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Evidence That Cd101 Is an Autoimmune Diabetes Gene in Nonobese Diabetic Mice

Daniel B. Rainbow,* Carolyn Moule,* Heather I. Fraser,* Jan Clark,* Sarah K. Howlett,* Oliver Burren,* Mikkel Christensen,§ Val Moody,* Charles A. Steward,† Javid P. Mohammed,‡ Michael E. Fusakio,§ Emma L. Masteller,§* Erik B. Finger,§* J. P. Houchins,|| Dieter Naf,§ Frank Koentgen,§ William M. Ridgway,** John A. Todd,§ Jeffrey A. Bluestone,** Laurence B. Peterson,**† Jochen Mattner,‡† and Linda S. Wicker*

We have previously proposed that sequence variation of the CD101 gene between NOD and C57BL/6 mice accounts for the protection from type 1 diabetes (T1D) provided by the insulin-dependent diabetes susceptibility region 10 (Idd10), a <1 Mb region on mouse chromosome 3. In this study, we provide further support for the hypothesis that Cd101 is Idd10 using haplotype and expression analyses of novel Idd10 congenic strains coupled to the development of a Cd101 knockout mouse. Susceptibility to T1D was correlated with genotype-dependent CD101 expression on multiple cell subsets, including Foxp3+ regulatory CD4+ T cells, CD11c+ dendritic cells, and Gr1+ myeloid cells. The correlation of CD101 expression on immune cells from four independent Idd10 haplotypes with the development of T1D supports the identity of Cd101 as Idd10. Because Cd101 has been associated with regulatory T and Ag presentation cell functions, our results provide a further link between immune regulation and susceptibility to T1D. *The Journal of Immunology, 2011, 187: 000–000.

Although the biological functions of CD101 remain unclear, and there are no known ligands for CD101, this transmembrane molecule having seven Ig-like domains is expressed by multiple subsets of immune cells including Foxp3+ regulatory T cells (Tregs), effector CD4 and CD8 T cells, granulocytes, dendritic cells, and monocytes in humans and mice (2–6). There are multiple lines of evidence using human cells suggesting that Cd101 modulates T cell activation either directly or indirectly via dendritic cells that express CD101 (5–8). CD101 expression levels on mouse Tregs were demonstrated to be positively correlated with functional suppression (3).

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; BAC, bacterial artificial chromosome; Idd, insulin-dependent diabetes; KO, knockout; MFI, mean fluorescence intensity; NGS, Next Generation Sequence; SNP, single nucleotide polymorphism; T1D, type 1 diabetes; Treg, regulatory T cell.
Materials and Methods

Mice

All mice were housed under specific pathogen-free conditions, and the appropriate institutional review committee approved experimental procedures. NOD/MikTac (NOD) and C57BL/6Tac (B6) mice were purchased from Taconic Farms (Germantown, NY). The NOD.B6 Cd101 (N16) strain (Tacoline 3538) was developed from the NOD.B6 Id101 (N12) strain (Tacoline 1100) (9) by backcrossing to NOD and genotyping progeny using NOD/B6 polymorphic markers to isolate the Id101 congenic segment. NOD:A1 Id101 (N10) and NOD:CAST Id101 (N9) were developed by backcrossing A1/J and CAST/1EJ mice that were obtained from The Jackson Laboratory (Bar Harbor, ME) to the NOD background using polymorphic markers near and in the Id101 region to define recombination events.

Genotyping

DNA extraction for genotyping and genotyping methods were described previously (9). Primer3 (10) was used to design primers for PCR that were then synthesized by Sigma-Genosys (Haverhill, U.K.). Sequences of D3Nds and D3Mit microsatellite markers are available at http://www-genome.cimr.cam.ac.uk/todd/public_data/mouse/NDS/NDSMicrosTop.html and http://mouse.ensembl.org, respectively. All remaining primers and probes used in this study are available in Supplemental Table I.

Generation of a CD101 null B6 strain

A 10-kb targeting vector was designed to the B6 CD101 sequence with a 4.6-kb 5’ homology arm and a 2.9-kb 3’ homology arm. The CD101 targeting vector contains one loxP site immediately before the ATG start codon. This loxP site is preceded by a second ATG start codon in frame with the CD101 coding sequence; the targeted locus will encode the CD101 protein with a 15-aa tag. A second loxP site is inserted distal of the PGK-neo selectable marker cassette. This loxP site is followed by three stop codons (TAATAATAA). Using Cre recombinease, the sequence between the two loxP sites can be removed, resulting in the deletion of exon 1 of CD101, but leaving the 15-aa tag, which is now in frame with the three stop codons. The CD101 gene was targeted in B6-derived Bruce4 cells, chimeras produced and mated with Cre deleter mice, and mice having one CD101 KO allele backcrossed to B6Tac mice for seven generations. Because Bruce4 ES cells have non-B6 regions derived from the strain used to donate the Thy1 congenic region present in the B6 strain from which the Bruce4 ES cells were derived (11) and the Cre deleter mice also have non-B6 regions (12), a 1449 SNP marker panel across 19 autosomes and the X chromosome, averaging a genetic interval of 5 Mb (1449 marker panel; Ta Momics Farms), was used to verify that non-B6 regions were not detected in the backcrossed CD101 KO mice. Sequences of primers used to genotype the wild-type and targeted CD101 alleles are included in Supplemental Table I.

Generation of an anti-mouse CD101 mAbs

RNA was isolated from B6 splenocytes (Absolutely RNA; Stratagene, La Jolla, CA), and cDNA was transcribed using reverse-transcriptase (SuperScript II; Invitrogen, Carlsbad, CA) and CD101-specific primers. The full-length extrachromosomal domain of CD101 was cloned by PCR (Pfu polymerase; Stratagene) from the cDNA and fused in-frame with the hinge and Fc domains of mouse IgG2a (mutated to alter complement and Fc binding epitopes), and fidelity was assured by sequencing. This CD101-Ig construct was subcloned into the pEFIREs-P expression vector (13) and stably transfected in CHO-K1 cells (Life Technologies; Invitrogen, Carlsbad, CA). The Ig fusion protein was purified by protein A-Sepharose absorption and quantified by detection of mouse IgG2a via ELISA (BD Pharmingen, San Diego, CA).

Purified protein was used for immunization and generation of rat hybridomas. Purified CD101-Ig (50 μg) and 80 μg Escherichia coli DNA protein were mixed and conjugated to amino-polystyrene beads (SpheroTech, Lake Forest, IL). The bead–protein conjugate was combined with RIBI adjuvant and used for immunization of a 2-month-old female rat. The animal was injected five times over a 15-d period with an aliquot of conjugate containing 5 μg CD101-Ig. On day 18, lymphocytes were harvested, fused to the X63-Ag8.653 cell line, and distributed into 10 96-well plates. Seven days later, wells were screened in a direct ELISA for reactivity with CD101-Ig and for lack of reactivity with an irrelevant Fc-fusion protein. Five anti-CD101 mAbs were developed, and one (clone 307707), a rat IgG2a, is available from R&D Systems (Minneapolis, MN). We have verified that the avidity of clone 307707 is identical for the B6 and NOD allotypes by observing that saturation of CD101 staining was achieved with the same concentration of clone 307707 and that a 30-fold titration of the reagent produced an identical stepwise reduction of staining on both allotypes. These results indicate that clone 307707 recognizes a monomorphic epitope on the CD101 protein, although which of the seven Ig domains present in the CD101 molecule is recognized by clone 307707 is unknown. Thus, the allele-specific expression differences described in this paper are not explained by differential binding of the mAb clone 307707 to the different allotypes.

Variant identification in Idd10

A portion of the Idd10 region has been resequenced using NOD bacterial artificial chromosome (BAC) clones selected and sequenced at the Wellcome Trust Sanger Institute and deposited into their NOD database (http://mouse.ensembl.org). NOD/MrkTac (NOD) and C57BL/6NTac (B6) mice were purchased from Taconic Farms (Germantown, NY). The NOD.B6 Idd10 Idd10(N16) strain (Tacoline 3538) was developed from the NOD.B6 Id101 (N12) strain (Tacoline 1100) (9) by backcrossing to NOD and genotyping progeny using NOD/B6 polymorphic markers to isolate the Id101 congenic segment. NOD:A1 Idd101 (N10) and NOD:CAST Idd101 (N9) were developed by backcrossing A1/J and CAST/1EJ mice that were obtained from The Jackson Laboratory (Bar Harbor, ME) to the NOD background using polymorphic markers near and in the Idd10 region to define recombination events.

Flow cytometry and intracellular cytokine staining

Single-cell suspensions were prepared from spleen, lymph nodes, thymus, and bone marrow. RBCs were not removed. Cell-surface expression of CD11c, B220, the CD45, and bone marrow. RBCs were not removed. Cell-surface expression of CD101 (clone 307707) was obtained from R&D Systems. Intracellular cytokine staining (IFN-γ, IL-2, IL-4, IL-5, IL-10, and tumor necrosis factor-α) was performed using standard techniques. Cells were stained with mAbs for 15 min at 4°C in the dark. After fixation, the cells were permeabilized using cytofix/cytoperm solution (BD Pharmingen, San Diego, CA). The Ig fusion protein was purified by protein A-Sepharose absorption and quantified by detection of mouse IgG2a via ELISA (BD Pharmingen, San Diego, CA). The log rank test (Prism4 software; GraphPad).
Although five pseudogenes and one noncoding RNA are notated in Ensembl in the Idd10 region, on inspection of the underlying data, evidence for these genes is inconclusive.

Cd101 was highlighted previously as an Idd10 candidate gene (1) because of the expression of its protein CD101 on multiple cell types in the immune system (2–5) and SNPs distinguishing the NOD and B6 Cd101 alleles that cause 10-aa substitutions in the CD101 protein, two of which are predicted to alter glycosylation (Supplemental Fig. 1).

Next Generation Sequence (NGS) for the entire genomes of 17 inbred strains has become available, including that of the NOD/ShiLtJ, A/J, and CAST/EiJ strains (http://www.sanger.ac.uk/resources/mouse/genomes/). In addition, NOD BAC-based sequence from selected regions of the NOD/MrkTac genome (http://www.sanger.ac.uk/Projects/M_musculus-NOD/), including a portion of the Idd10 region, is publicly available (NOD tile path in Supplemental Fig. 2). The variation determined by NGS (NOD/ShiLtJ) and the BAC-based methods (NOD/MrkTac) are essentially identical in this region (Supplemental Fig. 2), so we have made use of NGS to interrogate NOD/B6 variation through Idd10, which is displayed in the “NGS NOD SNPs” track in Fig. 1A.

Inspection of the B6/NOD variation present throughout the Idd10 region shows that only Man1a2 and Vtcn1 have areas of low SNP density when comparing the B6 and NOD haplotypes. Assessment of Idd10 sequence variation among inbred strains, originally by targeted hand-sequencing (data not shown), highlighted two Idd10 sequence from selected regions of the NOD/MrkTac genome (http://www.sanger.ac.uk/Projects/M_musculus-NOD/), including a portion of the Idd10 region, is publicly available (NOD tile path in Supplemental Fig. 2). The variation determined by NGS (NOD/ShiLtJ) and the BAC-based methods (NOD/MrkTac) are essentially identical in this region (Supplemental Fig. 2), so we have made use of NGS to interrogate NOD/B6 variation through Idd10, which is displayed in the “NGS NOD SNPs” track in Fig. 1A.
haplotypes, CAST and A/J, that could be potentially useful at defining disease-associated SNPs in the region. An SNP is considered to be disease associated when one allele at the SNP is associated with disease susceptibility and an alternate allele is associated with disease resistance. Although a single SNP can cause alteration in disease susceptibility, usually such SNPs are part of complex haplotypes in which many SNPs within a gene are coinherited. By determining the disease susceptibility phenotype

FIGURE 2. Idd10 congenic mouse strains. A. The introgressed regions present in the NOD.B6, NOD.CAST, and NOD.A/J Idd10 congenic strains are depicted together with the previously defined Idd10 interval and gene content. B. Zoomed-in region showing that the distal recombination points of the NOD.B6, NOD.CAST, and NOD.A/J Idd10 congenic strains and the proximal recombination point of R93 [a congenic strain that defined the distal boundary of Idd10 (1)], which all map within an ∼40 kb region. The distal and proximal boundaries of the NOD.A/J Idd10 and R93 strains, respectively, are defined by the same two markers, between which no additional polymorphisms are present; therefore, no region is indicated between the in and out markers for these two boundaries. The position of the markers is based on National Center for Biotechnology Information build 37.
of divergent haplotypes, the potential list of disease-causing SNPs can be reduced, as we did for the Idd3 and Idd5.1 regions (17, 18). SNPs between the B6 and CAST strains are shown on the “NGS CAST SNPs” track. The CAST strain from M. musculus castaneus is one of several inbred mouse strains isolated from the wild; such strains have the attribute of having a greater diversity of sequence variants than inbred laboratory strains (19–21). In the case of Idd3, the disease phenotype of an Idd3 haplotype isolated from the wild (from the CZECH strain) was instrumental in eliminating the hypothesized candidacy of B6/NOD amino acid variants in the IL-2 molecule that alter IL-2 glycosylation as being disease-causing (18). These results were later confirmed when the knockin of the B6 variants into the NOD wild; such strains have the attribute of having a greater diversity of sequence variants than inbred laboratory strains (19–21). In the case of Idd3, the disease phenotype of an Idd3 haplotype isolated from the wild (from the CZECH strain) was instrumental in eliminating the hypothesized candidacy of B6/NOD amino acid variants in the IL-2 molecule that alter IL-2 glycosylation as being disease-causing (18). These results were later confirmed when the knockin of the B6 variants into the NOD wild; such strains have the attribute of having a greater diversity of sequence variants than inbred laboratory strains (19–21). In the case of Idd3, the disease phenotype of an Idd3 haplotype isolated from the wild (from the CZECH strain) was instrumental in eliminating the hypothesized candidacy of B6/NOD amino acid variants in the IL-2 molecule that alter IL-2 glycosylation as being disease-causing (18). These results were later confirmed when the knockin of the B6 variants into the NOD wild; such strains have the attribute of having a greater diversity of sequence variants than inbred laboratory strains (19–21).

### Table I. Disease-associated SNPs in the coding regions of genes in the Idd10 region

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Based on NGS sequence from the B6, A/J, CAST, and NOD strains, all synonymous and nonsynonymous SNPs in the coding regions of the genes in the Idd10 region are detailed. An SNP is considered to be disease associated when the T1D-resistant B6 and A/J strains share an allele, and the NOD and CAST share a different allele.

Protection from T1D in NOD.B6 Idd10 and NOD.A/J Idd10 mice is equivalent, thereby supporting the hypothesis that Idd10 is the CD101 gene

NOD mice congenic for the Idd10 regions of B6, A/J, and CAST were generated (Fig. 2) to assess their ability to mediate protection from T1D (Fig. 3A). Of note, we found that the distal boundary of the A/J congenic strain occurs in the same 1705-bp region as the proximal boundary of the NOD.B6 R93 congenic strain (between the markers labeled Ptgfrn_Int1_SN52 and AL672281_7, Fig. 2B, which are both in the first intron of Ptgfrn) that, via truncation analysis, defined the distal boundary of Idd10 (23). Because we have identified an additional two independent congenic strains that
Cd101 provides equivalent protection from TID. The frequency of diabetes was determined in the indicated number (N) of females in each cohort. A, NOD, NOD.B6 Cd101, NOD.CAST Cd101, and NOD.AJ Cd101 congenic mice were bred using breeders homozygous for the NOD, B6, CAST, and AJ haplotypes, respectively. B, NOD and NOD.AJ Cd101 congenic mice were intercrossed and the resulting F1 progeny intercrossed to produce an F2 generation. Mice were genotyped at Cd101 following the development of diabetes or at the end of the 196-d observation period. C, NOD and NOD.CAST Cd101 congenic mice were used to generate an F2 cohort for assessing the development of diabetes as described in B.

recombine in this same 1705-bp region (data not shown), the first intron of Ptgfrn is likely to be a recombination hotspot (24). Because the Cd101 region was defined previously using congenic interval truncation analyses, this report is the first, to our knowledge, demonstrating the protection mediated by the isolated B6-derived Cd101 region (Fig. 3A). Consistent with the hypothesis that Cd101 is Cd101, the B6 and AJ Cd101 haplotypes, which are identical at Cd101 but not at other genes within Cd101, produced equivalent levels of protection from TID (Fig. 3A, 3B). In Fig. 3B, the AJ Cd101 haplotype was assessed using littermate progeny from heterozygous breeders to control for any residual AJ-derived gene regions remaining outside of the known congenic interval. Because the mice typed as NOD homozygous at Cd101 developed a NOD-like frequency (Fig. 3B, squares), this verifies the assumption that the protection observed in the NOD.AJ Cd101 congenic strain is due solely to the congenic interval. Although a trend to protection was observed, one dose of the protective AJ-derived Cd101 haplotype did not produce significant disease prevention, indicating that protection by Cd101 is recessive (Fig. 3B). In contrast to the potent disease protection mediated by the B6 and AJ haplotypes, the CAST Cd101 haplotype failed to provide significant protection from TID as compared with the NOD strain in one experiment (Fig. 3A, p = 0.08) and provided only a modest level of protection in another (Fig. 3C, p = 0.02). In a direct comparison, the CAST haplotype was less protective than both the AJ and B6 haplotypes (Fig. 3A, p = 0.0004 for both comparisons). In summary, the CAST haplotype at Idd10 provides a NOD-like susceptibility to T1D, whereas the AJ haplotype provides B6-like protection from disease.

Disease-associated SNPs in Cd101 and other genes in the Idd10 region

Based on the disease phenotypes determined from the TID frequency studies, we queried which SNPs held in common by the TID-protective B6 and AJ Cd101 haplotypes differed from those held in common by the susceptible CAST and NOD haplotypes (disease-associated SNPs). At least 445 disease-associated SNPs are found in the Idd10 region (Fig. 1A), the majority of which are most likely nonfunctional variants that are in linkage disequilibrium with functional ancestral polymorphisms (25). Of the 445 disease-associated SNPs, 16 occur in coding regions, and 9 of these 16 are nonsynonymous SNPs. There are disease-associated SNPs in Cd101 that confer nonconservative amino acid changes (H557D and N565D) and potentially alter N-linked glycosylation (N565D) (Supplemental Fig. 1, Table I). Two additional disease-associated SNPs in Cd101 confer conservative amino acid changes (V175A and V318A). Additional disease-associated SNPs conferring amino acid substitutions are present in two other Idd10 genes, Vtn1 and Ttf2 (Fig. 1, Table I). Disease-associated F263S in the Vtn1-encoded B7H4 protein occurs in an unstructured portion of the molecule, and T246P, T290A, E317G, and E438D are located within an unstructured portion of the Ttf2-encoded transcription factor. Disease-associated SNPs are located in the introns of all genes located in Idd10 except for Trim45 and could potentially cause allele-specific expression differences by altering splicing or transcription enhancer elements. In addition, 3’ and 5’ untranslated region disease-associated SNPs are present in most of the Idd10 region genes. Thus, although Cd101 is implicated as being disease-causing by the fact that the TID-resistant Cd101 haplotypes of the AJ and B6 strains are identical by descent only at the Cd101 gene within the Idd10 region, other genes in the region, with the exception of Trim45 and perhaps Man1a2, which has only two disease-associated SNPs in intron 1 (Fig. 1A), remain viable candidates because disease-associated SNPs are present in each of them. It is also important to note that even if Cd101 is Idd10, the causative SNP or SNPs are among 62 disease-associated SNPs in Cd101 (Fig. 1B). It is equally plausible that the causative SNP(s) could alter Cd101 function by amino acid replacement, by influencing Cd101’s expression, or both.

Development and initial characterization of a CD101 null mouse

To test further the possibility that the CD101 gene is Idd10, we have targeted the CD101 gene in the context of the B6 haplotype (Fig. 4A). Ultimately, we will test this Cd101 null allele for its influence on the frequency of TID following extensive backcrossing to the NOD background, a long-term experiment that is in progress. In this study, we have performed an initial charac-
terization of the B6 CD101 null strain, which has B6-like viability until at least 1 y of age (n = 30 males and 30 females); B6 CD101 null mice aged 1 y displayed no splenomegaly or lymphadenopathy (n = 20 males and 20 females). mAbs specific for CD101 were developed (see Materials and Methods) to characterize CD101 expression and confirm the lack of expression of the CD101 protein in B6 CD101 null mice (Fig. 4 B–E). The most prominent expression of CD101 in the tissues tested (bone marrow, spleen, lymph nodes, and thymus) is on Gr1+ cells in the bone marrow (Fig. 4B, upper left panel). Developing myeloid cells having the highest levels of Gr1 are those that coexpress CD101. As expected, expression of CD101 on bone marrow cells from CD101 null mice was not detected (Fig. 4B, upper middle panel); the minimal staining observed was equivalent to that obtained
using an isotype control for the directly conjugated anti-Cd101 mAb (Fig. 4B, upper right panel). We confirmed the CD101 expression reported recently (3) on a portion of CD4+ Foxp3+ Tregs (Fig. 4C, upper left panel) and CD44+ CD4+ T cells (Fig. 4C, lower left panel) and a small number of CD8+ T cells (data not shown). No expression of CD101 was observed on B or NK cells (Fig. 4D, 4E, respectively). In all cell types examined from CD101 null B6 mice (Fig. 4B–D, data not shown), CD101 expression was not detected, verifying that CD101 null mice do not express CD101 protein.

Genotype-dependent CD101 expression on Gr1+ bone marrow cells

Following the validation of the newly developed anti-Cd101 mAb, CD101 expression on cells types expressing CD101 was assessed on cells obtained from the bone marrow, spleen, lymph nodes, and thymus of NOD, NOD.B6 Idd10, NOD.AJ Idd10, and NOD.CAST Idd10 congenic mice to determine if genotype-determined CD101 expression could be correlated with disease susceptibility. The most obvious genotype-dependent CD101 expression observed was that among cells having the highest levels of CD101, which are Gr1+ cells in the bone marrow and spleen (Fig. 5, Supplemental Fig. 3). Within the Gr1+ cells in the bone marrow, CD101 was expressed on 38 and 34% of the Gr1+ bone marrow cells in the T1D-susceptible NOD and NOD.CAST Idd10 strains, respectively, whereas a higher percentage of cells were CD101 positive in the T1D-resistant NOD.B6 Idd10 and NOD.AJ Idd10 strains, 45 and 44%, respectively (Fig. 5A, 5B, Supplemental Fig. 3A). In addition, the mean fluorescence intensity (MFI) of the CD101-positive cells was ~50% higher for the T1D-resistant strains as compared with the susceptible strains (Fig. 5A, 5C). A further correlation that did not involve CD101 expression was observed: there were higher percentages of Gr1+ cells in the bone marrow cells of the T1D-resistant NOD.B6 Idd10 and NOD.AJ Idd10 strains, 58 and 59%, respectively, as compared with NOD and NOD.CAST Idd10 bone marrow cells, which were 46% were Gr1+ in both strains (Fig. 5A, 5D). No difference was detected in the absolute number of cells recovered from the bone marrow of the congenic strains, suggesting that the allelic status of Idd10 influences the likelihood of cells to become Gr1+ (data not shown).

Reduction of Gr1+ cells in the bone marrow of CD101 KO mice

Because a correlation exists in the NOD Idd10 congenic mice between protective haplotypes and higher CD101 expression on Gr1+ cells as well as an increased percentage of Gr1+ cells in the bone marrow, we tested the hypothesis that the percentage of Gr1+ cells would be altered in B6 mice lacking CD101 expression (Fig. 5E–H). Strikingly, the percentage of Gr1+ cells in the bone marrow of CD101 null mice, 30%, was substantially <48% Gr1+ cells in the bone marrow of wild-type B6 mice (Fig. 5E, 5F). The levels of Gr1+ cells in B6 mice expressing one copy of the wild-type CD101 gene (B6 CD101+/- mice) were intermediate (40% Gr1+) to those on the bone marrow cells of B6 and B6 CD101 null mice. As expected, the expression of CD101 on Gr1+CD101+ bone marrow cells of CD101 hemizygous mice (MFI of 76) was found to be ~50% of that observed on this subset in wild-type B6 mice (MFI of 141) (Fig. 5E, 5G). No difference in the percentage of Gr1+ cells that are CD101+ was detected between the two strains (Fig. 5E, 5H).

Genotype-dependent CD101 expression in spleen cells

As well as the bone marrow, high levels of CD101 expression on Gr1+ cells was also observed in the spleen, with the CD101 MFI ~50% higher on Gr1+CD101+ spleen cells obtained from mice having a protective Cd101 genotype (Supplemental Fig. 3B–D). Among the Gr1+ cells in the spleen, although the percent of Gr1+ CD101+ did not differ between strains (Supplemental Fig. 3E), there were more Gr1+CD101+ mice cells in mice with a Cd101 susceptible genotype (Supplemental Fig. 3F). Among splenic lymphocytes, the Foxp3+ population of CD4 T cells has the most notable expression of CD101 (Figs. 4C, 6A). Expression of CD101 was increased in Foxp3+ cells from strains having a protected haplotype (NOD.B6 Idd10 and NOD.AJ Idd10 strains) as compared with this regulatory subset in NOD and NOD.CAST Idd10 mice (Fig. 6A–C). CD101+ CD4 T cells not expressing Foxp3, although a much more rare population than the Foxp3+ CD4 T cells, also had higher expression of the CD101 in cells from mice protected from T1D (Fig. 6A, 6D).

Finally, investigation of CD101 expression on CD11c+ cells in the spleen showed that expression was predominant only on CD11chi dendritic cells (Fig. 6E). Most of the CD101-expressing CD11c+ cells belong to the myeloid dendritic cell subset because all coexpress CD11b and CD4, but <5% and 20% express CD8 and B220, respectively (Supplemental Fig. 4). We were surprised to find that CD101 expression was higher on CD11c+ cells from mice having the susceptible NOD and CAST Idd10 haplotypes as compared with the resistant B6 and AJ haplotypes (Fig. 6F, 6G), a genotype-dependent pattern opposite to that observed on Gr1+ cells and CD4+ T cells (Figs. 5, 6A–D).

Discussion

Using four independent Idd10 haplotypes, NOD, CAST, B6, and AJ, we have demonstrated correlations between T1D susceptibility and: 1) CD101 protein sequence variants and variants that potentially influence the expression of Cd101; 2) decreased CD101 expression on Foxp3+ CD4 T cells; 3) increased expression of CD101 on CD4+ CD11b+ CD11c+ dendritic cells; and 4) decreased CD101 expression on Gr1+ myeloid cells. The bone marrow cells of the T1D-susceptible NOD and NOD.CAST Idd10 strains also had a lower percentage of Gr1+ cells as compared with the two congenic strains protected from T1D, NOD.B6 Idd10 and NOD.AJ Idd10. Our data are consistent with the hypothesis that genetic variation of Cd101 is responsible for mediating Idd10’s T1D-modifying effects.

CD101 expression differences between susceptible and protective Cd101 haplotypes are modest, which is consistent with most variant genes that contribute to complex traits such as autoimmune disease in which a 2-fold difference in expression or function of a disease-associated protein is considered large (18, 19, 26–28). It is also notable that the direction of the expression differences is not the same in all tissues, and in some cell types, no difference in CD101 expression has been observed. This suggests that regulation of CD101 expression is mediated at least in part by tissue-specific transcription factors or that an extrinsic effect of Cd101 haplotypes influences CD101 expression in other cell types in a complex manner. Detailed analyses of mixed bone marrow chimeras will be required to determine if CD101 expression differences are extrinsic or intrinsic to each cell type.

In comparing the Cd101 genomic sequence of the two T1D-susceptible haplotypes, NOD and CAST, with the two T1D-protective haplotypes, B6 and AJ, the number of disease-associated SNPs in Cd101 was reduced from 125 (B6/NOD variation only) to 62 disease-associated SNPs (Fig. 1A, 1B). Notably, only 4 of the 10 B6/NOD amino acid differences previously reported (1) were shown to be disease-associated in the haplotype analysis (Table 1); the highest density of disease-associated SNPs was found in introns 3, 4, and 5 (Fig. 1B). Although no disease-associated Cd101 SNPs alter nucleotides known to be critical for
splicing (consensus sequences at the donor, acceptor, and branch sites), it is possible that the observed disease-associated CD101 expression changes are caused by one or more SNPs altering splicing efficiency and therefore total protein production. Mechanisms that could account for a reduction in splicing efficiency include one or more causal SNPs altering intronic or exonic splicing enhancer or silencer motifs as well as SNPs changing the secondary structure of the pre-mRNA in a manner by which the ordered removal of introns is made less efficient. Alternatively, disease-associated SNPs could alter the efficiency of Cd101 pre-mRNA transcription by influencing regulatory regions in the 5' and 3' untranslated region as well as transcriptional enhancer

FIGURE 5. Cd101 genotype-dependent phenotypes in bone marrow cells. A, Representative dot plots showing the expression of CD101 and Gr1 on bone marrow cells from NOD, NOD.B6 Idd10, NOD.A/J Idd10, and NOD.CAST Idd10 mice; gating strategy is shown in Supplemental Fig. 3A. B, Data for the percentage of CD101+ cells within the Gr1+ population are compiled from three independent experiments using groups of female mice 11–13 wk of age. Each point represents an individual mouse (n = 17, 20, 13, and 15 for the NOD, NOD.B6 Idd10, NOD.A/J Idd10, and NOD.CAST Idd10 strains, respectively). C, The MFI of CD101+ Gr1+ cells for one representative experiment of three is shown. D, The percentage of Gr1+ cells in the nonerythroid light scatter gate was compiled from the same mice described in C. E, Representative examples of CD101 and Gr1 expression on bone marrow cells from wild-type B6 mice (CD101+/-), B6 mice having one copy of the null allele of Cd101 (CD101+/-), and CD101 null B6 mice (CD101-/-). F, The percentage of Gr1+ cells in the nonerythroid light scatter gate was compiled from three independent experiments using groups of female mice 11–14 wk of age. Each point represents an individual mouse; n = 10 for B6 (CD101+/-), n = 8 for mice expressing only one allele of Cd101 (CD101+/-), and n = 8 for CD101 null B6 mice (CD101-/-). G, The MFIs of CD101+ Gr1+ cells for CD101+/+ and CD101+/- mice described in F are shown. H, The percentages of CD101+ cells within the Gr1+ population for the CD101+/+ and CD101+/- mice described in F are shown. Comparisons between groups were performed using the Mann–Whitney nonparametric test. Error bars indicate the SD of the mean.
Cd101 genotype-dependent CD101 expression in spleen cells. A, Splenocytes were gated on cells expressing the β-chain of the TCR and CD4 and assessed for CD101 and Foxp3 expression. Representative examples of the indicated congenic strains are shown. B, Representative examples of CD101 expression on Foxp3+ cells are shown for a NOD female (red histogram) and a NOD.B6 Idd10 female (blue histogram). Genotype-dependent expression of CD101 on Foxp3+ CD4 T cells (C) and Foxp3− CD4 T cells (D) from two independent experiments are compiled; three mice from each strain were tested on one day (closed circles) and one mouse from each strain on a different day (closed squares). A third experiment showed the same genotype-dependent expression differences in both T cell subsets (not shown). Comparisons between groups were performed using the Mann–Whitney nonparametric test. E, Representative dot plots showing expression of CD101 and CD11c on spleen cells from NOD, NOD.B6 Idd10, NOD.A/J Idd10, and NOD.CAST Idd10 mice (top panel) and the isotype control (bottom panel). F, Data for the percentage of CD101+ cells within the CD11c+ population. Each point represents an individual mouse (n = 15, 9, 6, and 9 for the NOD, NOD.B6 Idd10, NOD.A/J Idd10, and NOD.CAST Idd10 strains, respectively). G, The MFI of CD101+ CD11c+ cells was compiled from the same mice described in F. Comparisons between groups were performed using the Mann–Whitney nonparametric test.
regions that are present in introns. The additional complexity of genotype-dependent cell type-specific expression seen in this and a related study (6) suggests the possibility that the binding efficiency of tissue-specific transcription factors that influence splicing/transcription are influenced by polymorphisms that define distinct Cd101 haplotypes. Although none have been described, Cd101 isoforms created by alternative splicing could be expressed in a genotype-dependent manner. However, the Cd101-specific mAb used in the current study could fail to recognize one or more of the putative isoforms if the epitope recognized is missing due to splicing out of the sequence encoding the epitope or there is a conformational change induced by the deletion of nearby amino acids. If this scenario is true, we could be underestimating the differential expression of Cd101. Although we favor the hypothesis that the disease-causing sequence variation in Cd101 is causing an alteration in gene expression, it is conceivable that the four disease-associated amino acid changes in Cd101 (Table I) are causal, and the functional difference mediated by changing one or more of these amino acids drives the observed expression differences.

Results from B6 Cd101 KO mice support the hypothesis that Cd101 expression changes in Idd10 congenic strains are directly functional: in both models, reduced Cd101 expression on myeloid cells in the bone marrow is correlated with a reduction in the percentage of Gr1+ bone marrow cells. Because in the Cd101 KO model a change of amino acids is not required for the phenotypic change in the Gr1+ bone marrow population, this argues that it is the Cd101 expression change in the Idd10 congenic mice that influences the generation of Gr1+ cells. The role of Cd101 in myeloid cell development could extend beyond Gr1+ cells; myeloid lineage cells in the bone marrow are progenitors of multiple cell subsets in the periphery, including dendritic cells, macrophages, myeloid suppressor cells, and granulocytes. The detailed effect of Cd101 genotype on various precursor populations within the bone marrow and peripheral cell subsets awaits further definition, as does the question of how Cd101 contributes to the generation of Gr1+ cells in the bone marrow. It is possible that Cd101 influences the efficiency of maturation of Gr1+ cells via signaling or through Cd101-mediated cell–cell interactions.

Although the data presented do not prove the hypothesis that Cd101 is the Idd10 gene influencing the development of T1D, the observed differential expression of Cd101 on cell types important in immune regulation invites speculation on potential causal pathways influenced by the Idd10 region. Cd11c+ APCs and Foxp3+ Tregs are known to be essential for eliciting (29) and regulating (18, 30), respectively, the autoimmune response to islets. It is important to consider that these same cell types may or may not contribute to Novosphingobium aromaticivorans-mediated liver autoimmunity described by Mohammed et al. (6). As opposed to T1D pathogenesis, there is a likely causal role for differentially expressed Cd101 on Gr1+ cells in a disease process that is partially dependent on the clearance rate of infectious bacteria.

As is the case for Cd101, expression of most immune molecules is not restricted to one subset of cells. In regard to increased expression of Cd101 on Foxp3+ CD4 T cells found in mice having the disease-protective genotypes, it is notable that the expression of Cd101 on Foxp3+ CD4 T cells is correlated with an increased ability to suppress effector T cells (3). Punkosdy et al. (31) recently demonstrated that Cd101 expression on Foxp3+ Tregs is greatly increased during a chronic viral infection, extending the observation that there is dynamic regulation of Cd101 on this T cell subset. Multiple studies have demonstrated that Foxp3+ CD4 T cells can suppress T1D in the NOD model (18, 30, 32). It is tempting to speculate that NOD.B6 Idd10 and NOD.A/I3 Idd10 Foxp3+ CD4 T cells have an increased ability to mediate suppression because they express higher levels of Cd101 on this important regulatory cell population as compared with NOD and NOD.CAST Idd10 mice (Fig. 6E–H).

Although it is likely, as argued above, that the genetic variation in Cd101 accounts for T1D susceptibility, we have also demonstrated that other genes within the ~800 kb Idd10 region have potentially causal disease-associated sequence differences; thus, the gene responsible for the phenotypic effects of Idd10 must still be considered to be unknown. No genes in the syntenic region on human chromosome 1p13–1p12 show evidence of association with T1D, including Cd101 itself (http://www.t1dbase.org). We are currently developing a NOD.B6 Idd10 mouse lacking Cd101 expression by backcrossing B6 Cd101−/− mice to the NOD parental strain. If variants of Cd101 do indeed modify T1D progression by altering Cd101 expression or function, a prediction would be that the frequency of T1D in NOD.B6 Idd10 Cd101−/− mice would differ from that of NOD.B6 Idd10 mice. Furthermore, if the reduction of Cd101 expression on cell types such as Tregs contributes to the susceptibility phenotypes of the NOD and CAST Idd10 haplotypes, we would predict that NOD.B6 Idd10 Cd101−/− and NOD.B6 Idd10 Cd101+/− mice will be more susceptible to T1D than NOD.B6 Idd10 mice. However, if the increased expression of Cd101 on a subset of CD11c+ cells, which is observed in mice having a susceptible Idd10 haplotype, is causal for T1D susceptibility, an opposite prediction would be made: NOD.B6 Idd10 Cd101−/− mice would be expected to be protected from T1D. If Cd101 expression on both Tregs and CD11c+ cells contribute to T1D pathogenesis, the disease status of NOD.B6 Idd10 Cd101−/− mice would be difficult to predict.

The importance of Cd101 in the immune response has been recently demonstrated in a model of infection-induced liver autoimmunity in which disease can be induced on both the B6 and NOD backgrounds (6). NOD Idd10 congenic mice having the B6 and A/I T1D-protective haplotypes showed delayed clearance of N. aromaticivorans in the liver and subsequently developed more severe liver autoimmunity than NOD and NOD.CAST Idd10 mice. On the B6 background, reduction of Cd101 expression decreased bacterial clearance in the liver following infection and increased the autoimmune response.

Further elucidation of the roles of particular cell subsets expressing Cd101 in T1D and infection-induced liver autoimmunity will increase our understanding of the cellular functions that are modulated by this molecule. It is likely that the pleiotropic expression and function of Cd101 during immune homeostasis and immune challenge contributes to a complex functional balance of innate and adaptive effector and regulatory cell populations, a balance that likely differs depending on the target tissue. Thus, it would not be surprising if the pivotal Cd101-expressing cell type in T1D differs from that critical for influencing the progression of infection-induced liver autoimmunity.

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Disclosures
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References


