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Identification of the Inflammasome Nlrp1b as the Candidate Gene Conferring Diabetes Risk at the Idd4.1 Locus in the Nonobese Diabetic Mouse

Vinicius N. Motta,*1 Janet G. M. Markle,*‡1,1,2 Omid Gulban,* Steven Mortin-Toth,* Kuo-Chien Liao,‡ Jeremy Mogridge,‡ Charles A. Steward,§ and Jayne S. Danska*,§,†

Type 1 diabetes in the NOD mouse model has been linked to >30 insulin-dependent diabetes (Idd) susceptibility loci. Idd4 on chromosome 11 consists of two subloci, Idd4.1 and Idd4.2. Using congenic analysis of alleles in NOD and NOD-resistant (NOR) mice, we previously defined Idd4.1 as an interval containing >50 genes that controlled expression of genes in the type 1 IFN pathway. In this study, we report refined mapping of Idd4.1 to a 1.1-Mb chromosomal region and provide genomic sequence analysis and mechanistic evidence supporting its role in innate immune regulation of islet-directed autoimmunity. Genetic variation at Idd4.1 was mediated by radiation-sensitive hematopoietic cells, and type 1 diabetes protection conferred by the NOR allele was abrogated in mice treated with exogenous type 1 IFN-β. Next generation sequence analysis of the full Idd4.1 genomic interval in NOD and NOR strains supported Nlrp1b as a strong candidate gene for Idd4.1. Nlrp1b belongs to the Nod-like receptor (NLR) gene family and contributes to inflammasome assembly, caspase-1 recruitment, and release of IL-1β. The Nlrp1b of NOR was expressed as an alternative spliced isoform that skips exon 9, resulting in a premature stop codon predicted to encode a truncated protein. Functional analysis of the truncated NOR Nlrp1b protein demonstrated that it was unable to recruit caspase-1 and process IL-1β. Our data suggest that Idd4.1-dependent protection from islet autoimmunity is mediated by differences in type 1 IFN- and IL-1β-dependent immune responses resulting from genetic variation in Nlrp1b. The Journal of Immunology, 2015, 194: 000–000.

Abbreviations used in this article: B6, C57BL/6; BAC, bacterial artificial chromosome; BM, bone marrow; IDD, insulin-dependent diabetes; IFN, IFN-induced helicase; IL1RN, IL-1R antagonist protein; LeTx, anthrax lethal toxin; NLR, Nod-like receptor; NOR, NOD-resistant; TAP, tandem affinity purification; T1D, type 1 diabetes; T1IFN, type 1 IFN.

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Nlrp1b protein contains several domains crucial to its activity (18, 19). The NACHT and FIIND domains mediate oligomerization, resulting in proteolysis of procaspase-1 that is bound to the CARD domain (20, 21). Once freed, caspase-1 mediates cleavage and secretion of proinflammatory cytokines IL-1β, IL-18, and IL-33 (22). IL-1β mediates acute inflammatory responses (23, 24) and causes β cell death in human and rodent islets (25, 26). In the present study, we provide evidence that T1D resistance at Idd4.1 is mediated by a NOR-derived Nlrp1b allele encoding a truncated protein lacking the CARD domain and unable to stimulate IL-1β secretion.

Materials and Methods

Mice

All mice used in this study were maintained in a specific pathogen-free facility at the Hospital for Sick Children. Spontaneous T1D incidence at age 6 mo in NOD/Idd6 animals is 85% in females and 35% in males. NOR mice show no T1D. All procedures performed on these mice followed the guidelines of the Institutional Animal Care Committee.

Generation of new strains, DNA preparation, and microsatellite genotyping

NOD subcongenic strains were generated by extensive backcrossing of a previously reported NOD/NOR-Idd4 strain (13) to parental NOD mice, intercrossing their pups, and genotyping the progeny with a panel of high-density microsatellite markers (Supplemental Fig. 1). Genomic DNA was prepared from tail biopsies by digestion in tissue digest solution B (AutoGen) with proteinase K (Qiagen) prior to PCR amplification. Novel microsatellite markers were identified as previously described (15). To identify informative markers, DNA was amplified and the PCR products were resolved on the 3730xl DNA analyzer (Applied Biosystems). Alleles were sized, in comparison with size standards, by viewing their electro-photographs in GeneMapper (Applied Biosystems).

Spontaneous diabetes assessment

Blood and/or urine glucose levels were measured in NOD and Idd subcongenic females biweekly. Animals were classified as diabetic when blood glucose was >16 mmol/l or urine glucose was >250 mg/dl. Statistical analyses on TID lifetable data were performed using the log-rank (Mantel–Cox) test through Prism software (version 5.0b, GraphPad Software).

Preparation of bone marrow chimeras

Bone marrow (BM) cells were prepared from femurs and tibias and pooled from multiple age- and sex-matched donors of the same genotype. Experimental mice were backcrossed with NOD.C.B6-Il2rγnu (also known as NOD.CD45.2<sup>black</sup>) recipients, which have a T1D incidence comparable to NOD mice (http://jaxmouse.jax.org/strain/005616.html), were used to allow CD45 allotype discrimination of donor versus recipient cells and assessment of chimerism by FACS. After 240 d of observation, nondiabetic recipients were euthanized and all donor cells were recovered from BM and genotyped.

DNA samples from AJ, CAST/Eij, BALB/c, Akr/J, and DBA/2j mice were purchased from The Jackson Laboratory. DNA from NOD and NOR mice was prepared from tail biopsies (see the microsatellite typing method described above). Primers specific for intronic sequences flanking Nlrp1b exon 9 (Ensembl ENST00001298690) were designed: forward primer, 5′-TGTTATGACGGAGACACACAAG-3′, reverse primer, 5′-CATCACACAGGCTTGTTCC-3′. Owing to polymorphisms in the NOD intrinsic region, 5′ bp of the forward primer were replaced to allow perfect alignment and optimal PCR amplification. The NOD allele-specific forward primer was 5′-TGGCATGAAAGACAGAGGA-3′. DNA was PCR amplified, and the PCR product was purified (PCR purification kit, Qiagen). Purified PCR products were sequenced using the same primers as above, at TCAG.

Amplification of NOD and NOR Nlrp1b mRNA

BM cells from NOD and NOR mice were isolated as described above and cultured with GM-CSF for 7 d, with media replaced every other day. At day 7, suspended cells were removed and RNA was prepared from adherent cells by lysis in TRIzol followed by phenol/chloroform extraction and ethanol precipitation. cDNA synthesis with 1 μg RNA was performed with a SuperScript VILO cDNA synthesis kit (Invitrogen). Nlrp1b was amplified with previously published forward primer 5′-CCGAGGACTGAGCTGGCATGA-3′ and the reverse primer 5′-AGTTCGACTGTCAGTGAAGTCC-3′. The resulting cDNA fragment containing exon 9 was amplified, using the forward primer 5′-CGTCTCTCTCAGGTCGCT-3′ and the reverse primer 5′-CGTCTCTCTCAGGTCGCT-3′, which were designed using a Newbler gsAssembler version 2.3. All parameters were standard. Probes were designed to cover the region on chromosome 11 from 69,895,472 to 71,298,870 bp on the mouse reference sequence B6.D11Gul2671 and B6.D11Gul2721. DNA was subjected to digestion in tissue digest solution B (AutoGen) with proteinase K (Qiagen) prior to PCR amplification. Novel microsatellite markers were identified as previously described (15). To identify informative markers, DNA was amplified and the PCR products were resolved on the 3730xl DNA analyzer ( Applied Biosystems). Alleles were sized, in comparison with size standards, by viewing their electropherograms in GeneMapper (Applied Biosystems).

Sequencing alleles of Nlrp1b exon 9

Nlrp1b allele 1 (20) was amplified from cDNA prepared from RAW264.7 cells (derived from BALB/C mice) and Nlrp1b NOR allele was amplified from BM-derived macrophages. In each case, Nlrp1b cDNA was amplified using forward primer 5′-CCGGAGACTGAGCTGGCATGA-3′ and reverse primer 5′-AGTTCGACTGTCAGTGAAGTCC-3′. PCR products were digested with BamHI and XhoI and ligated into pNTAP-A (Strategene). Nlrp1b allele 3 (20) was cloned in two steps, as reported (19), using cDNA from TIB-47 cells (from Akr/J mice). The 5′ fragment was amplified with forward primer 5′-CCGAGGACTGAGCTGGCATGA-3′ and reverse primer 5′-AGTTCGACTGTCAGTGAAGTCC-3′. The resulting PCR product was digested with BamHI and XhoI and ligated into pNTAP-A. The second fragment of the Nlrp1b allele 3 gene was amplified using forward primer 5′-CCGGAGACTGAGCTGGCATGA-3′ and reverse primer 5′-AGTTCGACTGTCAGTGAAGTCC-3′. The resulting PCR product was digested with BamHI and XhoI and ligated into pNTAP-A.
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TAC-3° and reverse primer 5°-CGG GGG CCC TCTA GAA AGA GAC CCC ACC-3° and the product was digested with XhoI and Apal and ligated into the previous plasmid containing the 5° fragment of the Nlrp1b allele 3 gene.

**Procasapase-1–T7 plasmid**

AT7-tagged pcDNA3 vector was generated by inserting the T7 tag between the pcDNA3 Apal and Nhel restriction sites (19). The procasapase-1 cDNA was amplified with forward primer 5°-CGG GAA TTC TAT GCT GGA CAA GAT CCT GAG G-3° and reverse primer 5°-CGC CTC GAG AGA GAC TGG GAA CTA CAT GGT G-3° and the PCR product was digested with BamHI and XhoI and then ligated into pcDNA3-T7 (19).

**Pro–IL-1β-hemagglutinin plasmid**

The pro–IL-1β gene was amplified using the forward primer 5°-CGG GAA TTC ATG GCA ACT GTT CCT GAA CTC-3° and the reverse primer primers 5°-CGC CTC GAG AGA GAC CAC GGA CCT CAT GGT G-3°. The PCR product was digested with EcoRI and XhoI and then ligated into pcDNA3-hemagglutinin (HA) (19, 30).

**Cell culture of human fibroblasts and reagents**

HT1080 cells (American Type Culture Collection) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Hydrogen peroxide solution was purchased from Sigma-Aldrich and used at 1 mM.

**Detection of tandem affinity purification–tagged proteins**

One 10-cm dish of HT1080 cells were transfected with 1 μg plasmids encoding designated tandem affinity purification (TAP)-tagged proteins, 1 μg plasmids encoding procasapase-1–T7, and 1 μg plasmids encoding pro–IL-1β-HA. Twenty-four hours after transfection, cell pellets from each plate were lysed with EBC buffer (0.5% Nonidet P-40, 20 mM Tris [pH 8.1], 150 mM NaCl, 1 mM PMSF) at 4°C for 1 h. Cell lysates were incubated with streptavidin agarose (Thermo Scientific, no. 20349), and beads were washed with EBC buffer. Proteins were analyzed by immunoblots of SDS-PAGE with rabbit polyclonal antisera against an N-terminal epitope of Nlrp1b (Ab Solutions, Sunnyvale, CA).

**IL-1β secretion assay**

One million HT1080 cells were seeded the day before transfection. On the day of transfection, 1 μg each pNTPA-Nlrp1b, pcDNA3-procasapase-1–T7, and pcDNA3-pro–IL-1β-HA was transfected using polyethyleneimine. Twenty-four hours later cells were treated with 1 mM H2O2 for 3 h. The cell supernatant was mixed with anti-HA Ab (Sigma-Aldrich, no. H9658) overnight and then incubated with protein A-Sepharose (GE Healthcare). Bound proteins were eluted with SDS loading dye and subjected to SDS-PAGE and immunoblotting using a polyclonal HA Ab (Santa Cruz Biotechnology, no. sc805). Equivalent amounts of direct cell lysate control protein (30 μg) were subjected to SDS-PAGE and immunoblotted with anti-HA (Santa Cruz Biotechnology, no. sc805) and anti-β-actin (Sigma-Aldrich, no. A5441) Abs.

**Results**

**Fine mapping of the TID risk genes on chromosome 11**

A NOD-background congenic strain containing B6-derived alleles was previously reported to be protected from TID relative to NOD mice (12). This strain was later found to harbor B6 alleles on chromosome 1 within the Idd4 locus known to control insulitis and T1D (31, 32). Upon backcrossing to remove the chromosome 1 contaminant, diabetes protection was lost (http://jaxmicejax.org/strain/006809.html). In crosses of NOD with NOR mice, we showed that DBA/2-derived genes on chromosome 11 of the NOR strain confer a decreased incidence of T1D after administration of cyclophosphamide (13). Moreover, NOD-derived alleles in the Idd4.1 interval bounded by D11Gul2535–D11Gul2721 (69,948,613–71,337,812 bp) and containing 52 genes conferred TID susceptibility on the NOR background (15).

Within this Idd4.1 interval, the chemokine-encoding Cxcl16 was a promising candidate gene. Cxcl16 expression is induced by proinflammatory cytokines IFN-γ and TNF-α (33) and controls T cell recruitment (34). We identified several coding variations distinguishing NOD and NOR strains. To test this candidate gene, we generated a NOD.Cxcl16<sub>NOR</sub> transgenic strain by direct injection of the construct into the pronuclei of NOD fertilized eggs. Despite confirmed transgene expression, this transgenic strain showed no change in TID incidence or latency compared with NOD (data not shown), suggesting that Cxcl16 was not a strong candidate gene for Idd4.1.

**Spontaneous TID in NOD-background Idd4 subcongenics defines minimal Idd4.1 region**

We then generated four additional NOD.NOR-Idd4 recombinant congenic strains to further refine the Idd4.1 interval, with each strain capturing a different region of the NOR-derived locus (Fig. 1). A dense set of novel microsatellite markers was used to precisely define the boundaries of the NOR-derived region in each of these strains (Supplemental Fig. 1).

To determine the TID protection conferred by these NOR-derived chromosome 11 regions captured by our new Idd4 subcongenic lines, we assessed females of all strains for spontaneous TID relative to NOD. Consistent with prior reports, NOD females in our colony display a cumulative TID incidence of >80% by age 250 d (reviewed in Ref. 35). The D4 strain, encompassing the full-length NOR-derived Idd4 region, was protected from TID relative to NOD (p > 0.001, Fig. 2). The D4R1 and D4R2 strains were also protected relative to parental NOD mice (p < 0.001 for each, Fig. 2), suggesting that the genes mediating this effect lay between the markers D11Mit10 and D11Gul5. Strain D4R4 was also protected from TID (p = 0.001, Fig. 2) but did not show the same magnitude of protection as did congenic strains with a longer NOR-derived interval. Thus, the D11Gul2850–D11Gul5 interval might include genes that confer additional effects on diabetes. This interval displayed partial overlap with our previous defined Idd4.2 region (14). In contrast, strain D4R6 was not protected from TID relative to parental NOD mice (p = 0.975, Fig. 2). Because D4R6 mice carried NOR-derived alleles from D11Gul2522 to D11Gul2687, the genes in this region were excluded from the analysis. Collectively, these data refined a minimal Idd4 region bounded by the markers D11Gul2687 and D11Gul2850 containing NOR-derived genetic variations that conferred TID protection.

**Idd4 locus effect on TID is mediated by the hematopoietic compartment**

TID pathogenesis depends on the action of multiple hematopoietic immune cell types in addition to certain stromal cells, such as β islet cells. To determine whether the protective effect mediated by Idd4.1 alleles is acting through the BM-derived or radioresistant compartments, we generated a series of reciprocal BM chimeras. Lethally irradiated NOD.Cd45.2<sup>BALB/c</sup> recipients (45–60 d of age) were injected with donor BM cells from a variety of NOD.NORIdd4 (CD45.1) congenic strains and observed for 240 d. At termination of the experiments, flow cytometry analysis of the recipient peripheral LNs and spleen with Abs to CD45.1 and CD45.2 confirmed that the chimeras contained >95% donor-derived hematopoietic cells (data not shown). As expected, transplantation of NOD BM into NOD.Cd45.2<sup>BALB/c</sup> recipients resulted in 100% TID incidence by 32 wk posttransfer (Fig. 3). In contrast, BM from either NOD.NOR-D4 or NOD.NOR-D4R1 conferred significantly lower frequency of T1D in NOD.Cd45.2<sup>BALB/c</sup> recipients in the reciprocal chimera. NOD.NORIdd4 (D4) recipients of NOD.Cd45.2<sup>BALB/c</sup> BM were not protected from TID relative to NOD recipients (Supplemental Fig. 2), demonstrating that even the largest NOR-derived Idd4 interval did not confer TID protection via radioresistant tissues. In each of these chimeras,
the genotype of the BM cells recapitulated the TID incidence of the strain from which they were isolated (Fig. 3). These data demonstrated that variation within an interval centromeric to D11Gul5 (including Idd4.1) provided TID protection through the radiosensitive hematopoietic compartment.

**Exogenous IFN-β overcomes Idd4-mediated TID protection**

Previous work in BM-derived macrophages identified differential expression of several genes involved in the IFN response that map to Idd4 (15). Given the role of IFN in TID pathogenesis (reviewed in Ref. 36), these findings suggest that Idd4 modifies TID susceptibility by regulating the expression of IFN-responsive genes. To test whether exogenous IFN could alter the protective effect of Idd4, we administered recombinant IFN-β in PBS or PBS only (sham) by weekly injections to 3-wk-old NOD or NOD.NOR-Idd4 (D4) congenic females. The NOD sham and NOD treated cohorts had virtually identical TID incidence and kinetics (p = 0.7799), indicating that IFN-β did not alter disease course in NOD parental mice (Fig. 4). In contrast, we observed different survival curves for the sham versus IFN-β–treated NOD.D4 mice (p = 0.0257), demonstrating that exogenous IFN-β abrogated the NOR-Idd4–mediated TID protection. These results suggested that the TID protective effect of NOR alleles in Idd4 genes could be modified by the T1IFN pathway, and we focused our analysis on Idd4.1 locus genes relevant to the IFN-dependent inflammatory response.

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**FIGURE 1.** Fine mapping of the Idd4 locus. Novel subcongenic lines were generated from the NOD.NORIdd4 strain (D4) by backcrossing to NOD and then intercrossing progeny in a “speed congenic” approach. Progeny were genotyped at the microsatellite markers shown to determine locations of NOD-derived (white) and NOR-derived (black) regions. Unresolved boundaries are shown in gray gradient. Regions for which no polymorphic microsatellites could be found are shown in hatched lines, and regions identical in NOD and NOR are shown in gray. **Left**, View of chromosome 11 in NOD-background Idd4 subcongenic strains. **Right**, Expanded view of five subcongenic strains (genotypes shown) used to define the Idd4.1 locus boundaries.

Nlrp1 IS A CANDIDATE TID SUSCEPTIBILITY GENE
Genomic sequence analysis of Idd4.1 identified Nlrp1b as a candidate gene

A NOD reference sequence was available based on sequencing of BAC libraries that included the Idd4.1 region (http://www.sanger.ac.uk/sanger/HumPub_ChromoView_NodMouse). In contrast, NOR genomic reference sequences were not available. To compare the NOD and NOR genomic sequences across Idd4.1, we used an array-based sequence capture technology, with 385K custom-designed probes (60 bp in length) distributed across 1.5 Mb of chromosome 11, to enrich genomic DNA across the Idd4.1 interval and subjected the material to high-throughput sequencing.

The high-throughput sequencing data set from the captured genomic DNA was large and we reduced the number of sequence reads to 150,000 used for the NOR de novo assembly. This choice allowed assembly of an interval of 1,161,027 bp from 482 contigs (size range, 500–18,794 bp). When taking sequence gaps into consideration, this assembly approximated with the length of Idd4.1 in both the B6 and NOD reference genomes. We also captured and performed high-throughput sequencing of the Idd4.1 region of NOD to compare the specificity and efficiency of this approach to the NOD BAC reference sequence.

Sequence capture of the Idd4.1 region of NOD resulted in high-quality reads for most of the interval with poorer assembly at the 3′ end of the Idd4.1. This issue was anticipated because design of the array-based sequence capture technology over the 3′ end of the locus revealed many segments not covered by the probes. Thus, for most candidate genes of the Idd4.1 interval, including Rpain, C1qbp, Derf2, and Mxs32, the NOD sequence data were assembled into a single contiguous sequence. There were relatively few synonymous coding-region single nucleotide polymorphisms (SNPs) in theses genes when comparing the NOR sequence to NOD, and no predicted amino acid substitutions. Nup88 and Dhx33 produced high-quality contiguous sequences and showed one and two predicted amino acid changes, respectively, between NOD and NOR alleles (Table I). Nup88 encodes a highly conserved, broadly expressed member of the nuclear pore complex and was not a strong candidate for an immune-mediated phenotype. Dhx33 is member of the DEAD box protein family, involved in modulating RNA secondary structure to allow for translation initiation, ribosome assembly, and other cell processes. Dhx33 also interacts with the NLRP3 inflammasome complex after poly(I:C) stimulation (37), suggesting a possible contribution to innate immune recognition in the NOD model. We performed computational analysis to predict whether the amino acid substitutions in these two genes may affect protein function. The results of the variant effect predictor tool (Ensembl) in conjunction with the sorting tolerant from intolerant (SIFT) algorithm (38) indicated that these three amino acid variations are well tolerated (Supplemental Fig. 3B).

Additionally, multispecies alignments of the Nup88 and Dhx33 protein sequences of mouse, rat, Drosophila, and zebrafish demonstrated that the three amino acid residues differentiating NOD and NOR also vary between species (data not shown) and are therefore unlikely to be critical to protein function. Although these analyses do not rule out Nup88 and Dhx33 as candidate genes, they reduced their priority as variants for mediating T1D protection.

For two Idd4.1 candidate genes, Nlrp1a and Nlrp1b, de novo assembly of a high-quality, contiguous NOR sequence was not possible (Table I). This limitation resulted from low sequence coverage in some regions of the telomeric end of the interval where repeated sequences had been masked and excluded from the oligonucleotide capture probes array design. Nlrp1a and Nlrp1b are large genes spanning 50–70 kb and containing ~15 exons. Despite the lack of intergenic and intronic sequences, we did obtain the full coding sequence of both genes. The coding sequence of Nlrp1a displayed minimal polymorphism and only one amino acid variation between NOD and B6 (Supplemental Fig. 3). In contrast, Nlrp1b was highly polymorphic between NOD and B6, prompting additional molecular genetic investigation.

The NOR Nlrp1b allele produces a spliced variant lacking the CARD domain

Nlrp1b forms an inflammasome, a multiprotein complex that mediates caspase-1 activation, which in turn promotes the cleavage of pro–IL-1β into its mature form IL-1β (39). Moreover, T1IFN can alter Nlrp1 inflammasome activity via STAT1 (40). Inhibition of Nlrp1 results in diminished caspase-1–dependent IL-1β production (40). Taken together with our evidence that the T1D protection conferred by the NOR Idd4 allele was inhibited by rIFN-β treatment (Fig. 4), these data implicated Nlrp1b as a priority candidate for the Idd4.1 locus effect on T1D.

Nlrp1b is highly polymorphic among mouse strains—at least five alleles are found in standard laboratory inbred strains (20). Variant alleles of Nlrp1b have functional consequences, for example, they confer differential responses to anthrax lethal toxin (LeTx). LeTx exposure results in Nlrp1b self-association into an inflammasome that enables processing of pro- to the active form.
of caspase-1 (20, 41). Mouse strains carrying \textit{Nlrp1b} alleles 1 and 5 respond to LeTx-mediated pyroptosis whereas strains carrying alleles 2, 3, and 4 are unresponsive to the toxin (20).

A previously reported 5-bp deletion in DBA/2-derived \textit{Nlrp1b} (between 51 and 55 bp of exon 9) was suggested to cause a frame shift and premature stop codon (20). NOR mice inherited idd locus genes from B6 and DBA/2J ancestors, and they are DBA/2-like at the \textit{Idd4.1} interval (4, 5). We sequenced the genomic \textit{Nlrp1b} exon 9 from NOD and NOR, as well as mouse strains carrying the five known \textit{Nlrp1b} alleles, that is, BALB/c (allele 1), A/J (allele 2), AKR/J (allele 3), DBA/2J (allele 4), and CAST/EiJ (allele 5) (20). In agreement with a previous report (20), we found that AKR/J and NOD/Jsd mice both carry \textit{Nlrp1b} allele 3 (Fig. 5). However, we did not confirm the 5-bp deletion in exon 9 in either DBA/2J or NOR (allele 4) (Fig. 5). We did identify a spliced variant form of \textit{Nlrp1b} in the NOR mRNA that skips the entire exon 9 (Fig. 6, Supplemental Fig. 4). Interestingly, the resulting joining of exons 8–10 leads to a premature stop codon in the NOR \textit{Nlrp1b} sequence, which predicts a truncated protein that lacks the CARD signaling domain (Fig. 6C). These data agree with findings of a truncated \textit{Nlrp1b} transcript produced by the DBA/2J allele (20), which our data indicate results from exon 9 skipping and not from a 5-bp deletion in exon 9. Thus, both the NOR and DBA/2J strains have \textit{Nlrp1b} allele 4, which produces a shorter transcript and results in a truncated protein.

\textit{Nlrp1b} is the only member of the NLRP family with a carboxy-terminal extension containing FIIND and CARD domains (42). Whereas deletions of the LRR and NACHT domains of \textit{Nlrp1b} cause constitutive activation of caspase-1, deletion of the FIIND or CARD domains results in an inactive protein (19). To determine whether the exon skipping and premature stop codon identified in the NOR \textit{Nlrp1b} sequence resulted in a truncated transcript, RNA was isolated from NOD and NOR macrophages from which cDNA was synthesized. PCR amplification of the entire \textit{Nlrp1b} mRNA (Fig. 6A) or a shorter fragment of \textit{Nlrp1b} flanking exon 9 (Fig. 6B) was performed and the products were visualized by gel electrophoresis. The NOD full-length \textit{Nlrp1b} transcript migrated at 3300 bp, as expected, whereas the NOR product migrated faster, consistent with a shorter fragment (Fig. 6A). Amplification of an \textit{Nlrp1b} mRNA segment with primers flanking exon 9 illustrated the size difference even more clearly (Fig. 6B). The NOR allele migrated at the predicted size, whereas the NOD allele was appreciably shorter. The premature termination codon in the NOR allele is located <50 nt from a downstream splice junction, suggesting that it is unlikely that the NOR \textit{Nlrp1b} mRNA undergoes non-sense-mediated decay (43, 44). These results indicated that the NOR \textit{Nlrp1b} protein might lack the critical CARD domain, resulting in inability to recruit caspase-1 to the inflammasome complex.

The NOR truncation predicts a nonfunctional allele

Since CARD–CARD interactions are crucial to NLRP1 inflammasome assembly (42), and the CARD domain and adjacent 56 aa of \textit{Nlrp1b} mediate caspase-1 activation (19), we predicted that the NOR allele would be nonfunctional (Fig. 6C). To test this hypothesis, we first assessed the responsiveness of NOD and NOR macrophages to the known agonist LeTx. For comparison we used C3H-derived macrophages that carry \textit{Nlrp1b} allele 1 and are responsive to LeTx. In agreement with published results (20), the NOD macrophages were resistant (nonresponsive), whereas C3H macrophages were susceptible to LeTx-mediated pyroptosis. NOR macrophages also showed resistance to LeTx-induced pyroptosis (data not shown). The viability of macrophages from NOD, NOR, and NOR-\textit{Nod}-\textit{Idd4.1} congenic (NOR.D4) mice was not affected by LeTx treatment at all doses tested (data not shown). Therefore, it was necessary to devise an alternative \textit{Nlrp1b} inflammasome activation assay to assess whether \textit{Nlrp1b} allele 3 (NOD) and allele 4 (NOR) differed in ability their to recruit caspase-1 and mediate IL-1β processing.

\textit{NOR}-derived \textit{Nlrp1b} behaves as a null allele

Because reactive oxygen species have been implicated in the activation of the NLRP3 inflammasome (45), we developed an assay to test differential responsiveness of \textit{Nlrp1b} alleles to H2O2 and used it to determine whether \textit{Nlrp1b} alleles 3 (NOD) and 4 (NOR) displayed differential responses. HT1080 human fibroblast cells were transfected with a pNTAP vector containing either the NOD, NOR, or BALB/C (allele 1) \textit{Nlrp1b} cDNA. The transfected cells

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**Table 1. The NOR genomic sequence across \textit{Idd4.1} differs from NOD at a limited number of genes**

<table>
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<th>Start Position</th>
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<th>Number of NOR Contigs</th>
<th>Coding Region SNPs</th>
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<td>3</td>
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<td>3</td>
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</table>

Sequence comparison of all known protein-coding genes in the interval \textit{D11Gul2687–D11Gul2850} (the minimal \textit{Idd4.1} region) showing the number of contigs, number of coding region SNPs, and predicted amino acid changes from our NOR sequence data compared against the NOD Sanger reference sequence.

*The number of nonsynonymous SNPs in \textit{Nlrp1a} and \textit{Nlrp1b} could not be identified owing to incomplete alignment of the NOD and NOR sequences.
were lysed and TAP-tagged proteins precipitated. SDS-PAGE and Western blot analyses were performed to confirm the presence of ectopically expressed Nlrp1b (Fig. 7A). These immunoblots confirmed that the NOD allele has an apparent molecular mass of 133 kDa, whereas the NOR allele migrated at 100 kDa, reflecting the protein truncation at exon 9 (Fig. 7A). As previously reported, lysates from HT1080 transfected with Nlrp1b allele 1 produced two bands, suggesting that when ectopically expressed, Nlrp1b is autoproteolytically processed (19, 46). To assess the ability of the Nlrp1b allelic variants 1, 3, and 4 to activate caspase-1 and elicit IL-1β secretion, HT1080 cells were cotransfected with the plasmids encoding procaspase-1, pro–IL-1β-HA, and a pNTAP vector containing Nlrp1b alleles 1, 3, or 4. Approximately 24 h later, the cells were treated with H2O2 and the cell lysates examined for pro–IL-1β. Cell culture supernatant was then examined for HA-tagged IL-1β and pro–IL-1β. Pro–IL-1β was present in all Nlrp1b-transfected HT1080 cell lysates (Fig. 7B). Only cells transfected with Nlrp1b allele 1 produced the 17-kDa mature form of IL-1β in the supernatant, demonstrating IL-1β secretion upon H2O2 treatment. Cells containing Nlrp1b allele 3 (NOD) showed detectable IL-1β secretion, at lower levels than allele 1. In contrast, cells expressing the Nlrp1b allele 4 (NOR) had no detectable IL-1β secretion (Fig. 7C). Although pro–IL-1β was present in these cells, the protein encoded by Nlrp1b allele 4 was unable to stimulate caspase-1–dependent cleavage into mature IL-1β. These results indicated that the NOR-derived Nlrp1b allele lacking the CARD domain behaved as a nonfunctional variant. Such a loss of function may mediate the T1D protection conferred to NOD congenic mice by introgression of the NOR-Idd4.1 locus.

**Discussion**

Genome-wide association studies in human populations reveal complex inheritance patterns for T1D (47). Similar to human T1D, disease in the NOD mouse has been mapped to multiple Idd loci (3). The NOR mouse is significantly protected from T1D by the Idd4.1 and Idd4.2 regions on chromosome 11. In this study, we present high-resolution mapping of Idd4.1 to a 1.1-Mb region. The protective effect of NOR-Idd4.1 alleles was transferrable through BM transplantation from NOD.NOR-Idd4 congenic mice into NOD recipients, demonstrating that the effect of genetic variation at this locus was mediated through radiosensitive hematopoietic cells. Among the protein-coding genes in the minimal Idd4.1 locus, Nlrp1a and Nlrp1b show extensive coding region polymorphisms between NOD and B6 genomes (Supplemental Fig. 3). Because previous studies showed that B6-derived Idd4 confers T1D resistance in NOD congenic mice (10, 48), we prioritized genetic variations between these two strains. Sequence analysis of Nlrp1a allele revealed a single amino acid substitution between B6 and NOD allele (data not shown), whereas Nlrp1b displayed a high frequency of coding polymorphisms between NOD and B6 (20). The NOR strain is DBA/2-derived at Idd4.1. We identified an alternatively spliced isoform in NOR Nlrp1b that skips exon 9, which results in a frame shift and premature stop codon. The NOR Nlrp1b variant is predicted to produce a truncated protein that lacks the CARD signaling domain, resulting in an Nlrp1b unable to activate caspase-1–mediated cleavage of pro–IL-1β (Fig. 6).
The connection between innate receptors and metabolism (50), energy stress might signal Nlrp1b activation and contribute to T1D in NOD mice.

Mouse Nlrp1b differs from the human ortholog by the lack of the N-terminal pyrin domain, which interacts with the adaptor protein ASC that recruits and activates caspase-1. Upon activation, caspase-1 processes and releases IL-1β, IL-18, and IL-33 (51–53). Because the murine Nlrp1b protein lacks a pyrin domain, it must use an alternate mechanism to associate with caspase-1 and mediate proinflammatory IL-1β, IL-18, and IL-33 secretion. This interaction likely occurs through the C-terminal CARD domain, which we show is absent from the NOR Nlrp1b protein. This dysfunctional allele is predicted to decrease pyroptosis and caspase-1–mediated IL-1β, IL-33, and IL-18 processing. Thus, NOR-Idd4.1–derived T1D protection is likely conferred by the inability of Nlrp1b to respond to some perturbations in vivo.

IL-1β likely exerts both direct and indirect pathological effects on islets. Our group (54) and others (55, 56) have previously demonstrated expression of IL-1 in NOD islets. Rat β cells are highly sensitive to IL-1β, responding by limiting replication and, at higher concentrations, initiating apoptosis (57). The endogenous human IL-1R antagonist protein (IL1RN) inhibits binding of IL-1β to its receptor. Ectopic expression of IL1RN in isolated human islets can prevent IL-1β–mediated β cell apoptosis (58). Additionally, levels of IL1RN are positively associated with preserved β cell capacity in T1D patients (59). These data have led to the hypothesis that antagonism of IL-1β may have the potential to limit β cell death in T1D, an idea that showed promising results in a small, single-arm, open-label clinical trial (60), but was not supported by recent multicenter, randomized, double-blind, placebo-controlled trials (61). Although blocking the IL-1β pathway does not seem to be effective as a monotherapy, it may produce positive effects in combination with drugs that modify the adaptive immune response.

Similarly, blockade of IL-1β signaling alone by either genetic or pharmacological means is not sufficient to protect NOD mice from T1D. Although there is longer latency of T1D in Il1r-deficient compared with wild-type NOD mice, the final disease incidence is not affected (62). IL-18 can also be processed by NLRP1b-dependent caspase-1 activation (63) and administration of exogenous IL-18 to young NOD mice was shown to increase the development of diabetes (64). Because caspase-1 deficiency alone does not alter T1D incidence in NOD mice (65), we speculate that other proinflammatory caspases may interact with Nlrp1b to mediate secretion of IL-1β and IL-18 and other proinflammatory cytokines.

**FIGURE 6.** The NOR allele of Nlrp1b produces a spliced mRNA isoform that skips exon 9. BM-derived macrophage RNA was harvested from NOD and NOR mice, and cDNA was prepared using 1ug, 500ng, or 100ng of RNA. PCR products were produced by amplification of the entire Nlrp1b mRNA (A) or of a fragment of Nlrp1b flanking exon 9 (B). PCR reactions were performed with 30ng of cDNA from each of the cDNA templates. Both types of PCR products reflected a shorter transcript size in NOR compared with NOD cells. (C) Schematic representation of the NOD and NOR Nlrp1b protein. Sequence analysis of NOR Nlrp1b mRNA revealed that the NOR allele skips the entire exon 9 and generates a nonsense codon preceded by three amino acids changes. The shorter NOR protein is predicted to lack the last three exons including the CARD domain. Black lines represent amino acid changes in the NOR sequence in comparison with the NOD. Box indicates the site of the stop codon. Dashed lines indicate protein domains predicted to be absent from the NOR protein.
NLRPs are the largest subfamily of NLR genes in humans, with 14 known members (66). Several autoinflammatory disorders have been strongly associated with polymorphisms in inflammasome-forming NLRs, including celiac disease (67), rheumatoid arthritis (68), systemic lupus erythematosus (69) and Crohn’s disease (70). Recently, mutations in the NLRC4 gene, a crucial sensor for several Gram-negative intracellular bacteria, was linked to familial cold autoinflammatory syndrome. This link between NLRC4 and familial cold autoinflammatory syndrome highlights the importance of NLRC4 in the innate immune response to bacteria and in the development of inflammatory diseases (71, 72).

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An analysis of four SNPs in NLRP1 performed in cohorts of patients with autoimmune disorders and healthy controls provided evidence of association of the major allele of a coding SNP (rs12150220; Leu155His) with both T1D (odds ratio, 1.15; 95% confidence interval, 1.06–1.49; p = 0.007) and Addison’s disease (p = 0.005) and Addison’s disease (p = 0.007) (73). This study also reported a lack of association with either systemic lupus erythematosus or juvenile idiopathic arthritis, suggesting that association of these NLRP1 variants impacts certain organ-specific autoimmune diseases. Our data suggest that functional polymorphism in Nlrp1b influences T1D in NOD mouse model through the Nlrp inflammasome pathway, implicating innate immune sensing in the immunopathogenesis of this disease.

We show in the present study that administration of exogenous IFN-β overcomes the T1D-protective effect of the NOR Idd4.1 locus. Together with our previous work demonstrating that Idd4 controls the expression of several T1IFN genes in APCs (15), these data suggest an interaction between T1IFN and inflammasome-mediated production of IL-1β. At high concentrations, T1IFN including IFN-β suppresses pro–IL-1β transcription, but at low concentrations it can initiate IL-1β production via induction of caspase-11. B6 mouse macrophages treated with high concentrations of IFN-β showed decreased IL-1β release upon LeTx exposure (40), which was interpreted as evidence that T1IFN antagonizes Nlrp1b activity. At lower concentrations IFN-β did not inhibit IL-1β secretion (40). In fact, low doses of IFN-β initiate signaling in an STAT-1–dependent cascade that induces expression of caspase-11. Caspase-11 can physically interact with and cleave caspase-1 to promote its activation (74, 75). Therefore, in vivo administration of rIFN-β may represent an Nlrp1b-independent mechanism for caspase-1 activation and IL-1β release and may explain why we observed the loss of T1D protection in NOD.NOR-Idd4 congenic mice treated with rIFN-β. Further mechanistic analysis of this effect is required to determine whether rIFN-β administration provoked caspase-11 expression, for example by APCs within the pancreatic lymph nodes.

There is robust genetic and functional evidence that variants in an innate immune sensor that controls the IFN response control aspects of human T1D. Genome-wide studies identified an association between a coding variant in a cytoplasmic sensor of viral nucleic acid, the IFN-induced helicase (IFIH1), and T1D in both case control and family-based cohorts (76). Additionally, an IFIH1 variant was associated with the speed of progression from the appearance of anti-islet autoantibodies to T1D onset in a study of the children of T1D patients (77). Upon binding viral mRNA, IFIH1 activates several kinases that phosphorylate the IFN regulatory factors 3 and 7, which in turn activate transcription of an antiviral program including T1IFN. Resequencing of IFIH1 in T1D patients and controls further identified several rare variants that were predicted to alter the structure and expression of the protein (78). Collectively, these studies demonstrate an important role for genetic variants affecting the expression and function of
innate immune sensors and the T1IFN pathway in human T1D risk, as we have identified for the NOD mouse model.

Disclosures The authors have no financial conflicts of interest.

References


