Fas Deficiency Prevents Type 1 Diabetes by Inducing Hyporesponsiveness in Islet β-Cell–Reactive T-Cells

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Type 1 diabetes is an autoimmune disease wherein autoreactive T-cells promote the specific destruction of pancreatic islet β-cells. Evidence for a crucial role for Fas/FasL interactions in this destruction has been highly controversial because of the pleiotropic effects of Fas deficiency on the lymphoid and other systems. Fas-deficient mice are protected from spontaneous development of diabetes not because Fas has a role in the destruction of β-cells, but rather because insulitis is abrogated. Fas may somehow be involved in the series of events provoking insulitis; for example, it may play a role in the physiological wave of β-cell death believed to result in the export of pancreatic antigens to the pancreatic lymph nodes and, thereby, to circulating, naive, diabetogenic T-cells for the first time. To explore the implication of Fas in these events, we crossed the lpr mutation into the BDC2.5 model of type 1 diabetes to make it easier to monitor direct effects on the pathogenic specificity. We demonstrated that BDC2.5/NOD lpr/lpr mice have qualitatively and quantitatively less aggressive insulitis than do BDC2.5/NOD mice. In vitro proliferation assays showed that BDC2.5/NOD lpr/lpr splenocytes proliferated less vigorously than those from control mice in the presence of islet extracts, which reflects their inability to produce interleukin-2, resulting in weaker pathogenicity.

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ype 1 diabetes is an autoimmune disease in which the immune system specifically destroys insulin-producing β-cells of the pancreatic islets (1). A breakdown in the tolerance of T-cells toward pancreatic autoantigens results in infiltration of the islets by a cohort of lymphoid and other inflammatory cells. T-cells have been shown to play a critical role in this destruction (2,3), but the precise mechanisms involved remain elusive. It is not clear, for example, whether β-cells are killed directly by cytotoxic effector T-cells, indirectly by means such as cytokine secretion and/or activation of cytocidal activities in macrophages, or by a combination of the two.

In this context, much attention has been paid to Fas/FasL-mediated cell death and to the possibility that the triggering of “death-receptors” on β-cells might be the conduit for β-cell destruction. Engagement of the trimeric Fas molecule (CD95) on the cell surface rapidly induces cell death, the prototype of receptor-mediated apoptosis (4). The original evidence that the Fas/FasL axis might be involved in β-cell destruction came from the observation that introducing a mutation specifying Fas deficiency (the lpr mutation) protected NOD mice from diabetes development (5,6). Moreover, transfer of splenocytes from diabetic NOD mice into NOD+/+ recipients did not provoke diabetes, as it did in wild-type hosts (6). Fas-deficient NOD+/− mice did not develop diabetes either (7). Together, these findings suggest that the engagement of Fas on β-cells by FasL on effector T-cells might be a critical step in inducing β-cell death. On the other hand, closer examination of the mutant animals has revealed that insulitis is also abrogated in NOD+/− mice (6), implying that the lack of diabetes might not be due to direct impairment of β-cell killing, but rather to another effect of the mutation on the lymphoid system. One such possibility is that lymphocytes from lpr/lpr mice strongly upregulate FasL (8), rendering them potent killers of any cells displaying Fas (9). This might explain the inability of transferred potentially diabetogenic lymphocytes to exert their pathogenicity in NOD+/− mice. Indeed, this has been shown to be the case (9,10). Furthermore, islets from NO+/− recipients were not protected from autoimmune attack when transferred into diabetic wild-type recipients (10), casting strong doubt on the earlier interpretations.

Nonetheless, debate persists on the true role of Fas/FasL interactions in diabetogenesis. More recently, in vivo treatment of mice with an anti-Fasl antibody (11) or in whom a dominant-negative Fas mutation has been introduced (12) seems to suggest that Fas may contribute to the early stages of the disease. On the other hand, inactivation of the Fas gene, specifically in β-cells, has not prevented disease in a model where T-cells expressing a transgene-encoded T-cell receptor (TCR) specific for influenza hemagglutinin (HA) mediate diabetes in mice that express the HA peptide in β-cells (13).

These data do not explain the initial observation of diabetes prevention in NOD+/− mice, which may still be an important clue to understanding anti-islet autoimmunity. Does the mutation prevent formation of the autoreactive T-cell repertoire? Does it interfere with T-cell activation? We have recently shown that the ripple of physiological β-cell death that occurs in the first weeks of
life is an important initiator of islet antigen presentation to T-cells (14); might Fas be involved in this process?

To address these questions, we analyzed the impact of the lpr mutation in the BDC2.5/NOD model, a derivative carrying the rearranged TCR genes from a diabetogenic T-cell clone derived from a diabetic NOD mouse. BDC2.5/NOD mice harbor anti-islet T-cells at high frequency, thereby greatly facilitating the examination of events that modulate the activation and effector function of potentially diabetogenic T-cells (15). We found that the lpr mutation affects the progression of autoimmunity in BDC2.5/NOD mice, but that the mutation has an influence on T- and not on β-cells.

**RESULTS**

To identify the level at which a deficiency in Fas expression influences the development of autoimmune diabetes, we crossed the lpr mutation into the BDC2.5 TCR transgenic line. BDC2.5 mice express the transgene-encoded receptor on the majority of their T-cells, imparting reactivity to an unknown islet β-cell antigen in the context of the major histocompatibility complex II molecule Aβ7. Consequently, the selection and spreading of an autoreactive repertoire that occurs in the NOD mouse is experimentally shortened. BDC2.5 animals display very rapid and massive insulinitis by age 3 weeks, but progression to overt diabetes is subject to a variety of controlling elements (genetic influences, costimulatory control, regulatory cells) (19–21). An mAb antibody specific for the BDC2.5 clonotype has recently been generated, and >80% of the CD4+ T-cells in BDC2.5/NOD TCR transgenic mice were found to express various levels of the transgene-encoded TCR (17). The BDC2.5/NOD and NODlpr lines, both intensively backcrossed to the NOD/Lt background, were intercrossed twice to generate BDC2.5/NODlprlpr and control littermates (lpr/lpr or +/+).

We first asked whether the lpr mutation affected the selection of T-cells expressing the BDC2.5 TCR. The flow cytometric analyses shown in Fig. 1 demonstrated that BDC2.5/NODlprlpr mice are not defective in their ability to select T-cells displaying the BDC2.5 TCR. Clonotype-positive cells were present in the spleen and lymph nodes of these mice. We did not note any difference in the relative proportion of cells expressing high and intermediate levels of clonotype (Fig. 1 and data not shown). On the other hand, we noted an increase in the proportion of CD4+ cells. This increase was time dependent (Fig. 1B) and parallel to the expansion of CD4+ T-cells in lprlpr mice observed elsewhere (22–24). In any case, it was clear that the BDC2.5 specificity remained well selected in the absence of Fas.

To determine whether Fas deficiency influences the quantity or quality of insulinitis in the BDC2.5 model, we scored insulinitis in Fas-deficient and -sufficient animals. Insulitis appeared abruptly between ages 16 and 20 days in BDC2.5/NOD mice, and ~90% of the islets were infiltrated at age 24 days (19). In BDC2.5/NODlprlpr mice, the early-phase infiltration was clearly less aggressive than that seen in control littermates (Fig. 2A). The proportion of infiltrated islets was lower (Fig. 2A) and the lesions were typically of smaller size (Fig. 2B and C). This difference was not as obvious in older animals (age >56 days).
What are the functional consequences of the tepid insulitis in BDC2.5/NOD$^{lpr/lpr}$ mice? Because standard BDC2.5/NOD animals rarely progress to overt diabetes, we provoked them with CY. CY treatment induces diabetes in 100% of BDC2.5/NOD animals within a few days, with highly reproducible kinetics (25). We administered a single dose of 200 mg/kg CY to 5- to 8-week-old animals and continuously monitored the development of diabetes. Figure 3 shows that BDC2.5/NOD$^{lpr/lpr}$ mice were significantly, albeit not completely, protected from diabetes development. Thus, the mild insulitis of BDC2.5/NOD$^{lpr/lpr}$ mice did translate as a decreased propensity to convert to diabetes after CY challenge.

Collectively, these results indicated that, even when the T-cell repertoire is dominated by an autoimmune specificity, the Fas gene can exert a strong influence. It is easy to see how the partial reduction in diabetes incidence in the BDC2.5 model might translate to full protection in the NOD mouse, given that autoreactive T-cells are far less prevalent in the latter case.

**Reduced autoreactivity by T-cells from Fas-deficient BDC2.5 mice.** In theory, these influences of the $lpr$ mutation on insulitis progression and diabetes development could reflect an impact on the lymphocyte compartment (due to reduced responsiveness) or on the target cells (due to either decreased antigen release through apoptosis or increased resistance to infiltration and autoimmune destruction). To address this question, we used an in vivo T-cell stimulation assay in which BDC2.5/NOD cells were labeled with CFSE and then injected into a naive host; their proliferation was reflected as a halving of CFSE staining intensity with each cell division (26). In this system, BDC2.5 T-cells proliferated selectively in the pancreatic lymph nodes (PLNs) that drain the islets and not in...
To elucidate the effect of the lpr mutation, we performed a criss-cross experiment in which T-cells from BDC2.5/NOD^lpr/lpr or BDC2.5/NOD^+/+ donors were injected into NOD^lpr/lpr or NOD^+/+ recipients. The genotype of the host clearly made no difference (Fig. 4A), as proliferation was essentially identical in wild-type and Fas-deficient hosts. (This experiment could only be performed with BDC2.5/NOD^lpr/lpr donors, as previous studies have shown that upregulated FasL in lpr mice will induce the death of any transferred cells displaying Fas) (9). These results suggest that the lpr mutation does not interfere with the availability of the BDC2.5 autoantigen in the PLNs or the ability of dendritic cells to present it.

On the other hand, BDC2.5/NOD^lpr/lpr T-cells proliferated markedly less well than did T-cells from BDC2.5/NOD^+/+ mice when they were transferred into the standard NOD hosts (Fig. 4B; note that the absolute values of the controls in Fig. 4A cannot be directly compared with those of Fig. 4A, which were performed under slightly different conditions and with unrelated host and donors). Thus, the Fas deficiency affected the autoreactive T-cell rather than its target.

**Hyporesponsiveness in Fas-deficient autoreactive T-cells.** These results are reminiscent of published data suggesting a paradoxical role for Fas as a costimulator of T-cell responses to foreign antigen (28,29). To explore this possibility in our system, we performed proliferation assays using BDC2.5/NOD^lpr/lpr and BDC2.5/NOD^+/+ splenocytes stimulated with the BDC2.5 mimotope peptide 1040-63 (16) (Fig. 5A) or with extracts from purified islets (Fig. 5B). There was very little difference between the responses of lpr/lpr and wild-type T-cells to increasing concentrations of the 1040-63 BDC2.5 mimotope peptide, a very potent agonist, except that the mutant T-cells showed a continuing increase in proliferation at high peptide concentrations, concentrations at which the response of wild-type T-cells was tapering off. It is likely that this difference was due to the role of Fas in activation-induced cell death (30,31). On the other hand, Fas-deficient T-cells were very clearly hyporesponsive when challenged with the true autoantigen present in islet extracts. This finding held whether the islet extract was prepared from wild-type or Fas-deficient mice, thus ruling out the possibility that Fas might influence expression of the BDC2.5 antigen in β-cells (data not shown). These results indicated that the lpr mutation affected the responsiveness of autoreactive T-cells, but to a degree that seemed to vary with the T-cells' affinity/avidity for the peptide ligand.

To reveal the reason behind the impaired proliferation of the BDC2.5/NOD^lpr/lpr splenic T-cells when challenged with islet extracts, we measured IL-2 production in the cultures after stimulation (Fig. 5C and D). IL-2 release was severely impaired in BDC2.5/NOD^lpr/lpr splenocytes with either type of antigen, although the reduction was more extensive when islet extract was the antigenic stimulus. Thus, the decreased proliferation seen in BDC2.5/NOD^lpr/lpr splenocytes was accompanied by an impaired production of IL-2. To determine whether this defective IL-2 production was the root cause of the hyporesponsiveness, we tried to restore proliferative capacity by complementing the cultures with IL-2 (Fig. 5E). IL-2 addition had
no effect whatsoever on the proliferation of BDC2.5/NOD\textsuperscript{lpr/lpr} splenocytes to islet extract, indicating that the defect in IL-2 production did not, alone, account for the poor responses to islet antigen by Fas-deficient BDC2.5 T-cells. A stronger stimulus was provided by plates coated with mAbs directed against CD3 and CD28 (Fig. 5). When BDC2.5/NOD and BDC2.5/NOD\textsuperscript{lpr/lpr} splenocytes were challenged with this stimulus, little difference was observed, just as with the BDC2.5 agonist mimotope. Thus, Fas-deficient BDC2.5 T-cells responded poorly to the autoantigen in its natural state, but this deficit was at least partially overcome with high-affinity ligands.

**DISCUSSION**

By exploiting the performant read-out system afforded by the BDC2.5 TCR transgenic mouse model, this study aimed to elucidate the point of impact of the \textit{lpr} mutation on NOD mice, already clearly shown to protect them from insulitis and diabetes (5,6). A priori, it was thought that the absence of Fas could impinge on three levels of the diabetogenesis cascade. First, it might have prevented the early ripple of physiological cell death that occurs just...
before age 15 days that appears to be necessary for the priming of autoreactive T-cells in the PLN (33–36). Second, the Fas deficiency might have interfered with the T-cells themselves, directly or indirectly dampening their reactivity. Third, it might have protected β-cells from terminal destruction. The results presented here argue strongly in favor of the second option: although the antigen presentation function of NODprlpr mice seemed normal, the activation of BDC2.5 T-cells by pancreatic autoantigens was severely curtailed in the absence of Fas, both in vivo and in vitro (Figs. 4 and 5), resulting in slow and tapid insulitis despite the fact that the autoimmune cells were increased (Fig. 1). Most likely, this defective activation of autoreactive T-cells also explains the absence of insulitis and diabetes in NODprlpr mice, with the poor initial activation of islet β-cell–reactive T-cells resulting in an abortive anti-islet response.

The fact that BDC2.5/NODprlpr T-cells were clearly defective does not rule out the possibility that the third explanation holds as well and that Fas-induced apoptosis plays a direct role in β-cell destruction. On the other hand, Fas cannot be absolutely essential, as diabetes did occur in the mutant BDC2.5 mice (Fig. 3). Similarly, the expression in islet β-cells of a dominant-negative form of the Fas-associated death protein domain transducer molecule, the essential transducer of Fas signaling, also failed to prevent islet destruction (L.V., unpublished observations). These results are also consistent with the finding that abrogation of Fas expression specifically on β-cells did not influence diabetes development in another TCR transgenic model of type 1 diabetes (13).

Savinov et al. (12), on the other hand, have shown that Fas is involved in β-cell death in a model where diabetes is accelerated by FasL expression on β-cells. However, it is not clear how/whether this system translates to the normal context.

The requirement for Fas-mediated signals to engender full proliferative and cytokine responses to islet autoantigen has precedent in other antigen-driven responses (37,38). Conversely, FasL has been described as either a negative or a positive modulator of T-cell selection and activation (39–41), with opposite effects in CD4+ and CD8+ T-cells (39–41). In fact, FasL-deficient CD4+ T-cells have been claimed to exhibit more vigorous Ag-provoked proliferation than their wild-type counterparts (40–42). It is interesting that the requirement for Fas or FasL varied with the type and affinity of the signal transduced through the TCR, most visible with suboptimal ligands, but largely absent with high affinity/avidity ligands (41,42). This mirrors our present observations, where the impact of the Fas deficiency was more obvious with the natural autoantigen than with the high-affinity agonist peptide.

What is the mechanism by which the lpr mutation impairs activation of BDC2.5 T-cells? Noorchashm et al. (43) have proposed that the abundance of responding BDC2.5 T-cells might affect the efficiency of T-cell activation, already below par on the NOD background. This is unlikely, however, to account for the present observations, as the defective activation of BDC2.5/NODprlpr cells is already manifest at early times, before the accumulation of CD4+ cells typical of the lpr background. One interpretation is that a costimulatory signal is delivered through the Fas molecule itself. Another explanation might lie in the high levels of FasL displayed on T-cells in lpr mice. The cytoplasmic tail of FasL is rich in signal transduction motifs (44), and one can easily imagine that a high level of FasL at the cell surface might sequester important signal transduction molecules. Alternatively, FasL might engage another receptor in Fas-deficient mice. One candidate might be DcR3/TR6, a secreted protein that binds FasL with an affinity comparable with that of Fas, and competes functionally with Fas for FasL binding (45,46). Binding of DcR3/TR6 at a high level might prevent T-cell activation.

Finally, the preferential impact of the Fas-deficiency on T-cell stimulation by the natural autoantigen is also congruent with the idea that autoreactive T-cell responses are dominated by low-affinity reactivities (47). Low affinity responses to autoantigens might be those that are most attuned to qualitative or quantitative variation in Fas/FasL. Ineffective signaling through molecules of the death receptor family, or their ligands, would dampen T-cell autoreactivity to these targets, but at the cost of lymphoproliferative perturbation or β-cell–driven autoimmunity. Genetic polymorphisms at these loci in murine or human populations might navigate between these hazards.

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