions within mouse models.

similar to that in the NOD mouse with a similar mix of cell types, but it is clearly not as florid as in the mouse. However, studies of pancreata collected from long standing diabetics have shown that infiltrate can persist for a considerable length of time, suggesting it is an important feature of the human disease [81].

Analysis of insulitis and effector cells within the pancreas is hampered by the difficulties in obtaining samples for study. However, progress on the direct study of effector cells within the pancreas has been made recently. An elegant study by Coppietiers *et al*. utilized a large collection of frozen pancreas samples collected through the Net-

work for Pancreatic Organ Donors with Diabetes (nPOD), combined with some other rare cases obtained by the authors, to demonstrate definitively the presence of islet-reactive $CD8^+$ T cells within the islets of type 1 diabetic patients [82]. The authors used *in situ* tetramer staining of sections collected from HLA-A2 individuals with up to 8 years disease duration, to show specific reactivity of $CD8^+$ T cells within the insulitic lesion to six islet autoantigens, PPI, insulin, IGRP, GAD65, preproislet amyloid protein, and IA-2 [82]. These studies are important as they analyze directly the antigen specificity of T cells at the site of autoimmune beta-cell destruction. Within the limitations of human research, this provides excellent evidence for these T cell responses playing a direct role in beta-cell destruction.

5. Mechanisms of beta-cell destruction

5.1 The requirement for $CD8^+$ T cells

Autoreactive cytotoxic $CD8^+$ CTLs are dominant in destroying beta-cells. CTLs recognize peptide antigens presented on the beta-cell surface by MHC class I proteins that consist of a polymorphic heavy chain and a constant light chain, β2microglobulin. NOD mice deficient in β2-microglobulin lack MHC class I and $CD8^+$ T cells and are protected from diabetes [83-86]. Reconstitution of the beta-cell MHC class I expression results in diabetes following the transfer of diabetogenic splenocytes [87].

These experiments indicate a need for the direct interaction between beta-cell and $CD8^+$ T cell via MHC class I for beta-cell destruction. Overexpression of adenovirus E19 protein in beta-cells, which inhibits MHC class I expression, prevents CTL lysis [19]. Transgenic overexpression of the suppressor of cytokine signaling 1 (SOCS1) in beta-cells blocks CTL killing of beta-cells in part by reducing class I MHC expression and antigen presentation [88, 89]. However, the generation of NOD mice, containing a conditional deletion of class I specifically from the beta-cells (class I beta-bald mice), directly confirm the interaction between CTL and beta-cell is required for efficient disease progression [90].

Normally, $CD8^+$ T cells develop in these mice as MHC class I is expressed in the thymus and lymph nodes, but diabetes is significantly reduced. The direct interaction of $CD8^+$ T cell and beta-cell has recently been visualized using intra-vital 2-photon imaging [91]. Tracking of CTL movement into and within the pancreas in the RIP-LCMV model of diabetes, showed CTLs arresting at the junction with beta-cells and forming stable contacts of several hours duration [91]. Collectively, these studies demonstrate the dominant role of CTLs in beta-cell destruction. However, the lack of diabetes prevention in the class I beta bald mice indicates other type of immune cells, including $CD4^+$ T cells can also function as effectors [90].

5.2 Perforin and granzymes released by CTLs are the dominant effectors of beta-cell destruction

Granule exocytosis with release of perforin and granzymes is a major effector mechanism utilized by all CTLs. Diabetes incidence in NOD mice deficient in perforin was reduced to 17% compared to wild-type littermates and onset was delayed [92]. This observation was independently confirmed [93]. In both studies, a small percentage of mice still developed diabetes, which could be caused by other effector mechanisms or other effector cells. 8.3 TCR transgenic CTLs were reported to kill beta-cells independently of perforin, using the Fas/FasL pathway, suggesting perforin is not the dominant effector mechanism [93]. This was later clarified by Dudek *et al*. who used genetic deficiencies in both pathways to demonstrate in the 8.3 model that both perforin and Fas are operational and compensate for each other when one effector mechanism is removed [89]. However there is little evidence for this redundancy in NOD mice (see below). Overall the significant effect of perforin deficiency points overwhelmingly to its dominant role in CTL-mediated beta-cell destruction *in vivo* (**Figure 2**, **Table 1**). Perforin deficiency does not reduce diabetes mediated by $CD4^+$ T cells, demonstrating this is unlikely to be a dominant effector mechanism utilized by $CD4^+$ T cells [94, 95].

The main function of perforin is to facilitate the activation and delivery of granzymes into the target cell. Granzymes are a family of serine proteases that activate pathways within the cell to induce apoptosis. Recombinant granzymeB was able to induce beta-cell death *in vitro* by a BH3-interacting domain death agonist (Bid) dependent mechanism [96]. However, granzymeB was only effective in the presence of perforin. Given the dependence on perforin for granzyme activity, the perforin knockout mouse is also an effective knockout of granzymes. To investigate the role of granzymeB specifically, granzymeB knockout

NOD mice were recently generated (**Table 1**) [97]. Spontaneous diabetes in these mice was unaffected, although insulitis onset was delayed, indicating granzymeB is dispensable for diabetes, but may play a role in $CD8^+$ T cell activation. This suggests a redundancy for granzyme-mediated beta-cell killing, which is perhaps not surprising given that 11 different granzymes are present in mice and expression varies in CTL.

5.3 The Fas/FasL pathway is a minor effector of beta-cell destruction

Ligation of the Fas (CD95) death receptor by FasL on both effector CTLs and $CD4^+$ T cells is another proposed mechanism of beta-cell destruction (**Figure 2**). Beta-cells have low levels of Fas, but this can increase with exposure to inflammatory cytokines [98]. Ligation of Fas by FasL induces caspase activation and beta-cell apoptosis. FasL-induced beta-cell destruction has been characterized best *in vitro*. However, its pathogenic role in diabetes has been challenged by several studies. Fas-deficient NOD mice (NOD^{lpr}) were resistant to diabetes [99]. However, Fas-deficient islets transplanted into wild-type mice revealed only marginal protection of the islet graft, indicating a minor role for Fas in beta-cell destruction [100]. Transgenic NOD mice, expressing dominant negative Fas-associated death domain (FADD), had limited protection (**Table 1**). FADD is a component of the Fas signaling pathway that only occurs in beta-cells and transgenic mice containing a beta-cell-specific disruption of Fas [101, 102]. Similarly, islets deficient in Fas expression were not protected in CD4 T cell-induced diabetes, suggesting beta-cell destruction occurs in the absence of a functional Fas pathway [103, 104].

A drawback of these models is the overexpression of transgenes in beta-cells which can affect beta-cell function. To circumvent this, Mollah *et al*. investigated the incidence of diabetes in NOD mice deficient in Bid [105]. Bid is essential for Fas-induced apoptosis and Bid-deficient islets are protected from FasL-induced killing *in vitro* [106]. Development of spontaneous diabetes in NOD $Bid^{-/-}$ mice was similar to wild-type mice, indicating Bid-dependent Fas-induced apoptosis is not an effector mechanism utilized by T cells in the NOD mouse. Collectively, the impact of any of the mutations in the Fas-FasL pathway is relatively minor, demonstrating this pathway has only a small role in beta-cell destruction mediated by either CTLs or $CD4^+$ T cells (**Table 1**).

5.4 Effector mechanisms utilized by human auto-reactive CTLs

CTL clones recognizing several islet autoantigens have been isolated from the peripheral blood of both recent onset and long-term T1D patients [54, 107]. The increased expression of beta-cell MHC class I observed in pancreatic biopsies suggests that a direct interaction between CTLs and beta-cells is required for CTL killing in humans [75, 76, 82, 108]. Skowera *et al*. demonstrated that their isolated CTL clone specific for $PPI_{15\text{-}23}$ was able to kill beta-cells and required direct contact between CTL and beta-cell. This indicates that perforin or Fas are the likely candidate effector molecules.

Human islets are susceptible to killing by recombinant perforin and granzymeB *in vitro* [96]. To investigate which CTL effector mechanisms are utilized to kill beta-cells Campbell *et al*. used CTL clones specific for viral peptides presented by HLA-A2, HLA-B8, and human beta-cells pulsed with specific viral peptides [109]. This system circumvents the difficulties in isolating beta-cell antigen-specific CTLs. The CTLs killed beta-cells pulsed with viral peptides, dependent on MHC class I expression. Increasing the expression of MHC class I on the beta-cell surface increased the CTL killing. Beta-cell death was prevented by the presence of the perforin inhibitor concanamycin A. Therefore, killing of beta-cells was dependent on perforin. In contrast, neutralizing anti-Fas ligand antibody had no effect, suggesting the Fas-FasL pathway is not involved [108].

These findings have recently been confirmed using the $PPI_{15\text{-}24}$ clone and an additional HLA-A2-restricted PPI clone, $PPI_{3\text{-}11}$ [106]. These clones killed beta-cells by requiring a direct interaction via MHC class I and utilizing perforin and granzymes. The role of Fas/FasL was less clear, and there was no evidence for cytokine-dependent killing [106]. Overall, these studies demonstrate autoreactive CTLs in humans utilize the same effector mechanisms as those in the NOD mouse. There is a dominant role for perforin and granzymes, and a possible minor role for Fas in mediating beta-cell death.

5.5 Pro-inflammatory cytokines are critical regulators of effector function, but they are not important effectors of beta-cell destruction

Whilst $CD8^+$ T cells are the dominant effector cells, $CD4^+$ T cells are also involved in beta-cell de-

struction (**Figure 2**), even though beta-cells do not express MHC class II, and thus do not interact with CD4 T cells directly. Several mechanisms have been proposed to explain how $CD4^+$ T cells destroy beta-cells. Studies have suggested inflammatory cytokines such as IFNγ, TNFα, and IL-1 are important mediators of beta-cell death induced by $CD4^+$ T cells [110]. Pro-inflammatory cytokines are also secreted by activated CTLs. Treatment of islets with IFNγ and IL-1, or IFNγ and TNFα, leads to beta-cell death *in vitro*, probably through production of nitric oxide (NO) in beta-cells and activation of the intrinsic death machinery regulated by Bcl-2 family members [110, 111]. However, the *in vivo* role of inflammatory cytokines in beta-cell death remains controversial; extensive studies indicate that cytokines are not directly cytotoxic to beta-cells [103, 112, 113]. Instead, the actions of these cytokines are to mediate intracellular communication, recruit immune cells to islets, and/or increase beta-cell recognition by autoreactive CTLs (**Table 1**).

Experiments in knockout mice have shown that deficiencies in single cytokines or their receptors usually do not prevent diabetes. When they do, it is due to immunoregulatory effects (**Table 1**). Mice deficient in IFNγ or the IFNγ receptor subunits, IFNγR1 or IFNγR2, develop diabetes, demonstrating IFNγ is not required for spontaneous diabetes in NOD mice (reviewed in [113]). The onset of diabetes is also unaffected in NOD mice expressing a dominant negative form of IFNγR1 in the beta-cells (RIP-ΔγR NOD mice) (reviewed in [113]). IFNγ is responsible for the upregulation of MHC class I on beta-cells. This is observed in islets with insulitis in NOD mice, which promotes extravasation of $CD4^+$ and $CD8^+$ T cells through the vessel endothelium into islets (reviewed in [103, 113]). Similarly, the incidence of diabetes in IL-1 receptor-deficient NOD mice (NOD IL-R) is only slightly reduced and delayed. Comparing the destruction of wild-type and IL-1R-deficient islets in transplant experiments revealed that the IL-1R deficiency impacts on immune cells rather than beta-cells [112]. NOD mice express elevated levels of IL-1, which promotes the expansion of effector $CD4^+$ T cells and dilutes the number of $CD4^+CD25^+$ regulatory T cells (Tregs) [114]. In contrast, NOD mice deficient in the TNF receptor 1 subunit (NODTNFR1) are completely protected from diabetes, and develop a very mild peri-islet insulitis [115]. TNFR1 deficiency affects the immune system rather than protecting islets directly [116, 117]. Tregs from TNFR1-deficient mice are better able to suppress effector cells both *in vitro* and *in vivo* [116].

Blockade of several cytokines by overexpression of SOCS1 in beta-cells has a greater impact on diabetes mediated by $CD8^+$ T cells than a single cytokine deficiency has on diabetes development [88]. SOCS1 blocks signaling by multiple cytokines, including IFNγ. This approach completely prevents diabetes in the 8.3 $CD8^+$ T cell model, an effect linked to a reduction in beta-cell recognition by $CD8^+$ T cells [88, 89]. This result demonstrates one way in which the beta-cell contributes to its own demise. However, SOCS1 overexpression does not protect against $CD4^+$ T cell-induced diabetes [103]. Furthermore, IL-1 receptor knockout beta-cells overexpressing SOCS1 are destroyed *in vivo* as fast as wild-type beta-cells in mice carrying diabetogenic $CD4^+$ T cells [103]. In fact, only systemic, but not beta-cell-specific, deletion of cytokine signaling can delay or inhibit the development of $CD4^+$ or $CD8^+$ T cell-induced diabetes. This shows that inflammatory cytokines are critical regulators of the immune response, but are not required for beta-cell death.

5.6 The function of human autoreactive $CD4^+$ T cells

The strongest evidence for a role of $CD4^+$ T cells in humans originates from the genetic association between HLA class II region genes and T1D. Genetically, the HLA class II region has the strongest association with T1D. More than 90% of Caucasians with T1D carry the DR3-DQ2 (HLA DRB1*0301-DQB1*0201) or DR4-DQ8 (HLA DRB1*0401-DQB1*0302) haplotypes [118]. Surprisingly, individuals heterozygous for DR3-DQ2 and DR4-DQ8 haplotypes are at greater risk of T1D than those with either one of these haplotypes alone [119]. The role of $CD4^+$ T cells in human T1D is underscored by the observation that some HLA alleles, HLA DQB1*0602 for example, confer a reduced risk of T1D [120].

The effector mechanisms used by pathogenic human $CD4^+$ T cells are not fully understood, primarily because of the technical challenges associated with identifying beta-cell antigen-specific T cells in the peripheral blood [121]. The issues surrounding the identity of the antigens and epitopes recognized by pathogenic $CD4^+$ T cells add to the challenge. Nonetheless, it has been shown that the CD4/CD8 ratio is altered in patients with type 1 diabetes, and that $CD4^+$ T cells in patients means an increase in their activation status [122, 123].

Further studies with human T cells have shown that $CD4^+$ T cells with a Th17 phenotype are increased in T1D patients, with a concomitant increase in the concentration of IL-17 detectable in peripheral blood [124-127]. In addition, Treg function is decreased in T1D patients. Brusko *et al*. detected no change in the number of Tregs [128]. However, the suppressive function of Tregs isolated from patients is impaired, which may contribute to the loss of self-tolerance [126, 128, 129]. Collectively, declining Treg function and increasing effector $CD4^+$ T cell number could promote diabetes pathogenesis.

5.7 The emerging role of macrophages and NK cells

Evidence exists that macrophages take a decisive role in the destruction of pancreatic beta-cells. $CD4^+$ T cells may activate islet-infiltrating macrophages that then kill beta-cells (**Figure 2**). Diabetogenic $CD4^+$ T cells fail to induce diabetes in the absence of macrophages. Macrophages are in close proximity to beta-cells in insulitis. Depletion of macrophages using clodronate-loaded liposomes inhibits diabetes induced by diabetogenic $CD4^+$ T cells [13]. Activated macrophages isolated from infiltrated islets kill islet cells *in vitro* and *in vivo* [13]. Furthermore, the accumulation of macrophages within islets following chemokine expression by beta-cells can cause beta-cell death and diabetes, even in the absence of T cells [130]. Macrophages can directly exert cytotoxic effects through a variety of mechanisms. They are an important source of inflammatory cytokines, NO, and reactive oxygen species such as H_2O_2, all of which kill beta-cells *in vitro* [110]. Macrophages also have the ability to sense stressed cells and eliminate them by engulfment [131]. However, the *in vivo* mechanism of macrophage-mediated killing of beta-cells has not been established and remains an intriguing question.

Natural killer cells seem to play another complementary role in beta-cell destruction in autoimmune diabetes (**Figure 2**). It has been reported that a ligand for the NK cell receptor, NKp46, is expressed by beta-cells [14]. Interaction of NK cells and beta-cells could lead to the release of cytotoxic molecules from NK cells such as perforin and lysis of beta-cells. However, Angstetra *et al*. were unable to demonstrate that NK cells were directly cytotoxic to beta-cells, either *in vitro* or *in vivo* [132]. Instead, the study showed that depletion of NK cells in NOD4.1 mice reduced the incidence of diabetes, suggesting NK cells could maintain the right environment for the cytotoxicity of effector T cells [133]. This is consistent with an earlier report showing that the ablation of Tregs within islets results in activation of NK cells and the production of IFNγ, which promotes the function of effector $CD4^+$ T cells [133]. These two studies clearly indicate that the function of NK cells is to promote effector T cell function, as opposed to being directly cytotoxic to beta-cells (**Figure 2**).

6. The function of the islet environment

Recent studies have revealed that the progression of diabetes is driven by pathogenic events that occur within the islet microenvironment, including differentiation and proliferation of T cells. This suggests that the islet is not simply a passive target of autoimmune effector cells that develop within the lymph node.

6.1 Immune regulatory mechanisms within islets fail to control progression of insulitis

Tregs are readily identified within the sites of inflammation. The maintenance of Tregs at the site of inflammation is not simply the result of continued migration from lymphoid tissues. The inflammatory lesion also provides signals for Treg proliferation and survival, and $Ki67^+FOXP3^+$ Tregs have been detected within islets [26]. However, the progression of insulitis has been associated with a progressive reduction in the ratio of Tregs to effector T cells (Teffs) within islets [134]. While the events that lead to the failure of intra-islet Tregs to control insulitis remain to be fully elucidated, one contributor may be the insufficient local availability of IL-2 resulting in decreased Treg expression of Bcl2 and consequent apoptosis [134]. The NOD *Il2* gene is associated with reduced IL-2 production and reduced Treg number and function compared to the *Il2* gene of T1D resistant strains [135]. In addition, $CD4^+$ Teffs may acquire resistance to regulation, which further contributes to disease progression (**Figure 3**) [136]. Altered responsiveness of Tregs to IL-2 has also been observed in patients with T1D, and this leads to a decrease in the persistence of FoxP3 expression, declining function, and autoimmunity [137].

Reduced local IL-2 production is linked with high levels of IL-21 secretion within the islets of NOD mice. The Idd3 locus contains both the *Il2* and *Il21* genes, and both are highly expressed in NOD mice. However, the IL-2 mRNA is unstable, resulting in low level expression and a high level of

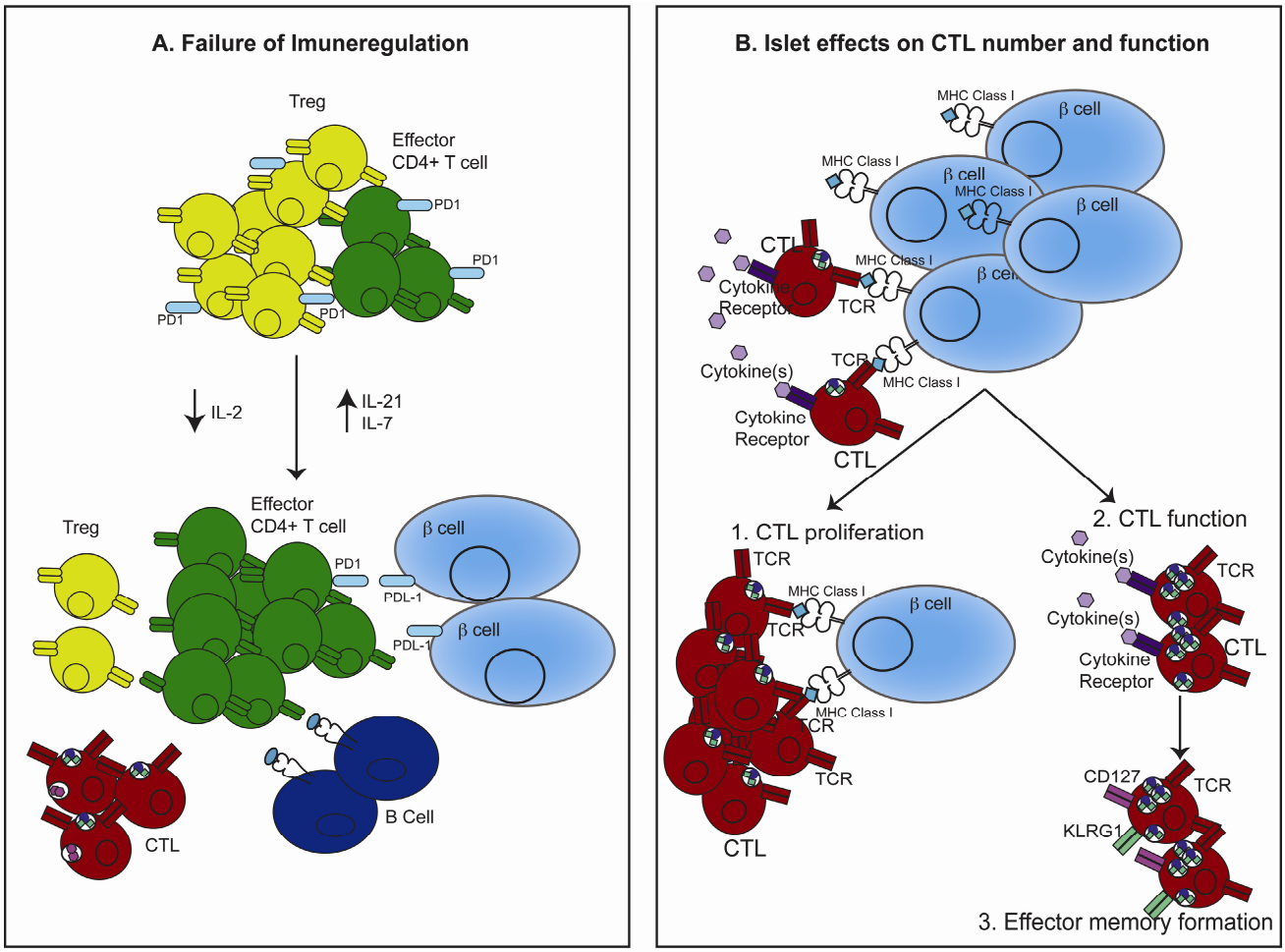

Figure 3. How the islet environment modifies the pathogenesis of diabetes. **A**: Immune regulatory mechanisms fail within the islet as diabetes progresses. Tregs present in the islet initially control the immune response. However, declining concentrations of IL-2 and increasing concentration of IL-21 result in decreased Treg number and function and increases in effector $CD4^+$ T cells. The high concentration of IL-21 also promotes CTL and B cell function in the islet. Increasing concentrations of IL-7 results in downregulation of PD-1 expression on Tregs and effector $CD4^+$ T cells, which promotes beta-cell destruction through failure of the PD-1/PDL-1 pathway. **B**: Islet environment promotes CTL function. Beta-cell antigen presentation promotes local proliferation of CTLs within the islet, increasing insulitis development. CTLs also respond to cytokines present in the islet, and this may increase the expression of cytotoxic markers, including granzymeB and IFNγ, and CTL function. CTLs within the islet are also stimulated to form an effector memory CTL pool.

IL-21 [138]. IL-21 is highly expressed by activated $CD4^+$ T cells and NKT cells. Deficiency in IL-21 signaling, either through genetic deficiency of the IL-21 receptor or through IL-21 neutralization prevents diabetes and insulitis [139-141]. Neutralization of IL-21 alters the function of $CD8^+$ T cells and B cells within the islet, suggesting this cytokine promotes CTL and B cell differentiation, and survival within islets [139]. Expression of IL-21 may also stimulate expression of CCR7 on DCs. This facilitates the migration of antigen-loaded DCs from islets into the PLN, where they can cross-present antigens to autoreactive $CD8^+$ T cells [27]. Thus, the imbalance between IL-2 and IL-21 within the islet environment results in declining Treg function, with a concomitant increase in effector T cell function (**Figure 3**).

Another common gamma chain cytokine, IL-7, may promote T cell function and progression of diabetes. Anti-IL-7 antibody treatment prevented

the development of spontaneous disease in NOD mice, and even reversed established disease [142, 143]. IL-7 neutralization appeared to increase the proportion of effector cells and Tregs expressing the inhibitory cell surface receptor programmed death 1 (PD-1) [142, 143]. PD-1 binds its ligand PDL-1 expressed on lymphocytes and beta-cells. The interaction between PD-1 and PDL-1 has previously been shown to be very effective at preventing diabetes [144, 145]. Therefore, the expression of IL-7 within the islets may suppress PD-1/PDL-1 interaction and promote pathogenic T cells to kill beta-cells (**Figure 3**).

6.2 Antigen presentation within the islet environment

There are at least four cell types within infiltrated islets that could act to present beta-cell antigens to $CD4^+$ and $CD8^+$ T cells. These include dendritic cells, endothelial cells, B cells, and the beta-cells themselves It is likely that the presentation to either $CD4^+$ or $CD8^+$ T cells utilizes different cell types. The importance of antigen presentation by beta-cells in CTL destruction is described above (section 5.1), and its role in promoting effector function is described below (sections 6.3 and 6.4). Likewise the function of endothelial cell antigen presentation is described above (section 3.2).

It has been suggested that B cells present antigens to both $CD4^+$ and $CD8^+$ T cells, although it is unclear whether this occurs within the islet environment or just within the PLN [10-12]. B cells have also been shown to promote the survival of autoreactive CTLs within islets. Depletion of B cells resulted in increased CTL apoptosis within islets, and indicated that B cells are required for intra-islet CTL survival and development of diabetes [146]. As described in sections 2.1 and 3.1 above, all islets contain intra islet dendritic cells, and these increase as insulitis progresses. Both $CD11b^+$ ($CD11c^+CD11b^+CD103^-$) and $CD103^+$ ($CD11c^+CD11b^{lo}CD103^+$) DCs are present in non-inflammed islets, they are more plentiful in the islets of NOD mice, and the $CD11b^+$ population expands during insulitis [16, 28]. These intra-islet dendritic cells contain beta-cell antigens, including insulin. The ability of these islet DCs to take up and present beta-cell antigens has been demonstrated by the capacity of freshly explanted islet DC to stimulate CD4 T cells [16, 17]). Cross-presentation by intra-islet DCs to CTLs within islets has not been demonstrated.

6.3 The local T cell response is driven by ongoing recruitment and intra-islet proliferation

Progression of insulitis is due both to $CD4^+$ and $CD8^+$ T cell recruitment from the peripheral circulation and proliferation of previously recruited cells within the islet environment. The sphingosine-1-phosphate receptor agonist, FTY720, that prevents the egress of lymphocytes from lymph nodes into the circulation, has been useful in determining which of these processes contributes to insulitis progression. FTY720 treatment effectively disconnects the islets from circulating lymphocytes. Continuous treatment of NOD mice with FTY720 prevents the development of diabetes [147]. Protection in the treated mice is not due to clearance of the islet infiltrate, but rather a stabilization of the existing insulitis. A two to three fold reduction in $CD4^+$ T cells within the islets was observed, but no change was seen in the numbers of infiltrating $CD8^+$ T cells present [147].

A similar approach in NOD8.3 mice identified that for $CD8^+$ T cells both recruitment from the circulation and local proliferation within the islet is required for the progression of insulitis [148]. Antigen presentation by the beta-cell was critical in stimulating CTL proliferation within the islet (**Figure 3**). These two studies suggest that $CD4^+$ and $CD8^+$ T cells use continuous recruitment and local proliferation differently during insulitis development. This clarifies the importance of the islet environment in enhancing the $CD8^+$ T cell response.

6.4 CTLs are armed within islets

The islet environment also promotes the differentiation of CTLs into effector memory T cells (**Figure 3**) [149]. Autoreactive CTLs found within the islets of diabetes-prone NOD mice display much higher expression of markers of cytotoxicity than recently activated cells, which are still residing in the PLNs. These markers included granzyme B, IFNγ, and surface expression of the degranulation marker CD107a. Their expression only increased when the $CD8^+$ T cells reached the islets. Pro-inflammatory cytokines appeared to provide the additional signals for CTL maturation rather than antigen presentation by beta-cells or intra-islet dendritic cells [149]. Adoptively transferred 8.3 $CD8^+$ T cells increased their expression of CD127 and KLRG1, two markers of effector memory differentiation. This phenotype was acquired within the islets (Chee, Kay & Krishnamurthy, unpublished 2012). These two studies

Table 2. Recent major discoveries in the pathogenic mechanisms of type 1 diabetes

Discovery	Importance	Specie	Reference
Proinsulin is the initiating antigen	Identification of a primary antigen target for the induction of tolerance	Mouse	[36]
Pathogenic type B insulin-specific CD4+ T cells	Provides a new model for the occurrence of autoimmunity	Mouse	[17, 48]
T cells within islets are antigen-specific	Entry and accumulation within islets is a tightly regulated process	Mouse	[64, 65]
Proinflammatory cytokines are not directly toxic to beta-cells in vivo	Identifies the function of these cytokines in diabetes	Mouse	[103, 112-114, 116]
IL-2/IL-21 cytokine expression and consequent changes to Treg/Teff cell rate in islets	Possible targets to restore Treg function within islets	Mouse	[134, 138, 139]
Effects of the islet environment on CTL maturation	Clarification of the mechanisms required for autoreactive CD8+ T cells to differentiate and mature to fully functional CTLs	Mouse	[148, 149]
CD8+ T cells within islets are specific for known beta-cell antigens	Direct analysis of antigen specificity at the site of autoimmune destruction	Human	[82]
Perforin is the dominant cytotoxic mechanism used by human autoreactive CTLs to kill beta-cells	Confirms data obtained in NOD mice is applicable to human CTLs. Provides a possible target to prevent beta-cell destruction	Human	[54, 107, 109]
Identification of T cell epitopes formed by post-translational modifications	Possible explanation for escape of autoreactive T cells from central and peripheral tolerance	Human/mouse	[57, 60]

Legend: CTL – cytotoxic T lymphocyte, IL-2 – interleukin 2, NOD – nonobese diabetic.

highlight the importance of the islet in CTL differentiation.

6.5 Post beta-cell destruction events

Our understanding of the events within the islets that occur following beta-cell destruction is limited due to difficulties in studying these events in both the NOD mouse and humans. Maintaining NOD mice post diabetes onset is problematic as they are moribund, and administration of insulin is complicated. In humans, obtaining biopsy specimens from patients is almost impossible. A recent study of diabetic patients whose duration of disease was 50 years or more (Joslin medalists) identified the presence of residual insulin-positive beta-cells [81]. This surprising finding suggests not all beta-cells are destroyed and/or beta-cells can regenerate over time.

Analysis of insulitis reveals that immune infiltration can persist, but it can also resolve leaving glucogon- and somatostatin-postive cells alone in pseudo-islets. Death of the T cells within the islet, once beta-cell destruction had occurred, was the mechanism proposed to cause the resolution of insulitis. However, recent studies have suggested that emigration from islets to the periphery, and formation of the peripheral effector memory T cells pool, is a more likely outcome. T cells specific for GAD65 and preproinsulin displaying an effector memory phenotype have been detected in the peripheral blood of patients [55, 150]. We have recently studied the formation of these cells in the NOD mouse. We found that they are formed within the islet, and then leave the environment to seed the periphery (Chee, Kay & Krishnamurthy, unpublished 2012). This is consistent with the visualization of T cells leaving the islets and with an older observation of autoreactive T cells appear-

ing in the blood in waves [91, 151]. Clearly, we still have much to learn about post beta-cell destruction events in both mice and humans, and this will be the focus of much research in the future.

7. Summary and conclusions

Much has been learned about the sequence of events that leads to type 1 diabetes, so that our current understanding is substantially different to that of a couple of decades ago. Yet, the more that we learn the more we realize we still do not know. In an overview, we understand that a process of aberrant immune recognition of self beta-cells by T cells occurs accompanied by autoantibody production. Immune infiltration of the pancreatic islets proceeds with loss of beta-cells and failure of insulin production. If regeneration of beta-cells occurs, then this is insufficient for metabolic function. Yet, within this schematic outline, we need to know more about the particular details for effective therapy to be developed. It is not possible at this time to clearly describe a current treatment with a very high likelihood of therapeutic benefit. In particular, we need to understand how much the great level of detail known about the NOD mouse is applicable to humans, and to what extent the sequence of pathogenic events varies between individual humans with type 1 diabetes.

Several recent major advances have been made (**Table 2**). They include identifying the islet as an important location of disease pathogenesis, not merely a passive target of events within the lymph nodes (**Figure 3**, **Table 2**). Technological innovation, for example those involving the use of class I and class II MHC tetramers, and advances in microscopy and other forms of imaging are allowing us to revisit and re-conceptualize events. Isolation of effector cell populations from T1D patients, and analysis of pancreatic samples from individuals with type 1 diabetes, mostly after death, are also providing important new information.

The discovery that perforin is the dominant effector mechanism utilized by human autoreactive CTL to kill beta-cells, and the identification of $CD8^+$ T cells specific for known beta-cell antigens present in islets of people with diabetes, are reassuring discoveries, as they mirror mechanisms described in the NOD mouse (**Table 2**). This overlap shows that analysis of the NOD mouse in conjunction with human samples will continue to be fruitful. We are yet to determine exactly how $CD4^+$ T cells act to kill beta-cells. The NOD model will be essential to understand this and many other questions. The very early pathogenic events remain difficult to study, especially in humans.

Of course, the target of the field is to understand and to treat human type 1 diabetes. Translation of findings from animal models, and a greater focus on studying human diabetes with the latest technology, should yield new insights and new opportunities for an effective future therapy to prevent or cure type 1 diabetes.

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