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Sex-Specific Effect of Insulin-Dependent Diabetes 4 on Regulation of Diabetes Pathogenesis in the Nonobese Diabetic Mouse

Evgueni A. Ivakine,†‡§ Casey J. Fox,†‡§ Andrew D. Paterson,‡ Steven M. Morton-Toth,† Angelo Canty,†‡§ David S. Walton,†‡§ Katarina Aleksa,¶ Shinya Ito,¶ and Jayne S. Danska2†§¶

Many human autoimmune diseases are more frequent in females than males, and their clinical severity is affected by sex hormone levels. A strong female bias is also observed in the NOD mouse model of type I diabetes (T1D). In both NOD mice and humans, T1D displays complex polygenic inheritance and T cell-mediated autoimmune pathogenesis. The identities of many of the insulin-dependent diabetes (Idd) loci, their influence on specific stages of autoimmune pathogenesis, and sex-specific effects of Idd loci in the NOD model are not well understood. To address these questions, we analyzed cyclophosphamide-accelerated T1D (CY-T1D) that causes disease with high and similar frequencies in male and female NOD mice, but not in diabetes-resistant animals, including the nonobese diabetes-resistant (NOR) strain. In this study we show by genetic linkage analysis of (NOD × NOR) F1 hybrid mice (7) that progression to severe islet inflammation after CY treatment was controlled by the nonobese diabetes-resistant (NOR) strain. In this study we show by genetic linkage analysis of (NOD × NOR) F1 hybrid mice (7) that progression to severe islet inflammation after CY treatment was controlled by the nonobese diabetes-resistant (NOR) strain.

Type 1 diabetes (T1D)1 is a complex disease of autoimmune etiology in which susceptibility is conferred by allelic variation at many loci. At least 20 insulin-dependent diabetes (Idd) loci have been identified in the disease-prone NOD mouse (1), and human genome scans have revealed similarly complex inheritance (2). In both species, the primary susceptibility locus, Idd1, is the MHC class II haplotype that controls Ag-specific T cell recognition (1). Evidence that the NOD Idd13.1 locus is a functional allele of β2-microglobulin (3) accords with human studies in which variation in MHC class I also contributes to T1D susceptibility (4). A strong association was reported between polymorphisms in Cita-4 and susceptibility to human T1D, Graves’ disease, and autoimmune thyroiditis. Interestingly, the abundance of a ligand-independent Cita-4 splice variant was shown to be 3–4 times lower in NOD compared with T1D-resistant C57BL10 mice (5), leading to differences in T cell responses between strains (6). The remaining Idd loci are not defined at the DNA sequence level. Because T1D is a clinical endpoint of the interaction of many genes in complex pathways, the influence of individual loci has been hard to resolve. We have examined the idea that preclinical phenotypes would display stronger inheritance with higher penetrance and involve contributions of fewer genes, enabling high resolution mapping and identification of T1D genes.

To focus on Idd loci predicted to exert strong effects on disease susceptibility, we compared the disease-prone NOD with the closely related, nonobese T1D-resistant (NOR) strain. NOR is a recombinant inbred strain that is 88% identical by descent with NOD including the Idd1 locus (H2n), but is protected from both spontaneous and cyclophosphamide (CY)-accelerated T1D (CY-T1D) by genes of C57BLKS/J (BKS) origin (7). The BKS strain was derived from C57BL/6 and DBA/2 stocks (8). Analysis of 19 Idd loci in NOD and NOR by our group (9) and others (10, 11) demonstrated that NOR differ only at Idd 4, 5, 9, and Idd13 (Fig. 1). Previously, we reported that NOR mice display benign, early stage insulitis with minimal T cell contribution (12) and mapped this preclinical trait to the Idd5 and Idd13 loci. These two intervals exerted strong control over T cell recruitment to islet lesions, an obligate step in insulitis progression (9).

CY accelerates T1D onset in both NOD male and female mice from months to only 3 wk (13). Similarly treated T1D-resistant strains (14), including NOR (7), remain disease free after CY treatment. First examined for prevention of T1D in NOD mice, this lymphotoxic drug paradoxically accelerated diabetes development...
in this strain, and both males and female mice were equally affected (13). Cases of T1D onset after CT treatment for lymphoma have also been reported in human patients with high risk MHC class II alleles (15). Although the mechanism of CY-accelerated T1D is not fully understood, the drug does not directly damage islet cells at the concentrations used in NOD mice (16), and the drug effect requires macrophages/dendritic cells and T cells similar to spontaneous disease (reviewed in Ref. 17). The sex difference in spontaneous T1D frequency (~85% NOD female and 15–40% of NOD males) (18) has largely confined study of the disease to NOD females. We took advantage of the similarly high frequency of T1D in both sexes after CY treatment (Ref. 13 and this study) to search for autosomal loci with possible sex-dependent effects. CY-accelerated insulitis progression was used as a trait in genetic analysis of a cohort of (NOD × NOR) F₁ × NOD backcross mice revealing significant linkage to Idd4 and Idd9. A series of reciprocal congenic strains were derived and tested for susceptibility to CY-accelerated and spontaneous T1D. These data validated linkage to Idd4 and Idd9 and revealed the contribution of a third locus, Idd5. Comparative analyses of Idd4, Idd9, and Idd5 congenic strains indicated that these loci operate at distinct stages in progression to T1D. Importantly, our data revealed striking sex specificity in the behavior of Idd4 alleles in CY-accelerated model of the disease.

Materials and Methods

Mice

All mice used in this study were maintained in a barrier facility at The Hospital for Sick Children. In our colony, the spontaneous diabetes incidence at age 6 mo in NOD/Jsd animals is 83% in females and 35% in males, and it is 0% in NOR and (NOD × NOR) F₁ mice.

Genomic DNA preparation

For microsatellite genotype analysis, genomic DNA was prepared from tail snips (0.6–0.8 cm long) using a DNA easy kit (Qiagen). Each preparation was diluted 1/20 for use in PCR amplification.

Genotyping of backcross progeny

Genotyping of (NOD × NOR) × NOD backcross progeny was performed as previously described (9). Sixty-six backcross animals were typed for microsatellite alleles at Idd4 (D11Mit51, D11Mit87, D11Mit339, D11Mit30, and D11Mit219), Idd5 (D4Mit46), Idd11 (D4Mit146, D4Mit11, D4Mit338, D4Mit72, D4Mit251, and D4Mit13), and Idd13 (D2Mit17). These mice were also typed at C57BLKS/J-derived regions in the NOR genome that do not contain known Idd loci on chromosomes 5 (D5Mit11), 7 (D7Mit105), 12 (D12Mit230), and 18 (D18Mit4 and D18Mit21). The order of these markers was obtained from the Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research). For most primer pairs, DNA was amplified for 35–40 cycles under the following conditions: 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C in 1.5 mM MgCl₂. For D11Mit30, D4Mit11, and D18Mit21, PCR was performed for 40 cycles (30 s at 94°C, 40 s at 50°C, and 1 min 10 s at 72°C in 2.5 mM MgCl₂). The amplification products were electrophoresed through 2% NuSieve (American Bioanalytical) and 2% agarose (Invitrogen Life Technologies) gels and were visualized with ethidium bromide.

Generation of congenic and double congenic mice

NOD.NOR-Idd4, NOD.NOR-Idd5, NOD.NOR-Idd9, and NOD.NOR-Idd13 congenic mice were developed by intercrossing with NOR/Lt mice purchased from The Jackson Laboratory, followed by a backcross with NOD/jsd mice with marker-assisted breeding. The following markers were used: D1Mit430, 3, 122, 212, 46, 44, and 305; D2Mit395, 338, 490, 412, 46, 44, and 305; D2Mit395, 338, 490, 412, Idd13-Idd9, NOD.NOR-Idd4-Idd9, NOD.NOR-Idd4-Idd5, NOD.NOR-Idd4-Idd9-Idd5. All congenic mice used in this study were maintained in a barrier facility at The Hospital for Sick Children. In our colony, the spontaneous diabetes incidence at age 6 mo in NOD/Jsd animals is 83% in females and 35% in males, and it is 0% in NOR and (NOD × NOR) F₁ mice.

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and 452; DAmi313, 33, 322, 219, 177, 157, 310, 217, 230, 151, 346, 16, 251, and 259; DSmi11; D7Mit105; D11Mit674, 340, 230, 135, 217, 310, 164, 157, 177, 4, 368, 30, 90, 364, 219, 322, and D11Bhm149; D12Mit76 and 230; and D18Mit4 and 21. For all markers, DNA was amplified with the following conditions: 10 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C in 1.5 mM MgCl2. NOR.NOD-Idd4, NOR.NOD-Idd5, NOR.NOD-Idd9, and NOR.NOD-Idd13 mice were generated using a similar strategy. All animals used for this study were of the NS-6 F3-6 generations. Marker analysis was initiated at the first backcross and then continued for each generation. NOR.NOD-Idd4 and NOR.NOD-Idd9 mice were intercrossed to produce NOR.NOD-Idd4/Idd9 double-congenic mice. Similarly, NOR.NOD-Idd4/Idd13 double-congenic mice were generated from the intercross between NOR.NOD-Idd5 and NOR.NOD-Idd13 single-congenic animals.

**CY treatment and insulitis assessment**

Thirty-day-old mice were injected i.p. with 300 mg/kg freshly dissolved cyclophosphamide (Procytox), purchased from ASTA Medica (Frankfurt, Germany). Pancreata were then removed from the mice 3, 7, or 14 days after injection; immediately immersed in TissueTec (Bayer Laboratories); snap-frozen in liquid nitrogen; and stored at −70°C. Preparation of frozen sections was performed with a CM 3050 Cryostat (Leica Canada). To maximize analysis of independent islet infiltrates, several 5-μm sections were prepared at least 300 μm apart. Pancreatic sections were stained with Mayer’s H&E and by the EBC (Sigma-Aldrich) to visualize leukocyte infiltration. Assessment of insulitis severity in pancreatic sections was performed as previously described (9). Briefly, ≥100 independent islets from three or more tissue depths were examined in each animal and graded according to the following criteria: 0, no visible infiltrates; 1, peri-insulitis, as indicated by perivascular and peri-islet infiltrates; 2, ≤25% of the islet interior occluded by leukocytes displaying invasive infiltrates (leukocytes evident in islet interior); 3, >25%, but ≤50%, of the islet interior occluded by leukocytes; or 4, complete infiltration, with the islet displaying invasive insulitis involving 50–100% of the islet field. For backcross analysis, animals were treated with a single dose of 300 mg/kg CY, as described above, pancreata from both male and female mice were removed 14 days after drug administration, and insulitis severity was determined using the same scoring scheme.

**CY treatment and diabetes assessment of congenic mice**

To induce diabetes, 10- to 12-wk-old mice of both genders were treated with two i.p. injections of freshly dissolved CY (Procytox), administered 14 days apart. The corresponding doses for the first and second injections were 300 and 200 mg/kg, respectively. Blood glucose levels were measured on days 14, 21, 24, 27, and 35 after the first injection using a Fast-Take blood glucose monitor (LifeScan Canada). Mice with blood glucose levels >16 mmol/L on two subsequent measurements were considered diabetic. Animals with high blood glucose on day 14 were monitored for an additional 3 days without a second CY injection. None of these mice reverted to normoglycemia; thus, all were included in the diabetic cohort. Animals with high blood glucose on day 14 were monitored for an additional 3 days without a second CY injection. None of these mice reverted to normoglycemia; thus, all were included in the diabetic cohort. Pancreata were collected from normoglycemic mice on day 35 after the first injection, and insulitis severity was assessed as described above.

**Assessment of spontaneous diabetes**

Blood glucose levels were measured in NOD and NOD.NOR-Idd4 congenic female mice biweekly using a FastTake blood glucose monitor (LifeScan Canada). Animals were classified as diabetic when blood glucose levels were ≥16 mmol/L for two subsequent measurements. Diabetic mice also displayed polyuria, polydipsia, and weight loss.

**Quantitation 4-hydroxycyclophosphamide (4-OH-CY) in plasma**

4-OH-CY was measured in mouse plasma by liquid chromatography/mass spectrometry using a currently unpublished method (D. Stempak, K. Aleksa, S. Baruchel, G. Koren, and S. Ito, unpublished observations). Blood samples were drawn 20 min after injection. Once drawn, 200 μL of blood was transferred into two capillary tubes containing 20 μL of 2 M O-methylhydroxylamine hydrochloride (Sigma-Aldrich Canada). The O-methylhydroxylamine hydrochloride forms a stable derivative with the aldophosphamide. Samples were s retrieval several times, then centrifuged, and the plasma was transferred to clean tubes. The tubes were left at room temperature for 24 h. Aldophosphamide was extracted with diethyl ether. The aqueous layer was removed and discarded, and the organic layer was dried under nitrogen. Samples were reconstituted with 100 μL of acetonitrile/water (1/1) before liquid chromatography/mass spectrometry analysis. The low limit of quantitation for OXIME was 2.5 fmol on the column. A standard curve was generated using 4-hydroxycyclophosphamide (provided by Dr. U. Niemeyer, ASTA Medica, Frankfurt, Germany).

**Statistical analysis**

Quantitative trait linkage analysis was performed as follows. A genetic map was built on the basis of the Whitehead Institute for Biomedical Research/Massachusetts Institute of Technology Center for Genome Research marker order, and markers were analyzed for trait linkage by the MAP-MAKER EXP (version 3.0) and QTL (version 1.1) suite of programs (19) (Genome Center software information and documentation). Multipoint analysis was conducted using recessive, dominant, and additive models. The logarithm of odds (LOD) scores, the proportion of the trait variance explained, and 1-LOD confidence intervals were determined with inclusion of all animals, and then separately by sex. Permutation analysis was performed for all animals using Map Manager QTxb20 (20, 21).

To examine strain or sex differences in the levels of 4-OH-CY, values from NOD and NOR mice, both males and females, were compared using one-way ANOVA. The difference in diabetes incidence between strains was assessed using Fisher’s exact test in SPSS for personal computer (version 8.01).

To test for sex-by-strain interaction effect in TID incidence, we performed logistic regression analysis in which the log odds were modeled as a linear function of the covariates. This analysis is similar to factorial ANOVA, commonly used in Drosophila genetics to study sex-dependent effects of loci (22–30). In our case, sex and strain were two discrete covariates. We consider there to be a sex-dependent effect of a locus if the ratio of the odds of diabetes in the parental strain and the odds of diabetes in the congenic strain is different for the two genders. To test for this, we fit a logistic regression model with three terms: a main effect for sex, a main effect for strain, and a sex-by-strain interaction term. We also fit a second model without the interaction term. This model assumes that the log odds ratio is the same in both sexes. A test statistic for the significance of the sex-dependent effect is twice the difference in log likelihood between these two models that has a χ2 distribution with a single degree of freedom if the null hypothesis of no interaction is true. The p values given in our results were obtained using this χ² distribution.

**Results**

**Cyclophosphamide-induced severe insulitis depends on Idd1**

We examined insulitis severity (IS) in age-matched NOD, NOR, (NOD × NOR) F1, as well as genetically unrelated BALB/c and C57BL/6 (B6) mice treated with CY. Insulitis was absent from CY-treated B6 and BALB/c animals and remained mild (IS <1.0) in treated NOR mice (Fig. 2a). This latter observation is consistent with the noninvasive insulitis previously reported in NOR animals (12). In contrast, IS in NOD mice rose dramatically after treatment (Fig. 2a), in agreement with a previous report (31), and all NOD animals maintained for 18 days post-treatment became diabetic (data not shown). Progression to severe insulitis and diabetes was not observed in CY-treated (NOD × NOR) F1 animals.

To address the contribution of the NOD-derived MHC (H2b) haplotype to the CY-associated phenotypes, we examined their appearance in congenic mice that lacked the NOD (Idd1) haplotype and retained the remaining NOD-derived Idd loci. These animals are resistant to both spontaneous and CY-accelerated diabetes (32). After CY administration, both NOD.H2b and NOD.H2a mice displayed mild or undetectable insulitis (Fig. 2b). Thus, CY-induced insulitis progression, like spontaneous diabetes, required the NOD-derived Idd1 haplotype. However, NOD mice, which are also H2a homozygotes, did not display this CY phenotype (Fig. 2a), supporting the oblique contribution of loci that are not identical by descent in NOR and NOD animals. Finally, NOD.Prkcscid mice were absolutely resistant to CY-induced insulitis, indicating that mature lymphocytes are required in the process (Fig. 2b).

**Biotransformation of CY in NOD and NOR mice**

An immune etiology of CY-accelerated TID is supported by previous studies (reviewed in Ref. 17) and the genetic analysis described above. However, the biological basis for differential susceptibility in NOD and NOR mice was unclear, because they are
mosome 4 (34–36). In this study we use Idd9 to denote this region of chromosome 4.

Next, we examined 66 (NOD × NOR) F1 × NOD backcross progeny for linkage of progression to severe insulitis to markers designating Idd4, 5, 9, and 13 as well as other C57BLKS/J-derived regions of the NOR genome (Fig. 1). Twenty-four percent of the backcross progeny exhibited progression to severe insulitis (average IS, 3.69) consistent with the involvement of two unlinked loci. Multipoint linkage analysis of the phenotype:genotype distribution revealed significant linkage (37) to markers overlying Idd4 and Idd9 (Fig. 3; LOD score, 4.9 (30% variance explained); LOD score, 7.4 (43% variance explained)). No other markers tested displayed significant linkage to the trait. Analysis of 10,000 permutations confirmed significance of the linkage to both Idd4 and Idd9 (p < 0.0001; data not shown). We constructed a general linear model using PROC GLM (SAS Institute; version 9.1) with insulitis as the outcome and sex, and genotypes at D11Mit219 and D4Mit338 as independent variables. As expected, genotype at D11Mit219 and D4Mit338 were significantly associated with insulitis (p < 0.0001). There was no significant difference in the linkage between males and females at the peak markers for Idd4 and Idd9 (F = 1.6; 1 df; p > 0.2).

Strikingly, progression to severe insulitis was only observed in backcross progeny homozygous for NOD alleles at both Idd4 and Idd9 loci. Animals homozygous at both Idd4 and Idd9 (n = 15) had significantly greater insulitis severity than animals with other genotypes at these two loci (Fig. 4). These interactions were significant (by ANOVA, p < 0.0005) and suggested a role for NOD-derived gene products at both Idd4 and Idd9 in CY-accelerated insulitis progression.

The linkage analysis provided evidence for 1 LOD confidence intervals at a 7.8-cM interval (37–44.8 cM from the centromere) for Idd4, and a 12.4-cM interval (53.6–66.0 cM from the centromere) for Idd9 (Fig. 3). Both locations were consistent with previous reports in which Idd4 was localized to an interval between 27–72 cM from the centromere on chromosome 11 (38), Idd9 to a 24-cM interval 58–82 cM (36), and Idd11 to a 12-cM region 59–71 cM from the centromere on chromosome 4 (39). The probable locations of both Idd4 and Idd9 were confirmed, and Idd4 was further refined by the linkage results presented in this study.

Analysis of Idd4 and Idd9 congenic strains

We next determined whether the impact of Idd4 and Idd9 on the CY-accelerated disease process could be observed when the regions were separated from other genes. Using marker-assisted breeding, we generated reciprocal congenic strains on the NOD and NOR backgrounds and examined their responses to CY (Fig. 5 and Table I). Idd4 congenic mice were made on the NOD and NOR backgrounds: NOR.NOD-Idd4 (33.8-cM interval), NOD.NOR-Idd4 long (27.8-cM interval), and NOD.NOR-Idd4 short (6.9-cM interval). A 26.7-cM interval including Idd9 was introgressed to produce NOR.NOD-Idd9 and NOD.NOR-Idd9 mice (Fig. 5). Finally, a double-congenic strain on the NOR background, NOR.NOD-Idd4/Idd9, was made to examine the combined effects of these two NOD-derived regions on the CY response (Fig. 5a).

As expected, NOD males and females progressed to T1D, and NOR mice of both sexes were relatively resistant (Table I). NOD.NOR-Idd4 (long) and NOD.NOR-Idd9 congenic females showed significant protection from disease (p = 0.005 and p = 0.002, respectively), as did NOD.NOR-Idd9 males (p = 0.002). In striking contrast, NOD.NOR-Idd4 males were not protected relative to NOD males (p = 0.96). These results demonstrate that NOR-derived alleles at both loci confer resistance to CY-accelerated T1D, with the effects of NOR alleles at Idd4 restricted to...
showing increased susceptibility (sensitive to CY-T1D compared with sex-matched NOR controls (double-congenic females and males were significantly susceptible). 0.0002 and 0.0015, respectively). NOR.NOD alleles in this background. Linkage analysis was performed on animals, probably reflecting the effects of additional NOR resistance loci in this background. Backcross progeny were not mapped for markers centromeric to D4Mit146 (a) or telomeric to D11Mit219 (b) because they are close to the boundary of the BKS-derived regions in NOR mice. Pairwise comparisons, using MAPMAKER QTL analysis, were performed (37).

![FIGURE 3. Idd4 and Idd9 mediate CY-induced invasive insulitis.](http://www.jimmunol.org/)

Markers and their position from centromere, cM

**FIGURE 3.** Idd4 and Idd9 mediate CY-induced invasive insulitis. The LOD scores computed for linkage of CY-accelerated invasive insulitis are shown relative to the distance in centimorgans from the centromere of chromosome 4 (a) and chromosome 11 (b). The bar beneath each graph depicts microsatellite markers used in the critical intervals. Marker order was adapted from (http://genome.wi.mit.edu) and was confirmed using the Ensembl mouse assembly ([http://ensembl.org/]). Backcross progeny were not mapped for markers centromeric to D4Mit146 (a) or telomeric to D11Mit219 (b) because they are close to the boundary of the BKS-derived regions in NOR mice. Pairwise comparisons, using MAPMAKER QTL analysis, were performed (37).

To further refine location of the Idd4 locus, we evaluated CY-T1D in females of the NOD.NOR-Idd4 short strain. They were significantly protected from the disease compared with NOD mice (p = 0.003), placing Idd4 within the 6.9-cM interval and defining the centromeric boundary of the locus at D11Mit30. Importantly, both long and short NOD.NOR-Idd4 strains were also protected from spontaneous T1D (Fig. 6), suggesting that common immunogenetic mechanisms involved in Idd4-mediated protection in both forms of the disease.

To determine whether NOD-derived Idd4 or Idd9 conferred T1D susceptibility on the NOR background, we analyzed NOR.NOD-Idd4, NOR.NOD-Idd9, and NOR.NOD-Idd4/Idd9 double-congenic mice (Table I). The NOD-derived loci enhanced T1D incidence compared with NOR mice. A strong effect was observed in NOR.NOD-Idd4 males (p = 0.0001), and a moderate effect was shown in females (p = 0.016). NOR.NOD-Idd9 females displayed significantly enhanced susceptibility (p = 0.002), with males of this strain also showing increased susceptibility (p = 0.03). NOR.NOD-Idd4/Idd9 double-congenic females and males were significantly more susceptible to CY-T1D compared with sex-matched NOR controls (p = 0.0002 and p = 0.001, respectively). However, in both sexes of NOR.NOD-Idd4/Idd9 mice, T1D incidence was lower than in NOD animals, probably reflecting the effects of additional NOR resistance alleles in this background. Linkage analysis was performed on (NOD × NOR) F1 × NOR backcross animals (Fig. 3), in which the contributions of NOR resistance loci with a recessive mode of inheritance were not detected. Because NOR mice carry Idd5 and Idd13 loci previously implicated in spontaneous T1D, these regions were considered likely candidates to contain the gene(s) responsible for the observed protection.

Analysis of Idd5 and Idd13 congenic strains

To evaluate possible contributions of Idd5 and/or Idd13 loci in response to CY, we generated NOD.NOR-Idd5 (18.2-cM interval), NOD.NOR-Idd13 (23.0-cM interval), NOD.NOD-Idd5 (18.2-cM interval), and NOD.NOD-Idd13 (13.2-cM interval) congenic and NOR.NOD-Idd5/Idd13 double-congenic mice using a marker-assisted speed congenic approach (Fig. 5). Both males and females of the NOD.NOR-Idd5 strain were protected from CY-T1D (p = 0.0005 and p = 0.0004, respectively), whereas NOD.NOR-Idd13 mice developed the disease as frequently as their NOD counterparts (Table I). To determine whether NOD-derived Idd5 and Idd13, either alone or in combination, could confer susceptibility to T1D, NOR.NOD-Idd5, NOR.NOD-Idd13, and NOR.NOD-Idd5/Idd13 mice were treated with CY. None of these strains showed enhanced CY-T1D susceptibility compared with NOR mice (Table I). Thus, despite strong disease protection conferred by the NOD-derived Idd5, the NOD-derived Idd5, alone or even in combination with the NOD-derived Idd13, was not sufficient to confer susceptibility to CY-T1D. Importantly, these data distinguish the effects of NOD alleles at Idd5 and Idd13 from those at Idd4 and Idd9 that did confer susceptibility to NOR mice with CY-accelerated disease.

![FIGURE 4. Insulitis severity in CY-treated backcross mice.](http://www.jimmunol.org/)

**FIGURE 4.** Insulitis severity in CY-treated backcross mice. Backcross progeny were parsed into all combinations of Idd4 (D11Mit219) and Idd9 (D4Mit11) genotypes (x-axis): homozygous (NN) for NOD-derived alleles at both loci, heterozygous (NB) at Idd4 and homozygous for NOD alleles at Idd9, heterozygous at Idd9 and homozygous at Idd4, and heterozygous at both loci. Only animals homozygous for NOD alleles at both Idd4 and Idd9 loci displayed progression to severe insulitis. , Males; , females.
Alleles at the Idd4 locus display sex-specific effect

Genotype-by-sex interaction can occur if a phenotypic trait affects only one sex (sex-specific effects), affects both sexes but to different degrees (sex-biased effects), or affects both sexes in opposite directions (sex-antagonistic effects) (reviewed in Ref. 22). Having noticed a striking effect of sex on susceptibility to CY-T1D in NOD.NOR-Idd4 congenic mice vs the parental NOD strain (p = 0.96 and p = 0.005 for males and females, respectively; Table I), we further investigated this matter using logistic regression analysis (Fig. 7). We used approaches developed in Drosophila complex trait genetics, where multiple sex-specific QTL have been described (22–30), to look for a sex-specific effect of the Idd loci in the current study. A sex-by-strain interaction effect of T1D incidence was only statistically significant when NOD and NOD.NOR-Idd4 mice were compared (p = 0.0194), supporting the idea of sex-specific behavior of alleles at the Idd4 locus. The sex-by-strain interaction effect was not observed in the NOR vs NOD.NOR-Idd4 comparison (p = 0.203), indicating that the sex-specific effect of Idd4 is influenced by genetic background. Alleles at neither Idd5 nor Idd9 displayed sex-specific behavior in any of the comparisons performed (Fig. 7), suggesting that of the three loci influencing susceptibility to CY-T1D, only alleles at the Idd4 locus behave in a sex-specific manner.

Possible mechanisms of CY-T1D protection in Idd4, Idd5, and Idd9 congenic strains

Having observed the impact of protective effects of NOR alleles at Idd4, Idd5, and Idd9 loci on CY-T1D incidence, we next examined the degree of islet inflammation in NOD and NOR congenic animals that remained normoglycemic 35 days after CY treatment. Ten of 62 (16%) CY-treated NOD mice did not become diabetic over the observation period. Histological analysis performed on pancreata from five of these 10 animals all displayed invasive insulitis (Fig. 8). Among NOD.NOR-Idd4 females, 14 of 35 (40%) remained normoglycemic at the conclusion of the experiment. As observed with NOD parental mice, seven of seven pancreata examined from these congenic mice displayed invasive insulitis (IS scores, 1.52–3.19). In contrast, nine of 25 randomly selected...
NOD.NOR-\textit{Idd9} mice that were normoglycemic after the observation period had only progressed to early peri-insulitis (IS scores, <1.0). Similarly, 13 of 15 NOD.NOR-\textit{Idd5} animals that were diabetic free at the end of the observation period had IS scores <1.0, indicating no progression to invasive insulitis. Ten of these mice displayed no insulitis (Fig. 8). These results suggested that NOR alleles at the \textit{Idd5} locus control early steps of insulitis progression in CY-treated animals, consistent with our previous evidence for control of early insulitis progression in spontaneous diabetes (9). The frequency of peri-insulitis in NOD.NOR-\textit{Idd9} differed from that in NOD mice (by Fisher’s exact test, \( p = 0.04 \), suggesting that NOR-derived alleles at this locus may limit progression from peri- to invasive insulitis. In contrast to \textit{Idd5} and \textit{Idd9}, which conferred an effect on insulitis progression, IS scores in nondiabetic, CY-treated, NOD.NOR-\textit{Idd4} mice were similar to those in NOR controls, suggesting that NOR \textit{Idd4} alleles may protect (females) from a step following invasive insulitis during progression to \( \beta \)-cell destruction.

**Discussion**

We found that \textit{Idd4}, \textit{Idd5}, and \textit{Idd9} loci control susceptibility to CY-TID, demonstrating that the drug-accelerated model is regulated by loci previously shown to impact spontaneous TID risk in NOD mice. \textit{Idd1} H2\textsuperscript{g7} was necessary, but insufficient, to confer CY-accelerated insulitis. MHC-identical NOR mice were resistant to the CY effect, indicating the role of other non-MHC-linked NOD \textit{Idd} alleles. Because no difference in biotransformation of CY into the active compounds was observed between NOD and NOR strains, the disease-promoting effect of the drug can now be ascribed to differential cellular responses to the active compound. Analysis of \((\text{NOD} \times \text{NOR}) \times \text{NOD}\) backcross animals produced significant evidence of linkage of insulitis to markers consistent with \textit{Idd9} and \textit{Idd4}. Importantly, only animals homozygous for NOD alleles at both loci displayed severe insulitis, suggesting interactions between these loci. Allelic variation at \textit{Idd4} or \textit{Idd9} displayed significant effects on CY-TID in congenic mice constructed on both the NOD and NOR backgrounds. Thus, two \textit{Idd} regions identified by linkage to an early step in disease progression displayed strong effects on disease outcome, validating the use of preclinical phenotypes for analysis of TID. In addition, we observed strong protection from CY-TID conferred by NOR alleles in NOD.NOR-\textit{Idd5} congenic strain. Allelic variation at \textit{Idd13} did not impact this disease model. NOR alleles at \textit{Idd4} provided strong protection from CY-TID for females, but not males. Strong sex-by-strain interaction effect was observed by comparison of NOD and NOD.NOR-\textit{Idd4} strains, clearly demonstrating a sex-specific effect of the \textit{Idd4} locus on CY-TID development. The distinction between sexually dimorphic traits and sex-specific locus effects in complex traits has emerged from analysis in model systems and now includes type 1 diabetes in the NOD mouse. Future studies will be required to determine whether allelic variation at \textit{Idd4} has an effect on the sexual dimorphism of spontaneous TID.

**\textit{Idd5}**

\textit{Idd5} was originally identified as a 34-cM interval on chromosome 1 that affected susceptibility to both spontaneous and cyclophosphamide-induced diabetes (40) as well as to peri-insulitis (41, 42). Analysis of recently developed \textit{Idd5} congenic strains in which portions of the C57BL/10 (B10) genome were introgressed into the NOD background suggested presence of two loci, \textit{Idd5.1} and \textit{Idd5.2}, each conferring protection from insulitis and TID ((43, 44); Fig. 9). Subsequent study further refined location of \textit{Idd5.1} to a small interval containing \textit{Ctla-4} (45), and recent work identified a \textit{Ctla-4} sequence variant affecting expression of a single splicing isoform between NOD and B10 mice (5). This study suggested that allelic variation at \textit{Ctla-4} mediates the TID protection provided by B10-derived \textit{Idd5.1} locus. A high resolution map of chromosome 1 in NOR mice indicates that \textit{Ctla-4} is NOR derived in this strain (9) and therefore can be excluded as a candidate gene conferring protection from CY-TID in the current study.

**FIGURE 6.** Cumulative frequency of spontaneous TID in NOD and NOD.NOR-\textit{Idd4} congenic females defining boundaries of the \textit{Idd4} interval. Diabetes development was monitored as described in Materials and Methods.
In an earlier study from our laboratory, a more telomeric portion of chromosome 1, defined as Idd5.3, was identified in an (NOD × NOR) F$_2$ intercross study as a locus that, together with Idd13, controlled islet invasiveness, specifically the transition from peri-insulitis to invasive insulitis (9). Idd5.3 is separated from Idd5.2 by ~12 cM. Both Idd5.2 and Idd5.3 intervals are BKs derived in the NOR strain, so genes within either or both of these loci may contribute to the protection from CY-T1D reported in this study. One attractive candidate is the natural resistance-associated macrophage protein (Nrampl) involved in the clearance of intracellular pathogens (46). This gene lies within the Idd5.2 region and is shown to be polymorphic between NOD and B10 strains (44). There are a number of genes without an obvious link to diabetes pathogenesis that map to the Idd5.3, including those for the nicotinic acetylcholine receptor, Acrg (47); collagen type VI, Col6a3 (48); the high density lipoprotein-binding protein, Hdlbp (49); and the receptor activity-modifying protein 1, Ramp1 (50).

**Idd9**

A large interval on the distal end of chromosome 4 was originally identified in the development of spontaneous diabetes in NOD mice (36, 38) (Fig. 9). In contrast to the current study, the previous reports did not observe linkage of CY-T1D to this region. However, in contrast to the data presented in this study, the cohort under study was 180 days old and free of spontaneous diabetes ever, in contrast to the data presented in this study, the cohort reports did not observe linkage of CY-T1D to this region. How-interestingly, Lyons et al. (34) also showed that despite diabetes resistance, NOD.B10-Idd9 mice displayed severe islet inflammation. Thus, for spontaneous disease, Idd9 appears to control a late step that affects β-cell death rather than the magnitude of islet inflammation.

Morahan et al. (39) reported a fourth generation (C57BL/6 × NOD) F$_1$ × NOD backcross analysis and concluded that heterozygosity at Idd11 (their designation) conferred protection from CY-accelerated disease despite homozygosity for NOD alleles at Idd11, 2, 3, 4, and 5. These data agree with our evidence that NOD alleles at Idd1, 4, and 9 drive susceptibility to CY-accelerated disease. The same group reported mapping B6-derived resistance to spontaneous T1D in congenic animals and suggested that the critical Idd11 interval lay between D4Mit31 (50.3 cM) and D4Mit204 (61.2 cM) (35) (Fig. 9). This location aligns with the 1-LOD confidence interval and was included in the Idd9 congenic strains reported in this study. Thus, previous studies and the data presented in this study identify potent Idd susceptibility genes between D4Mit331 and D4Nds13 that affect spontaneous T1D- and CY-accelerated insulinitis and T1D.

**Idd4 and genetic similarities between T cell-mediated autoimmune diseases**

Idd4 was first identified in a genome-wide linkage analysis of (NOD × B10.$^{H2g7}$) × NOD animals as a 30-cM region on chromosome 11 linked to early-onset T1D (38) (Fig. 9). Later studies examined linkage of this region to thymocyte proliferative unresponsiveness in NOD mice, a phenotype measured by in vitro ligation of the T cell Ag receptor with multivalent Ab (51). NOD thymocytes displayed lower proliferation than C57BL/6 and equal proliferation with DBA/2 thymocytes. The relationship of this phenotype to T1D susceptibility is not known. The same group later reported linkage of Idd4 with T1D pathogenesis in NOD.B6 congenic animals, suggesting that the maximal diabetes resistance was located within a 9.2-cM interval between D11Mit30 and D11Mit41 (52) (Fig. 9). Of several interesting candidate genes in this region, a favorite includes one that encodes inducible NO synthase (Nos2), an enzyme that produces NO, associated with β-cell inflammation.

**Sex-specific effect of Idd4**

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Sexual dimorphism in autoimmune disease

Sexual dimorphism in NOD T1D incidence was apparent from the first report of this strain (58) and could be manipulated by surgical castration of either sex (59). Previous genetic studies of spontaneous insulitis and diabetes only examined females and so were unable to detect sex-dependent effects of Idd alleles. The use of CY in the current study allowed evaluation of both sexes, demonstrating that allelic variation at the Idd4 region had distinct effects on male and female congenic mice (Table I). The sex specificity at this locus was dramatic, where NOR alleles protected females (p = 0.0194). Taken together with the previous reports and our finding (Fig. 6) that Idd4 impacts spontaneous T1D susceptibility, these observations provide a genetic basis for the sexual dimorphism in NOD T1D susceptibility and predict that the action of the critical genes at Idd4 will be sex dependent.

The existence of sex-influenced loci was recently established in several animal models of autoimmunity, such as EAE (60) and proteoglycan-induced arthritis (61) in mice and collagen-induced arthritis in rats (62). Interestingly, in both EAE and proteoglycan-induced arthritis models, linkage analysis to disease susceptibility identified several loci displaying differential behaviors in males vs females, with one of them mapping to the interval overlapping our Idd4. In the collagen-induced arthritis model, fine mapping of arthritis severity in congeneric rat strains revealed two loci, Cia3 and Cia5, located on chromosomes 4 and 10, respectively, with different effects in males and females (62). Cia5 was previously mapped to the telomeric portion of chromosome 10 (63) in an interval that is syntenic to the segment of mouse chromosome 11 where we positioned Idd4. Taken together with our work, these studies suggest a sex-dependent locus that either coincides with or is located in the close proximity to Idd4, affecting susceptibility to multiple autoimmune diseases involving distinct MHC haplotypes and tissue-specific reactions. Interestingly, a common functional variant in the protein tyrosine phosphatase (PTPN22) have been recently identified in T1D, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) patients (64–66). Future studies will be directed at genetic and genomic analyses of potentially shared susceptibility genes, and allelic variants for autoimmune diseases in rodent models and patient populations.

Many autoimmune diseases, including Sjogren’s syndrome, RA, SLE, MS, and autoimmune thyroiditis, occur at substantially higher frequencies in females than in males. The issue is more complex with T1D and is related to the substantial worldwide variation in incidence ranging from <1 to 36/100,000. Geographic regions with higher T1D incidence generally do not show a sex bias. However, in Japanese, Oceanic, and African-American populations with lower disease incidence, a higher T1D incidence has been observed in females (67–69). In addition to sex effects on destruction (53). The Scya cluster of monocyte-specific chemokine and chemokine receptor genes also maps in this region (54) and may help direct lymphocyte and monocyte recruitment to the islets. Interestingly, a locus (eae7) controlling susceptibility to experimental allergic encephalitis (EAE), a mouse model of autoimmune demyelinating disease with similarities to human multiple sclerosis (MS), also maps in the middle of the Idd4 region (55). Subsequent comparison of coding sequences of members of the Scya gene cluster between EAE-resistant and susceptible strains detected polymorphisms resulting in nonconservative amino acid substitutions in Scya1, 2, and 12 (56). Thus, allelic variation in the Idd4 region may affect susceptibility to multiple T cell-mediated autoimmune disorders. Higher resolution genetic analysis and direct tests of variants in transgenic models will be required to determine whether this reflects a true sharing of autoimmunity genes among different diseases. Immunological studies support similarities in the autoimmune responses in these diseases. For example, T cells in MS and T1D patients were found to target an overlapping set of autoantigens expressed in islet cells and the CNS. When treated with a protocol similar to that used to induce EAE, NOD mice displayed rapid, progressive CNS inflammation and demyelination (57). Thus, both genetic and functional evidence support shared mechanisms of T cell-mediated autoimmunity that ultimately targets different tissues.

FIGURE 8. Insulitis severity in CY-treated nondiabetic mice. a, NOD animals and mice of CY-T1D-resistant congenic strains were treated with CY twice as described in Materials and Methods. Pancreases from mice that did not become diabetic 35 days after first injection were collected, and insulitis severity was assessed (b). Mice with insulitis severity >1 were considered to progress to invasive insulitis. NOD.NOR-Idd4, NOD.NOR-Idd5, and NOD.NOR-Idd9 congenic strains displayed different levels of islet invasiveness, suggesting that these loci control separate steps of diabetes pathogenesis.
incidence, the presentation and severity of many autoimmune diseases differ between men and women. The strongest evidence that sex steroid hormones modulate the human immune response come from studies during pregnancy, when estrogen and progesterone levels rise and peak during the third trimester. Symptoms and signs of MS and RA frequently decline during pregnancy, but can flare in the postpartum period when levels of these hormones fall (70, 71). Conversely, pregnancy does not improve and often augments autoimmune activity in SLE (72). One explanation for these observations is that pregnancy induces a Th2-dominated immune response, enhancing Ab-mediated tissue damage in SLE and suppressing Th1 cell-mediated immune responses that drive MS and RA. However, there is little direct evidence for this idea, and the mechanisms of sexual dimorphism in autoimmune disease are probably more complex, involving neuroendocrine factors and epigenetic modification of gene expression.

Evidence from human populations and rodent models demonstrates that generation of an islet-reactive T cell repertoire depends upon MHC haplotype, and that destruction of islet β-cells requires coinheritance of multiple diabetes susceptibility loci. Diabetes susceptibility may be conferred by different constellations of genes in mice and humans as well as in genetically diverse human populations. The NOD model provides opportunities to analyze both the mechanisms of disease pathogenesis and their genetic control, thus elucidating shared immunological pathways within which lie potential targets for therapeutic intervention.

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Disclosures

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References


SEX-SPECIFIC EFFECT OF Idd4


