Idd9/11 Genetic Locus Regulates Diabetogenic Activity of CD4 T-Cells in Nonobese Diabetic (NOD) Mice

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OBJECTIVE—Although the $H2^{g7}$ major histocompatibility complex (MHC) provides the primary pathogenic component, the development of T-cell–mediated autoimmune type 1 diabetes in NOD mice also requires contributions from other susceptibility (*Idd*) genes. Despite sharing the $H2^{g7}$ MHC, the closely NOD-related NOR strain remains type 1 diabetes resistant because of contributions of protective *Idd5.2*, *Idd9/11*, and *Idd13* region alleles. To aid their eventual identification, we evaluated cell types in which non-MHC *Idd* resistance genes in NOR mice exert disease-protective effects.

RESEARCH DESIGN AND METHODS—Adoptive transfer and bone marrow chimerism approaches tested the diabetogenic activity of CD4 and CD8 T-cells from NOR mice and NOD stocks congenic for NOR-derived *Idd* resistance loci. Tetramer staining and mimotope stimulation tested the frequency and proliferative capacity of CD4 BDC2.5-like cells. Regulatory T-cells (Tregs) were identified by Foxp3 staining and functionally assessed by in vitro suppression assays.

RESULTS—NOR CD4 T-cells were less diabetogenic than those from NOD mice. The failure of NOR CD4 T-cells to induce type 1 diabetes was not due to decreased proliferative capacity of BDC2.5 clonotypic-like cells. The frequency and function of Tregs in NOD and NOR mice were also equivalent. However, bone marrow chimerism experiments demonstrated that intrinsic factors inhibited the pathogenic activity of NOR CD4 T-cells. The NOR *Idd9/11* resistance region on chromosome 4 was found to diminish the diabetogenic activity of CD4 but not CD8 T-cells.

CONCLUSIONS—In conclusion, we demonstrated that a gene(s) within the *Idd9/11* region regulates the diabetogenic activity of CD4 T-cells. ***Diabetes* 57:3273–3280, 2008**

In both humans and NOD mice, type 1 diabetes results from T-cell–mediated autoimmune destruction of insulin-producing pancreatic β-cells (1,2). Both CD4 and CD8 T-cells are essential for type 1 diabetes development in NOD mice (1,3). Similar to the case in humans, multiple genetic susceptibility (*Idd*) loci contribute to type 1 diabetes in NOD mice (1). The $H2^{g7}$ major histocompatibility complex (MHC) provides the primary component of type 1 diabetes susceptibility in NOD mice, but alone, it is insufficient for disease development (1). In conjunction with the $H2^{g7}$ MHC, other *Idd* genes are also required to interactively contribute to the development of diabetogenic T-cell responses in NOD mice (1). Although the identities of some non-MHC *Idd* genes have been revealed (4–6), most of them remain unknown.

A resource allowing inquiry to the function of non-MHC *Idd* genes is the NOR mouse (7). NOR mice share ~88% of their genome with the NOD strain, including the $H2^{g7}$ MHC (7). However, major disease resistance genetic loci on chromosomes (Chr) 1 (*Idd5.2*), 2 (*Idd13*), and 4 (*Idd9/11*) completely protect NOR mice from type 1 diabetes (8–10). *Ctla-4* most likely represents the *Idd5.1* subregion gene, but the susceptibility allele is shared by NOD and NOR mice (8,10). Conversely, the NOR strain appears to differ from NOD mice by the presence of a type 1 diabetes–protective *Idd5.2* sublocus. Allelic variants of β2-microglobulin (*β2m*) represent one of at least two polymorphic *Idd13* region genes respectively contributing to type 1 diabetes susceptibility or resistance in NOD or NOR mice (4). Polymorphic *β2m* variants induce conformational differences in MHC class I molecules, which influences their ability to positively select autoreactive CD8 T-cells (11). In addition, the pathogenic activity of NOR B-cells is significantly lower than those of NOD origin because of contributions from *Idd9/11* region genes on Chr4 (12). Type 1 diabetes–protective genetic polymorphisms in NOR mice also suppress the pathogenic activity of T-cells transgenically expressing T-cell receptors (TCRs) from the diabetogenic CD4 (NY4.1) and CD8 (NY8.3) clones (13). However, further congenic mapping of NOR genetic variants responsible for the greatly diminished pathogenic activity of these diabetogenic CD4 and CD8 T-cell clones has not been reported.

Multiple phenotypic or functional differences in several immunological components, including T-cells, dendritic cells, and macrophages, have also been reported between NOD and NOR mice (14–18). Eventual identification of unknown NOR *Idd* resistance genes can be aided by determining the cell types in which they individually or in combination exert disease protection effects. Thus, in the current study, we defined the role that specific NOR-derived *Idd* resistance loci may play in limiting the development of diabetogenic CD4 and CD8 T-cells.

RESEARCH DESIGN AND METHODS

NOD/ShiLtDvs and closely related NOR/Lt mice (7) are maintained by brother-sister mating at The Jackson Laboratory (Bar Harbor, ME). Stocks of NOD background mice carrying NOR-derived congenic intervals on Chr1 NOD.NOR-(*D1Mit532-D1Mit8*)/DvsJ (here designated NOD.$Chr1^{NOR}$) or Chr4 NOD.NOR-(*D4Mit31-D4Mit310*)/DvsJ (here designated NOD.$Chr4^{NOR}$) have been described previously (10). In the latter of these two stocks, the NOR-derived Chr4 congenic interval was found to extend more distally than previously reported (10) to also encompass the marker *D4Mit310*. The NOD.NOR-(*D2Mit63-D2Mit48*)/LtJ congenic stock (here designated NOD.$Chr2^{NOR}$) was termed NOD.$D2Mit490\text{-}Mit144^{NOR}$ in a previous study

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(11). Designation of this NOR-derived Chr2 congenic interval has been modified from that originally reported because of updated knowledge of marker positions. Previously described NOD congenic stocks respectively deficient in CD4 and CD8 T-cells (designated NOD.*CD4*^{null} and NOD.*CD8*^{null}) were also used (19,20). A NOD stock lacking B-cells (21) is now maintained at the N10 backcross generation (termed NOD.*IgH*^{null}). A NOD.*IgH*^{null} substock with B-cells that only express transgene-encoded HEL-specific Ig molecules (designated NOD.*IgHEL.IgH*^{null}) has been previously reported (21). NOD mice transgenically expressing the TCR from the diabetogenic CD8 T-cell clone AI4 (Vα8/Vβ2) and congenic for a functionally inactivated *Rag1* gene (NOD.*Rag1*^{null}.*AI4*) have also been described previously (22). Female mice were used for all experiments.

Adoptive transfer of AI4 T-cells. Mice (6–8 weeks old) were sublethally irradiated (600R from a ^{137}Cs source) and injected intravenously with 5–10 × 10^6 NOD.*Rag1*^{null}.*AI4* splenocytes (equivalent to 1–2 × 10^6 AI4 T-cells). Similar results were obtained over this dose range of NOD.*Rag1*^{null}.*AI4* splenocytes. In some experiments, AI4 T-cells were preactivated in vitro. Briefly, NOD.*Rag1*^{null}.*AI4* splenocytes were cultured at 5 × 10^6 cells/ml with 0.1 μmol/l of the previously described antigenic mimotope peptide (YFIENYLEL) (23) and 25 units/ml recombinant human interleukin (IL)-2 for 2 days. After activation, 1 × 10^6 viable AI4 T-cells were injected into the recipients. The comparative activation state of freshly ex vivo–isolated and in vitro antigen–stimulated AI4 T-cells were compared by flow cytometry using antibodies specific for the CD25 (PC61), CD69 (H1.2F3), and CD44 (IM7.8.1) markers. In each transfer experiment, standard NOD mice and other various test recipient stocks were compared side by side. Recipient mice were monitored for type 1 diabetes development over a 2-week period by daily monitoring of glycosuria onset with Ames Diastrix (Bayer, Diagnostics Division, Elkhart, NJ).

Generation of mixed bone marrow/T-cell chimeras. NOD.*CD4*^{null} mice at 4–7 weeks of age were lethally irradiated (1300R from a ^{137}Cs source) and reconstituted as previously described (24) with 5 × 10^6 of the indicated T-cell–depleted bone marrow cells isolated from 6- to 10-week-old donors. In some experiments, 5 × 10^6 T-cell–depleted NOD.*CD4*^{null} bone marrow cells were admixed with 5 × 10^6 purified CD4 T-cells as indicated. Splenic CD4 T-cells were purified from 6- to 9-week-old mice by depleting B220$^+$, CD8$^+$, and CD11b$^+$ cells with the previously described magnetic bead system (25). Biotinylated antibodies specific for B220 (RA3–6B2), CD8 (53–6.7), and CD11b (M1/70) were obtained from BD Bioscience. The purity of CD4 T-cells was routinely >92%, as determined by flow cytometry. A similar approach was used to study the diabetogenic activity of CD8 T-cells using lethally irradiated NOD.*CD8*^{null} mice as recipients. CD8 T-cells were purified with the same magnetic bead system by depleting B220$^+$, CD4$^+$ (clone GK1.5, BD Bioscience), and CD11b$^+$ cells. Type 1 diabetes was again monitored by onset of glycosuria.

Treg and BDC2.5 effector enumeration. The frequency of CD4 diabetogenic BDC2.5-like cells was determined by tetramer staining as previously described (26) with the exception that the tetramer reagent was used at a concentration of 4.0 μg/ml, and the staining was carried out at 37°C. Propidium iodide was used to gate out dead cells. Regulatory T-cells (Treg) were identified by surface staining with anti-CD4 (clone GK1.5) and intracellular staining with anti-Foxp3 (clone FJK-16s) using an intracellular staining kit from eBioscience. Stained cells were washed and analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

In vitro Treg assay. CD4 T-cells were purified as described above. The CD25$^+$ fraction was further isolated by staining with biotinylated anti-CD25 (clone 7D4; BD Bioscience) followed by streptavidin-conjugated microbeads (Miltenyi Biotec). CD4$^+$CD25$^+$ cells were then enriched by positive selection using an LS column (Miltenyi Biotec). The CD4$^+$CD25$^-$ fraction was used as the effector population. Effector cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) as previously described (27). Labeled effector cells (5 × 10^4) were cocultured in triplicate with indicated numbers of putative CD4$^+$CD25$^+$ Tregs in the presence of 2 × 10^5 NOD.*scid* splenocytes and 5 μg/ml anti-CD3 (clone 145–2C11, BD Bioscience) in round-bottomed 96-well tissue culture plates in a final volume of 200 μl culture medium as previously described (28). Proliferation of effector cells was determined after 3 days of culture by CFSE dilution.

BDC2.5 mimotope priming and recall assay. NOD, NOR, and NOD.*Chr4*^{NOR} females (8 weeks old) were immunized in a rear footpad with 20 μg BDC2.5 mimotope (AHHPIWARMDA) or a control IA^{g7} binding peptide (LSIALHVGFDH) emulsified in incomplete Freund's adjuvant (IFA). Ten days later, 1 × 10^6 cells from dispersed draining lymph nodes were seeded in triplicate into flat-bottomed 96-well tissue culture plates in a final volume of 200 μl culture medium as previously described (28) containing varying concentrations of BDC2.5 mimotope. Cells were cultured for 3 days and labeled with 1 μCi/well [^{3}H]thymidine during the last 20 h. Interferon-γ (IFN-γ), IL-4, and IL-10 were analyzed by ELISA kits (BD Bioscience).

RESULTS

Idd9/11-regulated events in CD4 T-cells and B-cells modulate the diabetogenic activity of AI4 CD8 T-cells. Autoreactive CD8 T-cells are essential to the initiation of type 1 diabetes development in NOD mice (29,30), but their efficient activation requires contributions from other immunological components. Thus, it seemed possible that some gene(s) contributing to type 1 diabetes resistance in the closely NOD-related NOR strain could do so by limiting the ability of other immunological component(s) to provide functions necessary for the efficient activation of pathogenic CD8 T-cells. CD8 T-cells transgenically expressing the AI4 TCR rapidly induce type 1 diabetes when adoptively transferred into sublethally irradiated standard NOD recipients (27). Therefore, we asked whether the AI4 adoptive transfer method could also induce type 1 diabetes in NOR mice or NOD stocks congenic for NOR-derived chromosomal regions carrying protective alleles. Although >75% of standard NOD mice receiving NOD.*Rag1*^{null}.*AI4* splenocytes developed type 1 diabetes within 2 weeks, all of the NOR recipients remained disease free over the same period of time (Table 1). Both NOD.*Chr1*^{NOR} and *NOD.Chr2*^{NOR} congenic strains were susceptible to AI4 T-cell–induced type 1 diabetes (Table 1). In contrast, no NOD.*Chr4*^{NOR} recipients developed type 1 diabetes under the same transfer conditions (Table 1).

We next asked what particular host lymphocyte populations modulate the diabetogenic activity of adoptively transferred AI4 T-cells. To do this, we transferred NOD.*Rag1*^{null}.*AI4* splenocytes into sublethally irradiated NOD mice lacking B cells (NOD.*IgH*^{null}), CD8 T-cells (NOD.*CD8*^{null}), or CD4 T-cells (NOD.*CD4*^{null}). Both host B-cells and CD4 T-cells, but not CD8 T-cells, were important for adoptively transferred AI4 T-cells to mediate type 1 diabetes development (Table 1). One important role of autoreactive B-cells for type 1 diabetes development in NOD mice is to function as a preferential antigen-presenting cell (APC) subset because of a unique ability to efficiently uptake autoantigens through cell surface Ig molecules (21). This allows these B-cells to subsequently preferentially present MHC class II–bound self-peptides to autoreactive CD4 T-cells. However, B-cells may also contribute to autoimmunity by mechanisms independent of membrane-bound or secreted Ig molecules (31). Furthermore, B-cells also reportedly exert functions facilitating the ability of dendritic cells to cross-present MHC class I–bound antigens to CD8 T-cells (32). To test these possibilities, we transferred NOD.*Rag1*^{null}.*AI4* splenocytes into sublethally irradiated NOD.*IgHEL.IgH*^{null} mice in which all B-cells express Ig molecules specific for the disease-irrelevant HEL protein. Similar to standard NOD recipients, NOD.*IgHEL.IgH*^{null} mice were highly susceptible to AI4 T-cell–mediated type 1 diabetes (Table 1). Therefore, antigen specificity of B-cells was not essential for their ability to facilitate the pathogenic function of AI4 T-cells. The majority of directly ex vivo–isolated splenic AI4 T-cells in the transfer inoculums displayed a naive phenotype (>99% CD69$^-$/CD25$^-$/CD44^{low}). Therefore, host CD4 T-cells and B-cells appear to facilitate the subsequent activation of adoptively transferred AI4 T-cells. AI4 T-cells can independently induce type 1 diabetes in T-cell– and B-cell–deficient NOD-*scid* recipients (33) but with significantly slower kinetics than observed in the present system

TABLE 1
Genetic control of susceptibility to AI4 T-cell–induced type 1 diabetes*

Experiment group	Transferred cells	Recipients	Diabetic	*P* value†
1	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	76.9 (13)	
		NOR	0 (11)	<0.001
2	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	60 (10)	
		NOD.*Chr1*^{NOR}	30 (10)	>0.05
3	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	57.1 (7)	
		NOD.*Chr2*^{NOR}	42.8 (7)	>0.05
4	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	70 (10)	
		NOD.*Chr4*^{NOR}	0 (10)	<0.005
5	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	75 (12)	
		NOD.*CD4*^{null}	0 (10)	<0.005
6	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	75 (8)	
		NOD.*CD8*^{null}	33.3 (9)	>0.05
7	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	61.5 (13)	
		NOD.*IgH*^{null}	0 (13)	<0.001
8	NOD.*Rag*^{null}.*AI4* splenocytes	NOD	60 (10)	
		NOD.*IgHEL.IgH*^{null}	70 (10)	>0.05
9	Activated AI4 T-cells	NOD	100 (3)	
		NOR	100 (3)	—
10	Activated AI4 T-cells	NOD	100 (4)	
		NOD.*Chr4*^{NOR}	100 (5)	—
		NOD.*CD4*^{null}	100 (5)	—
11	Activated AI4 T-cells	NOD	100 (5)	
		NOD.*IgH*^{null}	100 (5)	—

Data are percent (*n*). *NOD.*Rag*^{null}.*AI4* splenocytes or in vitro–activated AI4 T-cells were transferred into 600R irradiated female recipients. Type 1 diabetes was monitored for a period of 2 weeks. Mice within each experimental group received the same preparation of AI4 T-cells. †Statistical analysis by χ^2 test (vs. NOD recipients in the same experimental group).

when residual CD4 T-cells and B-cells remain in sublethally irradiated standard NOD recipients.

We next asked whether AI4 T-cells preactivated in vitro by mimotope peptide stimulation could induce type 1 diabetes in the absence of host CD4 T-cells or B-cells. Preactivated AI4 T-cells (>66% CD25$^+$CD69$^+$CD44^{hi}) induced type 1 diabetes development in CD4 T-cell– or B-cell–deficient NOD recipients (Table 1). In addition, NOR and NOD.*Chr4*^{NOR} mice rapidly developed type 1 diabetes when receiving preactivated AI4 T-cells (Table 1). Together, these results indicate that in this adoptive transfer system, CD4 T-cells and B-cells in NOD mice, but not NOR or NOD.*Chr4*^{NOR} mice, facilitate the pathogenic activation of AI4 CD8 T-cells. In the current study, we continued to analyze the mechanism by which *Idd9/11* genes regulate the activity of diabetogenic CD4 T-cells. The role of B-cells in regulating autoreactive CD8 T-cells will be the subject of future studies.

Reduced pathogenic activity of NOR CD4 T-cells. We next tested whether mature NOR CD4 T-cells remained unable to facilitate the initiation of type 1 diabetes development when all other components of the immune response were of NOD origin. The system used was to reconstitute lethally irradiated normally type 1 diabetes–resistant NOD.*CD4*^{null} mice (19) with syngeneic bone marrow mixed with purified CD4 T-cells from either NOD or NOR donors. NOD.*CD4*^{null} mice receiving syngeneic bone marrow alone did not develop type 1 diabetes (data not shown). Of the NOD.*CD4*^{null} recipients, >90% developed type 1 diabetes when reconstituted with syngeneic bone marrow and NOD CD4 T-cells (Fig. 1*A*). In contrast, NOD.*CD4*^{null} mice injected with syngeneic bone marrow, and NOR CD4 T-cells were highly resistant to type 1 diabetes development. NOR B-cells exert significantly less diabetogenic APC activity than those from NOD mice (12). Therefore, the variable capacity of transferred CD4 T-cells from NOD and NOR mice to facilitate type 1 diabetes development could solely result from differences in previous engagements with antigen-presenting B-cells capable of expanding pathogenic effectors. If this were the case, then compared with those of NOD or NOR origin, CD4 T-cells isolated from NOD.*IgH*^{null} mice should exhibit poor diabetogenic activity. Therefore, we reconstituted NOD.*CD4*^{null} mice with syngeneic bone marrow and CD4 T-cells from NOD.*IgH*^{null} mice. CD4 T-cells from NOD.*IgH*^{null} donors were as diabetogenic as those of NOD origin (Fig. 1*A*).

MHC class II A^{g7} tetramers were used to determine the frequency of BDC2.5-like diabetogenic CD4 T-cells in the spleens of NOR mice. Consistent with a previous report (26), comparable levels of BDC2.5-like CD4 T-cells developed in NOD and NOR mice (Fig. 1*B*). However, compared with both the NOD and NOR strains, BDC2.5-like T-cell levels are lower in the NOD.*IgH*^{null} stock, indicating that expansion and/or survival of these diabetogenic effectors is dependent on the presence of B-cells. The proliferation potential of BDC2.5-like cells in NOR mice was tested using a priming-recall assay. BDC2.5-like cells from NOD and NOR mice proliferated similarly in response to peptide stimulation (Fig. 1*C*). These collective results indicate that the inability of NOR CD4 T-cells to support type 1 diabetes development was not due to diminished numbers of potential β-cell autoreactive effectors.

Intrinsic factors inhibit the development of diabetogenic NOR CD4 T-cells. Although NOR CD4 T-cells have very limited diabetogenic activity, it was not known whether this is controlled intrinsically or modulated by functional differences in other cell types. One possible mechanism downregulating the diabetogenic activity of CD4 T-cells in NOR mice relative to the NOD strain is they

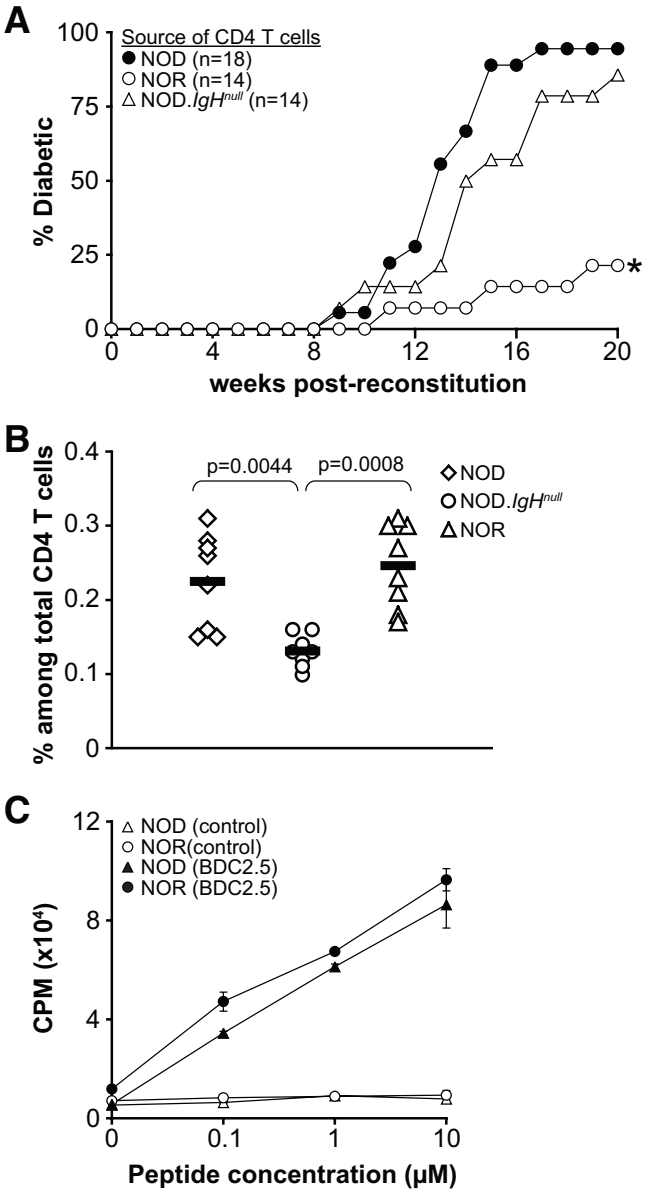

FIG. 1. Reduced diabetogenic activity of NOR CD4 T-cells. *A*: Incidence of type 1 diabetes in CD4 T-cell–reconstituted NOD.*CD4*^{null} mice. Lethally irradiated 4- to 7-week-old NOD.*CD4*^{null} mice were injected with equal numbers (5×10^6) of syngeneic bone marrow and purified CD4 T-cells from NOD, NOR, or NOD.*IgH*^{null} donors. Type 1 diabetes development was then followed for 20 weeks. *$P < 0.001$, significantly different from NOD CD4 T-cell recipients (Kaplan-Meier log-rank analysis). *B*: The frequency of BDC2.5-like diabetogenic CD4 T-cells in NOD, NOR, or NOD.*IgH*^{null} mice. Splenocytes from 6- to 9-week-old females were stained with CD4 antibodies and BDC2.5 MHC class II tetramers to identify BDC2.5-like cells. Each symbol represents an individual mouse. Horizontal bars indicate the means. The percentages of BDC2.5-like cells did not differ in NOD and NOR mice but were both significantly greater than in the NOD.*IgH*^{null} strain (Wilcoxon's rank-sum test). *C*: Functional analysis of BDC2.5-like cells in NOD and NOR mice. Groups of three mice were primed with 20 μg BDC2.5 mimotope or IA^{g7}-binding control peptide in IFA. After 10 days, cells from the draining lymph nodes of the same group were pooled, and all were restimulated in triplicate with indicated concentration of the BDC2.5 mimotope. The cultures were pulsed with [^{3}H]thymidine over the final 20 h of a 72-h incubation period. Results indicate the mean counts per minute (CPM) ± SE of the triplicates.

may have an increased proportion or enhanced function of Tregs. In the experiments described above, the splenic NOR-derived CD4 T-cells transferred to NOD.*CD4*^{null} recipients may have also included Tregs. Therefore, we compared the frequency of splenic Tregs in NOD and NOR

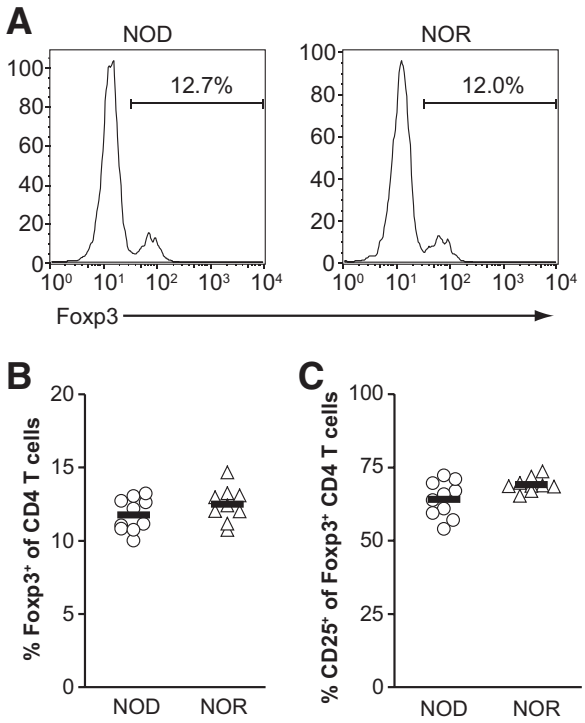

FIG. 2. Comparison of Treg frequencies in NOD and NOR mice. Splenocytes from 6- to 9-week-old mice were stained with antibodies against CD4, Foxp3, and CD25 to identify Tregs. *A*: Representative plots show the proportion of Foxp3-expressing CD4 T-cells. *B*: The percentages of CD4 T-cells expressing Foxp3. *C*: The percentages of CD25$^+$ cells among Foxp3$^+$ CD4 T-cells. Each symbol represents an individual mouse. Horizontal bars indicate the means. There is no statistically significant difference in the percentages shown in *B* and *C* between NOD and NOR mice (Wilcoxon's rank-sum test).

mice based on coexpression of CD4 and Foxp3. NOD and NOR mice did not differ in percentages of CD4 T-cells that coexpressed Foxp3 (Fig. 2*A* and *B*). In addition, the percentages of Foxp3$^+$ CD4 T-cells that coexpressed CD25 were similar in these two strains (Fig. 2*C*). Although numerically similar, it was possible that Tregs in NOD and NOR mice functionally differed. However, when tested in vitro, NOD and NOR CD4$^+$CD25$^+$ Tregs (both ~80% Foxp3$^+$) exhibited similar levels of suppressive activity (Fig. 3).

We next asked whether NOR CD4 T-cells support type 1 diabetes development when they differentiate from stem cells in an environment where most cells are NOD derived. Lethally irradiated NOD.*CD4*^{null} mice were reconstituted with a 4:1 mixture of syngeneic and NOR bone marrow cells. In this case, all CD4 T-cells were NOR derived with most other hematopoietic cells and all nonhematopoietic cells of NOD origin. Control chimeras consisted of NOD.*CD4*^{null} recipients repopulated with a 4:1 mixture of syngeneic and standard NOD bone marrow cells. These control chimeras developed a high incidence of type 1 diabetes (Fig. 4). In contrast, recipients of NOD.*CD4*^{null} and NOR bone marrow were completely type 1 diabetes resistant. NOR-derived leukocytes other than CD4 T-cells might have actively suppressed type 1 diabetes development in these bone marrow chimeras. To test this possibility, another set of control chimeras was reconstituted with a 4:1 mixture of NOD and NOR bone marrow. Similar to the recipients of NOD.*CD4*^{null} and NOD bone marrow, the second control group was type 1 diabetes susceptible (Fig. 4). These collective results indicate that an intrinsic

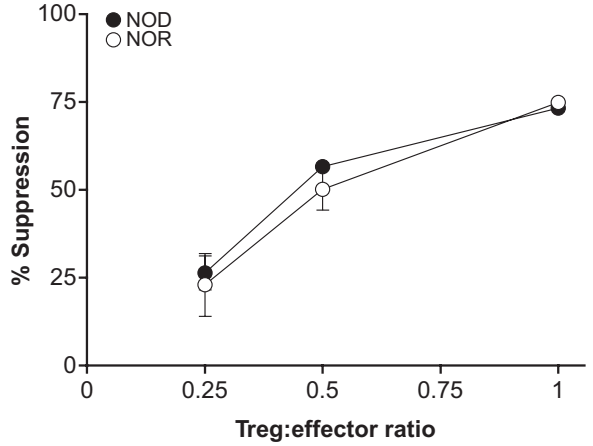

FIG. 3. Comparison of in vitro suppressive activities of NOD and NOR Tregs. Effector T-cells ($CD4^+CD25^-$) were labeled with CFSE and cocultured at indicated ratios with Tregs ($CD4^+CD25^+$) in triplicate in a 96-well plate in the presence of NOD.*scid* splenocytes (2×10^5) and 5 μg/ml anti-CD3 for 3 days. Proliferation of effector T-cells was determined by CFSE dilution. The percentage of suppression is defined by the percent reduction in the proportion of divided effector T-cells relative to that of the control without Tregs. Results indicate the means ± SE of the triplicates. Similar results were observed in another two experiments.

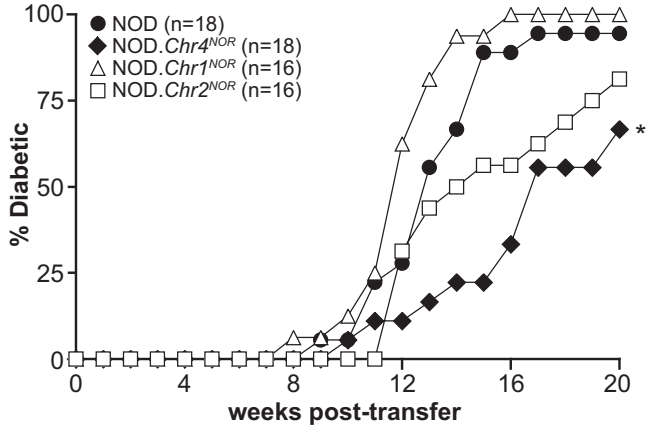

FIG. 5. The diabetogenic activity of CD4 T-cells is regulated by a gene(s) within the *Idd9/Idd11* region. CD4 T-cells (5×10^6) purified from NOD.$Chr1^{NOR}$, NOD.$Chr2^{NOR}$, or NOD.$Chr4^{NOR}$ mice and NOD.$CD4^{null}$ bone marrow cells (5×10^6) were mixed and injected into lethally irradiated 4- to 7-week-old NOD.$CD4^{null}$ recipients. Type 1 diabetes development was then followed weekly for 20 weeks. The same accumulated incidence of NOD CD4 T-cell recipients shown in Fig. 1*A* was also plotted here for comparative purposes. All CD4 T-cell transfer experiments, including those shown in Fig. 1*A*, were done in an overlapping fashion. *$P < 0.001$, significantly different from NOD CD4 T-cell recipients (Kaplan-Meier log-rank analysis).

factor(s) limits the ability of NOR CD4 T-cells to mediate diabetogenic responses.

Idd9/11 diabetes resistance loci control the pathogenic activity of CD4 T-cells. To dissect the genetic basis of diabetogenic CD4 T-cell development, we reconstituted lethally irradiated NOD.$CD4^{null}$ mice with syngeneic bone marrow and CD4 T-cells isolated from NOD.$Chr1^{NOR}$, NOD.$Chr2^{NOR}$, or NOD.$Chr4^{NOR}$ mice. Compared with those of NOD origin, CD4 T-cells isolated from NOD.$Chr4^{NOR}$ mice, but not NOD.$Chr1^{NOR}$ mice, demonstrated a reduced ability to support type 1 diabetes development (Fig. 5). There was a possible trend for CD4 T-cells from NOD.$Chr2^{NOR}$ mice to exert less diabetogenic activity than those of NOD origin, but this difference did not achieve statistical significance in the number of recipients analyzed (16–18 per group). Using the same bone marrow chimerism approach described in Fig. 4, we found that an intrinsic factor(s) limits the pathogenic potential of NOD.$Chr4^{NOR}$ CD4 T-cells (data not shown).

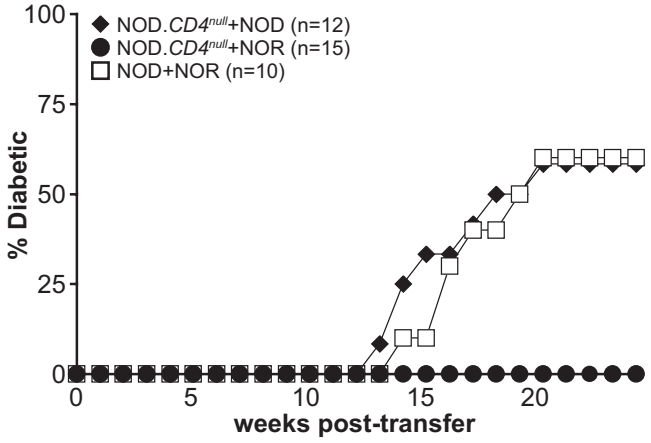

FIG. 4. Intrinsic factors control the diabetogenic activity of NOR CD4 T-cells. Lethally irradiated NOD.$CD4^{null}$ mice were reconstituted with a mixture of syngeneic bone marrow with NOD or NOR bone marrow at a 4:1 ratio. A control group was reconstituted with a 4:1 mixture of NOD and NOR bone marrow. Type 1 diabetes development was then analyzed weekly.

We analyzed the function of BDC2.5-like CD4 T-cells in NOD.$Chr4^{NOR}$ mice using the same priming-recall assay described in Fig. 1*C*. BDC2.5-like CD4 T-cells from NOD and NOD.$Chr4^{NOR}$ mice proliferated equivalently (data not shown). Production of IFN-γ, IL-4, and IL-10 by primed BDC2.5-like cells after antigenic peptide (10 μmol/l) restimulation was also assessed. NOD.$Chr4^{NOR}$ BDC2.5-like cells produced less IFN-γ than those of NOD origin (58.0 ± 7.1 and 109.3 ± 3.7 μg/ml, respectively). Therefore, the lower diabetogenic activity of NOD.$Chr4^{NOR}$ than NOD CD4 T-cells could result from reduced production of the inflammatory cytokine IFN-γ. However, on antigenic stimulation, lower levels of the immunosuppressive cytokine IL-10 were secreted by BDC2.5-like CD4 T-cells from NOD.$Chr4^{NOR}$ than NOD mice (755.9 ± 22.9 and 1,061.3 ± 38.1 pg/ml, respectively). Hence, *Idd9/11* region genes do not contribute to type 1 diabetes resistance in NOR mice through enhancing IL-10 production by CD4 T-cells. Both strains produced undetectable amounts of IL-4. These results indicated that BDC2.5-like CD4 T-cells in NOD and NOD.$Chr4^{NOR}$ mice have the same proliferative capacity but qualitatively differ in their effector responses.

Common pathways may contribute to the development of both autoreactive CD4 and CD8 T-cells. Hence, we assessed whether NOD.$Chr4^{NOR}$ CD8 T-cells are also less diabetogenic than those from NOD mice. Interestingly, the NOR-derived Chr4 congenic interval that inhibited diabetogenic CD4 T-cell responses did not diminish the pathogenic activity of CD8 effectors (Fig. 6). These results indicate an *Idd9/11* region gene(s) distinguishing NOD and NOR mice selectively controls the pathogenic activity of CD4 T-cells.

DISCUSSION

Studies using the 4.1 TCR transgenic system found thymic or peripheral deletion as well as anergy and ignorance do not contribute to CD4 T-cell tolerance induction in NOR mice (13). Similarly, we found the frequency and proliferative capacity of BDC2.5-like CD4 T-cells was comparable in NOD and NOR mice. The fact that NOR CD4 T-cells

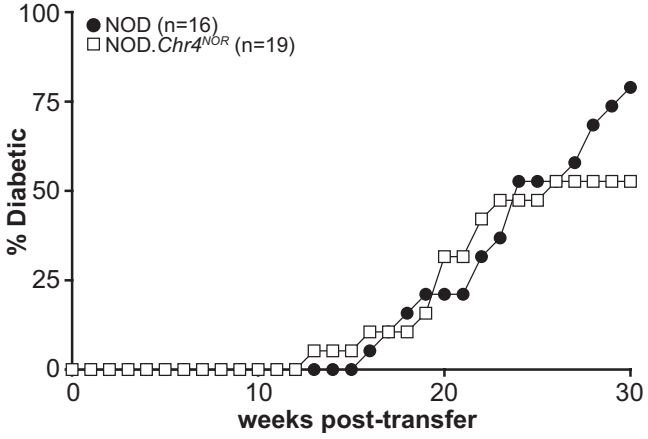

FIG. 6. The diabetogenic activity of NOD and NOD.*CD4*^{null} CD8 T-cells is comparable. Lethally irradiated NOD.*CD8*^{null} mice were injected with 5 × 10^6 syngeneic bone marrow cells and 5 × 10^6 CD8 T-cells purified from NOD mice or the NOD.*Chr4*^{NOR} congenic strain. Type 1 diabetes development was then analyzed weekly. The type 1 diabetes incidence between NOD and NOD.*Chr4*^{NOR} CD8 T-cell recipient groups is not significantly different (Kaplan-Meier log-rank analysis).

induced type 1 diabetes in some NOD.*CD4*^{null} recipients revealed the retention of at least minimal pathogenic activity. On the other hand, NOR T-cells are more susceptible than those from NOD mice to activation-induced cell death (AICD) (34). Therefore, abortive activation followed by AICD may limit the effector function of NOR diabetogenic CD4 T-cells. Another nonmutually exclusive possibility is that diabetogenic CD4 T-cells are more efficiently suppressed in NOR than NOD mice. However, we found no difference in the frequency or in vitro suppressive function between NOD and NOR Tregs. These results suggested that Treg function and the sensitivity of effector T-cells to suppression are similar in NOD and NOR mice at the age we tested. Our in vitro suppression assay did not consider Treg antigenic specificity. β-Cell antigen-specific Tregs are superior than those with a diverse repertoire in blocking type 1 diabetes development (35,36). Therefore, it is possible that there is higher frequency of β-cell–specific Tregs in NOR than NOD mice, which in turn more effectively suppress diabetogenic effectors in the former strain.

We previously demonstrated the NOR-derived *Idd9/11* region conferred type 1 diabetes resistance at the B-cell level (12). The current study found the same region also controls the pathogenic activation of diabetogenic CD4 but not CD8 T-cells. B-cells are important APCs for activating and expanding β-cell–autoreactive CD4 T-cells in NOD mice (37,38). However, it is unlikely that the diminished diabetogenic activity of NOR and NOD.*Chr4*^{NOR} CD4 T-cells is due to lower levels of disease-promoting B-cells than in NOD mice. This argument is supported by the fact that CD4 T-cells from B-cell–deficient NOD.*IgH*^{null} mice were as diabetogenic as those of NOD origin. Bone marrow chimerism studies also indicated that intrinsic mechanisms were responsible for inducing tolerance in NOR and NOD.*Chr4*^{NOR} CD4 T-cells. This indicates a NOR *Idd9/11* region gene(s) expressed in CD4 T-cells directly suppresses their diabetogenic potential.

A comparison of data depicted in Figs. 1*A* and 5 suggested that while not quite achieving statistical significance ($P = 0.06$), CD4 T-cells from NOR mice may be somewhat less diabetogenic than those from the NOD.*Chr4*^{NOR} strain. Thus, although representing a primary contributor, the *Idd9/11* locus may not be the sole genetic element suppressing diabetogenic CD4 T-cell responses in NOR mice. In particular, although not suppressive of diabetogenic CD4 T-cells by themselves, genes within the Chr1 (*Idd5.2*, *D1Mit532-D1Mit8*) and/or Chr2 (*Idd13*, *D2Mit63-D2Mit48*) congenic intervals analyzed here may interactively work with *Idd9/11* region genes to further inhibit the development or function of such pathogenic effectors. Alternatively, other genetic regions distinguishing NOR from NOD mice that were not analyzed in the current study may act independently or interactively with a *Idd9/11* region gene(s) to regulate the diabetogenic activity of CD4 T-cells.

The type 1 diabetes–protective congenic interval in NOD.*Chr4*^{NOR} mice does not contain the previously reported *Idd9.3* region (10,39). Therefore, the *Idd9.3* candidate gene *Cd137* cannot be a type 1 diabetes–protective factor in NOR mice. Another difference between the type 1 diabetes protective *Idd9* congenic interval originally reported by Wicker and colleagues (40) and that present in the NOD.*Chr4*^{NOR} stock is the donor strain. The type 1 diabetes–protective *Idd9* congenic interval described by Wicker and colleagues is derived from C57BL/10 (B10) rather than NOR mice. In NOR mice, ~12% of the genome is derived from C57BLKS/J, which itself is composed of genetic material mostly of B6 origin, but also from the DBA/2J and possibly the B10 and 129 strains (41). The Chr4 type 1 diabetes resistance region in NOR mice is complex and contains both B6 and DBA/2J genetic material (10). Therefore, it is possible that a DBA/2J-derived genetic component(s) is responsible for NOD.*Chr4*^{NOR} CD4 T-cells being less diabetogenic than those of NOD origin. Although the B6 and B10 genomes are quite similar, the distal region on Chr4 represents one region where they differ (42). As a result, different allelic variants may contribute to type 1 diabetes resistance in the B10-derived *Idd9* NOD congenic stock and the NOD.*Chr4*^{NOR} strain.

BDC2.5 T-cells represent a well-studied pancreatic β-cell autoreactive CD4 T-cell clone. However, BDC2.5 TCR transgenic NOD mice seldom develop type 1 diabetes when other endogenous TCR molecules are also expressed (43). Type 1 diabetes resistance in NOD BDC2.5 transgenic mice is attributed to the presence of Tregs (44). On the other hand, NY4.1 TCR transgenic CD4 T-cells induce accelerated type 1 diabetes in NOD mice also capable of expressing endogenously derived TCR molecules (45). These collective findings indicate different autoreactive CD4 T-cell clonotypes could be regulated by distinct mechanisms, perhaps because of variations in antigen specificity and TCR affinity. Thus, it is significant that under conditions where they can express a full array of TCR specificities, the development and/or function of the diverse diabetogenic CD4 T-cell repertoire normally generated in NOD mice is inhibited by the presence of a NOR-derived *Idd9/11* region gene(s). This NOR-derived *Idd9/11* gene(s) does not elicit increases in Treg numbers or activity that keeps β-cell autoreactive CD4 T-cells in check, but rather, it functions intrinsically to suppress the development or function of a broad range of such diabetogenic effectors. For this reason, the ultimate identification of the NOR-derived *Idd9/11* region gene(s) capable of directly suppressing development of a diverse repertoire of β-cell autoreactive CD4 T-cells could provide a target for future type 1 diabetes prevention and/or reversal therapies.

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