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*J Immunol* 2014; 193:3503-3512; Prepublished online 27 August 2014; doi: 10.4049/jimmunol.1400189

http://www.jimmunol.org/content/193/7/3503
The Mouse Idd2 Locus Is Linked to the Proportion of Immunoregulatory Double-Negative T Cells, a Trait Associated with Autoimmune Diabetes Resistance

Roxanne Collin,*† Véronique Dugas,*†,†1 Adam-Nicolas Pelletier,*† Geneviève Chabot-Roy,* and Sylvie Lesage*†,‡

Autoimmune diseases result from a break in immune tolerance. Various mechanisms of peripheral tolerance can protect against autoimmunity, including immunoregulatory CD4+CD8− double-negative (DN) T cells. Indeed, we have previously shown that diabetes-prone mouse strains exhibit a low proportion of DN T cells relative to that of diabetes-resistant mice, and that a single autologous transfer of DN T cells can impede autoimmune diabetes development, at least in the 3A9 TCR transgenic setting. In this study, we aim to understand the genetic basis for the difference in DN T cell proportion between diabetes-resistant and diabetes-prone mice. We thus perform an unbiased linkage analysis in 3A9 TCR F2 (NOD.H2k × B10.BR) mice and reveal that a locus on chromosome 9, which coincides with Idd2, is linked to the proportion of DN T cells in the lymph nodes. We generate two NOD.H2k.B10-Chr9 congenic mouse strains and validate the role of this genetic interval in defining the proportion of DN T cells. Moreover, we find that the increased proportion of DN T cells in lymphoid organs is associated with a decrease in both diabetes incidence and serum IgG Ab levels. Together, the data suggest that Idd2 is linked to DN T cell proportion and that a physiological increase in DN T cell number may be sufficient to confer resistance to autoimmune diabetes. Altogether, these findings could help identify new candidate genes for the development of therapeutic avenues aimed at modulating DN T cell number for the prevention of autoimmune diseases. The Journal of Immunology, 2014, 193: 3503–3512.

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toimmunity results from a break in immune tolerance that leads to the destruction of healthy organs, glands, joints, or the CNS. The triggers that cause lymphocytes to abnormally recognize and mount an immune response against self-Aggs remain to be fully elucidated. A prominent role in the prevention of autoimmunity has been attributed to the number and function of regulatory cell subsets. Indeed, several immunoregulatory T cell subsets carry the ability to inhibit inadequate immune responses and promote peripheral tolerance, including CD4+CD25Foxp3+ naturally occurring T cells, IL-10–producing CD4+CD25−Tr1 cells, TGF-β–producing CD4+Th3 cells, CD8+CD28−T cells, CD4+CD8− double-negative (DN) T cells, CD4+CD8+ T cells, and NKT cells (1–10). The specific contribution of these respective cell subtypes to the proper control of immune tolerance and prevention of autoimmune disease remains to be defined. Their diverse antigenic specificity and/or distinct mode of action provide strong hints that each of these cell subsets contribute differently to peripheral tolerance (1). Hence, there is a need to further examine the contribution of various T cell subsets to autoimmune predisposition.

Of these regulatory T cell subsets, DN T cells are of particular interest because they carry immunoregulatory properties in various pathological settings, including autoimmunity and transplantation (11). Indeed, regulatory DN T cells have been shown to promote allograft and xenograft tolerance (12–17), limit the incidence of graft versus host disease (17–20), as well as provide resistance to autoimmune diabetes development in different animal models (21–23). Specifically, autoimmune-prone mice carry fewer DN T cells, and injection of DN T cells has been shown to be sufficient to reduce the incidence of autoimmune diabetes in two distinct TCR transgenic mouse models (21, 22). Importantly, a low DN T cell proportion, rather than defects in DN T cell function, associates with diabetes susceptibility (22–24). Together, these observations suggest that variations in DN T cell proportion correlate with susceptibility to autoimmune diabetes. Therefore, identification of the genetic determinants defining the proportion of DN T cells may unravel key molecular targets to increase DN T cells in lymphoid organs, and thereby permit an increase in immune tolerance.

NOD mice spontaneously develop autoimmune diabetes, and this inbred strain has been a key tool to investigate the cause of this disease (25, 26). In addition, NOD congenic mice have proved useful in defining genetic loci linked to autoimmune diabetes susceptibility (27). To that effect, we have recently shown that the NOD.NOR-Idd13 congenic mice, which are resistant to diabetes progression (28), show a modest increase in DN T cell number relative to NOD mice, but that neither Idd3, Idd5, nor Idd9 con-

Abbreviations used in this article: DN, CD4+CD8− double-negative; HEL, hen egg lysozyme; HEL, HEL expressed under the rat insulin promoter; LOD, logarithm of odds; SNP, single nucleotide polymorphism.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1400189

Received for publication January 23, 2014. Accepted for publication July 28, 2014.

This work was supported by the Canadian Diabetes Association (Grant OG-3.13-4018 to S.L.), scholarships from Diabète Québec (to R.C. and A.-N.P.), the Université de Montréal (to R.C. and A.-N.P.), the Fonds de Recherche en Santé du Québec (to V.D.), and a New Investigator award from the Canadian Institutes of Health Research (to S.L.).

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Author Contributions: R.C. and A.-N.P. contributed equally to this work. R.C. and A.-N.P. designed the study and performed experiments. R.C. and A.-N.P. analyzed the data. R.C. and A.-N.P. wrote the manuscript. R.C. and A.-N.P. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. R.C. and A.-N.P. take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest: The editors in chief have approved the Journal of Immunology’s policies on author contributions, competing interests, and authorship (www.jimmunol.org/misc/ifora.htm). The submitted manuscript does not contain any information that has already been published and does not present irrelevant repetition of another publication. The authors declare that they have no relevant financial interests. The authors declare that they do not have a relationship with a company or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. The authors declare that they do not have any other relationship or any financial benefit that may be perceived to influence or have the potential to influence their work.

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tribute to this trait (24). Notably, the genetic determinants from the \textit{Idd13} locus only partially restored DN T cell number, strongly suggesting that other genetic loci also contribute toward defining the proportion of DN T cells.

In this study, we take advantage of the 3A9 TCR transgenic mouse model, in which the TCR recognizes a peptide from hen egg lysozyme (HEL) in the context of I-A\(^{k}\) (29), allowing the investigation of the genetic regulation of the proportion of DN T cells in secondary lymphoid organs. As we have previously demonstrated that the proportion of 3A9 DN T cells is significantly different in the lymph nodes of 3A9 TCR B10.BR and 3A9 TCR NOD.H2\(^d\) mice (22), we perform an unbiased linkage analysis on an F2 outcross from these two parental strains. We establish that the proportion of DN T cells is a complex trait, where the locus exhibiting the highest logarithm of odds (LOD) score is located on chromosome 9 and coincides with the \textit{Idd13} locus, also shows a suggestive linkage to 3A9 DN T cell proportion (24). We develop congenic mice to validate the contribution of the chromosome 9 locus in defining the proportion of DN T cells in peripheral lymphoid organs and explore the role of this locus in conferring resistance to autoimmune diabetes.

**Materials and Methods**

**Mice**

3A9 TCR transgenic and HEL transgenic mice, where HEL is expressed under the rat insulin promoter (iHEL), on B10.BR and NOD.H2\(^d\) backgrounds have been previously described (30). The congenic strains were obtained by backcrossing B10.BR \(\times\) NOD.H2\(^d\) F1 mice with the 3A9 TCR NOD.H2\(^d\) parental strain for six generations. All congenic mice were used at the sixth-generation backcross. Mice were genotyped by PCR at markers D9Mit129 and D9Mit328, and mice bearing at least one B10.BR allele at both markers were backcrossed to 3A9 TCR NOD.H2\(^d\) mice. The iHEL transgene was introduced at the fifth-generation backcross. At the sixth generation, a recombination event occurred between the D9Mit129 and D9Mit328 markers, where one of the mice presented with a B10.BR allele only at D9Mit129 marker. This mouse was the founder of the NOD.H2\(^d\)-Chr9S congenic strain, whereas the other mice, bearing B10.BR alleles at both D9Mit129 and D9Mit328 markers, were founders for the NOD.H2\(^d\)-Chr9L congenic strain. An Illumina medium-density linkage panel, obtained from The Centre for Applied Genomics at the Hospital for Sick Children, was performed on the tail DNA from congenic mice to delimit the boundaries of the B10.BR congenic interval. The 3A9 TCR F2 (B10.BR \(\times\) NOD.H2\(^d\)) mice were obtained by intercrossing F1 mice, with one of the parents bearing the 3A9 TCR transgene. All of the mouse strains were maintained at the Maisonneuve-Rosemont Hospital animal house facility. The Maisonneuve-Rosemont Hospital ethics committee, overseen by the Canadian Council for Animal Protection, approved the experimental procedures.

**Flow cytometry**

Seven- to 15-wk-old nondiabetic mice (Diastix negative) were analyzed. Lymph nodes and spleen were pressed through a 70-\(\mu\)m cell strainer (Thermo Fisher Scientific, Ottawa, ON, Canada). Spleen cell suspensions were treated with \(\text{NH}_4\text{Cl}\) to lyse RBCs. Single-cell suspensions were stained with different combinations of the following Abs to quantify 3A9 DN T cells: CD3e-FTTC (145-2C11; Biolegend, San Diego, CA), CD4-PE (GK1.5; Biolegend), CD8a-PerCP (53-6.7; Biolegend), CD19-PerCP (6D5; Biolegend), and anti-clonotypic 1G12 (homemade) (31) Ab followed by detection with IgG1-allophycocyanin (R7M1-1; Biolegend). Data were collected on a FACSCalibur (BD Biosciences, Mississauga, ON, Canada) and analyzed with FlowJo software (Tree Star, Ashland, OR).

**Linkage analysis**

Genomic DNA was isolated from the tails of the 171 3A9 TCR transgenic F2 (B10.BR \(\times\) NOD.H2\(^d\)) male and female mice aged between 7 and 15 wk by using the DNeasy blood and tissue kit (Qiagen, Toronto, ON, Canada). Single nucleotide polymorphisms (SNPs) were then detected from the F2 mice using the Illumina mouse low-density linkage panel served through The Centre for Applied Genomics at the Hospital for Sick Children. Additional genetic markers were used to delimit the interval on mouse chromosome 9, namely, D9Mit328 (41.7Mb), D9Mit330 (46.9Mb), D9Mit4 (51.9Mb), D9Mit21 (57.3Mb), D9Mit232 (58.2Mb), and D9Mit302 (67.0Mb). DNA from 3A9 TCR B10.BR, 3A9 TCR NOD.H2\(^d\), and 3A9 TCR F1 (B10.BR \(\times\) NOD.H2\(^d\)) mice were used as controls. Marker location (in Mb) was determined using the National Center for Biotechnology Information Build m37. The LOD scores were obtained through a single quantitative trait locus model using the R/qtl package (32) for the R software (version 2.11.1). To increase SNP resolution, we applied the Haley–Knott algorithm (33). LOD scores >3.5 were significant for single-dimensional analysis according to permutation tests (\(n = 10,000, p = 0.05\)), and LOD scores between 2.09 and 3.5 were considered suggestive. A Pearson’s \(\chi^2\) for allele frequencies was applied and SNP distributions that deviate from the Hardy–Weinberg equilibrium were removed. Notably, because the 3A9 TCR transgene is located on chromosome 5, we excluded the chromosome 5 data from our analysis.

**Diabetes incidence study**

Diabetes incidence was monitored daily in female 3A9 TCR:iHEL mice for overt signs of diabetes (wet cage, hunched posture) and every 2 wk for urine glucose levels using Diastix (Bayer, Toronto, ON, Canada) starting at 8–12 wk of age. After two successive positive Diastix tests, overt diabetes was confirmed by blood glucose levels >17 mmol/L. The age of diabetes onset is set at the first detection of increased urine glucose levels. The mice were sacrificed within 1 wk of detection of high blood glucose or when they reached >34 wk of age. At culling, tail DNA was collected to verify the genotype of

**FIGURE 1.** The proportion of 3A9 DN T cells is regulated by a complex trait. (A) Representative flow cytometry profiles for determining the proportion of 3A9 DN T cells in the lymph nodes of 3A9 TCR \(\times\) NOD.H2\(^d\) mice. Left panel shows CD4 versus 3A9 TCR on total live lymphocytes, where the 3A9 TCR is detected with the 1G12 clonotypic Ab. All of the 3A9 TCR\(^+\) cells are electronically gated to display CD4 versus CD8 profile, where the percentage of CD4 \(\times\) CD8\(^-\) T cells among 3A9 TCR\(^+\) cells is shown (right panel). The percentage of 3A9 DN T cells is calculated by multiplying the percentage of 3A9 TCR\(^+\) cells (left panel) to the percentage of CD4\(^+\) CD8\(^-\) T cells among 3A9 TCR\(^+\) cells (right panel). (B) The distribution of the F2 cohort (171 3A9 F2 mice aged from 7 to 15 wk) relative to the total proportion of 3A9 DN T cells in lymph nodes is shown.
the mouse. Also, the serum was collected and the pancreas was conserved in formalin for at least 48 h at room temperature before being sent for paraffin inclusion. The 3A9 TCR:iHEL NOD.H2\(^d\) female mice included in the diabetes incidence study are littermate controls from either 3A9 TCR:iHEL NOD.H2\(^d\)-Chr9L or 3A9 TCR:iHEL NOD.H2\(^d\)-Chr9S congenic mice that carried homozygous NOD alleles at both D9Mit129 and D9Mit328 markers.

**Histology**

H&E staining was performed on 5- to 7-\(\mu\)m pancreas cuts from paraffin blocks, for 2–4 nonsuccessive cuts per slide with one slide per mouse (Tables I and II). H&E slides were scored for infiltration as previously described (34), and according to the following scale: 0 = no infiltration, 1 = peri-insulitis, 2 = infiltration <50\%, 3 = infiltration >50\%, 4 = complete infiltration.

**ELISA**

Total IgG was measured by ELISA according to the manufacturer’s protocol (Bethyl Laboratories, Montgomery, TX). Serum anti-HEL IgG, IgM, and IgG1 levels were measured by ELISA on Nunc Maxisorp plates (Thermo Fisher Scientific) coated with 100 \(\mu\)g/ml HEL prepared in NaHCO\(_3\) at pH 9.5, developed with goat anti-mouse IgG-HRP (clone poly4053; Biolegend), goat anti-mouse IgM-HRP (VWR), or rat anti-mouse IgG1-biotin (clone RMG1-1; Biolegend) followed by avidin-HRP (Biolegend). A reference pool of sera from diabetic and nondiabetic TCR: iHEL mice was set to contain 1 arbitrary unit.

**Statistics**

Data for the various experiments were tested for significance using a nonparametric Mann–Whitney \(U\) test with a minimal threshold of 0.05. Estimation of the interval coordinates on chromosome 9 was obtained using a 95% Bayes interval test. Significance for the F2 genotype distribution differences was tested with a one-way ANOVA test. Statistical significance of the differences between the diabetes incidence curves was determined with the Mantel–Cox log rank test with a minimal threshold of 0.05. All statistical analyses and the F2 distribution were obtained using the SPSS 19.0 software (IBM, Montreal, QC, Canada).

**Results**

In nontransgenic mice, immunoregulatory DN T cells compose \(\sim 1–3\%\) of total T cells (22, 35–37). The low number of these cells not only presents a challenge for their isolation and characterization, but limits the sensitivity in detection necessary to perform a linkage analysis. In addition, the specific characterization of immunoregulatory DN T cells is further complicated by the fact that DN T cells share the CD4\(^+\) CD8\(^-\) TCR\(^{ab}\) phenotype with a subset of NKT cells (37, 38), which would need to be carefully excluded from our analyses. Because of these challenges, we instead opted to take advantage of the 3A9 TCR transgenic mouse model, in which the TCR recognizes a peptide from HEL in the context of I-A\(^b\) (29), allowing to investigate the genetic regulation of the proportion of DN T cells in secondary lymphoid organs. Indeed, as for other TCR transgenic systems (15, 38–43), the 3A9 TCR transgenes enhance the proportion of DN T cells in peripheral lymphoid organs and preclude the differentiation of potentially contaminating NKT cells, increasing the sensitivity of detection of DN T cells (22–24). We thus performed an F2 out-

**FIGURE 2.** Genome-wide linkage analysis for the proportion of 3A9 DN T cells. Genome-wide LOD score plot (R/Qtl) for the proportion of 3A9 DN T cells in the 171 F2 cohort is shown. The approximate position of the Idd2 locus is depicted. The full and dotted lines, respectively, indicate the significance threshold of \(p < 0.05\) and the suggestive threshold.

**FIGURE 3.** Some suggestive linkages for 3A9 DN T cell proportion coincide with autoimmune susceptibility loci. High-resolution map of the suggestive loci linked to 3A9 DN T cell proportion. Known Idd loci are depicted for each of these chromosomes. The position are based on the T1DBase database (http://www.t1Dbase.org) (59). The dotted lines indicate the suggestive threshold.
cross of the 3A9 TCR B10.BR and 3A9 TCR NOD.H2k parental strains, because they exhibit a significant difference in the proportion of 3A9 DN T cells in the lymph nodes (22).

We analyzed the proportion of 3A9 DN T cells in the lymph nodes of 171 3A9 TCR F2 (B10.BR × NOD.H2k) mice, where 3A9 TCR+ cells are detected with the 1G12 mAb. The 3A9 TCR+ cells are electronically gated to select for all 3A9 TCR+ T cells (Fig. 1A, left). The proportion of CD4+, CD8+, and CD4-CD8- T cells is shown among 3A9 TCR+ T cells (Fig. 1A, right panel). In this example, the total proportion of 3A9 DN T cells in lymph nodes is 18%, where we find 44.2% of CD4-CD8- cells among 40.6% of 3A9 TCR+ cells (44.2% × 40.6% = 18.0%). The proportion of 3A9 DN T cells in the 171 3A9 TCR+F2 (B10.BR × NOD.H2k) mice varies from 5 to 34% and presents with a Gaussian distribution (Fig. 1B). In agreement with our previous finding that the Idd13 locus only partially restores DN T cell proportion in NOD mice (24), the normal distribution suggests that more than one locus contributes toward defining the proportion of 3A9 DN T cells in the lymph nodes.

For the linkage analyses, we subjected the DNA of the 171 F2 mice to the Illumina Golden Gate low-density platform and performed a genome-wide SNP genotyping. Of note, because the 3A9 TCR transgene is located on chromosome 5 (44) and we select for 3A9 TCR+ mice for analysis, the data for chromosome 5 could not be included in the linkage analysis. The linkage of the genotypes to the phenotypes demonstrated that a locus on chromosome 9 is tightly linked to the proportion of 3A9 TCR DN T cells in the lymph nodes, presenting with a maximal LOD score >4 (Fig. 2). Interestingly, this region of chromosome 9 is comprised within the Idd2 diabetes-resistance locus. As we have previously shown that 3A9 DN T cells can confer resistance to diabetes (22), it is tempting to suggest that the regulation of DN T cell proportion contributes to the diabetes-resistant trait conferred by the Idd2 locus. Moreover, the distal region of chromosome 2, which encompasses the Idd13 locus known to partially restore DN T cell number (24), exceeded the suggestive threshold along with five other genetic regions, namely, on chromosomes 1, 4, 12, and two regions on chromosome 6 (Fig. 2). As for the loci on chromosomes 2 and 9, three of the five other intervals reaching the suggestive threshold also coincide with known autoimmune susceptibility loci, namely, the Idd9 locus on chromosome 4, the Iddb and Idd19 loci on distal chromosome 6, and the Nbwal quantitative trait locus on chromosome 12 (Fig. 3). Together, these data suggest that the proportion of 3A9 DN T cells is associated with more than one locus, which are linked to autoimmune susceptibility loci.

The most significant linkage for 3A9 DN T cell proportion was observed on chromosome 9. Although we have previously shown that the proportion of 3A9 CD4+ and CD8+ T cells are similar between both 3A9 TCR B10.BR and 3A9 TCR NOD.H2k parental strains (24, 30), this does not discount the possibility that the

![Figure 4](http://www.jimmunol.org/DownloadedFrom)  
**FIGURE 4.** Genome-wide linkage analysis for the proportion of 3A9 CD4+ T cells and 3A9 CD8+ T cells. Genome-wide LOD score plot (R/Qtl) for the proportion of (A) 3A9 CD4+ T cells and (B) 3A9 CD8+ T cells in the 171 F2 cohort is shown. The full and dotted lines, respectively, indicate the significance threshold of $p < 0.05$ and the suggestive threshold.
variation in 3A9 DN T cell proportion in the F2 mice results from secondary variations in either 3A9 CD4+ or CD8+ T cells. Therefore, to validate that this linkage was indeed specific to 3A9 DN T cells, we plotted the linkage analysis for both 3A9 CD4+ and CD8+ T cells (Fig. 4). We find that both 3A9 CD4+ T cells and 3A9 CD8+ T cells are respectively associated with significant linkages on chromosomes 7 and 3, but not on chromosome 9, demonstrating that the locus on chromosome 9 is linked to 3A9 DN T cell proportion and not to variations in other 3A9 T cell populations.

To better define the genetic interval on chromosome 9 linked to 3A9 DN T cell proportion, we generated a higher resolution map by including six additional genetic markers, namely, D9Mit328, D9Mit330, D9Mit4, D9Mit21, D9Mit232 and D9Mit302 (Fig. 5A). We find that five of these markers exceed the significance threshold. To determine the extent of the contribution of this locus toward determining the proportion of 3A9 DN T cells in the lymph nodes, we segregated the 171 F2 mice according to their genotype at the D9Mit232 marker, exhibiting the highest LOD score of 4.27. F2 mice carrying the homozygous B10.BR allele at the D9Mit232 marker presented with a proportion of 3A9 DN T cells ranging from 5 to 29%, with an average of 17% (Fig. 5B). However, F2 mice homozygous for the NOD allele at that marker showed a significantly reduced proportion of 3A9 DN T cells, with an average of 12% (Fig. 5B). This result suggests that the B10.BR alleles on chromosome 9 near the D9Mit232 marker contribute toward increasing the proportion of 3A9 DN T cells in the lymph nodes. Finally, F2 mice carrying a heterozygous genotype for the B10.BR/NOD allele at that marker showed a proportion of 3A9 DN T cells varying from 4 to 34%, with an average of 15%, not significantly different from mice that bear the B10.BR homozygous genotype (Fig. 5B). This result suggests that the B10.BR alleles, in relation to the NOD alleles at or near the D9Mit232 marker, act either in a dominant or codominant fashion for defining the proportion of 3A9 DN T cells in the lymph nodes.

Together, the earlier results strongly suggest that the genetic determinants near the D9Mit232 marker on mouse chromosome 9 contributes toward defining the proportion of 3A9 DN T cells. To validate this result, we generated 3A9 TCR NOD.H2k congeneric mice bearing B10.BR alleles on chromosome 9. We performed six backcrosses to the 3A9 TCR NOD.H2k background by genotyping for B10.BR alleles at D9Mit129 and D9Mit328 at each generation, where both markers were found near the highest LOD score in the preliminary linkage analysis composed of 93 F2 mice (data not shown). We obtained 3A9 TCR NOD.H2k-Chr9L congeneric mice, where L stands for “long.” During the sixth backcross, we also obtained a recombinant mouse that bore B10.BR alleles only at the D9Mit129 marker. We named this strain 3A9 TCR NOD.H2k-Chr9S, where S stands for “short.”

We crossed the mice bearing a heterozygous genotype at either the Chr9L or the Chr9S loci to obtain homozygous mice at each locus. The purity of the genetic background of the homozygous mice from both strains was interrogated using the Illumina Golden Gate medium-density platform to perform a genome-wide SNP genotyping. Apart from the chromosome 9 genetic locus, both strains showed >99% homology to the NOD.H2k parental strain, where none of the potentially B10.BR contaminating DNA was located within known Idd loci (data not shown). We did observe 0.3% of B10.Br alleles in both congeneric strains, which were located on chromosome 15 in an interval not linked to the proportion of 3A9 DN T cells (Fig. 2 and data not shown). With this genome-wide genotyping analysis, we also delimited the B10.BR congeneric intervals in both chromosome 9 congeneric lines as depicted on Fig. 6. Note that both the Chr9L and the Chr9S intervals at least partly coincide with a segment of the Idd2 diabetes-resistance locus.

**FIGURE 6.** Delimitation of the B10.BR interval from the two congeneric lines. The B10.BR congeneric intervals in the 3A9 TCR NOD.H2k-Chr9L and 3A9 TCR NOD.H2k-Chr9S strains are shown, based on a medium-density linkage analysis. (A) The chromosome 9 is fully depicted for all strains. (B) High resolution of the boxed interval in (A) to represent the Chr9 congeneric intervals. Marker position is based on National Center for Biotechnology Information build m37. The D9Mit129 and D9Mit328 markers, used for genotyping the mice for the backcross, are also included on the map.
With our newly generated 3A9 TCR NOD.H2\(^k\)-Chr9L and 3A9 TCR NOD.H2\(^k\)-Chr9S congenic mice, we determined the contribution of these loci in defining the proportion of 3A9 DN T cells (Fig. 7A). Importantly, we observed no difference in the proportion of 3A9 DN T cells between the parental 3A9 TCR NOD.H2\(^k\) and the 3A9 TCR NOD.H2\(^k\)-Chr9S congenic strains relative to that of 3A9 TCR B10.BR mice, where 3A9 TCR B10.BR mice present with ∼8 and 50% of 3A9 DN T cells in the spleen and lymph nodes, respectively (Fig. 7B). Still, because both the Chr9L and the Chr9S genetic intervals from the B10.BR strain demonstrate an equivalent potential to partially restore the proportion of 3A9 DN T cells in 3A9 TCR NOD.H2\(^k\) mice, this suggests that the genetic determinants defining the proportion of 3A9 DN T cells are likely to be encoded within the shorter Chr9S interval. Taken together, these data demonstrate that the Chr9S locus is sufficient to partially restore the proportion of 3A9 DN T cells in 3A9 TCR NOD.H2\(^k\) mice.

Because the Idd2 locus partially coincides with both the Chr9L and the Chr9S loci, we opted to define whether the complete Chr9L or the shorter Chr9S locus would contribute to autoimmune diabetes resistance. To address this, we took advantage of the fact that both 3A9 TCR NOD.H2\(^k\)-Chr9L and the 3A9 TCR NOD.H2\(^k\)-Chr9S congenic strains relative to that of 3A9 TCR B10.BR mice, where 3A9 TCR B10.BR mice present with ∼8 and 30% of 3A9 DN T cells in the spleen and lymph nodes, respectively (Fig. 7B). Specifically, both congenic strains exhibited an average of 2 and 6% of 3A9 DN T cells in the spleen and lymph nodes, respectively, representing almost a 2-fold increase over that of 3A9 TCR NOD.H2\(^k\) mice (Fig. 7B). Expectedly, because the proportion of 3A9 DN T cells is regulated by more than one locus and the linkage analysis explains only 9.8% of the variance, the proportion of 3A9 DN T cells was only partially restored in both the 3A9 TCR NOD.H2\(^k\)-Chr9L and the 3A9 TCR NOD.H2\(^k\)-Chr9S congenic strains relative to that of 3A9 TCR B10.BR mice, where 3A9 TCR B10.BR mice present with ∼8 and 30% of 3A9 DN T cells in the spleen and lymph nodes, respectively (Fig. 7B). Still, because both the Chr9L and the Chr9S genetic intervals from the B10.BR strain demonstrate an equivalent potential to partially restore the proportion of 3A9 DN T cells in 3A9 TCR NOD.H2\(^k\) mice, this suggests that the genetic determinants defining the proportion of 3A9 DN T cells are likely to be encoded within the shorter Chr9S interval. Taken together, these data demonstrate that the Chr9S locus is sufficient to partially restore the proportion of 3A9 DN T cells in 3A9 TCR NOD.H2\(^k\) mice.

FIGURE 7. 3A9 TCR NOD.H2\(^k\)-Chr9L and 3A9 TCR NOD.H2\(^k\)-Chr9S congenic mice exhibit an increase in the proportion of 3A9 DN T cells. (A) Representative flow cytometry profiles of 1G12 + (3A9 TCR) CD3 + cells among CD4 + CD8 + cells. The proportion of 3A9 DN T cells in double-transgenic mice are from the sixth-generation backcross. *p < 0.05, **p < 0.01.

We have previously shown that elevated proportions of 3A9 DN T cells in secondary lymphoid organs correlate with decreased diabetes susceptibility. We thus expected that 3A9 TCR:iHEL NOD.H2\(^k\)-Chr9L mice, which exhibit a higher proportion of 3A9 DN T cells, would be more resistant to autoimmune diabetes than both 3A9 TCR:iHEL NOD.H2\(^k\) and 3A9 TCR:iHEL NOD.H2\(^k\)-Chr9S mice. Indeed, by monitoring the diabetes incidence, we found that, similar to the 3A9 TCR:iHEL B10.BR mice, only 25% of 3A9 TCR:iHEL NOD.H2\(^k\)-Chr9L female mice progressed toward overt diabetes within 34 wk (Fig. 8A). However, although 3A9 TCR:iHEL NOD.H2\(^k\)-Chr9S mice seemed to exhibit a lower diabetes incidence than 3A9 TCR:iHEL NOD.H2\(^k\) mice, this did not reach statistical significance in our relatively small cohort (Fig. 8A). Expectedly, as a greater proportion of 3A9 TCR:iHEL NOD.H2\(^k\) mice progressed to overt diabetes, the degree of lymphocytic infiltration in mice from the diabetes incidence study was slightly more severe in this strain relative to the other strains (Fig. 8B, Table I). Altogether, these data support the view that elevated 3A9 DN T cell number correlates with autoimmune di-
male mice are littermate controls from either the 3A9 TCR:iHEL NOD.

H2k

Chr9L or the 3A9 TCR:iHEL NOD.

H2k

Chr9L (n elimination of activated B cells (22, 23). We thus assessed whether

leads to a reduction in autoantibody levels, likely because of the

transfer of 3A9 DN T cells in 3A9 TCR:iHEL diabetes-prone mice

autoantibody production (47). In addition, we have shown that

NOD.

mice and for female 3A9 TCR:iHEL B10.BR (p

was scored in nondiabetic mice

infiltration is shown for mice included in the diabetes incidence study and

genotype for the Chr9L or Chr9S interval was homozygous for NOD alleles.

n mice were analyzed for islet infiltration: B10.BR, age, after confirmation of the diabetes status. The following numbers of

mice (Fig. 8A). Interestingly, the decrease in HEL-specific IgG Abs in the 3A9 TCR:iHEL NOD.

H2k

Chr9L and 3A9 TCR:iHEL NOD.

H2k

Chr9S mice was not limited to HEL specificity, because total IgG levels were also reduced in these mice (Fig. 9A). In addition, a comparable degree of lymphocytic infiltration in the islets of these euglycemic mice was observed (Fig. 9B, Table II), demonstrating that the decrease in IgG levels was not due to a decrease in the autoimmune response toward islet Ags. It is rather likely attributed to the nature of this response. Together, these findings suggest that genetic determinants within the Chr9L locus lead to an increase in DN T cell number accompanied by a parallel decrease in IgG serum levels and a significant decrease in diabetes progression.

Discussion

Immunoregulatory DN T cells contribute to immune tolerance in various pathological settings, including autoimmune diabetes (11, 15, 21, 22, 37). The elevated proportion of DN T cells in autoimmune-resistant relative to autoimmune-prone mice has allowed us to perform a linkage analysis and uncover that the proportion of DN T cells is a complex trait. Importantly, we show that the diabetes-resistance locus, Idd2, is linked to this trait. In addition, we develop two congenic lines bearing B10.BR alleles on chromosome 9, in a locus that partially encompasses Idd2. Using these congenic lines, we demonstrate that the locus on mouse chromosome 9 impacts the proportion of immunoregulatory DN T cells, a trait associated with autoimmune diabetes resistance.

The Idd2 locus was initially linked to diabetes resistance in an NOD to NON outcross (48, 49). Interestingly, the NON strain exhibits a low proportion of total CD4+ and CD8+ T lymphocytes, and Tlf (for T lymphocyte frequency) coincides with Idd2 (50, 51). Using the B10.BR to NOD.

H2k

outcross, we found that Idd2 is linked to the proportion of a T cell subset, namely, DN T cells. However, we did not find that Idd2 is linked to the proportion of CD4+ or CD8+ T cells in the F2 outcross. The reason for the discrepancy between these results is likely due to the different parental strains used, because B10.BR mice, in contrast with NON mice, do not present with a low T cell proportion.

Initial studies attempting to generate NOD.NON-Idd2 congenic lines unfortunately lost the diabetes resistance trait because of a high frequency of recombination events between the original linkage and the trait (52). We have now successfully generated NOD.

H2k

Chr9L congenic mice, which are able to confer diabetes resistance at least in the 3A9 TCR:iHEL NOD.

H2k

mouse model.

Table I. Islet count details for mice included in diabetes incidence in Fig. 8B

|       | B10.BR | NOD.

H2k

| NOD.

H2k

Chr9L | NOD.

H2k

Chr9S |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>6</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Total islets counted per strain</td>
<td>170</td>
<td>171</td>
<td>173</td>
<td>107</td>
</tr>
<tr>
<td>Mean no. of islets per cut</td>
<td>9.07</td>
<td>5.7</td>
<td>6.91</td>
<td>7.13</td>
</tr>
</tbody>
</table>
To our knowledge, this is the first demonstration that an NOD congenic mouse strain for a genetic interval coinciding with the Idd2 locus is protected from diabetes onset. Because the Chr9L interval coincides, at least in part, with the Idd2 locus, we propose that genetic determinants encoded within the Chr9L interval define Idd2. The relevant genetic determinants would most likely be encoded outside of the Chr9S interval, which confers only modest diabetes resistance in the 3A9 TCR:iHEL NOD.H2S mouse model. Alternatively, genetic interactions between genes encoded with both the Chr9L and the Chr9S intervals could contribute to the diabetes resistance trait. Additional congenic mice will need to be generated to evaluate these hypotheses. In addition, we are currently performing an outcross of the 3A9 TCR:iHEL NOD.H2S-Chr9L to NOD mice, to ultimately generate nontransgenic NOD-Chr9L mice, in which we will be able to validate the contribution of this interval to diabetes resistance in the nontransgenic mice bearing the H2S MHC susceptibility locus.

As mentioned earlier, the proportion of DN T cells is defined by a complex trait, including Idd2, Idd13, and potentially five other suggestive loci. Using congenic mice, we have validated that both Idd2 and Idd13 partially contribute toward defining the proportion of DN T cells. Additional congenic mice will need to be generated to determine the contribution of the other genetic loci in defining the proportion of DN T cells. Moreover, double-congenic mice will need to be generated to establish whether each locus independently or synergistically contributes to the trait. To that effect, we are currently intercrossing 3A9 TCR NOD.H2S-Idd13 mice to 3A9 TCR NOD.H2S-Chr9L mice. This experiment will allow us to establish the potential genetic interactions between these two loci in defining the proportion of 3A9 DN T cells and conferring resistance to autoimmune diabetes.

By comparing the data from our two congenic strains, namely, 3A9 TCR:iHEL NOD.H2S-Chr9L and 3A9 TCR:iHEL NOD.H2S-Chr9S mice, we found that the proportion of DN T cells, the serum IgG levels, and the resistance to diabetes are likely to be defined by at least two genetic determinants within the Chr9L interval, where at least one is encoded within and one outside of the Chr9S interval. Indeed, although 3A9 TCR NOD.H2S-Chr9S mice exhibit the same proportion of 3A9 DN T cells to the 3A9 TCR NOD.H2S-Chr9L mice, the Chr9S interval only partially contributes to the phenotypes in the 3A9 TCR:iHEL double-transgenic setting. Interestingly, of the genes encoded within the Chr9S interval, we found genes of the CD3 complex, which could potentially contribute to the homeostatic regulation of DN T cells. Thy1 and Cbl, among many others, also present as likely candidate genes for influencing the proportion of DN T cells. However, these genes have also been associated with autoimmune phenotype (55–56), and 3A9 TCR NOD.H2S-Chr9S mice exhibit little, if any, resistance to autoimmune diabetes, suggesting that the gene contributing to the DN T cell phenotype in the Chr9S interval is likely to be distinct from these. Still, it is possible that the Chr9S interval would contribute to diabetes resistance in a setting other than in the 3A9 TCR transgenic model, such that these candidate genes cannot be readily discarded. Nevertheless, Ii10ra, also included within the Chr9S interval, has particularly grabbed our attention for defining the proportion of DN T cells. Indeed, we have previously shown that IL-10 limits the expansion of 3A9 DN T cells in vitro. We are currently attempting to define whether the Ii10ra B10.BR gene explains the increased DN T cell number in the 3A9 TCR NOD.H2S-Chr9S congenic mice. Among the candidate genes present within the Chr9L interval, but not in the Chr9S interval, we found Crium, which has been associated with diabetes induction in a mouse model of induced autoimmune diabetes and is expressed on some T cell populations (57). Estam, involved in hematopoiesis (58), is also of interest considering that

Table II. Islet count details for euglycemic mice included in Fig. 9B

<table>
<thead>
<tr>
<th></th>
<th>B10.BR</th>
<th>NOD.H2S</th>
<th>NOD.H2S-Chr9L</th>
<th>NOD.H2S-Chr9S</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Total islets counted per strain</td>
<td>176</td>
<td>102</td>
<td>197</td>
<td>255</td>
</tr>
<tr>
<td>Mean no. of islets per cut</td>
<td>8.38</td>
<td>4.96</td>
<td>8.33</td>
<td>9.2</td>
</tr>
</tbody>
</table>

FIGURE 9. Reduced IgG serum Ab levels in 3A9 TCR:iHEL NOD.H2S-Chr9L congenic mice. (A) Anti-HEL IgG, anti-HEL IgG1, anti-HEL IgM, and total IgG levels in the serum of 9- to 18-wk-old male or female nondiabetic congenic and control mice are shown relative to a pool of serum from 3A9 TCR:iHEL NOD.H2S mouse, which was set as a value of 1. Each dot represents data for one mouse. Data under detection threshold are excluded from the statistical tests. **p < 0.01. (B) The degree of islet infiltration is shown for euglycemic male and female mice aged between 7 and 18 wk. The following number of mice was analyzed for islet infiltration: B10.BR, n = 7; NOD.H2S, n = 8; NOD.H2S-Chr9L, n = 8; NOD.H2S-Chr9S, n = 9.
DN T cell proportion is regulated by bone marrow–intrinsic factors (24). Tlrap and Thbg1 are two other genes within the Ch9L interval known to play significant roles in the immune system, which could be candidate genes for defining the proportion of DN T cells and susceptibility to diabetes.

In summary, studying the genetic underpinnings of immunoregulatory DN T cells has revealed that, as for susceptibility to autoimmune diabetes, it is a complex trait. Interestingly, at least two Idd susceptibility loci are linked to the proportion of DN T cells, emphasizing their relevance in contributing to autoimmune diabetes resistance. Identifying the genetic determinants defining the proportion of DN T cells may unravel key molecular targets to increase DN T cells in lymphoid organs, and thereby permit an increase in immune tolerance to prevent autoimmune disease onset. The congenic mice developed in this study are important tools that will serve toward this goal.

Acknowledgments
We thank Dr. Edward Leiter for providing a critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


