[Frontiers in Bioscience 14, 644-656, January 1, 2009]

Interferon signalling in pancreatic beta cells

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1. ABSTRACT

Type 1 diabetes results from apoptotic destruction of insulin-producing beta cells by a range of effector molecules produced by immune cells that infiltrate pancreatic islets. Interferons are found within the inflammatory infiltrate of islets during progression to type 1 diabetes. Interferons can promote the action of effector cells that induce beta cell death. They can also act directly on islet cells to induce gene expression, and together with other cytokines they can cause beta cell death. Because of their pleiotropic nature, it was proposed that this family of cytokines may be involved in type 1 diabetes development. In the non-obese diabetic mouse model, interventions have been made at multiple points in the signalling pathways of interferons. This review aims to construct a clear picture of the outcomes of these interventions to determine how interferons are involved in the pathogenesis of type 1 diabetes.

2. INTRODUCTION

Type 1 diabetes (T1D) results from autoimmune destruction of pancreatic beta cells and subsequent loss of glucose homeostasis requiring daily insulin injections. There are an estimated 4.7 million sufferers of T1D worldwide and this number is increasing. Even with intensive insulin treatment, long term complications including blindness, kidney failure and heart disease occur. This results in an enormous economic and personal cost.

Because of the lack of clinical symptoms until almost all beta cells are destroyed, and the inaccessibility of the pancreas, it is difficult to study the pathogenesis of T1D in humans. For this reason, animal models of T1D have been developed. The most widely studied of these is the non-obese diabetic (NOD) mouse. NOD mice have many of the key features of the human disease (1). Like in humans, there is a complex genetic susceptibility, and a

long time course of disease, with mice developing clinical signs around 4-6 months of age. There is also a critical role for T cells in the pathogenesis of disease in NOD mice and humans alike.

2.1. Beta cell destruction

In T1D, beta cell destruction occurs by selective apoptosis of beta cells, leaving other islet cells including glucagon-producing alpha cells, intact. Apoptotic beta cells, and beta cells expressing molecules that induce apoptosis, such as the Fas death receptor, have been detected in sections of pancreas from mouse models of T1D and newly diagnosed human subjects (2-5). Beta cell apoptosis is induced by a range of effector molecules produced by immune cells that infiltrate the pancreas (6, 7).

A key feature of the pre-diabetic pancreas is the presence of an inflammatory infiltrate surrounding and subsequently invading the islets. In NOD mice, infiltration begins before three weeks of age with the first cells detectable being antigen presenting cells (APC) such as dendritic cells (DC) and monocytes (8). Lymphocytes (including B and T cells) arrive after weaning. There is excellent evidence for a role for T cells in the development of T1D in both humans and mice. Disease can be adoptively transferred from diabetic donors into immunocompromised recipients. Additionally, onset can be prevented in the absence of T cells, for example after treatment with T cell immunosuppressants or after neonatal thymectomy (9-12). In humans, biopsies show T cell infiltration with a predominance of $CD8^+$ T cells (13).

Spontaneous diabetes is a complicated process involving many cell types and quite likely multiple effector mechanisms. Both $CD4^+$ and $CD8^+$ T cells are important for disease and both can affect beta cell destruction. There are diabetogenic $CD4^+$ and $CD8^+$ T cell clones in mice that can cause disease on their own (14, 15). We and others, have used these clones to determine the killing mechanisms used by different types of T cells in NOD mice. $CD8^+$ T cells use predominately the granule exocytosis pathway, in the absence of which, they can use the Fas/Fas ligand (FasL) pathway (16). The mechanism used by $CD4^+$ T cell clones is less clear. It has been hypothesised that they kill beta cells using the Fas/FasL pathway and by secretion of pro-inflammatory cytokines that are toxic to beta cells, however a definitive role for either of these pathways in CD4-mediated beta cell destruction has not been determined. Nevertheless, there is a large amount of literature citing a role for pro-inflammatory cytokines in beta cell destruction.

2.2. Cytokines and their role in type 1 diabetes

It has long been known that cytokines can kill beta cells *in vitro (*17). Combinations of IFNγ with cytokines that activate the NFκB pathway including IL-1 and TNF are highly toxic to islets, inducing as many as 90% of islet cells to die in 2-6 days. This toxicity is due to cytokine-mediated upregulation of inducible nitric oxide (NO) synthase (iNOS), which contains both IFNγ and NFκB responsive elements in its promoter. NO is then produced at high levels by beta cells, leading to their death. Because cytokines appear to be present in islet lesions, and they are toxic to beta cells *in vitro*, cytokine-induced death has been studied by many as a possibly important mechanism of beta cell destruction leading to T1D. The actions and roles of inflammatory cytokines and NO have been extensively reviewed elsewhere (18). This review will focus on interferons and their role in the pathogenesis of T1D.

3. INTERFERON SIGNALLING

Interferons (IFNs) are a family of cytokines originally identified in the 1950s because of their antiviral properties. There are two major types of IFNs, type 1 (including IFNα, IFNβ and a host of other IFNs), and type 2 or IFNγ. These different IFNs have overlapping but distinct signalling pathways (Figure 1) and biological activities (reviewed in (19-21)).

Type 1 IFNs bind to the two receptor chains IFNα-R1 and IFNα-R2. After IFN binding, the receptor-associated tyrosine kinases Tyk2 and Jak1 are autophosphorylated and activated. This then leads to phosphorylation of the transcription factors Stat1 and Stat2. Stat1 homodimers or Stat1-Stat2 heterodimers form, and translocate to the nucleus together with the adaptor IRF9, to form a complex called ISGF3. ISGF3 binds to the IFN-stimulated response element (ISRE) DNA sequence in promoters of IFN-responsive genes to induce transcription. Stat1 homodimers bind to the γ-activated sequence (GAS) in gene promoters.

IFNγ binds the pre-assembled complex of IFNγ-R1 and IFNγ-R2. The signalling pathway of IFNγ is largely the same as that of type 1 IFNs, except that the ISGF3 complex is not formed, only Stat1 homodimers. Stat1 becomes phosphorylated on tyrosine residues by receptor-associated Jak1 and Jak2. Activated Stat1 dimers translocate to the nucleus and induce expression of genes that contain a GAS site in their promoter. These molecules and pathways are highly conserved across species including human and mouse.

One of the puzzles since the Jak/Stat signalling pathway was elucidated in the 1990's has been to determine how this single pathway can generate all of the pleiotropic effects of IFNs. IFNs induce hundreds of genes activating a variety of biological responses. Some genes have only an ISRE, some have only a GAS and some have both, thus different combinations of transcription factors may be required for transcription of different genes. In addition, it is increasingly clear that other pathways can be activated by IFNs, including among others, the MAPK (22) and PI3K (23) pathways, suggesting that the Jak/Stat pathway alone is not sufficient for all the biological effects of IFNs. Stat1 can also be phosphorylated on serine residues, and while this is not required for dimerization or binding to DNA elements, serine phosphorylation is crucial for some of the functions of Stat1, as was determined in gene targeting studies (24).

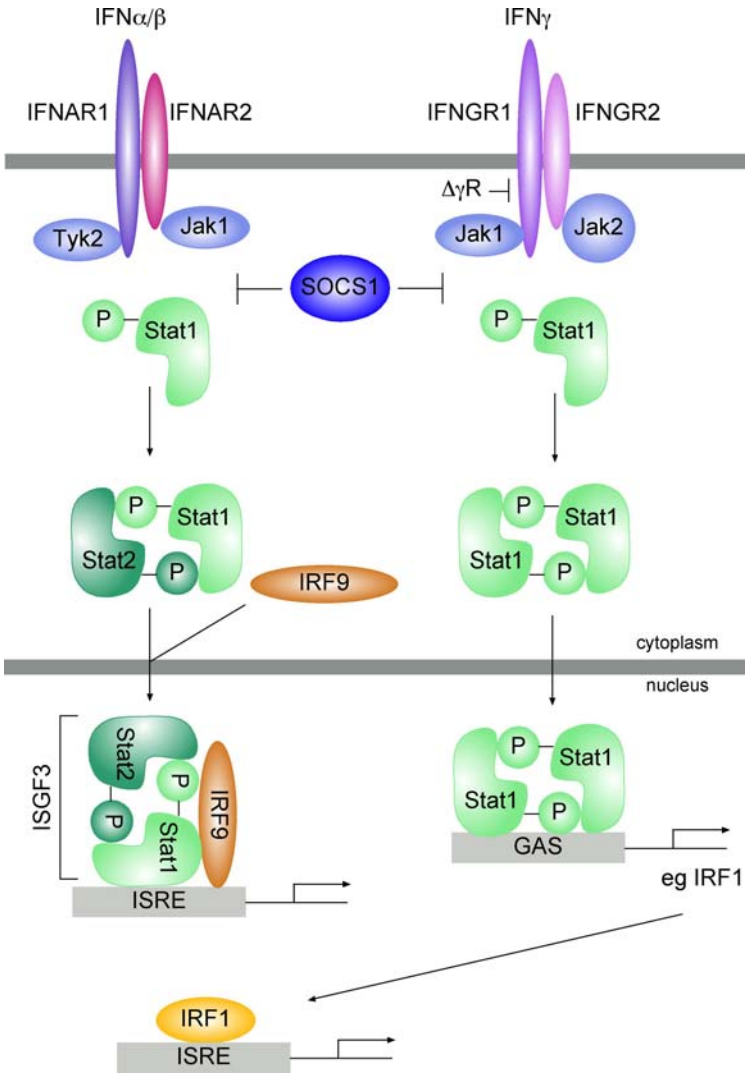

Figure 1. Interferon signalling pathways. Diagram outlining the signalling events that take place when type 1 (IFNα/β) or type 2 (IFNγ) bind to their specific cell surface receptors, IFNAR and IFNGR respectively. Also shown are points at which interventions have been made in NOD mice, including dominant negative IFNγ receptor (ΔγR) and suppressor of cytokine signalling-1 (SOCS1).

Interferon responsive factors (IRFs) are a family of transcription factors activated by IFNs (reviewed in (25, 26)). IRF1 is constitutively expressed in a variety of cell types, and its expression is greatly increased by viral infection or by IFNγ stimulation. Induction of IRF1 expression is mediated by Stat1 and NFκB binding to responsive elements in its promoter. IRF1 then binds to an ISRE to activate expression of genes including type 1 IFNs and others. IRF1 functions as a regulator of cellular responses to IFNs.

Uncontrolled IFN signalling is dangerous, particularly for the immune system. IFN signalling is largely regulated by suppressor of cytokine signalling (SOCS) molecules (27-29) and protein inhibitors of activated Stats (PIAS) (30). SOCS proteins suppress signalling by ubiquitin-mediated degradation of the signalling complex, by directly inhibiting Jak activity through a kinase-inhibitory region, or by preventing binding of Stats to the activated cytokine receptor. PIAS bind to Stats to inhibit Stat-mediated gene activation.

Expression of SOCS proteins is induced by cytokines, therefore they function in a classical negative feedback loop to inhibit cytokine signal transduction (31-33). While overexpression studies of these molecules have suggested that they have promiscuity in their activity, *in vivo* studies using gene targeting have demonstrated that their physiological roles are probably more limited. For example, SOCS1-deficient mice develop a multi-organ inflammation and die as neonates due to liver failure (34, 35). This disease can largely be prevented by combining SOCS1-deficiency with IFNγ-deficiency, suggesting that SOCS1 is a specific inhibitor of IFNγ signalling *in vivo* (36,

Table 1. Spontaneous diabetes in transgenic and knockout mice on a NOD genetic background

Strain	Onset	Incidence	Result	Reference
NOD[1]	10-20wk	70-100%	Standard	
IFNγ$^{-/-}$	25wk	35% (wt 50%)[2]	Delay	62
IFNγR1$^{-/-}$	19wk	75%	Standard	65
IFNγR2$^{-/-}$	15wk	85%	Standard	63
Stat1$^{-/-}$	N/A[3]	0%	Absent	74
IRF1$^{-/-}$	N/A[3]	0%	Absent	75
RIP-ΔγR	17wk	30% (wt 40%)[2]	Standard	68
RIP-SOCS1	17wk	11-45%	Reduced	67, 87

[1] Incidence for NOD mice is a range from individual experiments. [2] Wild type (wt) incidence was lower than the normal range for diabetes incidence. [3] Not applicable (N/A). Diabetes was absent therefore no age of onset.

37). In addition to regulation by PIAS and SOCS proteins, IFN signalling is also terminated by other intra-cellular regulators and by reduction in local extra-cellular IFNγ concentration.

IFNs regulate expression of a number of genes that may be involved in activation of T cells or beta cell destruction. Of particular relevance are class I MHC (and also genes in the class I antigen processing pathway) (38), Fas (4, 39), chemokines such as IP-10 (40), iNOS (41, 42) and SOCS genes (43, 44). Target genes of IRF1 include cytokines (in particular type 1 IFNs) and molecules in the class I MHC antigen processing pathway. Expression of type 1 and 2 IFNs, and genes regulated by these cytokines has been observed in the islets of both NOD mice and humans with recent diagnosis of T1D (45-47). Because of the range of genes that are regulated by IFNs, it was proposed that this family of cytokines may be involved in T1D development.

Many of the components of type 1 and type 2 IFN signalling have been knocked out and the knockouts investigated for susceptibility to diabetes. IFNγ, both chains of its receptor, Stat1, and IRF-1 have all been knocked out and back crossed to the diabetes susceptible NOD genetic background. The aim of this review is to examine the similarities and differences in the results of these experiments to construct a coherent picture of how IFNγ is involved in the pathogenesis of diabetes. Less has been done with type 1 IFNs. Additional features of Stat1 knockouts over IFNγ knockouts or IFNγ receptor knockouts provide some insight into the role of type 1 IFNs in the NOD mouse.

4. TYPE 1 INTERFERONS IN DIABETES

Type 1 IFNs are a family with multiple subtypes of IFNα, a single IFNβ as well as other less common subtypes. They exert a vast array of biological functions by modulation of hundreds of genes. Viruses have been implicated in the aetiology of T1D. Cytokines such as IFNα are produced in response to activation of toll like receptors (TLR) by viral double stranded RNA or the viral mimic poly I:C. IFNα can then lead to activation or acceleration of T1D by a number of different mechanisms including activation of DCs, proliferation of T cells, mitochondrial suppression or direct beta cell apoptosis (reviewed in (48-50)).

There is evidence that type 1 IFN can promote development of T1D both in mice and humans. Elevated levels of IFNα were detected in the serum (51) and pancreas of patients with T1D (45, 52). Transgenic expression of IFNα (53), IFNβ (54) or IFNκ (55) in beta cells leads to inflammatory infiltrates and beta cell destruction in non-autoimmune strains of mice. Neutralization of IFNα with a monoclonal antibody prevented diabetes in IFNα transgenic mice, demonstrating that IFNα was the cause of diabetes in these mice (53). Poly I:C induces diabetes in BB rats, in mice that express the costimulatory molecule CD80 on beta cells, and in streptozotocin-treated mice (56-58). BALB/c mice that are immunized with the insulin B-chain peptide B:9–23 and poly I:C develop insulin autoantibodies and insulitis (57). If IFNα is given in later stages of the disease, it can have protective effects. Poly I:C administration to NOD mice prevents diabetes (59). Oral IFNα delayed the onset of T1D in NOD mice (60) and to a lesser extent in human clinical trials (61).

Therefore if given early, type 1 IFNs or viral mimics can promote disease. IFNα may favour the development of a Th1-type response, leading to stimulation of IFNγ-producing CD4$^+$ T cells. IFNα can also upregulate class I MHC leading to better antigen presentation on beta cells.

5. IFNγ AND TYPE 1 DIABETES

Several studies using backcrossed IFNγ-, IFNγR1- and IFNγR2-deficient NOD mice demonstrated that an IFNγ/IFNγR interaction is not required for development of spontaneous diabetes in NOD mice (Table 1). IFNγ-deficient (62) and IFNγR2-deficient (63) NOD mice displayed a normal incidence of T1D with a slight delay in onset. IFNγR1-deficient mice, however, were completely resistant to disease, with only a few mice showing signs of mild insulitis (64). This result led to a great deal of speculation about the mode of action of IFNγ, until Kanagawa *et al* demonstrated that a genetic region from the 129 mouse strain was located next to but distinct from the IFNγR1 locus on chromosome 10 that was responsible for the association of this genetic region with diabetes (65). The diabetes resistance gene in this region has not yet been identified. When IFNγR1-deficient NOD mice with a congenic interval that excluded the disease resistance gene were backcrossed 13 times, mice developed

Table 2. Onset and incidence of diabetes after adoptive transfer of splenocytes from diabetic donor mice

Donor	Recipient	Onset	Incidence	Result	Reference
Diabetic NOD	NOD	2wk	100%	Standard	
	IFNγ$^{-/-}$	4wk	33%	Delayed + reduced	66
	IFNγR2$^{-/-}$	3wk	80%	Slightly delayed	66
	Stat1$^{-/-}$	4wk	80%	Delayed	74
	RIP-ΔγR	2wk	80%	Standard	68
	RIP-SOCS1	2-6wk	80-100%	Standard/slightly delayed	67, 87
Diabetic IFNγ$^{-/-}$	NOD	2wk	100%	Standard	66
	IFNγ$^{-/-}$	2wk	50%	Delayed + reduced	66
Diabetic IFNγR2$^{-/-}$	NOD	2wk	100%	Standard	66
	IFNγ$^{-/-}$	6wk	50%	Delayed + reduced	66

T1D with the same kinetics as NOD mice. These experiments highlight the importance of adequate backcrossing and analysis of genetic intervals when studying gene knockout mice backcrossed onto the NOD background.

Adoptive transfer experiments (Table 2) revealed a possible role for IFNγ in extravasation from vessel endothelium into islets. Splenocytes from diabetic donor mice deficient in IFNγ or IFNγR2 were able to efficiently transfer diabetes into wild type NOD or NOD.*scid* recipients, suggesting that IFNγ is not required for function of effector T cells (66). However when diabetogenic splenocytes were transferred into IFNγ- or IFNγR2-deficient recipients, diabetes was significantly delayed and reduced, even when the recipients were on a NOD.*scid* background (62, 66). Further analysis showed that there was a defect in homing of donor cells into the islets. Cells were accumulating around the entrance of the islet in the blood vessels to a much greater degree than in wild type recipients. Therefore IFNγ is acting on a host cell to promote the progression of insulitis and diabetes after adoptive transfer. This host cell is not a T or B cell, because adoptive transfer into NOD.*scid* mice is also reduced. It is also not the beta cell, because adoptive transfer of diabetes develops normally in models where IFNγ signalling has been eliminated only on the beta cell (67, 68). It remains unclear why this effect is not observed in spontaneous diabetes in mice deficient in IFNγ and its receptors, however diabetes in these models may occur by different mechanisms.

Cyclophosphamide administration to NOD mice can accelerate diabetes and has been used widely as a diabetes-inducing agent. How cyclophosphamide does this and the differences in mechanism between spontaneous diabetes and cyclophosphamide-induced diabetes are poorly understood. It is hypothesized that regulatory T cells may be especially sensitive to cyclophosphamide resulting in their preferential loss and increased disease progression (69). Alternatively destruction of many haemopoietic cells by cyclophosphamide may result in subsequent homeostatic proliferation of beta-cell specific T cells and increased T-cell activation. Neutralization of IFNγ with monoclonal Abs or soluble IFNγR prevents cyclophosphamide-induced diabetes (70-72). Although N13 backcross IFNγR1-deficient NOD mice developed diabetes normally, they remained resistant to cyclophosphamide-induced T1D (65). These data suggest that IFNγ is required for this form of diabetes, as originally suggested by systemic neutralization of IFNγ.

Global deficiency of IFNγ or its signalling molecules has made it difficult to interpret whether protective effects are due to IFNγ actions on immune cells or protection of beta cells from the effects of cytokines. Using inhibitors targeted to the beta cell with the insulin promoter it has been possible to ask specifically about the effects of IFNγ on the beta cell.

RIP-ΔγR mice express a dominant negative form of the IFNγR1 in their beta cells (68). This mutant receptor forms dimers with endogenous receptors, but does not transduce a signal, making the beta cells unresponsive to IFNγ. We made these mice to examine the direct effects of IFNγ on the beta cell, in a NOD mouse where the immune system was unaffected by lack of IFNγ or its receptor. These mice develop spontaneous diabetes with the same kinetics and incidence as wild type NOD mice (68), which is not surprising given the story that unfolded with IFNγ- and IFNγR-deficient NOD mice. RIP-ΔγR mice also develop diabetes normally after cyclophosphamide treatment. Together with the IFNγR-deficient NOD data, this suggests that IFNγ action on a non-beta cell, such as T cell or APC, is important for cyclophosphamide-accelerated diabetes.

Deficiency of Stat1, as expected, protects islets from the toxic effects of IL-1 + IFNγ or TNF + IFNγ *in vitro* (73, 74). Spontaneous T1D is completely absent in Stat1-deficient NOD mice, as is infiltration of immune cells into the islets (74). Similarly, deficiency of the transcription factor IRF1 completely prevented T1D (75). Immediately these data suggest a broader phenotype for Stat1 or IRF1 deficiency than for IFNγR deficiency because protection from diabetes was observed. The mechanism by which Stat1- and IRF1-deficient mice are protected has not been clearly elucidated but may involve blockade of responses to other cytokines including type 1 IFNs. Islet graft studies suggest that protection is at the level of the beta cell, however this was done in the cyclophosphamide model, where the mechanism of T1D is possibly different from spontaneous diabetes and more dependent on IFNγ (74). IRF1-deficient mice have defects in the generation of T cells, particularly $CD8^+$ T cells due to inefficient expression of class I MHC in the thymus, offering one potential explanation for the protection of these mice from T1D (75). Stat1-deficient mice have impaired anti-tumor

CTL and NK cell maturation (76). The ability of Stat1-deficient CTL to kill islet targets was not tested, nor the incidence of diabetes in a $CD8^+$ T cell-dependent model, but it is possible the acquisition of a cytolytic phenotype by CTL in Stat1-deficient NOD mice may be impaired. Stat1-deficiency was less protective in other models of T1D, including injection of multiple low dose streptozotocin (73), and after adoptive transfer of diabetogenic NOD splenocytes (74).

5.1. Class I MHC

Disruption of the β2-microglobulin gene (β2m), which is required for the transport of class I MHC molecules to the cell surface, results in a near complete lack of class I expression. This results in very few $CD8^+$ T cells developing because of lack of positive selection in the thymus. The absence of both insulitis and diabetes in NOD-$\beta2m^{null}$ mice suggests that $CD8^+$ T cells and class I MHC play a crucial role in the development of T1D in NOD mice (77-80). Adoptive transfer of diabetogenic splenocytes into irradiated NOD recipients proceeds slowly if beta cells do not express class I MHC (81). When β2m is deleted from beta cells but not other tissues so class I MHC surface expression on beta cells is negligible (82, 83), insulitis is not prevented in NOD mice but diabetes is substantially diminished, providing evidence for an important role for beta cell class I MHC in the effector phase of beta-cell destruction. This result also suggests that class I expression on the beta cell is not required for insulitis to develop.

Class I MHC is upregulated on beta cells by IFNγ (84). Using flow cytometry, we demonstrated that the upregulation of class I MHC on beta cells increases as NOD mice become older (68). RIP-ΔγR mice, with beta cells unresponsive to IFNγ, did not display increased class I MHC on beta cells, suggesting IFNγ is a major factor influencing class I MHC upregulation *in vivo*. These transgenic mice did, however, develop normal insulitis and diabetes, indicating that while basal class I MHC expression is needed, upregulation of class I MHC is not essential for diabetes in the NOD mouse. These data implied that class I MHC upregulation is a result of rather than a requirement for insulitis. However, more recent experiments have raised the possibility again that class I MHC expression levels and the density of class I MHC molecules bearing autoantigenic peptides may be an important regulator of recognition of beta cells by $CD8^+$ T cells at least *in vitro (*85).

6. SUPPRESSORS OF CYTOKINE SIGNALLING

Expression of SOCS molecules has been examined in mouse and human beta cells by RT PCR. These molecules are constitutively expressed at very low levels, and are upregulated by cytokine treatment *in vitro* (43, 44). In particular, SOCS1 is upregulated by IFNγ or by IFNα, and treatment with IL-1 or TNF together with IFNγ enhances expression (43). Other SOCS family members studied including CIS, SOCS2 and SOCS3 are also upregulated by IL-1, TNF and IFNγ and combinations of these in mouse and human islets (43, 44). *In vivo*, there is some expression of SOCS1 observed in sections of pancreas from donors with T1D but not normal donors (44). In NOD mice, SOCS proteins begin to be expressed as early as day 30 (CIS), day 50 (SOCS2) and day 70 (SOCS1), and expression increases as mice become older, suggesting that increasing SOCS gene expression correlates with the presence of cytokines in the islet lesion (86).

SOCS molecules have been overexpressed in beta cells as a method for inhibition of the action of pro-inflammatory cytokines including IFNs (67, 87). RIP-SOCS1 mice overexpress SOCS1 in beta cells under control of the insulin promoter. Similar to RIP-ΔγR mice, beta cells from RIP-SOCS1 mice are unresponsive to IFNγ, but in contrast they are also unresponsive to other cytokines regulated by SOCS1. *In vitro*, these include other cytokines that use the Jak/STAT signalling pathway such as type 1 IFNs and IL-12, cytokines that use the γc receptor (eg IL-2, IL-4, IL-7, IL-9 IL-15, IL-21), TNF, and other agents such as LPS and insulin (27). It is not known which of these factors are blocked *in vivo* in RIP-SOCS1 mice, but it is likely that they would all be if their actions were directed towards beta cells. However, beta cells do not express receptors for many of these ligands. As expected, class I MHC is not upregulated on beta cells of RIP-SOCS1 mice *in vivo* or after treatment with high concentrations of IFNγ *in vitro (*67).

Unlike RIP-ΔγR mice, RIP-SOCS1 mice show some protection from spontaneous T1D both in our studies and in others' (67, 87). SOCS1 transgenic islets were also able to delay allograft rejection (88). Studies on diabetes in RIP-SOCS1 mice suggest that the action on beta cells of a cytokine other than IFNγ plays a role in diabetes development, or that the extent of blockade of IFNγ effects is greater in RIP-SOCS1 beta cells. It is possible that SOCS1 overexpression on beta cells may influence the very early stages of disease, when type 1 IFN and engagement of TLRs may be important.

Islets deficient in SOCS1 do not have a more intense response to IFNγ, nor are they more sensitive to IFNγ, suggesting that physiologically, SOCS1 plays little role in the regulation of IFNγ signalling in islets (43). SOCS1-deficient islets do, however, have increased sensitivity to TNF (89). They are more susceptible to IFNγ+TNF-induced cytotoxicity, and to TNF-induced gene expression. This is at least in part due to dysregulated p38 MAPK activation in SOCS1-deficient islets. This unexpected ability of SOCS1 to control TNF, a cytokine that does not use the Jak/Stat pathway for signalling, suggests that overexpression of SOCS1 would protect beta cells from more cytokines than deficiency of Stat1. The lack of complete protection from diabetes in RIP-SOCS1 mice adds further weight to the suggestion that protection of Stat1-deficient mice from diabetes is not entirely at the level of the beta cell.

SOCS3 is also able to inhibit IFNγ signals when overexpressed. It is upregulated predominately by IL-6, and also by IL-1 and TNF but not by IFNγ (28). When SOCS3

is overexpressed in a beta cell line, it inhibits IFNγ-induced Stat1 activation, and also cytokine-induced apoptosis and iNOS expression (90). SOCS3 appears to be a better regulator of IL-1 effects on beta cells, and acts by inhibiting activity of the signalling molecules TAK1 and TRAF6 (91). Together SOCS1 and SOCS3 may be sufficient to eliminate many if not all of the effects of pro-inflammatory cytokines on beta cells, however whether this would influence the incidence of diabetes remains to be tested.

In summary, IFNγ or its receptors are not required for diabetes in NOD mice. Global deficiency of Stat1 and IRF-1 appear to have greater impact than deficiency of IFNγ alone, as would be expected. Similarly inhibition of the action of multiple cytokines on the beta cell by SOCS1 has greater effect than inhibition of IFNγ only.

6.1. SOCS1 and virus infection

Type 1 and to a lesser extent type 2 IFNs are important in the cellular defence against viruses. They are secreted from infected cells early in infection to lower the permissiveness of surrounding cells to infection through the induction of antiviral defence genes (92). Enterovirus infections, in particular Coxsackievirus have been linked with T1D development through direct infection of the beta cells (93-95). Islets from RIP-SOCS1transgenic mice or islets deficient in IFN receptors have increased susceptibility to Coxsackievirus infections and *in vivo* RIP-SOCS1 mice infected with Coxsackievirus developed rapid diabetes with extensive beta cell destruction (96, 97). Clearly beta cell responsiveness to IFNs is critical to the antiviral defence against Coxsackievirus infection. Recent studies in human islets have shown IFNs induce a similar range of antiviral defence genes in beta cells to lower permissiveness to Coxsackievirus infection (98). Inhibiting both type 1 and type 2 IFNs through SOCS1 overexpression is a promising strategy to block beta cell destruction and is potentially translatable to human islets. It will be important to address the caveat of increased susceptibility to virus infection to ensure success. It is illogical to protect islets from one form of attack while removing the beta cells' ability to arm itself against others. Alternative strategies could be to mimic SOCS1 protection by the combined loss of other pathways including IFNγ, IL-1 and TNF.

7. INTERFERONS IN CD8-DEPENDENT MODELS OF TYPE 1 DIABETES

TCR transgenic mice with beta cell-specific diabetogenic T cells simplify analysis of the diabetes process. In our laboratory, we have used a number of $CD8^+$ and $CD4^+$ T cell-dependent models to understand the mechanisms used by specific cell types to cause beta cell destruction.

NOD8.3 mice express the TCR gene rearrangements from the clone NY8.3, which was originally isolated from a diabetic NOD mouse (15). This clone recognizes the beta cell antigen IGRP (99), and is highly diabetogenic. NOD8.3 mice develop rapid onset diabetes starting around 40 days of age, until 100 days of age when most mice have developed disease. These mice, generated in the laboratory of Santamaria, have proven extremely valuable in understanding the pathogenesis of diabetes. We have used NOD8.3 mice to understand in detail the relationship between class I MHC, IFNγ action on beta cells, and development of diabetes in a CD8-dependent model.

NOD8.3 mice with RIP-SOCS1 on their beta cells are completely protected from hyperglycaemia (67). They do, however, develop insulitis. The protection in these mice was initially thought to be due to absence of beta cell Fas expression, because of unresponsiveness to cytokines that upregulate Fas (eg IFNγ, TNF). This was consistent with the report by Amrani *et al* suggesting that Fas was the major effector mechanism used by NOD8.3 T cells to kill beta cells (100). However, further investigation suggested that the mechanism of protection of SOCS1 overexpression in NOD8.3 mice was more complex (85). RIP-SOCS1 transgenic mice express basal levels of class I MHC on their beta cells, but this does not result in sufficient antigen presentation for T cell activation in the CD8-dependent model (although basal expression of class I MHC is sufficient in the standard NOD model – see above and (68)). Addition of exogenous antigen in the form of IGRP peptide increases the antigen presentation on beta cells to a level sufficient to make RIP-SOCS1 islets good targets for NOD8.3 CTL *in vitro*. These data emphasize that it is not just class I MHC expression levels that are important but also recognition complexes for specific T cells.

While absence of Fas expression is probably not the main mechanism of protection of RIP-SOCS1 beta cells from NOD8.3 T cells, Fas/FasL is nevertheless a mechanism that these and other CTL can use to kill beta cells *in vitro*. In studies using islet antigen-specific CTL, we found that perforin is the dominant mechanism used to kill beta cells. When perforin was absent, however, the Fas/FasL pathway became more important. This is true for NOD8.3 T cells (85), and also for ovalbumin-specific OT-1 CTL when killing islets transgenically expressing ovalbumin, or pulsed with the SIINFEKL peptide recognized by OT-1 cells (101). In this system, RIP-SOCS1 and RIP-ΔγR islets, and those lacking Fas (lpr) were resistant to *in vitro* killing by perforin-deficient OT-1 CTL. In addition, Fas was not upregulated on beta cells of these islets after incubation with OT-1 cells. It remains to be determined whether Fas and perforin are the only mechanisms used by CTL *in vivo*.

We have also found blocking IFNγ signalling on beta cells to be protective in the CD8-dependent LCMV-induced model of T1D (102, 103). In this model, mice transgenically express the lymphocytic choriomeningitis virus (LCMV) glycoprotein in their beta cells (RIP-GP). When transgenic mice are infected with LCMV, they develop $CD8^+$ T cells specific for LCMV-GP, and develop insulitis and diabetes 3 weeks after infection (reviewed in (104)). IFNγ-deficient RIP-GP mice do not develop diabetes (105). To determine if the protection is due to

action of IFNγ or other cytokines on the beta cell itself, we developed RIP-GP/RIP-ΔγR and RIP-GP/RIP-SOCS1 double transgenic mice. After LCMV infection, class I MHC expression was greatly induced on wild type islets, but not on RIP-GP/RIP-SOCS1 islets, suggesting that a cytokine blocked by SOCS1 is required for class I MHC expression (102). This cytokine may be IFNγ, however when we examined class I MHC expression on islets of RIP-GP/RIP-ΔγR mice after LCMV infection, it was similar to wild type islets (103), suggesting an alternative cytokine, perhaps type 1 IFN, TNF or both.

There is additional evidence for a role for type 1 IFN in upregulation of class I MHC on islets in the LCMV model. Lang *et al* found that IFNα production and upregulation of class I MHC occurred four days after LCMV infection due to ligation of TLR3 (106). It was concluded that beta cell class I MHC was required for cytotoxicity. Our own data suggests upregulation of class I MHC is not the only role of IFNs in this model. When RIP-GP/RIP-ΔγR and RIP-GP/RIP-SOCS1 mice were studied for diabetes development after virus infection, both were protected, despite class I MHC being like wild type in the former and basal in the latter mice (102, 103). It is still unclear exactly what the role of IFNγ is in the LCMV model, but it may be important for upregulating expression of chemokines such as IP-10 on beta cells to allow more efficient homing of activated CTL to the islets (40).

Therefore IFN action on beta cells, class I MHC expression and perhaps other IFN-stimulated genes play an important role in diabetes mediated by $CD8^+$ T cells.

8. IFNγ IN CD4-DEPENDENT MODELS OF TYPE 1 DIABETES

Kim *et al* found a significant reduction in diabetes incidence after transfer of wild type $CD4^+$ BDC2.5 TCR transgenic splenocytes into Stat1-deficient mice (74). CFSE-labelled BDC2.5 T cells proliferated normally in the pancreatic draining lymph node of Stat1-deficient mice, suggesting little requirement for Stat1 in T cells and APC in this model. When Stat1-deficient islets were transplanted into BDC2.5 transgenic mice made diabetic with cyclophosphamide, they were protected from inflammation and destruction, suggesting deficiency of Stat1 in islet cells is protective. The mRNA levels of cytokines induced by cyclophosphamide treatment of BDC2.5 mice have been measured (107). IFNγ mRNA increased, as did a range of other cytokines including IL-2, IL-12, IL-18, IL-1β and TNF. Neutralization of IFNγ delayed the onset of diabetes but most mice succumbed to disease. Neutralization of IL-1β, TNF or IL-12 abrogated disease in this model.

Pakala *et al* grafted genetically modified islets into NOD.*scid* mice, and adoptively transferred activated BDC2.5 into the grafted mice (108). This experiment examined the ability of pre-activated $CD4^+$ T cells to kill islets that lacked different effector mechanisms including Fas and IFNγR. Both Fas-deficient (lpr) and IFNγR-deficient islets were destroyed by BDC2.5 cells as efficiently as wild type islets, suggesting that these molecules are not involved in killing of islets by activated cells. We have generated similar data with RIP-SOCS1 transgenic mice after transfer of BDC2.5 splenocytes (unpublished). The main difference between the results of Pakala *et al* and those of Kim *et al* is the way in which diabetes was induced. When induced with cyclophosphamide, blocking IFNγ was protective, but when spontaneously induced, there was no protection in the CD4-dependent model.

NOD4.1 mice express the TCRαβ rearrangements of the I-A^{g7}-restricted $CD4^+$ T cell clone NY4.1, and develop diabetes in an accelerated, CD4-dependent manner (15). Amrani *et al* found that cytokines (IL-1 and IFNγ) upregulate Fas on islets from NOD4.1 mice, making them good *in vitro* targets for killing by NOD4.1 T cells (109). They suggested that Fas is the major mechanism used by NOD4.1 T cells to kill beta cells, and found that when crossed with Fas-deficient NOD.*lpr* mice, NOD4.1 mice were protected from diabetes. Fas is also expressed on beta cells from NOD4.1 mice *ex vivo* during the course of diabetes development (86). Our own findings suggest that NOD4.1 mice that express SOCS1 in beta cells are not protected from diabetes, despite absence of beta cell Fas expression (unpublished). It remains to be determined the exact mechanism used by $CD4^+$ T cells to kill beta cells, however evidence suggests that IFNγ and Fas are less important than originally thought.

In summary, IFNγ action on beta cells is not required for diabetes induced by BDC2.5 and 4.1 $CD4^+$ TCR transgenic cells. It is possible that diabetes following cyclophosphamide may be IFN-dependent in these models and IFNγ may be needed for homing of diabetogenic T cells.

9. PERSPECTIVE

It is now clear that global deficiency of IFNγ or its receptors is insufficient to reduce spontaneous diabetes in the NOD mouse, however, blocking multiple cytokines such as with Stat1- or IRF1-deficiency is significantly more protective. This suggests that stimuli other than IFNγ that activate Stat1 or IRF1 must be required for the pathogenesis of diabetes. The data suggest that normal development and function of CTL may require these factors ie that CTL may be the cells affected by Stat1 or IRF1 deficiency. With beta-cell specific blockade of IFNγ responses, no protection was observed, consistent with the lack of any effect of global IFNγ deficiency. Beta-cell SOCS1 overexpression partially protected from spontaneous diabetes in NOD mice, completely protected from diabetes in NOD8.3 mice and did not protect at all from diabetes in NOD4.1 mice. This has two implications: first, cytokines with effects inhibited by SOCS1 other than IFNγ must be acting on the beta cell to promote diabetes; candidates would include type 1 IFNs and these are worthy of further study. Second, the effect of SOCS1 overexpression is likely to be very similar to Stat1 deficiency in beta cells but protection from diabetes is

clearly less, perhaps consistent with the idea that the protection seen in Stat1 deficient mice is at least partly due to effects on cells other than the beta cell. These studies do not suggest that neutralisation of IFNγ would be clinically useful. Transcription factors such as Stat1 and IRF1 that are used by IFNs are widely used by the immune system, and adverse effects of blocking these on immune homeostasis would need to be considered. Reducing class I MHC expression on transplanted beta cells remains a possible therapeutic strategy suggested by RIP-SOCS1 mice and β2m-deficient mice.

10. ACKNOWLEDGEMENTS

This work was supported by the National Health and Medical Research Council of Australia, the Juvenile Diabetes Research Foundation and Diabetes Australia. We thank Dr Janette Allison for critical reading of the manuscript.

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Abbreviations: T1D: type 1 diabetes; NOD: non-obese diabetic; APC: antigen presenting cell; DC: dendritic cell; FasL: Fas ligand; IFN: interferon; TNF: tumor necrosis factor; IL: interleukin; NO: nitric oxide; iNOS: inducible NO synthase; SOCS: suppressor of cytokine signalling; CTL: cytotoxic T lymphocyte; β2m: β2 microglobulin; LCMV: lymphocytic choriomeningitis virus

Key Words Type 1 diabetes, Interferons, Beta cell, NOD mouse, Autoimmunity, Review

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