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Congenic mapping identifies a novel *Idd9* subregion regulating type 1 diabetes in NOD mice

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Abstract

Type 1 diabetes (T1D) results from complex interactions between genetic and environmental factors. The nonobese diabetic (NOD) mouse develops spontaneous T1D and has been used extensively to study the genetic control of this disease. T1D is suppressed in NOD mice congenic for the C57BL/10 (B10)-derived *Idd9* resistance region on chromosome 4. Previous studies conducted by other investigators have identified four subregions (*Idd9.1*, *Idd9.2*, *Idd9.3*, and *Idd9.4*) where B10-derived genes suppress T1D development in NOD mice. We independently generated and characterized six congenic strains containing B10-derived intervals that partially overlap with the *Idd9.1* and *Idd9.4* regions. T1D incidence studies have revealed a new B10-derived resistance region proximal to *Idd9.1*. Our results also indicated that a B10-derived gene(s) within the *Idd9.4* region suppressed the diabetogenic activity of CD4 T cells and promoted CD103 expression on regulatory T cells indicative of an activated phenotype. In addition, we suggest the presence of a B10-derived susceptibility gene(s) in the *Idd9.1/Idd9.4* region. These results provide additional information to improve our understanding of the complex genetic control by the *Idd9* region.

Keywords

type 1 diabetes; *Idd9*; regulatory T cells; NOD mouse

Type 1 diabetes (T1D) in both human and nonobese diabetic (NOD) mice is a polygenic autoimmune disease caused by T cell-mediated destruction of pancreatic β -cells (Driver et al. 2011; Todd 2010). Excess inflammation generated by innate immunity participates in the initiation of autoimmune diabetes in the NOD mouse model as well as in humans (Chen et al. 2014; Diana et al. 2013; Ferreira et al. 2014; Kallionpaa et al. 2014). This in turn leads to activation and accumulation of autoreactive T cells that contribute to subsequent stages of the disease. Both CD4 and CD8 T cells participate in the development of T1D (Christianson

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et al. 1993; DiLorenzo et al. 1998). Pathogenic CD4 T cells promote T1D development by secreting inflammatory cytokines, enhancing the immunogenic functions of antigen presenting cells, and providing help to cytotoxic CD8 T cells. Pathogenic CD8 T cells directly kill pancreatic β -cells through the recognition of peptides presented by major histocompatibility complex (MHC) class I molecules. Autoreactive T cells can be controlled by FOXP3⁺ regulatory CD4 T cells (Tregs) (Bettini and Vignali 2009; Jeker et al. 2012). Cellular and genetic defects associated with Tregs have been reported in both NOD mice and T1D patients (Garg et al. 2012; Gregg et al. 2004; Lindley et al. 2005; Long et al. 2010; McClymont et al. 2011; Pop et al. 2005; Tritt et al. 2008; Yamanouchi et al. 2007). NOD mice have been used extensively to study the genetic factors contributing to T1D development (Driver et al. 2011; Wicker et al. 2005). While the *H2^{g7}* haplotype provides the primary component of T1D susceptibility in NOD mice, it alone is not sufficient for disease development (Driver et al. 2011). In conjunction with the *H2^{g7}* MHC, other insulin dependent diabetes susceptibility (*Idd*) genes are also required to interactively contribute to the development of diabetogenic T cell responses in NOD mice (Driver et al. 2011). Similarly, human genome-wide association studies have also revealed more than 40 non-MHC genetic loci associated with T1D risk (Barrett et al. 2009; Onengut-Gumuscu et al. 2015).

The *Idd9/Idd11* genetic loci located on mouse chromosome 4 are particularly complex regions contributing to T1D susceptibility or resistance and have been the interest of many studies (Brodnicki et al. 2005; Cannons et al. 2005; Hamilton-Williams et al. 2013; Hamilton-Williams et al. 2010; Kachapati et al. 2012; Lyons et al. 2000; Tan et al. 2010; Yamanouchi et al. 2010). NOD mice congenic for the C57BL/10 (B10) derived *Idd9* region are highly resistant to T1D development when compared to NOD mice (Lyons et al. 2000). Subsequent analyses revealed that B10 *Idd9* mediated diabetes resistance is caused by the cumulative protective effect of at least four subregions named *Idd9.1*, *Idd9.2*, *Idd9.3*, and *Idd9.4* (Hamilton-Williams et al. 2013). Parallel to these *Idd9* mapping studies conducted by other laboratories, we independently backcrossed the previously reported line 905 (Hamilton-Williams et al. 2013) to NOD/LtDvs (NOD hereafter) mice, genotyped microsatellite and single nucleotide polymorphism (SNP) markers to screen for recombination, and generated 3 distinct subcongenic strains (Lines 1 to 3 in Figure 1). The B10-derived regions in Lines 1 to 3 overlap to various extents with the recently redefined *Idd9.1* and *Idd9.4* loci (Figure 1 and (Hamilton-Williams et al. 2013)). We redefined the proximal boundary of line 905 and confirmed it to be the same in Lines 1, 2, and 3 (between rs27499480 and rs27516145, NOD and B10 are identical by descent in this region, Figure 1). Diabetes incidence was followed in female mice by weekly testing urine glucose using Diastix (Bayer). Mice were considered diabetic after two consecutive readings of ≥ 250 mg/dL. Diabetes incidence was plotted by Kaplan-Meier curves and compared by the log-rank test using the Prism software (Graphpad). T1D development in Lines 1, 2, and 3 was significantly suppressed when compared to NOD mice ($p < 0.0001$, Figure 2a).

To further refine the diabetes resistance region(s) present in Lines 1 and 2 we backcrossed these stocks to NOD mice respectively to create Line 6 as well as Lines 4 and 5 (Figure 1). Diabetes incidence studies did not reveal any difference between Lines 4 to 6 and standard NOD mice (Figures 2b and 2c). Since T1D was suppressed in Line 2 but not in Lines 4 and

5, the results suggest a novel B10-derived region (between rs13477946 and D4Mit11) conferring diabetes resistance in NOD mice. However, we cannot completely rule out the possibility that the congenic region in Line 4 contains a resistance gene that is not sufficient to confer diabetes suppression on its own or its T1D protective effect is masked by another B10-derived susceptibility gene, similar to the case of Line 5 discussed below. Therefore, we define the newly identified B10 resistance region (tentatively named *Idd9.5*) as the segment between rs27516145 and D4Mit11 (Figure 1). We searched Mouse Genome Informatics (www.informatics.jax.org) for genes mapped to the *Idd9.5* region. The 3.47 Mb *Idd9.5* region contains 65 genes of which *Zfp69* has been shown to be differentially expressed between NOD and B10 alleles (Berry et al. 2015). *Zfp69* represents a potential candidate gene as it has been linked to obesity-associated diabetes and modulation of hepatic insulin sensitivity in mice (Chung et al. 2015; Scherneck et al. 2009). Although a role of *Zfp69* in the immune system has not been reported, its function in regulating insulin sensitivity may indirectly contribute to T1D.

The lack of T1D protection in Line 5 was unexpected since it harbors the entire *Idd9.1* region defined by Hamilton-Williams et al. in 2013 and *Idd11* mapped in Tan et al. in 2010 (Figure 1), and B10 and B6 alleles of the *Idd11* candidate gene *AK005651* (also known as *1700003M07Rik*) are identical. This observation suggested the presence of a B10 diabetes susceptibility region in Line 5 that masked the T1D protective effect of B10 *Idd9.1*. This possibility is also supported by the comparison between Lines 1 and 3, and between Lines 2 and 3. Although Lines 1 and 3 did not differ from each other, Line 2 was significantly less protected than Line 3 ($P = 0.0305$, Figure 2a), suggesting a B10 susceptibility region between rs27516779 and D4Mit71 that partially overlap with *Idd9.1* and *Idd9.4* (Figure 1). Analyses of additional congenic strains are required to confirm and further define this B10 susceptibility locus. Direct comparison between Lines 2 and 3 also indicates the B10 resistance interval between rs13459077 and rs3718220 (Figure 1), likely representing the previously reported *Idd9.4* region (Hamilton-Williams et al. 2013).

We have previously observed that genes within the NOR-derived *Idd9/Idd11* resistance region regulates the diabetogenic activity of CD4 T cells (Chen et al. 2008; Stolp et al. 2012). Other studies have also demonstrated that the B10 *Idd9* protective effect is in part mediated by CD4 T cells (Hamilton-Williams et al. 2010; Waldner et al. 2006; Yamanouchi et al. 2010). Therefore, we asked if the diabetogenic activity of CD4 T cells isolated from Lines 1, 2, and 3 was suppressed compared to that of the NOD counterparts. β -cell autoreactive CD8 T cells of the NY8.3 clonotype require CD4 T cell help to fully exert their diabetogenic function (Serra et al. 2003; Verdaguer et al. 1997). We obtained NOD.NY8.3 (stock# 005868) and NOD.*Rag1*^{-/-} (stock# 003729) mice from The Jackson Laboratory to subsequently generate through interbreeding the NOD.*Rag1*^{-/-}.NY8.3 strain. We then purified splenic CD4 T cells from 7 to 8-week-old NOD as well as Lines 1, 2, and 3 mice by the previously described magnetic bead system (Chen et al. 2008) and intravenously transferred them (5×10^6 cells) into 7 to 8-week-old NOD.*Rag1*^{-/-}.NY8.3 recipients to test their diabetogenic activity. Purity of the isolated CD4 T cells was routinely verified by flow cytometry to be higher than 92%. Female mice were used for both donors and recipients. T1D development in the CD4 T cell recipients was monitored as described above. CD4 T cells isolated from Line 3 was found to be significantly less diabetogenic than those from

standard NOD mice (Figure 3). On the other hand, B10-derived congenic regions in Lines 1 and 2 did not suppress the diabetogenic activity of the CD4 T cells (Figure 3). Further supporting the presence of a B10-derived disease susceptibility gene(s) in the Line 2 congenic region was the finding that adoptively transferred CD4 T cells from this stock were more diabetogenic than those from standard NOD mice but not the counterparts from Line 1 (Figure 3). Together these results indicate that B10-derived *Idd9.4* genes mediate T1D protection at the level of CD4 T cells.

The *Idd9.1* interval described earlier by Yamanouchi et al. in 2010 is within Line 3 (Figure 1). This region was shown to regulate the development and function of CD4+ FOXP3+ regulatory T cells (Tregs). Thus, we compared the expression of a panel of markers (CD25, CTLA4, CD73, GITR, OX40, LAG-3, GARP, ICOS, CD137, and CD103) on splenic Tregs (identified as CD4+ FOXP3+) in 8 to 10-week-old standard NOD and Line 3 congenic females by flow cytometry. We consistently observed a higher expression level of CD103, but not other markers, on Tregs in Line 3 than in NOD mice (data not shown). We further compared the frequencies of CD103+ Tregs in the spleens and pancreatic lymph nodes (PLNs) of Lines 1, 2, and 3 to map the genetic region that controls the accumulation of this regulatory subset. Compared to standard NOD mice, we did not observe any difference in the frequency of total Tregs in Lines 1, 2, and 3 (Figures 4a and 4c). Consistent with our earlier studies, the percentages of CD103+ Tregs were higher in both the spleens and PLNs of Line 3 than those in NOD mice (Figures 4b and 4d). On the other hand, the levels of CD103+ Tregs were comparable in spleens and PLNs between NOD mice and Line 1 (Figures 4b and 4d). Compared to NOD mice, the frequencies of CD103+ Tregs were found to be similar in the spleens but slightly lower in the PLNs of Line 2 (Figures 4b and 4d). CD103 has been shown as a marker of activated Tregs (Huehn et al. 2004; Zhao et al. 2008). Therefore, our results suggest that the *Idd9.4* region genes control Treg activation and this phenotype likely contributed to the reduced diabetogenic activity of Line 3 CD4 T cells in the adoptive transfer studies shown in Figure 3.

In summary, we identified a novel *Idd9* subregion (*Idd9.5*) that regulates T1D development in the NOD mouse model. Our studies also indicate that an *Idd9.4* gene(s) controls the diabetogenic activity of CD4 T cells, possibly through enhancing Treg activation. Lastly, we suggest the presence of a B10 susceptibility gene(s) in the *Idd9.1/Idd9.4* region. Our studies provide additional insight into the complexity of the *Idd9*-mediated T1D genetic control.

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Fig. 1.

Genetic map of the *Idd9* subcongenic lines used in this study. The intervals of the previously defined *Idd9.1* and *Idd9.4* regions are also indicated.

Fig. 2.

T1D Incidence of congenic lines and NOD mice. (a) NOD and Lines 1–3 females were monitored for T1D. (b) NOD and Lines 4 and 5 females were monitored for T1D. (c) NOD and Line 6 females were monitored for T1D. Statistical analysis was performed by the log-rank test.

P value	Line 1	Line 2	Line 3
NOD	0.8045	0.0215	0.021
Line 1		0.2691	0.0262
Line 2			< 0.0001

Fig. 3.

Idd9.4 region genes regulate the diabetogenic activity of CD4 T cells. NOD.*Rag1*^{-/-}.*NY8.3* mice receiving purified splenic CD4 T cells (5×10^6) from NOD or Lines 1–3 were followed for diabetes incidence. Statistical analysis was performed using the log-rank test.

Fig. 4.

Idd9.4 region genes modulate CD103 expression on Tregs. Splenocytes (a and b) and pancreatic lymph node cells (c and d) of 7-week-old females were analyzed for the frequency of Tregs (defined as CD4+ FOXP3⁺) and their expression of CD103. Each symbol represents an individual mouse. Horizontal lines represent the mean. Statistical analysis is performed using the Mann-Whitney test.