Congenic Mapping of the Diabetogenic Locus *Idd4* to a 5.2-cM Region of Chromosome 11 in NOD Mice

Identification of Two Potential Candidate Subloci

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Twenty diabetes susceptibility loci on 12 mouse chromosomes have been identified to control the development of type 1 diabetes at the level of either initiation of insulitis or progression from insulitis to overt diabetes or both. Previously, we demonstrated that the genetic control of T-cell proliferative unresponsiveness in nonobese diabetic (NOD) mice is linked to *Idd4* on mouse chromosome 11. Here, we show by congenic mapping of three newly generated NOD.B6*Idd4* diabetes-resistant mouse strains that *Idd4* is limited to a 5.2-cM interval of chromosome 11. This B6-derived region expressed in NOD.B6*Idd4A* mice maps between the D11Nds1 (43.8 cM) and D11Mit38/D11Mit325 (49.0 cM) markers and dramatically reduces the development of both insulitis and type 1 diabetes. NOD.B6*Idd4B* and NOD.B6*Idd4C* mice, which carry a smaller B6-derived segment of chromosome 11 that spans <5.2 cM distal to D11Nds1, exhibit protection against type 1 diabetes with the restoration of T-cell proliferation. Our findings suggest that diabetes resistance conferred by *Idd4* may be mediated by the *Idd4.1* and *Idd4.2* subloci. *Idd4.1* is localized in the D11Nds1 interval that influences both diabetes and insulitis. *Idd4.2* is localized within the D11Mit38/325 interval that mainly influences diabetes incidence and restores T-cell proliferative responsiveness. Three potential candidate genes, platelet activating factor acetylhydrolase Ib1, nitric oxide synthase-2, and CC chemokine genes, are localized in the 5.2-cM interval. *Diabetes* 51:215–223, 2002

The nonobese diabetic (NOD) mouse spontaneously develops autoimmune type 1 diabetes with an immunopathologic profile similar to the human disease (1). Type 1 diabetes in the NOD mouse is a complex, multigenic disease characterized by T-cell infiltration (insulitis) followed by a progressive destruction of pancreatic islet β-cells. Many immunologic and genetic studies have attempted to define various phenotypes that contribute to disease susceptibility. In both humans and NOD mice, genes that map to the major histocompatibility complex (MHC) are important in the disease process. Analyses of MHC congenic strains have shown that the NOD MHC (H-2^{g7}) is required for the development of type 1 diabetes. In NOD mice, this gene conferring susceptibility to type 1 diabetes is termed *Idd1* on chromosome 17 (2,3).

Despite the importance of the MHC to the disease process, non-MHC genes also contribute to the breakdown of self-tolerance and development of type 1 diabetes (4). Although the nature of these non-MHC genes is not well-defined in the human disease, genetic analyses of NOD mice have revealed that several genes are involved. The identification of such genes may be expected to facilitate our understanding of the molecular basis of type 1 diabetes. For analyzing the genetic control of the development of type 1 diabetes in NOD mice, congenic mouse strains have been produced based on linkage mapping. Protection from disease in such strains provides evidence of the existence of individual *Idd* loci. To date, 19 non-MHC–associated susceptibility loci have been identified (*Idd2-Idd12*, *Idd13a*, *Idd13b*, and *Idd14-Idd19*) on 12 chromosomes through crosses of NOD mice with various diabetes-resistant strains (5). These loci act in a collective, recessive manner to determine development of insulitis as well as disease frequency and severity. Some of these have been mapped to specific chromosomal locations, although their products have not been identified (6,7).

Previously, we showed that thymic and peripheral T-cells from NOD mice exhibit a proliferative hyporesponsiveness (anergy) upon cross-linking of the T-cell receptor (TCR)/CD3 complex (8,9). This T-cell hyporesponsiveness is genetically inheritable and is mediated by the reduced secretion of both interleukin (IL)-2 and IL-4 (8,10,11) as well as a defect in the protein kinase C/Ras/mitogen-activated protein kinase T-cell activation signaling pathway (9). Notably, there is an age-dependent correlation between the onset of this T-cell hyporesponsiveness and insulitis development in NOD mice, both of which persist until the onset of type 1 diabetes (10). On the basis of the hypothesis that a gene that regulates T-cell proliferation may affect type 1 diabetes susceptibility in NOD mice, we investigated the genetic control of this T-cell proliferative hyporesponsiveness trait. Our initial genome scan conducted by quantitative trait loci and genetic linkage anal-

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AH, acetylhydrolase; EAE, experimental allergic encephalomyelitis; IFN-γ, γ-interferon; IL, interleukin; MCP, monocyte chemotactic protein; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; NOS-2, nitric oxide synthase-2; Orch, autoimmune orchitis resistance; PAF, platelet activating factor; TCR, T-cell receptor.

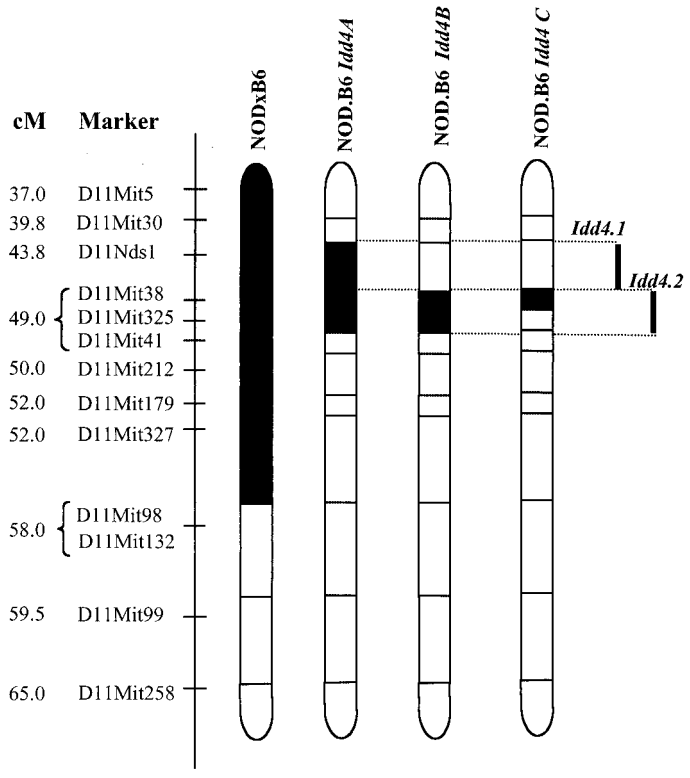

FIG. 1. Congenic mapping of *Idd4* loci on chromosome 11. NOD.B6 congenic mice were typed using genomic DNA and the complete panel of polymorphic microsatellite markers as described in RESEARCH DESIGN AND METHODS. Retained B6 loci (■) are shown with flanking markers, which were determined to be homozygous for NOD alleles (□). Map distance of microsatellite markers from the centromere is derived from the Mouse Genome Database (Mouse Genome Informatics, the Jackson Laboratory).

yses revealed that the control of NOD T-cell proliferative hyporesponsiveness genetically maps to a central region on mouse chromosome 11 that includes the *Idd4* locus (12). In the present study, we extended this genetic mapping of *Idd4* by generating and analyzing three novel congenic NOD.B6*Idd4* mouse strains that display a profound resistance to diabetes. These analyses limit the *Idd4* locus to a 5.2-cM region of chromosome 11 and raise the possibility that *Idd4* may be split into two subloci, *Idd4.1* and *Idd4.2*.

RESEARCH DESIGN AND METHODS

Mice. NOD/Del female mice were bred in a specific pathogen-free barrier facility at The John P. Robarts Research Institute (London, Ontario, Canada). In our colony of female NOD mice, islet infiltration begins at 4–6 weeks of age and progression to destructive insulitis and overt diabetes occurs by 4–6 months of age. The incidence of diabetes in female NOD mice is 40–50% at 20 weeks of age and >75% by 25 weeks. Severe combined immunodeficient NOD (NOD.*Scid*) mice, provided by Dr. L. Shultz (The Jackson Laboratory, Bar Harbor, ME), were bred in our colony and used as recipients in T-cell transfer experiments. C57BL/6J (B6) mice were obtained from Charles River Laboratories (Montreal, Quebec, Canada).

For developing novel NOD.B6*Idd4* congenic strains, (NOD × B6) (BXN) recombinant inbred mice previously generated between the parental B6 and NOD strains (12) were backcrossed three generations onto the NOD background. Mice were intercrossed to generate mice expressing different amounts of B6-derived chromosome 11 introgressed onto the NOD genetic background (Fig. 1). Genotyping was performed using the microsatellite markers D11NdsI, D11Mit38, and D11Mit325, which detect DNA polymorphisms on chromosome 11 between NOD and B6. Additional markers used to confirm the presence of NOD genes telomeric to *Idd4* on chromosome 11 are shown in Table 1. The chromosomal positions of these markers were determined according to the most current map of mouse chromosome 11 (Mouse Genome Database, Mouse Genome Informatics, the Jackson Laboratory [online at http//www.informatics.jax.org]). These analyses narrowed the region of interest to a chromosomal distance of ~5.2cM (from D11Nds1 to D11Mit38/325). For ensuring that *Idd* loci on chromosomes other than chromosome 11 are NOD in origin, the founding breeder pairs of each NOD congenic strain were genotyped for the *Idd* 1–10, 12–15 microsatellite markers, as follows: D17Mit21 (*Idd1*); D9Nds3 (*Idd2*); D3Nds42, D3Nds43, D3Mit3, D3Mit10 (*Idd3*); D11Mit38 (*Idd4*); D1Mit3, D1Mit26 (*Idd5*); D6Mit15 (*Idd6*); D7Mit105 (*Idd7*); D4Mit59 (*Idd8*); D3Mit10 (*Idd10*); D4Mit11, D4Mit199 (*Idd11*); D14Mit61 (*Idd12*); D2Mit257 (*Idd13*); D13Mit61 (*Idd14*); and D5Mit48 (*Idd15*). By breeding congenic mice, we generated three NOD.B6*Idd4* congenic mice strains on the NOD background containing ~5.2 cM (from D11Nds1 to D11Mit38/D11Mit325) of the introgressed B6-derived *Idd4* locus. Strain NOD.B6*Idd4A* carries the D11Nds1 and D11Mit38/325 intervals, strain NOD.B6*Idd4*B carries only D11Mit38/325, and strain NOD.B6*Idd4*C carries only D11Mit38 (Fig. 1). Mice of the desired genotype were intercrossed, and F4–F6 generation pups were used for phenotypic analysis.

TABLE 1
Chromosome 11 microsatellite marker mapping

Marker	cM	NOD.B6*Idd4A* genotype	NOD.B6*Idd4B* genotype	NOD.B6*Idd4C* genotype
D11Mit78	2.0	N	N	N
D11Mit151	13.0	N	N	N
D11Mit84	17.0	N	N	N
D11Mit367	19.5	N	N	N
D11Mit187	20.0	N	N	N
D11Mit313	28.0	N	N	N
D11Mit349	32.0	N	N	N
D11Mit5	37.0	N	N	N
D11Mit30	39.8	N	N	N
D11Nds1	43.8	B	N	N
D11Mit38	49.0	B	B	B
D11Mit325	49.0	B	B	N
D11Mit41	49.0	N	N	N
D11Mit212	50.0	N	N	N
D11Mit179	52.0	N	N	N
D11Mit327	52.0	N	N	N
D11Mit98	58.0	N	N	N
D11Mit132	58.0	N	N	N
D11Mit99	59.5	N	N	N
D11Mit258	65.0	N	N	N

The genotype at each marker found to be NOD (N) or B6 (B). The chromosome position for each marker is indicated (cM).

Assessment of diabetes. Mice were monitored for the development of type 1 diabetes by blood glucose levels, as described (13). Mice with a reading of >11.1 mmol/l (200 mg/dl) glucose on two consecutive occasions were indicative of the onset of type 1 diabetes.

DNA isolation and polymerase chain reaction amplification. Genomic DNA was isolated from mice tail samples using the QIAamp Tissue Kit (Qiagen, Chatsworth, CA), and mouse genotyping was performed by polymerase chain reaction amplification (12).

Adoptive T-cell transfer. Purified splenic T-cells isolated from 12-week-old female nondiabetic congenic mice and diabetic female NOD mice were transferred intraperitoneally (5×10^6 cells/recipient) to 6- to 8-week-old female NOD.*Scid* recipients (n = 10–15 mice/group) (13). Alternatively, 2.5×10^6 purified splenic T-cells from nondiabetic congenic mice were co-transferred with diabetogenic NOD T-cells (2.5×10^6) into NOD.*Scid* recipients.

Histopathology analysis. Pancreata were removed, fixed with 10% buffered formalin, embedded in paraffin, and sectioned at 5-μm intervals. The incidence and severity of insulitis and insulin immunostaining were analyzed as described (13). A minimum of 30 islets from each mouse were observed, and insulitis scores were determined blindly by two observers as follows: 0, normal; 1, peri-insulitis (mononuclear cells surrounding islets and ducts but no infiltration of the islet architecture); 2, moderate insulitis (mononuclear cells infiltrating, <50% of the islet architecture); and 3, severe insulitis (>50% of the islet tissue infiltrated by lymphocytes, accompanied by a reduction in insulin staining). Insulitis index (I) was calculated using the following formula: I = [(# islets with score of 0 × 0) + (# islets with score of 1 × 1) +

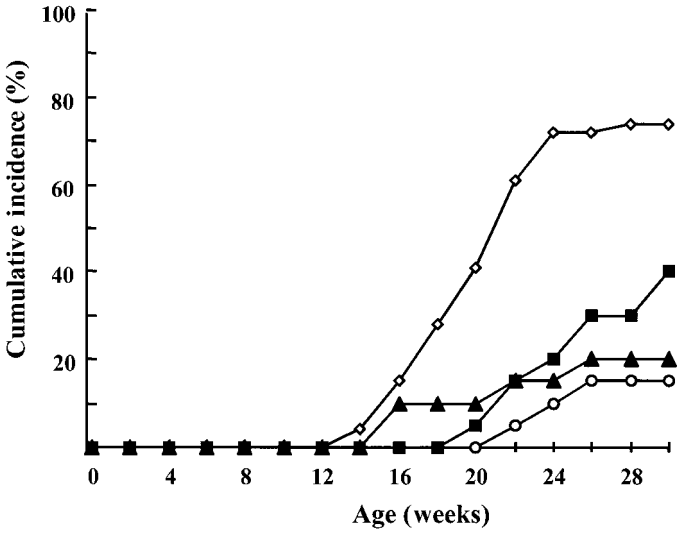

FIG. 2. Cumulative incidence of diabetes in congenic NOD.B6*Idd4* mice. Female mice were monitored weekly for blood glucose levels until 30 weeks of age. ◇, NOD (n = 45); ○, NOD.B6*Idd4A* (n = 20); ■, NOD.B6*Idd4B* (n = 20); ▲, NOD.B6*Idd4C* (n = 20).

(# islets with score of 2 × 2) + (# islets with score of 3 × 3)]/three × (total # islets).

T-cell proliferation. Splenic T-cells were isolated (11) from 8- to 10-week-old female mice; cultured (10^6/ml) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 10 mmol/l HEPES buffer, 1 mmol/l sodium pyruvate, 2 mmol/l L-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 μmol/l 2-ME (Life Technologies, Burlington, Ontario, Canada); and stimulated with the plate-bound 145–2C11 anti-CD3 monoclonal antibody (0.5 μg/ml; Cedarlane Laboratories, Hornby, Ontario, Canada). Cells were harvested after 72 h and were then assayed for the incorporation of [^{3}H] thymidine (1 μCi/well; Amersham, Oakville, Ontario, Canada) added during the last 18 h of culture.

Cytokine production assays. Culture supernatants collected after 72 h were assayed for T-cell cytokine production by enzyme-linked immunosorbent assay as described (13).

Statistical analysis. Results were compared using the Student's t test for splenic T-cell proliferation and cytokine production. Insulitis scores were compared by χ^2 analysis. For analyses of cumulative diabetes frequencies, the Wilcoxon's log rank test was used. Values with a $P < 0.05$ were considered to be significant.

RESULTS

Incidence of type 1 diabetes in NOD.B6*Idd4* mice. The cumulative incidence and age of onset of type 1 diabetes was monitored in wild-type NOD parental mice and NOD.B6*Idd4A* congenic mice up to 30 weeks of age. Whereas the onset of type 1 diabetes begins at 12–15 weeks of age and reaches an incidence of 75% by 30 weeks of age in wild-type female NOD mice, type 1 diabetes does not begin until 22 weeks of age ($P < 0.001$) and progresses to 15% incidence by 30 weeks ($P < 0.01$) in female NOD.B6*Idd4A* mice (Fig. 2). The incidence of type 1 diabetes was <5% in male NOD.B6*Idd4A* mice compared with a 45% incidence in male NOD mice at 30 weeks of age (data not shown). Thus, the D11Nds1 to D11Mit38/D11Mit325 interval of *Idd4* regulates protection from the spontaneous development of type 1 diabetes.

Analyses of two other NOD.B6*Idd4* congenic strains that do not carry the B6-derived D11Nds1 interval showed that female NOD.B6*Idd4B* and NOD.B6*Idd4C* mice possess a reduced incidence, with only 40% and 20% of mice, respectively, developing type 1 diabetes at 30 weeks of age (Fig. 2). The onset of type 1 diabetes is significantly delayed ($P < 0.001$) until 18–20 weeks in NOD.B6*Idd4B* mice compared with the 12- to 15-week age of onset in NOD mice. The incidence of type 1 diabetes in NOD.B6*Idd4A* and NOD.B6*Idd4B* mice is not statistically different at 30 weeks of age ($P = 0.08$). Although a small proportion of NOD.B6*Idd4C* mice develop type 1 diabetes as early as 16 weeks of age, this time of onset does not differ statistically from that observed in NOD.B6*Idd4A* and NOD.B6*Idd4B* mice ($P > 0.05$). However, note that the age of onset of type 1 diabetes in these NOD.B6*Idd4* strains is significantly delayed compared with that of NOD mice ($P < 0.05$). Thus, genes within the *Idd4* boundaries of D11Nds1 and D11Mit 325/38 influence the age of onset of type 1 diabetes. These observations further support a role for *Idd4* in conferring disease susceptibility and suggest that the absence of the B6-derived D11Nds1 interval in *Idd4* still provides considerable protection.

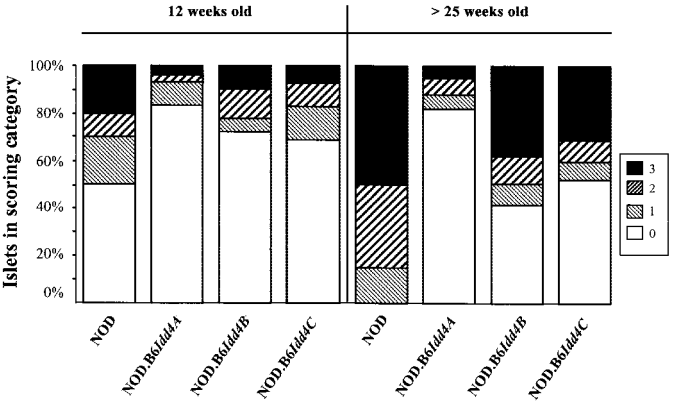

FIG. 3. Insulitis in NOD and congenic NOD.B6*Idd4* mice. Formalin-fixed pancreata were harvested from mice (n = 7–10/group) at the indicated time points. The number of islets (≥30) in each category is reported as a percentage of total islets observed. Scoring categories are as follows: 0, normal; 1, peri-insulitis (mononuclear cells surrounding islets and ducts but no infiltration of the islet architecture); 2, moderate insulitis (mononuclear cells infiltrating, <50% of the islet architecture); and 3, severe insulitis (>50% of the islet tissue infiltrated by lymphocytes, accompanied by a reduction in insulin staining).

Insulitis in NOD.B6*Idd4* mice. Linkage analysis of the NOD genome revealed that there are two classes of non-MHC genes; the first consists of genes with disease resistance alleles that reduce the development of both insulitis and type 1 diabetes, and the second is composed of genes that prevent type 1 diabetes without reducing the amount of insulitis (6). To determine whether protection from type 1 diabetes in NOD.B6*Idd4* mice correlates with a reduction of insulitis, we examined the pancreatic histology in the NOD.B6*Idd4* congenic strains at different ages. NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice were found to have similar percentages (>70%) of normal islets at 12 weeks of age and a significantly greater percentage than those in NOD mice (<50%; $P < 0.05$). However, at 25–30 weeks of age, the insulitis scores in NOD.B6*Idd4A* mice are significantly lower (<10% infiltrated islets, $I = 0.07$) than those in NOD.B6*Idd4B* and NOD.B6*Idd4C* mice (<45% infiltrated islets, $I = 0.3–0.4$; $P < 0.001$) (Fig. 3). Note that the insulitis scores in NOD.B6*Idd4B* and NOD.B6*Idd4C* mice are significantly lower than those in NOD mice (>80% infiltrated islets, $I > 0.5$; $P < 0.05$). Histological analyses also showed that pancreatic islets from >25-week-old NOD.B6*Idd4A* (Fig. 4*B*) and NOD.B6*Idd4C* (Fig. 4*C*) mice have a much lower

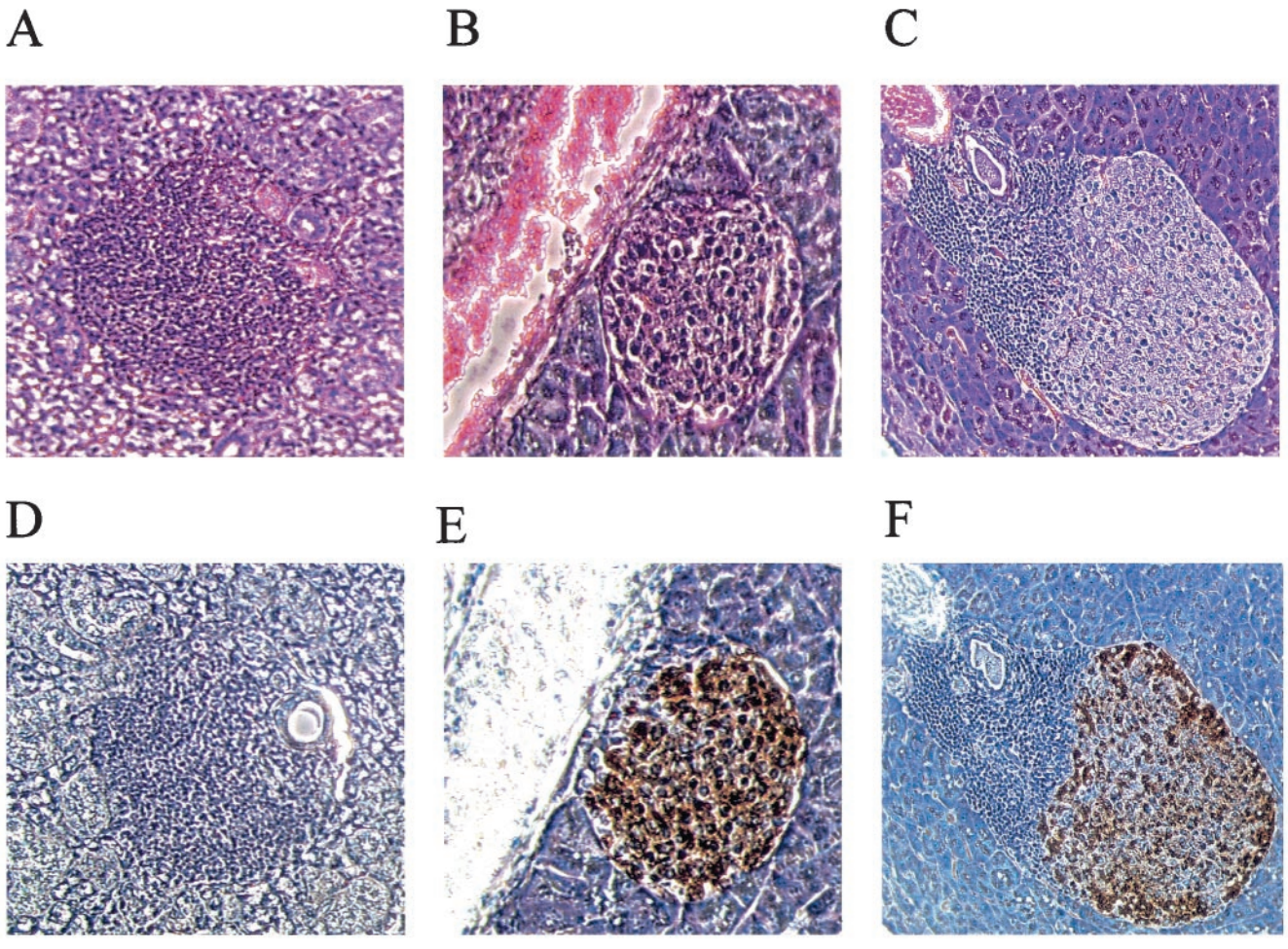

FIG. 4. Histopathology in congenic NOD.B6*Idd4* mice. Sections from the pancreas of >25-week-old NOD, NOD.B6*Idd4A*, and NOD.B6*Idd4C* mice were stained with hematoxylin and eosin (*A–C*) or insulin (*D–F*). NOD mice show severe destructive islet (*A*) with rare stored insulin (*D*); NOD.B6*Idd4A* mice have occasional periductal infiltrates and noninvasive peri-insulitis (*B*) but have healthy islets with insulin-producing β-cells (*E*); NOD.B6*Idd4C* mice show typical peri-insulitis as well as small invasive insulitis (*C*) but have healthy, well-granulated β-cells (*F*). Results from one of two studies are shown.

degree of islet infiltration than do NOD mice at this age (Fig. 4*A*). The patterns of infiltration in NOD.B6*Idd4B* mice (not shown) were similar to those in NOD.B6*Idd4C* mice in both age groups. Thus, the extent of islet infiltration in NOD.B6*Idd4* mice is significantly decreased compared with that in NOD mice of similar age. These observations indicate that the region encompassing D11Nds1 and D11Mit38/325 also plays a critical role in the regulation of the severity of insulitis.

Although NOD.B6*Idd4A* mice develop periductal infiltrates, infiltrating cells remain primarily in the peri-islet region, with only mild, nondestructive peri-insulitis until at least 25–30 weeks of age (Fig. 4*B*). Islet β-cells contain normal amounts of stored insulin, as assayed by immunohistochemical staining (Fig. 4*E*). NOD.B6*Idd4A* mice therefore display reduced invasive insulitis and β-cell destruction compared with NOD mice. However, NOD.B6*Idd4B* (not shown) and NOD.B6*Idd4C* mice develop more islet-associated infiltrates and invasive insulitis, although insulin storage seems to be preserved in these mice (Fig. 4*C* and *F*) relative to NOD.B6*Idd4A* mice (Fig. 4*B* and *E*). Thus, diabetes resistance controlled *Idd4* seems to arise by different mechanisms in NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice. The differences observed in the insulitis scores between NOD.B6*Idd4A* and either NOD.B6*Idd4B* or NOD.B6*Idd4C* mice may be associated with genes in the D11Nds1 region, which may significantly reduce insulitis and confer resistance to islet β-cell destruction.

Diabetogenic potential of splenic T-cells from NOD.B6*Idd4* mice. Adoptive transfer experiments conducted with recipient neonatal NOD, irradiated NOD, or immunodeficient NOD.*Scid* mice demonstrate the importance of splenic T-cells in the transfer of type 1 diabetes (14). To determine whether splenic T-cells from diabetes-resistant NOD.B6*Idd4* mice have a reduced diabetogenic potential in NOD.*Scid* recipients, we performed adoptive transfer experiments. Splenic T-cells from either 12-week-old female nondiabetic NOD.B6*Idd4* mice or diabetic female NOD mice were either transferred alone or co-transferred into NOD.*Scid* recipients. The onset of type 1 diabetes in recipients of co-transferred T-cells was not delayed significantly compared with that observed in recipients of transferred NOD T-cells (Fig. 5*A*). T-cells from NOD.B6*Idd4* mice are unable to suppress the diabetogenicity of NOD-derived T-cells. However, the transfer of NOD.B6*Idd4* T-cells alone yielded a reduced frequency (50%) of type 1 diabetes in NOD.*Scid* recipients at 70 days after transfer compared with recipients of NOD T-cells

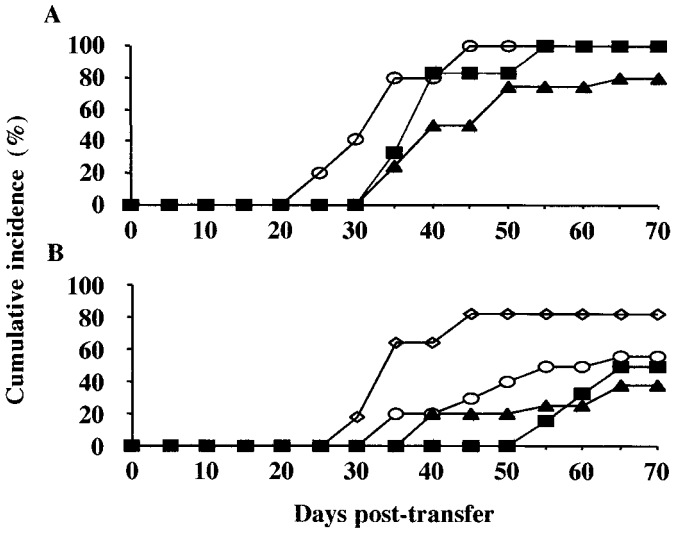

FIG. 5. Cumulative incidence of diabetes in NOD.*Scid* recipient mice after adoptive transfer. Mice were monitored for incidence of TID as in Fig. 2. Cumulative incidence was determined as a percentage of total number of recipient mice that developed diabetes at each time point. Mice (n = 10) were observed for 70 days after transfer. *A*: T-cells from donor NOD.B6*Idd4A* (○), NOD.B6*Idd4B* (■), or NOD.B6*Idd4C* (▲) mice co-transferred with purified T-cells from diabetic NOD mice. *B*: T-cells from donor NOD.B6*Idd4A* (○), NOD.B6*Idd4B* (■), or NOD.B6*Idd4C* (▲) mice, or T-cells from diabetic NOD mice (◇, n = 12). Results from one of two representative experiments are shown.

(80%; $P < 0.01$) (Fig. 5*B*). The age of onset of type 1 diabetes was delayed upon transfer of NOD.B6*Idd4B* T-cells, as type 1 diabetes was first detected at 55 days after transfer compared with 30 days after transfer for NOD T-cells ($P < 0.05$). Thus, genes encoded in the 5.2-cM fragment of chromosome 11 may influence the activation of effector T-cells involved in islet β-cell destruction.

The diabetogenic potential of T-cells was decreased in all NOD.B6*Idd4* congenic mice compared with NOD T-cells ($P < 0.01$). However, 50% of NOD.*Scid* mice still developed type 1 diabetes upon adoptive transfer compared with the 20–40% incidence of spontaneous type 1 diabetes observed in NOD.B6*Idd4* congenic mice. This result suggests that T-cells from NOD.B6*Idd4* congenic mice can develop into diabetogenic effector cells in the appropriate environment. Diabetogenic T-cell precursors may be unable to reach full maturation in NOD.B6*Idd4* mice and may require an additional step of maturation or activation in the NOD environment to become invasive and destructive.

Splenic T-cell proliferation and cytokine production in NOD.B6*Idd4* mice. The extent of TCR-stimulated T-cell proliferation was analyzed in NOD.B6*Idd4* mice. The magnitude of T-cell proliferation in NOD.B6*Idd4B* and NOD.B6*Idd4C* mice is similar to that observed in B6 mice ($P > 0.05$) and was significantly higher than that detected in NOD and NOD.B6*Idd4A* mice ($P < 0.01$) (Fig. 6*A*). These data further confirm our previous report that NOD T-cell hyporesponsiveness to TCR stimulation cosegregates with the D11Mit38/D11Mit325 markers in *Idd4* (12). Nonetheless, T-cell proliferative responses in NOD.B6*Idd4A* and NOD mice were very similar ($P > 0.05$).

To address further the mechanism of diabetes resistance in NOD.B6*Idd4* mice, we assayed the levels of cytokine production by NOD, B6, and NOD.B6*Idd4* anti-CD3–stimulated splenic T-cells. Consistent with the above T-cell proliferation results, similar levels of IL-2 production were detected for NOD.B6*Idd4A* and NOD T-cells, whereas B6-like levels of IL-2 secretion were noted for NOD.B6*Idd4B* and NOD.B6*Idd*4C T-cells (Fig. 6*B*). The level of IL-4 secretion by NOD.B6*Idd4* T-cells exceeded that of NOD T-cells ($P < 0.01$) (Fig. 6*C*), and the reverse was true for levels of γ-interferon (IFN-γ) secretion in NOD.B6*Idd4*A ($P < 0.05$) (Fig. 6*D*). Similar levels of IFN-γ production by NOD.B6*Idd4B*, NOD.B6*Idd4C*, and NOD T-cells were detected ($P > 0.05$). It seems that a gene(s) in the D11Nds1 interval polarizes T-cells toward increased IL-4 production, which elicits protection from type 1 diabetes in the absence of restoration of normal T-cell proliferative responsiveness. Conversely, a gene(s) in the D11Mit38/D11Mit325 interval triggers increased T-cell production of IL-2, which is accompanied by the restoration of normal T-cell proliferative responsiveness. Fluorescence-activated cell sorter analyses of CD69 and CD25 surface expression also indicate that, as expected, the levels of NOD.B6*Idd4A* and NOD T-cell activation are lower than those of NOD.B6*Idd4B* and NOD.B6*Idd4C* T-cells (M.G., Q.-S.M., C.M., and T.L.D., unpublished observations). Thus, the B6 allele in the D11Nds1 interval may regulate T-cell hyporesponsiveness, and coexpression of the NOD allele at D11Nds1 and the B6 allele at D11Mit38 seems to result in a normal (B6-like) T-cell proliferative response.

DISCUSSION

In this study, we localized the *Idd4* diabetes-susceptibility locus to a 5.2-cM region on mouse chromosome 11 by genetic analyses of three novel NOD.B6*Idd4* congenic mouse strains that we generated. This B6-derived region expressed in NOD.B6*Idd4A* mice maps between the D11Nds1 (43.8 cM) and D11Mit38/D11Mit325 (49.0 cM) markers and dramatically reduces the development of both insulitis and type 1 diabetes. NOD.B6*Idd4B* and NOD.B6*Idd4C* mice, which carry a smaller B6-derived segment of chromosome 11 that spans <5.2 cM distal to D11Nds1, exhibit protection against type 1 diabetes and normal T-cell proliferative responsiveness. The levels of TCR-induced T-cell proliferative responses in NOD.B6*Idd4B* and NOD.B6*Idd4C* mice is comparable to that in B6 mice, whereas the T-cell response in NOD.B6*Idd4A* mice is similar to the lower response characteristic of NOD wild-type mice. Splenic T-cells from the NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* type 1 diabetes–resistant congenic strains have reduced diabetogenic potential in NOD.*Scid* recipients but do not diminish the diabetogenicity of NOD T-cells upon cotransfer into NOD.*Scid* mice. Our data implicate at least two regions in *Idd4* that control B6 *Idd4*-mediated protection from type 1 diabetes. These loci are designated as *Idd4.1* and *Idd4.2* (Fig. 1), respectively. *Idd4.1* is located in the D11Nds1 interval and mainly contributes to a reduction in the number of islet infiltrating cells. *Idd4.2* is located in the D11Mit38/325 interval and contributes to a restoration of NOD T-cell proliferative responsiveness and a delay in the onset of type 1 diabetes.

The incomplete protection from type 1 diabetes (10–40% incidence) observed in NOD.B6*Idd4* mice was not surprising, as the development of type 1 diabetes is under polygenic control and is dependent on epistatic interac-

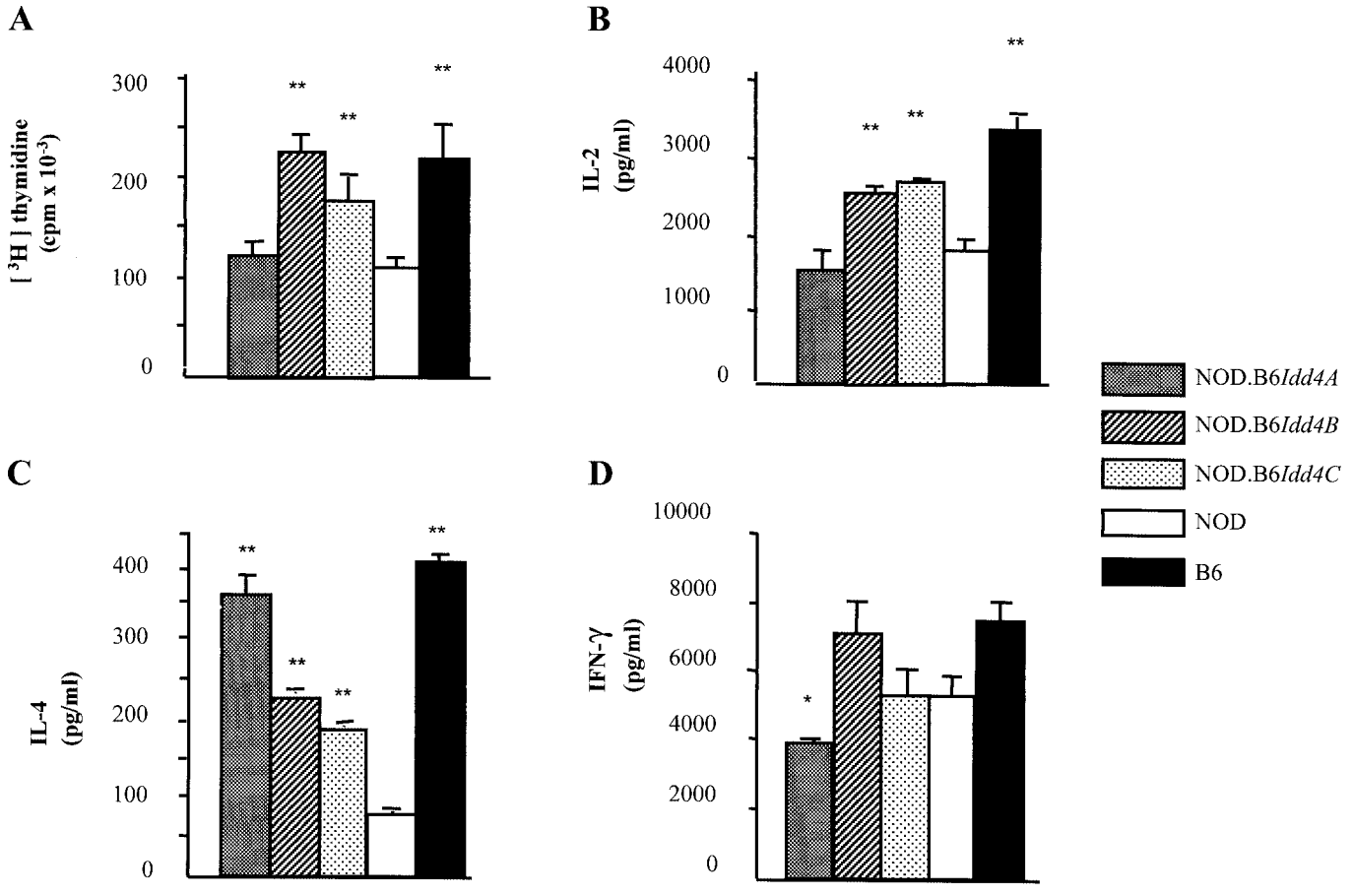

FIG. 6. Anti-CD3–induced proliferative responses of splenic T-cells. Splenic T-cells from 8- to 10-week-old NOD; B6; and congenic NOD.*B6Idd4A*, NOD.*B6Idd4B*, and NOD.*B6Idd4C* mice (n = 4–5) were activated by plate-bound anti-CD3. *A*: Cell proliferation was determined by [^{3}H]thymidine incorporation. The results of triplicate cultures are expressed as the means ± SD and are representative of three different experiments. *B–D*: Cytokine (IL-2, IL-4, and IFN-γ) levels in T-cell culture supernatants were determined by enzyme-linked immunosorbent assay as described in RESEARCH DESIGN AND METHODS. Results from one of three representative experiments are shown. (*P < 0.05, **P < 0.01 versus NOD mice).

tions between different *Idd* loci. Studies with congenic mice have shown that some *Idd* loci genes have varying degrees of penetrance and do not singly confer a high degree of resistance to disease. For example, the incidence of type 1 diabetes is 25% in NOD.B10*Idd3* mice (7), 40% in NOD.B10*Idd10* (15) and NOD.B10*Idd5* mice (16), and 7% in NOD.B10*Idd9* mice (6). However, the incidence of type 1 diabetes varies between 3% and 7% in NOD.B6*Idd3Idd10Idd18* and NOD.B6*Idd3Idd10Idd17Idd18* mice (15), whereas type 1 diabetes does not develop in NOD.B6*Idd3Idd10Idd17Idd18Idd9* mice (6). These results suggest that protection from type 1 diabetes is controlled by several loci and that these loci may function in a collective manner to determine the development of type 1 diabetes.

Previously, we demonstrated that a proliferative hyporesponsiveness occurs in TCR-stimulated NOD thymocytes and peripheral T-cells and that IL-4 and anti-CD28 treatments reverse NOD T-cell proliferative hyporesponsiveness in vitro and prevent the development of insulitis and type 1 diabetes in NOD mice in vivo (8,9,11,13,17). Our present analyses of NOD.B6*Idd4* congenic mice suggest the presence of two types of *Idd* loci that regulate the development of type 1 diabetes in NOD mice. The first includes genes that prevent type 1 diabetes without the restoration of T-cell proliferative responsiveness, and the second includes genes that prevent type 1 diabetes when accompanied by the restoration of T-cell proliferative responsiveness. We propose that the latter two types of *Idd* loci be added to the two *Idd* loci reported by Wicker and colleagues (6) to mediate protection from islet β-cell destruction by either reducing the number of islet-infiltrating cells or changing the cytokine profiles and pathogenic properties of islet-infiltrating cells.

The magnitude of the anti-CD3–induced T-cell proliferative responses observed in NOD.B6*Idd4B* and NOD.B6*Idd4C* mice (expressing the B6 *Idd4.2* locus) are identical to that of B6 T-cell responses. The latter two NOD.B6*Idd4* congenic mice display less severe insulitis and a high degree of protection from type 1 diabetes compared with NOD. It follows that although T-cell hyporesponsiveness may correlate with the development of type 1 diabetes, this hyporesponsiveness phenotype may not be the single causal factor leading to the onset of type 1 diabetes, because NOD.B6*Idd4A* mice express this phenotype and yet are diabetes-resistant. T-cell hyporesponsiveness is presumably a polygenic trait manifested via pleiotropic, epistatic, and/or penetrance effects. The T-cell hyporesponsive phenotype observed in NOD.B6*Idd4A* mice is associated with reduced IL-2 and IFN-γ production and elevated IL-4 production, which would be expected to lead to significantly reduced insulitis and pro-

tection from type 1 diabetes (13). These findings suggest that diabetes resistance in NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice may arise by different mechanisms. The differences in insulitis scores detected between NOD.B6*Idd4A*, NOD.B6*Idd4B*, and NOD.B6*Idd4C* mice seems to be associated with genes in the D11Nds1 region, which may significantly reduce insulitis and confer resistance to islet β-cell destruction. Thus, albeit T-cell hyporesponsiveness can be associated with the development of type 1 diabetes, this association may be a necessary but not sufficient condition that elicits the onset of type 1 diabetes.

What candidate genes are found in the vicinity of *Idd4*? Although further fine-mapping of *Idd4* is required, a number of candidate genes may be responsible for the phenotypes that we observed in the NOD.B6*Idd4* congenic mice. The candidate genes in *Idd4* may include platelet activating factor acetylhydrolase Ib1 (*PAF-AHIb1*) mapping at 44.0 cM, nitric oxide synthase-2 (*NOS2*) at 45.6 cM, and CC chemokines at 47.0–47.7 cM.

PAF-AH, a specific acetylhydrolase, can inhibit inflammatory responses by efficiently inactivating its substrate PAF (18). During inflammatory and allergic immune responses, PAF activates neutrophils, eosinophils, and macrophages, which may elicit microvascular leakage, vasodilation, smooth muscle contraction, and endothelial cell adhesion (19). It is interesting that levels of PAF in peripheral blood are elevated in type 1 diabetes but not type 2 diabetes patients, and PAF-AH activity is significantly decreased in the plasma of type 1 diabetes but not type 2 diabetes patients (20–22). PAF inhibitors can reduce insulitis and the frequency of type 1 diabetes in BB rats (23,24). Intraperitoneal injection of recombinant PAF-AH reduces the incidence of type 1 diabetes in DP-BB rats, and DP-BB rats protected from type 1 diabetes have a reduced severity of insulitis and an increased percentage of insulin-positive stained cells in pancreatic sections compared with that in diabetic rats (25). These results suggest that the elevated levels of PAF in type 1 diabetes could be due to a decrease in PAF acetylhydrolase activity. Thus, *PAF-AHIb1* may be a candidate gene in the *Idd4.1* interval that confers protection from insulitis and type 1 diabetes.

NOS2 encodes the inducible NOS enzyme, which is considered to be a candidate gene in autoimmune disorders because NO is known to play a pathogenic role in inflammatory responses (26,27). Inhibition of either *NOS2* or NO activity and its by-products, such as peroxynitrite, results in the blocking or ameliorating disease symptoms in both type 1 diabetes and experimental allergic encephalomyelitis (EAE) (28). In addition, transgenic mice expressing mouse *NOS2* cDNA under the control of the insulin promoter develop hypoinsulinemic diabetes by 4 weeks of age, and treatment with an inhibitor of *NOS2* prevents/delays the development of type 1 diabetes (29). Accordingly, the production of NO by islet β-cell NOS2 may play an important role in β-cell destruction.

Chemokines mediate innate and adaptive immune responses by their ability to recruit, activate, and costimulate T-cells and monocytes (30,31). Macrophage inflammatory protein-lα (MIP-lα), MIP-1β, and monocyte chemotactic protein-l (MCP-1) are members of the CC family of chemokines that act primarily upon monocytes and T-cells (30–32), and they all are localized in the *Idd4.2* interval. A role for CC chemokines, particularly MIP-lα, has been noted in experimental autoimmune diseases, such as EAE (33–35). Certain chemokines may be associated with predominant Th1 or Th2 cell responses, as evidenced by linkage of MIP-lα to a Th1 inflammatory response and of MCP-1 to a Th2 response (33,34,36). It is interesting that chemokines that promote the onset of type 1 diabetes are more abundant in islet-specific Th1 cells than Th2 cells (37). Recently, we found that MCP-1, MIP-1α, and MIP-1β may control the development of insulitis in NOD mice (38). An increased MIP-1α–to–MIP-1β ratio is associated with a greater severity of insulitis and more rapid progression to type 1 diabetes. Moreover, the incidence of destructive insulitis and type 1 diabetes is significantly reduced and delayed in MIP-1α–deficient NOD.MIP-1$\alpha^{-/-}$ mice compared with NOD.MIP-1$\alpha^{+/+}$ mice. IL-4 treatment of NOD mice elicits a high MIP-1α–to–MIP-1β expression ratio in splenic T-cells and the pancreas. These data implicate MIP-1α as a proinflammatory chemokine in the pathogenesis of type 1 diabetes (39). In contrast, our results are consistent with the hypothesis that MIP-1β may have an anti-inflammatory role in type 1 diabetes, because the expression of MIP-1β is upregulated in peripheral T-cells and pancreas of NOD mice protected from insulitis and type 1 diabetes by IL-4 treatment (13). MIP-1α can also affect T-cell selection and/or maturation, because it is expressed in the thymic cortex and is an inhibitor of hematopoietic stem cell development (40). Taken together, our data suggest that a CC chemokine gene(s) in *Idd4.2* may control the magnitude of T-cell proliferation and susceptibility to type 1 diabetes in NOD mice.

It is interesting that *Idd4* colocalizes with the *Eae7* and autoimmune orchitis resistance 3 (*Orch3*) loci on chromosome 11 (41). We suggest that *Idd4*, *Eae7*, and *Orch3* are susceptibility loci that regulate various autoimmune diseases. Furthermore, an association between *Idd4* and loci that control susceptibility to systemic lupus erythematosus in (NZB x NZW) mice has been reported (42). Also, previous quantitative trait loci analyses conducted using F1/F2 outcrosses between inbred NON/Lt (nonobese, nondiabetic) and NZO/HI (New Zealand obese) mice revealed a linkage of type 2 diabetes susceptibility to D11Mit41 at the position of 49 cM in chromosome 11. Mice with an NZO-derived allele at this marker display low levels of serum immunoreactive insulin (43). Note that *Idd3* on chromosome 3 mediates protection from both type 1 diabetes and EAE (44). These findings raise the possibility that non-MHC susceptibility genes may be divided into two broad classes: the first class genetically controls susceptibility to several autoimmune diseases, and the second class regulates susceptibility to a specific autoimmune disease.

In conclusion, we limited the *Idd4* locus to a 5.2-cM interval on mouse chromosome 11. Congenic mapping analyses shows that at least two subloci, *Idd4.1* and *Idd4.2*, may confer diabetes resistance. *Idd4.1* maps close to the D11Nds1 interval and is associated with reduced insulitis and protection from diabetes. *Idd4.2* maps close to the D11Mit38/325 interval, is associated with a normal

level of T-cell proliferative responsiveness, and confers protection against type 1 diabetes. Three sets of candidate genes, *PAF-AH*, *NOS2*, and a CC chemokine gene(s), are localized in the 5.2-cM interval. Additional fine mapping of this region and characterization of these loci and genes are required to further elucidate the mechanisms involved in the genetic control of diabetes resistance by *Idd4*.

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