Chapter 4
The Role of T-Cells in Beta Cell Damage in NOD Mice and Humans
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Type 1A diabetes (T1D) is a chronic disorder that results from the immune-mediated destruction of the insulin-producing \( \beta \)-cells of the pancreatic islets\(^1\). In its initial phase, which is clinically silent, T lymphocytes and other inflammatory cells invade the islets and eventually destroy them. The disease then becomes clinically evident with the pathological consequences (hyperglycemia, ketosis and long-term complications) resulting from the inability to maintain glucose and lipid homeostasis.

**Type 1 diabetes is a T-cell mediated disease**

The first indication that T1D is an autoimmune disease came from the results of a comprehensive histological examination of pancreata from diabetic patients who had died shortly after diagnosis. This showed that most of the subjects had significant lymphocytic infiltration of their islets concordant with loss of \( \beta \)-cell mass\(^2\). Subsequently, islet-cell antibodies (ICAs) and anti-pancreatic cell-mediated immunity were detected in recently diagnosed T1D patients\(^3-5\), suggesting that the lymphocytes accumulated as a result of attraction by antigens derived from pancreatic \( \beta \)-cells\(^6\). Consistent with this hypothesis, insulitis was only seen in islets containing \( \beta \)-cells. With the advent of monoclonal antibodies capable of identifying distinct lymphocyte sub-populations more detailed immunohistochemical examinations of islet infiltrates became possible. One of the earliest of such studies showed a predominance of CD8\(^+\) T-cells in the islets of a deceased 12-year old girl with newly diagnosed T1D, which, together with the observed up-regulation of MHC class I molecules by islet cells, implicated cytotoxic T-cells (CTLs) in \( \beta \)-cell destruction\(^7\). Additional studies of pancreas from patients with type 1 diabetes have confirmed preponderance of CD8 T-cells and the presence of B-lymphocytes related to extent of \( \beta \)-cell destruction\(^8\). The JDRF nPOD (Network for Pancreatic Organ Donors with Diabetes) program now allows viewing of pancreatic histology of cadaveric donors directly online (http://www.jdrfnpod.org/). Similarly, the strong association of T1D with particular MHC II haplotypes (see chapter 7) suggested a critical role for CD4\(^+\) T-cells in the disease process (reviewed by\(^9\)). Additional circumstantial evidence supporting a crucial role for T-cells and MHC-restricted self antigen recognition in diabetogenesis came from the reversal and recurrences of diabetes following twin to twin pancreatic isografts\(^10,11\), and the inadvertent transfer of disease between HLA-identical siblings by bone marrow transplantation\(^12\).

The mere presence of T-cells in infiltrates, though highly suggestive, does not by itself
establish a direct role for these cells in the development of T1D. However, the histological findings were subsequently followed by reports of T-cell reactivity to β-cell proteins, providing further support for the hypothesis. Thus, Roep and colleagues established CD4+ T-cell lines and clones restricted to HLA-DR from the peripheral blood of new-onset diabetics after stimulation in vitro with rat insulinoma cells. Of the eight clones examined, five appeared to recognize insulinoma membrane components, one of which was a 38kD protein later termed IMOGEN38. Surprisingly, after expression cloning IMOGEN38 was shown to be a broadly distributed mitochondrial protein (a probable subunit of the mitochondrial ribosome), and studies with the human ortholog suggested that the response was likely xenogeneic (JC Hutton personal communication). Nonetheless, the identification of several islet cell molecular targets allowed subsequent studies to be conducted using defined autoantigens, rather than crude fractions, and have suggested that the peripheral blood of diabetic subjects and their at-risk relatives contain elevated numbers of T-cells able to recognize epitopes from β-cell proteins. Such studies suggest that T-cells provide a legitimate therapeutic target for intervention, and results from clinical trials of new-onset diabetic patients with humanized anti-CD3 monoclonal antibodies delayed the deterioration of circulating C-peptide levels normally seen in the year following diagnosis in 9 of 12 subjects. Attempts to build upon these partial successes and improve the therapeutic regimen are currently in progress.

**Animal models of T1D**

### A. Spontaneous models

Since the target organs of T1D (islets and draining pancreatic lymph nodes) are inaccessible in human subjects, the study of T1D has been greatly facilitated by the availability of animal models such as the Biobreeding-diabetes prone (BB-DP) rat and the nonobese diabetic (NOD) mouse, which spontaneously develop diseases that mimic many features of human T1D. In particular, the NOD mouse has been the subject of extensive studies for over 20 years, and has provided key experimental evidence supporting several strategies to treat the human disease, some of which are showing promise in initial clinical trials. Numerous abnormalities have been reported in the immune systems of NOD mice, including defects in antigen presenting cells (APCs) and hyporesponsiveness of T lymphocytes, which together may compromise both central and peripheral tolerance to pancreatic β-cells. Similar abnormalities have been reported in human subjects (for example, and it has been proposed that T-cell hyporesponsiveness may be a general feature conferring susceptibility to inflammatory autoimmune disorders. However, it must be noted that the immune systems of humans and mice show several key differences (reviewed by, which must be kept in mind when extrapolating between these species. Indeed, there are notable differences between T1D in NOD mice and humans, not the least being that NOD mice only develop disease if kept in specific pathogen free conditions. Moreover, in these animals disease is associated with pronounced cellular infiltrates that surround the individual islets (Figure 1) that begin to form at least 7 weeks prior the onset of overt...
disease \textsuperscript{43, 44}. In contrast, pancreata obtained at post mortem from diabetic subjects who died shortly after clinical manifestation of T1D typically show a much less florid infiltration (e.g. \textsuperscript{7, 45}). In that NOD mice are inbred they can only be considered to be the equivalent of a single genotype "case study" of T1D and a genotype that is homozygous at all loci.

Although the precise sequence of events that lead to T1D in NOD mice remain uncertain, the central role of T-cells in diabetogenesis in these animals is incontrovertible. Thus, treatment of newly diabetic animals with anti-CD3 antibodies, which suppress immune responses by transient T-cell depletion and modulation of T-cell Receptor (TCR) signaling, induces long-term remission \textsuperscript{46, 47}. Moreover, diabetes can be transferred to immuno-compromised hosts by mixed T-cell populations, or in some instances, individual T-cell clones \textsuperscript{48-51}. For example, Haskins and colleagues isolated 8 CD4\(^+\) T-cell clones recognizing islet antigens (including chromagranin and islet amyloid polypeptide) \textsuperscript{52, 53} presented by the NOD MHC class II molecule, I-A\(^{\text{g7}}\), at least 5 of which are capable of inducing disease after adoptive transfer \textsuperscript{54, 55}. Transgenic mice expressing the T-cell receptor (TCR) of one of the diabetogenic clones, BDC2.5 (target peptide derived from chromogranin), have been generated and bred onto the NOD background \textsuperscript{56}. Interestingly, evaluation of the lymphoid compartment in NOD/BDC2.5 animals showed no sign of negative selection in the thymus, with normal peripheral T-cell reactivity. This was also true of transgenic C57BL/6-H-2\(^{\text{g7}}\) (B6\(^{\text{g7}}\)/BDC2.5) animals. However, autoimmune pathogenesis was highly dependent upon the genetic background of the animal \textsuperscript{57}. In both NOD and B6\(^{\text{g7}}\) transgenics there was no manifestation of disease in the first 2 weeks of life, with pancreatic islets completely free of infiltration. Insulitis appeared abruptly at 18 days, and subsequently progressed to eventually involve essentially all islets. However, whilst the B6\(^{\text{g7}}\)/BDC2.5 animals suffered a highly aggressive insulitis and the majority rapidly progressed to overt disease, paradoxically, NOD/BDC2.5 animals were significantly protected from
spontaneous disease, and exhibited a more benign "respectful" insulitis. Nevertheless, young NOD/BDC2.5 are significantly more sensitive to cyclophosphamide induced diabetes than their non-transgenic relatives \(^58\), and NOD/scid/BDC2.5 mice rapidly progress to overt T1D and typically die of diabetic complications on or before 33 days of age \(^59\). The precise mechanisms by which the un-manipulated NOD/BDC2.5 animals restrain their insulitis are currently uncertain, but may involve populations of T-cells expressing alternative \(\alpha\) T cell receptor chains due to incomplete allelic exclusion at this locus \(^56,60\).

In addition to TCR transgenic animals, targeted gene disruption and retrogenic \(^61\) technologies have also been used to study other features of T1D in NOD mice. For example, mice lacking expression of \(\beta_2\)-microglobulin (\(\beta_2\)M), that consequently do not express functional MHC class I molecules, do not develop insulitis \(^62-65\), implicating CD8\(^+\) T-cells in the initiation of disease. Interestingly, transgenic restoration of MHC class I expression in NOD-\(\beta_2\)M\(^-\) mice using different \(\beta_2\)M alleles provided contrasting results. Thus, animals reconstituted with the endogenous \(\beta_2\)M\(^a\) allele developed T1D, whilst those given the \(\beta_2\)M\(^b\) allele (which only differs at a single amino acid residue) did not \(^66\). At present the precise mechanism of protection is uncertain, although it may reflect subtle differences in the peptide-binding properties of the resultant MHC class I molecules \(^67\).

Targeted gene disruption has also provided evidence for a key role for proinsulin in diabetogenesis in NOD mice. In contrast to humans, mice have 2 non-allelic insulin genes (insulin 1 on chromosome 19, and insulin 2 on chromosome 7), and recently NOD mice with disruptions in either one have been created \(^68,69\). Surprisingly, the mice show contrasting phenotypes; insulin 1\(^-\) mice are markedly protected from diabetes (but do develop anti-insulin autoantibodies), while insulin 2\(^-\) animals show accelerated disease. In contrast, animals whose sole preproinsulin is a mutant form of insulin 2 in which the immunodominant B:9-23 epitope is disrupted are completely protected from diabetes \(^70\), although they do develop sialitis confirming the organ-specificity of the effect. Such results are at variance with those from related studies in which NOD mice lacking the autoantigens GAD65, IA-2, phogrin (IA-2\(\beta\)) and IGRP \(^71\) were produced and where disease occurrence was not significantly altered \(^72-74\). This suggests a central role for (pro) insulin in the disease process, although the precise mechanisms of acceleration, or protection from disease, remain to be determined. The potential central role of insulin and insulin peptide B:9-23 is supported by studies where both insulin genes are eliminated and an insulin mutated at position B16 (Y to A). These mice do not develop diabetes \(^70,75\). Krishnamurthy and coworkers have found that eliminating response to proinsulin eliminates the prominent CD8 T-cell response to IGRP \(^76,77\). Vignali and coworkers have introduced a new methodology to study T-cell receptor targeting of islet \(\beta\) cells, namely the creation of retrogenic mice \(^78,79\). The creation of retrogenic mice utilizes retroviruses to introduce T-cell receptors into bone marrow cells that are then transplanted into immunodeficient mice. This technology greatly accelerates studies of such T-cell receptors compared to creating transgenic mice. Within 8 weeks, the pathogenicity of T-cell receptors can be assessed. Studying a series of 17 retrogenes, those targeting GAD failed to induce diabetes. Insulin peptide
B:9-23 reactive TCR caused delayed diabetes and IA-2/phogrin TCR caused insulitis and TCRs targeting chromogranin (e.g. BDC 2.5) caused diabetes as did TCR’s targeting unknown molecules. (Diabetes induction TCR: BDC-10.1 > BDC-2.5 > NY 4.1 > BDC 6.9). Of note, a series of TCRs targeting GAD65 caused fatal encephalitis independent of the induction of anti-GAD autoantibodies. No insulitis was observed. Presumably this was related to the lack of GAD in mouse islets.

B. Experimental autoimmune diabetes (EAD)

As only a limited number of animal models exhibiting spontaneous disease are currently available, systems to induce EAD in non-autoimmune prone mice have also been developed. Typically these are based upon the transgenic β-cell expression of heterologous proteins under control of the rat insulin promotor (RIP). Such models have provided key insights into the establishment and breaking of tolerance to β-cell antigens. Initial studies showed that some lines of RIP-Tag C57BL/6 mice, which express the SV40 large T antigen (Tag) in their β-cells, were intolerant of the transgene, developing spontaneous autoimmunity. Tolerance, or autoimmunity, correlated with the presence or absence of embryonic expression of the transgene, with animals that did not express Tag until adulthood developing disease. Moreover, tolerant animals also expressed the transgene in the thymus, suggesting that, at least for some proteins, central tolerance can be established to apparently organ-specific antigens (reviewed in). However, central tolerance could be broken if the precursor frequency of peripheral autoreactive T-cells was too high. Thus, tolerant RIP-Tag mice crossed with transgenic mice showing low expression (~10% of peripheral T-cells) of the TCR of a Tag specific CTL were protected from spontaneous disease. In contrast, offspring of parents showing high expression (~90%) of the transgenic TCR were intolerant. The importance of central tolerance was also demonstrated in a model where CBA (H-2k) mice expressing the Kb MHC class I molecule under control of the RIP were crossed with transgenic mice expressing the TCR of an H-2k-restricted CTL clone recognizing Kb. In this case the progeny rejected skin grafts from H-2b animals, but were protected from T1D. However, crossing the double transgenic mice with RIP-IL-2 animals caused rapid onset disease. Neonatal replacement of the thymuses of the double transgenic animals with tissue from non-transgenic mice permitted high avidity Kb-specific T-cells to reach the periphery, which allowed disease to be triggered in animals primed either with allogeneic skin grafts or the injection of irradiated splenocytes from H-2b donors. Some animals required multiple primings, suggesting that the duration of the stimulus, as well as the avidity of the peripheral autoreactive T-cells, profoundly influences the course of disease.

The lack of spontaneous disease in RIP-Kb x Kb-TCR mice after thymus replacement, despite the presence of peripheral high affinity Kb-specific T-cells, indicates that protective mechanisms exist in the periphery which can act to negate a lack of central
tolerance. Two potential mechanisms have been proposed, namely immune ignorance and active tolerance, and evidence that both are involved in protection from T1D has come from RIP-based transgenic models. For example, two strains of mice secreting either high (RIP-Ova\textsuperscript{hi}) or low (RIP-Ova\textsuperscript{lo}) levels of ovalbumin from their β-cells were exposed to 5, 6-carboxy-succinimidyl-fluorescein-ester (CSFE) labeled OT-1 T-cells specific for ovalbumin. Analysis of the pancreatic draining lymph nodes 3 days after transfer revealed that the OT-1 cells had proliferated in the RIP-Ova\textsuperscript{hi} but not the RIP-Ova\textsuperscript{lo} mice, indicating that the level of antigen acquired by antigen presenting cells (APCs) in the RIP-Ova\textsuperscript{lo} animals was insufficient to trigger naive T-cells, and consequently that the immune system was ignorant of the presence of the neo-antigen. Both strains developed T1D if exposed to pre-activated OT-1 CTLs, confirming that the RIP-Ova\textsuperscript{lo} animals did indeed exhibit β-cell expression of the transgene. However, despite proliferation in the draining lymph nodes, adoptive transfer of even 10\textsuperscript{7} naive OT-1 cells to RIP-Ova\textsuperscript{hi} mice did not induce disease, suggesting that effector CTL generation was inefficient under these conditions. Nonetheless, tolerance to β-cell expression of ovalbumin could be broken if the efficiency of presentation was enhanced. Thus, studies with RIP-mOva mice, which express a membrane-anchored chimeric fusion protein derived from ovalbumin and the transferrin receptor, demonstrated that cell-associated ovalbumin was cross-presented to CD8\textsuperscript{+} T-cells ~50,000-fold more efficiently than the secreted form and adoptive transfer of 5 x 10\textsuperscript{6} naive OT-1 T-cells into non-irradiated RIP-mOva mice rapidly induced disease.

To analyze the fate of OT-1 cells following transfer into RIP-mOva mice without inducing disease these animals were crossed with bm1 mice (that have a mutation in the K\textsuperscript{b} molecule which prevents presentation to OT-1 T-cells). The resulting RIP-mOva.bm1 mice were lethally irradiated and reconstituted with bone marrow from wild-type C57BL/6 animals so that OT-1 cells could interact with hematopoietic, but not peripheral, cells. Analysis of draining lymph nodes 3 days after adoptive transfer of CSFE-labeled OT-1 T-cells confirmed that they had proliferated, but after 8 weeks transgenic T-cells comprised only ~1% of CD8\textsuperscript{+} T-cells recovered from the spleens and lymph nodes of B6→RIP-mOva.bm1 mice as compared to ~7.4% in B6→bm1 animals, suggesting that activation had led to peripheral deletion. Further studies indicated that deletion was dependent upon CD95 (Fas) expression by the OT-1 cells, and that surface expression of CD95 was up-regulated following cross-presentation in the draining lymph nodes. However, the presence of antigen-specific CD4\textsuperscript{+} T-cells impaired deletion of activated CTLs, and instead led to enhanced activation and expansion. Thus, neither 2 x 10\textsuperscript{8} OT-II cells (a CD4\textsuperscript{+} ovalbumin-specific clone), nor 2.5 x 10\textsuperscript{5} OT-I cells, by themselves were diabetogenic in RIP-mOva mice. In contrast, 68% of animals receiving both populations of cells became diabetic within 10 days of transfer, which corresponded with an ~10-fold increase in the numbers of OT-1 cells in their spleens and lymph nodes after 4 days as compared to animals that did not receive OT-II cells. Interestingly, experiments using mixed chimeras of bone marrow cells from I-A\textsuperscript{−/−}, I-A\textsuperscript{−/−}CD40\textsuperscript{−/−}, and bm1 mice demonstrated that it was not necessary for the same APC to activate both the OT-I and OT-II cells, but that T1D only resulted when the APC interacting with the naive OT-I cells had been activated via CD40 by activated OT-II cells expressing CD154 (CD40 ligand). Antigen-antibody immune complexes can also
"license" dendritic cells to efficiently prime CTLs \(^{95}\), suggesting a potential mechanism by which autoantibodies might contribute to the expansion of the autoimmune response.

The RIP-Ova based studies indicate that efficient mechanisms exist to maintain tolerance to β-cell proteins, but that these can be overcome if the peripheral frequency of high avidity T-cells exceeds a critical threshold, by co-operation between CD4\(^+\) and CD8\(^+\) T-cells, or by local expression of pro-inflammatory cytokines. Similar conclusions were reached in another EAD model based upon expression of the glycoprotein (GP) or nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV). Thus, RIP-LCMV mice do not develop spontaneous diabetes, but disease is triggered by LCMV infection \(^{96, 97}\). In contrast, spontaneous disease occurs in double transgenic mice co-expressing RIP-NP and RIP-IFN\(\alpha\) \(^{98}\), or RIP-GP and RIP-B7.1 \(^{99}\). As with the RIP-Tag mice, thymic expression of the LCMV proteins was not observed in all founders, which significantly influenced the rate and characteristics of disease in their progeny. Animals lacking thymic expression of the transgene rapidly developed T1D in a CD4\(^+\)-independent manner following infection, whilst thymic expression significantly slowed disease induction, which in this case was dependent upon the presence of both CD8\(^+\) and CD4\(^+\) T-cells \(^{100}\). RIP-NP x RIP-B7.1 double transgenic mice that had thymic expression of the viral protein and also express CD80 (B7.1) on their pancreatic β-cells, do not develop spontaneous disease, although T1D is accelerated in these animals following viral infection relative to their RIP-NP single transgenic littermates. Thymic expression of the transgene caused negative selection of high avidity CD8\(^+\) T-cells specific for the immunodominant epitope of NP \(^{101}\), although interestingly, the viral infection was cleared with essentially identical kinetics in the presence or absence of negative selection. Comparison of the T-cell responses to LCMV in RIP-NP and non-transgenic animals revealed a significant skewing of the anti-viral response towards a normally sub-dominant epitope in NP, suggesting that high avidity T-cells to sub-dominant or cryptic epitopes within autoantigens can escape negative selection. This may have important implications during the expansion of the auto-response in T1D.

In both CD4\(^+\)-dependent and independent RIP-LCMV models T1D induction depends upon infection with a virus capable of causing pancreatic inflammation \(^{102}\), highlighting a key role of viral tropism, and consequent local exposure to pro-inflammatory chemokines and cytokines, in overcoming peripheral tolerance to islet antigen in this model. Consistent with this conclusion immunization of RIP-LCMV mice with an LCMV-GP peptide caused expansion of autoreactive CTLs but did not induce T1D unless TLR ligands such as the viral mimic polyinosinic-polycytidylic acid (poly I/C) were co-administered \(^{103}\). Similarly, co-expression of IL-2 \(^{104}\) or the chemokine CXCL10 (IP10) \(^{105}\) in islets does not induce spontaneous disease, but enhances T1D development after initiation of the anti-self response. However, genetic factors influence the degree of "pre-lesioning" necessary to allow activated autoreactive CTLs to infiltrate islets and destroy β-cells \(^{106}\), suggesting that in at least some individuals susceptibility to T1D in humans likely includes hyper-responsiveness to pro-inflammatory stimuli.

Experiments using a panel of closely related viruses demonstrated a critical threshold for the frequency of high avidity cross-reactive CTLs that must be exceeded to induce
disease, and showed that variations between the primary sequences of the β-cell protein and its viral mimic either in the flanking residues of the immunodominant epitopes that affect their processing in APCs, or in the epitopes themselves, significantly influences whether or not this occurs. Interestingly, experiments conducted with combinations of LCMV and the distantly related Pichinde virus (PV), which mimics a sub-dominant epitope in LCMV, suggested that a mimicking low-affinity epitope could re-activate resting antigen-experienced CTLs under conditions where it did not activate naive CD8+ T-cells 107. Thus, exposure to PV alone did not induce disease in thymically-expressing RIP-NP mice, nor did it accelerate T1D in those animals subsequently given LCMV. In contrast, exposure to PV following prior infection with LCMV significantly enhanced the rate of onset of diabetes. This suggests that the order of exposure to two potentially auto-reactive viruses can significantly influence the nature of the immune response to self, and that disease might be triggered by reactivation of memory cells generated during a previous sub-threshold response to a potentially diabetogenic virus by a subsequent infection with a cross-reactive but otherwise non-diabetogenic one 108.

In contrast to the rapid-onset RIP-LCMV model, exposure of adult Ins-HA mice (whose β-cells, but not thymuses, express the influenza virus strain A/PR/8/34 hemagglutinin (HA) at high levels) to cognate virus did not induce disease 109. Although this may in part be due to the fact that this strain of influenza is unable to replicate in mice, there is also evidence that it represents the establishment of dominant peripheral tolerance to the neo-antigen following efficient cross-presentation of the antigen prior to immunization 110, 111, possibly via the expansion of antigen-specific regulatory or suppressor cells. Immunization of neonatal Ins-HA animals with influenza virus rapidly caused T1D 112, consistent with the observation that constitutive, but not inflammatory, cross-presentation is disabled in the pancreas of young mice 113. Similarly, rapid onset spontaneous disease was observed in H-2^d Ins-HA mice crossed with mice transgenic for the TCR of the K^d-restricted HA-specific CTL clone 4 114. Spontaneous T1D also occurred in approximately 30-40% of Balb/c (H-2^d) Ins-HA mice crossed with animals transgenic for the TCR of the I-E^d-restricted HA-specific clone 6.5 115, but not in the same mice crossed with HNT-TCR transgenic animals that recognize HA126-138 in the context of I-A^d 116. However, HNT-TCR T-cells are diabetogenic in B10.D2 Ins-HA mice, highlighting the importance of non-MHC genes in determining whether a pathogenic or protective response occurs. Peripheral tolerance is not always effective, as evidenced by the spontaneously diabetic RIP-Tag mice, and by the fact that, in contrast to the InsHA mice (none of whom develop spontaneous disease), a significant proportion (13-27%) of mice expressing the HA of influenza A/Japan/305/57 under control of the RIP developed spontaneous T1D 117.

EAD has also been induced using natural diabetic autoantigens. Thus, immunization with a peptide from the insulin B chain caused the development of insulin autoantibodies, but not insulitis in Balb/c (H-2^b), but not C57/BL6 (H-2^b), mice 118. Co-administration of peptide and poly I/C to Balb/c mice led to a predominantly CD4+ insulitis, but not hyperglycemia 119. However, immunization of RIP-B7.1 transgenic mice on an H-2^db or H-2^d/d background with either poly I/C or the insulin peptide alone induced autoimmune diabetes, which was accelerated by co-administration of both
agents. The mechanism(s) underlying disease induction are incompletely understood, and may vary slightly for the peptide and mimic. Given that under these circumstances the insulitis was predominantly comprised of CD8\(^+\) T-cells, and expression of B7.1 on pancreatic β-cells abrogates the requirement for CD4\(^+\) T-cells in diabetogenesis\(^{120}\), it appears likely that the peptide acts to promote expansion/activation of insulin-specific CD8\(^+\) T-cells similar to the H2-K\(^d\)-restricted clone G9C8\(^{121}\). In contrast, since poly I/C will induce the production of type 1 interferons by APCs, disease induction by the viral mimic is probably mechanistically similar to that induced by transgenic islet expression of IFN\(\alpha\)\(^{122}\), with bystander activation of APCs\(^{123}\) causing expansion of pre-existing auto-reactive CTLs\(^{124,125}\). T1D induction by poly I/C probably also involves direct stimulation of islet cells via TLR3 and consequent secretion of chemokines such as IP-10\(^{126}\), and will likely produce a more diverse initial CD8\(^+\) response than produced with the peptide, possibly including β-cell proteins such as IGRP as well as insulin. In each case β-cell expression of the co-stimulatory molecule will promote amplification of the response within the target organelle, and consequent destructive insulitis. Bystander activation has also been proposed as the underlying mechanism in disease induction by coxsackie virus B4 in NOD/BDC2.5 transgenic mice\(^{127}\), and is likely to be important in the accelerated T1D observed in NOD/RIP-B7.1 animals\(^{128}\), and the diabetes observed in RIP-B7.1 x RIP-TNF\(\alpha\) double transgenic mice\(^{129}\). EAD can also be induced in H-2\(^b/b\) RIP-B7.1 mice by vaccination with (pro) insulin cDNA, but not GAD65 cDNA\(^{130}\), further highlighting the importance of (pro) insulin in the initiation of disease.

**Checkpoints in the development of autoimmune diabetes**

Studies with NOD/BDC2.5 TCR transgenic mice and various congenic NOD strains have allowed investigators to define three major checkpoints in the pathogenesis of T1D\(^{131}\). Checkpoint 0 controls the development of an autoimmune prone T-cell repertoire, checkpoint 1 the onset of insulitis, and checkpoint 2 the switch from controlled insulitis to overt diabetes (Figure 2). Each appears to be regulated by multiple susceptibility loci\(^{132}\) and ill-defined epigenetic or environmental factors, such that passage through checkpoints 0 and 1 does not inevitably lead to clinical disease, and even an extensive and active insulitis can persist for long periods of time without significant β-cell depletion. For example, congenic NOD.B10 Idd9 mice develop an aggressive insulitis and high insulin autoantibody expression\(^{133}\) but rarely progress to diabetes. Although the natural history of human T1D is temporally much more variable than the mouse models, with symptomatic disease occurring at any time from the first year of life to well into middle-age, and does not show the same pronounced sex bias observed in most NOD colonies, it is believed that it also involves passage through similar checkpoints that mark key changes in the autoimmune process.

The molecular and cellular mechanisms that underlie these checkpoints are not just of academic interest since therapeutic interventions to prevent, or arrest human T1D prior to complete β-cell destruction, may stem from influencing such control mechanisms.
Moreover, the multiplicity of factors that regulate disease progression suggest that distinct therapeutic strategies are likely to be required to treat individuals at the various stages of T1D. Consequently they are currently the focus of considerable research. It has long been appreciated that the specific MHC class II alleles expressed by an individual are a crucial factor in controlling whether or not checkpoint 0 is passed (see Chapter 7), and that expression of alternative alleles in autoimmune prone mice can have a dominant protective effect. At present the precise mechanisms by which particular alleles increase or decrease susceptibility to specific autoimmune diseases (e.g. DRB1*1501-DQB1*0602 haplotypes confers susceptibility to multiple sclerosis but protection from T1D) remains unresolved, although this presumably reflects the nature of the trimolecular complex interaction between the MHC class II bound autoantigenic peptide and TCR of autoreactive T-cell. In this regard it is interesting to note that the recent crystal structures of 3 autoimmune TCRs bound to their cognate ligands showed unconventional interactions resulting in sub-optimal contact with the MHC bound self-peptide. This would presumably lead to a low functional avidity and increased likelihood of a failure of negative selection due to a consequent resistance to activation-induced apoptosis, especially if molecular defects in pro-apoptotic pathways were also present. However, it is now recognized that autoreactive T-cells can also be protective and that the same peptide/MHC class II glycoprotein combination can stimulate both diabetogenic and non-pathogenic T-cells, consistent with the notion that the intrinsic balance between protective and pathogenic T-cell subsets in an individual is only partly dependent upon their MHC haplotype.

**Figure 2. Probable checkpoints in the development of T1D**

Checkpoint 0

Genetic pre-disposition (hyporesponsive T-cells, hyper- or hypo-responsive APCs, β-cells hypersensitive to stress induced apoptosis)

Reduced negative selection of high avidity β-cell specific T-cells and/or insufficient generation of β-cell specific regulatory T-cells

Inherent Th1 v Th2 bias

![Diagram](image)

No Insulitis

Risk-conferring peripheral T-cell repertoire

Checkpoint 1

Proinflammatory environment in pancreas and draining pancreatic lymph nodes (Infection or β-cell necrosis)

Activation of β-cell specific CTLs and Th1 biased CD4+ T-cells
Controlled Insulitis

Sufficient intra-islet amounts of protective cytokines and/or regulatory T-cells to prevent uncontrolled β-cell apoptosis

β-cells retain functional and recovery/replicative capacity

Checkpoint 2

Avidity maturation of autoresponse and expansion via epitope spreading
Recruitment of additional effector cells
Breakdown of protective mechanisms (decrease in intra-islet anti-inflammatory chemokines/cytokines and regulatory cells)
Cytokine, oxidative, and/or hyperglycemic stress on β-cells and loss of functional and recovery/replicative capacity
Uncontrolled β-cell apoptosis (cell contact and/or stress dependent)

Destructive Insulitis and functional β-cell insufficiency

Overt T1D

Figure 4.2. Probable checkpoints in the development of T1D

The virtually co-incident temporal onset of peri-insulitis in NOD/BDC 2.5 TCR transgenic animals (where checkpoint 0 is by-passed), and their non-transgenic relatives, indicates that the observed 2-3 week delay does not represent the time required to establish an expanded auto-reactive peripheral repertoire per se, but rather reflects an inherent difference in the homing potential of the T-cells and/or “attractiveness” of the islets. This is likely to be due to multiple factors including increased expression of lymphocyte addressins (especially MadCAM-1 and PNAd) by the pancreatic blood vessel endothelium, chemokine-dependent activation of their ligands (e.g. α4β7 integrin) on the surface of activated lymphocytes (reviewed by), and presentation of islet antigens by endothelial cells. Together such changes will promote extravascularization of activated auto-reactive T-cells within the islet, although it remains uncertain precisely how such events are triggered. In this regard it should be noted that three immunologically important events coincide with the passage of NOD mice through checkpoint 1; weaning, a wave of islet cell apoptosis due to tissue remodeling, and the establishment of a preferential trafficking route from the gut to the pancreatic draining lymph nodes. The former is associated with significant changes in the intestinal flora and exposure to a large array of novel food antigens. This transiently induces a broad T-cell stimulation, likely involving both mesenteric and pancreatic lymph nodes, which in susceptible animals could lead to bystander damage of islets, or direct targeting of
pancreatic β-cells due to molecular mimicry \(^{131, 152}\).

Weaning is also associated with increased β-cell activity due to the switch to a more carbohydrate-rich diet. This probably renders the islets more susceptible to stress-related damage \(^{153}\), which in pre-disposed individuals could trigger the expression of the pro-apoptopic death receptor 4 ligand by stressed β-cells and their subsequent killing and phagocytosis by macrophages \(^{154}\), thereby providing a source of antigens for presentation to auto-reactive T-cells \(^{155}\). Similarly, the wave of remodeling-induced islet cell apoptosis could also provide a source of β-cell antigens for presentation by APCs \(^{156, 157}\). All of these events occur in both resistant, and diabetes prone animals, suggesting that they are not inherently diabetogenic. Indeed in normal circumstances they will probably result in peripheral tolerance \(^{157, 158}\), but in the context of intestinal stress or injury (for example due to a viral infection \(^{159}\) and/or intrinsic abnormalities in APCs and lymphocytes such as are observed in NOD mice \(^{160, 161}\), may lead to an immunogenic rather than tolerogenic response. The mouse models also indicate that the timing of exposure to a potential autoantigen during the development of the immune system during neonatal life can be critical in determining whether a protective or immunogenic response results, and interestingly, recent research has suggested that there may be a time window in infancy outside which initial exposure to cereals increases T1D risk in susceptible children \(^{162}\).

**The autoantigens**

At present the specificity, and antigenic diversity, of the diabetogenic T-cells whose activation at checkpoint 1 initiates the process that ultimately leads to disease remains uncertain. Given the tissue specificity of T1D it is tempting to speculate that the primary targets are β-cell specific and proinsulin is a prime target with ability of islets containing the B:9-23 epitope when transplanted to activate disease. Multiple other targets are present including peri-islet Schwann cells \(^{163}\). Similarly, although activation of T-cells in draining pancreatic lymph nodes is clearly critical for pathogenesis, and surgical removal of these structures from young (3 week old) NOD mice prevented T1D in at least 80% of the treated animals \(^{164}\), a recent study unexpectedly showed that lymphocytes isolated from mesenteric lymph nodes of 3 week-old NOD mice were almost 4-fold more diabetogenic than those from the pancreatic nodes of the same animals, following adoptive transfer into NOD.scid recipients \(^{165}\). Consistent with previous studies, Jaakko and colleagues also showed that, in contrast to the 3-week old animals, at 6 weeks of age lymphocytes from the pancreatic nodes were the most effective in transferring disease, whilst at later times the spleen contained the highest proportion of diabetogenic cells. Such results might indicate the presence of a significant proportion of regulatory cells in the pancreatic nodes of the 3 week-old animals \(^{166}\), but instead could also suggest that the initial priming is to a foreign antigen that occurs in the gut-associated tissue, and that this response is subsequently amplified and expanded to include islet cell antigens in the pancreatic nodes. This latter
hypothesis is consistent with the previously observed effects of blocking the mucosal addressin MAdCAM-1, and is not inconsistent with the fact that at early time points activated BDC2.5 TCR transgenic T-cells are only found in islets and their draining lymph nodes, if it is assumed that in a non-transgenic NOD mouse the equivalent cells are activated as part of the presumptive amplification stage, given that their cognate antigen is believed to be islet-specific. Thus it is possible that in NOD mice passage through checkpoint 1 occurs due to the temporal co-incidence of a wave of islet cell apoptosis that leads to enhanced presentation of β-cell antigens in the pancreatic lymph nodes, and exposure to novel antigens in the gut that activate APCs and/or Th1 polarized T-cells that subsequently migrate to the pancreas and perturb the response in the draining nodes such that an immunogenic response to islet cell antigens results. In the transgenic animals the wave of apoptosis alone would be sufficient to activate the diabetogenic cells due to their high frequency and the lack of appropriate regulatory cells, and so molecular mimicry would not be required. A similar involvement of post-natal islet cell apoptosis, congenital β-cell abnormalities, and dietary or enteroviral triggers, in human T1D have also been proposed, although at present their relative roles (if any), and generality as casual factors, remain controversial.

Nonetheless, whether the auto-immune response is initiated directly, or as a secondary consequence of a primary reaction to a foreign antigen, it is clear that islet cell antigens are critical to the disease process, and that a detailed knowledge of their molecular characteristics is essential both to the rational design of immunotherapies, and in the monitoring of at-risk individuals. Moreover, although disease can be driven by T-cells having single specificities in immuno-compromised animals, and there may be a restricted number of islet-cell reactive clones in early pancreatic infiltrates in NOD mice, natural disease progression appears to involve multiple autoantigens and both epitope spreading within, and avidity maturation of, the T-cell response. As T-cells with identical specificities can adopt either pathogenic or tolerogenic properties, and a monoclonal regulatory T-cell population can suppress a polyclonal diabetogenic response, it is likely that any diabetic autoantigen has the potential to be used therapeutically. Thus, considerable effort has been devoted to identifying the molecules themselves, and the epitopes within them, that interact with particular MHC glycoproteins. To date the majority of diabetic autoantigens that have been defined at the molecular level were discovered either by a candidate gene approach, or by the identification of antibody targets in human diabetic sera. Insulin, the 65 kilodalton form of glutamic acid decarboxylase (GAD65), the insulin granule membrane proteins ICA512 (IA-2), phogrin (IA-2β) and ZnT8 are major targets of circulating islet autoantibodies in man. Of these only insulin appears to be β-cell specific and ZnT8 islet specific, whereas the others are broadly distributed among neuroendocrine tissues such as the brain, pituitary and adrenal medulla. The humoral response per se probably contributes little to the pathogenesis of the disease of man, and although B-lymphocytes may be important for antigen presentation in T1D, they are not essential. Nonetheless, circulating autoantibodies provide useful pre-clinical markers for diabetic autoimmunity, and may also play a role in modulating T-cell responses through their effects as APCs. ZnT8, the islet zinc transporter, is the most
recently identified target of humoral immunity of man and studies of T-cells reacting with ZnT8 are underway\textsuperscript{185,186}.

Given that the production of high affinity antibodies is a T-dependent process, it is reasonable to suggest that molecules recognized by autoantibodies might also be the targets of autoreactive T-cells, although this need not be the case for particulate antigens due to the process of linked activation. Nonetheless, this hypothesis appears correct, at least for (pro)insulin, IA-2/phogrin and GAD65\textsuperscript{187} and specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell clones have been isolated from spleen, lymph nodes or islet infiltrates of pre- or newly diabetic NOD mice\textsuperscript{54,188-190} which do not correspond to any of the known serological markers. Similarly, HLA-DR restricted CD4\textsuperscript{+} T-cell lines have been isolated from new onset T1D patients that recognize currently unidentified β-cell antigens\textsuperscript{191}. This may indicate that their target antigen is a presently unidentified component of ICAs, or is intrinsically unable to elicit a humoral response, or in the case of CD8\textsuperscript{+} cells, the targeting of epitopes not present in a stable protein\textsuperscript{192,193}. Defining the cognate antigens for these “orphan” clones remains an important goal, and with the recent advances in proteomic techniques, now appears a realistic aim. Thus, using a combination of a sensitive bioassay and high-end liquid chromatography coupled to tandem mass spectrometry to analyze peptides eluted from H-2K\textsuperscript{d} molecules from NIT1 insulinoma cells the target of the well studied NY8.3 CD8\textsuperscript{+} clone was shown to be a peptide derived from the β-cell protein islet-specific glucose 6-phosphatase related protein (IGRP)\textsuperscript{194}. Similarly, screening of a combinatorial peptide library in a positional scanning format led to the identification of a peptide derived from dystrophia myotonica kinase (DMK) as the antigen for the AI4 T-cell clone\textsuperscript{195}. Target of BDC 2.5 (chromagranin) and BDC 5.2.9 (islet amyloid polypeptide) have similarly been identified.

Although the number of diabetic autoantigens identified in man and the NOD mouse is expected to continue to increase, currently the majority of attention is focused on the 6 proteins described in more detail below.

**Preproinsulin**

Insulin and its precursors are obvious target autoantigens in T1D since the hormone is the major constituent of β-cells (10-15% of total protein), and indeed preproinsulin reactive T-cells have been isolated from both diabetic subjects and NOD mice (reviewed by. Initial studies of islet reactive T-cells from infiltrates of pre-diabetic NOD mice revealed that insulin reactive clones predominated\textsuperscript{196}. Subsequently, the majority were shown to recognize amino acids 9-23 of the B chain (B:9-23), and when cloned, were capable of causing accelerated disease in young NOD mice or adoptive transfer of T1D to NOD.scid animals\textsuperscript{197}. More recently CD4\textsuperscript{+} T-cell hybridomas recognizing other epitopes within preproinsulin have been generated from pre-diabetic NOD mice\textsuperscript{198}, and spontaneous responses to proinsulin B24-C33 detected in splenocytes from both pre-diabetic and newly-diabetic animals\textsuperscript{199}. Similarly, multiple preproinsulin epitopes have
also been reported in immunized HLA DRB1*0401 transgenic mice \(^{200}\) and amongst CD4\(^+\) T-cells from the peripheral blood of diabetic patients and their antibody positive 1\(^{st}\) degree relatives \(^{178, 201-204}\). The relevance of these observations to diabetogenesis was supported by the recent demonstration that insulin-specific HLA-DR4-restricted CD4\(^+\) T-cells could be expanded from the pancreatic lymph nodes, but not spleen, of 2 long-term T1D subjects, but not from those of a DR4\(^+\) non-diabetic control \(^{205}\). Interestingly, TCR recognition appears to depend upon post-translational formation of a vicinal disulfide bond between the adjacent cysteine residues in the epitope (A:1-15) \(^{206}\). The finding that the highly diabetogenic CD8\(^+\) T-cell clone G9C8 recognizes amino acids 15-23 of the insulin B chain, demonstrated that insulin is also a target for CD8\(^+\) T-cells in NOD mice. Recently this has also been shown to be the case in human T1D \(^{207}\).

Anti-islet autoimmunity can be induced in some non-autoimmune prone mouse strains by immunization with insulin peptides. In particular, administration of B:9-23 to mice homozygous or heterozygous for H-2\(^d\) (e.g. Balb/c or Balb/c x C57BL/6 F1 mice) rapidly induces insulin autoantibodies. Induction is dependent upon the MHC of the animal, and they are not produced following immunization of H-2\(^b\) mice. Surprisingly, the induced insulin autoantibodies cannot be absorbed with the immunizing B:9-23 peptide, although anti-peptide antibodies are also produced. It appears likely that the B:9-23 peptide, when given subcutaneously in saline or incomplete Freund’s adjuvant (IFA), overcomes T-cell tolerance inducing autoantibodies from what appear to be an existing population of primed lymphocytes. Consistent with this hypothesis, proinsulin specific T-cells can be detected in draining pancreatic lymph nodes, but not the spleen, of unimmunized Balb/c mice. Although the immunized mice develop insulin autoantibodies they do not develop insulitis, which only occurs with additional inflammatory signals.

The ability to induce autoimmunity in non-autoimmune prone animals by peptide immunization raises important considerations when considering this as a potential prophylactic therapy. Nonetheless, studies using the NOD mouse have shown that insulin or B:9-23 given orally \(^{208, 209}\), subcutaneously, or intranasally \(^{210}\), prior to the onset of insulitis, delays the onset and decreases the incidence of diabetes. However, peptide immunization studies occasionally have unexpected results, as exemplified by the observation that treatment with the B24-C36 peptide failed due to the presence of an internal "cryptic" CD8\(^+\) T-cell epitope \(^{211}\). Similarly, whilst intraperitoneal immunization of 18-day old NOD mice with proinsulin, and subcutaneous immunization of 5-week old animals with insulin partially protected the recipients, subcutaneous administration of proinsulin to 5-week old mice accelerated disease. The mechanism(s) by which tolerance in successfully treated animals is restored are incompletely understood, but likely involve the generation of insulin-specific regulatory CD4\(^+\) \(^{212}\) and CD8\(^+\) T-cells. One mechanism may relate to the induction of cytotoxic T cells that kill antigen presenting cells expressing their cognate peptide \(^{213}\). It remains to be seen whether the success in modulating diabetes in the NOD mouse using insulin/proinsulin or derivatives can be translated to humans. The initial clinical trials of insulin therapy in pre-diabetic or newly diagnosed diabetic subjects were disappointing, with no general benefit being observed \(^{214, 215}\), although some response was detected in a subset of individuals having the highest titers of insulin autoantibodies \(^{216, 217}\) and progression to
diabetes of at risk relatives treated with oral insulin with high levels of insulin autoantibodies may have been slowed leading to current Trialnet oral insulin trial. However, there is an issue with the late timing of the interventions in these cases, and trials at earlier stages in genetically at risk 1st degree relatives of diabetic individuals who have a single islet autoantibody have been proposed, although even here the NOD mouse data might suggest that the autoimmune disease may be too advanced for this form of therapy to be effective by itself. Use of an altered peptide ligand (APL) of the B:9-23 peptide that has alanine substitutions at positions 16 and 19 has been studied with apparently no hypersensitivity reactions but no benefit.

The structure of the insulin promotor renders expression primarily β-cell specific (reviewed by 220). However, preproinsulin transcripts and immunoreactivity have also been reported in a minority of thymic and lymph node cells in both humans and rodents likely, at least in part, due to the action of the autoimmune regulator (AIRE) protein. Such expression probably plays a pivotal role in establishing central tolerance, and may be reduced in diabetes-prone individuals (reviewed by 224). Thus, expression of particular variable nucleotide tandem repeat (VNTR) alleles in the human promotor, which appear to control thymic expression, are associated with disease susceptibility (IDDM2). Similarly, NOD mice lacking preproinsulin 2, the predominant (if not exclusive) preproinsulin gene expressed in the thymus of mice, show accelerated diabetes, while thymic over-expression of preproinsulin is protective. At present the precise identities of extra-islet cells expressing proinsulin are a matter of debate, although recent attention has mainly focused upon medullary epithelia within Hassall's corpuscles. Nevertheless, the available data strongly suggest that the loss of tolerance to (pro)insulin is a critical risk-factor for T1D, both in NOD mice, and humans, and the establishment of recessive tolerance to this key molecule can protect from disease. Insulin gene expression has also been reported in multiple organs of diabetic rodents, although the significance of this finding remains unclear.

Immune responses to insulin, and even to the “same” B:9-23 epitope, can be both pathogenic and protective. A transgenic mouse with the 2H6 anti-B:9-23 T-cell receptor prevents diabetes dependent upon TGFβ production. A TCR transgenic (BDC 12-4.1) and retrogenic (BDC 12-4.4) cause disease (Note 12-4.4 sequence variants differ in pathogenicity). Of note, most of the T-cell receptors recognizing insulin B:9-23 utilize a conserved VαT-cell receptor segment (TRAVID-4*04). These alpha chain T-cell receptors can pair with multiple very different TCR beta chains and recognize the B:9-23 peptide. In addition, the N and V region alpha chain sequences targeting the B:9-23 peptide are variable. This has led to the hypothesis that propensity for insulin autocomponents may be genonomically determined by common Vα chain sequences targeting an invariant insulin peptide sequence (B:12-22). Genetically determined abnormalities of tolerance maintenance and environmental activation might act upon an immune system poised to target insulin.

There has been progress in defining human T-cells targeting proinsulin and insulin. Kent and coworkers detected DR4 restricted clonally expanded pancreatic lymph node T-
cells recognizing insulin A1-15 peptide. Peakman defined CD8 T-cells recognizing epitopes from the human preproinsulin leader sequence presented by HCA2 (PPI17-24 WGPDPAAA and PPI15-24 ALWGPDPAAA)\(^{141}\). By Y-interferon ELISPOT, approximately 50% of new onset patients had circulating CD8+ T-cells reacting with these peptides. Reactivity was greater than to other HLA-A2 studied proinsulin peptides (B10-18; B18-27; C22-30; C27-35; C31-A5; and A1-10). Three clones from a single patient all had same TCR β chain TRBV12-4 (two α chains TRAV 12-3 and TRAV 13-2). The clones recognizing PPI15-24 showed enhanced cytotoxicity when islets were cultured in high glucose. Durinovic-Bello identified a CD4 DRB1*0401 T-cell epitope (proinsulin 73-90) with core sequence LALEGSLQK (human cone 52c1)\(^{238}\). T-cells reacting with this epitope upon in vitro expansion can be isolated utilizing a DRB1*0401 tetramer. In evaluation of a series of proinsulin peptides (e.g. C19-A3 GSLQPLALEGSLQKRGIV) presented by DRB1*0401, Arif and coworkers find responses of both patients and controls but patient responses are proinflammatory (IFN-γ) while on ELISPOT controls produce IL-10 to some peptides.

Work by Stadinski et al. brought a new focus to the details of antigen presentation in type 1 diabetes\(^{239}\). This study used a method of register trapping to determine which “face” of B:9-23 epitope is recognized by T cells, and which binds to I-A\(^{97}\). The register in which a peptide binds to MHC is defined as the specific linear orientation of the peptide within the MHC groove. As one moves a peptide one amino acid up or down the groove of the MHC, the peptide has to rotate to bind to the pockets of the MHC, changing the side chains facing outward and interacting with T cell receptors. By fixing the way the insulin B:9-23 peptide binds to MHC II, Stadinski and coworkers showed that B:9-23 stimulated a panel of insulin-specific CD4+ T cell hybridomas only when bound in “register 3” (SHLVERLYLVCGEEG; the downwardly pointing residues are mutated anchor residues for p1 and p9 pockets of I-A\(^{97}\)). This directly contradicted findings by Levisetti et al. who proposed register 1 and 2 as being recognized by the two classes of T cell hybridomas\(^{240}\). Levisetti measured T cell responses to N- and C-terminally deleted versions of B:9-23, and interpreted diminished IL-2 production as elimination of B:9-23 binding in a specific register. However, changes in T cell responses could have also been explained as elimination of an important TCR epitope(s) instead.

Another paper from Kappler’s group showed that some truncations of the B:9-23 peptide might in fact increase T cell reactivity. For example, C-terminal truncation to B21 eliminates the arginine residue that conflicts with the I-A\(^{97}\) p9 binding pocket when bound in register 3, and truncation to B20 eliminates glutamic acid that interferes with recognition by some T cell clones\(^{241}\). Building on this knowledge, the group described generation of a series of B:9-23/ I-A\(^{97}\) tetramers that stained majority of insulin-specific CD4+ T cell hybridomas. These tetramers displayed slightly modified B:9-23 epitopes bound in register 3 to I-A\(^{97}\). In addition to the hybridomas, the B:9-23 tetramers could also stain about 5% of primary CD4+ T cells present in the pancreas of NOD mice. The authors also proposed a model explaining how the “register 3” peptide might be recognized as “de novo” antigen expressed specifically in the pancreas. According to this model, pancreatic β cells generate truncated version(s) of the peptide that are not
present in the thymus during maturation of thymocytes. Absent during negative selection, this peptide(s) cannot be used to delete autoreactive thymocytes recognizing insulin. Thus, this unique version of the peptide can be considered as “neo-antigen”. An ability of pancreatic β cells to process insulin has been previously described by Mohan et al.\textsuperscript{242}. This paper showed that small percentage of β cells had secretory granules containing B:9-23 peptide, and that this peptide could be “picked up” by resident dendritic cells. Therefore, it is conceivable that this unique “register 3” insulin peptide can be generated only in the pancreas.

Additional support for “register 3” recognition came recently from the lab of Harald von Boehmer\textsuperscript{243}. In this study, Daniel et al. used an insulin B:9-23 register 3 binding mimetope to prevent diabetes in NOD mice. They slowly infused low levels of the mimetope, and showed that this regimen generated insulin-specific regulatory T cells. They also showed that such treatment induced dominant tolerance since polyclonal responses to insulin were blocked.

The insulin peptide B:9-23 can be targeted by T cells not only in mice with I-A\textsuperscript{g7} but also by I-A\textsuperscript{b} in a model created by the laboratory of Massimo Trucco. In this model (ID-TEC: insulin deleted thymic epithelial cell) an insulin 2 knockout mouse is combined with Aire driven Cre insulin 1\textsuperscript{−/−} deleting insulin 1 in thymic epithelial cells. These C57 mice with I-A\textsuperscript{b} rapidly develop diabetes and have large numbers of T cells producing interferon gamma in response to stimulation by insulin or the B:9-23 peptide\textsuperscript{244}.

In summary, identification of register 3 presentation of B:9-23 peptide opened new avenues of research. First, the panel of I-A\textsuperscript{g7}/B9-23 tetramers will enable in vivo studying of insulin-specific, I-A\textsuperscript{g7} restricted CD+4 T cells in NOD mice. Second, the analogous approach might lead to generation of similar reagents for human system. Finally, Daniel's study shows that administration of insulin peptide mimetope designed to bind in relevant register can induce dominant tolerance that prevents autoimmune activation of T cells in the pancreas\textsuperscript{245}.

**Glutamic Acid Decarboxylase**

Like insulin, glutamic acid decarboxylase (GAD) is a major autoantibody target in human diabetic patients (reviewed by\textsuperscript{246}). It catalyzes the formation of the inhibitory neurotransmitter α-amino-butyric acid (GABA), and is expressed on synaptic vesicles in many regions of the central nervous system and multiple neuroendocrine tissues. GAD exists in two non-allelic forms, GAD65 and GAD67, which are 65% identical, differing primarily in their initial 250 residues, but the precise distributions of the isoforms differ between man and rodents. Thus, GAD65 is the major form expressed in the human pancreas, where it is predominantly localized to β-cells, whereas GAD67 is the major isoform expressed by mouse islets, albeit at barely detectable levels\textsuperscript{247,248}. Such differences provide important considerations for extrapolating data regarding GAD.
obtained from mouse models to the human disease.

Unlike humans, spontaneous antibody responses to GAD are rarely, if ever, detected in NOD mice, although T-cell responses are amongst the first autoreactivities detected in neonatal females, being apparent as early as 3-4 weeks of age. Initial responses to GAD in NOD mice are directed to the fragment GAD65 (509-543), which contains at least two overlapping I-A$^b$ restricted determinants (residues 524-538 and 530-543), each eliciting T-cells of distinct phenotypes and showing particular TCR V$\beta$ gene usage. Thus, spontaneous GAD-reactive CD4$^+$ T-cells from young NOD mice primarily recognize the immunodominant epitope 530-543 (p530). However, T-cells to the overlapping determinant 524-538 (p524) dominate the response after immunization with GAD65 (524-543). p530-responsive T-cells typically use the V$\beta$4 gene, whereas the V$\beta$12 gene is preferentially used to encode the TCR of p524-responsive T-cells. The p524 responsive T-cells appear to be regulatory and upon adoptive transfer to young NOD mice can inhibit diabetes development. During the course of disease determinant spreading occurs generating T-cells recognizing additional epitopes towards the N-terminus of GAD65. Similarly, immunization with recombinant GAD65 or GAD67 reveals additional epitopes, some of which are shared between the two isoforms. GAD-reactive CD8$^+$ T-cells have also been identified.

GAD65 is also a major target of T-cell autoimmunity in human T1D, with peripheral blood mononuclear cells (PBMCs) from approximately 50% of new onset patients responding to this autoantigen. HLA-DR4:GAD65$_{555-567}$ reactive CD4$^+$ T-cells can also be detected in the peripheral blood of healthy subjects, although in contrast to diabetic individuals these cells are primarily restricted to the naive pool. Intriguingly, in a cohort of at-risk 1$^{st}$ degree relatives there was an inverse relationship between cellular and humoral autoreactivity to GAD65. The significance of this observation remains uncertain, but it may reflect the fact that binding of some GAD65-specific antibodies to their target can suppress presentation of certain immunodominant T-cell epitopes, although the converse has also been reported. A number of CD4$^+$ epitopes within human GAD65 have been mapped using immunized transgenic mice or T-cells isolated from newly diabetic subjects and at-risk relatives. These studies have provided evidence for molecular mimicry to both exogenous and endogenous antigens. Thus, cross-reactivity of T-cells from recent onset HLADR3/4 positive patients between rubella virus envelope protein 1(157-176), RVE2 (87-107) and GAD65 (274-286) was observed. Similarly, a GAD65 (339-342) restricted T-cell clone from a pre-diabetic patient also responded to residues 674-687 of human cytomegalovirus major DNA-binding protein of 134kDa, an observation that appears especially relevant given the reported association of cytomegalovirus infection and T1D. Other apparent cross-reactivities that have been reported include GAD65 (247-279) with Coxsackie virus B3 P2-C protein (32-47), and GAD (506-518) and proinsulin (24-36), although these conclusions are disputed by a more recent study using cloned T-cells. As in the NOD mouse, GAD-reactive CD8$^+$ T-cells have also been identified in human T1D patients.

Like other autoantigens, the precise role of GAD65 in the onset and progression of
spontaneous T1D in humans and NOD mice remains uncertain. Thus, in one of three transgenic lines of NOD mice expressing an antisense construct shown to reduce the expression of both GAD65 and GAD67, not only was diabetes and insulitis abolished, but also T-cells derived from these mice were unable to transfer disease; a result which suggests that GAD65 plays a critical role in T1D development. There has been no follow up of this study and it is possible that disease prevention related to introduction of chromosomal region from knockout rather than effect of GAD knockout. Conflicting results have come from other studies. For example, NOD mice homozygous for a disrupted GAD65 gene still develop diabetes, and NOD mice rendered tolerant to GAD by transgenic expression of a modified form of the protein under control of the invariant chain promotor, still develop T1D with normal incidence. The reasons for the contradictory outcomes are unclear, and have been the subject of debate (e.g.), but it seems likely that the protection afforded by the antisense construct is not solely due to the immunological effects of suppression of GAD, and that GAD65 is unlikely to be essential for diabetes development in NOD mice, although given the differences in expression, this conclusion cannot be extended to humans.

Nonetheless, GAD65 is a legitimate target for immunotherapy, and administration of GAD65 intrathyrmically, orally, nasally or in the form of epitope peptides to 3-week old NOD mice protects against diabetes. Intramuscular injection of a recombinant adeno associated virus expressing GAD500-585 to 7-week old female NOD mice also induced tolerance. Similarly, phase 2 clinical trials of new onset type 1 patients classified as latent autoimmune diabetes of the adult with alum-formulated recombinant GAD65 demonstrated preservation C-peptide levels in the treated subjects at 24 weeks post-therapy. GAD67, though not a major diabetes autoantigen may also protect against disease, and GAD65 expressed at high levels in pancreatic β-cells under control of the rat insulin promoter was protective in one of two transgenic lines, likely due to an increase in the proportion of IL-10 producing T-cells. In contrast, widespread expression of GAD65 under control of the MHC class I promoter did not induce tolerance in NOD mice, and in one of three transgenic lines disease was exacerbated with a greater degree of insulitis in males and an increased overall incidence of T1D. Transgenic and retrogenic expression of T-cell receptors targeting GAD do not induce diabetes but can protect NOD mice and a human TCR transgenic developed insulitis. A recent study of anti-GAD retrogenics by Vignali and coworkers suggests that there is insufficient GAD in islets of mice to allow T cell targeting, with the anti-GAD retrogenic mice dying of encephalitis with no evidence of insulitis despite induction of GAD autoantibodies. A large trial of GAD in alum immunization failed to alter loss of immunization c-peptide of new onset patients.

IA-2 and phogrin

In addition to proinsulin and GAD65, another major autoantibody targets in human
diabetic subjects defined at the molecular level are the protein tyrosine phosphatase (PTP) superfamily members IA-2 (also known as ICA512 and PTPRN) and phogrin (also known as IA-2ß, IAR, ICAAR, PTPRP, and PTPRN2) (reviewed in 291, 292). Approximately 60-70% of new-onset T1D patients react to both molecules, as does 40-50% of 1st degree relatives 293-299, but less than 2% of controls. In multiple antibody positive individuals IA-2 is typically one of the last to become evident, and consequently is highly predictive for progression to disease 300, 301. Initially described as autoantibody-reactive 40 and 37 kDa tryptic fragments derived from non-GAD 64 kDa precursors immunoprecipitated from radio labeled β-cells 293, 298, 302, they were subsequently shown to be proteins that had previously been cloned independently by several groups using subtractive and expression screening strategies 294, 299, 303-310.

Like the GAD isoforms, IA-2 and phogrin show over-lapping but distinct expression patterns in the brain and multiple neuroendocrine cell types, but are expressed at much higher levels than GAD in islets, and are localized to dense core secretory granules rather than synaptic vesicles 305, 311. In addition, they each exist in a number of splice forms, which are differentially expressed in various tissues 312, JC Hutton, personal communication). Both IA-2 (979 amino acids, encoded on human chromosome 2q35-36.1) and phogrin (986 amino acids, encoded on human chromosome 7q36) are type 1 integral membrane glycoproteins, and have cytoplasmic domains (378 and 375 amino acids respectively) that are ~74% identical 291. In contrast there is only 27% indentify within their extracellular domains. All humoral responses so far identified map to the cytoplasmic domains 296, 313, 314, and 90% of IA-2 reactive sera also detect phogrin, whilst 99% of phogrin-reactive sera detect IA-2, yet other PTP family members are not targeted 315. Initially synthesized as ~130kDa glycosylated precursors, IA-2 and phogrin are cleaved at a consensus dibasic cleavage site in the late Golgi/granule to produce the mature 62-64kDa proteins 305, 316. Following glucose-stimulated insulin secretion IA-2, but not phogrin, is cleaved by µ-calpain within its cytoplasmic domain 317.

At present the precise functions of IA-2 and phogrin within pancreatic β-cells remain uncertain. The orientation of the mature proteins in the granule membrane (NH2 terminal ~200aa in the lumen/ ~400aa COOH terminal PTP domain in the cytoplasm) suggests possible roles as receptors that signal to the cytoplasm from either the granule lumen or the extracellular environment, however the nature of the presumptive ligands, and the signal transduction mechanisms, are unclear 311, 318. Changes in sequence surrounding the consensus active site in their PTP domains are predicted to preclude catalytic activity, and the proteins have little, if any, phosphatase activity towards common substrates 305. Nevertheless, they adopt a similar fold to active PTPs (JC Hutton, personal communication), and activity can be restored in either IA-2 isoform by mutagenesis of 2-3 amino acids 319. Given that several other receptor-type PTPs contain tandem PTP domains, one of which is active and the other inactive and likely regulatory 292 it has been proposed that IA-2 and phogrin may interact with an as yet unidentified active PTP, but at present this remains conjecture. Regulated interaction of IA-2 with the PDZ domains of β2-syntrophin, and nNOS has been demonstrated in rat insulinoma cells 317, 320, but to date no interaction partners have been defined for phogrin. Nonetheless disruption of either the IA-2 or phogrin genes in mice produces a
similar phenotype; mild glucose intolerance due to impaired insulin secretion \(^{72,321}\).

Unlike humans, NOD mice produce circulating antibodies to IA-2/phogrin at relatively low frequency, and they do not show disease specificity \(^{322}\). In contrast, T-cell responses to both autoantigens have been detected in both murine and human T1D \(^{301,323-326}\). To date attention has mainly focused upon CD4\(^+\) responses to the conserved cytoplasmic domains, although a potential epitope in the luminal domain of rat IA-2 was recently identified by pooled sequencing of peptides eluted from rat insulinoma cells transfected with cDNAs encoding HLA-DR4 \(^{327}\). Similar to studies of proinsulin by ELISPOT assays, patients with type 1 diabetes produce DRB1*0401 restricted proinflammatory responses to IA-2 (e.g. 652-80; 709-35; 752-75; 793-817; 853-72; 955-76) while controls respond but primarily with IL10 production. Thus, immunization of NOD mice with recombinant rat phogrin cytoplasmic domain in CFA induced CD4\(^+\) Th1 biased responses focused upon two major epitopes, amino acids 640-659, and amino acids 755-777 \(^{328}\). Subsequently, spontaneous proliferative responses to residues 755-777, but not 640-659, were detected in splenocytes and lymph nodes from young prediabetic NOD mice \(^{329}\). No responses were observed in splenocytes from Balb/c mice of the same age. Although none of the cloned phogrin-reactive T-cells accelerated disease when transferred to prediabetic NOD mice, some were able to destroy transplanted rat islets and cause diabetes in a NOD diabetes recurrence model \(^{325}\).

The phogrin 640-659 and 755-777 peptides also induced responses in immunized HLA-DQ8 transgenic mice, and the same specificities were detected in 17% and 35% respectively of ICA positive prediabetic individuals, but less than 5% of age- and HLA-matched controls \(^{330}\). Surprisingly, proliferative responses to the phogrin antigenic peptides were seen in PBMCs from some HLA-DQ8 negative individuals, suggesting that they might be “super-epitopes” recognized by multiple HLA-DR and –DQ molecules.

The cross-reactivity between IA-2 and phogrin autoantibodies and extensive sequence conservation, suggests that T-cells recognizing both PTP isoforms might also be generated. Initial studies did not support this hypothesis; none of the T-cell clones isolated from NOD mice immunized with the cytoplasmic domain of phogrin recognized epitopes derived from IA-2, and similarly, T-cells from IA-2 C-terminus immunized mice did not react to processed phogrin (K Kelemen personal communication). Consistent with this observation, the 2 immunodominant epitopes presented by I-A\(^\gamma\) from processed IA-2 (residues 685–701 and 725-741; JC Hutton and DAA Vignali personal communication) are not conserved between the 2 isoforms. Peptides containing these epitopes, which are 100% conserved between mouse and human IA-2, also induced responses when used to immunize NOD mice \(^{331}\), although the highest stimulation index (SI) was obtained following administration of the peptide linking these two epitopes which is conserved between IA-2 and phogrin, but was not detected in the initial studies using naturally processed antigen. However, more recently a T-cell hybridoma generated from NOD mice immunized with phogrin was shown to recognize a cross-reactive epitope of IA-2 (HW Davidson, unpublished observation) raising the possibility that this might also occur in human diabetic subjects. Like phogrin, spontaneous
responses to IA-2 have been detected in NOD mice, although in contrast to phogrin, IA-2 specific T-cells responded by cytokine secretion but not proliferation\textsuperscript{329, 332}. The epitope(s) spontaneously recognized have yet to be defined.

Spontaneous responses to IA-2 C-terminal epitopes have also been observed amongst\textsuperscript{326, 333, 334, 334}. For example, 6 sets of peptides nested around defined core sequences were eluted from HLA-DR4 molecules expressed by B lymphoblastoid cells that had processed recombinant protein. At least one of the peptides corresponding to these epitopes produced proliferative responses (SI>2) in PBMCs from 9/13 HLA-DR4 positive diabetic patients, but 0/8 -DR4 positive non-diabetic controls\textsuperscript{334}. Similarly, ELISPOT analyses conducted using a peptide library from the IA-2 cytoplasmic domain revealed a 17-fold increased frequency of IFN\_\gamma positive cells in PBMCs from DR4/DQ8 positive T1D subjects as compared to matched controls\textsuperscript{326}.

CD8\(^{+}\) T-cell responses to IA-2 have also been detected, although these appeared not to be disease specific\textsuperscript{335}. In this regard it should be noted that the phosphatase domains of phogrin and IA-2 show high evolutionary conservation with homologs in zebrafish (~80% identity), \textit{Drosophila} (FLYDA; ~60% identity) and \textit{C. elegans} (B0244.2 gene product IDA-1; ~50% identity)\textsuperscript{336, 337}, which are all greater than the conservation of IA-2 with other mammalian PTP domains (<40% identity)\textsuperscript{292}. This suggests that exposure to common multi-cellular pathogens could trigger cross-reactive T-cells through molecular mimicry, although this hypothesis remains to be established. Molecular mimicry with multiple viruses including rotavirus, Dengue, cytomegalovirus, measles, hepatitis C, rhinoviruses, hantaviruses, and flaviviruses, as well as the bacterium \textit{Haemophilus influenza}, and several milk, wheat, and bean proteins has also been proposed\textsuperscript{333}, although likewise direct evidence to support this suggestion is currently lacking.

Like GAD65, neither IA-2 nor phogrin is essential for T1D induction in NOD mice\textsuperscript{72, 73}, whilst attempts to target the molecules therapeutically have given mixed results. Thus, immunization of 3-4 week old NOD mice with phogrin peptide 755-777 in saline reduced the incidence of T1D (JC Hutton, personal communication), but administration of recombinant IA-2 in IFA exacerbated disease\textsuperscript{332}. Thymic expression of IA-2 has been documented\textsuperscript{312, 338}, and in common with other tissue-specific antigens, involves cells of the medullary epithelium. This suggests that most IA-2 restricted T-cells are likely to be subject to negative selection. However, the major splice-variant of IA-2 expressed in the thymus bears a deletion in exon 13\textsuperscript{312}, which could allow autoreactive cells directed to this region to escape negative selection. Interestingly, the exon 13 deleted variant, which also is expressed in pancreatic islets\textsuperscript{312}, lacks the transmembrane domain, and as a consequence the carboxy-terminal domain is predicted to enter the lumen of the endoplasmic reticulum. This might allow a potential site of N-linked oligosaccharide addition to be used (aa766-768), significantly affecting the processing of the molecule by APCs.

\textit{IGRP}
First identified as a β-cell specific protein in a subtractive hybridization screen that was performed to identify β-cell specific proteins that could be autoantigens, or regulators of insulin stimulus-secretion coupling, IGRP was recently identified as the target of the diabetogenic NY8.3 clone. It is expressed in a highly pancreatic β-cell specific manner yet appears to be controlled by a different set of transcription factors than those regulating other β-cell genes such as insulin and amylin. Due to its sequence homology with glucose 6-phosphatase (G6Pase) it has been investigated as a potential component of a glucose substrate cycle potentially controlling energy metabolism in the β-cell, however to date no catalytic activity has been demonstrated. The human IGRP gene is located on chromosome 2q 24-31, a short distance from the glucagon and GAD67 genes, in a region where IDDM7 and NIDDM and the Bardet Biedl genes map. Like the liver G6Pase, IGRP bears a carboxy-terminal KKXX sequence typical of endoplasmic reticulum resident transmembrane proteins, and most of its sequence appears buried in the membrane with only short cytoplasmic and luminal peptide loops. IGRP mRNA is abundant in mouse and human islets, but it is a pseudo-gene in the rat. Humoral autoreactivity to IGRP has been searched for in NOD mice and human diabetic subjects without success (JC Hutton, personal communication).

The NY8.3 CD8$^+$ T-cell clone was isolated over 10 years ago by Yoon and colleagues from islet infiltrates of acutely diabetic NOD/Lt mice. It is restricted to H2-K$^d$, and in the presence, but not absence, of CD4$^+$ T-cells can transfer disease to irradiated NOD mice. Circumstantial evidence suggests that the target of NY8.3 may be associated with the initial insulitic response. Thus, CD8$^+$ T-cells with closely related, or even identical, α chains to NY8.3 (V$\alpha$17 and J$\alpha$42 elements joined by the N-region sequence MR(D/E)), but distinct β chains, are prevalent in NOD insulitic lesions as early as 4-5 weeks of age, whilst NOD mice transgenic for the NY8.3 β chain (Vß8.1) show accelerated T1D, and the majority of infiltrating CTLs from diabetic animals express an endogenous α chain identical to that of NY8.3. Disease onset is further accelerated in NOD mice transgenic for both NY8.3 TCR chains in both male and female animals, although overall incidence is unchanged. NY8.3 TCR transgenic animals on a NOD/RAG2$^{-/-}$ background develop diabetes less frequently and at a slower rate than RAG2$^+$ 8.3-NOD animals (albeit to a much greater extent than non-transgenic NOD/RAG2$^{-/-}$ animals which are protected from T1D), but rapidly progress to disease following adoptive transfer of non-transgenic CD4$^+$ T-cells, further confirming the importance of CD4$^+$ cells in the recruitment of naïve CD8$^+$ cells to islets. Despite the high frequency of CD8 T-cells targeting IGRP, induction of tolerance to the molecule does not influence progression to diabetes and even the TCR anti-IGRP transgenic requires immune response to insulin for spontaneous diabetes. Nevertheless, peptides of IGRP can be used to prevent the development of diabetes of NOD mice.

A mimotope was defined for the NY8.3 TCR (NRP), and subsequent cytotoxicity assays indicated that almost 50% of the CD8$^+$ T-cells from islet infiltrates of non-transgenic animals recognized this peptide. Similarly, H2-K$^d$ tetramers bearing an NRP variant showing higher agonistic properties (NRP-V7) stain up to 37% of the CD8$^+$ positive
T-cells present in NOD islet infiltrates from 11-14 week old animals, and a significant percentage in the circulation. More recently, H2-K^d-NRP-V7 coated superparamagnetic nanobeads have been used to selectively label NY8.3 TCR transgenic T-cells, and their recruitment to islets studied in real-time by magnetic resonance imaging. Tetramers bearing residues 206-214 of mouse IGRP, the epitope identified by sequencing of peptides from NIT1 insulinoma cells, similarly stain a significant proportion of islet infiltrating cells (JC Hutton, personal communication). Interestingly, studies using NRP and its derivatives demonstrated a time-dependent increase in the functional avidity of the peripheral clonotypic response that may contribute to the progression from benign to destructive insulitis. Subsequent analyses indicated that the observed avidity maturation correlated with differential usage of 3 distinct V_α17 elements that created TCRs with differing binding affinities for the natural ligand. Thus, at 9 weeks a significant population of low affinity V_α17.6 expressing cells were present, whilst by 20 weeks these had been replaced by high affinity V_α17.5 expressing clonotypes, presumably reflecting differential peripheral expansion of the high avidity clones at the expense of their low avidity counter-parts. Interestingly, some altered peptide ligands targeting IGRP_{206-214} specific T-cells could prevent T1D, but only under conditions that spared low avidity clonotypes; higher doses or affinities that caused virtually complete ablation of this response led to increased recruitment of alternative, sub-dominant specificities to islets and disease progression. There is evidence for targeting of IGRP by patients.

ZnT8

Autoantibodies to the islet zinc transporter ZnT8 are prominent in man but apparently absent in NOD mice. The ZnT8 molecule associated with beta cell secretory granules transports zinc from the cytoplasm to the granule where it complexes with insulin forming insulin crystals. Multiple epitopes of ZnT8 are recognized by autoantibodies including a polymorphic amino acid associated with Type 2 diabetes risk. Patients who target only the arginine variant of ZnT8 are homozygous for the arginine polymorphism, while those targeting the tryptophan variant are homozygous for the tryptophan variant of molecule. This confirms the autoimmune nature of targeting self in Type 1 diabetes. ZnT8 autoantibodies disappear most rapidly after onset of diabetes. In man, multiple different peptides are recognized by T lymphocytes.

Chromogranin A
Haskins and coworkers have recently identified chromogranin A \(^{53}\) as the target of the classic T cell BDC 2.5 clone that has been utilized to study the pathophysiology of islet autoimmune for more than 20 years \(^{56, 169, 361}\). This neuroendocrine molecule is widely expressed and is processed to yield multiple peptides. To date, no autoantibodies reacting with chromogranin A are described. Given its widespread neuroendocrine distribution, it is remarkable that tissue destruction by the BDC 2.5 clone appears to be islet specific. As reported, the recognition by the BDC 2.5 T cell receptor is very specific reacting with a specific neuroendocrine produced peptide of chromogranin (WE-14) such that the peptide does not fill all of the I-A\(^{9}\) groove and a relatively long c-terminal extension of the peptide projects from the MHC groove \(^{53}\). Adding the native amino acids at the N-terminus to the groove or removing c-terminus amino acids of the EL-14 peptide abrogates BDC 2.5 reactivity. Thus it appears that only tissue specific processing of chromogranin can create the relevant recognized sequence.

**Molecular recognition of target autoantigens**

A developing consensus is that the peptide determinants driving autoimmune disorders may be recognized by their cognate T cell receptor in unusual conformations such as not completely filling the MHC groove \(^{53}\), in low affinity MHC binding registers \(^{239}\), with T cell receptors bound at unusual angles \(^{362}\) and with post-translational modifications \(^{363}\). In addition, tissue specific peptides may be generated in the specific target cell in a manner unlikely to be recapitulated in antigen presenting cells or thymic epithelial cells \(^{53, 242}\). All the above properties suggest mechanisms that can contribute to autoreactive T cells escaping negative thymic selection. The processing of chromogranin A to the specific peptide recognized by the BDC2.5 T cell receptor is a prominent example of only partially filling the groove of I-A\(^{9}\) and requiring specific neuroendocrine processing \(^{53}\). The B:9-23 peptide studied by Unanue and coworkers and Keppler and coworkers is another example \(^{242}\).

**ICA69**

Originally identified by an expression cloning approach using sera from pre-diabetic ICA positive individuals who subsequently progressed to overt disease \(^{364}\), the product of the ICA1 gene was recently shown to be an arfaptin-related protein associated with the Golgi apparatus \(^{365}\). Like GAD65 and IA-2, ICA69 is expressed in multiple neuronal and endocrine cell types, but may also be expressed in other peripheral tissues including the heart and exocrine pancreas \(^{366, 367}\), and antibodies reacting with ICA69 are found in patients suffering from several other immune disorders besides T1D. To date no group
has established a specific ICA69 auto-antibody assay to assess their predictive power in pre-diabetic subjects. Tissue-specific expression is controlled by alternative core promotors \(^{368}\), and in NOD mice, thymic expression of ICA69 is greatly decreased relative to non-autoimmune prone strains \(^{369}\), although the significance of this is unclear. Disruption of the ICA1 gene does not protect NOD mice from spontaneous T1D, but intriguingly ICA69null animals resist disease acceleration by cyclophosphamide \(^{370}\).

T-cell responses to ICA69 have been detected in diabetic subjects \(^{371, 372}\) and NOD mice \(^{373}\). Interestingly, the immunodominant epitope of ICA69 (residues 36-47) shows immunological cross-reactivity with the ABBOS epitope in BSA \(^{374}\), which together with epidemiological studies, has prompted speculation that BSA from cow’s milk might be a triggering antigen in human T1D \(^{375}\). However, responses to ABBOS are observed in PBMCs from both diabetic subjects and healthy controls \(^{376}\), casting doubt on this suggestion. Interestingly, T-cell responses to ICA69 in diabetic subjects showed a positive correlation with HLA-DR3, but inverse relationship with antibody positivity \(^{371}\), and increased tendency to anergy relative to other T1D autoantigens \(^{377}\).

**Carboxypeptidase E**

Carboxypeptidase E (CPE) is a neuroendocrine specific prohormone processing enzyme that selectively removes carboxy-terminal basic residues from precursor proteins and peptides (reviewed by \(^{378, 379}\)). Also known as carboxypeptidase H, CPE is a major component of insulin secretory granules \(^{380}\), and is co-secreted with insulin by pancreatic β-cells \(^{381}\). It was shown to be a serological autoantigen in man, but is not a humoral autoantigen in NOD mice. Presumptive T-cell responses have not been defined, although a recent study using HLA-DR4 transfected INS1 cells identified potential epitopes within the CPE molecule \(^{327}\).

**Amylin**

Also known as islet amyloid polypeptide (IAPP), amylin is a secretory granule protein localized primarily in pancreatic β-cells, but which is also expressed to a limited extent in gastric endocrine tissue (reviewed by \(^{382}\)). Like insulin it is a disulfide-linked heterodimer that is initially synthesized as a single-chain precursor \(^{383, 384}\), from which the mature protein is excised by prohormone convertases \(^{385}\). At present the precise physiological role(s) of amylin are uncertain, although it is probably involved in controlling food intake and body weight \(^{382}\). The human protein is capable of forming amyloid fibrils, and has been implicated in the pathogenesis of type 2 diabetes.
Recently, an HLA A*0201 restricted epitope within the signal sequence of amylin, which is cleaved in the endoplasmic reticulum, was identified using PBMCs from human diabetic subjects 386.

The initial indication that IAPP might play a role in NOD mice came from mapping studies showing genetic linkage between IAPP locus and a candidate antigen for the BDC6.9 NOD CD4+ T-cell clone 387. The same group reported that islets from NOD.IAPP-deficient mice could not stimulate BDC6.9 clone, but the authors failed to identify the relevant stimulatory peptide 52. However, the same paper presented convincing evidence that another clone, BDC 5.2.9, recognizes KS20 peptide derived from the sequence located between propeptide 1 and propeptide 2 of IAPP protein. Given the highly diabetogenic nature of BDC5.2.9 clone, and the data mentioned above, one might conclude that IAPP serves as an autoantigen in NOD mice, and that it might contribute to the human disease.

**Hsp 65**

Heat shock protein of 65kD (Hsp65), now commonly referred to as Hsp60, is a member of the broadly expressed, and evolutionarily highly conserved, HSP60/GroEL/chaperonin family. Although most of the research relating to this protein has focused upon its role in protein folding, it has recently attracted considerable interest as a potential stress cytokine283, 284, and has been implicated in several inflammatory autoimmune diseases388. In mammals the HSP60 family comprises cytosolic and mitochondrial isoforms. The cytosolic form is released from stressed cells 286 and during necrosis 287, and may also be a component of secretory granules in neuroendocrine cells389. The precise role of secreted Hsp60 in innate immunity is currently a matter of controversy, with reports of pro-inflammatory properties mediated through CD14, Toll-like receptor 2 (TLR2) or TLR4 (e.g. 289 being recently ascribed to contamination with TLR ligands such as LPS390. A primarily anti-inflammatory role has also been proposed391.

Bacterial Hsp60 is highly immunogenic, and induces antibodies that cross-react with mammalian Hsp60 and are pro-inflammatory. However, in pathogen-free NOD mice spontaneous T-cell responses to Hsp60 that precede detectable humoral responses to this antigen, can be observed prior to the onset of insulitis392, 393. Hsp65-specific T-cells transfer diabetes to young NOD mice 392, and immunization of young animals with the antigen in IFA triggers disease, although this effect is transient and the mice show long term protection 392. In contrast, immunization of some, but not all, non-autoimmune prone strains of mice with residues 437-460 of Hsp65 (p277; 394) conjugated to ovalbumin or BSA induces a non-remitting T1D which could be transferred to naïve animals by p277-specific T-cell lines 291. Protection from spontaneous T1D is provided by immunization of young NOD mice with the protein in PBS392, and of
cyclophosphamide accelerated T1D by DNA vaccination with Hsp65 cDNA \textsuperscript{292}. Peptide immunization may also reverse established disease in NOD mice \textsuperscript{395}, although this result has been disputed \textsuperscript{396}. Moreover, treatment of donor animals with p277 prevents adoptive transfer of disease to NOD.scid animals by T-cells from islet infiltrates \textsuperscript{397}, and arrests streptozotocin-induced disease \textsuperscript{398}, likely due to the induction of a Th2 biased response \textsuperscript{296, 297}. However, intra-thymic administration of p277 did not protect NOD mice from spontaneous disease, but tended to exacerbate it \textsuperscript{298}. In addition, a study by Bowman and colleagues failed to replicate studies in NOD mice showing protection from diabetes with P277 peptide \textsuperscript{396}. Surprisingly, administration of the Th1 p277-restricted clone C3.5 protected NOD mice from spontaneous disease \textsuperscript{399}, presumably due to attenuation of the clone as was previously reported for the related C9 line \textsuperscript{400}. Hsp65 is also a T-cell autoantigen in humans \textsuperscript{401-403} although it is uncertain if this response is disease-specific. Interestingly, although no correlation between IFNγ producing Hsp65 specific T-cells and diabetes risk was observed in discordant monozygotic twins, low-risk (ICA negative) individuals showed significantly enhanced IL-10 producing Hsp65 specific T-cells \textsuperscript{300}. On the basis of the protection afforded to NOD mice \textsuperscript{395} a limited phase II clinical trial with a humanized version (DiaPep277) has been conducted in new onset adult patients. Interestingly, this showed preservation of C-peptide levels for at least 10 months, with reduced exogenous insulin requirement, in the treated individuals \textsuperscript{404}. Further phase II and III trials were conducted with inconclusive results \textsuperscript{405, 406}.

**Primacy and diversity of the molecular targets of T-cells in diabetes**

As discussed above, the concept that diabetic autoimmunity in humans is initiated by T-cell mediated attack directed at a single β-cell specific molecule, while attractive is as yet unproven though with considerable evidence that insulin/proinsulin may be such a primary target. That this can be the case is clearly demonstrated by the various mouse models in which neo-antigens are expressed under control of the RIP (for example \textsuperscript{80, 96, 97} or where activated diabetogenic T-cell clones are transferred to susceptible lymphopenic animals (for example \textsuperscript{51, 308}). However, it is uncertain if it is the case in the natural disease, where the triggering event remains ill-defined, although it does appear likely that the initial response targets only a limited number of autoantigens. In NOD mice there is a restricted islet-reactive TCR repertoire in early insulitic lesions although surprisingly, neither GAD65 nor insulin-specific clones predominate \textsuperscript{173, 407}. Similarly, CD8\textsuperscript{+} T-cells from the earliest insulitic lesions are clearly cytotoxic to islet cells, and show a recurrent amino acid sequence motif in the complementarily determining region 3 of their α chains, with a prevalence of Va17 frequently joined to the Ja42 segment, although there is a diverse TCR Cα and Cβ repertoire \textsuperscript{408}. The latter observation suggested that the majority of CD8\textsuperscript{+} T-cells participating in the initial phase of autoimmunity recognize a limited number of MHC class I-peptide complexes expressed
by β-cells, although subsequent studies indicate that more than one antigenic specificity is present. Nonetheless, it remains possible that expansion of the autoreactive repertoire had already occurred prior to these analyses being conducted. It is also possible that multiple autoantigens participate in the initiation of the autoimmune response, but that disease progression requires the subsequent activation of an autoresponse to a "primary" (major but not initial) antigen such as proinsulin. Such an hypothesis would reconcile the apparently conflicting facts that the earliest insulitic lesions contain few if any T-cells that recognize proinsulin, yet proinsulin 1/- NOD mice are almost all protected from disease. It must be noted, however, that a few proinsulin 1/- NOD mice do develop T1D, indicating that though important, it is not the only key auto antigen in these animals, a conclusion that was also reached in a recent study in which preproinsulin 2 was expressed in NOD APCs under control of a modified invariant chain promoter. However, the possibility that residual T1D in preproinsulin 1/- NOD mice required autoreactivity to proinsulin 2, and that diabetes in mice tolerized to preproinsulin 2 stemmed from reactivity to preproinsulin 1, has gained some support from a recent study in which a modified preproinsulin 2 transgene with a substitution within the immunodominant B:9-23 epitope was expressed in NOD mice lacking expression of both wild-type preproinsulin genes. Animals lacking the B:9-23 epitope do not develop diabetes with suppressed insulitis and insulin autoantibodies, highlighting the importance of this epitope in disease induction in these animals. In addition, tolerance to proinsulin blocks all NOD diabetes and expression of IGRP reactive T-cells. The primacy of any one autoantigen in triggering human T1D is a matter of debate. Serological studies of young at-risk children indicate that, on average, autoimmunity to insulin appears before GAD65 which in turn precedes IA-2/phogrin autoantibodies, but only by a matter of months. At the level of the individual, however, any of these three autoantibodies may present first, and in older subjects insulin autoantibodies appear only in a minority of patients at disease onset. Even if there is a primary autoantigen, it is reasonable to hypothesize that any molecule that is a major target of autoimmunity in type 1 diabetes is a candidate for use in antigen-based therapy. Effective tolerization strategies in NOD mice based on immunization with the native epitopes of insulin, or GAD65 or insulin B-chain cDNA, appear to bear this out, although whether these therapies can be effectively transferred to human subjects remains a matter of debate.

Epitope spreading and the progression to overt disease

Although invasive insulitis appears to be a necessary precursor to disease, overt T1D does not necessarily occur even in the presence of profound lymphocytic accumulation. Histologic studies indicate that beta cell loss in the NOD mouse proceeds over a considerable time period, namely beta cell destruction is chronic and likely balanced by beta cell proliferation. Experiments using congenic mouse strains have suggested that passage through the final barrier that precedes clinical disease
(checkpoint 2) is mainly under control of non-MHC loci \(^{57, 418}\), whilst those using transgenic BDC2.5 TCR mice have demonstrated that epitope spreading is not a pre-requisite (although it may be important in the “natural” disease \(^{419}\)). Although expansion of the immune response to include novel targets, and avidity maturation of individual clonotypes \(^{352}\) may play an important role, recent studies have suggested that the transition to the destructive phase is dependent upon the breakdown of key regulatory mechanisms, including both decreased sensitivity of diabetogenic cells to negative signaling \(^{420, 421}\), and functional depletion of regulatory T-cell populations \(^{422, 423}\). Subsequently the breakdown in peripheral tolerance might lead to further changes in the Th1/Th2 balance \(^{424-427}\) (though fewer), up-regulation of T-cell effector molecules (such as FasL \(^{428}\) or inflammatory cytokines), recruitment of additional effector cells (including activated macrophages and cytotoxic T-cells \(^{429}\)), or cytokine-driven changes in β-cell function (such as up-regulation of MHC class I molecules \(^{430}\) or increased sensitivity to oxidative damage \(^{431, 432}\) ) (Figure 2), all of which might contribute to the uncontrolled destruction of pancreatic β-cells. Though in the NOD mouse checkpoint 2 is often discussed as a relatively acute event in both men and mouse, there is evidence for asynchronous destruction of beta cells within individual islets over time and in man, metabolic deterioration usually occurs over years \(^{1, 433}\).

Multiple interventions can prevent the progression to T1D in neonatal NOD mice, an increasing list of agents are effective once checkpoint 2 has been passed. Transplantation with sustained immunosuppression, non-specific T-cell depleting or modulating antibodies and fusion proteins have proven effective for long-term abrogation of established disease in mice (reviewed by \(^{434}\)). Successful agents include polyclonal anti-lymphocyte serum \(^{26164}\) \(^{314}\), non-depleting anti-CD3 \(^{46, 47}\), sICAM/Ig \(^{315}\), and depleting anti-CD4 with \(^{435}\), or without \(^{316}\), anti-CD8. In each case the studies indicate a critical window of effectiveness, namely the first 7-14 days following overt hyperglycemia, and cannot provide long-term protection for allografts introduced into spontaneously diabetic animals. Reversal of new onset diabetes has been achieved recently with multiple agents and experimental designs emphasizing treatment with the initial increase in glucose immediately after the onset of hyperglycemia. Therapies have included tyrosine kinase inhibitors \(^{436}\), injection of substance P \(^{437}\), antithymocyte globulin, adaptive T regulatory cells \(^{438}\), exendin-4 and lysofylline \(^{439}\), dendritic cells \(^{440}\), and insulin coupled to antigen presenting cells \(^{441}\).

The success of the anti-CD3 therapy in NOD mice has led to recent clinical trials. The first, using a non-mitogenic derivative of OKT3 (OKT31 Ala Ala; \(^{442}\)) preserved β-cell mass in 9/12 newly-diabetic subjects in the 12 months following diagnosis \(^{20, 443}\). The effect was maintained for at least 2 years, although it diminished during the second year of follow-up \(^{318}\). The second trial, using an aglycosylated derivative of a rat antibody (ChAglyCD3; \(^{444}\)) also preserved residual β-cell function for at least 18 months \(^{26167}\), although in this case efficacy was limited to a subgroup of the treated patients who had the highest initial residual function. In both humans and mice anti-CD3 therapy likely functions in 2 phases, initially acting to clear the insulitis, and subsequently decreasing the effector : regulatory T-cell ratio to promote long-term tolerance \(^{445, 446}\). Larger phase III trials have been less encouraging \(^{21}\). Interestingly, expansion of IL-10 producing
proinsulin-specific T-cells was observed in a case of spontaneous remission of T1D following insulin therapy. A complete understanding of the therapeutic mechanism of anti-CD3 treatment is still lacking, but the insight so far gained provides the basis for improved strategies to increase effectiveness in human subjects. For example, clearing of the insulitis presumably prevents further autoimmune β-cell damage, and, likely at least in part due to the alleviation of cytokine-mediated inhibition of insulin secretion, allows a rapid restoration of normoglycemia, reducing β-cell stress, and potentially allowing islet regeneration. Consequently, strategies to combine T-cell depletion with agents to promote β-cell growth may be more effective than treatment with either agent alone. Similarly, improved methods of generating human antigen-specific regulatory T-cells either in situ or ex vivo should lead to a more effective therapy.

**Mechanism and mediators of β-cell death**

At present, the precise molecular mechanisms that lead to β-cell death in the destructive phase of T1D remain largely unresolved. It is generally accepted that death primarily occurs by apoptosis (316-319), but the relative contributions of direct effector cell:β-cell contact, and of secreted or shed soluble mediators, in the destructive process are matters of some debate, and may vary between mouse and human T1D. Thus, experiments investigating the involvement of proteins involved in contact-dependent killing such as perforin, Fas, or FasL have produced inconclusive results. For example, NOD/lpr mice (which are deficient in Fas expression) are protected from spontaneous diabetes, consistent with a critical role for Fas-FasL interactions in β-cell killing. However, as NOD/lpr animals show little insulitis it is possible that the lymphadenopathy seen in these animals significantly alters their T-cell responses to diabetic autoantigens, or that Fas is required for priming of the autoresponse, but is redundant at later times. Similarly, IL-1β mediated induction of Fas by β-cells can be demonstrated in vitro, but is not seen in spontaneous T1D in NOD mice, although β-cell expression of Fas is observed in inflamed human islets, and is readily detectable following accelerated T1D in mice. Likewise, perforin-deficient NOD mice show a reduced incidence and delayed onset of spontaneous disease, but perforin-deficient NY8.3 TCR transgenic NOD mice develop diabetes more frequently than their perforin-competent littermates.

Similarly, despite circumstantial evidence supporting a role for NO-mediated oxidative damage in triggering β-cell death (e.g., iNOS is not essential for diabetes in NOD mice or for cytokine-mediated destruction of human islets). Some of the apparent discrepancies between individual studies may stem from the fact that most of the effectors tested have pleiotrophic, and often conflicting, effects. For example, NO may be directly cytotoxic to β-cells, but also favors the generation of a Th2 biased immune response, which is likely to be less pathogenic to islets, whilst ligation of Fas can trigger either apoptosis or proliferation of target cells depending upon context. Alternatively the aforementioned inconsistencies may simply reflect functional redundancy within the immune system, and the likelihood that in the natural disease terminal β-cell destruction...
involves multiple effector mechanisms, which can compensate for each other's absence. However, it is also possible that the key effector mechanism for β-cell death in T1D remains to be elucidated.

**T-cell subsets in T1D**

In both humans and NOD mice multiple cell-types are recruited to islet infiltrates during the progression to T1D, including CD4+ and CD8+ T-cells, B cells, macrophages, dendritic cells, and NK cells. In NOD mice macrophages and dendritic cells precede T lymphocytes into the islet, and play essential roles in the disease process. Presumably this is also true for the human disease. However, as stated above, T-cells are considered to be the primary mediators of T1D in both humans and mice. The relative importance of the CD4+ and CD8+ subsets in the diabetogenic process has been a matter of some debate. Both CD4+ and CD8+ T-cells are required for adoptive transfer of disease from diabetic NOD mice to irradiated hosts, and antibodies to either subset can prevent disease at early time points. However, whereas treatment of NOD mice with antibodies to CD4 can arrest T1D progression at late stages, and may even reverse established disease, anti-CD8 therapy is only effective when given to young (2-5 week old) animals. Moreover, diabetes can be transferred to NOD.scid mice lacking expression of MHC class I by splenocytes from overtly diabetic, but not pre-diabetic donors, and in several instances to immuno-compromised animals by individual CD4+ T-cell clones. This suggests that CD8+ T-cells are essential for the initiation of diabetogenesis, but are dispensable for the destructive phase. However, most NOD mice selectively deficient in pancreatic β-cell expression of MHC class I molecules, due to co-transgenesis for human insulin promotor driven Cre and loxP flanked β2-microglobulin, develop insulitis but not hyperglycemia, challenging this conclusion.

Despite their obvious importance to diabetes induction in un-manipulated NOD mice, CD4+ T-cells are dispensable for disease induction in some other animal models of T1D. Thus, in contrast to several other CD8-restricted TCR transgenic mice, those expressing the TCR of the AI4 CTL develop spontaneous disease in the absence of CD4+ T-cells. Similarly, RIP-LCMV mice that do not express the transgene in their thymuses rapidly develop T1D following LCMV infection even when depleted of CD4+ T-cells. Together, the mouse models suggest that circumstances can be contrived under which either subset is unnecessary. However, it seems most reasonable to conclude that in the “natural” disease both subsets contribute significantly to diabetogenesis, and that treatment of established disease will likely require modulation of both CD4+ and CD8 restricted T-cell responses.

In mice, the CD4+ T-cell subset can be broadly classified into 6 subgroups on the basis of cytokine secretion profile and effector function, namely Th1, Th17, Th2, Th3, Tr1, and Treg. Th1, which secrete IFNγ, TNF-α, and IL-2, and Th17 (IL17) are primarily associated with cellular immunity, and Th1 has been strongly implicated in diabetogenesis. Moreover, ectopic islet expression of the signature cytokines can promote the development of T1D (for example, but see also), and the systemic treatment of NOD mice with IL-12, a potent inducer of Th1 cells, leads to accelerated diabetes.
In contrast, Th2 cells, which secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, and are mainly involved in humoral immune responses and allergy, seem either to be relatively innocuous, or to provide protection from T1D, and it is generally believed that an increase in the Th1:Th2 ratio promotes diabetogenesis. Consistent with this hypothesis, RIP-IL-4 NOD mice that over-express the signature cytokine in their islets are protected from diabetes. However, NOD mice deficient in IL-4 (the primary polarizing cytokine for Th2 development) exhibit similar disease incidences to regular NOD mice. Transfer experiments with Th2 cells generated from BDC2.5 transgenic mice have provided conflicting results. Naive T-cells cultured in the presence of IL-4 and an anti-IFN-γ monoclonal antibody for 4-6 days and then transferred to neonatal NOD recipients, invaded the islets, but neither provoked disease, nor provided significant protection from co-injected Th1 polarized cells, although they did induce diabetes when transferred to NOD.scid animals. However, in the latter case the inflammatory lesion resembled an allergic inflammation, and the kinetics of disease were markedly different from T1D induced by Th1 cells. In contrast, naive BDC2.5 T-cells cultured for an extended period under Th2 polarizing conditions, and subsequently cloned, generated effectors capable of accelerating disease in neonatal NOD recipients, but which were unable to induce diabetes in NOD.scid mice unless a second Th1 polarized β-cell specific T-cell clone was co-transferred. The reason for these obviously contradictory results is not immediately apparent, but might reflect differences in the relative amounts of the various Th2 cytokines generated by the T-cells polarized under the 2 conditions. Thus, the diabetes induced in NOD.scid mice by Th2 polarized T-cells could be prevented by treatment with neutralizing antibodies to IL-10, but not IL-4, suggesting that it might share some features with the accelerated T1D observed in RIP-IL10 NOD mice, which also exhibit an atypical islet infiltrate. In contrast, T1D induced by the BDC2.5 Th2 polarized clones may reflect IL-4 mediated activation of APCs, and their consequent shift from a tolerizing to priming state. Together, these results suggest that under specific circumstances Th2 polarized T-cells are also capable of promoting diabetogenesis, which raises important concerns regarding potential therapies based upon altering the Th1/Th2 balance or administration of Th2 cytokines, particularly for treating lymphopenic individuals. Nonetheless, numerous studies using NOD mice have indicated a beneficial effect of increasing systemic IL-4 and/or IL-10 levels through administration of recombinant protein or gene therapy. At present this therapy has not been tried in prediabetic patients, and it must be noted that the strict dichotomy between IFNγ and IL-4 or IL-10 secretion by mouse CD4+ T-cell subsets is not recapitulated in the human immune system.

Both Th1 and Th2 subsets can properly be regarded as immune effectors. However, it is now generally accepted that multiple populations of regulatory T-cells also exist, and are critical to maintaining peripheral tolerance (reviewed in). Thus, the outcome of any immune response, including autoimmunity, depends upon the balance between activated effector and suppressor cells at critical anatomical sites. Since a numerical or functional deficiency in regulatory T-cells will confer susceptibility to autoimmunity, considerable recent interest has been focused on defining their properties and ontogeny. Operationally, regulatory T-cells cells can be divided into two basic classes;
innate and induced \(^{476}\). The repertoire and numbers of the innate class, which include NKT cells (reviewed by \(^{477}\) and Foxp3 expressing CD4\(^+\)CD25\(^hi\) T\(_{\text{reg}}\) cells (reviewed by \(^{478}\), may be genetically determined \(^{479, 480}\), although this remains a matter of debate \(^{481, 482}\). Nonetheless, peripheral \(^{476}\) deficiencies in both NKT and resting CD4\(^+\)CD25\(^+\) T-cells have been reported in human diabetic subjects, and NOD mice are deficient in NKT cells \(^{402}\). The spontaneous population of Foxp3 expressing CD4\(^+\)CD25\(^hi\) T\(_{\text{reg}}\) cells develop in the thymus, and are presumably specific to self-antigens expressed by the thymic epithelium or its resident APCs \(^{483}\). Thus, abnormally low thymic expression of a peripheral antigen, such as may occur in individuals with class I preproinsulin promoter VNTR alleles (the IDDM2 locus) \(^{225, 228}\), may confer disease susceptibility either by decreased production of organ-specific T\(_{\text{reg}}\) cells, defective negative selection, or by a combination of these 2 probable consequences. A modest numerical deficiency of CD4\(^+\)CD25\(^hi\) T\(_{\text{reg}}\) cells in NOD mice has been reported \(^{403}\), although this conclusion was subsequently disputed following comparisons with a wider range of non-autoimmune prone strains \(^{481}\). However, more recently an age-dependent decline in the proportion of FoxP3\(^+\) CD4\(^+\)CD25\(^hi\) T\(_{\text{reg}}\) cells in the PLN and islet infiltrates of female NOD mice was reported, suggesting a defect in their maintenance and/or expansion in these animals \(^{404}\).

Besides constitutive expression of CD25, several other surface markers have been used to define spontaneous regulatory T-cells including CD62L, CD45RB\(^lo\), and CD103 \(^{339, 340}\). However, there is heterogeneity for these markers within the CD4\(^+\)CD25\(^hi\) subset, suggesting that multiple populations of T\(_{\text{reg}}\) cells are generated, and that these may differentially protect particular organs \(^{341}\). In this regard it should be noted that protection of BDC2.5NOD.scid mice from spontaneous T1D was provided by transfer of polyclonal CD4\(^+\)CD62L\(^+\) T-cells rather than by polyclonal CD4\(^+\)CD25\(^+\) T-cells \(^{484}\), although in vitro expanded monoclonal BDC2.5 CD4\(^+\)CD25\(^hi\) T-cells can also prevent diabetes in other systems \(^{180, 181}\). Foxp3 expressing T\(_{\text{reg}}\) cells can also be generated from peripheral CD4\(^+\)CD25\(^+\) cells by activation in the presence of TGF-\(\beta\) \(^{343, 344}\), although whether the precursors are truly naive or are pre-programmed in the thymus, remains a matter of discussion (reviewed by \(^{485}\)). In addition to Foxp3 expressing T\(_{\text{reg}}\) cells, at least 2 other subsets of inducible, antigen-specific, CD4\(^+\) regulatory T-cells are found in the periphery, namely Th3 and Tr1 cells. The former subset provides "help" for IgA production, mainly secretes TGF-\(\beta\), and is primarily involved in establishing mucosal tolerance \(^{486, 487}\). In contrast, Tr1 cells secrete multiple cytokines including IL-10, IL-5, IFN\(\alpha\) and TGF-\(\beta\), but little IL-2 or IL-4 \(^{488}\), and are induced during chronic antigenic exposure. Tr1 cells can also be induced by some subsets of "tolerogenic" APCs \(^{489}\), and by T\(_{\text{reg}}\) cells \(^{490}\), and regulatory cells mediate the long-term protective effects of anti-CD3 therapy \(^{445}\) in NOD mice. Both Th3 and Tr1 cells secrete immunosuppressive cytokines and can cause bystander suppression, although they can also act by a contact-mediated mechanism. The innate CD4\(^+\)CD25\(^hi\) T\(_{\text{reg}}\) cells, by contrast, exert their suppressive effect in a cytokine-independent fashion, but can act both directly upon activated T-cells, as well as indirectly through direct effects on APCs \(^{491, 492}\). In addition to regulatory CD4\(^+\) T-cells, subsets of CD8\(^+\) suppressor T-cells have also been identified \(^{492, 493}\), and may play a role in CD3-mediated preservation of \(\beta\)-cell mass in humans \(^{347}\).
Although CD4^+CD25^hi T\textsubscript{reg} cells were initially regarded as being anergic to \textit{in vitro} stimulation, conditions have since been defined allowing their \textit{ex vivo} expansion (for example 180), and it is now clear that they can proliferate extensively \textit{in vivo} 348, 349, 403. Moreover, the fact that they are able to control an ongoing autoimmune response suggests that the transfer of autologous $\beta$-cell specific T\textsubscript{reg} cells represents a rational strategy for therapeutic intervention in pre-diabetic and possibly new-onset diabetic, subjects 494. However, the low frequency of antigen-specific T\textsubscript{reg} cells in peripheral blood, and the need to carefully select the target antigen(s) to reduce the risk of unwanted side-effects such as increased tumor susceptibility 495 provide important caveats. Similarly, although treatment with the NKT cell ligand $\alpha$-galactosyl ceramide can protect NOD mice from T1D 352, 353, doubts have been expressed about its efficacy in humans unless organ-specific NKT cells can be identified and targeted 496.

It is clear that B-lymphocytes and potentially antibodies contribute to the development of diabetes of the NOD mouse and likely man 322. Anti-CD20 therapy of NOD mice prevents diabetes associated with induction of regulation and in new onset patients delays loss of c-peptide 497.

\textit{Problems associated with the detection of T-cell reactivity to $\beta$-cell antigens in human subjects - Conclusions from International T-Cell Workshops}

There is a considerable amount of direct evidence that T-cells are primary mediators of $\beta$-cell destruction in the NOD mouse. In contrast, there is much less certainty that this is also the case in human subjects, with much of the available evidence being circumstantial. A major obstacle to characterizing ongoing islet-specific T-cell responses in humans comes from the fact that the only practical source of starting material is peripheral blood. However, it is generally accepted that the frequency of islet-specific T-cells in the peripheral blood of prediabetic or newly diabetic patients is very low 498, and newly diagnosed T1D patients are often lymphopenic 499. Moreover, there is relatively high percentage of monocytes, which may influence \textit{in vitro} proliferation assays. Consequently it is technically difficult to isolate and expand auto-antigenic T-cells, although the recent appreciation that the effector and regulatory subsets have overlapping, or even identical, specificities, suggests that the actual precursor frequencies might be higher than originally appreciated 500. Despite the technical challenges, many investigators have obtained evidence that diabetic subjects have T-cell reactivity to $\beta$-cell associated antigens, and recent studies have suggested that examination of autoimmunity within different T-cell subsets might provide insight into the T-cell responses of different clinical groups of patients. Thus, in newly diagnosed T1D patients autoimmune T-cell responses were primarily present amongst activated T-cells (CD45RA^+RO^+), whereas those with longer disease duration reacted to autoantigens with memory cells (CD45RO^+).

The first international workshop for the standardization of the T-cell assays organized by the Immunology of Diabetes Society in 1999 aimed to identify, and suggest solutions to, the problems associated with autoreactive T-cell assays in T1D 501. The workshop
defined a series of candidate autoantigens that were distributed to 26 participating laboratories around the world where T-cell proliferative responses of diabetic and non-diabetic individuals were analyzed worldwide in 26 laboratories. Three conclusions were reached:

1) The quality of the recombinant antigens required improvement.

2) All laboratories were able to detect T-cell responses to the control antigen (tetanus toxoid) although with significant inter-laboratory variation in sensitivity.

3) Significant differences in T-cell proliferative responses to diabetic autoantigens between diabetics and non-diabetic individuals were not consistently observed.

To follow-up this initial study a second international T-cell workshop was held which focused on the identification of more suitable antigens and the development of standardized assays. Various preparations of GAD65, proinsulin, and IA-2 were evaluated for endotoxin content, their ability to stimulate T-cell clones, and any inhibitory effects they had on proliferation to control antigens. Subsequently, recommendations were made for the preparation of antigens with optimal quality.

_T-cell assay development._

The difficulties associated with human studies using T-cell assays based upon proliferation have stimulated the development of alternative procedures, with considerable effort being devoted to developing ELISPOT and tetramer based assays. The former technique is capable of defining both the frequency and cytokine profile of autoresponsive cells, and was the focus of the third international T-cell workshop that demonstrated its potential for detecting low-level autoreactive T-cell responses. For example, ELISPOT analysis has provided evidence for IFNγ-producing insulin B:9-23 reactive T-cells in peripheral blood from new onset and prediabetic patients. These observations were confirmed in the recent fourth international T-cell workshop that used a panel of coded samples and antigens in a fully blinded study.

Synthetic tetramers in which biotinylated MHC class I or class II molecules loaded with a specific T-cell epitope are multimerized using fluorescent streptavidin provide another tool to follow autoreactive lymphocytes. Thus, H-2K^d_ MHC class I tetramers combined with insulin B:15-23 or NRP-V7, a mimetope peptide to the NY8.3 CTL clone, have been used to study CD8^+ T-cells in NOD insulinic lesions. Interestingly, B:15-23 reactive T-cells were readily detectable in islet infiltrates at an early age (4-5 weeks) but not later, while NRP-V7 reactive T-cells were found only in older mice (11-18 weeks). Using the NY8.3 mimetope peptide evidence has been obtained for “avidity maturation” of the CD8^+ T-cell response to islets during the development of disease. Moreover, analysis of NRP-V7-reactive T-cells in peripheral blood from pre-diabetic
NOD mice revealed a significant increase immediately prior to disease onset, providing the first assay capable of accurate temporal prognosis of T1D. In contrast, B:15-23 reactive CD8+ T-cells were not detected in peripheral blood.

CD4+ T-cells can also be analyzed by tetramers. When the specific target antigen of the BDC2.5 T-cell clone was unknown, tetramers combining I-A with mimetopes of the peptide for the BDC2.5 TCR efficiently stained both BDC2.5 transgenic T-cells, and thymic CD4+ T-cells from NOD mice. In contrast, T-cells recognizing I-A tetramers loaded with GAD65 peptides could only be detected in immunized mice, although HLA-DRB1*0401 tetramers containing an immunodominant peptide from GAD65 can detect CD4+ autoreactive T-cells in peripheral blood of T1D patients. With register trapping, Kappler's laboratory has developed tetramers recognizing insulin B:9-23 T cells of the NOD mouse. The possibility of using tetramers for therapeutic, as well as diagnostic, applications is also being actively pursued. A very interesting approach is the use of nanoparticles with MHC-peptide immunization generating CD8 T cells that can kill antigen presenting cells expressing islet autoantigens.

Summary

The NOD mouse has been available to research community for over 20 years and in that time tremendous advances have been made in understanding the role of the immune system in development of T1D. It is clear that β-cell destruction is due to an adaptive immune response with both CD4+ and CD8+ T-cells playing important roles. Moreover, NOD mice, as well as other rodent models of T1D, have provided key insights into how tolerance can be broken and restored. It should be kept in mind that development of diabetes in the NOD mouse differs from that in humans in several respects, including a marked sex bias in females and very high concordance (80-90% in NOD female mice). Nevertheless, the NOD mouse provides a very important model for understanding diabetes pathogenesis in humans, and for developing potential therapeutic strategies. There is a developing consensus that the immune system response to insulin may be primary with elimination of other target antigens dispensable for disease. If there is a primary autoantigen and epitope (e.g. insulin B:9-23) understanding at a structural level, the trimolecular complex (I-A – peptide – TCR) may lead to novel deletional therapies. Even if there is a primary antigen induction of T regs to multiple islet antigens, with suppression of autoimmunity can clearly suppress disease. Thus, multiple different antigen specific therapies are candidates for diabetes prevention.

The data obtained from investigations of human subjects, though not conclusive, are highly suggestive of a direct role for T-cells in initiating β-cell destruction, although it remains uncertain to what extent data from the various mouse models can be extrapolated to the human condition. However, it is hopefully only a matter of time before advances in techniques will allow a definitive assessment of the roles of the various T-cell subsets in human T1D, and the development of effective strategies for
accurate diagnosis and antigen-specific therapeutic intervention to prevent or retard disease progression.

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