The *Idd9/11* genetic locus regulates diabetogenic activity of CD4 T-cells in non-obese diabetic (NOD) mice

Yi-Guang Chen, Felix Scheuplein, Melissa A. Osborne, Shirng-Wern Tsaih, Harold D. Chapman, and David V. Serreze

The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609

**Address correspondence to:**

Dr. David V. Serreze

Professor

The Jackson Laboratory

Bar Harbor, Maine 04609

USA

[Email-dave.serreze@jax.org](mailto:Email-dave.serreze@jax.org)

Submitted 10 June 2008 and accepted 25 August 2008.
Idd9/11 regulates diabetogenic CD4 T-cells

ABSTRACT

**Objective:** While the H2^g7^ major histocompatibility complex (MHC) provides the primary pathogenic component, the development of T-cell-mediated autoimmune type 1 diabetes (T1D) in NOD mice also requires contributions from other susceptibility (Idd) genes. Despite sharing the H2^g7^ MHC, the closely NOD-related NOR strain remains T1D resistant due to 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 (BM) 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 T1D 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, BM chimerism experiments demonstrated 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.

**Conclusion:** In conclusion, we demonstrated that a gene(s) within the Idd9/11 region regulates the diabetogenic activity of CD4 T-cells.
In both humans and NOD mice, type 1 diabetes (T1D) results from T-cell-mediated autoimmune destruction of insulin-producing pancreatic β-cells (1; 2). Both CD4 and CD8 T-cells are essential for T1D development in NOD mice (1; 3). Similar to the case in humans, multiple genetic susceptibility (Idd) loci contribute to T1D in NOD mice (1). The $H2^{67}$ major histocompatibility complex (MHC) provides the primary component of T1D susceptibility in NOD mice, but alone is insufficient for disease development (1). In conjunction with the $H2^{67}$ MHC, other Idd genes are also required to interactively contribute to the development of diabetogenic T-cell responses in NOD mice (1). While 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 $H2^{67}$ 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 T1D (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 T1D protective Idd5.2 sublocus. Allelic variants of β2-microglobulin ($\beta2m$) represent one of at least two polymorphic Idd13 region genes respectively contributing to T1D susceptibility or resistance in NOD or NOR mice (4). Polymorphic $\beta2m$ 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 due to contributions from Idd9/11 region genes on Chr4 (12). T1D-protective genetic polymorphisms in NOR mice also suppress the pathogenic activity of T-cells transgenically expressing T-cell receptors (TCR) 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.

**MATERIALS AND METHODS**

*Mice.* 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 Chr 1 NOD.NOR-(D1Mit532-D1Mit8)/DvsJ (here designated NOD.Chr1NOR) or 4 NOD.NOR-(D4Mit31-D4Mit310)/DvsJ (here designated NOD.Chr4NOR) have been described previously (10). In the latter of these two stocks, the NOR derived Chr 4 congenic interval was found to extend more distally than previously reported (10). In the latter of these two stocks, the NOR derived Chr 4 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.Chr2NOR) was termed NOD.D2Mit490-Mit144NOR in a previous study (11). Designation of this NOR derived Chr 2 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 or CD8 T-cells (designated NOD.CD4null and NOD.CD8null) were also utilized (19; 20). A NOD stock lacking B-cells (21) is now maintained at the N10 backcross generation (termed NOD.IgHnull). A NOD.IgHnull substock with B-cells that only express transgene encoded HEL specific immunoglobulin molecules (designated NOD.IgHEL.IgHnull) 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.Rag1null.AI4) have also been described (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 137Cs source) and injected i.v. with 5-10x10^6 NOD.Rag1null.AI4 splenocytes (equivalent to 1-2x10^6 AI4 T-cells). Similar results were obtained over this dose range of NOD.Rag1null.AI4 splenocytes. In some experiments, AI4 T-cells were pre-activated in vitro. Briefly, NOD.Rag1null.AI4 splenocytes were cultured at 5x10^6 cells/ml with 0.1 μM of previously described antigenic mimotope peptide (YFIENYLEL) (23) and 25 U/ml recombinant human IL-2 for 2 days. Following activation, 1x10^6 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 T1D 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.CD4null mice at 4-7 weeks of age were lethally irradiated (1300R from a 137Cs source) and reconstituted as previously described (24) with 5x10^6 of the indicated T-cell-depleted bone marrow (BM) cells isolated from 6-10 week old donors. In some experiments, 5x10^6 T-cell-depleted NOD.CD4null BM cells were admixed with 5x10^6 purified CD4 T-cells as indicated. Splenic CD4 T-cells were purified from 6-9 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 higher than 92% as determined by flow cytometry. A similar approach was used to study the diabetogenic activity of CD8 T-cells using lethally irradiated NOD.CD8null 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. T1D 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, BD Bioscience), and CD11b+ cells. TID was again monitored by onset of glycosuria.

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 a LS column (Miltenyi Biotec). The CD4^+CD25^- fraction was used as the effector population. Effector cells were labeled with CFSE as previously described (27). Labeled effector cells (5x10^5) were co-cultured in triplicate with indicated numbers of putative CD4^+CD25^- Tregs in the presence of 2x10^5 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 of the previously described culture medium (28). Proliferation of effector cells was determined after three 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 of BDC2.5 mimotope (AHHPIWARMDA) or a control IAg7 binding peptide (LSIALHVGFDH) emulsified in incomplete Freund’s adjuvant (IFA). Ten days later, 1x10^6 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 of the previously described culture medium (28) containing varying concentrations of BDC2.5 mimotope. Cells were cultured for 3 days and labeled with 1 μCi / well of [3H]-thymidine during the last 20 hours. IFNγ, 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 A14 CD8 T-cells.** Autoreactive CD8 T-cells are essential to the initiation of T1D 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 T1D 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 A14 TCR rapidly induce T1D when adoptively transferred into sublethally irradiated standard NOD recipients (27). Therefore, we asked if the A14 adoptive transfer method could also induce T1D in NOR mice or NOD stocks congenic for NOR-derived chromosomal regions carrying protective alleles. While more than 75% of standard NOD mice receiving NOD.Rag1null.A14 splenocytes developed T1D within 2 weeks; all 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 A14 T-cell induced T1D (Table 1). In contrast, no NOD.Chr4^NOR^ recipients developed T1D under the same transfer conditions (Table 1). We next asked what particular host lymphocyte populations modulate the diabetogenic activity of adoptively transferred A14 T-cells. To do this, we transferred NOD.Rag1null.A14 splenocytes into sublethally irradiated NOD mice lacking B cells (NOD.IgHnull), CD8 T-cells (NOD.CD8null), or CD4 T-cells (NOD.CD4null). Both host B-cells and CD4 T-cells, but not CD8 T-cells, were important for adoptively transferred A14 T-cells to mediate T1D development (Table 1). One important role of autoreactive B-cells for T1D development in NOD mice is to function as a preferential APC subset due to a unique ability to efficiently uptake autoantigens through cell surface immunoglobulin (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...
Idd9/11 regulates diabetogenic CD4 T-cells 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.Rag1null AI4 splenocytes into sublethally irradiated NOD.IgHEL.IgHnull mice in which all B-cells express Ig molecules specific for the disease irrelevant HEL protein. Similar to standard NOD recipients, NOD.IgHEL.IgHnull mice were highly susceptible to AI4 T-cell mediated T1D (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 inoculum displayed a naive phenotype (>99% CD69-/CD25-/CD44low). Therefore, host CD4 T-cells and B-cells appear to facilitate the subsequent activation of adoptively transferred AI4 T-cells. Indeed, AI4 T-cells can independently induce T1D in T-cell and B-cell deficient NOD-scid recipients (33), but with significantly slower kinetics than observed in the present system when residual CD4 T-cells and B-cells remain in sublethally irradiated standard NOD recipients.

We next asked if AI4 T-cells pre-activated in vitro by mimotope peptide stimulation could induce T1D in the absence of host CD4 T-cells or B-cells. Pre-activated AI4 T-cells (>66% CD25+CD69+CD44hi) induced T1D development in CD4 T-cell or B-cell deficient NOD recipients (Table 1). In addition, NOR and NOD.Chr4NOR mice rapidly developed T1D when receiving pre-activated AI4 T-cells (Table 1). Together, these results indicate that in this adoptive transfer system, CD4 T-cells and B-cells in NOD, but not NOR or NOD.Chr4NOR 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 if mature NOR CD4 T-cells remained unable to facilitate the initiation of T1D development when all other components of the immune response were of NOD origin. The system used was to reconstitute lethally irradiated normally T1D resistant NOD.CD4null mice (19) with syngeneic BM mixed with purified CD4 T-cells from either NOD or NOR donors. NOD.CD4null mice receiving syngeneic BM alone did not develop T1D (data not shown). More than 90% of the NOD.CD4null recipients developed T1D when reconstituted with syngeneic BM and NOD CD4 T-cells (Fig. 1A). In contrast, NOD.CD4null mice injected with syngeneic BM and NOR CD4 T-cells were highly resistant to T1D 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 T1D development could solely result from differences in previous engagements with antigen presenting B-cells capable of expanding pathogenic effectors. If this was the case, then compared to those of NOD or NOR origin, CD4 T-cells isolated from NOD.IgHnull mice should exhibit poor diabetogenic activity. Therefore, we reconstituted NOD.CD4null mice with syngeneic BM and CD4 T-cells from NOD.IgHnull mice. CD4 T-cells from NOD.IgHnull donors were as diabetogenic as those of NOD origin (Fig. 1A).

MHC class II Ag7 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. 1B). However, compared to both the NOD and NOR strains, BDC2.5 like T cell
levels are lower in the NOD.IgH\textsuperscript{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. 1C). These collective results indicate the inability of NOR CD4 T-cells to support T1D development was not due to diminished numbers of potential β-cell autoreactive effectors.

**Intrinsic factors inhibit the development of diabetogenic NOR CD4 T-cells.** While NOR CD4 T-cells have very limited diabetogenic activity, it was not known if 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 may have an increased proportion or enhanced function of Tregs. Indeed, in the experiments described above, the splenic NOR derived CD4 T-cells transferred to NOD.CD4\textsuperscript{null} recipients may have also included Tregs. Therefore, we compared the frequency of splenic Tregs in NOD and NOR mice based on co-expression of CD4 and Foxp3. NOD and NOR mice did not differ in percentages of CD4 T-cells that co-expressed Foxp3\textsuperscript{+} (Fig. 2A, B). In addition, the percentages of Foxp3\textsuperscript{+} CD4 T-cells that co-expressed CD25 were similar in these two strains (Fig. 2C). While numerically similar, it was possible that Tregs in NOD and NOR mice functionally differed. However, when tested in vitro, NOD and NOR CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs (both ~80% Foxp3\textsuperscript{+}) exhibited similar levels of suppressive activity (Fig. 3).

We next asked if NOR CD4 T-cells support T1D development when they differentiate from stem cells in an environment where most cells are NOD-derived. Lethally irradiated NOD.CD4\textsuperscript{null} mice were reconstituted with a 4:1 mixture of syngeneic and NOR BM cells. In this case, all CD4 T-cells were NOR-derived with most other hematopoietic, and all non-hematopoietic cells of NOD origin. Control chimeras consisted of NOD.CD4\textsuperscript{null} recipients repopulated with a 4:1 mixture of syngeneic and standard NOD BM cells. These control chimeras developed a high incidence of T1D (Fig. 4). In contrast, recipients of NOD.CD4\textsuperscript{null} and NOR BM were completely T1D resistant. NOR-derived leukocytes other than CD4 T-cells might have actively suppressed T1D development in these BM chimeras. To test this possibility, another set of control chimeras were reconstituted with a 4:1 mixture of NOD and NOR BM. Similar to the recipients of NOD.CD4\textsuperscript{null} and NOD BM, the second control group was T1D susceptible (Fig. 4). These collective results indicate an intrinsic 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\textsuperscript{null} mice with syngeneic BM and CD4 T-cells isolated from either NOD.Chr1\textsuperscript{NOR}, NOD.Chr2\textsuperscript{NOR}, or NOD.Chr4\textsuperscript{NOR} mice. Compared to those of NOD origin, CD4 T-cells isolated from NOD.Chr4\textsuperscript{NOR}, but not NOD.Chr1\textsuperscript{NOR} mice demonstrated a reduced ability to support T1D development (Fig. 5). There was a possible trend for CD4 T-cells from NOD.Chr2\textsuperscript{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/group). Using the same BM chimerism approach described in Fig. 4, we found an intrinsic factor(s) limits the pathogenic potential of NOD.Chr4\textsuperscript{NOR} CD4 T-cells (data not shown).
We analyzed the function of BDC2.5-like CD4 T-cells in NOD.$\text{Chr}^\text{NOR}$ mice using the same priming-recall assay described in Fig. 1C. BDC2.5-like CD4 T-cells from NOD and NOD.$\text{Chr}^\text{NOR}$ mice proliferated equivalently (data not shown). Production of IFN$\gamma$, IL-4, and IL-10 by primed BDC2.5-like cells following antigenic peptide (10$\mu$M) re-stimulation was also assessed. NOD.$\text{Chr}^\text{NOR}$ BDC2.5-like cells produced less IFN$\gamma$ than those of NOD origin (58.0$\pm$7.1$\mu$g/ml and 109.3$\pm$3.7$\mu$g/ml, respectively). Therefore, the lower diabetogenic activity of NOD.$\text{Chr}^\text{NOR}$ than NOD CD4 T-cells could result from reduced production of the inflammatory cytokine IFN$\gamma$. However, upon antigenic stimulation, lower levels of the immunosuppressive cytokine IL-10 were secreted by BDC2.5-like CD4 T-cells from NOD.$\text{Chr}^\text{NOR}$ than NOD mice (755.9$\pm$22.9pg/ml and 1061.3$\pm$38.1pg/ml, respectively). Hence, Idd9/11 region genes do not contribute to T1D resistance in NOR mice through enhancing IL-10 production by CD4 T-cells. Both strains produced undetectable amounts of IL-4. These results indicated BDC2.5-like CD4 T-cells in NOD and NOD.$\text{Chr}^\text{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 if NOD.$\text{Chr}^\text{NOR}$ 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 NOR CD4 T-cells induced T1D in some NOD.$\text{CD}^\text{null}$ 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 non-mutually exclusive possibility is 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 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 T1D development (35; 36). Therefore, it is possible 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 T1D 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 APC for activating and expanding β-cell-autoreactive CD4 T-cells in NOD mice (37; 38). However, it is unlikely the diminished diabetogenic activity of NOR and
Idd9/11 regulates diabetogenic CD4 T-cells

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 CD4 T-cells from B-cell deficient NOD.Igh^null^ mice were as diabetogenic as those of NOD origin. BM chimerism studies also indicated 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 Figures 1A 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, while 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, while 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 T1D-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 T1D-protective factor in NOR mice. Another difference between the T1D protective Idd9 congenic interval originally reported by L. Wicker and colleagues (40) and that present in the NOD.Chr4^NOR^ stock is the donor strain. The T1D-protective Idd9 congenic interval described by L. Wicker and colleagues is derived from C57BL/10 (B10) rather than NOR mice. About 12% of the genome in NOR mice 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 T1D resistance region in NOR mice is complex and contains both B6 and DBA/2J genetic material (10). Therefore, it is possible a DBA/2J derived genetic component(s) is responsible for NOD.Chr4^NOR^ CD4 T-cells being less diabetogenic than those of NOD origin. While 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 T1D 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 T1D when other endogenous TCR molecules are also expressed (43). T1D 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 T1D 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 due to 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/Idd11 region gene(s). This NOR derived Idd9/11 gene(s) does not elicit increases in Treg numbers or activity that keep β-cell autoreactive CD4 T-cells in check, but rather functions intrinsically to suppress the T1D responses.
$Idd9/11$ regulates diabetogenic CD4 T-cells 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 $\beta$-cell autoreactive CD4 T-cells, could provide a target for future T1D prevention and/or reversal therapies.

**ACKNOWLEDGMENTS**

We thank Luc Teyton and Thomas Stratmann for providing us BDC2.5 MHC class II tetramers. This work was supported by National Institutes of Health grants DK46266, DK51090 (to D.V. Serreze), and DK077443 (to Y.-G. Chen); as well as by grants from the Juvenile Diabetes Research Foundation International (to Y.-G. Chen and D.V. Serreze). F. Scheuplein is a recipient of a postdoctoral fellowship from the Juvenile Diabetes Research Foundation International.
REFERENCES

1. Serreze DV, Leiter EH: Genes and cellular requirements for autoimmune diabetes susceptibility in nonobese diabetic mice. *Curr Dir Autoimmun* 4:31-67, 2001
2. Anderson MS, Bluestone JA: The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23:447-485, 2005
3. DiLorenzo TP, Serreze DV: The good turned ugly: immunopathogenic basis for diabetogenic CD8+ T cells in NOD mice. *Immunol Rev* 204:250-263, 2005
4. Hamilton-Williams EE, Serreze DV, Charlton B, Johnson EA, Marron MP, Mullbacher A, Slattery RM: Transgenic rescue implicates beta2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice. *Proc Natl Acad Sci U S A* 98:11533-11538, 2001
5. Vijayakrishnan L, Slavik JM, Illes Z, Greenwald RJ, Rainbow D, Greve B, Peterson LB, Hafler DA, Freeman GJ, Sharpe AH, Wicker LS, Kuchroo VK: An autoimmune disease-associated CTLA-4 splice variant lacking the B7 binding domain signals negatively in T cells. *Immunity* 20:563-575, 2004
6. Yamanouchi J, Rainbow D, Serra P, Howlett S, Hunter K, Garner VE, Gonzalez-Munoz A, Clark J, Veijola R, Cubbon R, Chen SL, Rosa R, Cumiskey AM, Serreze DV, Gregory S, Rogers J, Lyons PA, Healy B, Smink LJ, Todd JA, Peterson LB, Wicker LS, Santamaria P: Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet* 39:329-337, 2007
7. Prochazka M, Serreze DV, Frankel WN, Leiter EH: NOR/Lt mice: MHC-matched diabetes-resistant control strain for NOD mice. *Diabetes* 41:98-106, 1992
8. Fox CJ, Paterson AD, Mortin-Toth SM, Danska JS: Two genetic loci regulate T cell-dependent islet inflammation and drive autoimmune diabetes pathogenesis. *Am J Hum Genet* 67:67-81, 2000
9. Serreze DV, Prochazka M, Reifsnyder PC, Bridgett MM, Leiter EH: Use of recombinant congenic and congenic strains of NOD mice to identify a new insulin-dependent diabetes resistance gene. *J Exp Med* 180:1553-1558, 1994
10. Reifsnyder PC, Li R, Silveira PA, Churchill G, Serreze DV, Leiter EH: Conditioning the genome identifies additional diabetes resistance loci in Type I diabetes resistant NOR/Lt mice. *Genes Immun* 6:528-538, 2005
11. Serreze DV, Bridgett M, Chapman HD, Chen E, Richard SD, Leiter EH: Subcongeneric analysis of the Idd13 locus in NOD/Lt mice: evidence for several susceptibility genes including a possible diabetogenic role for beta 2-microglobulin. *J Immunol* 160:1472-1478, 1998
12. Silveira PA, Chapman HD, Stolp J, Johnson E, Cox SL, Hunter K, Wicker LS, Serreze DV: Genes within the Idd5 and Idd9/11 diabetes susceptibility loci affect the pathogenic activity of B cells in nonobese diabetic mice. *J Immunol* 177:7033-7041, 2006
13. Verdaguer J, Amrani A, Anderson B, Schmidt D, Santamaria P: Two mechanisms for the non-MHC-linked resistance to spontaneous autoimmunity. *J Immunol* 162:4614-4626, 1999
14. Serreze DV, Gaskins HR, Leiter EH: Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. *J Immunol* 150:2534-2543, 1993
15. Piganelli JD, Martin T, Haskins K: Splenic macrophages from the NOD mouse are defective in the ability to present antigen. *Diabetes* 47:1212-1218, 1998
16. O'Brien BA, Huang Y, Geng X, Dutz JP, Finegood DT: Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. *Diabetes* 51:2481-2488, 2002
Idd9/11 regulates diabetogenic CD4 T-cells

17. Steptoe RJ, Ritchie JM, Harrison LC: Increased generation of dendritic cells from myeloid progenitors in autoimmune-prone nonobese diabetic mice. *J Immunol* 168:5032-5041, 2002
18. O’Keeffe M, Brodnicki TC, Fancke B, Vremec D, Morahan G, Maraskovsky E, Steptoe R, Harrison LC, Shortman K: Fms-like tyrosine kinase 3 ligand administration overcomes a genetically determined dendritic cell deficiency in NOD mice and protects against diabetes development. *Int Immunol* 17:307-314, 2005
19. Graser RT, DiLorenzo TP, Wang F, Christianson GJ, Chapman HD, Roopenian DC, Nathenson SG, Serreze DV: Identification of a CD8 T cell that can independently mediate autoimmune diabetes development in the complete absence of CD4 T cell helper functions. *J Immunol* 164:3913-3918, 2000
20. Pearson T, Markees TG, Serreze DV, Pierre MA, Marron MP, Wicker LS, Peterson LB, Shultz LD, Mordes JP, Rossini AA, Greiner DL: Genetic disassociation of autoimmunity and resistance to costimulation blockade-induced transplantation tolerance in nonobese diabetic mice. *J Immunol* 171:185-195, 2003
21. Silveira PA, Johnson E, Chapman HD, Bui T, Tisch RM, Serreze DV: The preferential ability of B lymphocytes to act as diabetogenic APC in NOD mice depends on expression of self-antigen-specific immunoglobulin receptors. *Eur J Immunol* 32:3657-3666, 2002
22. DiLorenzo TP, Lieberman SM, Takaki T, Honda S, Chapman HD, Santamaria P, Serreze DV, Nathenson SG: During the early prediabetic period in NOD mice, the pathogenic CD8(+) T-cell population comprises multiple antigenic specificities. *Clin Immunol* 105:332-341, 2002
23. Serreze DV, Holl TM, Marron MP, Graser RT, Johnson EA, Choisy-Rossi C, Slattery RM, Lieberman SM, DiLorenzo TP: MHC class II molecules play a role in the selection of autoreactive class I-restricted CD8 T cells that are essential contributors to type 1 diabetes development in nonobese diabetic mice. *J Immunol* 172:871-879, 2004
24. Serreze DV, Leiter EH: Development of diabetogenic T cells from NOD/Lt marrow is blocked when an allo-H-2 haplotype is expressed on cells of hemopoietic origin, but not on thymic epithelium. *J Immunol* 147:1222-1229, 1991
25. Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tisch RM: B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. *J Immunol* 161:3912-3918, 1998
26. Stratmann T, Martin-Orozeo N, Mallet-Designe V, Poirot L, McGavern D, Losyev G, Dobbs CM, Oldstone MB, Yoshida K, Kikutani H, Mathis D, Benoist C, Haskins K, Leyton L: Susceptible MHC alleles, not background genes, select an autoimmune T cell reactivity. *J Clin Invest* 112:902-914, 2003
27. Chen YG, Choisy-Rossi CM, Holl TM, Chapman HD, Besra GS, Porcelli SA, Shaffer DJ, Roopenian D, Wilson SB, Serreze DV: Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol* 174:1196-1204, 2005
28. Serreze DV, Leiter EH: Defective activation of T suppressor cell function in Nonobese Diabetic mice. Potential relation to cytokine deficiencies. *J. Immunol.* 140:3801-3807, 1988
29. Serreze DV, Chapman HD, Varnum DS, Gerling I, Leiter EH, Shultz LD: Initiation of autoimmune diabetes in NOD/Lt mice is MHC class I-dependent. *J Immunol* 158:3978-3986, 1997
30. DiLorenzo TP, Graser RT, Ono T, Christianson GJ, Chapman HD, Roopenian DC, Nathenson SG, Serreze DV: Major histocompatibility complex class I-restricted T cells are required for all but the end stages of diabetes development in nonobese diabetic mice and use a
Idd9/11 regulates diabetogenic CD4 T-cells. Proc Natl Acad Sci U S A 95:12538-12543, 1998

31. Silveira PA, Grey ST: B cells in the spotlight: innocent bystanders or major players in the pathogenesis of type 1 diabetes. Trends Endocrinol Metab 17:128-135, 2006

32. Diaz-de-Durana Y, Mantchev GT, Bram R J, Franco A: TACI-BLys signaling via B-cell-dendritic cell cooperation is required for naive CD8+ T-cell priming in vivo. Blood 107:594-601, 2006

33. Takaki T, Lieberman SM, Holl TM, Han B, Santamaría P, Serreze DV, DiLorenzo TP: Requirement for both H-2Db and H-2Kd for the induction of diabetes by the promiscuous CD8+ T cell clonotype A14. J Immunol 173:2530-2541, 2004

34. Yang W, Hussain S, Mi QS, Santamaría P, Delovitch TL: Perturbed homeostasis of peripheral T cells elicits decreased susceptibility to anti-CD3-induced apoptosis in prediabetic nonobese diabetic mice. J Immunol 173:4407-4416, 2004

35. Masteller EL, Warner MR, Tang Q, Tarbell KV, McDevitt H, Bluestone JA: Expansion of functional endogenous antigen-specific CD4+CD25+ regulatory T cells from nonobese diabetic mice. J Immunol 175:3053-3059, 2005

36. Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM: CD25+CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. J Exp Med 199:1467-1477, 2004

37. Bouaziz JD, Yanaba K, Venturi GM, Wang Y, Tisch RM, Poe JC, Tedder TF: Therapeutic B cell depletion impairs adaptive and autoreactive CD4+ T cell activation in mice. Proc Natl Acad Sci U S A 104:20878-20883, 2007

38. Greeley SA, Moore DJ, Norochashm H, Noto LE, Rostami SY, Schlachterman A, Song HK, Koeberlein B, Barker CF, Naji A: Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of B cell-deficient nonobese diabetic mice. J Immunol 167:4351-4357, 2001

39. Cannons JL, Chamberlain G, Howson J, Smink LJ, Todd JA, Peterson LB, Wicker LS, Watts TH: Genetic and functional association of the immune signaling molecule 4-1BB (CD137/TNFRSF9) with type 1 diabetes. J Autoimmun 25:13-20, 2005

40. Lyons PA, Hancock WW, Denny P, Lord CJ, Hill NJ, Armitage N, Siegmund T, Todd JA, Phillips MS, Hess JF, Chen SL, Fischer PA, Peterson LB, Wicker LS: The NOD Idd9 genetic interval influences the pathogenicity of insulitis and contains molecular variants of Cd30, Tnfr2, and Cd137. Immunity 13:107-115, 2000

41. Mao HZ, Roussos ET, Peterfy M: Genetic analysis of the diabetes-prone C57BLKS/J mouse strain reveals genetic contribution from multiple strains. Biochim Biophys Acta 1762:440-446, 2006

42. McClive PJ, Huang D, Morahan G: C57BL/6 and C57BL/10 inbred mouse strains differ at multiple loci on chromosome 4. Immunogenetics 39:286-288, 1994

43. Gonzalez A, Andre-Schmutz I, Carnaud C, Mathis D, Benoist C: Damage control, rather than unresponsiveness, effected by protective DX5+ T cells in autoimmune diabetes. Nat Immunol 2:1117-1125, 2001

44. Herman AE, Freeman GJ, Mathis D, Benoist C: CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. J Exp Med 199:1479-1489, 2004

45. Schmidt D, Verdaguer J, Averill N, Santamaria P: A mechanism for the major histocompatibility complex-linked resistance to autoimmunity. J Exp Med 186:1059-1075, 1997
**Figure 1.** Reduced diabetogenic activity of NOR CD4 T-cells. (A) Incidence of T1D in CD4 T-cell-reconstituted NOD $CD4^{null}$ mice. Lethally irradiated 4-7 week old NOD $CD4^{null}$ mice were injected with equal numbers ($5 \times 10^6$) of syngeneic BM and purified CD4 T-cells from NOD, NOR, or NOD $IgH^{null}$ donors. T1D 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 6-9 weeks 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 rank sum test). (C) Functional analysis of BDC2.5-like cells in NOD and NOR mice. Groups of 3 mice were primed with 20 μg of BDC2.5 mimotope or IAg7-binding control peptide in IFA. After 10 days, cells from the draining lymph nodes of the same group were pooled and all re-stimulated in triplicate with indicated concentration of the BDC2.5 mimotope. The cultures were pulsed with $^3$H-thymidine over the final 24 hr of a 72 hr incubation period. Results indicate the mean CPM±sem of the triplicates.
Idd9/11 regulates diabetogenic CD4 T-cells

A

Source of CD4 T cells
- NOD (n=16)
- NOR (n=14)
- NOD.igH\textsuperscript{nu} (n=14)

% Diabetic

weeks post-reconstitution

B

% among total CD4 T cells

\( p=0.0044 \)
\( p=0.0008 \)

C

CPM \( \times 10^4 \)

Peptide concentration (µM)
Figure 2. Comparison of Treg frequencies in NOD and NOR mice. Splenocytes from 6-9 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 rank sum test).
**Figure 3.** Comparison of *in vitro* suppressive activities of NOD and NOR Tregs. Effector T-cells (CD4⁺CD25⁻) were labeled with CFSE and co-cultured at indicated ratios with Tregs (CD4⁺CD25⁺) in triplicate in a 96-well plate in the presence of NOD.scid splenocytes (2x10⁵) 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 % reduction in the proportion of divided effector T-cells relative to that of the control without Tregs. Results indicate the mean±sem of the triplicates. Similar results were observed in another two experiments.
Figure 4. Intrinsic factors control the diabetogenic activity of NOR CD4 T-cells. Lethally irradiated NOD.\textit{CD4}^{null} mice were reconstituted with a mixture of syngeneic BM with NOD or NOR BM at a 4:1 ratio. A control group was reconstituted with a 4:1 mixture of NOD and NOR BM. T1D development was then analyzed weekly.
Idd9/11 regulates diabetogenic CD4 T-cells

Figure 5. The diabetogenic activity of CD4 T-cells is regulated by a gene(s) within the Idd9/Idd11 region. CD4 T-cells (5x10^6) purified from NOD.Chr1^NOR, NOD.Chr2^NOR, or NOD.Chr4^NOR mice and NOD.CD4^{null} BM cells (5x10^6) were mixed and injected into lethally irradiated 4-7 week old NOD.CD4^{null} recipients. T1D development was then followed weekly for 20 weeks. The same accumulated incidence of NOD CD4 T-cell recipients shown in Figure 1A was also plotted here for comparative purposes. All CD4 T-cell transfer experiments including those shown in Figure 1A were done in an overlapping fashion. *P<0.001, significantly different from NOD CD4 T-cell recipients (Kaplan–Meier log-rank analysis).
Figure 6. The diabetogenic activity of NOD and NOD\textit{CD4}^{null} CD8 T-cells is comparable. Lethally irradiated NOD\textit{CD8}^{null} mice were injected with $5 \times 10^6$ syngeneic BM cells and $5 \times 10^6$ CD8 T-cells purified from NOD mice or the NOD\textit{Chr4}^{NOR} congenic strain. T1D development was then analyzed weekly. The T1D incidence between NOD and NOD\textit{Chr4}^{NOR} CD8 T-cell recipient groups is not significantly different (Kaplan–Meier log-rank analysis).
**Table 1.** Genetic control of susceptibility to AI4 T cell induced T1D

| Exp. group | Transferred cells | Recipients       | % Diabetic | p value<sup>b</sup> |
|------------|-------------------|------------------|------------|---------------------|
| 1          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 76.9 (n=13) | <0.001              |
|            |                   | NOR              | 0 (n=11)   |                     |
| 2          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 60 (n=10)  | >0.05               |
|            |                   | NOD.*Chr<sub>1</sub><sup>NOR</sup> | 30 (n=10)  |                     |
| 3          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 57.1 (n=7) | >0.05               |
|            |                   | NOD.*Chr<sub>2</sub><sup>NOR</sup> | 42.8 (n=7) |                     |
| 4          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 70 (n=10)  | <0.005              |
|            |                   | NOD.*Chr<sub>4</sub><sup>NOR</sup> | 0 (n=10)   |                     |
| 5          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 75 (n=12)  | <0.005              |
|            |                   | NOD.*CD<sub>4</sub><sup>null</sup> | 0 (n=10)   |                     |
| 6          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 75 (n=8)   | >0.05               |
|            |                   | NOD.*CD<sub>8</sub><sup>null</sup> | 33.3 (n=9) |                     |
| 7          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 61.5 (n=13)| <0.001              |
|            |                   | NOD.*IgH<sup>null</sup> | 0 (n=13)   |                     |
| 8          | NOD.*Rag<sup>null</sup> AI4 splenocytes | NOD              | 60 (n=10)  | >0.05               |
|            |                   | NOD.*IgHEI.IgH<sup>null</sup> | 70 (n=10)  |                     |
| 9          | Activated AI4 T cells | NOD              | 100 (n=3)  |                     |
|            |                   | NOR              | 100 (n=3)  |                     |
| 10         | Activated AI4 T cells | NOD              | 100 (n=4)  |                     |
|            |                   | NOD.*Chr<sub>4</sub><sup>NOR</sup> | 100 (n=5)  |                     |
|            |                   | NOD.*CD<sub>4</sub><sup>null</sup> | 100 (n=5)  |                     |
| 11         | Activated AI4 T cells | NOD              | 100 (n=5)  |                     |
|            |                   | NOD.*IgH<sup>null</sup> | 100 (n=5)  |                     |

<sup>a</sup> NOD.*Rag<sup>null</sup> AI4 splenocytes or in vitro activated AI4 T cells were transferred into 600R irradiated female recipients. T1D was monitored for a period of 2 weeks. Mice within each experimental group received the same preparation of AI4 T cells.

<sup>b</sup> Statistical analysis by Chi-square test (versus NOD recipients in the same experimental group).