The *Idd9.1* locus controls the suppressive activity of FoxP3+CD4+CD25+ Regulatory T-cells

**Running title:** Mechanisms of T1D suppression by *Idd9.1*

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Objective: The ∼45 cM Insulin-dependent diabetes 9 (Idd9) region on mouse chromosome 4 harbors several different type 1 diabetes (T1D)-associated loci. Nonobese diabetic (NOD) mice congenic for the Idd9 region of C57BL/10 (B10) mice, carrying anti-diabetogenic alleles in three different Idd9 sub-regions (Idd9.1, Idd9.2 and Idd9.3), are strongly resistant to T1D. However, the mechanisms remain unclear. This study aimed to define mechanisms underlying the T1D resistance afforded by B10 Idd9.1, Idd9.2 and/or Idd9.3.

Research Design and Methods: We used a reductionist approach that involves comparing the fate of a T1D-relevant autoreactive CD8+ T-cell population, specific for residues 206–214 of islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP206-214), in non-congenic versus B10-Idd9-congenic (Idd9.1+Idd9.2+Idd9.3, Idd9.2+Idd9.3, Idd9.1, Idd9.2 and Idd9.3) TCR-transgenic (8.3) NOD mice.

Results: Most of the protective effect of Idd9 against 8.3-CD8+ T-cell-enhanced T1D was mediated by Idd9.1. Although Idd9.2 and Idd9.3 afforded some protection, the effects were small, and did not enhance the greater protective effect of Idd9.1. B10 Idd9.1 afforded T1D resistance without impairing the developmental biology or intrinsic diabetogenic potential of autoreactive CD8+ T-cells. Studies in T- and B-cell-deficient 8.3-NOD.B10 Idd9.1 mice revealed that this anti-diabetogenic effect was mediated by endogenous, non-transgenic T-lymphocytes in a B-cell-independent manner. Consistent with this, B10 Idd9.1 increased the suppressive function and anti-diabetogenic activity of the FoxP3+CD4+CD25+ T-cell subset, both in TCR-transgenic and non-transgenic mice.

Conclusions: A gene(s) within Idd9.1 regulates the development and function of FoxP3+CD4+CD25+ Treg cells and, in turn, the activation of CD8+ effector T-cells in the pancreas draining lymph nodes, without affecting their development or intrinsic diabetogenic potential.
Type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice is the result of a complex T-cell-mediated autoimmune process against the pancreatic beta cells. Putative poorly defined environmental triggers conspire with a constellation of genetic elements scattered throughout the genome to elicit a multifactorial autoimmune response that involves virtually every cell type of the immune system. Genome-wide association studies in humans and mice have identified at least fifteen different chromosomal regions harboring candidate T1D-associated gene loci, in addition to the HLA and H-2 complexes on human and mouse chromosomes 6 and 17, respectively (1-4).

The ~45 cM insulin-dependent diabetes 9 (Idd9) region on mouse chromosome 4 has a major effect on T1D development (5). NOD mice congenic for the Idd9 region of C57BL/10 (B10) mice are highly resistant to T1D (5). However, the underlying mechanisms remain unclear. Initial work correlated protection from T1D with recruitment of anti-diabetogenic CD30/IL-4-expressing cell types into pancreatic islets (5). Subsequent studies, however, suggested that alternative mechanisms might be at play. For example, using NOD and NOD.B10 Idd9 mice expressing an insulin promoter-driven influenza hemagglutinin (HA) transgene, Martinez et al. found that the B10 Idd9 region affords tolerance to HA-specific CD8+ T-cells through a mechanism that does not inhibit their activation in the pancreatic lymph nodes (6). Another study suggested that Idd9 controls the recruitment of autoreactive CD4+ T-cells to the pancreas (7). A third, employing NOD mice congenic for the Idd9/11 region of NOR mice, which partially overlaps B10 Idd9, supported the CD4+ T-cell intrinsic effect(s) of B10 Idd9 reported by Waldner et al. (8).

Additional investigations on congenic NOD mice carrying chromosome 4 fragments around the Idd9.1 region of different lengths and donor strains reported effects of Idd9 on iNKT cell (9), B-cell (10; 11) or dendritic cell biology (12), and even on susceptibility of β-cells to cell death (13). This rather extensive assortment of mechanisms seemingly unrelated to one another clearly indicates that the effects of Idd9 on T1D are complex and suggest that the phenotypes are probably determined by more than one gene within the Idd9 region. In fact, it has been established from congenic strain mapping that the B10 Idd9 region contains at least three T1D-associated genes: Idd9.1, Idd9.2 and Idd9.3 (5). A congenic strain having the Idd9.2 + Idd9.3 protective subregions (R11) was more susceptible to T1D than the strain having all three congenic subregions (R28). However, the R11 strain was more protected from T1D than the R35 strain that has a T1D-protective B10-derived allele at only Idd9.3.

This study was initiated to dissect mechanisms underlying the T1D resistance afforded by B10 Idd9 loci. We used a reductionist experimental approach (14) that involves comparing the fate and diabetogenicity of a T1D-relevant autoreactive CD8+ T-cell population in B10 Idd9-congenic versus non-congenic, TCR-transgenic NOD mice. Our data show that the B10-derived Idd9.1 subregion has a powerful suppressive effect on the diabetogenic activity of a prevalent monospecific CD8+ T-cell population without impairing its developmental biology or intrinsic diabetogenic potential. We find that this anti-diabetogenic effect is mediated by endogenous, non-transgenic T-lymphocytes but not B-cells, and show that it is associated with enhanced regulatory activity of CD4+CD25+ T regulatory cells not only in TCR-transgenic, but also non-transgenic NOD.B10 Idd9.1 mice.
RESEARCH DESIGN AND METHODS

**Mice.** 8.3-NOD and 8.3-NOD.*RAG2*−/− mice have been described (15). NOD.B10 *Idd9R28* (line 1104), NOD.B10 *Idd9R11* (line 1105), and NOD.B10 *Idd9.3* (line 1106) congenic mice were developed as described previously (5). Development of the NOD.B10 *Idd9.1* (line 1565) and NOD.B10 *Idd9.2* (line 1566) strains and the fine-mapping of *Idd9.1* and *Idd9.2* will be the subjects of future reports. 8.3-NOD.B10 *Idd9R28*, 8.3-NOD.B10 *Idd9R11*, 8.3-NOD.B10 *Idd9.1*, and 8.3-NOD.B10 *Idd9.2* mice were established by crossing 8.3-NOD mice with NOD.B10 *Idd9R28* (line 1104), NOD.B10 *Idd9R11* (line 1105), NOD.B10 *Idd9.1* (line 1565), NOD.B10 *Idd9.2* (line 1566), and NOD.B10 *Idd9.3* (line 1106) mice, respectively. The line number associated with each strain refers to the unique line designation for strains bred at Taconic (Germantown, New York). Lines 1565, 1566 and 1106 are available from Taconic through the Emerging Models Program. Lines 1104 and 1105 are no longer extant. Figure 1A shows a map of the congenic strains used in this study in comparison to *Idd9*-congenic strains described by others (9; 16; 17). Lines 1565, 1566 and 1106 contain the B10 *Idd9.1*, *Idd9.2* and *Idd9.3* regions alone, respectively. The markers that define the boundaries of the lines described here are given in Supplemental Table 1 (which is available at http://diabetes.diabetesjournals.org), along with the primer sequences of novel markers developed for this study. Supplementary Figure 1 lists the SNPs distinguishing B10, B6 and NOD in the *Idd9.1* region defined by line 1565. All genes containing polymorphisms are noted. Also noted are the regions of overlap of the B10-*Idd9* congenic line with R201 of Ueno et al and the two Brodnicki et al B6 *Idd9/Idd11*-congenic lines defining the smallest *Idd11* interval published to date. Information on the congenic strains is also available at http://www.t1dbase.org/page/DrawStrains (select the strain of interest). Note that the B10-*Idd9* fragment in line 1565 overlaps the distal 30% of the B10-*Idd9.1* fragment carried by the R201 strain from Ueno et al. (9), and that Brodnicki's *Idd11*-congenic strains were produced using B6 donor mice (16; 17). 8.3-NOD.*Igu*−/−, 8.3-NOD.B10 *Idd9.1/Igu*−/−, and 8.3-NOD.B10 *Idd9.1/RAG2*−/− mice were established by crossing 8.3-NOD and 8.3-NOD.B10 *Idd9.1* with NOD.*Igu*−/− and NOD.*RAG2*−/− mice, respectively, followed by backcrossing 8.3-TCR+, mutant heterozygous F1 mice to 8.3-NOD.B10 *Idd9.1* mice and intercrossing 8.3-TCR+, mutant heterozygous, B10 *Idd9.1* homozygous littermates. All mice were housed in SPF conditions.

**Peptides and antibodies.** Peptides (NRP-A7 and TUM) were purchased from Mimotopes (Clayton, Victoria, Australia). All monoclonal antibodies (mAbs) were purchased from PharMingen (San Diego, CA, USA), unless indicated otherwise. Anti-mouse GITR, anti-mouse/rat FoxP3, and anti-folic receptor 4 (FR4) were from eBiosciences (San Diego, CA, USA). Streptavidin-PerCP was from Becton-Dickinson (San Jose, CA, USA).

**Preparation of DCs.** MLN and PLN DCs were purified from collagenase digested MLNs and PLNs using anti-CD11c mAb-coated magnetic beads (Miltenyi Biotec). Purified DCs were pulsed with NRP-A7 or TUM peptides (1 µM) for 1 h at 37°C and then used in proliferation and cytokine secretion assays employing 8.3-CD8+ T-cells as responders.

**Diabetes and insulitis.** Diabetes was monitored by measuring urine glucose with Diastix (Miles, Ontario, Canada); animals were considered diabetic after two readings ≥3+. Differences between diabetes survival curves were compared with the Kaplan-Meier log-rank test using Prism software (Graphpad).
Insulitis scores were measured on 14 H&E stained pancreas sections 150 µm apart, using the following criteria: 0: intact islet; 1: peri-insulitis; 2: up to 25% of the islet infiltrated; 3: 25-50% infiltration; 4: >50% infiltration.

**Proliferation and cytokine secretion assays.** Splenic CD8+ T-cells (2×10^4/well) were incubated with NRP-A7/TUM peptide-pulsed (0.0001-1 µM) APCs (10^5 irradiated splenocytes/well) for 2 or 3 days (for cytokine and proliferation assays, respectively) at 37°C in 5% CO2. Cytokines in the supernatants were measured by ELISA. The 3-day cultures were pulsed with 1 µCi [3H] thymidine during the last 18 h and harvested. The regulatory activity of CD4+CD25+ T-cells was measured by adding 0.125–1×10^4 CD4+CD25+ T-cells pre-activated with anti-CD3 mAb and rIL-2 to CD8+ cell:APC co-cultures for 48 h (IFNγ content measurements) or 72 h (proliferative activity, assessed by [3H] incorporation during the last 18 h of culture).

**CTL differentiation and 51Cr release assays.** Splenic CD8+ T-cells purified from 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice using anti-CD8-coated microbeads (Miltenyi Biotec) (2×10^4 cells/well) were stimulated with NRP-A7-pulsed irradiated NOD splenocytes (10^5 cells/well) for 3 days and expanded in 0.5 U/ml of rIL-2 (Takeda, Osaka, Japan) for 7–10 days. Cytolytic activity of CTLs was measured using NRP-A7- or TUM-pulsed (1 µM) RMA-SKd cells.

**T-cell transfers.** Splenic CD8+ T-cells were purified using iMAG CD8 beads (BD Bioscience) following the manufacturer’s protocols, labeled with CFSE (2.5 µM), and injected i.v. (10^7 CD8+ T-cells) into 9-11 week-old hosts (NOD or NOD.B10 Idd9.1). Hosts were sacrificed 6 days later and their PLN and MLN examined for presence of CD8+ CFSE+ cells. To purify CD4+CD25+ cells, lymph node and/or splenic cells were enriched for CD4+ cells, incubated with anti-CD25-PE, and separated using anti-PE mAb-coated beads (Miltenyi Biotec). The purity was >85% for CD4+CD25+ cells. The mice were injected i.v. with 2×10^5 cells and monitored for diabetes for 15 weeks.

**Statistical analyses.** Data were compared using log rank, Mann-Whitney U, or two-way ANOVA tests.

**RESULTS**

**The B10 Idd9 region suppresses CD8+ T-cell-induced diabetes predominantly via Idd9.1.** To determine if the B10-derived Idd9 region that protects from spontaneous T1D can also protect from disease accelerated by the presence of a diabetogenic T-cell receptor (TCR), we introgressed two copies of the B10 Idd9.1–9.3 and Idd9.2–Idd9.3 intervals (Figure 1A) into transgenic NOD mice expressing a diabetogenic T-cell receptor, 8.3 (15). This TCR is representative of a large fraction of islet-associated CD8+ T-cells in NOD mice that use highly homologous TCRα chains (15; 18; 19) and recognize the mimotopes NRP-A7 and NRP-V7 in the context of the MHC molecule H-2Kd (20). These T-cells are already a significant component of the earliest NOD islet CD8+ infiltrates (19-21), are pathogenic (15; 18), target a peptide from islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP206-214, similar to NRP-A7) (22), and are unusually frequent in the periphery (>1/200 CD8+ T-cells) (23).

8.3-NOD.B10 Idd9R28 mice, carrying the complete B10 Idd9 interval that affords >95% protection from spontaneous T1D (5), displayed a significantly reduced frequency of diabetes as compared to their non-congenic 8.3-NOD counterparts (~40% vs. 80%) (Figure 1B). The shorter B10 Idd9.2–Idd9.3 (Idd9R11) interval, which provides 60% protection from spontaneous T1D (5) was also anti-diabetogenic, but to a much lesser extent (Figure 1B). To ascertain whether the higher anti-diabetogenic effect of Idd9 was due to additive/synergistic effects of loci
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contained in different sub-regions, we developed 8.3-NOD mice congenic for each of the individual Idd9 intervals (Idd9.1, Idd9.2 and Idd9.3); each of the individual Idd9 congenic regions affords non-TCR-transgenic NOD mice protection from spontaneous T1D as shown in Figure 1C. The fine-mapping of the Idd9.1 and Idd9.2 regions is in progress and will be the subject of separate reports (Rainbow et al and Hamilton-Williams et al, manuscripts in preparation). The Idd9.3 region is 1.2 Mb with only 15 genes, including the prime candidate Tnfrsf9, which encodes 4-1bb (also known as CD137) (24-26). As shown in Figure 1D, the incidence of diabetes in 8.3-NOD.B10 Idd9.1 mice was virtually identical to that seen in 8.3-NOD.B10 Idd9R28 mice, suggesting a minor contribution of the B10 Idd9.2 and Idd9.3 alleles on 8.3-CD8+ T-cell-enhanced T1D. In agreement with these observations, the diabetes survival curves corresponding to 8.3-NOD.B10 Idd9.2 and 8.3-NOD.B10 Idd9.3 mice were remarkably similar to that seen in 8.3-NOD.B10 Idd9R11 mice. This is in contrast to what occurs in non-transgenic NOD mice, where Idd9.2 and Idd9.3 augment the anti-diabetogenic effects of each other and of Idd9.1 (5). Since the Idd9.1 interval is as protective in 8.3-NOD mice as it is in non-TCR-transgenic NOD mice (5), these data suggest that expression of the diabetogenic TCR overwhelms the anti-diabetogenic properties of Idd9.2 and Idd9.3, but not the effects of Idd9.1.

The B10 Idd9.1 allele suppresses 8.3-CD8+ T-cell-enhanced diabetes and insulitis without compromising 8.3-CD8+ T-cell development or function. Since the anti-diabetogenic effect of the B10 Idd9.1 region in 8.3-NOD.B10 Idd9.1 mice was associated with slower progression of insulitis (Figure 2A), we asked if this region's anti-diabetogenic activity was mediated by effects on the developmental biology of 8.3-CD8+ T-cells. Cytofluorometric studies indicated that the thymi and spleens of both types of mice contained similar absolute and relative numbers of double-positive CD4+CD8+ thymocytes and/or single-positive CD4+ or CD8+ T-cells, respectively (Figure 2B and data not shown). In addition, the transgenic CD8+ T-cells of both types of mice expressed comparable levels of the transgenic Vβ8.1+ TCR and CD8 (Figure 2C), and bound NRP-V7d/Kd and IGRP_206-214/Kd tetramers (recognized with high and intermediate avidity, respectively) with similar frequency (Figure 2D) and mean fluorescence intensity (Figure 2E).

Functional in vitro assays revealed that the splenic CD8+ T-cells of both types of mice proliferated equally well and secreted similar levels of interferon gamma (IFN-γ) and interleukin-2 (IL-2) in response to NRP-A7 peptide stimulation over a range of concentrations (Figure 3A). Likewise, in vitro-differentiated 8.3-CD8+ T-cells from both strains killed peptide-pulsed target cells with similar efficiency (Figure 3B).

Similar results were obtained in vivo. CFSE-labeled 8.3-CD8+ T-cells from 8.3-NOD.B10 Idd9.1 mice proliferated in the pancreatic (but not mesenteric) lymph nodes (PLN and MLN, respectively) of wild-type NOD hosts as efficiently as those derived from 8.3-NOD donors (Figure 3C).

Taken together, these observations indicated that the B10 Idd9.1 allele does not impair the development or function of IGRP_206-214-reactive CD8+ T-cells.

CD8+ T-cell-extrinsic inhibition of 8.3-CD8+ T-cell activation by B10 Idd9.1 in vivo. Whereas CFSE-labeled 8.3-CD8+ T-cells proliferated equally well in vivo regardless of the Idd9.1 genotype of the donor mice, CFSE-labeled 8.3-CD8+ T-cells from both 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice proliferated significantly less in the PLNs of NOD.B10 Idd9.1 hosts than in the PLNs of NOD hosts (Figures 3C and 3D), without any obvious effects on the relative or absolute
numbers of recruited/retained 8.3-CD8+ T-cells (Figure 3E). This suggested that B10 Idd9.1 somehow impairs the cross-priming of 8.3-CD8+ T-cells by endogenous autoantigen-loaded dendritic cells. This was not due to inter-strain differences in autoantigen loading of DCs in vivo but rather to impaired PLN DC function because whereas ex vivo-NRP-A7-pulsed DCs purified from the MLNs of NOD and NOD.B10 Idd9.1 mice could stimulate naive 8.3-CD8+ T-cells with similar efficiency, NRP-A7-pulsed DCs purified from the PLNs of NOD.B10 Idd9.1 mice could not do so as efficiently as NRP-A7-pulsed DCs purified from the PLNs of NOD mice (Figure 3F).

Suppression of 8.3-CD8+ T-cell-enhanced diabetes by the B10 Idd9.1 region requires endogenous T- but not B-lymphocytes. To ascertain whether the CD8+ T-cell extrinsic effects of B10 Idd9.1 on 8.3-CD8+ T-cell cross-priming in vivo were associated with B10 Idd9.1’s anti-diabetogenic activity, we introduced a recombination activating gene (Rag2)-deficiency into 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice. Although penetrance of T1D in 8.3-NOD.RAG2-/- mice is significantly lower than in 8.3-NOD mice (owing to lack of CD4+ T-cell help) (15), the incidence of diabetes in 8.3-NOD.RAG2-/- and 8.3-NOD.B10 Idd9.1/RAG2-/- mice were essentially the same (Figure 4A), indicating that the RAG-2 deficiency had completely abrogated the anti-diabetogenic effects of the B10 Idd9.1 allele. Because these mice only export 8.3-CD8+ T-cells (but not CD4+ T-cells or B-cells) to the peripheral lymphoid organs (15), we reasoned that the protective activity of B10 Idd9.1 was mediated by endogenous T-cells and/or B-lymphocytes. To distinguish between these two possibilities, we introduced an immunoglobulin μ heavy chain gene (Igμ)-deficiency into 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice and followed both types of B-cell-deficient mice for development of diabetes. B-cell-deficient 8.3-NOD.Igμ-/- mice developed diabetes essentially like B-cell-competent 8.3-NOD mice, indicating that T1D development in the 8.3-NOD model, unlike the case in non-transgenic NOD mice, is B-cell-independent (Figure 4B). In contrast, 8.3-NOD.B10 Idd9.1/Igμ-/- mice remained protected from T1D despite lacking B-cells, indicating that the protective effects of the B10 Idd9.1 region are B-cell-independent and mediated by endogenous T-cells.

The B10 Idd9.1 region enhances the development and function of FoxP3+CD4+CD25+ T regulatory cells. Prompted by these findings and the observation that the protective effect of the B10-Idd9.1 interval of line 1565 was not associated with increased development of thymic iNKT cells (9) (Supplementary Figure 2), we investigated whether B10 Idd9.1 might enhance the development and/or function of FoxP3+CD4+CD25+ T regulatory (Treg) cells. We compared the relative size of the FoxP3+CD4+ T-cell subset in thymuses, spleens, MLNs and PLNs of 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice. The lymphoid organs of 8.3-NOD.B10 Idd9.1 mice contained higher percentages (albeit not absolute numbers) of FoxP3+CD4+ T-cells than those of 8.3-NOD mice, although the differences were relatively small and statistically significant only for thymuses and MLNs (Figures 5A and B, and Supplementary Figure 3A). These differences were not a peculiarity of TCR-transgenic mice, because the thymuses and MLNs of non-transgenic NOD.B10 Idd9.1 mice also contained significantly higher percentages of these T-cells as compared to NOD mice (Figure 5C). As expected, there were no differences in the percentages of FoxP3+ T-cells within the CD4+CD25+ T-cell subsets (Figures 5A and C). These CD4+CD25+ T-cells had all the hallmarks of CD4+CD25+ Treg cells described in other strains: they expressed
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GITR, FR4, CD134, and TGF-β (Figure 5D), and produced IFN-γ, IL-4, and IL-10 when stimulated with plate-bound anti-CD3 mAb in the presence, but not absence, of rhIL-2 (Figure 5E). The CD4+CD25+ T-cells of transgenic and non-transgenic NOD and NOD.B10 Idd9.1 mice expressed similar levels of all these markers, including CD25 and FoxP3 (Figure 5F and data not shown).

We next asked whether these B10 Idd9.1-associated differences in the relative size of the FoxP3+CD4+CD25+ Treg subset were accompanied by differences in suppressive activity. We first compared the ability of CD4+CD25+ (and CD4+CD25-) T-cells purified from NOD.B10 Idd9.1 and NOD mice to suppress the activation of naive 8.3-CD8+ T-cells from 8.3-NOD mice in response to NRP-A7 peptide-pulsed APCs. The CD4+CD25+ T-cells purified from 8.3-NOD.B10 Idd9.1 or NOD.B10 Idd9.1 mice had significantly higher regulatory activity than the CD4+CD25+ T-cells purified from 8.3-NOD or NOD mice, respectively, at different CD4+CD25+:CD8+ T-cell ratios (Figures 6A and 6B, and Supplementary Figure 3B). The CD4+CD25- T-cells of both types of mice were not suppressive in these assays (data not shown), as described previously (33). These strain differences in Treg function were also true in vivo. Whereas 8.3-NOD.RAG2−/− hosts transfused with CD4+CD25+ T-cells from wild-type NOD donors developed spontaneous diabetes essentially like non-manipulated 8.3-NOD.RAG2−/− mice (15), 8.3-NOD.RAG2−/− hosts transfused with CD4+CD25+ T-cells from NOD.B10 Idd9.1 mice remained diabetes-free throughout the follow-up period (Figure 6C).

Taken together, these data are consistent with the idea that the diabetes resistance afforded by the B10 Idd9.1 interval is mediated, at least in part, by enhanced development and function of CD4+CD25+ regulatory T-cells.

DISCUSSION

Analyses of Idd-congenic NOD mice, in which T1D-associated chromosomal regions are replaced by the corresponding regions found in non-diabetes-prone strains of mice, such as B6 and B10, have demonstrated the existence of more than 20 non-MHC-linked T1D-associated regions/loci (1; 27). The nature of the genes that are responsible for these associations has been resolved for only a handful of Idd regions, including Idd16–β2m (28), Idd3–Il2 (14), Idd5.1–Ctla4 (29; 30) and Idd5.2–Nramp1 (29; 31; 32). Furthermore, with the exception of Idd3 (14), which controls Treg development and function, the mechanisms by which most of these loci contribute to the diabetogenic process remain unclear.

The current study aimed to elucidate mechanisms underlying the resistance to T1D afforded by B10 Idd9 (5), to help pave the way towards eventual identification of the responsible gene. We compared the fate and diabetogenicity of a T1D-relevant autoreactive CD8+ T-cell population in B10-Idd9-congenic versus non-congenic TCR-transgenic-NOD mice. We find that this reductionist system, which has enabled us to solve Idd3 (14), is not informative to dissect mechanisms underlying Idd9.2- and Idd9.3-linked suppression, presumably because the large size of the transgenic CD8+ T-cell population born by these mice largely overwhelms the corresponding anti-diabetogenic mechanisms. This, however, was not the case for Idd9.1. Our data show that Idd9.1 regulates the suppressive activity and anti-diabetogenic potential of FoxP3+CD4+CD25+ Treg cells. Tregs arising in B10 Idd9.1-congenic NOD mice were significantly more suppressive and anti-diabetogenic than those arising in NOD mice. Since FoxP3+CD4+ T-cells suppress diabetogenic T-cell responses, at least in part, by inhibiting DCs in the pancreas-draining
lymph nodes (33), these observations are consistent with the suboptimal ability of autoantigen-loaded (in vivo) and peptide-pulsed (ex vivo) PLN DCs from NOD.B10 Idd9.1 mice to support 8.3-C8+ T-cell activation, both in vitro and in vivo; selective suppression of PLN DCs in these mice may result from cognate suppression of autoantigen-loaded DCs, which predominantly (if not exclusively) reside in the PLNs, by local autoregulatory CD4+CD25+ Treg cells. Accordingly, we propose that B10 Idd9.1-enhanced Tregs contribute to B10 Idd9.1-linked T1D inhibition by suppressing the activation of CD8+ (and possibly other) effector T-cells in the pancreas draining lymph nodes, without affecting their developmental biology or intrinsic diabetogenic potential. These data might explain the observations of Chamberlain et al. in mice expressing a rat insulin promoter-driven TNFα transgene, in which B10 Idd9.1 somehow suppressed the ability of CD8+ T-cells to respond to islet-infiltrating APCs (34). They are also compatible with the DC phenotype described by O’Keeffe et al. (12), with the caveat that this congenic strain, unlike the one described here, was produced using B6, as opposed to B10 donor mice, and that B10 and B6 DNAs differ substantially in the distal region of chromosome 4 (Supplemental Figure 1). These observations, however, do not exclude, and are compatible with possible effects of Idd9.1 polymorphisms on other lymphocyte populations. For example, it has been reported that a B10 chromosome 4 region partially overlapping the B10 Idd9.1 fragment studied here restores impaired iNKT cell development and an iNKT cell-related DC phenotype described in NOD mice (9). Our congenic line 1565, however, bears a B10 chromosome 4 fragment that only overlaps the distal 30% of the B10 chromosome 4 fragment present in Ueno et al.'s R201 line. Furthermore, line 1565 does not display Ueno et al.'s iNKT cell phenotype, suggesting that this phenotype is encoded in a gene that is not in the overlapping region. Also, our work does not exclude possible effects of genes within Idd9.1, Idd9.2 and/or Idd9.3 on peripheral tolerance of autoreactive T-cell clones other than those expressing the 8.3-TCR (35), such as for example CD8+ T-cells recognizing IGRP206-214 with higher avidity (36).

It is intriguing that the effects of the B10 Idd9.1 allele on Treg function and T1D susceptibility observed in this study are remarkably similar to those we have described recently for B6 Idd3, where Treg development and function are enhanced by increased transcriptional activity of Il2 (14). Accordingly, it is tempting to suspect that regulatory lymphocytes, including the FoxP3+CD4+CD25+ Tregs, may be common targets of T1D-associated chromosomal regions. This hypothesis is compatible with what we know about the T1D-gene associations in humans. In addition to the IL2-IL21 region on human chromosome 4q27 (2; 4; 37), and IL2RA on chromosome 10p15.1, which encodes CD25, the high-affinity receptor for IL-2 expressed by activated T-cells and FoxP3+CD4+CD25+ Tregs (37-40), several other candidate human T1D-associated loci play a role in Treg development and function. For example, the T1D susceptibility gene CTLA4 on chromosome 2q33.2, encodes a negative regulator of T-cell mediated immunity (30; 41; 42). The susceptibility allele generates reduced levels of an alternatively spliced transcript that encodes the soluble form of CTLA-4, which have been correlated with variations in the peripheral frequency of CD4+CD25+ T-regulatory (Treg) cells, which constitutively express CTLA-4 (43). PTPN22 on chromosome 1p13.2, associated with susceptibility to a number of autoimmune diseases, including T1D (40; 44-46), encodes lymphoid tyrosine-phosphatase (LYP), another negative regulator of T-cell activation.
A non-synonymous polymorphism (R620W) abolishes the binding of Csk, a negative regulatory kinase, to LYP. Consequently, the R620W LYP variant results in a gain of function that presumably enables LYP to more effectively suppress T-cell signaling as compared to the non-T1D-associated variants. It has been suggested that PTPN22 polymorphisms contribute to autoimmune disease susceptibility by impairing negative selection of autoreactive thymocytes and by decreasing the number and function of CD4+CD25+ Tregs (47; 48). Another locus possibly implicated in autoimmune disease susceptibility by modulating T cell signaling is PTPN2 on chromosome 18p11.21, a non-receptor type 2 tyrosine phosphatase that is a negative regulator of T-cell signaling (2).

The fine-mapping of Idd9.1 has not progressed sufficiently to productively comment on candidate genes present in the region. The B10-derived congenic interval present in the Idd9.1 strain used in this study (line 1565), has 329 annotated genes in this 18.5 Mb segment. The Idd11 region as defined by B6-derived congenic strains (16) is an ~8 Mb region that is wholly contained within the Idd9.1 interval defined by the congenic strain used in this study. However, B6 and B10 are not identical-by-descent throughout the overlapping region (http://phenome.jax.org); from SNP analyses, B6 and B10 differ at 32.3 % of the SNPs within the 8 Mb Idd9.1/Idd11 interval (Supplemental Figure 1). Notably, within this 8 Mb, there are small regions that appear to be identical-by-descent for B6 and B10 including approximately 200 kb surrounding Lck, a previously highlighted candidate gene in the Idd9.1 region (16), where the ancestral haplotype shared by the B6 and B10 strains differs from that present in NOD mice (Supplemental Figure 1). Further fine-mapping and gene expression studies are required to define the genes that are responsible for Idd9.1- and Idd11-mediated T1D protection and to determine if the protective alleles are indeed shared by the B6 and B10 strains in this region.

In conclusion, we have shown that the B10 Idd9.1 locus affords diabetes resistance, at least in part, by promoting the function of CD4+CD25+ regulatory T-cells without altering the developmental biology of effector CD8+ T-cells.

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FIGURE LEGENDS
FIGURE 1. Anti-diabetogenic effects of different B10 Idd9 intervals on 8.3-CD8+ T-cell-enhanced T1D. A, Chromosome 4, B10 Idd9-congenic NOD lines used in this and other studies (9; 16). B, Incidence of diabetes in female 8.3-NOD (n=38), 8.3-NOD.B10 Idd9R28 (n=22) and 8.3-NOD.B10 Idd9R11 mice (n=31). C, Incidence of diabetes in NOD (n=67) as compared to NOD.B10 Idd9.1 (n=58), NOD.B10 Idd9.2 (n=57) and NOD.B10 Idd9.3 (n=67) female mice. D, Incidence of diabetes in female 8.3-NOD (n=38), 8.3-NOD.B10 Idd9.1 (n=100), 8.3-NOD.B10 Idd9.2 (n=94) and 8.3-NOD.B10 Idd9.3 mice (n=85). The Kaplan-Meier survival curves were compared with Log-Rank test.

FIGURE 2. Effects of B10 Idd9.1 on insulitis and 8.3-CD8+ T-cell development. A, Insulitis scores of 8.3-NOD (n=6) and 8.3-NOD.B10 Idd9.1 (n=5) at 10 weeks of age. B, Flow cytometry profiles of thymocytes (left) and splenocytes (right) in 8.3-NOD (n=7) and 8.3-NOD.B10 Idd9.1 mice (n=6). Values correspond to the means ± standard errors. C, Representative anti-CD8 mAb/IGRP206-214/Kd and anti-CD8 mAb/NRP-V7/Kd tetramer staining profiles of PLN cells. D, Percentages of thymic, splenic MLN and PLN CD8+ T-cells binding IGRP206-214/Kd and NRP-V7/Kd tetramers. Data (means ± standard errors) correspond to 4 mice per strain. E, Representative TUM/Kd (negative control), IGRP206-214/Kd (intermediate avidity) and NRP-V7/Kd (high avidity) tetramer staining profiles of splenic T-cells from 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice.

FIGURE 3. Effects of B10 Idd9.1 on the function of naive 8.3-CD8+ T-cells. A, Proliferation of, and cytokine (IFN-γ and IL2) production by CD8+ splenocytes purified from 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice in response to NRP-A7 peptide-pulsed irradiated NOD splenocytes. Data are representative of two separate experiments. B, CTL differentiation potential and cytolytic activity of in vitro-differentiated 8.3-CTL from 8.3-NOD and 8.3-NOD.B10 Idd9.1 mice. Naive 8.3-CD8+ T-cells (n=3 for each strain) were stimulated with NRP-A7 (1 µM)-pulsed APCs for 3 days and cultured in the presence of rhIL-2 for 4 additional days. Cytotoxicity assays were done using NRP-A7- and TUM-pulsed (1µM) RMA-SKd cells as targets, at different effector:target ratios. Background responses against TUM-pulsed targets were subtracted. C and D, CFSE-labeled 8.3-CD8+ T-cells were injected into 9-11 week-old NOD (n=9; five 8.3-NOD donors and four 8.3-NOD.B10 Idd9.1 donors) and NOD.B10 Idd9.1 hosts (n=9; six 8.3-NOD donors and three 8.3-NOD.B10 Idd9.1 donors) and analyzed for dividing (CFSE low) CD8+ T-cells in pancreatic (PLN) and mesenteric lymph nodes (MLN) 6 days later. C Shows representative flow cytometry profiles and D provides average quantitative data. E, Percentages and total numbers of 8.3-CD8+ T-cells in the PLNs and MLNs of NOD and NOD.B10 Idd9.1 hosts (the histogram legend is the same as in D). F, Proliferation of, and secretion of IFNγ by naive 8.3-CD8+ T-cells in response to NRP-A7-pulsed DCs isolated from the PLNs or MLNs of NOD and NOD.B10 Idd9.1 mice. Bars in A, B, D, E and F correspond to the standard errors of the means. P values were calculated with Mann-Whitney U.

FIGURE 4. The anti-diabetogenic effects of B10 Idd9.1 are effector T-cell-extrinsic and mediated by endogenous T-cells in a B-cell-independent manner. A, Incidence of diabetes in female 8.3-NOD.RAG2-/- (n=106) and 8.3-NOD.B10 Idd9.1/RAG2-/- mice (n=19). B, Incidence of diabetes in female 8.3-NOD.Igr-/- (n=33) and 8.3-NOD.B10 Idd9.1/Igr-/- mice (n=47). The Kaplan-Meier survival curves were compared with Log-Rank test.
Mechanisms of T1D suppression by \textit{Idd9.1}

FIGURE 5. Effects of B10 \textit{Idd9.1} on the size and phenotype of the regulatory FoxP3+CD4+CD25+ Treg subset. A, Representative FoxP3/CD25 flow cytometry profiles. B, Percentages of FoxP3+ cells in the CD4+ and CD4+CD25+ T-cell subsets in the MLNs, PLNs, spleens (Spl) and Thymuses (Thy) of 8–11 week-old 8.3-NOD (n=12) and 8.3-NOD.B10 \textit{Idd9.1} mice (n=11) mice. Values correspond to the means ± standard errors. P values were calculated with Mann-Whitney U test. C, Percentages of FoxP3+ in the CD4+ and CD4+CD25+ T-cell subsets in the spleen, MLNs, PLNs, and Thymus of 8–11 week-old NOD (n=9 for spleens, MLNs and PLNs; n=5 for Thymuses) and NOD.B10 \textit{Idd9.1} mice (n=8 for spleens, MLNs, PLNs; n=4 for Thymuses). D, GITR, FR4, CD134 and surface TGF-β flow cytometry profiles of CD4+CD25+ and CD4+CD25- cells from NOD and NOD.B10 \textit{Idd9.1} mice. Values correspond to the means ± standard errors of 2-4 experiments/strain type. E, Proliferation and cytokine production by CD4+CD25+ cells from NOD and NOD.B10 \textit{Idd9.1} mice in response to plate-bound anti-CD3 mAb in the absence/presence of rhIL-2. Values correspond to the means ± standard errors of 3 independent experiments. F, Comparison of FoxP3 and CD25 mean fluorescence intensities for FoxP3+CD4+CD25+ T-cells from both types of mice.

FIGURE 6. Effects of B10 \textit{Idd9.1} on the \textit{in vitro} and \textit{in vivo} suppressive activity of CD4+CD25+ T regulatory cells. A and B, IFN-γ production by naive 8.3-CD8+ T-cells from 8.3-NOD mice in response to NRP-A7-pulsed APCs in the presence of CD4+CD25+ T-cells from 8.3-NOD and 8.3-NOD.B10 \textit{Idd9.1} mice (A) or NOD and NOD.B10 \textit{Idd9.1} mice (B), at different Treg:responder ratios. Graphs are representative of 5 (A) and 3 (B) independent experiments. C, Incidence of spontaneous diabetes in 8.3-NOD.\textit{RAG2}−/− hosts (n=28) transfused with 2 x 10^5 CD4+CD25+ T-cells from NOD (n=16) or NOD.B10 \textit{Idd9.1} donors (n=12). P values for panels A and B were calculated with two-way ANOVA, and with Log-Rank for panel C.
Mechanisms of T1D suppression by *Idd9.1*

**REFERENCES**


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**Figure 1**

![Diagram](image)
Figure 2

A

\[ \text{Insulitis score} \]

\[ 0.0 \quad 1.0 \quad 2.0 \quad 3.0 \quad 4.0 \]

\[ \text{P} = 0.0087 \]

B

\[ \begin{align*}
\text{Thymus} & \quad \% \text{CD4-CD8-} \\
\text{Spleen} & \quad \% \text{CD4-CD8-}
\end{align*} \]

\[ \begin{align*}
\text{CD4-CD8-} & \quad \% \text{CD4-CD8-} \\
\text{CD4-CD8-} & \quad \% \text{CD4-CD8-}
\end{align*} \]

C

\[ \begin{align*}
\text{TUM} & \quad \text{IGRP}_{206-214} \\
\text{NRP-V7} & \quad \text{NRP-V7}
\end{align*} \]

\[ \begin{align*}
\text{8.3-NOD} & \quad \text{8.3-NOD.B10 } \text{Idd9.1}
\end{align*} \]

\[ \begin{align*}
0.0087 & \quad 0.26 \\
90.1 & \quad 91.6
\end{align*} \]

D

\[ \begin{align*}
\% \text{VR8+} & \quad \% \text{VR8+} \\
\text{CD4-CD8-} & \quad \text{CD4-CD8-}
\end{align*} \]

\[ \begin{align*}
\% \text{VR8+} & \quad \% \text{VR8+} \\
\text{CD4-CD8-} & \quad \text{CD4-CD8-}
\end{align*} \]

E

\[ \begin{align*}
\text{8.3-NOD} & \quad \text{TUM} \\
\text{IGRP}_{206-214} & \quad \text{NRP-V7}
\end{align*} \]

\[ \begin{align*}
\text{8.3-NOD.B10 } \text{Idd9.1}
\end{align*} \]
Mechanisms of T1D suppression by $Idd9.1$

**FIGURE 3**

**A**

- 8.3-NOD
- 8.3-NOD.B10 $Idd9.1$

**B**

- Specific lysis (%)

- Effector: Target ratio

**C**

8.3-NOD -> NOD
8.3-NOD.B10 $Idd9.1$ -> NOD.B10 $Idd9.1$

**D**

- 8.3-NOD -> NOD
- 8.3-NOD.B10 $Idd9.1$ -> NOD
- 8.3-NOD -> NOD.B10 $Idd9.1$
- 8.3-NOD.B10 $Idd9.1$ -> NOD.B10 $Idd9.1$

**E**

- % of CFSE+ cells
- Number of CFSE+ cells ($10^7$)

**F**

- Proliferation of 8.3-CD8+ (CPM)
- IFN-γ by 8.3-CD8+ (pg/ml)
Mechanisms of T1D suppression by *Idd9.1*

Figure 4

**A**

- 8.3-NOD.Rag2-/-
- 8.3-NOD.B10 *Idd9.1/Rag2-/-

\[ P = 0.1138 \]

**B**

- 8.3-NOD.Igµ-/-
- 8.3-NOD.B10 *Idd9.1/Igµ-/-

\[ P < 0.0001 \]
Mechanisms of T1D suppression by *Idd9.1*

**Figure 5**

A  
8.3-NOD  
8.3-NOD.B10 *Idd9.1*

B  
8.3-NOD  
8.3-NOD.B10 *Idd9.1*

C  
NOD  
NOD.B10 *Idd9.1*

D  
% GITR+  
% FR4+

E  
IFN-γ (pg/ml) Proliferation (CPM)

F  
MFI on CD4+CD25+

- IL-2  
+ IL-2  
anti-CD3
Mechanisms of T1D suppression by Idd9.1

**A**

![Graph A](image1.png)

**B**

![Graph B](image2.png)

**C**

![Graph C](image3.png)

**FIGURE 6**

CD4+CD25+: 8.3-CD8+ ratio