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# Subcongenic Analyses Reveal Complex Interactions between Distal Chromosome 4 Genes Controlling Diabetogenic B Cells and CD4 T Cells in Nonobese Diabetic Mice

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Autoimmune type 1 diabetes (T1D) in humans and NOD mice results from interactions between multiple susceptibility genes (termed *Idd*) located within and outside the MHC. Despite sharing ~88% of their genome with NOD mice, including the *H2<sup>g7</sup>* MHC haplotype and other important *Idd* genes, the closely related nonobese resistant (NOR) strain fails to develop T1D because of resistance alleles in residual genomic regions derived from C57BLKS mice mapping to chromosomes (Chr.) 1, 2, and 4. We previously produced a NOD background strain with a greatly decreased incidence of T1D as the result of a NOR-derived 44.31-Mb congenic region on distal Chr. 4 containing disease-resistance alleles that decrease the pathogenic activity of autoreactive B and CD4 T cells. In this study, a series of subcongenic strains for the NOR-derived Chr. 4 region was used to significantly refine genetic loci regulating diabetogenic B and CD4 T cell activity. Analyses of these subcongenic strains revealed the presence of at least two NOR-origin T1D resistance genes within this region. A 6.22-Mb region between *rs13477999* and *D4Mit32*, not previously known to contain a locus affecting T1D susceptibility and now designated *Idd25*, was found to contain the main NOR gene(s) dampening diabetogenic B cell activity, with *Ephb2* and/or *Padi2* being strong candidates as the causal variants. Penetrance of this *Idd25* effect was influenced by genes in surrounding regions controlling B cell responsiveness and anergy induction. Conversely, the gene(s) controlling pathogenic CD4 T cell activity was mapped to a more proximal 24.26-Mb region between the *rs3674285* and *D4Mit203* markers. *The Journal of Immunology*, 2012, 189: 1406–1417.

Autoreactive T cells are responsible for destroying pancreatic  $\beta$  cells during the development of type 1 diabetes (T1D) in humans and the NOD mouse model (1, 2). A pathogenic role for B cells in this disease first became evident when NOD mice made deficient in this population through introduction of an inactivated *Igμ H chain* gene (NOD.*Igμ<sup>null</sup>*) or

chronic treatment with anti-IgM Abs were strongly protected from T1D (3, 4). More recent studies showed that temporal B cell depletion in NOD mice at different stages of disease, using Abs or chimeric proteins targeting CD20, CD22, or the B lymphocyte stimulator (Blys/BAFF), could be effective at delaying, inhibiting, or reversing the onset of T1D (5, 6). These findings were mirrored in patients with recent-onset T1D treated with the B cell-depleting CD20-specific rituximab mAb, who, on average, exhibited decreased loss of endogenous insulin production and reduced requirement for exogenous insulin over a 1-y follow-up period (7). This confirmed the role of B cells in the pathogenesis of human T1D, as well as showed that targeting this population could be a potentially effective therapeutic strategy.

Multiple lines of evidence indicate that the primary pathogenic function of B cells in T1D consists of acting as APCs for CD4 T cells. First, NOD.*Igμ<sup>null</sup>* mice were shown to produce poor CD4 T cell responses to T1D-associated autoantigens (8–10). Second, autoreactive CD4 T cell responses and the development of insulinitis and T1D could only be restored by reconstituting NOD.*Igμ<sup>null</sup>* mice with NOD B cells and not autoantibodies (9). Finally, strong T1D protection was observed in NOD bone marrow (BM) chimera mice whose B cells were made specifically deficient in MHC class II molecules (11). The pathogenic APC function of B cells was shown to be particularly dependent on their capacity to specifically capture Ags via surface Ig (12, 13). Thus, like T cells, B cells contributing to T1D must arise from defects in self-tolerance mechanisms, leading to the generation and activation of autoreactive clones. Indeed, defects in various tolerance-induction mechanisms, including receptor editing (14), anergy (15–17), and peripheral deletion of self-reactive clonotypes (15, 18), were shown to contribute to the generation of autoreactive B cells in NOD mice.

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Abbreviations used in this article: B6, C57BL/6; B10, C57BL/10; BKS, C57BLKS; BM, bone marrow; Chr., chromosome; FL, full length; FO, follicular; HEL, hen egg lysozyme; IgHEL, hen egg lysozyme-specific Ig; MZ, marginal zone; NOR, non-obese resistant; NR4, NOD.*NOR-Chr4*; q-rtPCR, quantitative real-time PCR; SBM, syngeneic bone marrow; sHEL, soluble hen egg lysozyme; SNP, single nucleotide polymorphism; T1D, type 1 diabetes; tg, transgenic; TJL, The Jackson Laboratory.

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Similar defects in B cell tolerance were recently reported in humans developing T1D (14, 19, 20). Given the important contribution of B cells to T1D, it was likely that their pathogenic capacity would be controlled by genes conferring susceptibility to this disease.

Predisposition to T1D in both NOD mice and humans is determined by multiple susceptibility genes located within and outside the MHC (21, 22). In NOD mice, these genes have been mapped to >40 loci (termed *Idd*) on 16 chromosomes (Chr.) (21). Although the *MHC class II* genes within the NOD *H2<sup>g7</sup>* haplotype (*Idl1*) are the most influential determinants of T1D in this strain, contributions by non-MHC susceptibility genes are also necessary for disease development (21). This is highlighted by the closely related nonobese resistant (NOR) mouse strain, which, despite sharing ~88% of its genome with NOD mice, including the *H2<sup>g7</sup>* MHC haplotype, exhibits strong protection from T1D (23). This protection is encoded within C57BLKS (BKS)-derived genetic regions (itself a combination of C57BL/6 [B6] and DBA/2 genomes) in NOR mice on Chr. 1, 2, and 4 (23–26). The NOR-derived T1D resistance locus on Chr. 2, termed *Idl13*, had not been observed in outcrosses of NOD mice with other strains. Conversely, the NOR-derived T1D resistance genes on Chr. 1 and 4 were mapped to similar regions as were the previously identified *Idd5.2/5.3* and *Idd9.1/9.2/11* loci, respectively, which were localized using NOD outcrosses with C57BL/10 [B10] or B6 disease-resistant strains (27–29). However, given their diverse origin from the BKS strain, it is also possible that the NOR-derived T1D resistance genes on Chr. 1 or 4 are not completely overlapping with those of B6 or B10 origin.

Our previous studies showed that reconstitution of lethally irradiated NOD.*Igμ<sup>null</sup>* mice with syngeneic BM (SBM) and purified NOD B cells prevented rejection of grafted B cells by host CTL and restored their susceptibility to T1D (9, 30). In contrast, those reconstituted with SBM only remained resistant to disease. Interestingly, reconstitution of NOD.*Igμ<sup>null</sup>* recipients with equivalent levels of B cells from NOR mice, instead of NOD mice, resulted in a significantly reduced incidence of disease (30). To identify the NOR-derived *Idd* resistance loci responsible for dampening the diabetogenic activity of B cells, NOD.*Igμ<sup>null</sup>* mice were reconstituted with B cells from NOD stocks containing NOR-derived congenic regions on Chr. 1, 2, or 4 (30). Only those B cells containing a congenic region on Chr. 4 (NOD.NOR-Chr4 [NR4]), spanning from 104.99 to 149.30 Mb (26), recapitulated the T1D resistance conferred by NOR B cells (30). Thus, genes within the *Idd9.1/9.2/11* regions, or alternatively a previously unrecognized locus, are important for inhibiting diabetogenic B cell activity in NOR mice.

NOD and NR4 B cells were subsequently compared for a number of characteristics to establish potential mechanisms by which polymorphic genes within the distal region of Chr. 4 controlled their diabetogenic activity. Although a previous study reported that genes within distal Chr. 4 contributed to exaggerated marginal zone (MZ) B cell development in NOD mice (31), splenic B cell subsets were comparable in NOD and NR4 mice (30). In addition, deletion of splenic transitional-1 B cells stimulated through the BCR was equivalent between strains. However, mature B cells from lymph nodes (mainly of the follicular [FO] subset) or spleen (FO and MZ subsets) of NR4 congenic mice proliferated more vigorously in response to BCR stimulation than did those from NOD mice; this was accentuated by CD40 costimulation (30). The NR4 congenic region was also shown to contribute to the maintenance of energy in self-reactive B cells (30). Thus, in contrast to *hen egg lysozyme (HEL)-specific Ig (IgHEL)* transgenic (tg) B cells on the NOD background, which showed equivalent levels of proliferation

following stimulation through the BCR and CD40, irrespective of whether they developed in the presence or absence of their cognate soluble neo-self-Ag (soluble HEL [sHEL]), similar B cells from the NR4 stock anergized properly in response to self-Ag exposure. A subsequent study found that the NR4 congenic region also had the capacity to intrinsically dampen the pathogenic activity of CD4 T cells contributing to T1D (32). It remains to be elucidated whether the CD4 T cell effect is controlled by the same or a distinct NOR gene(s) as that controlling B cells.

The NOR-derived congenic interval in NR4 mice is large (44.31 Mb, containing 889 known or predicted genes), making identification of genes regulating the pathogenic activity of B and CD4 T cells within this region difficult. In this study, we describe the generation of a series of novel subcongenic strains for the NOR-derived Chr. 4 region. B and CD4 T cells from subcongenic strains were used in various experimental strategies to significantly refine genetic regions within distal Chr. 4 that modulate development of T1D by controlling the pathogenic function of these lymphocyte populations.

## Materials and Methods

### Mice

All mice were housed at the Garvan Institute or The Jackson Laboratory (TJL) animal facilities under standard specific pathogen-free conditions with unrestricted access to food and acidified water. NOD/Lt mice were purchased from Australian BioResources (Moss Vale, NSW, Australia) or produced at TJL. Derivation of the NR4 strain (at N5) using a speed-congenic strategy was described previously (26). The proximal boundary of the congenic region in these mice lies between *rs3674285* (104.99 Mb) and *D4Mit31* (106.78 Mb). Screening of additional genetic markers identified a more distal boundary for the congenic region than originally described, which lies between *D4Mit129* (148.30 Mb) and *D4Mit127* (149.30 Mb). Seven new subcongenic strains (NR4S1–S4 and NR4S6–7 at N7 and NR4S5 at N8) containing smaller NOR-derived congenic regions on Chr. 4 were developed by outcrossing the NR4 strain with NOD mice and then backcrossing heterozygous progeny with NOD mice for two or three additional generations. Progeny of the first backcross were screened for recombinations within the original congenic interval by determining the origin of microsatellite markers at the proximal (*D4Mit31*) and distal (*D4Mit129*) boundaries. Those that were NOD homozygous at one of these markers were screened with an additional 22 microsatellite (primer sequences obtained from the Mouse Genome Informatics Web site [http://www.informatics.jax.org/] or Ref. 27) or single nucleotide polymorphism (SNP) markers (carried out by KBioscience, Hoddesdon, U.K.) to refine the location of recombinations. The NR4S5 strain, containing two recombinations within the original NR4 congenic interval, was generated by an additional backcross to NOD of a subcongenic mouse that was NOD/NOR heterozygous between microsatellites *D4Mit203* and *D4Mit129*. Mice with informative recombinations underwent a final backcross to NOD to produce male and female offspring with the same recombination, which were intercrossed to fix the congenic regions to homozygosity. Generation of NOD.*Igμ<sup>null</sup>* (3), NOD.*CD4<sup>null</sup>*, NOD.*A14αβ-tg* (33), and NOD.*B6-Ptprc<sup>b</sup>* (NOD.*CD45.2*) (34) mice, as well as NOD and NR4 mice expressing *IgHEL* and *sHEL* transgenes (12, 15, 30), were described previously. Only female mice were used in experiments, which were approved by the Garvan Institute/St. Vincent's Hospital or TJL Animal Ethics Committees.

### Assessment of T1D development

T1D development was assessed by measuring glycosuric values weekly with Ames Diastix (Bayer Corporation Diagnostics Division, Elkhart, IN). Values ≥500 mg/dl were considered indicative of overt diabetes.

### Mixed BM and B cell chimeras

CTLs in otherwise unmanipulated NOD.*Igμ<sup>null</sup>* mice destroy passively transferred B cells (9). Therefore, B cell reconstitution was achieved by lethally irradiating NOD.*Igμ<sup>null</sup>* mice between 4 and 5 wk of age with two 550-rad split doses from an x-ray source, followed by i.v. injection with  $3 \times 10^6$  anti-CD4 (GK1.5) and anti-CD8 (53-6.7, eBioscience, San Diego, CA)-coated SBM cells admixed with  $7 \times 10^6$  purified splenic B cells from the indicated strains. Splenic B cells were purified using the previously



described (9) MACS negative-depletion system (Miltenyi Biotec, Bergisch Gladbach, Germany), which routinely achieved >93% purity, as determined by flow cytometry. BM/B cell recipients were monitored weekly between 8 and 23 wk postreconstitution for T1D development. Upon disease onset or at the end of the incidence study (23 wk postreconstitution), spleens from recipient mice were assessed by flow cytometry to determine the percentage reconstitution of B cells and CD4 T cells using previously published methods and reagents (9). Flow cytometry was also used to establish reconstitution levels of lymphocytes expressing CD45.2 or CD45.1 allotypic markers in NOD.*Igμ<sup>null</sup>* recipients using the 104 and A20 Ab clones (BD Biosciences), respectively.

#### Mixed BM and CD4 T cell chimeras

NOD.*CD4<sup>null</sup>* mice were lethally irradiated (two 650-rad split doses from a [<sup>137</sup>Cs] source) and reconstituted i.v. with  $5 \times 10^6$  anti-CD8 (53-6.7; eBioscience)-coated SBM cells admixed with  $5 \times 10^6$  purified CD4 T cells from the indicated strains. Splenic CD4 T cells were purified using the previously described MACS negative-depletion strategy (32), which routinely achieved >92% purity, as determined by flow cytometry. BM/CD4 T cell recipients were monitored weekly between 8 and 20 wk postreconstitution for T1D development.

#### Adoptive transfer of A14 T cells

At 6–8 wk of age, the indicated recipient mice were sublethally irradiated (600 rad from a [<sup>137</sup>Cs] source) and injected i.v. with  $5 \times 10^6$  NOD.*Rag1<sup>null</sup>*.A14αβ-tg splenocytes ( $\sim 1 \times 10^6$  A14 T cells). T1D development was monitored daily for up to 2 wk posttransfer.

#### B cell-proliferation assays

B cells were purified from pooled spleens of three mice of the indicated strains (6–8 wk of age) using the EasySep Mouse B cell enrichment kit, followed by separation using an EasySep magnet, according to the manufacturer's instructions (StemCell Technologies, Vancouver, BC, Canada). This method routinely achieved >98% purity of B cells, as determined by flow cytometry. Triplicate aliquots of  $1 \times 10^5$  B cells were cultured in complete RPMI 1640 medium with 10 μg/ml LPS (Sigma-Aldrich, St. Louis, MO) or 10 μg/ml AffiniPure goat anti-mouse IgM F(ab')<sub>2</sub> fragments (Jackson ImmunoResearch Laboratories, West Grove, PA) and 5 μg/ml CD40-specific mAb HM40-3 (BD Biosciences, San Jose, CA), alone or in combination. Control wells contained no stimulatory agents. Proliferation of cells was assessed by addition of 1 μCi/well [<sup>3</sup>H]thymidine (Amersham Pharmacia Biotech, Uppsala, Sweden) in the final 24 h of a 72-h incubation period using previously described methods (12).

#### Microarrays and quantitative real-time PCR

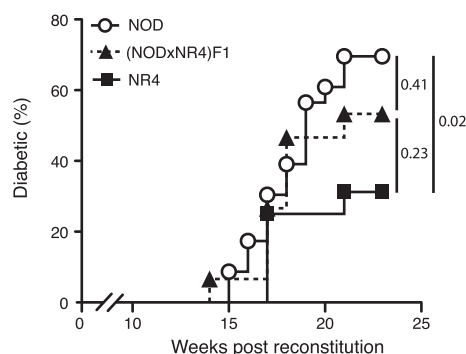
For microarrays, B cells from three independent lots of seven or eight pooled spleens from NOD or NR4 mice, respectively, were purified using a previously described MACS negative-depletion strategy (9). B cells from each lot were resuspended at  $1 \times 10^7$  cells/ml for 2 h in complete RPMI 1640 medium, alone or with 10 μg/ml AffiniPure goat anti-mouse IgM F(ab')<sub>2</sub> fragments (Jackson ImmunoResearch Laboratories). Total RNA was then purified from B cells using TRIzol reagent (Invitrogen, Carlsbad, CA) and subjected to one-cycle linear amplification, biotin labeling, and fragmentation, according to the protocol provided, by the GeneChip Expression 3'-Amplification One-cycle kit (Affymetrix, Santa Clara, CA). Ten micrograms of biotin-labeled and fragmented cRNA from two BCR-stimulated and three unstimulated NOD B cell samples plus three BCR-stimulated and three unstimulated NR4 B cell samples were hybridized to Mouse Genome 430 2.0 Chips (Affymetrix) for 16 h at 45°C and scanned with a GeneChip Console Scanner (Affymetrix). All microarray data were submitted to the Gene Expression Omnibus public database (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE37294. Analysis of expression data was performed using GeneSpring 12.0 software (Agilent Technologies, Santa Clara, CA). All samples were normalized by Robust Multiarray Analysis on a per-gene basis to the median of the total samples. Differential expression of genes was determined using one- or two-way ANOVA tests, with Benjamini and Hochberg false detection rate correction (q-value) to account for multiple testing. Gene symbols used in the figures and tables are those prescribed by the Mouse Genomic Nomenclature Committee. For quantitative real-time PCR (q-rtPCR), B cells were purified from three pools of three spleens each from the indicated strains of mice (independent of microarray samples), using the aforementioned EasySep Mouse B cell enrichment kit. RNA was then immediately extracted from each sample using TRIzol reagent (without culture) and transcribed into cDNA using M-MuL V reverse transcriptase (New England BioLabs, Ipswich, MA), via the first-strand synthesis protocol pro-

vided by the manufacturer. Levels of gene transcripts were determined with a PRISM7900 HT machine (Applied Biosystems, Foster City, CA) using triplicate q-rtPCR reactions containing 50–100 ng cDNA and 0.9 μM of each primer in LightCycler-RNA SYBR Green I Master Mix (Roche, Mannheim, Germany). Primers for each gene were designed such that one would bind across an exon–exon boundary (sequences in Supplemental Table I). The capacity of primers to quantify target genes by q-rtPCR was initially established using triplicate sets of 4-fold serial dilutions (1/1, 1/4, 1/16, 1/64, and 1/256) of NR4 or NOD spleen cDNA. Amplification efficiency of all primers ranged between 95.7 and 99.6% (data not shown). Expression of target genes in B cell samples were normalized to the *Hprt* housekeeping gene.

## Results

### Determining the mode of inheritance of the NOR-derived Chr. 4 gene(s) that dampens the diabetogenic activity of B cells

In a previous study, we showed that, in contrast to the low incidence of T1D exhibited by NR4 mice (25% by 30 wk), mice that were NOD/NOR heterozygous at this congenic region developed a higher incidence of disease that was not significantly different from NOD homozygous littermates (71 versus 92% by 30 wk, respectively; NS, log-rank test) (26). This result indicated that a NOR-derived allele(s) in the original full-length (FL) Chr. 4 congenic interval must be homozygous to confer significant resistance to T1D. To establish whether the gene(s) decreasing the diabetogenic capacity of B cells in the NR4 congenic region was consistent with this mode of inheritance, we compared the T1D incidence of lethally irradiated NOD.*Igμ<sup>null</sup>* mice reconstituted with SBM plus MACS-purified B cells from NOD, NR4, or (NOD×NR4)F1 donors (Fig. 1). The T1D incidence of NOD.*Igμ<sup>null</sup>* recipients reconstituted with NR4 B cells differed significantly from those reconstituted with NOD B cells after 23 wk (31.3% [ $n = 16$ ] versus 69.6% [ $n = 23$ ];  $p < 0.05$ , log-rank test). However, T1D development did not differ in a statistically significant manner in recipients reconstituted with (NOD×NR4)F1 or NOD B cells. This indicated that a NOR-derived allele(s) on distal Chr. 4 must be homozygous to significantly suppress diabetogenic B cell development. Differences in T1D susceptibility between cohorts was unlikely due to expansion of contaminating CD4 or CD8 T cells from the purified donor B cell preparations. This was made evident in two lethally irradiated NOD.*Igμ<sup>null</sup>* mice (expressing CD45.1) reconstituted with SBM plus B cells purified from NOD.*CD45.2* congenic mice, whose spleens, 6 wk postengraftment, contained 1.2 and 6.0% CD45.2<sup>+</sup> CD4 T cells, 2.4 and 6.4%



**FIGURE 1.** NOR-derived allele(s) on distal Chr. 4 must be homozygous to significantly suppress diabetogenic B cell development. T1D incidence was monitored weekly for up to 23 wk in cohorts of female NOD.*Igμ<sup>null</sup>* mice that were lethally irradiated at 4–5 wk of age and reconstituted with  $3 \times 10^6$  SBM admixed with  $7 \times 10^6$  splenic B cells from NOD ( $n = 23$ ), NR4 ( $n = 16$ ), and (NOD×NR4)F1 ( $n = 15$ ) mouse strains. The  $p$  values for comparison of incidence curves by log-rank analysis are shown.

CD45.2<sup>+</sup> CD8<sup>+</sup> T cells, and 99.3 and 99.2% CD45.2<sup>+</sup> B cells, respectively (data not shown).

Generation of novel subcongenic lines of the NR4 mouse strain

To facilitate identification of the NOR-derived distal Chr. 4-region gene(s) controlling T1D resistance, as well as the decreased pathogenic activity of B cells in the original FL NR4 congenic strain, subcongenic lines were generated as outlined in *Materials and Methods*. This resulted in the generation of five novel subcongenic NR4 mouse lines (referred to as NR4S1–S5) containing smaller homozygous NOR-derived regions spanning different sections of the original FL 44.31-Mb congenic interval. The genetic boundaries of the NOR-derived regions in the NR4S1–S5 subcongenic strains, relative to the original NR4 strain and previously identified *Idd9* and *Idd11* genes (27, 28), are illustrated in Fig. 2A.

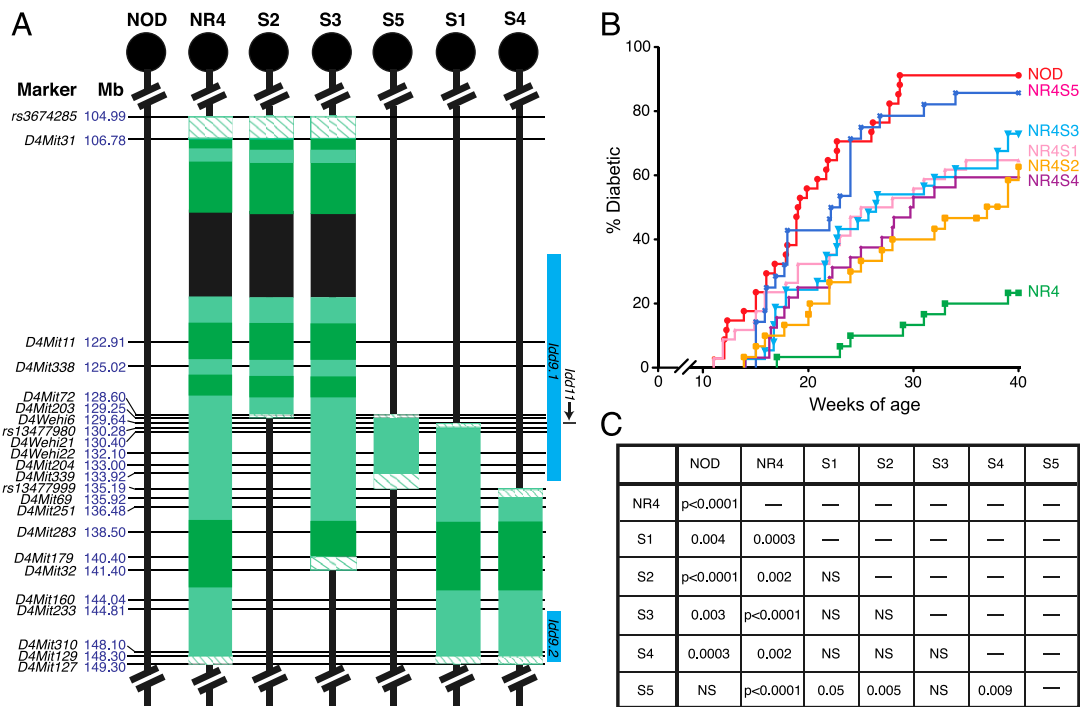
T1D susceptibility in NR4 subcongenic mouse lines

To refine the NOR-derived region(s) on Chr. 4 responsible for conferring protection from T1D, the natural incidence of disease in female mice of the NR4S1–S5 subcongenic lines was compared with that of the NOD and the original FL NR4 congenic strain through 40 wk of age (Fig. 2B). More than 90% of female NOD mice developed T1D by 40 wk of age compared with only 23% of the NR4 stock ( $p < 0.0001$ , log-rank analysis, Fig. 2C), consistent with the results of a previous study on the same strains performed in an independent animal facility (26). The NR4S1 (65%,  $n = 33$ ), NR4S2 (63%,  $n = 29$ ), NR4S3 (73%,  $n = 36$ ), and NR4S4 (59%,  $n = 31$ ) mouse strains all showed an intermediate incidence of T1D (Fig. 2B) that differed significantly from both the NOD and FL

NR4 strains but not each other (Fig. 2C). In contrast, the NR4S5 mouse strain developed T1D at a similar incidence and rate (86%,  $n = 27$ ) as did NOD mice (Fig. 2B), indicating that the central section of the NR4 congenic region between *D4Mit72* and *rs13477999* (Fig. 2A), which encompasses the *Idd11* locus (27), does not contain a NOR origin gene conferring resistance to T1D development. Our observation that none of the subcongenic strains showed the same level of T1D protection as did the original FL NR4 congenic strain provides evidence of more than one distal Chr. 4-region gene contributing to disease resistance in NOR mice. This was also confirmed by the fact that there was no overlap in the NOR-derived regions of two subcongenic strains showing T1D resistance: NR4S4 and NR4S2 (Fig. 2A, 2B). Therefore, these two subcongenic strains define at least two loci containing NOR T1D-resistance genes on Chr. 4, which span the intervals between the markers *rs3674285* (104.99 Mb) and *D4Mit203* (129.25 Mb) and *rs13477999* (135.18 Mb) and *D4Mit127* (149.30 Mb). These regions partially overlap with the T1D-protective *Idd9.1* and *Idd9.2* loci, respectively, which were found to be present in the B10 strain (28). However, given that the T1D-protective genes in the distal region of NOR Chr. 4 originally derive from the BKS strain, the possibility cannot be excluded that their identity differs from those responsible for the *Idd9.1* and/or *Idd9.2* effects.

Diabetogenic capacity of B cells in NR4 subcongenic mouse lines

We next refined the location of the distal Chr. 4-region gene(s) suppressing the diabetogenic capacity of NOR B cells. T1D de-



**FIGURE 2.** Generation of NR4 subcongenic strains reveals the presence of more than one T1D-resistance gene in the distal Chr. 4 region of NOR mice. **(A)** Schematic illustration showing boundaries of NOR-derived congenic regions on Chr. 4 in the NR4 and NR4S1–S5 subcongenic mouse lines. Orientation of Chr. 4 is marked by a circle symbolizing the centromere. Rectangular boxes within the broken lines represent NOR-derived congenic intervals. Boundaries of congenic intervals were determined by the polymorphic microsatellite and SNP markers shown. Markers are ordered according to their physical position in megabases according to Ensembl (<http://www.ensembl.org/>), with the exception of WEHI markers, whose positions were obtained from Ref. 27. Colors within the congenic regions mark NOR genome of NOD (black), B6 (light green), or DBA/2 (dark green) origin, as previously defined by Reifsnnyder et al. (26). Regions of unknown origin between markers are represented by hatched lines. For comparison, the most current positions of the *Idd9.1* (116.08–134.49 Mb), *Idd9.2* (144.97–149.10 Mb), and *Idd11* (129.63–129.64 Mb) loci are shown (27, 28). **(B)** Cumulative incidence of T1D in female cohorts of NOD ( $n = 33$ ), NR4 ( $n = 29$ ), NR4S1 ( $n = 33$ ), NR4S2 ( $n = 29$ ), NR4S3 ( $n = 36$ ), NR4S4 ( $n = 31$ ), and NR4S5 ( $n = 27$ ) mice through to 40 wk of age. **(C)** The  $p$  values from comparisons of T1D incidence curves using log-rank analysis.

velopment was assessed in NOD.*Igμ<sup>null</sup>* mice that were lethally irradiated and reconstituted with SBM plus splenic B cells from the T1D-resistant subcongenic strains NR4S1–S4 (Fig. 3A). Disease incidence in these groups up to 23 wk was compared with control cohorts of NOD.*Igμ<sup>null</sup>* recipients reconstituted with SBM and splenic B cells from NOD or FL NR4 strains. Mirroring our previous study (30), NOD.*Igμ<sup>null</sup>* mice reconstituted with NOD B cells developed a high incidence of T1D (68%,  $n = 33$ ), which was reduced by approximately half (36%,  $n = 30$ ) in recipients of NR4 B cells (Fig. 3A) despite similar levels of B cell reconstitution between groups (Table I). Compared with those receiving NOD B cells, there was a significant reduction in the incidence of T1D shown by NOD.*Igμ<sup>null</sup>* mice reconstituted with either NR4S3 (20%,  $n = 10$ ) or NR4S4 (35%,  $n = 17$ ) B cells (Fig. 3A). In addition, these two groups of recipients developed a delayed onset of T1D (NR4S3:  $21.2 \pm 0.9$  wk, NR4S4:  $21.5 \pm 0.5$  wk) compared with the NR4 B cell-reconstituted groups ( $18.5 \pm 0.6$  wk), suggestive of an enhanced level of protection (Fig. 3B). The difference in mean onset time of T1D was significant between NOD and NR4S4 B cell-reconstituted recipients ( $p = 0.006$ , Student *t* test). Given that only two NOD.*Igμ<sup>null</sup>* mice containing NR4S3 B cells developed T1D in this study, the difference in mean time of disease onset compared with NR4 B cell recipients was not statistically significant; however, the trend for delay was still strong ( $p = 0.08$ ). In contrast, NOD.*Igμ<sup>null</sup>* mice reconstituted with B cells from NR4S1 (50%,  $n = 16$ ) and NR4S2 (54%,  $n = 13$ ) subcongenic strains showed no significant decrease in T1D incidence compared with recipients of NOD B cells (Fig. 3A).

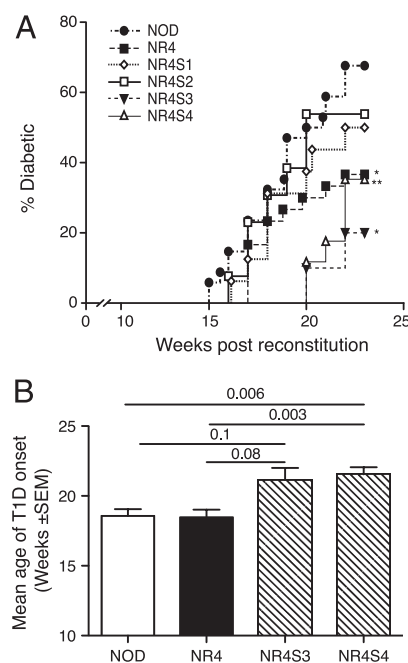
Two-way ANOVA tests were performed to examine differences in B cell or CD4 T cell reconstitution levels in spleens of diabetic

and nondiabetic mice of the various recipient groups (Table I). The strain of B cell donor contributed to variance in B cell ( $p = 0.03$ ), but not CD4 T cell (NS), reconstitution levels in the different recipient groups. However, this effect was not strong given that Bonferroni posttests did not identify a significant difference in the percentages of B cells between recipients of any two specific B cell donors in either the diabetic or nondiabetic groups. Added to this, it is unlikely that differences in T1D susceptibility between recipient groups were a consequence of variance in B cell reconstitution levels, because in all transfers, there was no significant difference in B cell proportions in spleens of diabetic versus nondiabetic mice (Table I; NS, two-way ANOVA, Bonferroni posttests). Furthermore, recipient groups with a higher incidence of T1D (i.e., NOD, NR4S1, and NR4S2 donors) did not necessarily have higher mean levels of B cell reconstitution than did those with a lower incidence (i.e., NR4, NR4S3, and NR4S4 donors) (NS, Student *t* test).

The results of these incidence studies indicate that the main NOR-derived gene(s) responsible for dampening the diabetogenic activity of B cells lies within the 6.22-Mb overlap of the NR4S3 and NR4S4 congenic regions (i.e., between *rs13477999* and *D4Mit32*, Fig. 2A). This region of Chr. 4 has not been previously identified to contain an *Idd* locus. Thus, we now designate this newly identified locus on distal Chr. 4 differentially controlling the development of diabetogenic B cells in NOD and NOR mice as *Idd25*. However, it is interesting that the protective B cell phenotype mediated by the *Idd25* resistance variant seems to be abrogated by a combined interaction between at least two other NOR genes within the intervals bound by *D4Wehi6* to *D4Mit69* and *D4Mit179* to *D4Mit127*, which are both present in the NR4S1 strain but do not coexist in the NR4S3 and NR4S4 strains (Fig. 2A). The original FL NR4 congenic region also dampens diabetogenic B cell activity, despite containing all of the above-mentioned subregions. Thus, it is likely that another NOR origin gene(s) in the most proximal region, bound by the markers *rs3674285* and *D4Mit203*, also contributes to the dampened diabetogenic activity of B cells. However, this NOR gene(s) does not mediate a reduction in pathogenic B cell activity when inherited alone, given that B cells from the NR4S2 congenic stock are as diabetogenic as those from standard NOD mice (Fig. 3A).

#### Hyperresponsiveness of B cells in NR4 subcongenic mouse lines

B cells from FL NR4 mice undergo significantly higher levels of proliferation upon BCR or BCR plus CD40 stimulation than do those from NOD mice (30). This difference in responsiveness to stimulation was evident in B cells from spleen (FO and MZ subsets) or lymph nodes (FO subset only). To identify the genetic region(s) within the original FL NR4 congenic region that is responsible for this B cell hyperproliferative phenotype, we subjected purified splenic B cells from the NR4S1–5 congenic strains to in vitro stimulation with anti-IgM-F(ab')<sub>2</sub> fragments or anti-CD40 Abs alone or in combination, or LPS. Consistent with our previous study, NR4 B cells proliferated significantly more than those from NOD mice when stimulated with anti-IgM-F(ab')<sub>2</sub> or anti-IgM-F(ab')<sub>2</sub> plus anti-CD40 ( $1.3 \pm 0.07$ - and  $1.5 \pm 0.1$ -fold higher, respectively, Fig. 4A, 4B). This difference was probably the direct consequence of variability in signaling downstream of the BCR, given the observed equivalent responses between B cells stimulated through CD40 alone or through the TLR4 receptor with LPS (Fig. 4C, 4D). Of the subcongenic B cells, only those isolated from the NR4S1 stock exhibited the hyperproliferative phenotype of the FL NR4 strain in response to stimulation with BCR, with or without CD40 costimulation ( $1.5 \pm 0.1$ - and  $1.4 \pm 0.2$ -fold higher



**FIGURE 3.** B cells from NR4S3 and NR4S4 subcongenic mouse strains exhibit decreased diabetogenic capacity. (A) T1D incidence was monitored for up to 23 wk in cohorts of female NOD.*Igμ<sup>null</sup>* mice that were lethally irradiated (1100 rad) at 4–5 wk of age and reconstituted with  $3 \times 10^6$  SBM admixed with  $7 \times 10^6$  purified splenic B cells from NOD ( $n = 33$ ), NR4 ( $n = 30$ ), NR4S1 ( $n = 16$ ), NR4S2 ( $n = 13$ ), NR4S3 ( $n = 10$ ), or NR4S4 ( $n = 17$ ) strains. \* $p < 0.05$ , \*\* $p < 0.01$  compared with NOD B cell reconstituted group (log-rank analysis). (B) Graph comparing mean time of disease onset in diabetic NOD.*Igμ<sup>null</sup>* mice reconstituted with NOD ( $n = 23$ ), NR4 ( $n = 11$ ), NR4S3 ( $n = 2$ ), or NR4S4 ( $n = 6$ ) B cells. The  $p$  values between groups are shown (Student *t* test).



Table I. Levels of B220<sup>+</sup> and CD4<sup>+</sup> lymphocytes in SBM plus B cell-reconstituted NOD.*Igμ<sup>null</sup>* recipients

B Cell Donor Used To Reconstitute NOD. <i>Igμ<sup>null</sup></i> Mice <sup>a</sup>	n	Diabetic (%; Mean ± SEM)		n	Nondiabetic (%; Mean ± SEM)	
		B220 <sup>+</sup> <sup>b</sup>	CD4 <sup>+</sup> <sup>c</sup>		B220 <sup>+</sup> <sup>b</sup>	CD4 <sup>+</sup> <sup>c</sup>
NOD	22	41.8 ± 6.9	44.9 ± 6.0	11	37.8 ± 3.4	40.8 ± 2.4
NR4	11	43.2 ± 8.0	29.6 ± 4.9	19	47.8 ± 3.7	28.9 ± 2.8
NR4S1	8	40.6 ± 3.6	39.4 ± 3.0	8	37.3 ± 3.9	31.8 ± 3.2
NR4S2	7	26.0 ± 4.3	38.5 ± 3.7	6	23.3 ± 2.6	44.2 ± 3.0
NR4S3	2	31.3 ± 7.4	34.9 ± 7.2	8	22.6 ± 1.3	38.9 ± 3.4
NR4S4	6	28.0 ± 4.3	43.4 ± 3.9	11	31.2 ± 2.5	36.0 ± 2.8

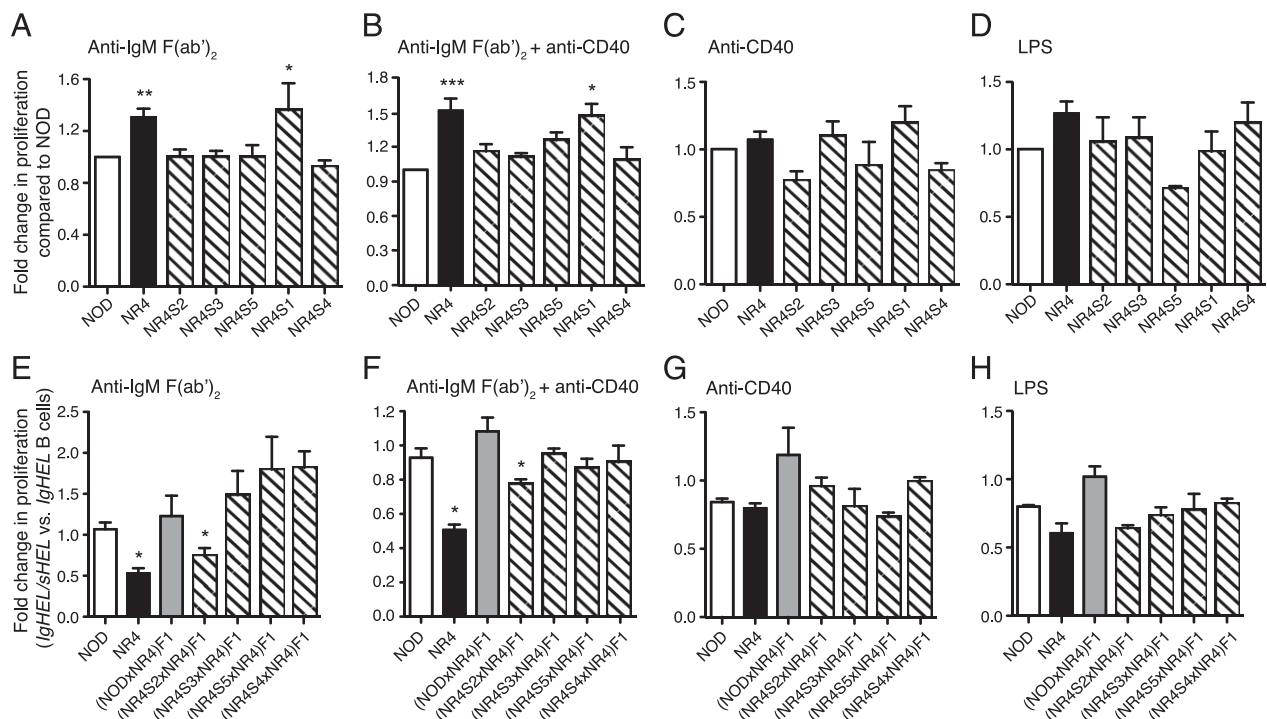
<sup>a</sup>NOD.*Igμ<sup>null</sup>* mice were lethally irradiated at 4–5 wk of age and reconstituted with  $3 \times 10^6$  SBM and  $7 \times 10^6$  splenic B cells from the indicated strain. Proportions of B220<sup>+</sup> B cells and CD4<sup>+</sup> T cells in spleens of each recipient mouse were determined by flow cytometry after T1D onset or at the end of the study (23 wk postreconstitution).

<sup>b</sup>Two-way ANOVA test of B220<sup>+</sup> reconstitution dataset did not find significant interaction between diabetes status and B cell donor. Furthermore, diabetes status did not contribute significantly to variation in B220<sup>+</sup> reconstitution. In contrast, the strain of B cell donor was found to significantly contribute to variation in B220<sup>+</sup> reconstitution ( $p = 0.03$ ). However, Bonferroni posttests did not identify a significant difference between any two specific B cell donors in diabetic or nondiabetic groups.

<sup>c</sup>Two-way ANOVA test of the CD4<sup>+</sup> T cell reconstitution dataset did not find significant interaction between disease status and B cell donor. In addition, neither diabetes status nor B cell donor contributed significantly to variation in CD4<sup>+</sup> T cell reconstitution.

proliferation than NOD, respectively, Fig. 4A, 4B). NR4S4 and NR4S3 B cells did not exhibit a hyperproliferative response to BCR stimulation. This suggested that the hyperproliferation phenotype of NR4S1 B cells is the result of an interaction between NOR genes located in two regions bound by *D4Wehi6* and *D4Mit69*

and *D4Mit179* and *D4Mit127* that are not coexistent in the NR4S4 and NR4S3 lines. Interestingly, these were also the two regions that combined to lessen the ability of the NOR gene(s) within the *rs13477999–D4Mit32* region to suppress diabetogenic B cell activity (Figs. 2A, 3A). These results dissociate the induction of



**FIGURE 4.** Hyperresponsive and anergy phenotypes are not necessary for decreased diabetogenic activity of NR4 B cells. (**A–D**) To test hyperresponsiveness, B cells were purified from pooled spleens of three 6–8-wk-old female NOD, NR4, NR4S1, NR4S2, NR4S3, NR4S4, and NR4S5 mice. Triplicate aliquots of  $1 \times 10^5$  B cells were stimulated in culture with 10  $\mu$ g/ml anti-IgM-F(ab')<sub>2</sub> (A), 10  $\mu$ g/ml anti-IgM-F(ab')<sub>2</sub> and 5  $\mu$ g/ml anti-CD40 (B), 5  $\mu$ g/ml anti-CD40 (C), or 10  $\mu$ g/ml LPS (D). Proliferation over the final 24 h of a 72-h incubation period was measured by [<sup>3</sup>H]thymidine incorporation and is shown as the mean ( $\pm$  SEM) fold change compared with NOD B cells in two to four independent experiments for each congenic strain. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with NOD B cells (one-way ANOVA, Tukey posttest). (**E–H**) To test anergy induction, B cells were purified from pooled spleens of three 6–8-wk-old female *IgHEL* single-tg or *IgHEL/sHEL* double-tg mice with NOD, NR4, (NOD  $\times$  NR4)F1, (NR4S2  $\times$  NR4)F1, (NR4S3  $\times$  NR4)F1, (NR4S4  $\times$  NR4)F1, or (NR4S5  $\times$  NR4)F1 genetic backgrounds. Triplicate aliquots of  $1 \times 10^5$  B cells were stimulated in culture with 10  $\mu$ g/ml anti-IgM-F(ab')<sub>2</sub> (E), 10  $\mu$ g/ml anti-IgM-F(ab')<sub>2</sub> and 5  $\mu$ g/ml anti-CD40 (F), 5  $\mu$ g/ml anti-CD40 (G), or 10  $\mu$ g/ml LPS (H). Proliferation over the final 24 h of a 72-h incubation period was measured by [<sup>3</sup>H]thymidine incorporation. Bars represent the mean ( $\pm$  SEM) fold change in proliferation of B cells from *IgHEL/sHEL* double-tg mice compared with single *IgHEL* counterparts in two to four independent experiments for each subcongenic background, whereas the experiments using NOD, NR4, and (NOD  $\times$  NR4)F1 background controls were performed five, four, and eight times, respectively. \* $p < 0.05$  compared with NOD and (NOD  $\times$  NR4)F1 background group (one-way ANOVA, Tukey posttest).

a stronger BCR-signaling response from the ability of a NOR-derived Chr. 4 gene(s) to dampen diabetogenic B cell activity.

#### *B cell anergy in NR4 subcongenic mouse lines*

Our previous study demonstrated that homozygous expression of the FL NOR-derived Chr. 4 congenic region on the NOD background resulted in the correction of faulty B cell anergy induction in the *IgHEL/sHEL* tg model (30). Based on the observation that the NOR-derived FL NR4 congenic interval must be inherited in a homozygous manner to significantly suppress diabetogenic B cell development (Fig. 1), an array of F1 hybrids was made to fix the various NR4 subcongenic intervals to homozygosity to assess their effects on B cell anergy induction. The strategy consisted of crossing the previously developed FL NR4 congenic strain expressing both *IgHEL* and *sHEL* transgenes (30) with the NR4S2, NR4S3, NR4S4, or NR4S5 subcongenic mouse lines. The resulting hybrid progeny were homozygous at the NOR-derived region shared between the NR4 and subcongenic strain but heterozygous throughout the remainder of the FL congenic region, while also expressing a combination of hemizygous *IgHEL* and *sHEL* transgenes. Anergy was assessed by measuring the proliferative response of B cells from *IgHEL/sHEL* double-tg mice versus *IgHEL* single-tg mice from each hybrid cross following in vitro stimulation with LPS or anti-IgM-F(ab')<sub>2</sub> fragments and anti-CD40 Abs, alone or in combination (Fig. 4E–H). The occupancy of HEL on the majority of BCRs of B cells of double-tg, but not single-tg, mice precluded us from using specific Ag stimulation (17). B cells from NOD, (NOD×NR4)F1, and NR4 background mice expressing the same transgenes were used as controls. Consistent with our previous study (30), HEL-specific B cells in NOD mice were not effectively anergized when maturing in the presence of *sHEL*, and thus proliferated at similar levels as did naive B cells in culture, irrespective of the stimuli (Fig. 4E–H). Conversely, a significant reduction in proliferation was observed in NR4 B cells from *IgHEL/sHEL* double-tg mice compared with *IgHEL* single-tg mice in response to anti-IgM-F(ab')<sub>2</sub> with or without anti-CD40 (Fig. 4E, 4F). Such a reduction was not evident when B cells were stimulated with LPS or anti-CD40 alone (Fig. 4G, 4H). B cells from (NOD×NR4)F1 mice expressing *IgHEL* and *sHEL* transgenes showed a similar defect in B cell anergy as did NOD background mice (Fig. 4E, 4F), highlighting the requirement for homozygous expression of NOR alleles in the FL NR4 congenic region to correct this immunoregulatory process. Of the hybrid subcongenic progeny, only B cells homozygous for the NR4S2 region exhibited a significant decrease in proliferation following stimulation with anti-IgM-F(ab')<sub>2</sub> alone or in combination with anti-CD40 after having developed in the presence, as opposed to the absence, of their cognate Ag (Fig. 4E, 4F). This decrease (~30%) was not as distinct as that observed for *IgHEL/sHEL* double-tg B cells from the FL NR4 strain (~50%). This indicated that the NR4S2 subcongenic region contributes to a partial decrease in the responsiveness of self-reactive B cells but is not the sole region affecting this phenotype in the original FL NR4 congenic mouse strain. In this regard, it is worth highlighting the result of tg B cells derived from hybrid mice homozygous for the NR4S3 region. Despite also being homozygous throughout the NR4S2 region, NR4S3 B cells showed equivalent levels of proliferation in response to anti-IgM-F(ab')<sub>2</sub> and anti-IgM-F(ab')<sub>2</sub> plus anti-CD40 Abs, whether they matured in the presence or absence of their cognate self-Ag, similar to NOD background mice (Fig. 4E, 4F). This result implies that the NOR region differentiating the NR4S2 and NR4S3 strains (between *D4Mit203* and *D4Mit32*, Fig. 2A) contains a gene(s) that contributes to the aberrant induction of anergy in self-reactive

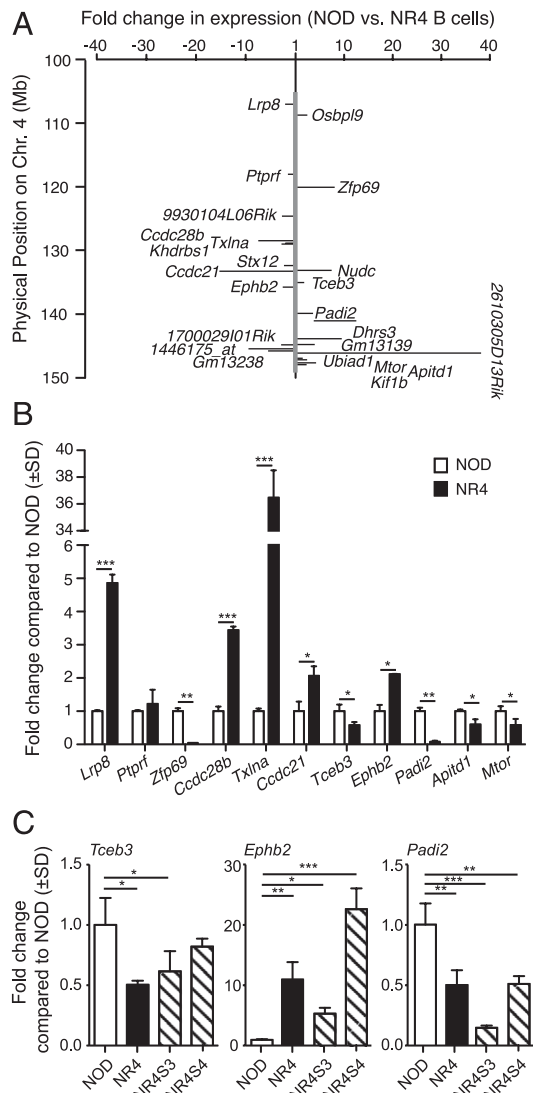
B cells. Moreover, the clear difference in responsiveness of self-reactive B cells in mice homozygous for the NR4S3 subcongenic region compared with those carrying the FL NR4 congenic segment indicates that a NOR-derived region between *D4Mit179* and *D4Mit127* (Fig. 2A) must also contribute to the correction of B cell anergy induction. The gene within this region can only mediate its effect on B cell anergy when inherited in combination with a homozygous NOR-derived gene(s) in the NR4S2 congenic interval. This conclusion is supported by the finding that strains homozygous for NOR genes in the distal region alone (i.e., NR4S1 and NR4S4 hybrid strains) displayed defects in B cell anergy induction that were indistinguishable from that in standard NOD mice (Fig. 4E, 4F). B cells showing partially corrected tolerance induction in the NR4S2 line were as diabetogenic as those from standard NOD mice, whereas NR4S3 and NR4S4 B cells with dampened pathogenic activity exhibited anergy defects equivalent to those from NOD mice (Figs. 3A, 4E, 4F). Thus, it appears that homozygous expression of NOR genes in both the *rs3674285* to *D4Mit203* and *D4Mit179* to *D4Mit127* intervals, which combinatorially restore anergy-induction processes, does not independently suppress diabetogenic B cell activity but may augment this process.

#### *Differentially expressed genes from microarray analysis of NOD versus NR4 B cells*

Microarrays were conducted on B cells purified from spleens of NOD and NR4 mice to highlight differentially expressed genes within the distal Chr. 4 locus. B cells were either cultured in media alone (unstimulated) or with BCR cross-linking anti-IgM-F(ab')<sub>2</sub> fragments (stimulated) for 2 h before RNA was extracted for transcript analysis. Several significant expression differences were noted between NOD and NR4 B cell samples using a one-way ANOVA test, and, as expected, most of these genes (24/32 mapped probes) were situated within the distal Chr. 4 region encompassed by the FL NR4 congenic interval (Fig. 5A, Supplemental Fig. 1A, 1B, Supplemental Table II). These 24 differentially expressed genes represented 1.8% of the total probe sets ( $n = 1299$ ) that detect transcripts within the Chr. 4 congenic region. Interestingly, none of the genes that were differentially expressed between NOD and NR4 B cells changed significantly upon 2 h of BCR cross-linking (Supplemental Fig. 1A, 1B). Nevertheless, polymorphic genes within the distal Chr. 4 region had the capacity to differentially alter stimulation-responsive genes located at other sites, given that more than approximately half of the genes that changed significantly after BCR cross-linking ( $\geq 2$ -fold change and  $q < 0.1$ , one-way ANOVA) were not shared by the NOD and NR4 strains (Supplemental Fig. 1A, Supplemental Table III).

Of the 24 genes within distal Chr. 4 that were deemed to be differentially expressed between NOD and NR4 B cells (Fig. 5A), 11 were chosen for further examination by q-rtPCR because of their potential involvement in tolerance mechanisms (e.g., cell death), BCR signaling, and lymphocyte function or as a consequence of large differences in expression levels between mouse strains. Differential expression of these genes was analyzed in an independent set of cDNA derived from purified NOD and NR4 splenic B cells that were not subjected to culture. Consistent with the results of the microarrays, the expression of the selected genes *Lrp8*, *Ccdc28b*, *Ccdc21*, *Ephb2*, and *Txlna* were higher in B cells from NR4 mice than from NOD mice, whereas *Zfp69*, *Siii*, *Tceb3*, *Padi2*, *2610305D13Rik*, and *mTOR* were more highly expressed by B cells from NOD mice compared with NR4 mice (Fig. 5B). The only gene that was not consistent with the results of the microarrays was *Ptprf*, which did not show a significant difference in expression between NOD and NR4 B cells by q-rtPCR.





**FIGURE 5.** Differentially expressed genes in NOD versus NR4 B cells within the distal Chr. 4 locus. **(A)** Differentially expressed genes between NOD and NR4 splenic B cells ( $q < 0.05$  false detection rate, one-way ANOVA), which map within the region of distal Chr. 4 and distinguish the two strains, were determined using Affymetrix Mouse 430 2.0 microarray chips after a 2-h culture in media alone ( $n = 3$  for NOD,  $n = 3$  for NR4) or with  $10 \mu\text{g/ml}$  of anti-IgM-F(ab')<sub>2</sub> fragments ( $n = 2$  for NOD,  $n = 3$  for NR4). None of the Chr. 4 gene probes showed significant changes in expression with stimulation (see Supplemental Fig. 1A, 1B). Therefore, each line represents the mean fold change in expression between combined NOD ( $n = 5$ ) versus NR4 ( $n = 6$ ) B cell groups for each differentially expressed gene probe plotted against its physical position (in megabases) according to the Ensembl database. The location of the congenic region in NR4 mice is demarcated by the thick gray line. All differentially expressed genes reside in genomic areas that are B6 derived in NR4 mice, with the exception of *Padi2* (underlined), which is in a DBA/2-derived region. **(B)** Differentially expressed genes in microarray analyses were confirmed by performing q-rtPCR of selected genes using cDNA from three independent lots of purified NOD and NR4 splenic B cells that were not subjected to culture. q-rtPCR was conducted on triplicate aliquots of cDNA and normalized to expression of the housekeeping gene *Hprt*. Data are presented as mean fold change differences in expression in three NR4 samples relative to the mean of the NOD B cell samples.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  (Student *t* test). **(C)** q-rtPCR was performed for the *Tceb3*, *Ephb2*, and *Padi2* genes using cDNA obtained from three independent lots of splenic B cells from 6–8-wk-old female NOD, NR4, NR4S3, and NR4S4 mice. Gene expression was normalized to *Hprt*. Data for each strain are presented as the mean fold change difference in expression relative to the mean of the NOD B cell samples.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , compared with NOD (one-way ANOVA, Tukey posttest).

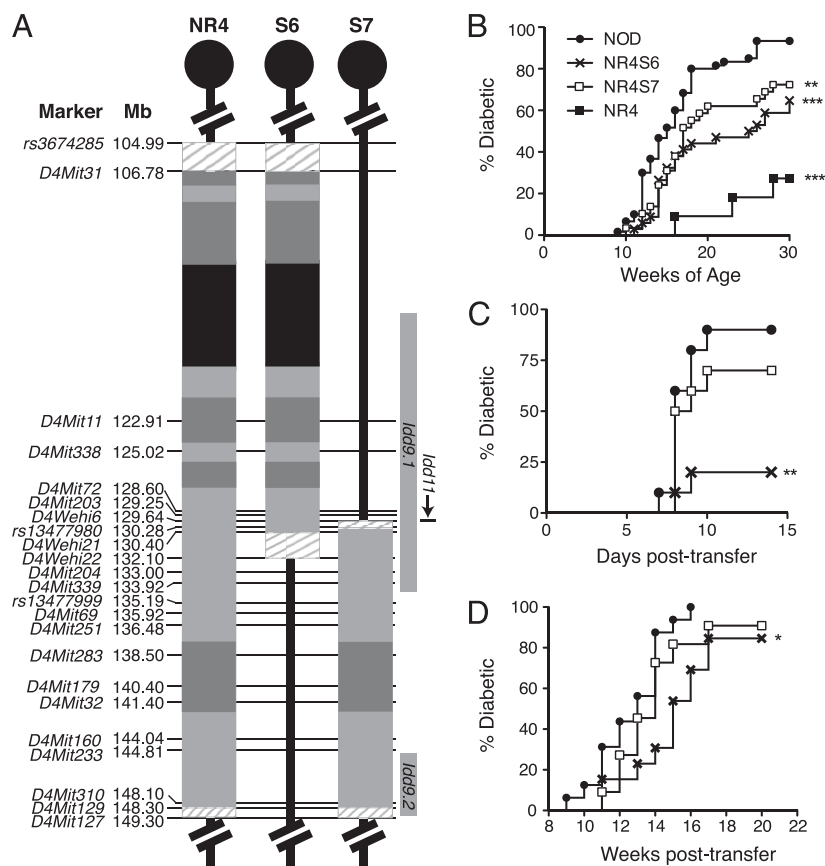
B cells from the subcongenic NR4S3 and NR4S4 strains showed a decrease in diabetogenic activity (Fig. 3A). Of the genes confirmed to be differentially expressed between NOD and NR4 B cells by microarray, only *Tceb3*, *Ephb2*, and *Padi2* are located within the overlap of the NR4S3 and NR4S4 congenic regions (between *rs13477999* and *D4Mit32*, Fig. 2A). Therefore, we assessed the differential expression of these genes by q-rtPCR in NR4S3 and NR4S4 B cells compared with those from NOD and NR4 mice (Fig. 5C). Consistent with their expression in NR4 B cells, we observed a significant decrease in the expression of *Padi2* and a significant increase in the expression of *Ephb2* in NR4S3 and NR4S4 B cells compared with those from NOD mice. Although a significant decrease in *Tceb3* expression was also observed in NR4S3 B cells relative to NOD B cells, this difference was not evident in the comparison with NR4S4 B cells. Confirmation of differential *Padi2* and *Ephb2* expression within the NR4S3 and NR4S4 mouse strains implicates these genes as potential *Idd25* candidates for the control of diabetogenic B cell activity in NOD mice.

#### Subcongenic analysis of the NR4 region at the level of CD4 T cells

We previously showed that the FL NR4 congenic interval also conferred T1D resistance at the level of CD4 T cells (32). The complex interaction of the NR4-region genes described above raised the possibility that some of the B cell phenotypes are influenced to some extent by the quality of CD4 T cell help. Conversely, B cell phenotypes controlled by polymorphic NR4-region genes could also differentially effect activation of diabetogenic CD4 T cells. In an independent effort carried out at TJL, two additional NR4 subcongenic lines (NR4S6 and NR4S7) were generated to further define the critical interval that regulates the diabetogenic activity of CD4 T cells (Fig. 6A). T1D-incidence studies performed at TJL demonstrated that both subcongenic lines were less susceptible to disease than were standard NOD mice (Fig. 6B). However, neither subregion alone was sufficient to confer the same level of T1D protection provided by the FL NR4 congenic interval. These results are consistent with the observations at the Garvan Institute and confirm that at least two genes contribute to T1D resistance in NR4 mice.

We next asked whether any of the NOR-derived Chr. 4 subregions could suppress the diabetogenic activity of CD4 T cells. We previously showed that the ability of adoptively transferred  $\beta$  cell-autoreactive *A14- $\alpha\beta$ TCR-tg* CD8<sup>+</sup> effectors to induce rapid T1D onset in sublethally irradiated NOD recipients was enhanced by help provided by pathogenic CD4 T cells (32). Therefore, the susceptibility of NR4S6 and NR4S7 mice to A14-induced T1D development was examined to initially evaluate the pathogenic helper activity of their CD4 T cells. As shown in Fig. 6C, the ability of A14 T cells to induce the rapid onset of T1D was significantly reduced in NR4S6 mice, but not in NR4S7 mice. These results suggested that CD4 T cells from the NR4S6 stock were less diabetogenic than were those from the standard NOD mice. This was indeed the case, as demonstrated in a combined BM/CD4 T cell-reconstitution system. NR4S6 CD4 T cells had a reduced ability to promote T1D development in *NOD.CD4<sup>mut</sup>* recipients compared with those from standard NOD mice (Fig. 6D). In contrast, NR4S7 and NOD CD4 T cells had comparable T1D-promoting capacity in *NOD.CD4<sup>mut</sup>* recipients. The NR4S6 congenic region partially overlaps that in NR4S5 mice (Figs. 2A, 6A). Because the congenic interval in the NR4S5 stock did not confer T1D resistance (Fig. 2B), it appears that the NOR-derived region regulating diabetogenic CD4 T cells can be defined in a 24.26-Mb region between the markers *rs3674285* and *D4Mit203*. This region

**FIGURE 6.** Genes within the NR4S6 subcongenic region regulate T1D development by modulating the pathogenic activity of CD4 T cells. **(A)** Polymorphic microsatellite and SNP markers were genotyped to define the boundaries of the NR4S6 and NR4S7 subcongenic lines developed at TJL. Colors within the congenic regions mark NOR genome of NOD (black), B6 (light gray), or DBA/2 (dark gray) origin. For comparison, the most current positions of the *Idd9.1* (116.08–134.49 Mb), *Idd9.2* (144.97–149.10 Mb), and *Idd11* (129.63–129.64 Mb) loci are shown (27, 28). **(B)** Cumulative incidence of T1D in female cohorts of NOD ( $n = 60$ ), NR4 ( $n = 11$ ), NR4S6 ( $n = 34$ ), and NR4S7 ( $n = 29$ ) mice was monitored through to 30 wk of age at TJL.  $**p < 0.01$ ,  $***p < 0.001$ , compared with NOD group (log-rank analysis). **(C)** Five million NOD.*Rag1*<sup>null</sup>.A14-tg splenocytes were transferred into the indicated sublethally (600 rad) irradiated female recipients ( $n = 10$ /group). T1D development was monitored daily for up to 2 wk post-transfer.  $**p < 0.01$ , compared with NOD recipients (log-rank analysis). **(D)** T1D development in NOD.*CD4*<sup>null</sup> females lethally irradiated (1300 rad) at 4–6 wk of age and reconstituted with equal numbers ( $5 \times 10^6$ ) of SBM and purified CD4 T cells from NR4S6 ( $n = 13$ ), NR4S7 ( $n = 11$ ), or NOD mice ( $n = 16$ ) was followed for 20 wk.  $*p < 0.05$ , compared with NOD CD4 T cell recipients (log-rank analysis).



partially overlaps with the previously defined *Idd9.1* locus (Fig. 6A) (28). In the B cell studies, the same region was shown to promote energy induction. Collectively, these results suggest that genes within this region may regulate T1D through modulating the interplay between B and CD4 T cells.

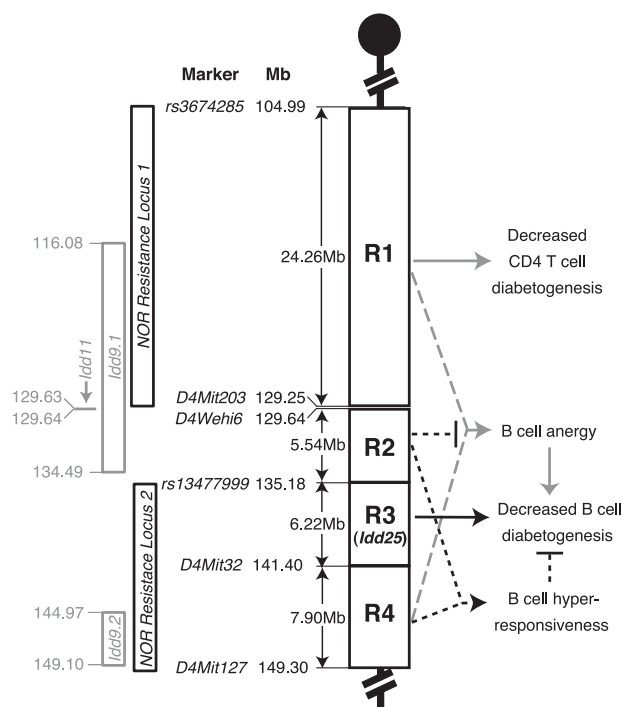
## Discussion

Congenic NOD mouse strains have facilitated the discovery of various T1D-susceptibility genes in this model (e.g.,  $\beta 2$  microglobulin, *Il2*, and *Ctla4*) (24, 35, 36), which have provided important insights into the molecular and cellular mechanisms influencing the human disease (21, 22). We previously described the generation of the NR4 strain (26), which contained a 44.31-Mb NOR-derived congenic region on distal Chr. 4 that decreased susceptibility to T1D in NOD background mice by dampening the pathogenic activity of B and CD4 T cells (30, 32). To significantly refine the location of genes contributing to these phenotypes, we generated seven novel subcongenic NOD mouse lines (NR4S1–S7) with truncated NOR-derived regions spanning different parts of the original FL NR4 congenic interval (Figs. 2A, 6A). T1D-incidence studies revealed that six of the seven subcongenic strains displayed some protection from disease, although none exhibited the same level of resistance seen in the original NR4 congenic mouse strain (Figs. 2B, 6B). This implied that the NOR-derived region contained more than one gene controlling T1D resistance, which was confirmed by the fact that the NR4S2 and NR4S4 lines containing nonoverlapping subcongenic regions afforded similar levels of disease protection. The result of these studies mapped the NOR genes conferring T1D resistance within the Chr. 4 locus to two regions: *rs3674285* (104.99 Mb) to *D4Mit203* (129.25 Mb) and *rs13477999* (135.18 Mb) to *D4Mit127* (149.30 Mb) (Fig. 7). These partly overlap with the previously described *Idd9.1* (128.37–131.18 Mb) and *Idd9.2*

(144.97–149.10 Mb) susceptibility loci, respectively, mapped using B10 congenic regions on the NOD background (28). Although the NOR and B10 strains may share T1D-resistance alleles at these loci, the distinct origin of the NOR genome, which is primarily derived from NOD, B6, and DBA/2 strains, appears to also endow it with other novel genes regulating disease (23–26).

Of the seven subcongenic strains described in this study, only the NR4S5 strain, with a NOR-derived congenic interval spanning from 128.60 to 135.19 Mb (Fig. 2A), did not contribute any protection from the development of T1D (Fig. 2B). This result was surprising, given that refinement of the locus housing the *Idd11* gene using NOD mice with B6-derived congenic regions showed that significant resistance to T1D was conferred within a 6.9-kb interval between 129.63 and 129.64 Mb that lies within a predicted gene of unknown function named *Ak005651* (27). Given that the NOR strain was found to have an identical sequence to B6 mice over this 6.9-kb interval (27), we would have predicted the NR4S5 strain to show significant protection from disease. This result may be suggestive of genetic drift in the B6-derived region surrounding the *Idd11* locus in NOR mice, which may possess polymorphisms that decrease the penetrance of the protective *Ak005651* allele.

The other incentive for generating NR4 subcongenic mouse lines was to map the NOR gene(s) within the distal end of Chr. 4 that reduced the diabetogenic activity of B cells, as well as to determine whether B cell hyperresponsiveness and the restoration of B cell energy remained linked to this gene. Our experiments with the subcongenic strains demonstrated that the original NR4 congenic interval could be divided into four subregions (termed R1–R4) that control different aspects of B cell function (Fig. 7). B cells from the NR4S3 and NR4S4 subcongenic strains had a reduced capacity to induce T1D in NOD.*Igμ*<sup>null</sup> mice compared with those from standard NOD donors (Fig. 3A). This led us to hypothesize



**FIGURE 7.** Model delineating the distinct segments controlling pathogenic B and CD4 T cell function in the distal Chr. 4 locus of NOR mice. Results of our experiments in the NR4S1–S7 subcongenic strains identified four regions (R1–R4) on distal Chr. 4 that contain genes controlling pathogenic B and CD4 T cell function. Markers demarcating the boundaries of each region are shown. T1D is independently controlled by NOR-derived genes in the R1 region and the R3–R4 region. The hyperresponsive phenotype of NR4 B cells to BCR stimulation is the result of an interaction of genes within R2 and R4. The maintenance of anergy in NR4 B cells is controlled by genes within R1 and R4, with counter-balancing negative regulation by R2. R3 contains the gene(s) responsible for dampening the diabetogenic activity of NR4 B cells. The penetrance of this gene is inhibited by genes within R2 and R4, potentially by making B cells hyperresponsive to BCR stimulation. Genes within R1 and R4 could also contribute to the dampening of B cell diabetogenic activity in the presence of the R3 region, potentially through their effects on B cell anergy. Finally, the R1 region contains a gene(s) responsible for dampening the diabetogenic function of NR4 CD4 T cells. For comparison, the most current positions of the *Idd9.1*, *Idd9.2*, and *Idd11* resistance loci (in megabases) from B10 or B6 mice are shown (27, 28).

that the principal gene(s) controlling the diabetogenic capacity of B cells is encompassed within the overlap of the NR4S3 and NR4S4 congenic regions. This subregion, termed R3, consists of a 6.22-Mb interval located between 135.18 and 141.40 Mb (Fig. 7). This region has not previously been associated with the development of T1D and, thus, is likely to represent a distinct susceptibility gene from those mapped to *Idd9.1*, *Idd9.2*, and *Idd11* loci, which we now designate as *Idd25*. Interestingly, the NOD. *Igμ<sup>null</sup>* recipients reconstituted with NR4S3 and NR4S4 B cells also exhibited a delayed onset of disease compared with those reconstituted with NR4 B cells (Fig. 3B). Hence, the gene(s) dampening diabetogenic B cell activity in the NR4S3 and NR4S4 strains may have been separated from NOR genes with opposing effects that exist within the FL congenic region.

Importantly, neither NR4S3 nor NR4S4 B cells exhibited the hyperresponsiveness (Fig. 4A, 4B) or the restoration of anergy phenotype (Fig. 4E, 4F) that characterize B cells from the NR4 strain (30). This confirmed that these phenotypes are not directly associated with the NOR gene(s) dampening diabetogenic B cell activity. Nevertheless, our experiments suggest that genes con-

trolling these associated phenotypes may have the capacity to modulate the diabetogenic activity of B cells. In this regard, it is worth noting that B cells from the NR4S1 strain, whose congenic interval also encompasses the R3 region (Fig. 2A), showed a similar diabetogenic capacity as those from standard NOD mice (Fig. 3A). The potential reason for this is that, in addition to R3, the congenic interval in NR4S1 mice contains two additional regions located between 129.64 and 135.18 Mb (R2) and between 141.40 and 149.30 Mb (R4) (Figs. 2A, 7). Our experiments showed that the combination of NOR genes within the R2 and R4 regions was necessary for B cells to display hyperresponsiveness to BCR stimulation (Fig. 4A, 4B). NR4S3 and NR4S4 B cells were not hyperresponsive because they only contained the R2 or R4 region, respectively (Figs. 2A, 7). From these experiments, we predict that the hyperresponsive phenotype encoded by NOR genes within R2 and R4 leads to increased diabetogenic activity of B cells, overriding the protective B cell phenotype encoded by R3.

Despite containing the R2, R3, and R4 regions (and the hyperresponsive phenotype), B cells from the original NR4 congenic mouse were less diabetogenic than were those from NR4S1 donors (Fig. 3A). This difference indicated that an additional region on NOR Chr. 4, between 104.99 and 129.25 Mb (R1) (Fig. 7), was capable of decreasing the diabetogenic activity of B cells. Nonetheless, it is important to note that genes within the R1 region are incapable of independently decreasing diabetogenic B cell activity, as demonstrated by the high incidence of disease in NOD. *Igμ<sup>null</sup>* mice reconstituted with NR4S2 B cells (Fig. 3A). NOR genes within R1, as well as the R4 region, were shown to contribute to the decreased responsiveness (i.e., anergy) of self-reactive B cells to BCR stimulation (Fig. 4E, 4F). Such a finding may explain the capacity of an R1-region gene(s) to reduce the diabetogenic capacity of B cells in an augmentative, but not independent, fashion.

In addition to their effects on B cells, NOR genes in the R1 region were shown to contribute to T1D resistance by dampening the pathogenic activity of CD4 T cells. This raised the possibility that modulation of CD4 T cells by R1 genes may indirectly regulate B cell diabetogenic activity and anergy. We do not believe this to be the case, given that our previous experiments showed that the B cell anergy defect in NOD mice is an intrinsic trait controlled by genes within Chr. 1 and 4 loci (17) and, as previously mentioned, the R1 region on its own had little effect on the diabetogenic activity of B cells (Fig. 3A). Alternatively, although the congenic region in NR4S4 mice was shown to have a strong dampening effect on the diabetogenic activity of B cells (Fig. 3A), a virtually identical congenic interval in the NR4S7 strain (Fig. 6A) had no effect on CD4 T cell pathogenesis (Fig. 6C, 6D). From these studies, we conclude that the NOR distal Chr. 4 region contains distinct sets of resistance alleles that mediate independent effects on B and CD4 T cells contributing to T1D. Inheritance of both sets of NOR genes is likely to significantly affect the interplay between these cell types, leading to greater protection from T1D.

Through our analyses of subcongenic mouse strains, we managed to reduce the region containing a gene(s) controlling the diabetogenic activity of B cells from 44.31 Mb (containing 889 genes) to a 6.22-Mb region (containing 152 genes) encompassing the newly identified *Idd25* locus contributing to this phenotype. The smaller size of this region makes it much more amenable to sequencing, which has the capacity to reveal all SNPs between NOD and NOR origin genes that may be responsible for the *Idd25* effect regulating B cell diabetogenic function. In the meantime, our microarray (Fig. 5A) and q-rPCR studies (Fig. 5B, 5C) identified two promising *Idd25* candidate genes that were differentially expressed between NOD and NR4 B cells in this 6.22-Mb



region: *Ephb2* and *Padi2*. *EphB2* encodes a protein tyrosine kinase receptor with the capacity to bind various Ephrin B ligands on neighboring cells (37). Although the function of *Ephb2* has been studied most extensively in the nervous system (38), a role for this receptor was more recently revealed in T cells, where it was shown to potentially act as a costimulatory molecule that alters TCR-mediated signaling (39). As a result, mice containing a deletion of *EphB2* exhibit defects in T cell development (40) and peripheral T cell responses (41, 42). As we also found in this study, expression of *EphB2* was reported for B cells, although its function in this population remains to be elucidated (37). *Padi2* encodes the  $\text{Ca}^{2+}$ -dependent enzyme peptidylarginine deaminase-2 that catalyzes the conversion of arginine residues to citrulline (43). This posttranslational conversion results in an ablation of the positive charge in proteins, potentially altering protein structure and function, as well as their recognition and presentation by the immune system (44–46). In humans, *PADI2* and its closely related family member, *PADI4*, were identified as susceptibility genes for multiple sclerosis and rheumatoid arthritis, respectively (47, 48). In both diseases, excess citrullination of proteins caused by overexpression of *PADI2* or *PADI4* leads to a breakdown in B and T cell tolerance (43, 47, 49, 50). Excessive expression of *Padi2* by NOD B cells could play a similar role in the breakdown of tolerance to pancreatic  $\beta$  cell Ags.

In conclusion, the refinement of the large congenic region in NR4 mice into smaller subcongenic regions revealed a complexity of interactions between multiple genes on Chr. 4 that regulate susceptibility to T1D by controlling the pathogenic activity of B and CD4 T cells. Identifying these gene(s) can provide significant insights into the mechanisms underlying the development of T1D and have the potential to highlight new molecular pathways that can be targeted as a disease-intervention approach with greater specificity than current pan-B or T cell-depleting drugs (6, 51). The distinct set of polymorphisms in the NOR background also makes our subcongenic mice useful for mapping allelic variants affecting several other immune defects in NOD mice that have been linked to the distal Chr. 4 region (21).

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## Disclosures

The authors have no financial conflicts of interest.

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