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

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*J Immunol* 2004; 172:871-879; doi: 10.4049/jimmunol.172.2.871
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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

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Development of autoreactive CD4 T cells contributing to type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice is either promoted or dominantly inhibited by particular MHC class II variants. In addition, it is now clear that when co-expressed with other susceptibility genes, some common MHC class I variants aberrantly mediate autoreactive CD8 T cell responses also essential to T1D development. However, it was unknown whether the development of diabetogenic CD8 T cells could also be dominantly inhibited by particular MHC variants. We addressed this issue by crossing NOD mice transgenically expressing the TCR from the diabetogenic CD8 T cell clone A14 with NOD stocks congenic for MHC haplotypes that dominantly inhibit T1D. High numbers of functional A14 T cells only developed in controls homozygously expressing H2g7 molecules. In contrast, heterozygous expression of some MHC haplotypes conferring T1D resistance energized A14 T cells through decreased TCR (H2b) or CD8 expression (H2g). Most interestingly, while A14 T cells exert a class I-restricted effector function, H2nb1 MHC class II molecules can contribute to their negative selection. These findings provide insights to how particular MHC class I and class II variants interactively regulate the development of diabetogenic T cells and the TCR promiscuity of such autoreactive effectors.


Type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice results from T cell-mediated autoimmune destruction of insulin-producing pancreatic β cells and is under complex polygenic control (reviewed in Refs. 1 and 2). However, particular MHC haplotypes provide the primary genetic component for susceptibility or resistance to T1D. Within the human MHC, particular combinations of HLA-DQ and DR class II alleles provide a large proportion of T1D susceptibility by mediating the selection and functional activation of β cell-autoreactive CD4 T cells (reviewed in Ref. 3). In contrast, other class II variants, in particular DQ6, provide dominant T1D resistance (reviewed in Ref. 3). Similarly, T1D development in NOD mice is also dependent upon the class II variants characterizing their H2g7 MHC haplotype (reviewed in Refs. 1 and 2). This includes both the expression of H2-Ag7 and an absence of H2-E class II molecules on NOD APC. Such a class II expression pattern contributes to T1D in a recessive manner because disease is inhibited in NOD mice carrying transgenes encoding H2-A variants derived from other MHC haplotypes or that restore H2-E expression (4–9). Reported mechanisms of T1D resistance mediated by dominantly protective MHC class II alleles transgenically or congenically expressed in NOD mice include both the intrathymic deletion of β cell-autoreactive CD4 T cells (10–12) and the selection of regulatory T cells (7).

It is becoming increasingly clear that certain MHC class I alleles can also contribute to susceptibility or resistance to T1D. Indeed, while they represent common alleles found in many strains lacking autoimmune proclivity, when the Kd and Db class I variants characterizing the H2g7 haplotype are expressed in NOD mice, they aberrantly mediate CD8 T cell responses against pancreatic β cells that are absolutely essential to T1D development (13–16). Similarly, there is increasing evidence from extended HLA haplotype analyses that the risk of T1D development in humans is further increased when certain common MHC class I variants are expressed in conjunction with particular class II alleles (17–23). It was subsequently shown that when transgenically expressed in NOD mice, the common human HLA-A2.1 class I variant mediates β cell-autoreactive T cell responses that accelerate the onset of T1D (24). This provided the first functional evidence that, in the proper genetic context, some common human MHC class I variants can aberrantly exert diabetogenic activities. However, T1D was suppressed in NOD mice transgenically expressing the human HLA-B27 class I variant (24). Hence, there is clearly a need to more completely understand how the development and function of MHC class I-restricted diabetogenic CD8 T cells is regulated.
In the present study, a previously developed genetic resource was employed to gain an increased understanding about the MHC regulation of diabetogenic CD8 T cell development and function. This resource is a stock of NOD mice transgenically expressing the TCR from the CD8 T cell clone, AI4, originally identified as a Kd MHC class I-restricted effector contributing to the earliest phases of autoimmune β cell destruction leading to T1D (designated NOD.AH4β transgenic (Tg) mice) (16, 25). Due to allelic exclusion, these NOD.AH4β Tg mice mainly produce AI4 clonotypic CD8 T cells that are capable of independently and rapidly mediating T1D development (25). T cells expressing the AI4 TCR can be easily followed by flow cytometry. This allowed us to cross NOD.AH4β Tg mice with a series of other NOD stocks congenic or transgenic for non-H2Kβ MHC alleles to determine whether they could influence the development or function of a diabetogenic CD8 T cell clonotype. It was found that when forced to mature in the presence of APC expressing various collections of MHC genes conferring resistance to T1D, the presence of APC expressing various collections of MHC genes influence the development or function of a diabetogenic CD8 T cell clone of T1D-resistant NOD-Scid mice (26) are maintained at the N11 background generation. Previously described stocks (27, 28) of T1D-resistant NOD mice congenic for either the H2Ab1 (Kb, Anb1, Eβ, Dp) or H2β (Kb, Aβ, Eβ, Fβ, Dp) haplotypes are both maintained at the N21 backcross generation. A previously described stock (29) of T1D-resistant NOD mice congenic for the H2β (Kb, Aβ, Eβ, Dp) haplotype is maintained at the N15 backcross generation. T1D-resistant NOD mice congenic for a previously described (30) class II-deficient H2β haplotype (Kb, Anβ1, Eβ, Dp) are maintained at the N11 backcross generation (designated NOD.H2β-Aβ1/mice). NOD mice transgenically expressing the AI4 TCR (Va8/N/V2, and a substock congenic for a functionally inactivated Rag1 gene (designated NOD.AH4β Tg or NOD.Rag1tm1/fl-AH4β Tg mice) have also been previously described (25, 31). A NOD stock transgenically expressing the murine H2Kβ class I variant was generated for use in some experiments (designated NOD-Kβ Tg). The H2-Kβ transgene was derived from a 7.5-kb genomic clone (32). Dormant 150-bp loxP sites were inserted into the HincII site in exon 3 of the NOD.Kβ Tg. The H2-Kβ transgene construct was microinjected directly into NOD embryos as previously described (33). Expression of the Kβ transgene does not alter the incidence of T1D development in NOD mice.

**Materials and Methods**

**Mice**

NOD/LtJds mice (H2Dβ = Kβ, Aβ1, Eβ, Dp) are maintained at The Jackson Laboratory by brother-sister mating. Currently, T1D develops in 90% of female and 63% of male NOD mice before a year of age. T and B lymphocyte-deficient NOD-Scid mice (26) are maintained at the N11 background generation. Previously described stocks (27, 28) of T1D-resistant NOD mice congenic for either the H2Ab1 (Kb, Anb1, Eβ, Dp) or H2β (Kb, Aβ, Eβ, Fβ, Dp) haplotypes are both maintained at the N21 backcross generation. A previously described stock (29) of T1D-resistant NOD mice congenic for the H2β (Kb, Aβ, Eβ, Dp) haplotype is maintained at the N15 backcross generation. T1D-resistant NOD mice congenic for a previously described (30) class II-deficient H2β haplotype (Kb, Anβ1, Eβ, Dp) are maintained at the N11 backcross generation (designated NOD.H2β-Aβ1/mice). NOD mice transgenically expressing the AI4 TCR (Va8/N/V2, and a substock congenic for a functionally inactivated Rag1 gene (designated NOD.AH4β Tg or NOD.Rag1tm1/fl-AH4β Tg mice) have also been previously described (25, 31). A NOD stock transgenically expressing the murine H2Kβ class I variant was generated for use in some experiments (designated NOD-Kβ Tg). The H2-Kβ transgene was derived from a 7.5-kb genomic clone (32). Dormant 150-bp loxP sites were inserted into the HincII site in exon 3 of the NOD.Kβ Tg. The H2-Kβ transgene construct was microinjected directly into NOD embryos as previously described (33). Expression of the Kβ transgene does not alter the incidence of T1D development in NOD mice.

**Assessment of T1D development**

T1D development in the indicated mice was defined by glycosuric values of ≥3 as assessed with Ames Diastix (kindly supplied by Miles Diagnostics, Elkhart, IN).

**T cell subset enumerations**

The proportion of PBL or splenocytes from the indicated mice that expressed the transgenic AI4 TCR was assessed by multicolor flow cytometric techniques (FACScan; Becton Dickinson, San Jose, CA) using the appropriate selection mixture included a biotinylated Ab specific for CD4 (GK1.5), anti-CD8 (B20.6) elements. Other studies enumerated various T cell populations within spleens and thymi of the indicated bone marrow chimeras. Three-color flow cytometric analysis of thymocytes was performed using Abs directed against CD4, CD8, and TCRβ to compare T cell differentiation in each chimeric class. CD8 expression was detected with the mAb 53-6.72 conjugated to a green fluorescent FITC tag. CD4 expression was detected with biotinylated mAb GK1.5 that was developed subsequently with a red fluorescent streptavidin-Red-670 tag (Life Technologies, Rockville, MD). Presence of a rearranged TCRαβ complex on CD4/CD8 double-positive thymocytes was detected with the mAb H57-597 conjugated to a red fluorescent PE tag whose fluorescence intensity can be readily distinguished from that of Red-670. For detection of total peripheral T cells, splenocytes were stained with the FITC-conjugated TCRαβ-specific mAb. Total thymocytes were further differentiated for CD8 expression with the mAb GK1.5 conjugated to the red fluorescent tag Cy3.18-Osu (Cy3; Biological Detection Systems, Pittsburgh, PA) or for CD8 expression with the mAb 53-6.72 conjugated to PE whose red fluorescence intensity can easily be distinguished from that of Cy3. Splenic or peripheral blood CD8 T cells expressing the transgenic AI4 TCR were detected by containing with the FITC-labeled CD8-specific Ab and the PE-conjugated TCR Vα8 element. Less than 1% of CD8 T cells in standard NOD mice express the TCR Vα8 element.

**Bone marrow chimeras and secondary adoptive transfer of CD8 T cells**

The indicated mice were lethally irradiated (1200 rad from a 137Cs source) at 4–6 wk of age and reconstituted as previously described (34) with single or 1:1 mixtures of various populations of bone marrow cells (5 × 106 cells of each type). Chimeras were assessed for diabetes development through 6 wk post bone marrow reconstitution. Upon diabetes development or a 6-wk observation period, the chimeras were assessed for proportions of various splenic T cell populations as described above. In some experiments, CD8 T cells were purified from spleens of the indicated chimeras and assessed for ability to adoptively transfer T1D to NOD-scid recipients (106 CD8 T cell recipients). CD8 T cells were purified from the previously described magnetic bead-based negative selection approach (35). The only change was that the negative selection mixture included a biotinylated Ab specific for CD4 (GK1.5) rather than CD8. Flow cytometric analyses indicated CD8 T cell purity was >95%.

**T cell proliferation assay**

CD8 T cells (>90% expressing the AI4 TCR) were purified from spleens of the indicated mice by the magnetic bead system described above. Triplicate aliquots of 5 × 104 CD8 T cells were then seeded into flat-bottom 96-well plates in 200 μl of the previously described medium (16) also containing 2 × 105 irradiated (2000 rad) NOD splenic leukocytes and an AI4 mitompeptide (Y1F13,13; S.M.L. and T.P.D., manuscript in preparation) at concentrations ranging from 0 to 100 nm. Following a 48-h incubation at 37°C in a 95% air/5% CO2 humidified atmosphere, the cultures were pulsed with 1 μCi/well [3H]thymidine for an additional 24 h. The cultures were then harvested and [3H]thymidine incorporation determined using a LKB Betaplate 1205 system (LKB Instruments, Gaithersburg, MD). Data are presented as mean cpm ± SEM of the triplicate cultures.

**MHC interaction assay**

The ability of various MHC gene products associated with T1D resistance to interact with the AI4 TCR was assessed through an in vitro mixed leukocyte assay system. Triplicate aliquots of 5 × 105 splenic leukocytes from NOD.Rag1tm1/fl-AH4β Tg mice were seeded in flat-bottom 96-well plates either in 200 μl of medium alone or with an equal number of irradiated (2000 rad) splenic leukocytes from the indicated NOD MHC congenic stocks and then incubated for 5 days at 37°C. In some experiments, the stimulatory splenocytes were pretreated with mAbs directed against the indicated MHC variants. The extent of activation through the AI4 TCR was then assessed by measuring IFN-γ levels in pooled supernatants from each triplicate culture with a commercially available ELISA kit (PharMingen, San Diego, CA).

**Results**

**Heterozygous expression of the diabetes protective H2m1 MHC haplotype blocks the development of AI4 T cells**

One mechanism by which expression of a non-H2Kβ MHC haplotype dominantly inhibits T1D development in NOD mice is by inducing intrathymic negative selection of class II-restricted β cell-autoreactive CD4 T cells (10, 11). However, it was unknown if T1D protection elicited by expression of non-H2Kβ MHC gene products also entails the negative selection of class I-restricted β cell-autoreactive CD8 T cells. To initially test this possibility, we determined if heterozygous expression of the H2m1 haplotype led to decreased development of AI4 T cells. As expected based
haplotypes other than H2 g7 expression of statistically differ from the positive controls. Hence, heterozygous hemopoietically derived cells including thymic epithelium. The significantly lower levels of AI4 CD8 T cells among PBL (0.93 ± 0.38%) than in the standard NOD.AI4β Tg controls (20.9 ± 1.0%). However, PCR analyses confirmed these F1 hybrids carried both the AI4 TCR α- and β-chain transgenes. Two experiments indicated the ability of the H2nb1 haplotype to block the development of AI4 T cells is unlikely to be a superantigen-mediated event. Both were based on previous reports that any given endogenous superantigen preferentially engages and induces the deletion of all T cells expressing particular TCR Vβ elements (reviewed in Ref. 36). However, heterozygous H2nb1 expression did not induce a reduction in T cells only expressing the transgenic Vβ2 chain of the AI4 TCR (data not shown). Furthermore, the total proportion of T cells expressing the TCR Vβ2 element in nontransgenic (NOD.H2nb1 × NOD)F1 hybrids (5.3 ± 0.5%, n = 4) was actually slightly higher than in NOD parental controls (4.5 ± 0.2%, n = 4). These collective findings indicated one mechanism by which MHC haplotypes other than H2nb1 confer dominant protection against T1D in NOD mice is by limiting the production of pancreatic β cell-autoreactive CD8 T cells such as the AI4 clonotype.

Heterozygous H2nb1 expression on hemopoietically derived APC results in the negative selection of AI4 T cells

A bone marrow chimera system was employed to determine whether heterozygous H2nb1 expression limited the development of AI4 T cells through impaired positive selection or enhanced negative selection. The approach taken was to determine whether AI4 T cell development was inhibited when H2nb1 molecules were heterozygously expressed on thymic epithelium or bone marrow-derived APC that respectively are the most efficient mediators of positive and negative selection (37). Positive controls consisted of NOD mice reconstituted with NOD.AI4β Tg marrow. As expected, at 6 wk postreconstitution this positive control group was characterized by high numbers of AI4 clonotypic CD8 T cells in the spleen (Fig. 1A). Negative controls consisted of (NOD.H2nb1 × NOD)F1 mice reconstituted with (NOD.H2nb1 × NOD.AI4αβ Tg)F1 marrow. These negative controls heterozygously expressed H2nb1 on both thymic epithelium and APC and were characterized by significantly fewer splenic AI4 CD8 T cells than in the positive controls. One test group consisted of (NOD.H2nb1 × NOD)F1 mice reconstituted with NOD.AI4αβ Tg marrow. In this group, developing AI4 T cells only encountered H2nb1 molecules on non-hemopoietically derived cells including thymic epithelium. The number of splenic AI4 CD8 T cells detected in this group did not statistically differ from the positive controls. Hence, heterozygous expression of H2nb1 molecules on thymic epithelium does not limit the positive selection of AI4 CD8 T cells. The second experimental group consisted of NOD mice reconstituted with (NOD.H2nb1 × NOD.AI4αβ Tg)F1 marrow. In this case, developing AI4 T cells only encountered H2nb1 molecules on hemopoietically derived cells including thymic APC. The number of splenic AI4 CD8 T cells in this latter group was significantly less than in the positive controls. Thus, heterozygous expression of H2nb1 on hemopoietically derived cells appeared to induce the negative selection of AI4 CD8 T cells.

Negative selection normally occurs at the CD4/CD8 double-positive stage of T cell development in the thymus when TCR expression first occurs (37). Thus, as a further test of whether heterozygous expression of H2nb1 molecules on hemopoietic cells results in the negative selection of AI4 T cells, we also enumerated

### Table I. Heterozygous H2nb1 expression blocks the development of T1D and AI4 clonotypic T cells in NOD.AI4αβ Tg mice

<table>
<thead>
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<th>Female T1D Incidence</th>
<th>% ± SEM AI4 TCR+ Cells Among PBL</th>
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<tbody>
<tr>
<td>NOD.AI4αβ Tg</td>
<td>9/10 (90%)</td>
<td>0.38% (n = 6)</td>
</tr>
<tr>
<td>(NOD.H2nb1 × NOD.AI4αβ Tg)F1</td>
<td>0/5 (0%)</td>
<td>0.93 ± 0.38 (n = 5)</td>
</tr>
</tbody>
</table>

a PBL from 5- to 6-wk-old mice were assessed for proportions of AI4 TCR-positive CD8 T cells by containing with Vα8- and CD8-specific Abs.

b Female T1D incidence through 10 wk of age.

c Female T1D incidence through 30 wk of age.

FIGURE 1. Heterozygous H2nb1 expression on hemopoietically derived APC results in the negative selection of AI4 T cells

A bone marrow chimera system was employed to determine whether heterozygous H2nb1 expression limited the development of AI4 T cells through impaired positive selection or enhanced negative selection. The approach taken was to determine whether AI4 T cell development was inhibited when H2nb1 molecules were heterozygously expressed on thymic epithelium or bone marrow-derived APC that respectively are the most efficient mediators of positive and negative selection (37). Positive controls consisted of NOD mice reconstituted with NOD.AI4β Tg marrow. As expected, at 6 wk postreconstitution this positive control group was characterized by high numbers of AI4 clonotypic CD8 T cells in the spleen (Fig. 1A). Negative controls consisted of (NOD.H2nb1 × NOD)F1 mice reconstituted with (NOD.H2nb1 × NOD.AI4αβ Tg)F1 marrow. These negative controls heterozygously expressed H2nb1 on both thymic epithelium and APC and were characterized by significantly fewer splenic AI4 CD8 T cells than in the positive controls. One test group consisted of (NOD.H2nb1 × NOD)F1 mice reconstituted with NOD.AI4αβ Tg marrow. In this group, developing AI4 T cells only encountered H2nb1 molecules on non-hemopoietically derived cells including thymic epithelium. The number of splenic AI4 CD8 T cells detected in this group did not statistically differ from the positive controls. Hence, heterozygous expression of H2nb1 molecules on thymic epithelium does not limit the positive selection of AI4 CD8 T cells. The second experimental group consisted of NOD mice reconstituted with (NOD.H2nb1 × NOD.AI4αβ Tg)F1 marrow. In this case, developing AI4 T cells only encountered H2nb1 molecules on hemopoietically derived cells including thymic APC. The number of splenic AI4 CD8 T cells in this latter group was significantly less than in the positive controls. Thus, heterozygous expression of H2nb1 on hemopoietically derived cells appeared to induce the negative selection of AI4 CD8 T cells.

Negative selection normally occurs at the CD4/CD8 double-positive stage of T cell development in the thymus when TCR expression first occurs (37). Thus, as a further test of whether heterozygous expression of H2nb1 molecules on hemopoietic cells results in the negative selection of AI4 T cells, we also enumerated

![Image](http://www.jimmunol.org/content/ji/168/7/873/F1a.png)

**A**. AI4 T cell numbers in NOD, thymic epithelium+APC, thymic epithelium, and APC reconstituted mice at 9 wk posttransplantation. The number of splenic AI4+CD8 T cells was significantly reduced in thymic epithelium+APC reconstituted mice compared to all other groups. B. AI4 T cell numbers in NOD, thymic epithelium+APC, thymic epithelium, and APC reconstituted mice at 9 wk posttransplantation. The number of splenic AI4+CD8 T cells was significantly reduced in thymic epithelium+APC reconstituted mice compared to all other groups.
by three-color FACS analysis the number of TCRαβ-expressing CD4/CD8 double-positive thymocytes that were present in all four experimental groups. As shown in Fig. 1B, the numbers of such thymocytes were only significantly reduced from levels seen in the positive controls when H2\(^{ab1}\) was expressed on cells of hemopoietic origin. Collectively, these results indicate that inducing the negative selection of MHC class I-restricted β cell-autoactive T cells such as the AI4 clonotype represents one mechanism by which heterozygous expression of non-H2\(^{\alpha}\) MHC haplotypes can dominantly mediate TID-protective effects.

The H2\(^{ab1}\) class I-matched H2\(^{b}\) and H2\(^{b}\)-Al\(^{ab}\) haplotypes do not mediate the negative selection of AI4 T cells

Given the fact they are a CD8 clonotype, we originally hypothesized it was a class I variant encoded within the H2\(^{ab1}\), but not the H2\(^{\alpha}\) haplotype, that provided a negatively selecting ligand for AI4 T cells. Kb represents the only known positive CD8 T cells by costaining with V8/11003/H11001/H2\(^{b}\)-Ab\(^{0}\) transgene construct (NOD-H2\(^{b}\)-Ab\(^{0}\)) (38). Thus, we tested if heterozygous expression of another K\(^{b}\)-encoding MHC haplotype (H2\(^{b}\)) could also mediate the negative selection of AI4. Surprisingly, AI4 T cell numbers were not reduced in (NOD.H2\(^{b}\) × NOD.AI4αβ Tg)F\(_1\) mice (Table II). This unexpected outcome led us to hypothesize that while the peripheral effector function of AI4 T cells is MHC class I restricted, during thymic maturation the AI4 TCR can engage class II variants encoded by the H2\(^{ab1}\) but not the H2\(^{b}\) haplotype in a negatively selecting fashion.

To test our hypothesis, we determined if AI4 T cells continued to mature under conditions where they were exposed to K\(^{b}\) class I molecules, but no class II variants differing from the sole A\(^{\beta}\) gene product expressed by NOD mice. NOD.AI4αβ Tg mice were crossed with NOD stocks either congenic for a class II-deficient H2\(^{b}\) haplotype (designated NOD.H2\(^{b}\)-Ab\(^{0}\)) or that expressed a genomic K\(^{b}\) transgene construct (NOD-K\(^{b}\) Tg mice). AI4 T cell numbers were not reduced in either (NOD.H2\(^{b}\)-Ab\(^{0}\) × NOD.AI4αβ Tg)F\(_1\) or (NOD-K\(^{b}\) Tg × NOD.AI4αβ Tg)F\(_1\) mice (Table II). Collectively, these data provided further evidence that the expression of different class II variants accounts for the ability of the H2\(^{ab1}\) but not the H2\(^{b}\) haplotype to induce the negative selection of AI4 T cells that express a class I-restricted diabetogenic effector function in NOD mice.

Expression of a second TCR does not account for the negative selection of class I-restricted AI4 diabetogenic T cells by an H2\(^{ab1}\) class II variant

Because the process of allelic exclusion is incomplete, T cells from TCR Tg mice, including the NOD.AI4αβ Tg stock, can express an additional endogenously derived TCR (25, 39–41). Hence, it was possible that engagement of such a second TCR allowed AI4 T cells to be negatively selected by an H2\(^{ab1}\) class II variant. To address this issue, we generated a NOD.AI4αβ Tg stock that was also homozygous for a functionally inactivated Rag1 allele that blocks all endogenous TCR gene rearrangements. The diabetes frequency by 6 wk of age in NOD.Rag1\(^{null}\).AI4αβ Tg female mice was found to be 88.2% (30/34). A mixed bone marrow chimera system was then employed to determine whether T cells only expressing the AI4 TCR were negatively selected by hemopoietically derived APC-expressing H2\(^{ab1}\) class II or other MHC variants.

Experimental chimeras consisted of female F\(_1\) hybrids between NOD mice and NOD stocks congenic for the indicated MHC haplotypes that were reconstituted with a 1:1 mixture of syngeneic F\(_1\) and NOD.Rag1\(^{null}\).AI4αβ Tg bone marrow cells, both obtained from female donors. This was done to eliminate any possibility of residual host vs graft responses that might limit the engraftment of APC heterozygously expressing non-H2\(^{\alpha}\) MHC gene products with a potential to modulate AI4 T cell development. Positive controls consisted of NOD mice reconstituted with a 1:1 mixture of NOD and NOD.Rag1\(^{null}\).AI4αβ Tg bone marrow. It had been our experience in similar studies that maximal, stable levels of chimerization are achieved by 6 wk postreconstitution (42). However, eight of 12 mice in this positive control group developed TID before the 6-wk postreconstitution time point (Fig. 2A). At the time of disease onset, the proportion of CD8 T cells that expressed the AI4 TCR in these eight control mice ranged from 28 to 52%. As expected, in the four control chimeras that did not develop TID by 6 wk postreconstitution, approximately half (43.5 ± 7.6%) of the CD8 T cells expressed the AI4 TCR.

One experimental group consisted of (NOD × NOD.H2\(^{ab1}\))F\(_1\) hybrids reconstituted with a 1:1 mixture of syngeneic F\(_1\) and NOD.Rag1\(^{null}\).AI4αβ Tg bone marrow. Only one of five mice in this group developed TID by 6 wk reconstitution (Fig. 2B). In the single diabetic chimera, ~23% of the CD8 T cells expressed the AI4 TCR. However, the four of five chimeras in this group that remained diabetes free had a significantly lower proportion of CD8 T cells expressing the AI4 TCR (6.3 ± 1.4%) than in the positive controls. This finding indicated that the AI4 TCR itself can engage a H2\(^{ab1}\)-encoded MHC structure in a negatively selecting fashion. To test whether this negatively selecting structure was a class II variant, we evaluated (NOD × NOD.H2\(^{ab1}\)-Ab\(^{0}\))F\(_1\) hybrids reconstituted with a 1:1 mixture of syngeneic F\(_1\) and NOD.Rag1\(^{null}\).AI4αβ Tg bone marrow. In this group, three of seven chimeras developed TID by 6 wk postreconstitution (Fig. 2C).

The proportion of CD8 T cells expressing the AI4 TCR in these diabetic chimeras ranged from 22 to 38%. Given how rapidly TID developed in these three chimeras (2 wk postreconstitution), it is possible the disease-inducing AI4 T cells were not newly derived from precursors, but rather were already mature effectors within the transferred bone marrow, and therefore had not encountered potentially tolerogenic H2\(^{\alpha}\) molecules during their maturation. In the four of seven chimeras remaining diabetes free through 6 wk postreconstitution, the proportion of CD8 T cells expressing the AI4 TCR (49.3 ± 3.8%) was not significantly different than in the positive controls. These results further indicated that while AI4 T cells exert a H2\(^{\alpha}\) class I-dependent diabetogenic effector function, they can be negatively selected if they encounter particular class II structures during their development.

We also analyzed (NOD × NOD.H2\(^{ab1}\))F\(_1\) hybrids reconstituted with a 1:1 mixture of syngeneic F\(_1\) and NOD.Rag1\(^{null}\).AI4αβ Tg bone marrow. All four of these chimeras remained diabetes free through 6 wk postreconstitution (Fig. 2D). The proportion of CD8 T cells expressing the AI4 TCR in these chimeras (57.3 ± 6.9%) was also not different from that of the positive controls. These

Table II. Heterozygous H2\(^{b}\), H2\(^{b}\)-Al\(^{ab}\), H2\(^{a}\), or K\(^{b}\) Tg expression does not block the development of AI4 clonotypic T cells

<table>
<thead>
<tr>
<th>Stock</th>
<th>AI4 TCR +</th>
<th>CD8 Cells Among PBL* (% ± SEM)</th>
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<tbody>
<tr>
<td>NOD (n = 6)</td>
<td>0.50 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>NOD.AI4αβ Tg (n = 6)</td>
<td>22.7 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>(NOD.H2(^{b}) × NOD.AI4αβ Tg)F(_1) (n = 5)</td>
<td>20.3 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>(NOD.H2(^{b})-Ab(^{0}) × NOD.AI4αβ Tg)F(_1) (n = 4)</td>
<td>25.9 ± 3.4</td>
<td></td>
</tr>
<tr>
<td>(NOD-K(^{b}) Tg × NOD.AI4αβ Tg)F(_1) (n = 2)</td>
<td>26.2 ± 5.3</td>
<td></td>
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</table>

* PBL from 5- to 6-wk-old mice were assessed for proportions of AI4 TCR-positive CD8 T cells by containing V8/11003/H11001/H2\(^{b}\)-Ab\(^{0}\) specific Abs.
AH T cells are anergized when maturing in the presence of H2b-Ab0- and H2b-expressing APC

While present at equivalently proportional levels, it was unclear why the AI4 T cells that developed in H2b-Ab0- or H2b-expressing chimeras elicited a lower frequency of TID (43 and 0%) than those in the positive controls (67%). Furthermore, while AI4 T cells were not deleted in the (NOD.H2b × NOD.AI4αβ Tg)F1 or (NOD.H2b-Ab0- × NOD.AI4αβ Tg)F1 mice depicted in Table II, none of them developed T1D. One possibility was that in both the chimeras and F1 hybrids, H2b-Ab0- or H2b-expressing APC did not block the development of AI4 T cells, but such effectors failed to become efficiently activated because in each case the β cell targets, and perhaps other tissues such as pancreatic APC, expressed H2z7 MHC class I molecules in a heterozygous rather than homozygous fashion. To test this possibility, we assessed the ability of 10^6 CD8 T cells purified from the spleens of chimeras with H2b-Ab0- or H2b-expressing APC to adoptively transfer T1D to female NOD-scid recipients, which unlike the donors homozgyously expressed H2z7 MHC molecules on pancreatic β cells and other tissues such as APC. As shown in Fig. 2, about half of the adoptively transferred CD8 T cells from these chimeras were of NOD.Rag1null.AI4αβ Tg origin. Hence, positive controls consisted of NOD-scid recipients of 0.5 × 10^6 CD8 T cells directly transferred from NOD.Rag1null.AI4αβ Tg female donors. These positive control AI4 T cells transferred T1D to four of five NOD-scid recipients (Table III). In contrast, T1D failed to develop in any NOD-scid recipients infused with Rag1null AI4 T cells that had developed in H2b-Ab0- (0/3) or H2b (0/3)-expressing chimeras. This ruled out our original hypothesis that it was heterozygous rather than homozygous expression of H2z7 on pancreatic β cells and perhaps other sites that prevented AI4 T cells from efficiently activating and inducing T1D in these donor chimeras.

An alternative hypothesis was suggested by the finding that Rag1null AI4 T cells maturing in the presence of H2b- or H2b-Ab0-expressing APC-repopulated NOD-scid recipients at lower levels than those only encountering H2z7 molecules (Table III). Based on these results, we hypothesized that AI4 T cells were totally or partially anergized when maturing in the presence of H2b-Ab0- or H2b-expressing APC. Hence, we assessed whether AI4 T cells that had matured in the presence of H2b-Ab0- or H2b-expressing APC had a suppressed ability to proliferate in response to a normally stimulatory mimotope peptide. CD8 T cells of which >90% expressed the AI4 TCR were purified from the spleens of both (NOD.H2b × NOD.AI4αβ Tg)F1 and (NOD.H2b-Ab0- × NOD.AI4αβ Tg)F1 mice. As shown in Fig. 3, following stimulation with the agonist peptide, AI4 T cells that had matured in the presence of H2b-Ab0- or H2b-expressing APC in the F1 hybrids proliferated at significantly lower levels than those isolated from standard NOD.AI4αβ Tg mice. These results indicated that while AI4 CD8 T cells are not deleted when maturing in the presence of H2b-Ab0- or H2b-expressing APC they are partially anergized. Such an induction of partial anergy could also explain why the ability of AI4 T cells to trigger T1D development was reduced, but not completely ablated, in the chimeras with H2b-Ab0-expressing APC. Furthermore, because AI4 T cells maturing in the presence of H2b-Ab0-expressing APC are anergized, this effect is likely mediated by a class I variant encoded within the H2b but not the H2z7 haplotype.

Down-regulation of TCR expression represents one mechanism by which tolerance can be achieved through the induction of anergy, rather than by clonal deletion (reviewed in Ref. 43). Hence, we determined if the partially anergic state of AI4 T cells that had matured in the presence of H2b-Ab0- or H2b-expressing APC was associated with decreased levels of TCR expression. This did seem

results indicated the AI4 TCR can engage class II variants encoded by the H2znull but not the H2b haplotype in a negatively selecting fashion.
Similar to the case with the through the down-regulation of CD8 expression in NOD.AI4 T cells are anergized when maturing in the presence of H2\(^b\)- or H2\(^{nb1}\)-expressing APC

\(\text{FIGURE 3.}\) AI4 T cells are anergized when maturing in the presence of H2\(^{nb1}\)- and H2\(^b\)-expressing APC. Triplicate aliquots of 5 \(\times\) 10\(^5\) CD8 T cells from NOD.AI4αβ Tg (.), NOD.H2\(^b\) × NOD.AI4αβ TgF\(_1\) ( ), or (NOD.H2\(^{nb1}\)-Ab\(^b\) × NOD.AI4αβ TgF\(_1\) ( ) mice were cocultured with 2 \(\times\) 10\(^5\) irradiated (2000 rad) NOD splenic leukocytes and the indicated concentration of the AI4 mimotope peptide (YFIELNYEL). After 48 h of incubation, the cultures were pulsed with \(^3\)H]thymidine for an additional 24 h. Proliferative responses are presented as mean cpm ± SEM. Asterisks designate a significantly higher (p < 0.05, Student’s t test) proliferative response by CD8 T cells from NOD.AI4αβ Tg than (NOD.H2\(^{nb1}\)- Ab\(^b\) × NOD.AI4αβ TgF\(_1\) or (NOD.H2\(^b\)-Ab\(^b\) × NOD.AI4αβ TgF\(_1\) mice. Similar results were obtained in a second experiment.
variant encoded by the $H2^{nb1}$ haplotype. However, pretreatment of NOD.$H2^{nb1}$ splenocytes with the $E_{k}$-binding Ab 14-4-4 also abrogated their ability to stimulate NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg T cells (Fig. 5B). The ability of NOD.$H2^{nb1}$ splenocytes to activate NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg T cells was abrogated to a far lesser extent by pretreatment with a mAb (28-13-3) directed against the K$^b$ class I variant rather than one of the class II molecules encoded by this MHC haplotype (Fig. 5B). Taken together with the data depicted in Fig. 5A, these results indicated that co-expression of the $H2^{nb1}$ class I and class II variants results in an additive effect that strongly stimulates diabetogenic AI4 CD8 T cells to the level required to induce their negative selection. On the other hand, APC that share the $K^b$ class I but not the class II variants of the $H2^{nb1}$ haplotype stimulate AI4 T cells to a much lower level that is apparently insufficient to trigger their deletion but does induce functional anergy through the down-regulation of TCR expression. Thus, it can be concluded that while AI4 T cells recognize a $\beta$ cell autoantigen in a $H2^{nb1}$ MHC class I-restricted fashion, the presence or absence of particular class II variants also determine the extent to which this diabetogenic clonotype develops.

NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg T cells also secreted significant levels of IFN-$\gamma$ when stimulated with NOD.$H2^{b}$ splenocytes (Fig. 5A). As described earlier, AI4 T cells seed the periphery of (NOD.$H2^{b}$-Ab 0 -o r $V_{b}$-specific Ab) splenic leukocytes from NOD mice or the indicated NOD MHC congenic stocks. The extent of activation through the AI4 TCR was then assessed as described above with irradiated (2000 rad) splenic leukocytes from NOD mouse or the indicated NOD MHC congenic stocks. The extent of activation through the AI4 TCR was then assessed by ELISA measurements of IFN-$\gamma$ levels in pooled supernatants from each culture. Data are presented as the mean IFN-$\gamma$ concentration ± SEM of each sample assayed in triplicate. Similar results were obtained in two other experiments. B, IFN-$\gamma$ production by NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg T cells stimulated as described above with irradiated NOD.$H2^{nb1}$ splenocytes in the presence and absence of mAbs (10 $\mu$g/ml) previously shown to recognize $K^b$ class I (28-13-3), $K^d$ class I (SF1-1.1), or $A^{b,d,q}$ E$d,k$ (M5/114) and $E^{b,k}$ (14-4-4) MHC class II molecules.

**FIGURE 5.** Signaling through the AI4 TCR is strongly stimulated by engagement of a $H2^{nb1}$ class II variant or an $H2^{b}$-encoded ligand. A, Triplicate aliquots of $5 \times 10^5$ splenic leukocytes from NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg mice were cocultured for 5 days with an equal number of irradiated (2000 rad) splenic leukocytes from NOD mice or the indicated NOD MHC congenic stocks. The extent of activation through the AI4 TCR was then assessed by ELISA measurements of IFN-$\gamma$ levels in pooled supernatants from each culture. Data are presented as the mean IFN-$\gamma$ concentration ± SEM of each sample assayed in triplicate. Similar results were obtained in two other experiments. B, IFN-$\gamma$ production by NOD.$Rag1^{null}$,AI4$\alpha\beta$ Tg T cells stimulated as described above with irradiated NOD.$H2^{nb1}$ splenocytes in the presence and absence of mAbs (10 $\mu$g/ml) previously shown to recognize $K^b$ class I (28-13-3), $K^d$ class I (SF1-1.1), or $A^{b,d,q}$ E$d,k$ (M5/114) and $E^{b,k}$ (14-4-4) MHC class II molecules.
Discussion

Until now it has been unknown if similar to the case with MHC class II-restricted effectors, whether class I-restricted diabetogenic T cell responses could also be dominantly inhibited by the expression of particular MHC variants. The current study indicates this is indeed the case. Specifically, we show AI4 T cells that exert a class I-restricted β cell-autoreactive effector function in NOD mice are negatively selected or functionally anergized by the expression of some MHC genes that confer dominant T1D resistance. Unexpectedly, we found that while AI4 T cells exert a class I-restricted diabetogenic effector function, the expression of particular MHC class II molecules on APC can contribute to the intrathymic negative selection of this pathogenic clonotype. Such class II molecules include a variant encoded within the H2\textsuperscript{d} haplotype, most likely H2\textsuperscript{d}\textsuperscript{b}, that is not produced by the closely related H2\textsuperscript{d}\textsuperscript{a} haplotype. The ability of T cells that mediate a class I-restricted effector function to be negatively selected by some class II variants is not unprecedented (44–46). However, ours is the first demonstration that some class II variants can regulate the development of T cells that contribute to autoimmunity in a class I-restricted fashion. This ability to contribute to the deletion of pathogenic CD8 as well as CD4 T cells (10, 11) adds to the mechanisms by which some MHC class II variants can mediate dominant resistance to T1D.

In addition to being deleted in the presence of some class II variants, we also found that AI4 T cells could be functionally anergized through interactions with class I molecules encoded by the H2\textsuperscript{b} but not the NOD H2\textsuperscript{nb1} haplotype. This process entailed a down-regulation of TCR expression. While the H2\textsuperscript{a-b} and H2\textsuperscript{b} haplotypes do not share class I variants whose additional presence can trigger the deletion of AI4 T cells, they are class I matched. Hence, it appears that certain combinations of MHC class I and class II variants can interactively confer protection against T1D by mediating the negative selection of pathogenic CD4 and CD8 T cells and, at least in the case of the latter class of effectors, anergize those escaping this deletional process. While unable to assign the function to class I or class II variants, AI4 T cells maturing in the presence of H2\textsuperscript{b} MHC molecules were also functionally impaired due to greatly diminished levels of CD8 coreceptor expression. However, this may not represent true anergy, but might rather be a by-product of an incomplete negative selection process that only spared AI4 T cells expressing low levels of CD8.

The fact it can engage class II as well as class I MHC structures indicates the AI4 TCR is quite promiscuous. Such promiscuity has been reported to be a feature of other autoreactive TCRs (10–12, 47). If expression of a promiscuous TCR is a common feature of autoreactive but not foreign Ag-reactive T cells, one consequence is that during their development the former would have a greatly increased probability of encountering negatively selecting ligands. Under this scenario, particular autoreactive T cells would only develop in individuals unfortunate enough to lack thymic expression of any of the multiple ligands that can normally contribute to negative selection of the clonotype in question. Of course, genetically controlled defects in APC function, such as those documented in NOD mice (reviewed in Ref. 2), could augment this process by limiting the tolerogenic presentation of the multiple ligands that might normally engage a promiscuous autoreactive TCR. While it may normally increase their probability of being negatively selected during thymic development, expression of a promiscuous TCR on any T cells that do reach the periphery could make them highly dangerous. Indeed, previous studies have indicated the promiscuity and pathogenicity of diabetogenic CD8 T cells in NOD mice may further increase with age due to TCR avidity maturation that results from peripheral RAG gene re-expression (47–49).

In conclusion, our results provide further insights to the identity of MHC variants that can dominantly inhibit T1D development and the mechanisms by which they do so. We verify that certain class II variants can provide protection against T1D, but unexpectedly do so by contributing to the negative selection of β cell-autoreactive CD8 as well as CD4 T cells. Furthermore, while other studies indicate certain common MHC class I alleles can provide an increased risk for T1D development when expressed in the right genetic context, our results demonstrate other such variants also exert disease-protective effects. In the current study, we found that one mechanism of MHC class I-mediated T1D resistance is an ability to anergize β cell-autoreactive CD8 T cells. However, it cannot be ruled out that some MHC class I variants also exert other T1D-protective effects. Our results also indicate some MHC haplotypes, such as H2\textsuperscript{d-b}, encode separate gene products that in an additive fashion can induce the negative selection or functional inactivation of autoreactive diabetogenic CD8 T cells. Establishing mixed hemopoietic engraftment of APC expressing allogeneic MHC genes blocks the development of β cell-autoreactive T cells and T1D in NOD mice (34, 42, 50). Not surprisingly, resistance is more efficiently established when APC express multiple rather than a single MHC gene with T1D-protective effects. As safer preconditioning regimens are developed, it may be possible to consider the use of mixed hemopoietic chimerization to prevent T1D development in humans at future risk for this disease. Our current results indicate extended MHC haplotype analyses in humans should not only be used to identify highly pathogenic combinations of T1D susceptibility genes, but also strongly interactive resistance variants. Such information would aid identification of the hemopoietic donor cells that would provide most efficacious T1D protection in any chimerization protocols ultimately approved for clinical use.

References
