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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Type 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 induction (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$^{lpr/lpr}$ recipients did not provoke diabetes, as it did in wild-type hosts (6). FasL-deficient NOD$^{gld/gld}$ 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$^{lpr/lpr}$ 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$^{lpr/lpr}$ mice. Indeed, this has been shown to be the case (9,10). Furthermore, islets from NOD$^{lpr/lpr}$ mice 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$^{lpr/lpr}$ 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

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AP, avidin-alkaline phosphatase; CFSE, 5,6-carboxyfluorescein diacetate succinimidyl ester; CY, cyclophosphamide; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; IL, interleukin; PLN, pancreatic lymph node; TCR, T-cell receptor.

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.

RESEARCH DESIGN AND METHODS

BDC2.5 TCR transgenic mice have been previously described (15). The BDC2.5/NOD mice used in these experiments have been backcrossed for more than 30 generations onto the NOD genetic background. NOD$^{lpr/+}$ mice were generated by backcrossing the *lpr* mutation (a kind gift of Dr. A. Strasser and Dr. J. Allison, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) for >15 generations onto the NOD/Lt background, followed by a cross to BDC2.5/NOD mice to introduce the BDC2.5 TCR transgenes, and intercrosses to obtain BDC2.5/NOD$^{lpr/lpr}$ animals and heterozygous littermates. The line was kept as balanced crosses, so that all *lpr*/+ and +/+ (wild-type) control mice were littermates of *lpr/lpr* animals, eschewing the risky practice of maintaining an isolated homozygous mutant line. Mice were typed for the BDC2.5 transgenes by cytofluorimetric analysis of blood (anti-Vβ4 staining) and for the Fas wild-type and *lpr* alleles by PCR analysis of tail DNA with the following primers: FasF: 3′-GTAAATAATTGTGCTTCGT CAG-5′; FasRV1: 3′-TAGAAAGGTGCACGGGTGTG-5′; and FasRV2: 3′-CAAATCTAGGCATTAACAGTG-5′ (generating bands at 184 bp for the wild-type allele and 212 bp for the lpr allele on a 2% agarose gel).

Cyclophosphamide (CY) (Sigma) dissolved in PBS was injected intraperitoneally at 200 mg/kg to induce type 1 diabetes; disease onset was evaluated by measuring glucose levels in the urine with Uristix (Bayer Diagnostics). Two consecutive measurements >10 g/l was considered indicative of diabetes; disease status was then confirmed by a blood glucose test (considered positive if >200 mg/dl). Mice were age 5–8 weeks at the time of CY injection.

Insulitis scoring. Thin sections from Bouin-fixed, paraffin-embedded pancreases were examined for the presence of insulitis after hematoxylin and eosin staining. Sections were made at four different levels through the pancreas, and a minimum of 40 islets were examined for each mouse. In very young animals, all of the available islets were analyzed.

Proliferation assays. Splenocytes depleted of erythrocytes in 0.83% NH_4Cl were cultured at 2×10^5 cells/well in 96-well flat-bottomed plates in 200 μl Dulbecco's modified Eagle's medium, 10% FCS, penicillin-streptomycin, nonessential amino acid, L-Glu, and β-mercaptoethanol for 3 days, either with the BDC2.5 mimotope peptide 1040-63 (16) (Peptron, Daejeon, South Korea) or with freeze-thaw extracts from purified islet preparations. Proliferation was assessed by incorporation of ^{3}H-thymidine in the last 16 h of culture.

Enzyme-linked immunosorbent assay. The primary anti–interleukin (IL)-2 antibody (18161D; BD Pharmingen, San Diego, CA) was diluted in PBS to 1 μg/ml to coat 96-well microtiter plates (3912; BD Falcon, Bedford, MA) for 16 h at 4°C. After five washes with enzyme-linked immunosorbent assay (ELISA) buffer (0.1% Tween 20, 2% BSA, 0.002% NaAzide in PBS) to block nonspecific binding, 50 μl of test supernatants (3-day-old cultures) were added for 16 h at 4°C. The detecting IL-2 biotinylated antibody (18172D; BD Pharmingen; 0.5 μg/ml in ELISA buffer) was then added for 1 h at room temperature. After six washes, 50 μl/well of avidin-alkaline phosphatase (AP; Sigma Immuno Chemicals) diluted 1:400 in blocking solution were added and incubation was allowed for 30 min. After six washes, color was developed with an AP reaction solution (9.6% diethanolamide, 0.25 mmol/l $MgCl_2$ [pH 9.8] with AP substrate tablets [1 AP tablet per 5 ml of AP reaction solution]; Sigma). Plates were incubated for 10 min at room temperature, and the optical density 405 nm was measured by plate spectrophotometry. Determinations were performed in triplicate and quantitated by comparison with a standard curve obtained with rIL-2 (BD Pharmingen).

CFSE labeling and adoptive transfer. Single-cell suspensions were prepared from pooled spleens of BDC2.5/NOD mice depleted of erythrocytes in 0.83% NH_4Cl, adjusted to 10^7/ml in PBS, and incubated for 10 min at 37°C in the presence of 2.5 mmol/l 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) prepared as a 5 mmol/l stock solution in DMSO. After being incubated, the cells were washed twice with RPMI supplemented with 5% FCS, adjusted to 10^8/ml, and 200 μl was injected intraperitoneally. After 66 h, lymph nodes were removed and analyzed by flow cytometry for the presence of CFSE-labeled cells (counterstaining with anti-CD4 and anti-Vβ4)

Quantitative PCR analysis of anergy-related genes. Splenocytes from BDC2.5/NOD$^{lpr/lpr}$ mice were stained with biotin-conjugated BDC2.5 clonotype mAb (17), anti-CD4, and anti-B220. Gating on CD4$^+$ B220$^-$ cells, we sorted (MoFlo cell sorter; Cytomation, Denver, CO) the cells into two groups, clonotype high and clonotype low, and then prepared RNA from them (~10^6 cells/group) using the Trizol method (Invitrogen, Carlsbad, CA). DNAse treatment was performed (Ambion, Austin, TX) and then reverse transcription with avian myeloblastosis virus reverse transcriptase (Roche, Indianapolis, IN). Sybr green PCRs were performed, with the resulting cDNA using the protocol and primers previously described (18). *L32B* and *HPRT* were used as housekeeping control genes for normalization of the amount of cDNA/sample. The *HPRT* primer sequence we used was forward: 3′-GACCGGTCCCGT-CATGC, and reverse: 3′-CAGTCCATGAGGAATAAACACTTTTTC-5′.

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 A^{g7}. Consequently, the selection and spreading of an autoreactive repertoire that occurs in the NOD mouse is experimentally shortcutted. BDC2.5 animals display very rapid and massive insulitis 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 NOD^{lpr} lines, both intensively backcrossed to the NOD/Lt background, were intercrossed twice to generate BDC2.5/NOD$^{lpr/lpr}$ and control littermates (*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/NOD$^{lpr/lpr}$ 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. 1*B*) and parallel to the expansion of CD4$^+$ T-cells in *lpr/lpr* 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 insulitis in the BDC2.5 model, we scored insulitis 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/NOD$^{lpr/lpr}$ mice, the early-phase infiltration was clearly less aggressive than that seen in control littermates (Fig. 2*A*). The proportion of infiltrated islets was lower (Fig. 2*A*) and the lesions were typically of smaller size (Fig. 2*B* and *C*). This difference was not as obvious in older animals (age >56 days).

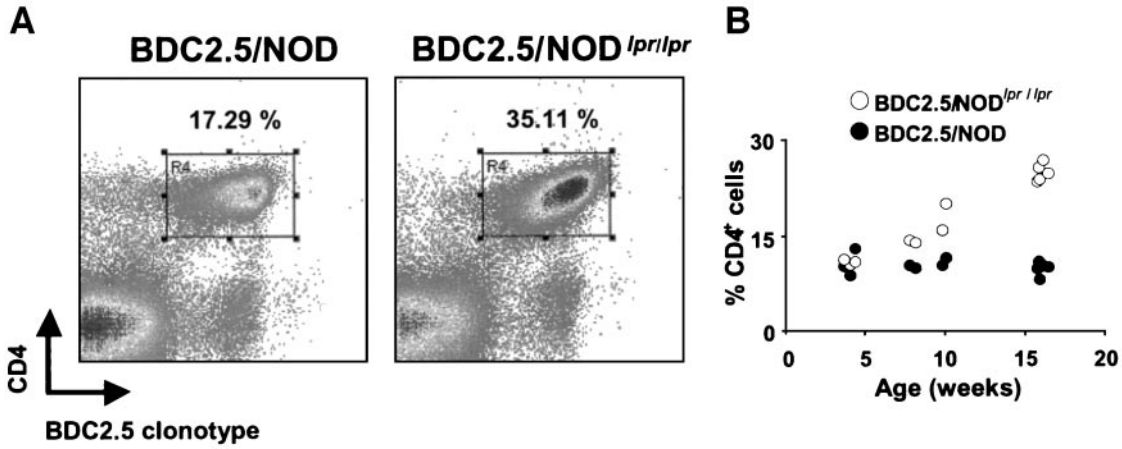

FIG. 1. Staining of BDC2.5/NOD or BDC2.5/NOD$^{lpr/lpr}$ splenocytes with the BDC2.5 clonotypic antibody. *A*: Splenocytes from adult BDC2.5/NOD or BDC2.5/NOD$^{lpr/lpr}$ mice were stained with anti-CD4, anti-B220, and anti-BDC2.5 clonotype antibodies to permit the estimation of the proportion of T-cells expressing the BDC2.5 TCR transgene. *B*: Proportion of CD4$^+$ cells in BDC2.5/NOD $^{lpr/lpr}$ mice compared with in BDC2.5/NOD mice.

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 β-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

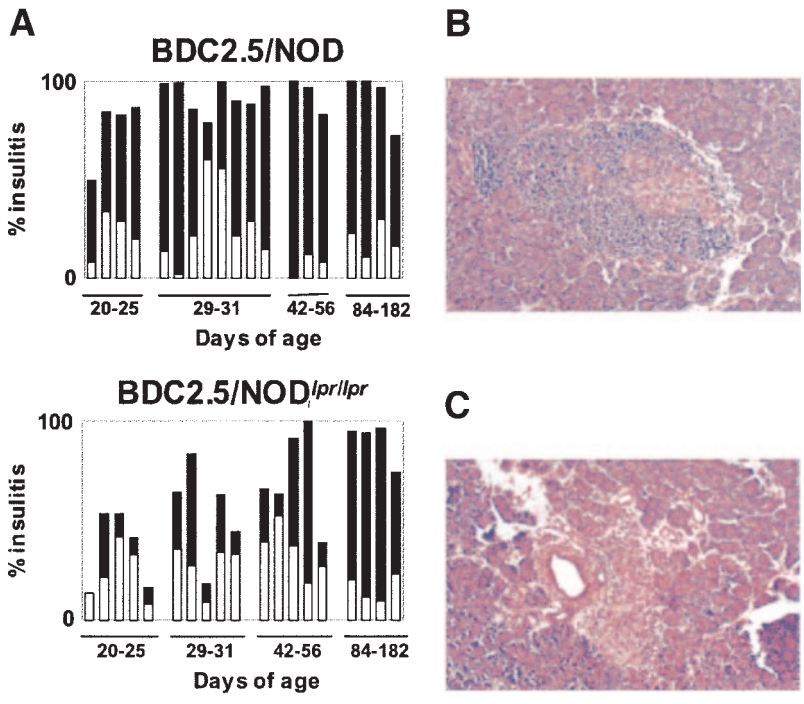

FIG. 2. Insulitis in BDC2.5/NOD$^{lpr/lpr}$ mice. *A*: BDC2.5 transgenic mice heterozygous (wild-type) or homozygous (*lpr/lpr*) for the *lpr* mutation were killed at the indicated ages; their pancreas sections were then prepared and scored as described in RESEARCH DESIGN AND METHODS. □, periinsulitis; ■, insulitis. Representative histology of islets from a 30-day-old BDC2.5/NOD (*B*) or BDC2.5/NOD$^{lpr/lpr}$ (*C*) animal is shown.

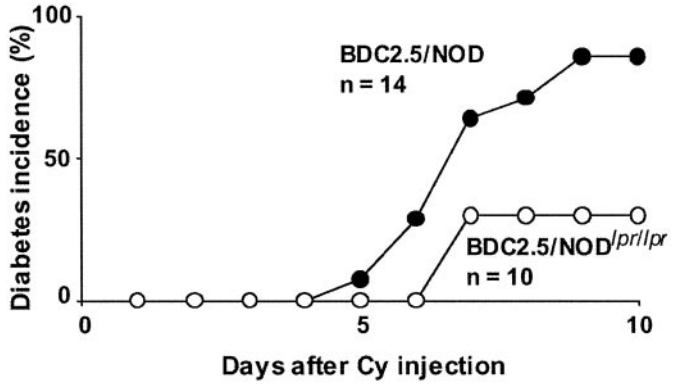

FIG. 3. CY-induced diabetes in BDC2.5/NOD$^{lpr/lpr}$ mice. On day 0, CY was injected at 200 mg/kg into BDC2.5/NOD$^{lpr/lpr}$ (○) and BDC2.5/NOD (●) mice, and diabetes development was monitored.

other gut or subcutaneous lymph nodes (27). 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. 4*A*), 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. 4*B*; note that the absolute values of the controls in Fig. 4*B* cannot be directly compared with those of Fig. 4*A*, 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$^{lpr/+}$ splenocytes stimulated with the BDC2.5 mimotope peptide 1040-63 (16) (Fig. 5*A*) or with extracts from purified islets (Fig. 5*B*). 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

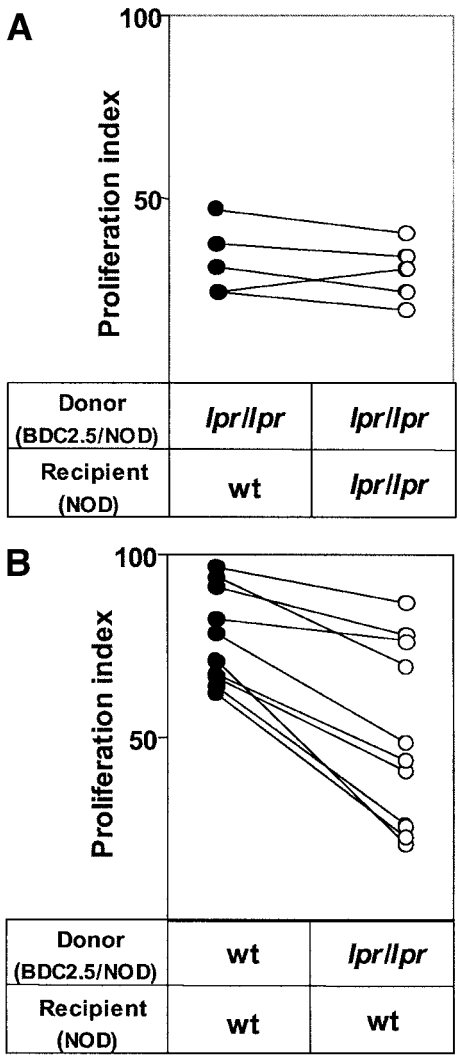

FIG. 4. Criss-cross transfer experiment. *A*: CFSE-labeled BDC2.5/NOD$^{lpr/lpr}$ splenocytes were transferred into NOD$^{lpr/lpr}$ or standard NOD mice. *B*: CFSE-labeled BDC2.5/NOD$^{lpr/lpr}$ or BDC2.5/NOD splenocytes were transferred into standard NOD mice. BDC2.5 splenocytes were isolated from 5-week-old mice; the recipients of 20 × 10^6 CFSE-labeled splenocytes were 25-day-old mice. PLNs were harvested 66 h after transfer and stained with CD44-CYCHR and CD4-PE Texas red. After gating on CD4$^+$ cells, we plotted the proportion of cells having diluted the CFSE (dividing cells). Each dot represents one mouse. wt, wild-type.

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. 5*C* 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. 5*E*). IL-2 addition had

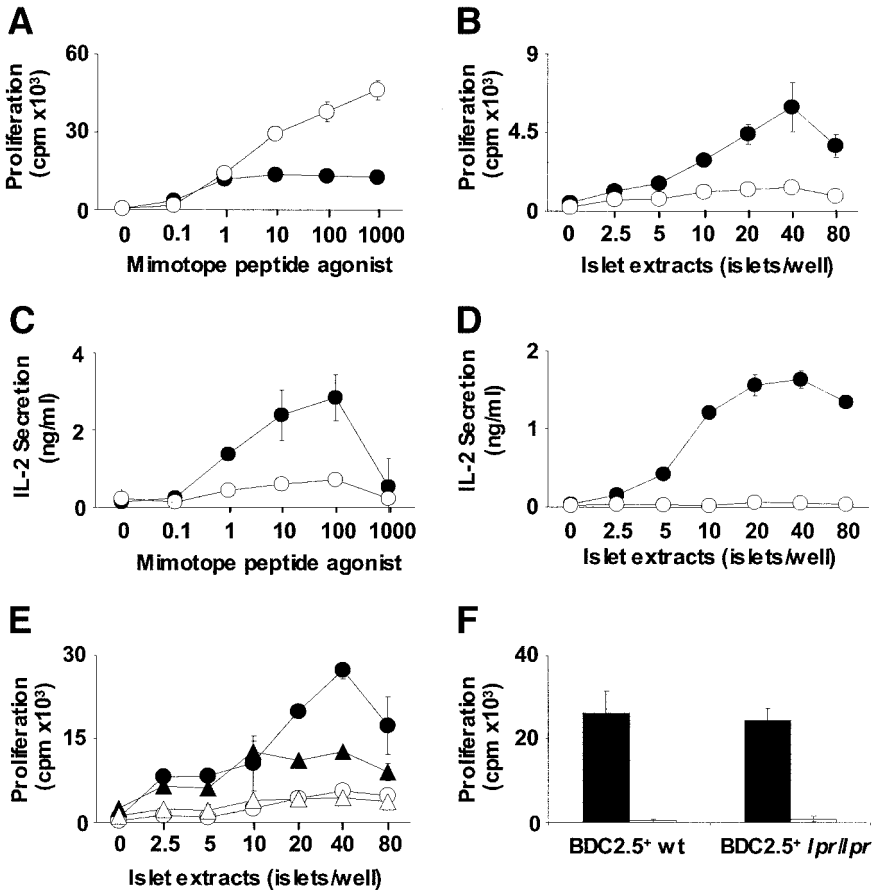

FIG. 5. In vitro assays of proliferation and IL-2 production. BDC2.5 splenocytes carrying (○, △) or not carrying (●, ▲) the *lpr* mutation were challenged with the BDC2.5 mimotope 1040-63 (*A*) or islet extracts (*B*) for 3 days. A pulse of radioactive thymidine was given to the cells 16 h before they were harvested. For quantitation of IL-2 production, splenocytes were challenged with the BDC2.5 mimotope 1040-63 (*C*) or islet extracts (*D*) for 3 days, and an IL-2 ELISA was performed with the supernatants. *E*: IL-2 complementation did not re-establish normal levels of proliferation by BDC2.5/NOD$^{lpr/lpr}$ splenocytes. △ and ○, BDC2.5/NOD$^{lpr/lpr}$; ▲ and ●, BDC2.5/NOD; △ and ▲, 10 ng/ml IL-2; ○ and ●, without IL-2. *F*: BDC2.5/NOD$^{lpr/lpr}$ splenocytes proliferated at the same rate as BDC2.5/NOD splenocytes with a strong stimulus (anti-CD3 plus anti-CD28).

no effect whatsoever on the proliferation of BDC2.5/NOD$^{lpr/lpr}$ 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*F*). When BDC2.5/NOD and BDC2.5/NOD$^{lpr/lpr}$ 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.

Anergy-specific genes' expression in the BDC2.5 *lpr/lpr* splenocytes. To explore the possibility that a defined state of anergy (32) might explain the inability of BDC2.5/N$^{lpr/lpr}$ T-cells to respond to cognate autoantigen, we performed an analysis by real-time quantitative PCR of genes that have been implicated in the anergic phenotype (18). We measured levels of expression of caspase-3, Ikaros, Grg-4, Jumonji, SOCS-2, CTLA-4, IL-10, and mGRAIL, a subset of the genes described by Macian et al. (18) to be particularly representative of the anergic response. Figure 6 illustrates that there was no significant difference in the expression of these anergy-related genes in BDC2.5/NOD$^{lpr/lpr}$ compared with BDC2.5/NOD$^{+/+}$ control splenocytes. These results, together with the inability of IL-2 complementation to overcome BDC2.5/NOD$^{lpr/lpr}$ T-cell hyporesponsiveness, suggest that conventional anergy is not the mechanism by which these T-cells are kept from proliferating.

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 *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 β-cell death that occurs just

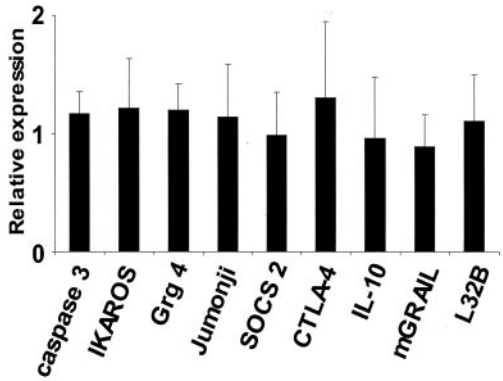

FIG. 6. Sybr green analysis of genes involved in anergy. mRNA was isolated from sorted BDC2.5^{high} *lpr/lpr* and BDC2.5^{high} wild-type splenocytes. "Relative expression" refers to the level of transcription of a gene in BDC2.5^{high} *lpr/lpr* splenocytes compared with in BDC2.5^{high} wild-type splenocytes. *HPRT* was used to normalize gene expression levels. *LB32* is a housekeeping gene. Three independent experiments were performed with pooled mice age 16 weeks (two to four mice/pool).

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 NOD$^{lpr/lpr}$ 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 tepid 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 NOD$^{lpr/lpr}$ 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/NOD$^{lpr/lpr}$ 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/NOD$^{lpr/lpr}$ 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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