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Polymorphism in the Innate Immune Receptor SIRPα Controls CD47 Binding and Autoimmunity in the Nonobese Diabetic Mouse

Andrea Sut Ling Wong,*† Steven Mortin-Toth,† Michael Sung, † Angelo J. Canty, ‡ Omid Gulban, † David R. Greaves,§ and Jayne S. Danska*†,‡

The signal regulatory protein (SIRP) locus encodes a family of paired receptors that mediate both activating and inhibitory signals and is associated with type 1 diabetes (T1D) risk. The NOD mouse model recapitulates multiple features of human T1D and enables mechanistic analysis of the impact of genetic variations on disease. In this study, we identify Sirpa encoding an inhibitory receptor on myeloid cells as a gene in the insulin-dependent diabetes locus 13.2 (Idd13.2) that drives islet inflammation and T1D. Compared to T1D-resistant strains, the NOD variant of SIRPα displayed greater binding to its ligand CD47, as well as enhanced T cell proliferation and diabetogenic potency. Myeloid cell–restricted expression of a Sirpa transgene accelerated disease in a dose-dependent manner and displayed genetic and functional interaction with the Idd5 locus to potentiate insulitis progression. Our study demonstrates that variations in both SIRPs sequence and expression level modulate T1D immunopathogenesis. Thus, we identify Sirpa as a T1D risk gene and provide insight into the complex mechanisms by which disease-associated variants act in concert to drive defined stages in disease progression. The Journal of Immunology, 2014, 193: 4833–4844.

Type 1 diabetes (T1D) is caused by autoreactive T cells that infiltrate and destroy pancreatic β cells, resulting in loss of insulin production and dependence on exogenous insulin. The NOD mouse develops spontaneous autoimmune T1D that shares many features with the human disease (1). In humans and NOD mice, T1D is a complex disease, resulting from interaction between multiple susceptibility genes and poorly defined environmental modifiers. The greatest genetic risk factor for T1D in humans and NOD mice is MHC class II (MHC II) haplotypes, in which the two species share remarkably similar amino acid residues that influence peptide binding and Ag presentation to T cells. In addition to the MHC II, known causal variants include regulator of immune homeostasis IL-2 and IL-2R and inhibitors of T cell activation CTLA-4 and LYP (2). These disease-causing variants are common functional alleles involved in maintaining the balance between tolerance and immunity, suggesting that T1D develops due to dysregulation of the immune response.

Genome-wide association studies (GWAS) in humans have identified >40 single nucleotide polymorphisms (SNPs) associated with T1D susceptibility, including polymorphisms in the signal regulatory protein (SIRP) locus that encodes a family of activating and inhibitory receptors (3). Members of the paired receptor family have similar extracellular regions but different signaling potentials that may have evolved to maintain the balance between tolerance and immunity in response to evolutionary pressure such as pathogens (4, 5). The majority of T1D-associated human SNPs have small effect sizes in which each confers modest impact on the phenotype: to date, few causal genetic variations have been identified. Collectively, GWAS fail to fully explain the heritability of T1D likely because this approach did not capture the contribution of gene–gene and gene–environment interactions underlying the complex disease phenotype (6). As shown in this study, the NOD mouse model enables analysis of complex features of T1D heritability through enriched preclinical phenotypes, access to immune cell compartments, and germline manipulations to test the effects of both individual and combined genetic variations on disease.

Diabetes onset in NOD mice is preceded by islet inflammation (insulitis) by multiple leukocyte subsets. Insulitis begins with peri-islet accumulation of myeloid cells and T cells, which then invades the β-cell–rich interior (invasive insulitis), leading to eventual loss of insulin secretion. In contrast, the nonobese T1D-resistant (NOR) strain displays only peri-islet infiltration by APC that does not invade the islet interior or compromise β cell function (7). The NOR strain is 88% identical by descent to NOD, shares the high risk Mhc haplotype, but is protected from T1D by genomic inheritance from C57BLKS/J mice (8). Previously, we showed that progression from peri-islet to invasive insulitis in NOD mice was regulated by two Idd loci, Idd5 and Idd13 (7). In this study, we report high-resolution genomic, genetic, and functional analyses supporting Sirpa, encoding the signal inhibitory receptor protein (SIRPα) as the candidate gene underlying the complex disease phenotype (6). As shown in this study, the NOD mouse model enables analysis of complex features of T1D heritability through enriched preclinical phenotypes, access to immune cell compartments, and germline manipulations to test the effects of both individual and combined genetic variations on disease.
at Idd13.2, which interacts with the Idd5 locus to drive insulin progression and T1D.

Materials and Methods

Mice

All mice used in this study were maintained in specified pathogen-free conditions at The Hospital for Sick Children (Toronto, Ontario, Canada) and followed the guidelines for the institutional animal care committee.

Generation of Idd13 congenic mice

All NOD.NOR-Idd13 congenic mice were generated by microsatellite marker-directed selection of breeders (Supplemental Table I). NOD.NOR-Idd5, NOD.NOR-Idd13, and NOD.NOR-Idd5.Idd13 congenic mice were generated using similar strategies (Supplemental Table II). Microsatellite markers were developed using diNucleotide Tandem Repeat Repeat (http://www.chghelpdesk.ca/servers/services.php), and genotyping was performed as previously described (9).

Generation of Sirpa transgenic mice

Sirpα cDNA was generated by reverse transcription using RNA isolated from NOD and NOR macrophages (Mφ) and cloned into XbaI site in pCDNA3 vector (10). Founders were identified by PCR with four pairs of primers: 1) 5'-GACGGAATTCTGC-3' and 3') 5'-GTCTCTTCCTCTTGAGTCCC-3'. These pairs were crossed to NOD.Idd5, and pups were genotyped for Sirpα transgene and microsatellite markers (Supplemental Table II). To generate NOD.NOR-SirpaTg homo, hemizygous mice were intercrossed and screened by real-time PCR with primers 5'-CCTCACAATCATGGCCTGG-3' and 5'-TACCAGTCTTCGCTCT-3'. Sirpα transgene copy number was normalized to RAG1, detected with primers 5'-GCCGAATTCTGC-3' and 5'-GTCTCTTCCTCTTGAGTCCC-3'. Amplion was quantified by SYBR Green on an ABI/PRISM 7900 HT Cycler (Life Technologies).

Sequence analysis of Idd13.2

NOD- and C57BL/6-derived Chr.2 sequence from Next Generation Sequencing was downloaded from the Sanger database (ftp://ftp.sanger.ac.uk/pub/NODmouse/) and formatted as a BLAST database. B6-derived sequences for Idd13.2 were downloaded from the Ensembl Genome Browser and aligned with NOD sequences. The resulting alignment was queried for exonic SNPs.

Insulitis assessment

Pancreata were fixed in formalin, paraffin embedded, and 5-μm sections were prepared at 200-μm spacing. Pancreatic sections were stained with MAb 9.32 (Sigma-Aldrich) to visualize cell infiltration. Each islet was scored as: 0 indicates no infiltration, 1 for perivascular/periductal infiltration touching islet perimeters but not penetrating, 2 for penetration of up to 25% islet mass, 3 for penetration up to 75% islet mass, and 4 for <20% of islet mass remaining. The number of islets scored were NOD.IGE-Tg néy (975 islets; n = 30), NOD.NOR-SirpaTg (1048 islets; n = 28), NOD.NOR-Idd5 (776 islets; n = 28), NOD.NOR-SirpaTg.NOD-Idd5 (1713 islets; n = 26), NOR.IGE-Tg néy (1535 islets; n = 26), NOD.NOR-Idd5 (1151 islets; n = 43), and NOD.NOR-Idd5.Idd13 (1778 islets; n = 38).

Statistical analysis of insulitis scores

To increase the power to detect differences between mouse strains, the distribution of insulitis scores, rather than a single mean insulitis score, was examined for each mouse. We used a Poisson model in which the log count at each time point was modeled as a linear mean function of the number of islets scored for that mouse as well as indicators for whether the mouse carried the NOD Sirpα transgene and/or the NOD Idd5 locus. The pairwise analyses included one of these indicators (the other was fixed) and assessed the evidence of differences between strains by comparing the increase in the log-likelihood caused by inclusion of the indicator, to the asymptotic χ² distribution with four df. To assess the evidence for interaction, we compared all mice together with both indicators and also the product of the indicators. If this product was different from 0 for any count, there was evidence of an interaction between the transgenic Sirpα status and the NOD Idd5 congenic status. Assessment of the defect of the interaction term was done by comparing the increased in log-likelihood caused by its inclusion to the asymptotic χ² distribution with four df (11). All statistical test analyses were carried out using R 2.13.1 available at http://www.R-project.org/ (12).

Generation of mouse CD47-Ig domain–human IgG-Fc fusion protein and human CD47-Fc

The plasmid pAP369 containing mouse CD47 was a gift from Dr. Eric Brown (13). FreeStyle 293-F cells (Life Technologies) were transfected according to the manufacturer’s guidelines. The mouse CD47-IgV domain–human IgG-Fc fusion protein (mCD47-Fc) was purified on protein G Sepharose (Pierce) and conjugated to biotin for flow cytometry–based binding assay. Human CD47-Fc protein (hCD47-Fc) was previously described (14).

Flow cytometry

For analysis of Sirpα expression, cells were stained with Abs specific for CD19, CD4, CD8α, plasmacytoid dendritic cell (DC) Ag-1 (PDCA-1), CD11b, and CD11c from ebioscience; P84 and CD3 from BD Biosciences; and hCD47-biotin, detected with streptavidin–PE-Cy7 (Caltag Laboratories). Data were collected on the LSR II (BD Biosciences), and analysis was performed using FlowJo (Tree Star). The gating strategy used is detailed in the following sections. In all flow cytometry analysis, live cells were identified by propidium iodide exclusion. To exclude doublets, live single cells were further identified based on their forward and side scatter profile.

mCD47-Fc binding assay

For flow-based mCD47-Fc binding assay, cells were blocked with anti-CD16/32 followed by preclustering of cell-surface Sirpα with unconjugated P84. Cells were stained with Abs specific for CD11b, F4/80-FITC (Cedarlane Laboratories), and biotinylated mCD47-Fc detected with Streptavidin–PE (Molecular Probes). Cells were gated on propidium iodide–negative, forward versus side scatter, and CD11b+ F4/80+.

Plate-based mCD47-Fc binding assay was performed on bone marrow–derived Mφ, cultured as previously described (14). Cells were blocked with anti-CD16/32 (2.4-62) and stained with mCD47-Fc. Binding was detected with HRP-conjugated anti-human IgG (Bethyl Laboratories) and chromogenic peroxidase substrate tetramethylbenzidine (Mendell Scientific). Nonlinear regression analysis with one site binding equation was performed to generate a ligand-binding curve and calculate equilibrium constant Kₐ using GraphPad Prism (GraphPad).

T cell proliferation assay

Splenic DC from 8–10-wk-old mice were isolated using anti-CD11c magnetic beads (Miltenyi Biotec), and a purity of ≥85% was routinely obtained. CD4+ cells from spleen and peripheral lymph nodes (LN) of 8–15-wk-old NOD.BDC2.5 transgenic mice (stock #004460; The Jackson Laboratory) were isolated using the CD4+ T cell isolation kit II (Miltenyi Biotec) and labeled with CFSE (Molecular Probes). A total of 2.5 × 10⁵ DC and 5 × 10⁴ BDC2.5 T cells was cocultured with BDC2.5 1040-63 peptide (RTRPLWVRME; AnaSpec) and proliferation measured 3 d later.

Adoptive transfer

LN were harvested from 12-wk-old NOD mice. Total T cells were isolated using the Pan T cell isolation Kit II (Miltenyi Biotec), and a purity of >90% was routinely obtained. A total of 10 × 10⁶ T cells was delivered via i.v. injection into 4–5-wk-old NOD.SCID and NOD.NOR-Idd13.SCID recipients. Recipients were aged up to 21–27 wk posttransfer and diabetes assessed by urinary glucose test using Chemstrip from Roche. Pancreatic LN (PLN) of disease-free NOD.NOR-Idd13.SCID recipients at the end of the study were analyzed for presence of T cells by staining for Abs specific for CD3, CD4, and CD8. NOD.NOR-Idd13.SCID recipients that received T cells (+T cells) were compared with NOD.NOR-Idd13.SCID mice that did not receive any donor T cells (−T cells).

Results

Refined genomic mapping of the Idd13.2 locus

Idd13 was originally mapped to a 4-cM region of chromosome 2 in linkage analysis of TID in (NOD × NORT12)2 mice in which the NOR allele conferred TID resistance (15). To refine the Idd13 locus to a tractable size for candidate gene analyses, we generated a series of congenic strains by introgression of NOR-derived Idd13 onto the NOD background (Fig. 1A). We developed an algorithm to identify
informative, high-density, microsatellite markers across the Idd13 region (Supplemental Table I) (9). The NOD.NOR-D2Jyh442-D2Mit452 congenic line (NOD.NOR-Idd13; Fig. 1A) encompassed the entire chromosome 2 interval previously defined by linkage analysis (7) and provided robust diabetes protection compared with parental NOD mice (p < 10^{-4}; Fig. 1B). A prior study reported that two subregions, Idd13.1 and Idd13.2, contributed to diabetes risk and proposed β2m-microglobulin (β2m) as a candidate gene at Idd13.1 based on evidence that NOD.β2m−/− mice were T1D resistant (15). In our congenic series, the NOD.NOR-D2Jyh443-D2Gul482 strain (NOD.R1) was protected from T1D (p < 10^{-4}), whereas the NOD.NOR-D2Jyh443-D2Jyh1192 strain (NOD.R4) was not (p = 0.64; Fig. 1A, 1B). Because NOD.R1 and NOD.R4 share a NOR-derived centromeric segment of the locus, these data refined the NOR-derived, T1D-protective region to an interval between markers D2Jyh1192 and D2Gul482 (designated Idd13.2) and excluded β2m as a candidate gene of Idd13.2. To further refine the Idd13.2 region, we generated NOD.NOR-Ila-D2Gul482 (NOD.R7), which was resistant to T1D (p = 0.007; Fig. 1A, 1B). In contrast, NOD.NOR-D2Gul1849-D2Gul482 (NOD.R12) was T1D susceptible, thus restricting the Idd13.2 region to a 0.8-Mb interval. NOR-Idd13.2 did not fully recapitulate the protective effect conferred by full-length NOR-Idd13, suggesting the presence of additional subregions in Idd13 as previously described (15). This observation that the locus first detected by linkage analysis consisted of multiple subregions is not unique to Idd13 and was also previously reported for Idd4 and Idd5 (9, 16).

Molecular genetic analysis of Idd13.2 genes

Based upon the Ensembl database, this 0.8-Mb interval contained six genes that were prioritized based on genomic sequence variation, gene expression, and functional annotation (Table I). The NOR genome regions of nonidentity with NOD is derived from C57BLKS/J, a recombinant strain consisting of C57BL/6J (B6), DBA/2J, and other strains (17). We identified 2191 SNP markers across the Idd13 interval from NOD, NOR, B6, and DBA/2J mouse strains from http://cgd.jax.org/datasets/popgen/diversityarray/yang2011.shtml. Comparison of SNPs across the interval revealed that NOR-Idd13 is of B6 origin. We then performed comparative exonic sequence analysis in B6 and NOD for all Idd13.2 genes. The genome sequence of 17 inbred strains, including NOD, has recently become available and was used as a source of NOD-derived Idd13.2 sequence in which the average depth of coverage was >25. No coding variations were identified in Il1b and Stk35 between NOD and B6. Synonymous-coding SNPs between strains were found in the F830045P16Rik and Il1a (Table I). A nonsynonymous SNP was observed in Pdyn, but this gene was given lower priority because neither its patterns of expression nor known functions are associated with hematopoietic or pancreatic cells (Table I).

We had previously identified the innate immune inhibitory receptor Sirpa as the genetic determinant of successful xenotransplantation of human blood stem cells in NOD.SCID mice (14). NOD and NOR-derived Sirpa displayed extensive polymorphism resulting in 18-aa variations in the extracellular N-terminal Ig variable (IgV)–like domain responsible for binding the ligand CD47 (18). Sirpαs signaling also control self-recognition functions of including clearance of red blood (19) and apoptotic cells by macrophages (20, 21) and homeostasis of DC (22) as well as their ability to trigger Th cell responses (23, 24). Given this sequence variation and its role in the immune response, we prioritized Sirpa as a candidate gene underlying the Idd13.2 locus.

**FIGURE 1.** Refined genomic mapping of Idd13.2 locus in NOD.NOR-Idd13 congenic strains. (A) Schematic representation of chromosome 2 in NOD.NOR-Idd13 congenic lines. Congenic mice were defined by genotyping novel and publicly available microsatellite markers (Supplemental Table I). Chromosomal intervals are depicted as NOD- (white) and NOR-derived (black) regions. Boundaries for each congenic interval are depicted in shaded gray. Susceptibility (S) or resistance (R) to T1D, relative to parental strain NOD, was determined based on survival curve comparisons shown in (B). Candidate genes within the refined Idd13.2 locus are shown. (B) TID incidence curves of NOD parental and NOD.NOR-Idd13 congenic lines. All mice were aged to a minimum of 240 d. Survival curve for each congenic line was compared with NOD using log-rank statistical test. After Bonferroni correction, p value ≤0.01 was considered significant.
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<td>Il1a</td>
<td>IL-1α</td>
<td>Activated monocytes and macrophages</td>
<td>Proinflammatory cytokine</td>
<td>Two synonymous SNPs</td>
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<td></td>
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<td>Promote recruitment of immune cells to inflammation sites</td>
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<td>Induces production of inducible NO synthase and cyclooxygenase 2</td>
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<td>Released from macrophages following activation via the TLR and activation of caspase 1 by nucleotide-binding oligomerization domain-like receptors</td>
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<td>ENSMUSG00000027398</td>
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<td>Pro-IL-1β is inactive and converted to mature form by protease cleavage</td>
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<td>RIKEN cDNA F830045P16 gene</td>
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<td>One synonymous SNP</td>
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<td></td>
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<td>Predicted to contain three IgC domains and interact with DAP12</td>
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<td>ENSMUSG00000037902</td>
<td>Sirpa</td>
<td>Signal regulatory protein α</td>
<td>Myeloid cells, neurons, and endothelial cells</td>
<td>Member of SIRP family of paired receptors</td>
<td>20 aa polymorphisms: 18 in IgV domain and 2 in IgC domain</td>
<td>(14)</td>
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<td>Contain ITIM domains allowing for association with cytoplasmic SHP-1 and SHP-2 phosphatase</td>
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<td>Attenuates various immune functions such as FcR-mediated phagocytosis</td>
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<td>Critical genetic determinant of HSC xenotransplantation in NOD.Scid</td>
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<td>ENSMUSG00000027400</td>
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<td>Brain</td>
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<td>R141Q</td>
<td>(48)</td>
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The Ensembl database was queried to identify candidate genes located between Il1a and microsatellite marker D2NGul1849. The NOD genome has been sequenced at high coverage and the data are publicly available. The NOR interval corresponding to Idd13 was determined to be of B6 origin by high-density SNP analysis of NOD, NOR, B6 and DBA/2J genomes across the Idd13 interval. Exonic sequence analysis for candidate genes in Idd13 compared NOD-derived sequences to B6 (NCBI37).

SHP: Src homology region 2 domain-containing phosphatase.
Sirpa alleles confer differential binding to mouse CD47

We previously showed that in contrast to NOR and B6, the NOD variant of Sirpα IgV domain polymorphisms conferred binding to hCD47 (14). Therefore, we questioned whether NOD and NOR Sirpα would display differential binding to mouse CD47, which is not polymorphic between these strains. We generated a mCD47-Fc for flow cytometric and chemiluminescent cell-based binding assays. Sirpα expression was highly expressed on myeloid cells including Mφ, neutrophils, and DC (25). Mφ isolated from NOD and NOR Nod-13 congenic mice (Fig. 1) were stained with either mCD47-Fc or control human IgG-Fc and assessed by flow cytometry. NOD and NOD.Nod-13 Mφ displayed similar surface expression of Sirpα based upon staining with anti-mouse Sirpα Ab (Mab P84; Fig. 2A); however, NOD Mφ displayed greater binding to mCD47-Fc at all concentrations tested compared with NOD.Nod-13 congenic Mφ (Fig. 2B). To ensure that the observed differential binding of mCD47-Fc was not influenced by other NOD background genes, we also compared B6 and B6.Nod-13 congenic Mφ and observed a similar dependence of mCD47-Fc binding on Nod-13 genotype (Fig. 2C, 2D).

mCD47-Fc binding to Mφ was also examined with a plate-based assay using the conversion of chemiluminescent substrates by HRP-conjugated anti-human IgG Ab. Mφ from NOD and NOD.R7 were incubated with a range of mCD47-Fc concentrations to calculate a ligand-binding curve (Fig. 2E). Under these conditions, the dissociation constant (Kd) of NOD Sirpα expressed on Mφ and mCD47-Fc was approximately three times lower than for NOD Sirpα (171 ± 12 versus 460 ± 69 nM; Fig. 2E). Taken together, these data show that compared with the NOD protein, NOD variant of Sirpα displayed a greater affinity for mouse CD47, suggesting a potential functional impact of these sequence variations.

Sirpa variation on DC impacts T cell proliferation and transfer of diabetes

SIRPα−CD47 interactions have been suggested to enhance T cell activation in response to APC (24, 26). CD47 is a broadly expressed protein that is often tightly associated with integrins (27). CD47 cell-surface expression on T cells is regulated during activation (28, 29) and delivers a positive signal during encounters with cognate Ag (30, 31). We asked whether the observed differential binding of NOD versus NOR SIRPα to mCD47 impacted T cell–APC interaction and T cell activation and proliferation. To test islet Ag-specific proliferation of T cells, we used the NOD.BDC2.5-Tcr (BDC2.5) TCR-transgenic mice, which expresses the TCR from a diabetogenic CD4+ T cell clone specific for Chromogranin A, a protein found in pancreatic β-cells and neuroendocrine tissues (32). CFSE-labeled CD4+ BDC2.5 T cells were cocultured with CD11c+ DC from NOD or NOD.R7 mice (Fig. 3A). A titration of the BDC2.5 mimotope peptide (1000–37 ng/ml) was added to the T cell–DC culture (33) and T cell proliferation measured as CFSE dilution by flow cytometry. Although DC from NOD and NOD.R7 mice had similar levels of costimulatory molecules such as CD86 and MHC II (data not shown), a higher fraction of proliferating BDC2.5 T cells (CFSElo) cells was observed in cultures with NOD-derived compared with NOD.R7-derived DC, particularly at low peptide concentrations (Fig. 3B). These data were consistent with prior evidence that CD47 conveys costimulatory effects under conditions of suboptimal TCR ligation (31).

Because BDC2.5 T cells cocultured with NOR compared with DC resulted in greater proliferation, we asked whether this difference affected T cell pathogenicity in vivo. Previous work established that NOD-derived T cells can transfer T1D to immunodeficient NOD.SCID mice (34). To examine whether the genotype of recipient myeloid cells affected the ability of NOD T cells to transfer T1D, we generated NOD.NOR-Idd13.SCID mice homozygous for NOR-derived Sirpa (14). NOD splenic T cells were adoptively transferred into either NOD.SCID or NOD.NOR-Idd13.SCID recipients. After 150 d posttransfer, NOD.NOR-Idd13.SCID recipients of NOD T cells had significantly lower T1D incidence than NOD.SCID recipients (p = 0.0013; Fig. 3C). At the study end point, PLN of T1D-free NOD.NOR-Idd13.SCID recipients were analyzed for T cell markers and found to be successfully engrafted (data not shown). These results suggested that, compared with the NOR variant, expression of NOD Sirpα on myeloid cells enhanced T cell–mediated transfer of diabetes. Taken together, these data demonstrated that NOD Sirpα expressed on myeloid cells is associated with enhanced T cell proliferation and diabetogenicity.

Generation of NOR.NOD-SirpaTg mice

We previously showed by genetic linkage analysis that Idd13 acts in concert with Idda (chromosome 1) to regulate progression of peri-insulitis to invasive insulitis. We tested whether NOD Sirpa was an Idd13 gene responsible for driving insulitis progression using a gain of function approach. NOR.NOD-Idd13 congenic and NOR.NOD-SirpaTg transgenic mice were generated to examine the impact of the NOD-Sirpa allele on insulitis progression.

NOR.NOD-Idd13 congenic mice were generated from (NOD × NOR)F1 mice followed by backcrossing to NOR with marker-directed selection of breeders at each generation (Supplemental Fig. 1, Supplemental Table II). To produce Sirpa transgenic mice with a myeloid-specific expression emulating the normal expression of the gene, NOD-derived Sirpa cDNA was cloned downstream of hCD68 previously reported to drive myeloid-lineage specific expression in mice (10). Fluorescence in situ hybridization identified the genomic insertion site of the NOD Sirpa transgene on chromosome 10C1 (data not shown), where there are no known Idd loci.

To characterize transgene expression, we compared the percentages of splenic lymphocytes (CD3+/CD19+), Mφ (CD11b+CD11c+), conventional DC (CD11c+PDCA-1−), and plasmacytoid DC (CD11c+PDCA-1+) in transgenic (NOR.NOD-SirpaTg) and transgene-negative littermates (NOR-Tg neg; Fig. 4A). The conventional DC subset was further refined using CD11b and CD8α markers (Fig. 4A, bottom panel). The frequency of these leukocyte subsets was not altered by the presence of the transgene (Fig. 4A). We previously showed that hCD47-Fc fusion protein binds to the NOD but not to NOR Sirpα, so we used this reagent to distinguish expression of the NOD Sirpa transgene from endogenous NOR-derived Sirpα (Fig. 4B). Expression of both the NOR and NOR variants of Sirpα was detected with mAb P84 (Fig. 4C). The NOR Sirpa transgene was not expressed on T or B cells (Fig. 4B1), just as we observed for expression of endogenous Sirpα (Fig. 4C1). In contrast, the product of the NOR Sirpa transgene was detected on Mφ and all DC subsets (Fig. 4B2–6). Although NOR Sirpa transgene expression was robust in Mφ and all DC subsets, the total Sirpα expression levels in NOR.NOD-SirpaTg were mostly unaltered compared with NOR-Tg neg mice (Fig. 4C2–4). Only a subset of the CD11b+CD8α+ DC displayed a higher level of total Sirpα expression in the NOR.NOD-SirpaTg compared with NOR-Tg neg mice (Fig. 4C5). Sirpα expression on bone marrow Mφ and neutrophils did not differ between NOR.NOD-SirpaTg and NOR-Tg neg strains (data not shown). Thus, with the exception of greater expression in CD11b+CD8α+ DC, the NOD Sirpa transgene was expressed at similar levels to the endogenous locus and did not affect the frequency of myeloid cell populations.
FIGURE 2. Sirpa alleles confer differential binding to mCD47. (A) NOD and NOD.NOR-Idd13 MΦ (CD11b+F4/80+) expressed similar levels of SIRPa. Peritoneal MΦ from NOD (gray) and NOD.NOR-Idd13 (black) mice were stained with anti-SIRPa and assessed by flow cytometry. (B) MΦ from NOD mice displayed greater binding to mCD47 than those from NOD.NOR-Idd13 mice. Peritoneal MΦ were stained with soluble mCD47-Fc fusion protein (solid black line) or isotype control human IgG-Fc reagent (gray shaded) and analyzed by flow cytometry. (C) B6 (black) and B6.NOD-Idd13 (gray) MΦ expressed similar levels of SIRPa. Experiment performed as described in (A). (D) B6.NOD-Idd13 MΦ exhibited enhanced binding to mCD47 compared with B6 MΦ. Experiment was performed as described in (C). (E) Chemiluminescence assay confirmed that NOD MΦ displayed enhanced mCD47 binding compared with NOD.R7. Bone marrow–derived MΦ were stained with a range of mCD47-Fc concentrations. Duplicates were performed for each sample, and data are representative of three independent experiments.
Sirpa variation impacts T cells and transfer of diabetes. (A) Ag-specific proliferation of BDC2.5 TCR transgenic T cells in response to peptide-pulsed DC from NOD (black) and NOD.R7 (gray) mice. T cell proliferation was measured by CFSE dilution and flow cytometry. Cells were gated on Thy1.2^CD4^ markers, and CFSE^hi^ (dividing) or CFSE^lo^ (nondividing) cells were identified within this gate. Data are representative of four independent experiments with one to two biological replicates in each experiment. (B) Enhanced fraction of proliferating BDC2.5 T cells in response to NOD-derived DC. The percentages of CFSE^hi^ cells identified in (A) for each peptide concentration are plotted. Data are pooled from four independent experiments (n = 9), and values are mean ± SEM. The Mann–Whitney U test resulted in a statistically significant difference between NOD and NOD.R7 DC (111 ng/ml of peptide; p = 0.04). (C) Adoptive transfers of NOD T cells into NOD.SCID (n = 13) and NOD.NOR-Idd13.SCID (n = 13) recipients. Survival curves of recipients aged to 150 d are shown. Log-rank statistics and χ² analysis were applied, and p = 0.0011 and p = 0.0013, respectively, were obtained.

**FIGURE 3.** Sirpa variation impacts T cells and transfer of diabetes. (A) Ag-specific proliferation of BDC2.5 TCR transgenic T cells in response to peptide-pulsed DC from NOD (black) and NOD.R7 (gray) mice. T cell proliferation was measured by CFSE dilution and flow cytometry. Cells were gated on Thy1.2^CD4^ markers, and CFSE^lo^ (dividing) or CFSE^hi^ (nondividing) cells were identified within this gate. Data are representative of four independent experiments with one to two biological replicates in each experiment. (B) Enhanced fraction of proliferating BDC2.5 T cells in response to NOD-derived DC. The percentages of CFSE^hi^ cells identified in (A) for each peptide concentration are plotted. Data are pooled from four independent experiments (n = 9), and values are mean ± SEM. The Mann–Whitney U test resulted in a statistically significant difference between NOD and NOD.R7 DC (111 ng/ml of peptide; p = 0.04). (C) Adoptive transfers of NOD T cells into NOD.SCID (n = 13) and NOD.NOR-Idd13.SCID (n = 13) recipients. Survival curves of recipients aged to 150 d are shown. Log-rank statistics and χ² analysis were applied, and p = 0.0011 and p = 0.0013, respectively, were obtained.

**NOD Sirpa transgene cooperates with NOD Idd5 to potentiate insulitis progression**

Given our previous report that *Idd13* and *Idd5* loci act together to regulate insulitis progression, we examined the impact of the NOD *Sirpa* transgene to the NOD-derived *Idd13* (NOR.NOD-*Idd13*) congenic interval on this phenotype. NOR.NOD-*Idd5* congenic mice were generated by microsatellite marker-directed breeding. The *Idd5* locus includes multiple subloci (Supplemental Fig. 1, Supplemental Table II): *Idd5.1* (*Ctla4*), *Idd5.2* (*Slc11a1* or *Nramp1*), and *Idd5.4* (16). NOD and NOR are identical by descent at *Idd5.1* excluding a contribution from *Ctla4*. Our NOR.NOD-*Idd5* congenic strain included NOD-derived *Idd5.2* and most of *Idd5.4* (Supplemental Fig. 1). NOR.NOD-*SirpaTg* mice were bred to NOR.NOD-*Idd5* congenic mice to generate NOR.NOD-*SirpaTg.NOD-*Idd5* animals. In addition, NOR.NOD-*Idd5* and NOR.NOD-*Idd13* congenic strains were intercrossed to produce NOR.NOD-*Idd5.Idd13* double-congenic strain (Supplemental Fig. 1, Supplemental Table II). Cohorts of each strain were aged to 120–140 d, and ≥30 islets in each pancreas were assessed for insulitis severity using a well-established scoring system (7), and a statistical analysis was used to evaluate the distribution of islet scores between samples (Fig. 5). Compared to transgene-negative littersmates, the NOD *Sirpa* transgene alone did not alter insulitis severity on the NOR background (p = 0.37; Fig. 5A, 5B). In contrast, the NOD-*Idd5* congenic interval enhanced insulitis progression compared with parental NOR mice (p = 5 × 10⁻⁶; Fig. 5A, 5B). When combined with the NOD *Idd5* congenic interval alone, addition of the NOD *Sirpa* transgene further enhanced insulitis severity compared with the transgene-negative littermate (NOR-*Tgneg.NOD-*Idd5*) controls (p = 5 × 10⁻⁶; Fig. 5A, 5B). A test for genetic interaction between the NOD *Sirpa* transgene and *Idd5* congenic interval confirmed a genetic interaction between these genes (p = 0.0085; Fig. 5C). Parallel results were obtained for the insulitis profile of NOR.NOD-*Idd5* compared with the NOR.NOD-*Idd5.Idd13* double-congenic strain (p < 1 × 10⁻¹⁰; Fig. 5D, 5E). At this point, too, we observed genetic interaction between
the Idd13 and Idd5 loci ($p = 2 \times 10^{-9}$; Fig. 5F), recapitulating the observation for NOR.NOD-SirpaTg.NOD-Idd5 insulitis severity. Thus, like the Idd13 congenic interval, the NOD Sirpa transgene displayed strong interaction with the Idd5 locus, supporting Sirpa as the gene responsible for insulitis progression at the Idd13.2 locus.

The NOD Sirpa transgene effect was evident in the microanatomy of pancreatic sections from NOR.NOD-SirpaTg.NOD-Idd5 compared with transgene-negative littermates (Fig. 5G, 5H). Although NOR-Tgneg.NOD-Idd5 islets displayed mild peri-insulitis with some invasive insulitis, NOR.SirpaTg.NOD-Idd5 mice displayed extensive islet infiltration (score $\geq 2$) accompanied by periductal and perivascular inflammation. Strikingly, we observed progression to T1D onset in 50% of NOR.NOD-SirpaTg.NOD-Idd5 that aged to 270 d (Fig. 5I). Although the T1D outcome in the small cohort could not reach statistical significance, these observations supported the potent interaction between the NOD Sirpa transgene and NOD Idd5 in driving $\beta$-cell–destructive insulitis.

Taken together, these results supported Sirpa as a causal gene at the Idd13.2 locus by demonstrating that it collaborates with NOD Idd5 to drive insulitis progression, a key step in diabetes pathogenesis.

Diabetes kinetics is altered by Sirpa gene dosage

Although CD47 is not polymorphic between NOD and NOR, other studies show that CD47 expression varies during T cell activation and differentiation. These observations suggested that in addition to the qualitative, genotype-dependent difference in SIRPaα–CD47.
binding, quantitative changes in SIRPα expression may also impact insulitis and T1D. To examine this quantitative mechanism, we constructed a NOR Sirpa transgene and produced NOD.NOR-SirpaTg mice by microinjection of NOD eggs. The NOR Sirpa transgene inserted at chromosome 1A1 (data not shown) was determined by fluorescence in situ hybridization, where there are no known Idd loci. We reasoned that NOD.NOR-SirpaTg mice would display effects of increased SIRPα expression level conferred by the NOR Sirpa transgene on T1D phenotypes. Cohorts of transgene-hemizygous (NOD.NOR-SirpaTg hemi) and homozygous (NOD.NOR-SirpaTg homo) mice were generated to evaluate the impact of Sirpa gene dosage. Hemizygous and homozygous NOD.NOR-SirpaTg genotypes were determined by quantitative PCR amplification (Fig. 6A), validating a Sirpa allele series from parental NOD to NOD.NOR-SirpaTg hemi and finally NOD.NOR-SirpaTg homo mice. T1D incidence was characterized in cohorts of each of these strains. The diabetes incidence in NOD.NOR-SirpaTg hemi mice was indistinguishable from parental NOD mice (Fig. 6B). In contrast, doubling the NOR-Sirpa transgene dosage (NOD.NOR-SirpaTg homo) accelerated diabetes onset and increased T1D incidence (to ~100%) compared with either parental NOD or NOD.NOR-SirpaTg hemi mice (p < 0.001; Fig. 6B). To evaluate the effect of NOR-Sirpa transgene copy number on SIRPα expression, splenocytes from NOD, NOD.NOR-SirpaTg hemi, and NOD.NOR-SirpaTg homo mice were analyzed by flow cytometry. Using the previously described gating strategy (Fig. 4A), increased SIRPα expression was observed on CD11b+CD8α+ DC population (Fig. 6C) with a mean fluorescence intensity ∼2-fold greater in NOD.NOR-SirpaTg homo compared with NOD.NOR-SirpaTg hemi and 4-fold greater compared with parental NOD cells (Fig. 6D). Thus, greater expression of NOR SIRPα increased T1D incidence on the NOD strain background providing independent genetic gain of function evidence that Sirpa is the T1D candidate gene at the Idd13.2 locus. Taken together, data from NOD and NOR Sirpa transgenic mice suggested that the impact of SIRPα–CD47 signaling on islet inflammation and diabetes was modulated by both sequence polymorphisms and the level of SIRPα expression.

Discussion

We used high-resolution genetic mapping and genomic sequence analysis, together with functional analyses of congenic and transgenic

FIGURE 5. NOD Sirpa transgene cooperates with NOD Idd5 to potentiate invasive insulitis. (A) Distribution of insulitis scores from cohorts of age-matched mice: NOR.NOD-SirpaTg, NOR-Tg neg, NOD.NOR-SirpaTg.NOD-Idd5, and NOR-Tg neg.NOD-Idd5. Each islet was given a leukocyte infiltration score of 0–4 (described in Materials and Methods). A total of n = 17–28 genotypes was scored. (B) Pairwise comparisons of insulitis scores shown in (A) based on a Poisson log-linear model for the distribution of scores. Asymptotic χ² likelihood ratio tests were used to generate a p value for each pairwise comparison. (C) Statistical interaction test between NOD Idd5 and NOD Sirpa transgene for effects on the distribution of insulitis scores using an asymptotic χ² likelihood ratio test. (D) Distribution of insulitis scores for NOR, NOR.NOD-Idd13, NOR.NOD-Idd5, and double-congenic NOR.NOD-Idd5, Idd13 mice. A total of n = 26–53 genotypes was scored. (E and F) Statistical tests described in (B) and (C) were applied to insulitis scores in (D). (G) Representative pancreatic sections from NOR.Tg neg.NOD-Idd5 (∼10 original magnification). (H) Representative pancreatic sections from NOR.NOR-SirpaTg.NOD-Idd5 (∼10 original magnification). (I) Diabetes incidence in NOR.NOR-SirpaTg.NOD-Idd5 and NOD.NOR-Idd5.Idd13 aged up to 270 d. Parental strains NOD and NOR were included as comparison. pd, periductal; pv, perivascular.
mice to identify Sirpa as a causal gene at Idd13.2 that regulates insulinitis progression and diabetes susceptibility in the NOD model. NOR-derived Idd13, previously shown to confer T1D protection to NOD mice, included Idd13.1 and Idd13.2 subregions (15). In this study, we mapped Idd13.2 to a 0.8-Mb interval containing six genes for which priority was defined by genomic sequence variation, gene expression pattern, and functional annotation. SIRPα displayed 18-aa variations in the IgV domain between NOD and NOR strains. These coding variations exerted functional effects on binding to the ligand CD47 and on the T cell response to an islet Ag. Transgenic gain-of-function studies on both the NOR and NOD strain backgrounds demonstrated that this gene impacted insulinitis progression and diabetes, further supporting Sirpa as the candidate gene at Idd13.2.

Genetic studies in humans and in the BioBreeding rat model also implicate a role for SIRP signaling in diabetes pathogenesis. Recent linkage analysis in the BioBreeding rat defined an interval termed Iddm27 that controls T1D susceptibility and islet integrity and is orthologous to mouse Idd13 region including SIRP genes (35). The T1D Genetics Consortium identified significant T1D association with an SNP in human SIRPα (p = 1.027) encoding an SIRP family receptor that also binds CD47 (3). Association with T1D was observed between imputed genotypes at an SNP in SIRPG intron 5 that displays linkage disequilibrium with SNP in SIRPG introns 4 and 5 and with a missense variant of unknown functional significance in SIRPG exon 4. Large duplicon and deletion alleles around neighboring SIRPB made this gene refractory to genotyping with GWAS platforms. Thus, these human T1D-association signals may reflect variants in SIRPB, SIRPG, or both genes. The qualitative and quantitative Sirpa-dependent effects we report in this study provide a framework to identify putative causal variations underlying T1D association with the human SIRP locus.

Previous studies suggested that β2m was a candidate gene for T1D risk at Idd13.1 based on evidence that NOD.β2mnull mice were T1D resistant (36). Given the critical role of MHC class I–restricted CD8+ T cells in T1D immunopathogenesis, disease protection in β2mnull NOD mice was not surprising. In subsequent

**FIGURE 6.** Sirpa gene dosage determines T1D incidence in NOD.NOR-SirpaTg mice. (A) Determination of the NOR Sirpa transgene copy number in NOD.NOR-SirpaTg hemizygous (hemi) and NOD.NOR-SirpaTg homozygous (homo). Genomic DNA from NOD, NOD.NOR-SirpaTg hemi, and NOD.NOR-SirpaTg homo were analyzed for transgene copy number by real-time PCR. The NOR Sirpa transgene copy number is displayed normalized to a single-copy gene Rag1. Data are representative of three independent experiments. (B) T1D incidence life tables for cohorts of NOD parental, NOD.NOR-SirpaTg hemi, and NOD.NOR-SirpaTg homo transgenic mice. A log-rank test for diabetes incidence was applied. (C) SIRPα cell-surface expression on Mφ and DC subsets from PLN of NOD parental (green), NOD.NOR-SirpaTg hemi (blue), and NOD.NOR-SirpaTg homo (red) mice. Mφ and DC subsets were identified using the gating strategy presented in Fig. 4A. Fluorescence-minus-one staining (black) contained all fluorescently labeled markers except P84. (D) Increased SIRPα cell-surface expression on the CD11b+CD8α+ population from cohorts of NOD, NOD.NOR-SirpaTg hemi, and NOD.NOR-SirpaTg homo mice. Median fluorescence intensity (MFI) of P84 staining on CD11b+CD8α+ DC subset defined in (C) is displayed. Results are pooled from two independent experiments, and values are mean ± SEM and Mann–Whitney U test was applied (*p < 0.01, **p < 0.001).
analyses, it was demonstrated that transgenic expression of NOD-β2m restored T1D incidence on the NOD-β2mnull background, but expression of a NOR-β2m transgene resulted in lower T1D incidence (37). In our hands, NOD mice congenic for a NOR-derived β2m-containing interval (NOD.R4; Fig. 1A), but not carrying NOR-derived alleles at Idd13.2, were diabetes susceptible, suggesting that β2m allelic variation alone was insufficient to protect the mice. A likely explanation for these disparate findings is that the NOD-β2mnull strain reported previously harbored non-NOD–derived genomic regions extending well beyond β2m. To address this possibility, we performed high-resolution genotyping of the Idd13 region of the NOD-β2mnull genome and found that the 129/SV-derived β2mnull gene was flanked by $\pm 1.96$ Mb of B6 and 129/SV-derived segments that included all the genes in Idd13.2 interval (Supplemental Table III). The likely origins of this region is from the 129/SV-derived embryonic stem cells used to generate the β2mnull mutation (38) and subsequent crosses to the B6 strain. Thus, the discrepancy between the T1D susceptibility of the NOD.R4 congenic strain reported in this study and the T1D resistance previously reported in NOD,β2mnull.NOR-β2m transgenic mice is that the protection conferred by NOR-β2m also reflected the protective 129/SV- and B6 derived-Idd13.2 locus. This predicted β2m-Idd13.2 “gene × gene” interaction highlights the complex genetic architecture of T1D.

The T1D phenotype reflects interactions between multiple susceptibility genes and environmental factors that collectively regulate the immune response. Of the human T1D genes in which plausible causal variations have been reported (e.g., INS and PTPN22), they are common, functional variants (2). Such functional variations are distinct from loss of function mutations observed in monogenic forms of autoimmunity such as the transcription factor autoimmune regulator responsible for the rare autoimmune polyendocrine syndrome type 1 (39). The odds ratios computed for most T1D-associated SNPs identified by GWAS are <2 (3). Variants with low genetic effect sizes likely confer their effects on the complex disease phenotype through gene–gene (and gene–environment) interactions. As we show in this study, the NOD model enables mechanistic analysis of gene–gene interactions under controlled environmental conditions that are far more difficult to demonstrate in genetically heterogeneous human subjects. In this study, we show that the effect of Sirpa is greatly potentiated by functional interaction with the Idd5 locus. Genetic interaction was observed between the NOD Idd13 locus and NOD Sirpsa transgene with NOD Idd5, supporting Sirpsa as the causal gene of Idd13.2 that drives insulitis progression together with a gene(s) at Idd5. Other examples of interlocus effects have been reported in the NOD model for Idd3+Idd10 and also Idd5.1/Idd5.2+Idd3 (40). In the context of the current study, the relevant regions of the Idd5 locus in this study include Idd5.2 (Sirpa1a1) and part of Idd5.4 for which a strong candidate gene is not yet defined (Supplemental Fig. 1). Further mapping efforts to identify the Idd5 sub loci interacting with Sirpa will provide a useful framework for identification and mechanistic analysis of causal variants in human T1D. Prioritizing T1D associated variants that act in convergent pathways may provide more effective therapeutic strategies.

SIRPsα–CD47 interaction is also required in other T cell–mediated autoimmune syndromes including collitis (23, 41) and experimental autoimmune encephalomyelitis (EAE) (24). Mutant mice lacking the SIRPsα cyttoplasmic signaling domain were resistant to myelin oligodendrocyte glycoprotein–induced EAE and displayed reduced DC stimulation of myelin oligodendrocyte glycoprotein–dependent T cell proliferation (24). Similarly, CD47−/− mice were also EAE resistant due to impaired T cell responses (42). These observations highlight role of SIRPsα–CD47 signaling in regulating DC function and T cell activation (43).

We report in this paper that NOD variant of SIRPsα displayed enhanced CD47 binding and was associated with greater islet Ag–specific T cell proliferation compared with the NOR protein. Moreover, NOD SIRPsα expressed on a recipient’s myeloid cells enabled greater diabeticogenic potential of adaptively transferred NOD T cells. Collectively, these results suggest that polymorphisms affecting SIRPsα–CD47 binding exerts control over spontaneous NOD diabetes through a T cell–mediated mechanism. Our study describes mechanisms through which Sirpsα impacts islet-autoimmunity and is one of multiple T1D-associated genes that alter the balance between lymphocyte activation and attenuation.

GWAS reveal that many common human variants associated with complex diseases reflect regulatory rather than coding variations (44). This study of Sirpsα in the NOD model provides an example of effects on autoimmunity conferred by coding polymorphisms impacting receptor–ligand binding and also by genetic variations that alter protein expression. Despite the lower level of CD47 binding by the NOR compared with NOD SIRPsα, an increase in NOR-Sirpsα transgene dosage accelerated diabetes kinetics and increased susceptibility, suggesting that SIRPsα–CD47 signaling outcomes are also subjected to quantitative influences. Identification of T1D-associated variants in immune receptors and functional analysis of their effects may provide targets to attenuate anti-Islet immunity and improve outcomes of β-cell replacement strategies in persons with diabetes.

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Disclosures

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