Unmasking Genes in a Type 1 Diabetes–Resistant Mouse Strain That Enhances Pathogenic CD8 T-Cell Responses

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OBJECTIVE—Nominally resistant mouse strains such as C57BL/6 (B6) harbor latent type 1 diabetes susceptibility genes uncovered in outcross to disease-susceptible NOD mice. However, identification of possible recessively acting B6-derived susceptibility genes is limited because very few F2 progeny derived from outcrossing this strain with NOD develop spontaneous autoimmune diabetes. Thus, we assessed whether a transgenic T-cell receptor (TCR) disease transfer model allowed the mapping of recessively acting B6 genetic loci that in the proper context contribute to diabetes.

RESEARCH DESIGN AND METHODS—CD8 T-cells transgenically expressing the diabetogenic A4 TCR were transferred into 91 (NODxB6.H2g7)F1xB6.H2g7 first-backcross (BC1) females. A genome-wide scan was performed for loci affecting clinical diabetes and insulitis severity.

RESULTS—A major locus on chromosome 11 in tight linkage with the marker D11Mit48 (logarithm of odds score = 13.2) strongly determined whether B1 progeny were susceptible to A4 T-cell–mediated diabetes. Mice homozygous versus heterozygous for B6 markers of this chromosome 11 genetic locus were, respectively, highly susceptible or resistant to A4-induced insulitis and diabetes. The genetic effect is manifest by host CD4 T-cells. Microarray analyses of mRNA expression identified a limited number of candidate genes.

CONCLUSIONS—The distal region of chromosome 11 in B6 mice harbors a previously unrecognized recessively acting gene (s) that can promote autoreactive diabetogenic CD8 T-cell responses. Future identification of this gene(s) may further aid the screening of heterogeneous humans at future risk for diabetes, and might also provide a target for possible disease interventions. Diabetes 60:1354–1359, 2011

Although particular major histocompatibility complex (MHC) haplotypes are the strongest contributor to T-cell–mediated autoimmune type 1 diabetes development in both humans and NOD mice, disease pathogenesis requires interactions with multiple other susceptibility (Idd) genes (1). This is illustrated by the fact that C57BL/6 background mice congenic for the NOD-derived H2g7 MHC haplotype (B6. H2g7) are normally diabetes-resistant (2). However, certain T-cell receptor (TCR) molecules contributing to diabetes development in NOD mice paradoxically exert even greater pathogenic activity when transgenically expressed in the B6. H2g7 strain (3,4). This finding, along with previous linkage analyses and congenic approach studies (5,6), indicate normally diabetes-resistant strains harbor some genes that in the proper combination can actually contribute to aggressive disease development.

Analyses of P2 rather than first backcross (BC1) progeny is the preferable approach for mapping diabetic genes, because this allows for the identification of both susceptibility or resistance variants from NOD mice or the outcross partner strain. However, identifying possible recessively acting B6-derived susceptibility genes after the outcross to NOD has been hampered because very few F2 progeny develop spontaneous diabetes even when the H2g7 MHC is fixed in all segregants (7). Nevertheless, analyses of progeny from a first backcross to B6. H2g7 mice revealed recessive alleles from this strain on chromosomes 1, 2, 7, and 15 promoting the diabetogenic activity of CD4 T-cells transgenically expressing the BDC2.5 TCR (3). We now report a similar BC1 strategy using another transgenic TCR that reveals at least one additional B6 origin recessive Idd susceptibility gene on chromosome 11 contributing to the peripheral activation of pathogenic CD8 T-cells.

RESEARCH DESIGN AND METHODS

Mice. NOD/ShiLtDvs mice are maintained in a specific pathogen-free research colony. B6.H2g7 mice are maintained at the N8 backcross generation (4). NOD mice transgenically expressing the TCR from the diabetogenic CD8 T-cell clone A4 plus a functionally inactivated Rag1 gene (NOD.Rag1null.AI4) have been described (8).

Adoptive transfer of diabetes. Indicated female mice were sublethally irradiated (600 R) and injected intravenously with 1 × 10⁹ NOD.Idd.14 splenocytes to induce diabetes. In one experiment, recipients were injected intraperitoneally with 250 µg/mouse of the CD25-depleting PC61.5 antibody 1 day before A4 T-cell transfer. In another experiment, donor cells were prelabeled with 2.5 µmol/L 5-bromo-2′-deoxyuridine carboxyl fluoroscein succinimidyl ester (CFSE). After 4 days, viable A4 T-cells from collagenase-digested spleen and pancreatic lymph nodes were identified by flow cytometry using CFSE and a CD8-specific antibody (53–6.7). Expression of various T-cell surface markers was assessed using antibodies specific for CD44 (IMB-8.1), CD25 (PC6.5.1), and CD62L (MEL-14). Another experiment used female mice injected with A4 T-cells preactivated in culture for 3 days with 100 nmol/L antigenic mimotope peptide YFIENYLEL and 50 units/mL interleukin 2.

In a separate experiment, female B6.H2g7 mice were lethally irradiated (1,200 R) and injected intravenously with 5 × 10⁶ bone marrow cells from BC1 progeny heterozygous or homozygous for the microsatellite marker D11Mit188. Fourteen weeks later, recipient mice were sublethally irradiated and injected intravenously with 1 × 10⁷ NOD.Rag1null.AI4 splenocytes to induce diabetes. In another study, B6.H2g7, NOD and F1 mice all homozygous for the Rag1null mutation were injected with 1 × 10⁷ NOD.Rag1null.AI4 splenocytes. Finally, magnetic bead purified CD4 T-cells from D11Mit188+/-/Dvs or D11Mit188−/−/Dvs BC1 progeny were cotransferred with 1 × 10⁷ NOD.Rag1null.AI4 splenocytes into B6.H2g7 Rag1null recipients. Recipients were either killed at the indicated time or monitored for diabetes development.

Assessment of diabetes and insulitis. Diabetes was assessed by daily monitoring of glycosuria with Ames Diastix (Bayer, Diagnostics Division, Elkhart, IN), with disease onset defined by two consecutive values of ≥3. Previously described criteria (9) were used to establish insulitis scores ranging from 0 (individual islet with no leukocytic infiltration, normal β-cell mass) to 4 (complete destruction) for the indicated mice.

Genotyping and linkage analyses. BC1 progeny were genotyped as previously described (10) for 131 single nucleotide polymorphisms (SNP) at

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approximately 20-Mb intervals across the genome (National Center for Biotechnology Information [NCBI] build 37 marker positions). Linkage markers for genes controlling insulitis severity and diabetes development in response to AI4 T-cell transfer were identified as previously described (11). PCR typing of the polymorphic D11Mit48 microsatellite marker was also done.

**Microarray analyses.** CD4 T-cells were purified from BC1 progeny either heterozygous or homozygous for D11Mit48 and cotransferred into female B6.H2b7.Rag1null recipients with 1 × 10⁷ NOD.Rag1null.AI4 splenocytes. RNA was isolated from CD4 T-cells sorted from spleens of these recipients by flow cytometry ~3 weeks after adoptive transfer. Three biologic replicates were generated for both genotypes, with each sample emanating from purified CD4 T-cells pooled from two donor mice. Microarray analysis of comparative CD4 T-cell gene expression was conducted using the Affymetrix 430v2 GeneChip array (Santa Clara, CA). R/Bioconductor software summarized the probe intensities for each gene using the robust multiaverage method (12,13). R/MAANOVA software (Churchill Group, Bar Harbor, ME) was used to generate lists of differentially expressed genes between the tested samples (14). Differentially expressed genes were identified by using tₙ, a modified t statistic incorporating shrinkage estimates of variance components from within the R/MAANOVA (15).

**FIG. 1.** NOD and B6.H2b7 but not (NODxB6.H2b7)F1 mice succumb to AI4 T-cell–induced diabetes. A: Incidence of diabetes in 6- to 8-week-old female NOD, B6.H2b7, F1, and (F1xB6.H2b7)BC1 recipient mice that were sublethally irradiated (600 R) and injected intravenously with 1 × 10⁷ NOD.Rag1null.AI4 splenocytes. Incidence of diabetes in BC1 recipients was significantly different (P < 0.0001) from NOD, B6.H2b7, and F1 mice. B: Insulitis scores (0 = no insulitis to 4 = no remaining islet cell mass) for surviving nondiabetic NOD.Rag1null.AI4 splenocyte recipients. Insulitis severity was significantly greater in surviving NOD mice compared with F1 and BC1 recipients according to the Mann-Whitney test. C: In vivo proliferation and activation of CFSE-labeled NOD.Rag1null.AI4 T-cells at 4 days after transfer in pancreatic lymph nodes of NOD, B6.H2b7, and F1 mice. D: Incidence of diabetes in 6- to 8-week-old sublethally irradiated female NOD and F1 recipients of in vitro activated AI4 T-cells.
RESULTS

We previously developed NOD mice transgenically expressing the TCR from the diabetogenic AI4 CD8 T-cell clone and also homozygous for the Rag1null mutation (NOD.Rag1null.AI4). Adoptively transferred AI4 T-cells from such donors rapidly induce diabetes in both sublethally irradiated NOD and B6.H2b7 mice (16). Thus, we were surprised AI4 T-cells failed to transfer diabetes or significant levels of insulitis to sublethally irradiated (NODxB6.H2b7)F1 hybrids (Fig. 1A and B). This difference was not due to varying post-transfer stimulation of AI4 T-cells within pancreatic lymph nodes, because the proliferation of such effectors at this site in F1 hybrids was similar to that of NOD mice and even greater than in B6. H2b7 recipients (Fig. 1C). Furthermore, no differences in expression of CD44, CD25, and CD62 L activation markers were detected between AI4 T-cells isolated from the parental or F1 mice (Fig. 1C and data not shown). However, AI4 T-cells preactivated in culture rapidly transferred diabetes to F1 recipients (Fig. 1D). Hence, the F1 genetic environment allows for less efficient initial pathogenic activation of diabetogenic CD8 T-cells than in either parental strain.

F1 hybrid resistance to AI4-induced diabetes indicates the NOD and B6.H2b7 genomes harbor separate recessively acting alleles supporting pathogenic CD8 T-cell activation. To map such unrecognized recessive B6 alleles, NOD.Rag1null.AI4 splenocytes were transferred into 91 sublethally irradiated female (NODxB6.H2b7)F1xB6.H2b7 BC1 progeny that were then monitored for diabetes development and also genotyped. Diabetes developed in 40% of BC1 segregants at a rate similar to both NOD and B6. H2b7 mice (Fig. 1A). Nondiabetic BC1 mice were examined for insulitis levels. Similar to F1 hybrids, insulitis levels were low in nondiabetic BC1 mice (Fig. 1B). This sharp dichotomy among BC1 progeny suggested that rather than contributions from multiple loci, which would result in a broad spectrum of insulitis scores, a limited number of genes rendered B6. H2b7 mice susceptible to AI4 T-cell–induced diabetes. Indeed, a SNP-based genome-wide one-dimensional scan of BC1 progeny revealed only one B6 genomic region on chromosome 11 that was highly linked to diabetes susceptibility and insulitis (logarithm of odds [LOD] score = 13.2; Fig. 2A).

On the basis of original typing of SNP markers only, the 95% CI for the region of interest was originally narrowed to a 3.6-Mb segment at the distal end of chromosome 11 (112.6–116.2 Mb), ~40 Mb below the previously identified Idd4 locus (Fig. 2B and C). Susceptibility to AI4 T-cell–induced diabetes/insulitis was associated with homozygosity for B6 markers within this region of chromosome 11, with LOD scores increasing up to and including the most distal NOD/B6 distinguishing SNP (rs3675087 at 116.2 Mb) that was typed. However, it remained possible that a gene(s) controlling susceptibility to AI4 transfer resided distally to the 116.2-Mb position. Thus, BC1 progeny were regenotyped for the polymorphic microsatellite marker D11Mit48 located at 117.76 Mb. Linkage between B6 homozygosity at D11Mit48 and diabetes/insulitis was very similar to that for rs3675087 (Supplementary Table 1). Sanger sequence analysis (www.sanger.ac.uk) indicated there are NOD/B6 polymorphisms in protein-encoding regions of two genes distal to D11Mit48, but these were not covered by the SNP typing panel available to us. For this reason, we redefined the support interval controlling differential sensitivity to type 1 diabetes induced by transferred AI4 T-cells to between 112.6 Mb and the end of chromosome 11 (121.8 Mb; Fig. 2C).

We tested whether genotyping BC1 mice for D11Mit48 alone could predict susceptibility to AI4 T-cell–induced diabetes. An additional cohort of 31 BC1 mice was genotyped for the D11Mit48 polymorphism before receiving
FIG. 3. A polymorphic gene(s) in close linkage with the D11Mit48 microsatellite marker controls susceptibility to AI4 T-cell–induced diabetes through effects on a CD4 T-cell population other than CD25+ Tregs. A: Mice homozygous for the B6 allele (B6/B6) vs. heterozygous (NOD/B6) for D11Mit48 were, respectively, highly susceptible and resistant to AI4 T-cell–induced diabetes. Results represent three independent experiments. B: Insulitis scores are shown for nondiabetic heterozygous and homozygous BC1 NOD.Rag1null.AI4 splenocyte recipients. C and D: B6.H2<sup>q7</sup> mice previously reconstituted with bone marrow from D11Mit48<sup>B6/B6</sup> or D11Mit48<sup>NOD/B6</sup> BC1 segregants are, respectively, susceptible or resistant to diabetes and insulitis induced by subsequently infused AI4 T-cells. E: Incidence of diabetes in 6- to 8-week-old female B6.H2<sup>q7</sup>.Rag1<sup>null</sup>, NOD. Rag1<sup>null</sup>, and F1.Rag1<sup>null</sup> recipients of 1<sup>3</sup>10<sup>7</sup> NOD.Rag1null.AI4 splenocytes. F: Incidence of diabetes in 6- to 8-week-old sublethally irradiated NOD and F1 recipients of 1<sup>3</sup>10<sup>7</sup> NOD.Rag1null.AI4 splenocytes. Recipients were also injected intraperitoneally with a CD25-depleting antibody (PC61 250 μg/mouse) 1 day before AI4 T-cell transfer. G and H: B6.H2<sup>q7</sup>.Rag1<sup>null</sup> mice infused with purified CD4<sup>+</sup> T-cells from D11Mit48<sup>B6/B6</sup> or D11Mit48<sup>NOD/B6</sup> BC1 segregants are, respectively, susceptible or resistant to diabetes and insulitis induced by subsequently infused AI4 T-cells.
NOD.Rag1null.AI4 splenocytes. None of the 15 heterozygous (D11Mit48NOD/B6) animals developed diabetes and usually no more than mild peri-insulitis (Fig. 3A and B). Conversely, 14 of 16 homozygous (D11Mit48B6/B6) mice developed diabetes, and the remaining two mice were severely insulitic (Fig. 3A and B). This simple segregation pattern strongly indicates that a recessively acting B6 origin gene(s) tightly linked to D11Mit48 is a primary contributor to the pathogenic activation of diabetogenic CD8 T-cells, whereas the NOD allelic variant actually dominantly suppresses this process. However, the protective effect of this NOD chromosome 11 allelic variant must normally be masked by the large number of other diabetes susceptibility genes characterizing this strain.

To determine whether the chromosome 11 gene(s) controls diabetes susceptibility through effects on hematopoietic cells, NOD.Rag1null.AI4 splenocytes were transferred into B6.H2^g7 mice previously reconstituted with bone marrow from D11Mit48B6/B6 or D11Mit48NOD/B6 BC1 progeny. Only recipients of D11Mit48B6/B6 but not D11Mit48NOD/B6 bone marrow developed diabetes (Fig. 3C) or significant levels of insulitis (Fig. 3D). Therefore, the D11Mit48-linked gene(s) controlling susceptibility to AI4 T-cell-mediated diabetic functions through a hematopoietic cell population(s).

Next, we established this gene(s) controls type 1 diabetes susceptibility through a lymphocyte population(s). This was determined by demonstrating NOD.Rag1null.AI4 splenocytes transferred diabetes with equal efficiency to B6.H2^g7, NOD, and F1 mice all homozygous for the Rag1null mutation, eliminating all endogenous lymphocytes (Fig. 3E). We tested whether regulatory T-cells (Tregs) were the lymphocyte population rendering F1 mice resistant to AI4-mediated diabetes. NOD and F1 mice were treated with a CD25-depleting antibody 1 day before receiving NOD.Rag1null.AI4 splenocytes. This eliminated most CD4+CD25+ Tregs for the duration of the 15-day postadoptive transfer period during which AI4 T-cells normally induce diabetes (Supplementary Fig. 1). Anti-CD25-treated F1 mice remained resistant to AI4-induced diabetes (Fig. 3F).

We next tested whether the chromosome 11 gene(s) controls type 1 diabetes susceptibility through effects on a CD4 T-cell population other than Tregs. Total CD4 T-cells purified from D11Mit48B6/B6 or D11Mit48NOD/B6 BC1 progeny were cotransferred with NOD.Rag1null.AI4 splenocytes into B6.H2^g7.Rag1null recipients. Only recipients of D11Mit48B6/B6 but not D11Mit48NOD/B6 CD4 T-cells developed AI4 T-cell–induced diabetes or high levels of insulitis (Fig. 3G and H). Therefore, the D11Mit48-linked gene(s) controlling susceptibility to AI4 T-cell–mediated diabetic functions through a non-Treg CD4 T-cell population(s).

We used microarray-based comparisons of mRNA transcript levels to identify candidates for a CD4 T-cell–expressed gene(s) within the chromosome 11 support interval regulating pathogenic activation of diabetogenic AI4 CD8 T-cells. This was accomplished by recovering CD4

### TABLE 1

List of polymorphic genes within the chromosome 11 interval 112.6 to 121.8 Mb that are differentially expressed in CD4 T-cells purified from D11Mit48B6/B6 vs. D11Mit48NOD/B6 genotyped BC1 progeny

| Gene          | Position (Mb)* | Description                                      | Relative fold change: NOD/B6 over B6/B6 | Fs  | P     | Function†               |
|---------------|----------------|-------------------------------------------------|----------------------------------------|-----|-------|-------------------------|
| Socs3         | 117.8          | Suppressor of cytokine signaling 3              | −1.4                                   | 0.0529 | 0.0088 | Inhibits activation and/or differentiation pathways in macrophages, dendritic cells, and T-cells |
| Mfsd11        | 116.7          | Major facilitator superfamily domain containing 11 | −1.4                                   | 0.0116 | Unknown | Unknown |
| 1110017F19Rik | 115.8          | RIKEN cDNA                                       | −1.3                                   | 0.0403 | 0.0387 | Multifunctional adaptor protein, recruiting cytoplasmic signaling proteins and membrane receptors/transporters into functional complexes. Defective regulation is linked with susceptibility to psoriasis |
| Slc9a3r1      | 115.0          | Solute carrier family 9                         | 1.1                                    | 0.0088 | 0.0088 | Calcium ion binding, nucleotide metabolism |
| Cant1         | 118.3          | Calcium-activated nucleotidase 1                | 1.2                                    | 0.0210 | 0.0326 | Unknown |
| Nt5c          | 115.4          | 5',3'-nucleotidase, cytosolic                   | 1.2                                    | 0.0023 | 0.0023 | GTPase expressed in mast cells |
| Nploc4        | 120.2          | Nuclear protein localization 4 homolog          | 1.2                                    | 0.0210 | 0.0326 | Unknown |
| Rab37         | 115.0          | RAB37, member of RAS oncogene family            | 2.1                                    | 0.0072 | 0.0392 | Unknown |
| Fig5l1        | 116.9          | Fidgetin-like 1                                 | 2.1                                    | 0.0072 | 0.0392 | Unknown |
| BC018473      | 116.6          | cDNA sequence BC018473                         | 2.6                                    | 0.0089 | 0.0089 | Member of an immunoglobulin superfamily gene cluster that may serve as an inhibitory receptor to regulate the maturation and differentiation of immune cells, helping to contain inflammation |
| Cd300lf       | 115.0          | CD300 antigen like family member F              | 2.6                                    | 0.0055 | 0.0055 | Component of the transmembrane laminin glycoprotein receptor |
| St6galnac1    | 116.6          | ST6 (α-N-acetyl-neuraminyl 1,2,3β-galactosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 1 | 2.6                                    | 0.0055 | 0.0055 | |

*Marker positions were taken from NCBI build 37.1 (www.ncbi.nlm.nih.gov). †See http://harvester.fzk.de/harvester.
T-cells purified from *D11Mit48<sup>B6/B6</sup>* and *D11Mit48<sup>NOD/B6</sup>* BC1 progeny that were previously cotransferred into B6. *H<sup>2<sup>d</sup></sup>*-*Rag<sup>1<sup>null</sup></sup>* recipients with NOD-*Rag<sup>1<sup>null</sup></sup>*-*Al<sup>4</sup>* splenocytes. CD4 T-cells were repurified from spleens once all recipients of *D11Mit48<sup>B6/B6</sup>* CD4 T-cells developed diabetes (all *D11Mit48<sup>NOD/B6</sup>* CD4 T-cell recipients were disease-free). Table 1 describes 12 genes mapping within the chromosome 11 support interval (112.6–121.8 Mb) that were differentially expressed by these two classes of CD4 T-cells and also characterized by NOD/B6 polymorphisms (www.sanger.ac.uk). Interestingly, three of these genes (*Rab37*, *Slc9a3r1*, and *CD300lf*) map to the PSORS2 psoriasis susceptibility locus in humans (17–19).

**DISCUSSION**

Although the NOD genome contains a collection of genes supporting development of spontaneous diabetes, additional susceptibility alleles remain masked within normally disease-resistant mouse strains. Indeed, we found the distal region of chromosome 11 in B6-*H<sup>2<sup>d</sup></sup>* mices harbors what is likely a single recessive gene acting through CD4 T-cells that more strongly promotes pathogenic activation of diabeticogenic CD8 T-cells than the NOD allelic variant. Presumably, the normal efficient thymic deletion of pathogenic CD8 T-cells in B6-*H<sup>2<sup>d</sup></sup>* mices (4,11) masks the activity of their prodiasabetic gene(s) on chromosome 11. Determination of diabetes susceptibility genes hidden in normally resistant mouse strains might ultimately aid the identification of individuals among heterogeneous humans who are at high risk of future disease.

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J.P.D. researched data and wrote the manuscript. Y.-G.C. contributed to discussion. W.Z. carried out statistical analyses. S.A. researched data. D.V.S. directed research and wrote the manuscript.

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