Interferon-γ Receptor Signaling Is Dispensable in the Development of Autoimmune Type 1 Diabetes in NOD Mice

David V. Serreze, Cristina M. Post, Harold D. Chapman, Ellis A. Johnson, Binfeng Lu, and Paul B. Rothman

There have been two previous conflicting reports that the development of T-cell-mediated autoimmune diabetes (type 1 diabetes) was respectively unaffected or inhibited in NOD mice genetically deficient in the T-helper (Th) 1 cytokine interferon (IFN)-γ or the α-chain subunit of its receptor. Our goal was to resolve this conundrum by congenically transferring, from a 129 donor strain to the NOD background, a functionally inactivated gene for the β-chain signaling (located on chromosome 16) rather than the α-chain ligand binding domain (located on chromosome 10) of the IFN-γ receptor. These NOD.\(\text{IFNγRBnull}\) mice were characterized by normal patterns of leukocyte development and T-cells that produced greatly enhanced levels of the putatively type 1 diabetes–protective Th2 cytokine interleukin (IL)-4. However, despite being unable to respond to the primary Th1 cytokine IFN-γ and having T-cells that produce greatly enhanced levels of IL-4, NOD.\(\text{IFNγRBnull}\) mice remained highly susceptible to type 1 diabetes. This result indicated that the previously reported inhibition of type 1 diabetes in NOD mice carrying a functionally inactivated IFN-γ receptor α-chain gene may have been due to a closely and previously unidentified diabetes resistance allele. Furthermore, our results indicate that the pathogenicity of diabetogenic T-cells in NOD mice is not dampened by an inability to respond to IFN-γ and a concurrent shift to greatly enhanced Th2 cytokine production. This finding calls into question whether clinical protocols designed to shift β-cell autoreactive T-cells from a Th1 to Th2 cytokine production profile will truly be safe and efficacious in blocking the development of type 1 diabetes in humans. Diabetes 49:2007–2011, 2000

Type 1 diabetes in the NOD mouse model results from autoimmune destruction of insulin-producing pancreatic β-cells mediated by both CD4 and CD8 T-cells (1). The development of these autoreactive diabetogenic T-cell responses in NOD mice is controlled by complex interactions between multiple susceptibility (\(\text{Idd}\)) genes both within and outside of the \(\text{H}2\) major histocompatibility complex (2,3). \(\text{Idd}\) gene interactions have been proposed to elicit type 1 diabetes through several non–mutually exclusive pathogenic mechanisms. These include defects at both the T-cell and antigen-presenting cell level that preferentially inhibit the apoptotic deletion, but not the functional activation, of autoreactive effectors (1). However, it has also been widely believed that after avoiding deletional mechanisms, the pathogenic potential of β-cell autoreactive CD4 T-cells in NOD mice is respectively enhanced or inhibited, depending on whether they produce cytokines of the Th-helper (Th) 1 (primarily interferon [IFN]-γ) or Th2 type (primarily interleukin [IL]-4 and IL-10). These conclusions are primarily based on reports that Th1 to Th2 cytokine shifts are often observed among β-cell–infiltrating T-cells of NOD mice that are paradoxically protected from overt type 1 diabetes by many antigen-specific or non-specific immunostimulation protocols (4–6). An explanation for such putatively protective shifts may be provided by a report that at very high stimulation levels, CD4 T-cells can deviate from a Th1 to Th2 profile (7).

Whereas the induction of type 1 diabetes resistance by many different protocols has been associated with Th1 to Th2 cytokine shifts, several other factors call into question whether the pathogenicity of β-cell autoreactive CD4 T-cells in NOD mice can be strictly compartmentalized on the basis of these currently defined cytokine production profiles. These profiles include reports of Th1 and Th2 clonotypic T-cells isolated from NOD mice that, contrary to expectations, had a respective ability to inhibit or promote type 1 diabetes development (8–11). There is also uncertainty as to how essential IFN-γ, the prototypic Th1 cytokine, is to type 1 diabetes development in NOD mice. Hultgren et. al. (12) reported that type 1 diabetes development was not significantly retarded in NOD mice congenic for a functionally disrupted IFN-γ gene, suggesting that this Th1 cytokine does not play an obligate pathogenic role. In contrast, another group concluded that signals induced by IFN-γ do play an important diabetogenic role, since disease development was inhibited in NOD mice congenic for a functionally inactivated IFN-γ receptor α-chain gene (13). However, data from a subsequent study indicated that type 1 diabetes was not inhibited by the functionally inactivated IFN-γ receptor α-chain gene but rather by a closely linked and previously unidentified \(\text{Idd}\) resistance allele within the chromosome 10 congenic interval transferred to the NOD background from the 129 donor strain (14). The goal of the present study was to resolve these conflicting sets of observations.
We reasoned that there could be several explanations for the apparently contradictory findings that type 1 diabetes development is respectively unaffected or reduced in NOD mice made genetically deficient in IFN-γ or the α-chain subunit of its receptor. The first of these explanations is that the retained type 1 diabetes susceptibility of NOD mice genetically deficient in IFN-γ can be explained by the presence of an unknown alternative ligand(s) that can initiate signaling through the IFN-γ receptor. A second possibility was that Kanagawa et al. (14) were correct in their conclusion described above that type 1 diabetes was not inhibited by the functionally inactivated IFN-γ receptor α-chain gene but rather by a closely linked Idd resistance allele from the 129 congeneric donor strain. In addition to the ligand binding α-chain subunit, the IFN-γ receptor also consists of a β-chain signaling subunit for which structural gene resides on chromosome 16 (15). If the alternative ligand hypothesis was correct, we reasoned that NOD mice deficient in the β-chain subunit of the IFN-γ receptor would be characterized by the same type 1 diabetes resistance originally reported for those mice lacking the α-chain subunit. In contrast, if IFN-γ receptor β-chain–deficient NOD mice remained susceptible to type 1 diabetes, this would support the conclusion that disease inhibition in mice lacking the α-chain subunit was actually the result of a linked Idd resistance gene.

RESEARCH DESIGN AND METHODS

Mice. NOD/Lt mice are maintained in a specific pathogen-free research colony at the Jackson Laboratory. Currently, type 1 diabetes develops in 90% of female and 63% of male NOD/Lt mice by 1 year of age. A previously described IFN-γ receptor β-chain allele (16) functionally disrupted by insertion of a neomycin resistance (neo) gene (official designation Ifngr2tm1Cmb and here as IFN-γRBnull for simplicity) was transferred to the NOD background by our “speed congenic” approach (17). At the N8 backcross generation, mice heterozygous for the IFN-γRBnull allele and fixed to homozygosity for linkage markers delineating all known Idd loci of NOD origin were intercrossed. The frequency of type 1 diabetes development in the resulting intercross progeny that were homozygous for the IFN-γRBnull allele was compared with that of IFN-γRB intact mice (pooled +/- and +/- segregation). During the course of establishing this NOD congenic stock, carriers of the disrupted IFN-γRBnull allele were identified by polymerase chain reaction (PCR) using the primer set 5′-GCTATTCCGCTATGCTGGG-3′ and 5′-GAAGGGGATAGAAGGCAGT-3′, which generates a 706-bp product from within the neo’ insert. Presence of the intact wild-type IFN-γRB allele was typed by PCR with the primer set 5′-TCGTTCTCGCCATGGGC-3′ and 5′-CAAGGTATATCCACCTGTA-3′, which generates a 132-bp product. This latter primer pair does not amplify a PCR product from the IFN-γRBnull allele. Thus, typing N8 intercross mice with both PCR primer sets allowed us to distinguish segregants homozygous for the IFN-γRBnull allele from those homozygous or heterozygous for an intact IFN-γRB gene.

Assessment of diabetes development. Mice were monitored for development of glycosuria with Ames Dia-stix (supplied by Miles Diagnostics, Elkhart, IN). Glycosuric values of ≥3 were considered diagnostic of diabetes onset. Flow cytometric analysis of leukocyte subsets. Proportions of various splenic leukocyte subsets in the indicated mice were determined by multicolor flow cytometric techniques (FACScan; Becton Dickinson, San Jose, CA). The monoclonal antibody 145-2C11, specific for the CD3 component of the T-cell receptor (TCR) and conjugated to a green fluorescent fluorescein isothiocyanate (FITC) tag, was used to detect T-cells. These T-cells were then further characterized for CD4 expression using the monoclonal antibody GK1.5 conjugated to the red fluorescent tag Cy3.18-Osu (Cy3; Biological Detection Systems, Pittsburgh, PA) or for CD8 expression with the monoclonal antibody 53-6.72 conjugated to a red fluorescent phycoerythrin Tag. B-cells were detected with a FITC-conjugated goat polyclonal antiserum specific for mouse Ig (Southern Biotechnology Associates, Birmingham, AL). As FITC-conjugated Gr-1 specific monoclonal antibody (RB68C5) was used in combination with a phycoerythrin-conjugated Mac-1 specific monoclonal antibody (M1/70) to delineate macrophages/dendritic cells and granulocytes. Macrophages/dendritic cells stain with the Mac-1 antibody only, whereas granulocytes co-stain with both the Mac-1 and Gr-1 antibodies.

RESULTS

Leukocyte subset development is not altered in NOD.IFN-γRBnull mice. A previous study demonstrated that the IFN-γRBnull mutation did not elicit any alterations in the proportions of four splenic leukocyte subsets in non–autoimmune prone mice with a mixed 129 and C57BL/6 (B6) genetic background (16). However, the NOD genetic background promotes an overaccumulation of T-cells that may be critical for the development of autoimmune diabetes (5) and that could conceivably be dependent on IFN-γ signaling. Thus, we examined whether the relative proportions of any splenic leukocyte subset in N8 NOD backcross segregants with a functionally disrupted IFN-γRB gene differed from those of standard NOD mice. However, as shown in Table 1, NOD.IFN-γRBnull mice were not characterized by significant changes in the development of any leukocyte subset.

NOD.IFN-γRBnull mice are characterized by enhanced Th2 responses. It is widely believed that a propensity for Th1 cytokine production by CD4 T-cells is an important component for the development of autoimmune type 1 diabetes in NOD mice. Based on previous observations in mice with a mixed 129 and B6 genetic background (16), we reasoned that the loss of IFN-γ–mediated signaling might impair the development of such Th1 responses in NOD.IFN-γRBnull mice. Hence, we compared the pattern of cytokines produced by anti-CD3–stimulated splenic T-cells from the NOD.IFN-γRBnull stock with those from standard NOD mice. As shown in Fig. 1, the levels of IFN-γ produced by anti-CD3–stimulated T-cells from NOD.IFN-γRBnull mice was actu-

<table>
<thead>
<tr>
<th>Strain</th>
<th>% CD4 T-cells</th>
<th>% CD8 T-cells</th>
<th>% B-cells</th>
<th>% Macrophages/dendritic cells</th>
<th>% Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD</td>
<td>29.6 ± 1.4</td>
<td>13.8 ± 1.2</td>
<td>45.2 ± 1.2</td>
<td>4.8 ± 0.4</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>NOD.IFN-γRBnull</td>
<td>29.6 ± 1.1</td>
<td>12.2 ± 0.5</td>
<td>47.7 ± 3.4</td>
<td>7.3 ± 0.9</td>
<td>4.0 ± 0.1</td>
</tr>
</tbody>
</table>

Data are mean ± SE of each splenic leukocyte population. Splenic leukocytes from standard NOD and NOD.IFN-γRBnull female mice were characterized by flow cytometry as described in RESEARCH DESIGN AND METHODS.
ally slightly higher than that of standard NOD T-cells. This could be due to an absence of negative feedback inhibition in NOD .IFNγRB null mice. Alternatively, the enhanced levels of IFN-γ in culture supernatants of anti-CD3–stimulated T-cells from NOD .IFNγRB null mice might represent a cytokine that is normally bound up in an autocrine fashion by IFNγR intact cells. However, of greater significance was the finding that the Th2 indicator cytokine, IL-4, was produced at much higher levels by anti-CD3–stimulated T-cells from NOD .IFNγRB null than from standard NOD mice.

**NOD .IFNγRB null mice remain susceptible to type 1 diabetes.** Based on their inability to respond to IFN-γ and an enhanced ability to produce Th2 cytokines, it might be expected that NOD .IFNγRB null mice would be rendered resistant to type 1 diabetes. However, whereas there was a slight delay in the initial time of onset, by 30 weeks of age, the female incidence of type 1 diabetes in NOD .IFNγRB null mice did not differ from segregants with a functionally intact

**FIG. 1.** Comparison of Th1 and Th2 cytokine secretion by anti-CD3–stimulated splenic T-cells from IFNγRB intact and deficient NOD females. Splenic T-cells underwent anti-CD3 stimulation as described in RESEARCH DESIGN AND METHODS, and culture supernatants were assessed by ELISA for concentrations of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4. Data represent the mean cytokine concentration ± SD produced by splenic T-cells from two mice of each strain. Similar results were obtained in a second experiment.

**FIG. 2.** Female incidence of type 1 diabetes through 30 weeks of age in an N8 backcross stock of NOD mice homozygous for a functionally disrupted IFNγRB allele compared with littermate controls with an intact IFNγRB gene (pooled +/+ and +/− segregants). N8 mice in each genotypic class were fixed to homozygosity for linkage markers delineating all known NOD Idd loci. Cumulative incidence of type 1 diabetes at 30 weeks of age did not statistically differ (P > 0.9, χ² analysis) between IFNγRB intact and deficient segregants.

**DISCUSSION**

Our present results indicate that signals mediated by the Th1 cytokine IFN-γ are dispensable in the development of type 1 diabetes in the NOD mouse. These results, along with results from another recent study (14), also help resolve the conundrum resulting from previous conflicting reports that type 1 diabetes onset is retarded, but not prevented, in IFN-γ-deficient NOD mice, whereas the overall incidence of disease is inhibited in a NOD stock genetically deficient in the IFN-γ receptor (12,13). One possible explanation for these apparently conflicting results could have been that the presence of an alternative unknown ligand accounted for the retained type 1 diabetes susceptibility of NOD mice genetically deficient in IFN-γ. However, this possibility is ruled out by our finding that NOD mice genetically deficient in the β-chain signaling unit of the IFN-γ receptor also remain susceptible to type 1 diabetes. Instead, our current findings support the conclusion reached by Kanagawa et al. (14), who used a different experimental system, that the basis for the previous contradictory reports described above

**IFNγRB gene (Fig. 2).** These results essentially duplicate those previously reported for a stock of NOD mice with a functionally inactivated IFN-γ gene (12).
is that type 1 diabetes was not inhibited in NOD mice by the presence of a functionally inactivated IFN-γ receptor α-chain gene but rather by a closely linked Idd resistance allele from the 129 congenic donor strain. However, there could also be another explanation for the finding that type 1 diabetes development is inhibited in NOD mice lacking the α-subunit but not the β-subunit of the IFN-γ receptor. This explanation is that the α-subunit could pair with an alternative unknown signaling molecule in our NOD. IFN-γRBnull mice.

Another significant aspect of our present results is that they call into question whether the pathogenicity of β-cell autoreactive CD4 T-cells in NOD mice can be dampened by skewing them toward a Th2 cytokine production profile. In addition to being unable to reach the key Th1 cytokine IFN-γ, NOD. IFN-γRBnull mice are also characterized by greatly enhanced T-cell secretion of the Th2 cytokine IL-4. Hence, based on the widely known paradigm that the pathogenicity of β-cell autoreactive CD4 T-cells in NOD is respectively enhanced or inhibited when producing Th1 versus Th2 cytokines, it might be expected that NOD. IFN-γRBnull mice would be strongly resistant to type 1 diabetes. This is clearly not the case because NOD. IFN-γRBnull mice actually develop type 1 diabetes at a very high rate. Hence, β-cell autoreactive T-cells from NOD mice retain their full pathogenic potential even in a greatly enhanced Th2 cytokine environment. In this regard, it should be noted that the Th1 to Th2 cytokine shifts that have been reported to characterize NOD mice protected from type 1 diabetes by numerous protocols (4–6) may actually be an outcome rather than the cause of disease resistance. One frequently overlooked factor that could lead to the secondary appearance of such shifts is that higher rates of activation-induced cell death occur among Th1 than Th2 cytokine-producing CD4 T-cells (20,21). Hence, some protocols may actually protect NOD mice by partially deleting β-cell autoreactive T-cells in a way that preferentially spares those producing Th2 cytokines. Such an "unmasking" process could give a secondary appearance of a Th1 to Th2 cytokine shift. However, it is also possible that because they were characterized by increased production of both IFN-γ and IL-4, T-cells from NOD. IFN-γRBnull mice are in a general hyperactivated state. We consider this latter possibility to be unlikely because if their T-cells were in a general hyperactivated state, type 1 diabetes development would be expected to occur more rapidly in NOD. IFN-γRBnull mice than in standard NOD mice, which is clearly not the case.

In conclusion, our results indicate that signals mediated by the Th1 cytokine IFN-γ are not required for the development of T-cell–mediated autoimmune type 1 diabetes in NOD mice. Furthermore, the pathogenicity of diabeticogenic T-cells in NOD mice is not dampened by an inability to respond to IFN-γ and a concurrent shift to greatly enhanced Th2 cytokine production. These results do not exclude the possibility that IFN-γ is an important effector cytokine in the spontaneous development of type 1 diabetes in standard NOD mice. Indeed, this possibility is supported by the fact that whereas genetic ablation of IFN-γ or the β-subunit of its receptor does not reduce the final frequency of type 1 diabetes in NOD mice, it does delay the initial time of disease onset. However, our results do indicate that the NOD mouse also brings a formidable array of other damaging cytokines into the pancreatic β-cell environment that can contribute to their destruction in the absence of IFN-γ–mediated events. Furthermore, the present results also call into question whether clinical protocols designed to shift β-cell autoreactive T-cells from a Th1 to Th2 cytokine production profile will truly be safe and efficacious in blocking the development of type 1 diabetes in humans deemed to be at high future risk for this disease.

ACKNOWLEDGMENTS

Work at the Jackson Laboratory was supported by National Institutes of Health Grants DK51090, DK46266, and AI41469; Cancer Center Support (CORE) Grant CA34196; and grants from Juvenile Diabetes Foundation International. Work at Columbia University was supported by National Institutes of Health Grant AI39675 and American Cancer Society Grant IM783.

Holly Savage is thanked for excellent animal care assistance.

REFERENCES

19. Christianson SW, Shultz LD, Leiter EH: Adoptive transfer of diabetes into...