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Review

The role of perforin and granzymes in diabetes

HE Thomas*[,1,2], JA Trapani[3] and TWH Kay[1,2]

Type 1 diabetes results from autoimmune destruction of pancreatic β-cells by CD8$^+$ T cells. The requirement for CD8$^+$ T cells implicates perforin and granzymes as effectors of tissue destruction. Diabetogenic cytotoxic T cells kill β-cells by the perforin/granzyme pathway *in vitro*. In the non-obese diabetic mouse model of type I diabetes, perforin deficiency results in a highly significant reduction in disease, indicating a direct role for perforin in β-cell death *in vivo*, although other cell death pathways must account for the residual diabetes in perforin-deficient mice. Perforin and granzyme B are also important in allogeneic destruction of islets. The dominant role of the perforin/granzyme pathway in β-cell destruction in type I diabetes and allogeneic islet graft rejection make this pathway an important target for blockade in future therapies for type I diabetes. In addition, granzymes have a newly recognized role in inflammation, a feature of both type I and II diabetes, suggesting their role should be further explored in both the common forms of diabetes.
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Diabetes is caused by insulin deficiency resulting from autoimmune destruction of insulin-producing β-cells in the case of type I diabetes, or insulin resistance and declining β-cell function in type II diabetes. It is associated with long-term complications that affect the eyes, heart, kidney and nervous system. These complications cause very significant morbidity and premature death. All forms of diabetes have high direct and indirect economic costs. The number of people with diabetes worldwide was estimated to be 171 million in 2000, with this number expected to be more than double by 2030.[1]

Although much is known about the role of perforin and granzymes in clearing viral infections and in tumor rejection,[2] and their involvement in the pathogenesis of diabetes was identified some 20 years ago,[3] devising strategies to prevent perforin and granzyme-dependent β-cell damage has only recently come under investigation. Nonetheless, the findings to date suggest that significant progress will be made in the coming years. This review will primarily focus on the role of perforin and granzymes in type I diabetes and islet allograft rejection because of their dependence on CD8$^+$ T cells. However, there is an increasingly recognized role for inflammation in type II diabetes, and the newly described role for granzymes in inflammation may implicate them in both the common forms of diabetes.

Type I Diabetes

Type I diabetes is a common chronic disease often diagnosed in childhood and increasingly recognized in adults (reviewed in Pietropaolo *et al.*[4]). It is an autoimmune disease in which the insulin-producing β-cells of the pancreas are destroyed in a highly specific manner by T lymphocytes, resulting in insulin deficiency and lifelong reliance on insulin injections. The non-obese diabetic (NOD) mouse is a spontaneous model of type I diabetes with substantial similarities to human disease. In the NOD mouse, lymphocytic infiltration of the pancreas, called insulitis, begins soon after weaning. Typically, the islet infiltrate is composed of antigen-presenting cells, B lymphocytes, and CD4$^+$ and CD8$^+$ T lymphocytes. In NOD mice and humans developing type I diabetes, there is a long pre-clinical period during which autoantibodies to islet proteins can be detected in circulation, but β-cell mass remains sufficient to maintain normoglycaemia. Eventually, a point is reached at which the clinical signs of diabetes appear, implying that a significant proportion of β-cell mass has been destroyed.

CD8$^+$ T Cells Destroy β-Cells in Type I Diabetes. The major cell type that destroys β-cells in type I diabetes is the CD8$^+$ cytotoxic T lymphocyte (CTL) that directly recognizes peptide antigens presented by class I major histocompatibility complex (MHC) proteins on the surface of β-cells.[5] In human subjects with type I diabetes, CD8$^+$ T cells predominate in affected islets.[6–8] However, most evidence for the role of CTL comes from NOD mice. Class I MHC proteins are expressed at high levels on β-cells during insulitis and CD8$^+$ T cells predominate in islets during β-cell destruction.[9] NOD mice deficient in β2-microglobulin, and

[1]Department of Immunology and Diabetes, St Vincent's Institute, 41 Victoria Parade, Fitzroy, Melbourne 3065, Australia; [2]Department of Medicine, The University of Melbourne St. Vincent's Hospital, 41 Victoria Parade, Fitzroy 3065, Australia and [3]Cancer Immunology Program, Research Division, Peter MacCallum Cancer Centre, East Melbourne 3002, Australia
*Corresponding author: HE Thomas, Immunology and Diabetes, St Vincent's Institute, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia. Tel: +61 3 9288 2480; Fax: +61 3 9416 2676; E-mail: hthomas@svi.edu.au
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Abbreviations: NOD, non-obese diabetic; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; APC, antigen-presenting cells; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; RIP, rat insulin promoter; SOCS-1, suppressor of cytokine signaling-1; LCMV-GP, lymphocytic choriomeningitis virus-glycoprotein; lpr, lymphoproliferation; BH3, Bcl-2 homology 3; XIAP, X-linked inhibitor of apoptosis; TCR, T-cell receptor; PLN, pancreatic lymph node
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therefore lacking surface class I MHC and CD8$^+$ T cells, do not develop insulitis or diabetes.[10–13] Adoptive transfer of spleen cells from diabetic NOD mice causes β-cell destruction efficiently in recipient NOD mice that express class I MHC proteins on the surface of β-cells but not on any other cell type, and diabetes is reduced if class I MHC is deleted specifically from β-cells, suggesting that β-cell-CD8$^+$ T-cell interactions are required.[14,15] These so-called 'β-bald' mice with conditional deletion of class I MHC from β-cells develop insulitis normally suggesting that β-cell-CD8$^+$ T-cell interaction is not needed for this pre-diabetes pathology (Table 1).[14] Both diabetes and insulitis are absent in mice specifically lacking class I MHC on antigen-presenting cells (APC), suggesting that APC–CD8$^+$ T-cell interactions are required for insulitis.[16] CD8$^+$ T-cell clones or cells from CD8$^+$ T-cell receptor transgenic mice can efficiently transfer diabetes to non-diabetic class I MHC compatible recipients[17–20] and diabetes is inhibited by expression in β-cells of viral proteins able to downregulate class I MHC.[21] Together these data provide strong evidence that CD8$^+$ T cells are important in diabetes development. Most important for this review, CTL are required for efficient β-cell killing, implicating CTL effector molecules like perforin and granzymes in the pathogenesis of diabetes (Figure 1).

β-Cell Destruction in Experimental Models of Type 1 Diabetes

There are undoubtedly multiple effector mechanisms capable of killing β-cells in the NOD mouse and also in humans. Effector mechanisms likely to be important in CTL-mediated β-cell death include perforin, death receptor molecules of the TNF receptor family such as Fas, and pro-inflammatory cytokines including the interferons and interleukins (Figure 2).[22] Genetically modified mice have provided the opportunity to definitively test the role of these effector mechanisms, particularly transgenics made directly in the NOD strain. We have studied the *in vivo* role of effector molecules including Fas/FasL and cytokines in several animal models of type I diabetes, in particular the NOD mouse. Because of redundancy of effector mechanisms, preventing Fas signaling in β-cells,[23–25] or deficiency of IFNγR[26,27] or IL-1R[28] has very little impact on type I diabetes in NOD mice. Deficiency of TNFR1 prevents diabetes in NOD mice, but this is most likely because of its effects on immune activation as opposed to direct β-cell death.[29,30] In addition to signals from immune cells, the β-cell itself is increasingly recognized to be actively involved in its own demise through cross-talk with immune cells and promoting local inflammation and apoptosis.[31]

Perforin-deficient mice. There is substantial evidence for a dominant role for the granule exocytosis pathway mediated by perforin. The pore-forming protein perforin is critical for the delivery of granyzmes into target cells triggering cell death after immunological synapse formation. Perforin-deficient mice were backcrossed on to the NOD genetic background soon after they were produced by Hengartner *et al*.[32] Only 16% of homozygous perforin-deficient NOD mice developed diabetes compared with 77% of wild-type NOD mice, and diabetes in perforin-deficient mice occurred at a much older age than in wild-type mice.[33] This pattern was reproduced

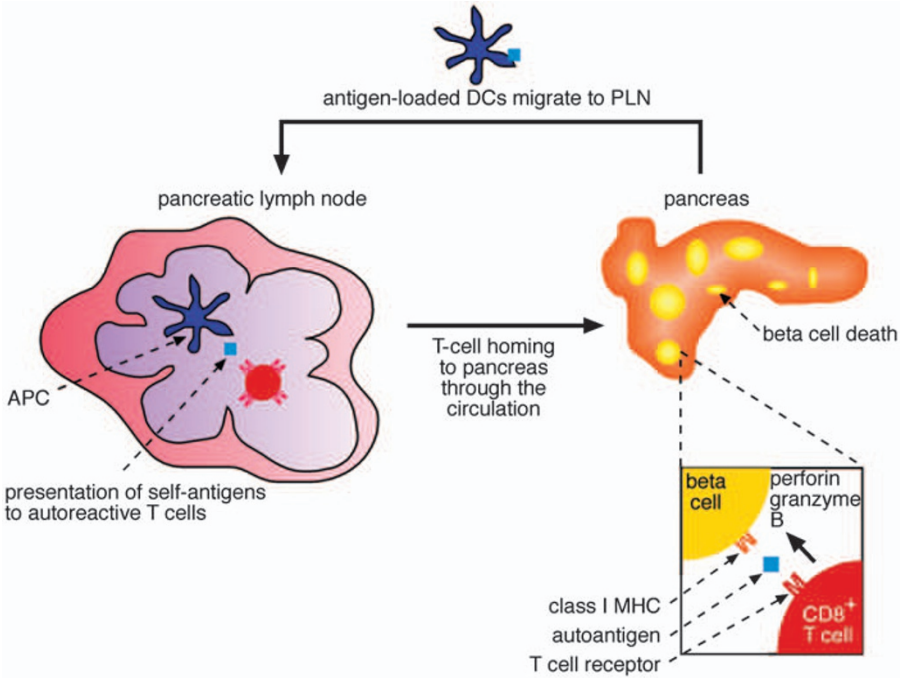

Figure 1 Autoreactive CD8$^+$ T-cell development. Autoreactive T cells escape deletion in the thymus and are exposed to self antigens in the periphery. β-cell antigens are presented to autoreactive T cells in the pancreatic lymph node (PLN), then T cells migrate through the circulation to the pancreas where they destroy β-cells. Antigen in the pancreas is taken up by dendritic cells, which migrate back to the pancreatic lymph node to complete the circuit

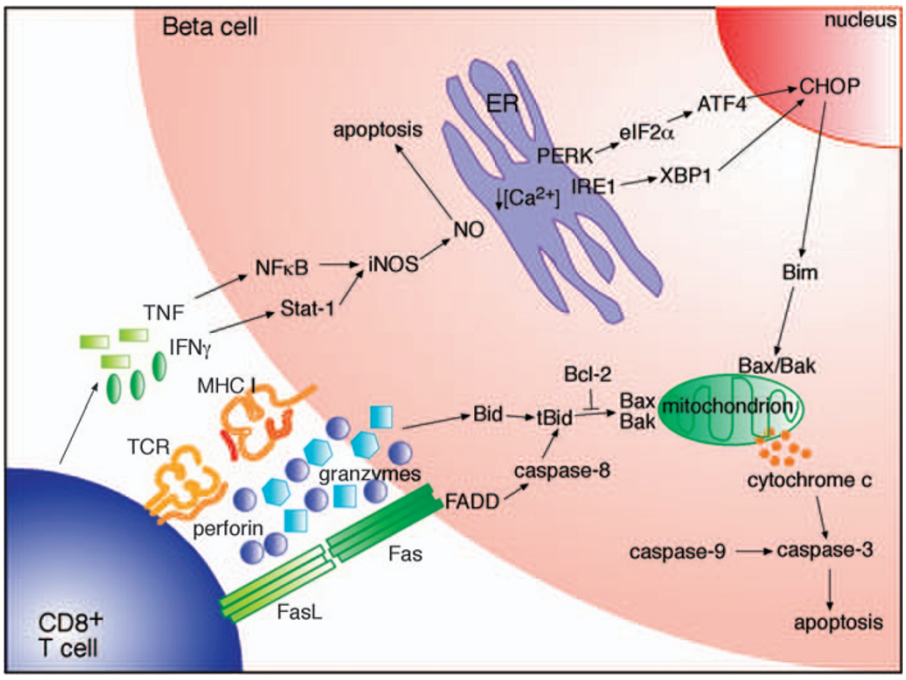

Figure 2 CD8$^+$ T-cell-dependent death of human β-cells. CD8$^+$ T cells recognize β-cells through class I MHC-T cell receptor (TCR) interactions. Mechanisms of killing include perforin and granzymes or Fas/FasL that induce the mitochondrial or Bcl-2-regulated apoptosis pathway; and inflammatory cytokines that induce free radical production and ER stress

and confirmed independently by another group.[34] A dose-effect was seen in two independent backcrosses of perforin-deficient NOD mice both of which had significantly reduced diabetes in heterozygous mice compared with wild-type mice and lowest frequency of all in homozygous perforin-deficient mice.[33,34]

We found that insulitis in perforin-deficient NOD mice is significantly reduced,[35] although originally reported to be the same as wild-type NOD mice.[33] Reduced insulitis is an unexpected result if the mechanism of protection is decreased β-cell death, raising the possibility that other factors such as impaired lymphocyte homeostasis, previously reported in perforin-deficient mice,[36] contributes to the reduced pathology. Another interpretation is that β-cell destruction is needed for full development of insulitis, and this is not observed in perforin-deficient mice. Reduced β-cell damage may result in insufficient antigen release to fuel the fire of autoimmunity.

Perforin is acknowledged to be the dominant molecular effector mechanism that destroys β-cells in NOD mice and the only effector mechanism for which genetic deficiency is protective from diabetes. However, there is clearly a role for other death pathways, because a small proportion of perforin-deficient NOD mice still develop diabetes. Also, anti-CD8 therapy is less effective in older mice suggesting other cells, such as CD4$^+$ T cells, become important.[37] To block the multiple effector mechanisms of diabetes in NOD mice, we made perforin-deficient NOD mice that overexpress suppressor of cytokine signaling (SOCS)-1 in β-cells (RIP–SOCS-1 mice).[35] SOCS-1 blocks signaling through multiple cytokine receptors,[38] the most relevant ones to type I diabetes being type 1 and 2 interferons. IL-1 may also be important in type I diabetes, but its signaling in β-cells does not appear to be inhibited by overexpression of SOCS-1. By blocking IFNγ, SOCS-1 overexpression also prevents upregulation of Fas on beta cells because its expression depends on IFNγ signaling.[39] Therefore β-cells from RIP–SOCS-1/perforin$-/-$ mice are resistant to effects of perforin, FasL and inflammatory cytokines, which constitute all of the well characterized mechanisms of killing β-cells. Surprisingly, these mice developed diabetes at the same low rate and onset as perforin-deficient NOD mice.[35] These data strongly suggest that there are alternative effector mechanisms that can kill β-cells in the absence of perforin, FasL and cytokines and cause diabetes in a small proportion of individual mice. Such alternative mechanisms remain unknown, but are likely to be mediated by macrophages or CD4$^+$ T cells.

Perforin and Granzyme Gene Expression in Islet-Infiltrating T Cells. Of the granzyme family of serine proteases, granzyme A and B are the most common in human and mouse. Granzyme B has a well-described caspase-dependent pro-apoptotic function by cleaving substrates after aspartate residues. Granzyme A induces single-strand DNA breaks through cleavage of SET complex components. Other granzymes including H, K and M in humans and C, D, E, F, G, K, L, M and N in mice are less well characterized.[2] The specific roles of the granzymes in β-cell destruction *in vivo* are not yet known, but will become clearer when individual granzyme genes are knocked out in NOD mice.

Perforin and granzymes, as well as cytokines including TNFα and IFNγ, have been detected in CD8$^+$ T-cells infiltrating islets of NOD mice by *in situ* hybridization, immunohistochemistry and flow cytometry.[3,40] An increased

number of serine protease-positive lymphocytes was also observed in biobreeding rats developing diabetes compared with control rats.[41] The expression of perforin, granzyme A and granzyme B do not appear to be uniform in infiltrating T cells, with as few as 3–10% of CD8$^+$ T cells expressing these molecules at detectable levels at any one time.[3,42] This is perhaps not surprising as it has been shown by single-cell PCR that individual T cells, even those within a clone, differ widely in their expression patterns of perforin, granzymes A–C and IFNγ.[43]

Granzymes as Markers of Lymphocyte Activation. During viral infection, naive CD8$^+$ T cells are activated in secondary lymphoid tissues such as the draining lymph node, resulting in generation of antiviral effector CD8$^+$ T cells. These activated CD8$^+$ T cell rapidly acquire the ability to produce effector molecules including IFNγ, TNFα and granzyme B in response to antigen stimulation. Granzyme B production is closely associated with cell division, with the ability to produce granzyme B being acquired after the first division and increasing thereafter.[44] In type I diabetes, CD8$^+$ T cells are also activated in the pancreatic lymph node, and acquire the ability to produce effector cytokines. We showed that *in vitro*, antigen-specific CD8$^+$ T cells produce granzyme B after stimulation with islets,[45] and this is also the case *in vivo* (K Graham and T Kay, unpublished).

Killing Pathways *in vitro*. Using a number of different model systems, the killing mechanisms used by diabetogenic CTL have been studied *in vitro* in conventional ^{51}Cr release assays. Overall the results suggest that when non-β-cell targets are used *in vitro*, CTL can effectively use either the perforin or Fas/FasL pathways to kill. Both pathways can also be used by CTL to kill β-cell targets, however, the perforin pathway is dominant, and a role for the Fas/FasL pathway is only observed in the absence of perforin, shown either with the inhibitor concanamycin A or CTL from perforin gene-disrupted mice (Figure 3).

TCR Transgenic Mouse Models of Type I Diabetes. The precursor frequency of β-cell antigen-specific T cells in NOD mice is very low, making it difficult to study these cells *in vitro*. Therefore transgenic mice expressing T-cell receptors (TCR) of diabetogenic T-cell clones have been very useful in the

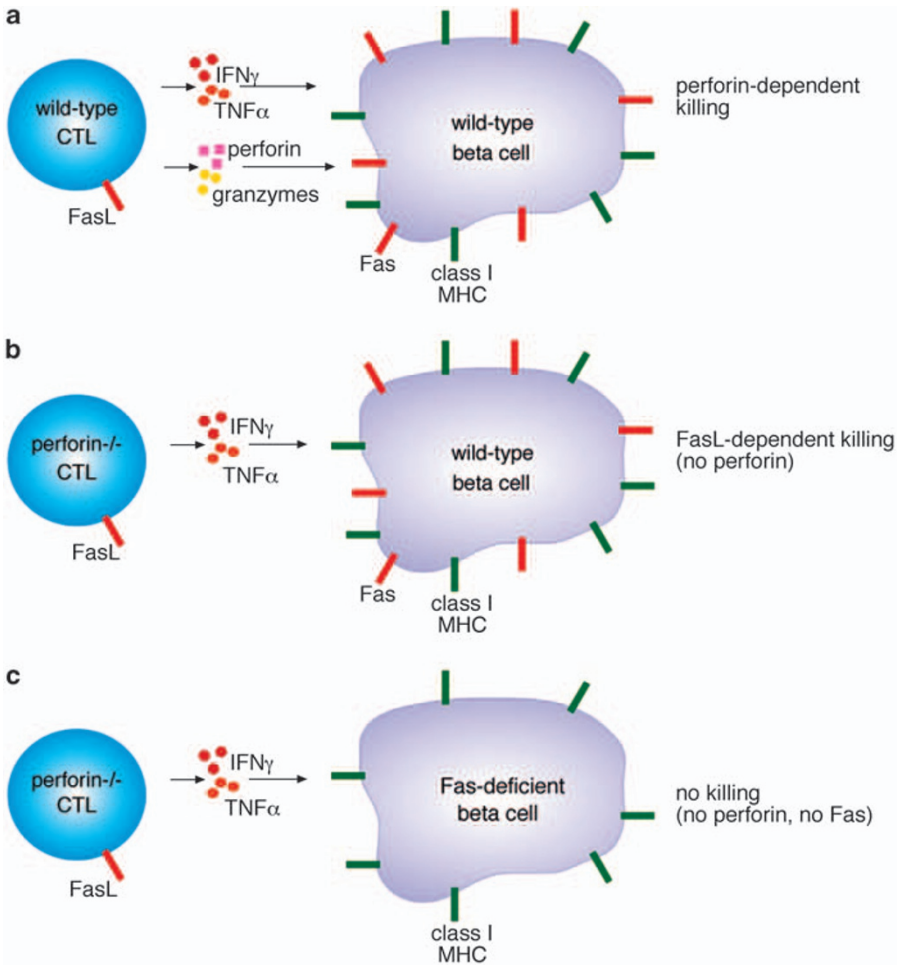

Figure 3 *In vitro* killing of β-cells. *In vitro*, activated CTL produce inflammatory cytokines, IFNγ and TNF that induce upregulation of Fas and class I MHC on β-cells. (**a**) Wild-type CTL kill β-cells using perforin and granzymes. (**b**) In the absence of perforin, CTL use the Fas/FasL pathway to kill β-cells. (**c**) *In vitro*, absence of both perforin in CTL and Fas on β-cells prevents killing. It remains likely that *in vivo*, other mechanisms are able to kill β-cells in the absence of functional Fas and perforin pathways

study of effector mechanisms of β-cell destruction. TCR transgenic NOD8.3 CTL recognize the β-cell antigen islet-specific glucose-6-phosphatase catalytic subunit-related protein.[19,46] NOD8.3 T cells develop and mature normally in the absence of perforin, and proliferate normally in response to islet antigens.[34] Although originally reported to use Fas-dependent killing, we found that NOD8.3 T cells remained capable of killing Fas-deficient NOD$^{lpr/lpr}$ islets, but had reduced ability to kill β-cells overexpressing SOCS-1.[45] This is likely to be because of reduced recognition of these transgenic β-cells that have basal but not upregulated levels of class I MHC on their surface, because of their inability to respond to IFNγ. Overexpression of SOCS-1 did not affect the ability of islets to be killed by recombinant perforin and granzyme B. *In vivo*, perforin-deficient NOD8.3 mice develop type I diabetes with normal incidence, suggesting that diabetes can occur independent of perforin.[34] However, Fas-deficient islets were effectively destroyed when grafted into NOD8.3 mice, suggesting β-cell destruction can occur independent of the Fas/FasL pathway also.[45] The exact mechanism by which CTL kill β-cells *in vivo* remains to be elucidated, and is a very important question to aid the design of therapies that can block β-cell destruction. The most straightforward interpretation of the data in NOD8.3 mice is that more than one mechanism has a role. When one is removed, another is able to complete β-cell destruction. Although it is possible that these mechanisms are perforin/granzyme and FasL/Fas, the data do not exclude other additional pathways.

Effector mechanisms of CTL have also been tested in systems where β-cells express a neo-antigen enabling them to be killed by antigen-specific TCR transgenic CTL. Such models include β-cells expressing ovalbumin (RIP-mOVA), hemagglutinin (Ins-HA) or the glycoprotein from lymphocytic choriomeningitis virus (LCMV-GP). Islets from these transgenic mice have been used as targets for CTL specific for these antigens. Using ovalbumin-specific CTL from OT-1 transgenic mice, we found that perforin was the dominant mechanism of killing; however, in the absence of perforin, there was residual killing that could be prevented by blocking the Fas/FasL pathway either by genetic deficiency of functional Fas (lpr), or preventing Fas upregulation (e.g., with RIP–SOCS-1).[47] Similarly, clone-4 CTL recognizing influenza hemagglutinin killed Ins-HA transgenic islets using both perforin and Fas/FasL pathways, but *in vivo* 30-fold more perforin-deficient CTL were required to induce diabetes.[48]

When LCMV–GP mice are infected with LCMV they develop autoimmune diabetes because of activated LCMV-specific CD8$^+$ T cells recognizing β-cells expressing the viral glycoprotein.[49] Perforin-deficient LCMV–GP mice do not develop diabetes.[50] However, these mice die prematurely because of failure to efficiently clear the viral infection. To bypass this, antigen-specific T cells deficient in perforin were transferred into LCMV–GP mice and these also failed to induce diabetes. Although the mechanisms used by a T-cell clone may not be the same as endogenous T cells, these data suggest that perforin has an essential role in the destruction of beta cells in this virus-specific model. However, perforin is not the only player, because LCMV–GP mice deficient in IFNγ, lacking functional IFNγ receptors on β-cells, or overexpressing SOCS-1 in β-cells also do not develop diabetes, suggesting an important role for IFNγ.[51–53]

In an effort to elucidate the killing pathways used by human CTL to kill human islets, we devised a model system using CTL specific for viral peptides and human islets loaded with specific peptide. We used HLA-A2-restricted CTL specific for the matrix peptide of influenza virus, and HLA-B8-restricted Epstein–Barr virus-specific CTL. Using both of these clones, we showed that in a short term (5 h) assay *in vitro*, killing of human islets is perforin dependent.[54] Although this model system may not be the same as genuine autoreactive CTL, and there are likely to be differences in affinity of the CTL for viral peptides compared with autoantigens, the system has the potential to test the efficacy of blocking killing pathways in human islets.

Genetic Association

Putative inactivating missense mutations in the perforin gene (*PRF1*) in humans (A91V and N252S) have recently been associated with type I diabetes,[55] although these alleles were found in the heterozygous state and a plausible mechanism to explain the association has not been proposed. As it is proposed that both alterations of protein sequence result in reduced perforin function, it is not intuitively clear how this would result in greater β-cell destruction. Although A91V-perforin clearly has reduced activity[56] and has been associated with an increased risk of familial hemophagocytic lymphohistiocytosis, another study failed to show a functional abnormality for the N252S perforin variant.[57] The association of genes encoding perforin and Fas[58] with type I diabetes suggest that predisposition to autoimmunity can occur when there are defects in genes controlling β-cell destruction as well as immune homeostasis and downregulation of the immune response, as is the case for both of these genes.

Allogeneic Islet Graft Rejection

The development of allogeneic islet transplantation as a treatment for type I diabetes[59] has opened up the possibility of testing therapies for improvement of islet survival. Although technical and ethical issues will need to be sorted out, the modification of islets either chemically or genetically before transplantation is an attractive method of improving the outcome of islet transplantation.

Allogeneic islet grafts are destroyed by CD8$^+$ T cells in a class I MHC-dependent manner. In the absence of CD8$^+$ T cells, graft rejection proceeds, although at a slower rate.[60] *In vitro*, perforin deficiency prevents allogeneic rejection of the insulinoma cell line NIT-1 and primary islet target cells in an 'early phase' of killing (5 h assay). When CTL are incubated with target cells for longer (24 h), killing by perforin-deficient CTL is restored, and this killing is at least in part because of TNFα-dependent mechanisms.[61]

Islet allografts are rejected in a perforin and Fas-independent manner *in vivo*. Transplantation of Fas-deficient (lpr) islets into perforin-deficient recipients resulted in efficient graft rejection.[62] There is evidence that allogeneic rejection of islets is contact-dependent because grafts consisting of mixed syngeneic and allogeneic islets resulted in the destruction of

Table 1 Effect of class I MHC modification in NOD mice

Modification	Class I MHC expression	$CD8^+$ T cells	Insulitis/Diabetes	Reference
$\beta_2$microglobulin−/−	No class I MHC	None	No insulitis No diabetes	10–13
RIP-β_2m/β_2m−/−	Class I MHC only on β-cells	None	Reduced insulitis No diabetes	15
β-bald	Class I MHC everywhere except β-cells	Normal	Normal insulitis Reduced diabetes	14
APC-bald	Class I MHC everywhere except APC	None	Reduced insulitis No diabetes	16
RIP-SOCS1	Basal levels of class I MHC on β-cells	Normal	Normal insulitis Reduced diabetes	45,85
RIP-E3	Reduced class I MHC on β-cells	Normal	Reduced insulitis Reduced diabetes	21

β_2m, $\beta_2$microglobulin; APC, antigen-presenting cells; RIP, rat insulin promoter; SOCS1, suppressor of cytokine signalling-1; E3, adenovirus E3 protein.

only the allogeneic islets.[63] It remains unclear how allografts are destroyed *in vivo*, but clearly perforin-independent mechanisms exist.

Elevated CTL gene expression in peripheral blood and urine has been reported to correlate with clinical renal allograft rejection.[64,65] Han *et al*.[66] performed similar studies in recipients of human islet allografts to predict rejection. Granzyme B, perforin and FasL were detected preceding allograft rejection (return to hyperglycemia and insulin requirement), with granzyme B being the best predictor of islet graft rejection. These studies suggest that granzyme B may be a useful predictor of islet allograft loss before onset of clinical symptoms.

Perforin/Granzyme Signaling in β-Cells

Details of how perforin and granzymes induce cell death have been significantly clarified in recent years (reviewed in Bolitho *et al*.[2]). Perforin is absolutely required for granzymes to function by allowing access to their targets but it does not simply create a membrane hole that granzymes pass through. Granzymes enter the cytoplasm by endocytosis. They cleave target molecules and trigger cell death by apoptosis.

We were the first to study the molecular events in the β-cell in response to recombinant perforin/granzyme B.[67] We showed that *in vitro*, dispersed islet cells are lysed by high concentrations of perforin that are sufficient to induce direct plasma membrane damage without apoptosis. However, whole islets are relatively insensitive to perforin, and this is because of the inability of the highly lipophylic perforin to penetrate the islet to affect the cells in the center. Granzyme B, on the other hand, efficiently enters islet cells and whole islets. These findings are of less significance when these molecules are delivered in a contact-dependent manner by CTL, but indicate that local delivery of perforin is important, with very little bystander effect.

When islet cells are incubated with sublytic doses of perforin plus recombinant human granzyme B, loss of insulin secretory function and apoptosis occurs.[67] This apoptosis is caspase dependent, involving the release of cytochrome *c* from the mitochondrial outer membrane. Release of cytochrome *c* was not affected by inhibition of caspase activation with zVAD.fmk. This is in contrast to the release of cytochrome *c* induced by FasL, which was blocked by caspase inhibition. Caspase inhibition prevented apoptosis induced by perforin and granzyme B, indicating that caspase activation is downstream of the disruption of mitochondrial function (Figure 2).

The involvement of the mitochondria suggests requirement for the Bcl-2 family of pro- and anti-apoptotic molecules (reviewed in Youle and Strasser[68]). We found that the BH3-only protein Bid is activated by incubation of islet cells with perforin and human granzyme B, and deficiency of Bid prevents granzyme B-dependent apoptosis.[67] We also showed that Bid-deficient islets are protected from Fas/FasL-induced apoptosis, indicating that Bid is activated in β-cells in response to death receptor stimulation (Figure 2).[69] The requirement for Bid for these two important effector mechanisms of β-cell death in diabetes has implications for designing protective strategies for human islets. Because multiple cytotoxic stimuli appear to signal through a similar pathway, one therapeutic intervention might be sufficient.

Although Bid is efficiently cleaved by human granzyme B, it is not cleaved as efficiently by mouse granzyme B, thus favoring activation of apoptosis through direct caspase activation.[70–72] In fact, mouse granzyme B is approximately 30-fold less cytotoxic than human granzyme B.[72] This difference in the substrate specificity and intracellular-signaling pathways in response to granzyme B is important when using mouse models of diabetes; however, the role of Bid in apoptosis induced by perforin and granzyme B remain valid for the prevention of human islet cell death by CTL. Blocking Bid cleavage and activation in human islets would be an effective strategy to test in prevention of both granzyme B- and FasL-mediated apoptosis.

Strategies to Prevent Perforin/Granzyme-Mediated β-Cell Death

CTL are protected from their own granule contents. A better understanding of the mechanism of this protection would suggest ways to transgenically protect the β-cell. A molecule capable of protecting β-cells from the granule contents of CTL would be potentially useful if it could be delivered to the right place without adverse effects on β-cell function.

Serine protease inhibitors or serpins regulate the serine protease family, including granzyme B. SERPINB9 or PI-9 in human and Spi6 in mouse are expressed in the cytoplasm of CTL and NK cells and confer resistance to granzyme B with species specificity (reviewed in Mangan *et al.*[73]). It is believed that these inhibitors exist in cytotoxic lymphocytes to prevent apoptosis because of misdirected granule contents, and indeed mice lacking Spi6 have impaired survival of CTL because of granzyme B-mediated apoptosis.[74] The use of serpins in preventing granzyme B-mediated apoptosis of β-cells is attractive; however, overexpression of PI-9 in human islets would need to be accompanied by inhibitors of other pathways of apoptosis such as FasL or pro-inflammatory cytokines. Therefore, the use of such a specific apoptosis inhibitor for prevention of islet graft rejection is perhaps limited.

The central role for Bid in islet apoptosis, as mentioned above, makes it a good candidate for protection of islets from immune-mediated killing. The species specificity of granzyme B in its ability to cleave Bid – -human granzyme B can cleave Bid whereas mouse granzyme B cannot – make this difficult to test in mouse models. However, the increase in human islet allotransplantation worldwide has made human islets readily available for research, making it possible to test many protective strategies in human systems, at least *in vitro*.

Caspase inhibition is an attractive method for prevention of β-cell apoptosis because most of the known mediators of β-cell death activate caspases. The caspase inhibitor XIAP has been used to prevent islet cell death *in vitro* mediated by pro-inflammatory cytokines or by immunosuppressive drugs, which are toxic to islets.[75] *In vivo*, XIAP overexpression in mouse islets provided protection from allograft rejection,[76] suggesting that it is able to block the onslaught of killing mechanisms, including perforin and granzymes. This strategy warrants further testing *in vivo*.

A Potential Role for Granzymes in Type II Diabetes

Although the idea that inflammation is associated with obesity and type II diabetes is not new, it has recently gathered momentum.[77] It is proposed that cytokines, adipokines and chemokines are produced within adipose tissue, and these activate intracellular-signaling pathways that promote insulin resistance and type II diabetes. IL-1, IL-6 and TNFα have attracted attention as mediators of insulin resistance. In particular, IL-1 can impair insulin signaling, and may act with other cytokines to directly induce β-cell damage.[78]

The recent discovery that granzymes, in particular granzyme A, can promote the release of pro-inflammatory cytokines such as IL-1β, and therefore promote inflammation,[79] suggest a new potential role for granzymes in the development of insulin resistance and type II diabetes. Granzyme A can be detected in the serum of patients with inflammatory disorders such as rheumatoid arthritis,[80] and this suggests granzyme A may be able to act independent of perforin and its delivery by cytotoxic cells.[81] It is not yet known if granzyme A can be detected in patients with insulin resistance or type II diabetes.

Conclusions

Although the importance of perforin in β-cell destruction has generally been accepted by the diabetes research community, there are very few studies to follow up the findings in perforin-knockout NOD mice that are now many years old. Many questions need answering such as the factors that influence the expression of perforin and the role of perforin expression in determining the rate of progression to diabetes, whether specific granzymes are important in β-cell death, whether perforin-dependent killing can be blocked, and whether human diabetes is perforin-dependent.

There is no question that clinical application of this information about β-cell destruction from mouse models is made problematic by redundancy of mechanisms, but the levels of protection seen in NOD mice with perforin deficiency are clearly worthwhile, if incomplete, and may be fully effective with other interventions. As perforin is directly implicated in β-cell damage, both directly and through facilitating the delivery of granzymes, it is potentially an important target for pharmacological inhibition. The first small molecule inhibitors of perforin were recently described,[82] and await evaluation in mouse models of type I diabetes and other perforin-dependent immunopathologies. Long-term generalized inhibition of perforin is unlikely to be a therapeutic option based on the clinical condition called familial hemophagocytic lymphohistiocytosis because of perforin deficiency.[83,84] However, the significant reduction in diabetes observed with heterozygous perforin deficiency suggests that partial perforin blockade, or inhibition of perforin action at the right time would be efficacious. As an alternative, shorter-term perforin inhibition or modulating its toxic effects on β-cells may be more feasible in the protection of transplanted cadaveric human islets from allo rejection.

Conflict of interest

The authors declare no conflict of interest.

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