T-Cell Tolerance by Dendritic Cells and Macrophages as a Mechanism for the Major Histocompatibility Complex–Linked Resistance to Autoimmune Diabetes

Shari Thiessen, Pau Serra, Abdelaziz Amrani, Joan Verdaguer, and Pere Santamaria

For poorly understood reasons, the development of autoimmune diabetes in humans and mice is dominantly inhibited by major histocompatibility complex (MHC) class II molecules with diverse antigen-binding sites. We have previously shown that thymocytes expressing a highly diabetogenic I-A\textsuperscript{g7}–restricted T-cell receptor (TCR) (4.1-TCR) undergo negative selection in mice carrying one copy of the antidiabeticogenic H-2\textsuperscript{b} haplotype in an I-A\textsuperscript{d}–dependent but superantigen-independent manner. Here, we show that 4.1-TCR–transgenic thymocytes undergo different forms of tolerance in NOD mice expressing antidiabeticogenic I-A\textsuperscript{d}, I-A\textsuperscript{d7PP}, or I-E\textsuperscript{b} transgenes. The ability of protective MHC class II molecules to induce thymocyte tolerance in 4.1-TCR–transgenic NOD mice correlates with their ability to prevent diabetes in non–TCR-transgenic mice and is associated with polymorphisms within positions 56–67 of their \( \beta_1 \) domains. The 4.1-thymocyte tolerogenic activity of these MHC class II molecules is mediated by dendritic cells and macrophages but not by B-cells or thymic epithelial cells and is a peptide-dependent process. Antidiabeticogenic MHC class II molecules may thus afford diabetes resistance by presenting, on dendritic cells and macrophages, tolerogenic peptides to a subset of highly diabetogenic and MHC-promiscuous CD4\textsuperscript{+} T-cells that play a critical role in the initiation of diabetes.

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Genetic susceptibility and resistance to autoimmune disorders is associated primarily with polymorphisms of genes of the major histocompatibility complex (MHC) and, to a lesser extent, with multiple non–MHC-linked modifiers (1). Much of what is currently known about MHC-linked susceptibility and resistance to autoimmunity has been learned from studies of insulin-dependent diabetes (type 1 diabetes), a spontaneous autoimmune disease that results from selective destruction of the insulin-producing pancreatic \( \beta \)-cells by T-lymphocytes (2). In humans, the MHC-linked susceptibility and resistance to autoimmune diabetes is primarily associated with polymorphisms at the HLA-DQ\textsuperscript{B1} locus. Alleles encoding DQB chains with serine, alanine, or valine at position 57 provide risk, whereas those encoding DQ\( \beta \) chains with aspartic acid at this position provide protection with different degrees of dominance (3,4). In mice, susceptibility and resistance to diabetes are also linked to the MHC. The NOD mouse is homozygous for a unique H-2 haplotype. This haplotype (H-2\textsuperscript{67}) carries a silent I-E\textsuperscript{g} gene and encodes an I-A\textsuperscript{\alpha}/I-A\textsuperscript{\beta} heterodimer in which the proline and aspartic acid found at positions 56 and 57 in most I-A\( \beta \) and I-E\( \beta \) chains are replaced by histidine and serine, respectively (3,4). Studies of NOD mice expressing single copies of non-NOD MHC haplotypes and NOD mice expressing I-E\textsuperscript{\alpha}, I-E\textsuperscript{\alpha} \textsuperscript{k}, modified I-A\textsuperscript{\alpha} \textsuperscript{g7} (I-A\textsuperscript{g7PP}), encoding proline and aspartic acid at positions 56 and 57), I-A\textsuperscript{\alpha}/I-A\textsuperscript{\beta}, I-A\textsuperscript{\alpha} \textsuperscript{k}, or I-A\textsuperscript{\alpha} \textsuperscript{b} transgenes have proven that MHC class II molecules play a direct role in providing resistance to type 1 diabetes (3,4). However, because recognition of \( \beta \)-cell autoantigens by diabetogenic T-cells is restricted by pro-diabetogenic MHC class II molecules (i.e., I-A\textsuperscript{g7}), it is difficult to understand how the development of autoimmune diabetes can be dominantly inhibited by so many different MHC class II molecules.

Although it has been conclusively established that the factors responsible for the MHC-linked resistance to autoimmune diabetes reside in the bone marrow (5–11), the underlying mechanisms remain elusive. A number of early studies of congenic and transgenic NOD mice expressing antidiabeticogenic MHC class II molecules found evidence for the existence of T-cell autoreactivity in the peripheral immune systems of these mice. These observations demonstrated that protective MHC class II molecules do not afford diabetes resistance by systematically deleting all autoreactive T-cell specificities (11–17). However, they could not rule out a role for selective tolerance (i.e., of certain autoreactive T-cells). Studies of mice expressing a highly diabetogenic I-A\textsuperscript{g7}–restricted T-cell receptor (TCR) (4.1) later provided direct support for this possibility. We found that thymocytes expressing the 4.1-TCR undergo complete deletion in mice carrying a single copy of the antidiabeticogenic haplotype H-2\textsuperscript{b} in an I-A\textsuperscript{d}–dependent but superantigen-independent manner (18). Like the diabetes resistance afforded by protective MHC class II molecules in non–TCR-transgenic mice, the diabetes resistance afforded by the I-A\textsuperscript{\beta}–induced deletion of thymocytes in...
4.1-TCR–transgenic mice was mediated by bone marrow–derived cells (18,19).

These observations suggested that in nontransgenic mice, protective MHC class II molecules might afford diabetes resistance by tolerizing certain highly pathogenic T-cell specificities. This hypothesis predicted that the 4.1-T-cell tolerogenic activity of I-A<sup>k</sup> would be shared by other antidiabetogenic MHC class II molecules and that it would be mediated by dendritic cells, macrophages, and B-cells. Here, we have tested these predictions by investigating the ability of three different MHC class II molecules (I-E<sup>a</sup>/I-<sup>β</sup>B<sup>7</sup>, I-A<sup>k</sup>, and I-A<sup>47PD</sup>) and different antigen-presenting cell (APC) types to tolerize 4.1-CD4<sup>+</sup> T-cells and/or to prevent 4.1-CD4<sup>+</sup> T-cell–induced diabetes. We show that 4.1-CD4<sup>+</sup> T-cells undergo different forms of tolerance in 4.1-TCR–transgenic NOD mice expressing I-E<sup>α</sup>/I-<sup>β</sup>B<sup>7</sup>, I-A<sup>k</sup>, or I-A<sup>47PD</sup> molecules. Surprisingly, T-cell tolerance in these mice is triggered by peptide/MHC class II–specific T-cells on dendritic cells and macrophages but not by peptide/MHC class II expressed on B-cells. These data provide but one explanation to the puzzling ability of 4.1-NOD mice expressing nonpathogenic 4.1-like CD4<sup>+</sup> peptide/MHC class II molecules to allow tolerance to these T-cells and thus to present a model for the study of pathogenic CD4<sup>+</sup> T-cell–induced diabetes and for the development of immunotherapies for diabetes.

**RESEARCH DESIGN AND METHODS**

**Mice.** We used 4.1-NOD mice and recombination activating gene-2 (RAG-2)–deficient 4.1-NOD mice expressing a transgenic, I-A<sup>g7</sup>–restricted, β-cell–reactive TCR derived from a pancreatic islet-associated CD4<sup>+</sup> T-cell clone (NY1.1), as previously described (18). The fine antigenic specificity of this TCR is unknown, but 4.1-CD4<sup>+</sup> T-cells do not recognize recombinant GAD65, GAD66, preinsulin, or I-A<sup>k</sup>–specific 4.1-NOD mice (19). NOD transgenic C57BL/6/129 mice (12) (Jackson Laboratory) with NOD mice for 10 generations were typed for microsatellites linked to known I<sup>dd</sup> regions. N8 mice were typed for homozygosity at known microsatellites linked to I<sup>dd</sup> regions. 4.1-TCR–transgenic NOD mice expressing an I-A<sup>g7</sup>–linked transgene of BALB/c (20) (from O. Nakagawa, Washington University, St. Louis, MO) onto the NOD background for at least seven generations and typed for homozgyosity at known I<sup>dd</sup> regions.

**RESULTS**

**Deletion of 4.1-thymocytes by transgenic and wild-type I-E<sup>k</sup> molecules.** Thymocytes expressing the 4.1-TCR undergo deletion in H-2<sup>b</sup>/b mice by engaging I-A<sup>k</sup> molecules on bone marrow–derived APCs (18,19). To investigate whether this phenotype could also be triggered by other antidiabetogenic MHC class II molecules, we followed the fate of the 4.1-TCR in 4.1-TCR–transgenic NOD mice expressing an I-E<sup>α</sup><sup>k</sup> transgene (4.1-NOD.I-E<sup>α</sup><sup>k</sup>). Nontransgenic NOD mice transcribe and translate I-E<sup>β</sup><sup>7</sup> molecules but cannot express I-E<sup>α</sup><sup>k</sup> heterodimers because they carry a nonproductive I-Eα gene (22). Because the introduction of I-E<sup>α</sup><sup>k</sup> transgenes in NOD mice restore I-E expression and renders the mice completely or partially resistant to diabetes (17,23–26), we wondered whether I-E<sup>k</sup> molecules could also trigger the deletion of 4.1-thymocytes.
One-third of 4.1-NOD.I-Eαk mice (7 of 21) had thymocyte and splenic cytofluorometric profiles that were compatible with complete negative selection of 4.1-thymocytes, as compared with other TCR-transgenic models (27–30). These mice (herein referred to as “deleting”) displayed a significant reduction in the percentage of CD4+.CD8− thymocytes, a reduction in the percentage of CD4+.CD8− thymocytes expressing the transgene-encoded Vβ11 element, and an increase in the percentage of CD4+.CD8− thymocytes when compared with 4.1-NOD mice (Table 1 and Fig. 1A and B, upper panels). In the spleen, deleting 4.1-NOD.I-Eαk mice had significantly fewer CD4+ T-cells and CD4+.Vβ11+ T-cells than 4.1-NOD mice (Table 1 and Fig. 1A and B, lower panels). The few Vβ11+ CD4+ T-cells that matured in deleting mice expressed significantly lower levels of the transgenic TCRβ chain on the cell surface than the Vβ11+.CD4+ T-cells that matured in 4.1-NOD mice (Table 1, Fig. 1A and B, and data not shown). This suggested that in deleting 4.1-NOD.I-Eαk mice, most CD4+ T-cells were selected on endogenous TCR chains that had bypassed allelic exclusion. The remaining two-thirds of 4.1-NOD.I-Eαk mice (14 of 21) displayed thymocyte and splenic profiles that were similar to those seen in 4.1-NOD mice (Table 1 and Fig. 1A and B), where 4.1-thymocytes undergo positive selection. Individual mice were classified as deleting if the values corresponding to the parameters described above were at least 2 SDs above or below the corresponding values in 4.1-NOD mice. Therefore, I-Eαk/I-Eβk heterodimers can trigger massive deletion of 4.1-thymocytes, albeit only in a fraction of the mice.

Proliferation assays using splenic CD4+ T-cells from deleting 4.1-NOD.I-Eαk mice as responders and irradiated NOD or NOD.I-Eαk islet cells as a source of antigen and APCs confirmed the absence of β-cell–reactive CD4+ T-cells in these mice (Fig. 2A). Surprisingly, the splenic CD4+ T-cells of nondeleting 4.1-NOD.I-Eαk mice also displayed proliferative unresponsiveness to islet stimulation in vitro (Fig. 2B, left panel). This unresponsiveness was caused by anergy (rather than deletion) because the addition of rIL2 to the cultures restored the CD4+ cells’ proliferative responsiveness to antigen (Fig. 2B, right panel). The splenic CD4+ T-cells from both 4.1-NOD and 4.1-NOD.I-Eαk mice did not express activation markers, including CD25, CD69, or CD44 (not shown), suggesting that they are naive and quiescent.

Studies of 4.1-TCR-transgenic (NODxC58/j) F1 mice, which are H-2k/heterozygous and can thus express I-Eαk/I-Eβk and I-Eαk/I-Eβk heterodimers, indicated that these two phenotypes (deletion and anergy) were not an artifact of transgenesis. As shown in Table 1 and Fig. 1C, 4.1-NODxC58/j F1 mice (also referred to as 4.1-H-2k/heterozygous mice) displayed the same two phenotypes. Altogether, 8 of 14 mice had a phenotype compatible with complete deletion of 4.1-thymocytes, whereas the remaining 6 mice had a phenotype compatible with positive selection (Table 1 and Fig. 1C; mice classified into “deleting” or “nondeleting” groups based on the criteria described above). The prevalence of deleters in the 4.1-NODxC58/j F1 strain was slightly greater than in the 4.1-NOD.I-Eαk strain, but the differences did not reach statistical significance. The thymocyte deletion in 4.1-NODxC58/j F1 mice was triggered by endogenous I-Eαk molecules and/or I-Eαk/I-Eβk heterodimers, rather than by I-Ak molecules, because deletion was not seen in I-Aαk/I-Aβk–transgenic 4.1-NOD mice (Table 1 and Fig. 1D). Deletion of 4.1-thymocytes in 4.1-NODxC58/j F1 and 4.1-NOD.I-Eαk mice was not mediated by Vβ11-binding superantigens, because non-TCR-transgenic (NODxC58/j) F1 and NOD.I-Eαk mice had as many thymic and splenic Vβ11+.CD4+ T-cells as NOD mice (data not shown). This is not surprising because neither (NODxC58/j) F1 nor NOD.I-Eαk mice express

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<th>CD8−</th>
<th>Vβ11+</th>
<th>CD4+</th>
<th>DP</th>
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Data are means ± SD unless otherwise indicated. Mice were studied at 8–16 weeks of age. The average CD4+CD8− (DN) and CD4+/CD8− (DP) ratios were obtained by averaging the corresponding ratios from individual mice; because of the variability, these values are not necessarily the same as those obtained by dividing the average percentage of CD4+ versus the average percentage of DN or CD8+ cells. DP, double positive; T, thymus; S, spleen. (Statistics (Mann-Whitney U test): a vs. b P = 0.0055; a vs. c P = 0.0007; a vs. d P = 0.0002; c vs. e P = 0.0002; i vs. j P = 0.0002; i vs. k P = 0.0007; j vs. l P = 0.0008; m vs. n P = 0.0054; m vs. o P = 0.0004; n vs. p P = 0.0136; i vs. k P = 0.0136; r vs. s P = 0.0012; r vs. t P = 0.0014; r vs. u P = 0.0020; r vs. v P = 0.0012; i vs. j P = 0.0002; i vs. k P = 0.0002; i vs. l P = 0.0002; i vs. m P = 0.0002; i vs. n P = 0.0002; i vs. o P = 0.0002; i vs. p P = 0.0002; i vs. q P = 0.0002; i vs. r P = 0.0002; i vs. s P = 0.0002; i vs. t P = 0.0002; i vs. u P = 0.0002; i vs. v P = 0.0002; i vs. w P = 0.0002; i vs. x P = 0.0002; i vs. y P = 0.0002; i vs. z P = 0.0002.)
FIG. 1. Representative CD4, CD8, and Vβ11 profiles of thymocytes and splenocytes from 4.1-NOD, 4.1-H-2^g7/k, 4.1-NOD.I-A^k, and 4.1-NOD.I-E^a mice. A–D: Upper panels correspond to CD4 vs. CD8 contour plots of cells stained with anti–CD8-PE, anti–Vβ11-FITC, and anti–CD4-biotin plus streptavidin-PerCP; and lower panels correspond to Vβ11 fluorescence histograms of each T-cell subset after electronic gating. DP, double-positive cells; DN, double-negative cells.
mouse mammary tumor virus superantigens that are tolerogenic for Vβ/H925211/H11001 T-cells (31). Furthermore, deletion required expression of both chains of the 4.1-TCR, because 4.1-TCR/H9252–transgenic (NODxC58/j) F1 mice had as many Vβ/H925211/H11001 CD4/H11001 thymocytes as 4.1-TCR/H9252–transgenic NOD mice (73/8 vs. 75/3%, respectively). Together, these data indicate that I-Ek/H9251/I-Eg7 molecules, regardless of whether they are transgene-encoded, can efficiently tolerize thymocytes.

I-E–induced deletion of 4.1-thymocytes in vivo is induced by hematopoietic cells and is a stochastic process. To investigate whether the I-Ek–induced deletion of 4.1-thymocytes was triggered by hematopoietic cells, we followed the fate of 4.1-CD4/H11001 thymocytes in lethally irradiated NOD mice transfused with marrow from deleting or nondeleting 4.1-NOD. I-Ek mice. The phenotype of the bone marrow donors was a good, but imprecise, predictor of the phenotype seen in the chimeras. Altogether, 7 of 8 chimeras reconstituted with marrow from nondeleting donors developed nonpathogenic insulitis, whereas 16 of 20 chimeras reconstituted with marrow from nondeleting donors displayed nondeleting flow cytometric profiles. Therefore, the phenotype of the chimeras is largely imprinted in the donor marrow, but marrow from single nondeleting or deleting donor mice can give rise to both nondeleting and deleting chimeras.

Deleting and nondeleting 4.1-NOD.I-Eαk mice are diabetes resistant. To determine the effects of thymocyte tolerance on 4.1-CD4+ T-cell–induced diabetes, we monitored 4.1-NOD.I-Eαk mice for the development of diabetes (Table 2). As shown in Table 2, 4.1-NOD.I-Eαk mice were nearly as diabetes-resistant as non–TCR-transgenic NOD.I-Eαk mice, regardless of whether they were deleting or nondeleting.

Nondeleting 4.1-NOD.I-Eαk mice develop nonpathogenic insulitis. Histological studies confirmed that deleting 4.1-NOD.I-Eαk mice did not develop insulitis, as expected (Table 2 and Fig. 3D). Surprisingly, however, nondeleting 4.1-NOD.I-Eαk mice developed significant insulitis (Table 2 and Fig. 3E), despite the fact that their peripheral CD4+ T-cells were unresponsive to antigenic
stimulation in vitro (see above). The insulitis lesions of nondeleting 4.1-NOD.I-Eαk mice were caused by 4.1-CD4+ T-cells rather than by endogenous (nontransgenic) T-cells, because non–TCR-transgenic NOD.I-Eαk mice are completely resistant to insulitis (Table 2 and Fig. 3B). To ascertain whether these lesions were caused by an accumulation of anergic, nonproliferating 4.1-CD4+ T-cells in islets, we investigated whether the islet-infiltrating cells of nondeleting 4.1-NOD.I-Eαk mice could incorporate exogenous BrdU in situ. Surprisingly, islets from BrdU-injected, nondeleting 4.1-NOD.I-Eαk mice contained as many BrdU+ cells as islets from BrdU-injected 4.1-NOD mice (10 ± 1.4 vs. 8.6 ± 0.8%) (Fig. 3F and G). Thus, in nondeleting 4.1-NOD.I-Eαk mice, 4.1-CD4+ T-cells are responsive to antigenic stimulation in vivo, yet they are unable to cause disease.

To determine whether the inability of the islet-infiltrating T-cells of these mice to trigger β-cell damage was caused by the recruitment of immune-deviated 4.1-CD4+ cells, we compared the ability of their islet-associated T-cells to secrete IL-2, IFN-γ, and IL-4 upon stimulation with PMA and ionomycin. The islet-associated T-cells of

<p>| TABLE 2 | Insulitis and diabetes in 4.1-NOD.I-Ak, 4.1-NOD.I-Eαk, 4.1-NOD.I-Ad, and 4.1-NOD.I-Ag7PD versus non-MHC–transgenic 4.1-NOD littermates |
|----------|-------------|----------------|-----------------|----------------|</p>
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Data are means ± SD unless otherwise indicated. Type 1 diabetes was followed in female mice only. Insulitis: 15–30 islets/mouse were scored (scored at 12–15 weeks of age except a and j, which were scored at 6 weeks). a vs. b P = 0.0089; a vs. c P = 0.0305; d vs. e P = 0.0004; d vs. f P = 0.0030; d vs. g P = 0.0010; d vs. h P = 0.0002; d vs. i P = 0.0019; j vs. k P < 0.0001; j vs. l P = 0.045.

FIG. 3. Insulitis in deleting and nondeleting 4.1-NOD.I-Eαk mice. Representative H/E stained islets from NOD (A), NOD.I-Eαk (B), 4.1-NOD (C), deleting 4.1-NOD.I-Eαk (D), and nondeleting 4.1-NOD.I-Eαk (E) mice and anti-BrdU-stained samples from BrdU-treated 4.1-NOD (F) and nondeleting 4.1-NOD.I-Eαk mice (G) are shown. Magnification: ×200 (A–D, F, and G) or ×100 (E).
4.1-NOD and nondeleting 4.1-NOD.I-E\(\alpha^k\) mice produced IL-2 and IFN-\(\gamma\) (232 \(\pm\) 130 vs. 60 \(\pm\) 15 pg/ml and 1,387 \(\pm\) 735 vs. 205 \(\pm\) 43 pg/ml, respectively) but virtually undetectable levels of IL-4 (9 \(\pm\) 5 vs. 6 \(\pm\) 1 pg/ml). These results were confirmed by intracytoplasmic staining of islet-associated CD4\(^+\) T-cells with anti-IL-2, anti–IFN-\(\gamma\), and IL-4 mAbs (data not shown). Thus, despite their ability to proliferate against islet antigen in vivo and to secrete pro-inflammatory cytokines, the “anergic” 4.1-CD4\(^+\) T-cells of nondeleting 4.1-NOD.I-E\(\alpha^k\) mice cannot cause clinically significant \(\beta\)-cell damage in vivo. This is in contrast to the exquisite diabetogenic activity of the islet-associated 4.1-CD4\(^+\) T-cells of 4.1-NOD mice, which are highly cytotoxic to \(\beta\)-cells, even in the absence of B-cells and endogenous T-cells (32).

**Deletion of 4.1-thymocytes by I-\(A^d\) molecules.** We next asked whether the ability of I-\(A^b\) (18) and I-E to induce 4.1-thymocyte tolerance was shared by other antidiotypic class II molecules (i.e., I-\(A^d\)). We crossed 4.1-NOD mice (expressing I-A\(\alpha^d\) and I-\(\beta^b\)) with I-\(A^b\)-transgenic NOD mice (NOD.I-A\(^d\)) to produce 4.1-NOD.I-A\(^d\) mice (expressing I-A\(\alpha^d\), I-\(\beta^b\), and I-\(A^b\)) molecules. As shown in Table 1 and Fig. 4, these mice displayed cytofluorometric profiles compatible with negative selection of 4.1-thymocytes. When compared with 4.1-NOD mice, 4.1-NOD.I-A\(^d\) mice had significant reductions in the percentages of CD4\(^+\)CD8\(^-\) and V\(\beta\)11\(^+\)CD4\(^+\)CD8\(^-\) thymocytes and a significant increase in the percentage of CD4\(^+\)CD8\(^-\) thymocytes (Table 1 and Fig. 4A, left panels). Deletion was incomplete but was seen in all of the mice that were studied. As was the case for 4.1-NOD.I-E\(\alpha^b\) mice, deletion of 4.1-thymocytes in 4.1-NOD.I-A\(^d\) mice was not triggered by endogenous superantigens because non–TCR-transgenic NOD.I-A\(^d\) and NOD mice had comparable numbers of V\(\beta\)11\(^+\)CD4\(^+\)CD8\(^-\) thymocytes and splenocytes (not shown). Studies of bone marrow chimeras indicated that I-\(A^d\)–induced deletion of 4.1-thymocytes was also triggered by bone marrow–derived APCs (eight of eight 4.1-NOD.I-A\(^d\) mice had a deleting phenotype versus none of seven chimeras generated with marrow from 4.1-NOD mice, \(P < 0.001\)).

**Proliferative unresponsiveness of nondeleted 4.1-CD4\(^+\) T-cells from 4.1-NOD.I-A\(^d\) mice in vitro but not in vivo.** The splenic CD4\(^+\) T-cells of 4.1-NOD.I-A\(^d\) mice did not proliferate in response to islet antigen, even in the presence of rIL-2 (Fig. 4B). This suggested that the peripheral frequency of mature 4.1-CD4\(^+\) T-cells in these mice was too low to be detected in short-term proliferation assays. To determine whether 4.1-thymocyte deletion in 4.1-NOD.I-A\(^d\) mice affected the natural history of diabetes, we followed 19 female 4.1-NOD.I-A\(^d\) mice for the development of diabetes. As shown in Table 2, very few mice developed diabetes, and those that did, did so significantly later than 4.1-NOD mice (Table 2). Surprisingly, the mice that did not become diabetic developed insulitis (Table 2). The insulitic T-cells of these mice were actively proliferating in situ because they efficiently incorporated BrdU (data not shown). However, they were not immune-deviated because their cytokine profiles were similar to those displayed by the insulitic T-cells of 4.1-NOD mice (described above and data not shown). Therefore, 4.1-NOD.I-A\(^d\) mice export a very small number of 4.1-CD4\(^+\) T-cells to the periphery, and although these cells can trigger insulitis, they cannot efficiently cause diabetes.

**I-A\(^8\) molecules carrying proline and aspartic acid at I-\(\beta\) positions 56 and 57 (I-A\(^8\)) are antidiotypic in 4.1-NOD mice.** Comparison of the amino acid sequences of the class II molecules that can tolerate 4.1-thymocytes suggested an association between tolerogenic activity and the presence of certain residues between positions 56 and 67 of the different \(\beta\)-chains. No other regions in I-\(\alpha\) or I-\(\beta\) chains had sequence motifs that were either shared by most deleting class II molecules or absent from nondeleting class II molecules. This association was intriguing because this motif contains proline and aspartic acid at positions 56 and 57, which are epidemiologically associated with the ability of protective MHC molecules to afford diabetes resistance in both humans and mice (3,4).

To investigate the independent contribution of these two residues to the 4.1-tolerogenic activity of antidiotypic MHC class II molecules, we followed the fate of the 4.1-TCR in 4.1-NOD.I-A\(^8\)-transgenic 4.1-NOD mice (4.1-NOD.I-A\(^8\)). Unlike I-A\(^8\) molecules, I-A\(^8\) molecules carry proline and aspartic acid at I-\(\beta\) chain positions 56 and 57 and have a strong antidiotypic activity in NOD mice (3,4). Flow cytometry studies of 4.1-NOD.I-A\(^8\)-transgenic mice revealed that, unlike I-A\(^b\), I-E, and I-A\(^d\), I-A\(^8\) did not cause massive deletion of 4.1-thymocytes (Table 1 and Fig. 4A, right panels). However, these mice had reduced CD4\(^+\)-to-CD4\(^-\)CD8\(^-\) thymocyte ratios when compared with 4.1-NOD mice (Table 1 and Figs. 1 and 4), and their peripheral CD4\(^+\) T-cells were less responsive to antigenic stimulation than the peripheral CD4\(^+\) T-cells of 4.1-NOD mice (in the absence but not the presence of rIL-2) (Fig. 4B). Interestingly, 4.1-NOD.I-A\(^8\) mice developed a significantly reduced incidence and a significantly delayed onset of diabetes when compared with 4.1-NOD mice (Table 2). The mice that did not develop diabetes developed insulitis. As was the case for 4.1-NOD.I-A\(^b\) and nondeleting 4.1-NOD.I-E\(\alpha^b\) mice, the insulitic T-cells of these mice were not immune-deviated (not shown). Therefore, the presence of proline and aspartic acid at I-\(\beta\) positions 56 and 57 has a protective effect against 4.1-CD4\(^+\) T-cell–induced diabetes through a mechanism that does not involve massive deletion of 4.1-thymocytes or their immune deviation.

I-A\(^8\), but not I-A\(^d\) or I-E\(^d\), can mediate the positive selection of 4.1-thymocytes on thymic epithelial cells. The above data show that different MHC class II molecules can tolerate a single, highly diabetogenic I-A\(^8\)–restricted TCR. We have previously shown that one of these class II molecules, I-A\(^b\), can trigger the deletion of 4.1-thymocytes in the thymic medulla but cannot restrict their positive selection in the thymic cortex (19). To determine whether the same was true for I-A\(^d\), I-E\(^d\), and I-A\(^8\), we investigated whether these molecules could mediate the positive selection of 4.1-thymocytes on thymic epithelial cells. We followed the fate of the 4.1-TCR in lethally irradiated NOD (I-A\(^e\)), BALB/c (I-A\(^d\) and I-E\(^d\)), or BALB/c.I-A\(^8\)-transgenic mice (I-A\(^8\)) that had been reconstituted with 4.1-NOD marrow. These chimeras expressed I-A\(^e\), I-A\(^8\), and/or I-A\(^d\) and I-E\(^d\) molecules on radioreistant thymic epithelial cells but only expressed I-A\(^8\) on the radiosensitive, bone marrow–derived APCs of the thymic medulla.

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FIG. 4. Development of 4.1-CD4\(^+\) T-cells in 4.1-NOD.I-A\(^d\) and 4.1-NOD.I-Ag\(^7PD\) mice. A: Representative CD4, CD8, and V\(\beta\)11 profiles of thymocytes and splenic cells. See legend to Fig. 1 for details. B: In vitro proliferation of splenic CD4\(^+\) T-cells against NOD islet cells. Bars show the SE of the mean.
As shown in Fig. 5A (upper right panel), thyrm of 4.1-NOD → BALB/c chimeras had profiles that were similar to those seen in MHC class II–deficient 4.1-NOD mice (4.1–I-A<sup>b</sup> in Fig. 5A, lower right panel), where 4.1-thymocytes undergo massive developmental arrest. In contrast, thyrm from 4.1-NOD → BALB/c.I-A<sup>g7PD</sup> mice, which expressed I-A<sup>g7PD</sup>, I-A<sup>b</sup>, and I-E<sup>α</sup> molecules on thymic epithelial cells and I-A<sup>b</sup> on marrow-derived APCs, had profiles compatible with positive selection of the 4.1-TCR; 4.1-NOD → BALB/c.I-A<sup>g7PD</sup> chimeras had more thyrmocytes (22 ± 4 vs. 5 ± 0.7 × 10<sup>6</sup>, P < 0.003) and greater CD4<sup>+</sup>CD8<sup>+</sup>-to-CD4<sup>+</sup>CD8<sup>+</sup> thyrmocyte ratios than 4.1-NOD → BALB/c chimeras (3.2 ± 0.5 vs. 1 ± 0.3; P < 0.007) (Fig. 5A). As expected, splenic CD4<sup>+</sup> T-cells from 4.1-NOD → BALB/c.I-A<sup>g7PD</sup> chimeras proliferated almost as well as splenic CD4<sup>+</sup> T-cells from 4.1-NOD mice in response to islet antigen, both in the presence and absence of rIL-2 (Fig. 5B). Therefore, 4.1-thymocytes can recognize I-A<sup>g7PD</sup> (but not I-A<sup>d</sup> or I-E<sup>α</sup>) molecules on thymic epithelial cells. This demonstrates that, as was the case for I-A<sup>b</sup> (19), deleting class II molecules can restrict the negative selection of 4.1-thymocytes in the medulla but not their positive selection in the thymic cortex.

**Presentation of tolerogenic peptide/MHC class II complexes to 4.1-thymocytes by dendritic cells and macrophages but not B-cells.** To determine the nature of the tolerogenic APC population, we compared the ability of purified thyrm dendritic cells, splenic dendritic cells, splenic B-cells, and peritoneal exudate macrophages from several mouse strains to downregulate CD4 and CD8 co-receptors from monospecific 4.1-CD4<sup>hi</sup>CD8<sup>hi</sup> thyrmocytes (an ex vivo assay of thyrmocyte tolerance referred to as “dulling” [33–35]). We focused these studies on H-2<sup>b</sup> mouse strains because I-A<sup>b</sup> triggers complete deletion of the 4.1-TCR in 100% of the mice. As shown in Fig. 6A and B, dendritic cells from C57BL/6 (I-A<sup>b</sup>) mice, but not from NOD (I-A<sup>b</sup>), I-A<sup>b</sup>–deficient C57BL/6 (I-A<sup>b<sup>c</sup></sup>) or C57BL/10.H<sup>2g7</sup> (I-A<sup>g7</sup>) mice, triggered dulling of double-positive thyrmocytes from RAG-2–deficient 4.1-NOD mice, which can only express the 4.1-TCR. To ascertain whether the dulling activity of I-A<sup>b</sup> molecules was peptide-specific, we also tested thyrm and splenic dendritic cells from H-2Ma–deficient C57BL/6 mice. Professional APCs from H-2Ma–deficient C57BL/6 mice express normal levels of I-A<sup>b</sup> (Fig. 6D), but these molecules present a very limited repertoire of peptides (mostly invariant chain-derived [CLIP]). As shown in Fig. 6A, H-2Ma–deficient dendritic cells could not downregulate CD4 and CD8 from 4.1-CD4<sup>+</sup>CD8<sup>+</sup> thyrmocytes. Thus, the ability of 4.1-thymocytes to recognize I-A<sup>b</sup> molecules on dendritic cells is peptide-specific.

Subsequent experiments with macrophages and splenic B-cells indicated that the ability of dendritic cells from C57BL/6 mice to downregulate CD4 and CD8 from 4.1-CD4<sup>+</sup>CD8<sup>+</sup> thyrmocytes was not a property of all professional APCs. Dulling was efficiently induced by peritoneal exudate macrophages but not by splenic B-cells (Fig. 6C), despite the fact that the levels of I-A<sup>b</sup> on these two APC types were virtually identical (Fig. 6C). Finally, prolifera-tion and cytokine secretion assays using splenic 4.1-CD4<sup>+</sup> T-cells from 4.1-NOD mice as responders and the different APC types discussed above revealed that the peptide/I-A<sup>b</sup> complex that triggers dulling of immature 4.1-thymocytes cannot trigger functional responses from mature 4.1-CD4<sup>+</sup> T-cells (data not shown). Together, these data suggest that 4.1-thymocytes undergo deletion by recognizing a tolerogenic peptide/I-A<sup>b</sup> complex on dendritic cells and macrophages but not B-cells.

**DISCUSSION**

Bone marrow–derived APCs expressing the antidiabetic complex II molecule I-A<sup>b</sup> trigger the complete deletion of thyrmocytes bearing the highly diabetogenic, I-A<sup>g7</sup>–restricted 4.1-TCR (18,19). Because the diabetes resistance afforded by protective MHC class II molecules (including I-A<sup>d</sup>) in non–TCR-transgenic mice is also triggered by bone marrow–derived cells (5–11,17), the above observation suggested a general mechanism for the MHC class II–linked resistance to autoimmunity. Here, we have tested the ability of three different antidiabetic complex II molecules (I-E<sup>k</sup>/I-EB<sup>β7</sup>, I-A<sup>d</sup>, and I-A<sup>g7PD</sup>) to tolerate 4.1-CD4<sup>+</sup> T-cells and/or to prevent 4.1-CD4<sup>+</sup> T-cell–induced diabetes. We show that 4.1-CD4<sup>+</sup> T-cells undergo different forms of tolerance in mice expressing I-E<sup>k</sup>, I-A<sup>d</sup>, or I-A<sup>g7PD</sup> molecules, owing to the ability of immature 4.1-CD4<sup>+</sup>CD8<sup>+</sup> thyrmocytes to recognize protective peptide/MHC class II complexes on dendritic cells and macrophages but not on B-cells or thymic epithelial cells. These data provide but one explanation as to how structurally diverse MHC class II molecules might provide dominant resistance against type 1 diabetes.

Expression of transgenic (and wild-type) I-E<sup>k</sup> molecules in 4.1-NOD mice restored I-E expression and triggered the deletion of 4.1-CD4<sup>+</sup> thyrmocytes in approximately one-third of the mice. The remaining two-thirds of mice positively selected 4.1-CD4<sup>+</sup> T-cells, but these cells were unresponsive to antigenic stimulation in vitro. Experiments with bone marrow chimeras indicated that the deletion of 4.1-thymocytes in 4.1-NOD.I-E<sup>k</sup> mice was a partially stochastic process that was mediated by bone marrow–derived cells. The mechanisms underlying the incomplete penetrance of this phenotype (deletion) are unclear. However, because the incomplete penetrance of the deleting phenotype seen in 4.1-NOD.I-E<sup>k</sup> mice was also seen in 4.1-(NODxC58/j) F<sub>1</sub> mice, it is unlikely that it can be accounted for by introduction of a genetic contaminant derived from the I-E<sup>k</sup> donating strain. Furthermore, we have indirect evidence suggesting that it may be the result of differences in the number of APCs expressing I-E molecules (data not shown). It is also worth noting that whereas overexpression of an I-E<sup>d</sup> transgene completely prevented diabetes in non–TCR-transgenic NOD mice, expression of this transgene at physiological levels did not completely abrogate diabetes susceptibility in NOD mice (17).

As expected, deletion of 4.1-thymocytes in deleting 4.1-NOD.I-E<sup>k</sup> mice afforded the mice resistance to both insulitis and diabetes. Although nondelleting 4.1-NOD.I-E<sup>d</sup> mice (bearing anergic 4.1-CD4<sup>+</sup> T-cells) were also diabetes resistant, they developed insulitis. Surprisingly, the insulitis lesions of these mice were caused by actively proliferating 4.1-CD4<sup>+</sup> T-cells. This indicated that 4.1-CD<sup>+</sup> T-cells maturing in nondeleting 4.1-NOD.I-E<sup>d</sup> mice were only anergic to in vitro but not in vivo stimuli. The diabetes resistance of nondelleting 4.1-NOD.I-E<sup>d</sup> mice...
FIG. 5. I-A\(^{g7PD}\) molecules can positively select immature 4.1-thymocytes in the thymic cortex. A: CD4, CD8, and V\(\beta\)11 profiles of thymocytes from bone marrow chimeras. Data are average values of six to seven chimeras/group. The thymocyte profiles of the chimeras are compared with those of MHC class II–deficient (I-A\(^{0/0}\)) 4.1-TCR–transgenic mice, where 4.1-thymocytes undergo developmental arrest. 4.1-NOD → BALB/c chimeras had fewer thymocytes (5 ± 0.7 vs. 22 ± 4 × 10\(^6\) cells; \(P < 0.003\)) and lower double-positive cell-to-double-negative cell ratios (1 ± 0.3 vs. 3.2 ± 0.5; \(P < 0.007\)) than 4.1-NOD → BALB/c I-A\(^{g7PD}\) chimeras. B: Proliferation of splenic CD4\(^+\) T-cells from 4.1-NOD → Balb/c I-A\(^{g7PD}\) chimeras and 4.1-NOD mice against islet cells. Bars show the SE of the means. DP, double-positive cells; DN, double-negative cells.
FIG. 6. Dulling activity of different APC types on 4.1-CD4⁺CD8⁺ thymocytes from RAG-2–deficient 4.1-NOD mice. A–C: Dulling assays. These were done by co-culturing 10⁵ APCs with 10⁵ thymocytes from RAG-2–deficient 4.1-NOD mice for 20 h, followed by staining with anti-CD4-PE, anti-CD8-FITC, and anti-CD11c-, anti-CD11b-, or anti-B220-biotin plus streptavidin-PerCP mAbs. Each panel (A–C) corresponds to a different experiment. The figure shows ungated profiles, but similar results were obtained when CD11c⁺, CD11b⁺, and B220⁺ cells were electronically gated out. The values shown correspond to the percent of CD4⁺CD8⁺ thymocytes within the CD4⁺CD8⁺ subset. The figure is representative of one of three independent experiments.

D: Levels of I-Aβ on thymic dendritic cells, peritoneal macrophages, and splenic B-cells from NOD, C57BL/6, and H-2Ma⁻⁻ C57BL/6 mice. Cells were stained with anti-I-Aβ-biotin (AF6–120.1) and streptavidin PerCP.
was not caused by MHC-induced differentiation of 4.1-CD4⁺ T-cells into Th2 cells or by recruitment of regulatory Th2 cells to islets, because their islet-associated T-cells produced IL-2 and IFN-γ but not IL-4. Furthermore, it was not caused by the inability of 4.1-CD4⁺ T-cells to recognize islet autoantigen, because 4.1-CD4⁺ T-cells from 4.1-NOD recognized NOD.I-Eaçõesk (I-Aγ7+/I-Eγ⁺) and NOD (I-Aγ7+/I-Eγ−) islets equally well (S.T., P.Se., A.A., J.V., P.Sa., unpublished observations). These data suggested that in nondeleting 4.1-NOD.I-Eaçõesk mice, the anergyizing stimulus was sufficiently strong to prevent the differentiation of 4.1-CD4⁺ T-cells into β-cell–cytotoxic T-cells but not strong enough to prevent their proliferation in response to strong in vivo stimuli (i.e., autoantigen-loaded dendritic cells). The fact that nondeleting 4.1-NOD.I-Eaçõesk mice develop insulitis (unlike non–TCR-transgenic NOD.I-Eaçõesk mice) does not necessarily argue against a role for thymocyte tolerance as a mechanism for the MHC-linked resistance to diabetes in non–TCR-transgenic mice. The high frequency of 4.1-thymocytes in 4.1-TCR–transgenic mice may, in some mice, overwhelm the tolerogenic machinery that in non–TCR-transgensics would target a significantly smaller population of 4.1-like T-cells.

Although the overall three-dimensional structure of I-Ab and I-Eb molecules is similar, the amino acid sequences of their α- and β-chains are very different (36). This prompted us to investigate whether the ability of these two MHC molecules to tolerate 4.1-CD4⁺ thymocytes might be shared by other antidiabetogenic MHC class II molecules, such as I-Aa and I-Aγ7PD. The 4.1-NOD.I-Aaçõesk mice displayed a phenotype that was similar to that seen in deleting 4.1-NOD.I-Eaçõesk mice, with two major differences—the deleting phenotype of 4.1-NOD.I-Aaçõesk mice was incomplete, but its penetrance through the mouse population was complete. Unlike deleting 4.1-NOD.I-Eaçõesk mice, 4.1-NOD.I-Aaçõesk mice developed insulin, and a small percentage of mice even developed diabetes. The mechanisms underlying these phenotypic differences are unknown but may be related to differences in the levels or timing of expression of I-E and I-A on thymic APCs. Subsequent studies of 4.1-NOD.I-Aγ7PD mice suggested that this ability of protective class II molecules to prevent 4.1-CD4⁺ T-cell–induced diabetes was not unique to I-Aγ7 and I-Aγ7PD, but not I-Aγ7. The 4.1-NOD.I-Aγ7PD mice displayed reduced CD4⁺–to-CD4⁺ CD8⁻ thymocyte ratios when compared with 4.1-NOD mice, and 4.1-CD4⁺ T-cells maturing in 4.1-NOD.I-Aγ7PD mice consistently showed a slight reduction in proliferative responsiveness to antigen, which could be corrected by the addition of IL-2. The 4.1-NOD.I-Aγ7PD mice developed moderate insulitis but a significantly reduced incidence (and delayed onset) of diabetes. It could be argued that the antidiabetogenic effect of the transgenic MHC class II molecules tested here was an artifact of transgenesis (i.e., by causing a reduction in the percentage of B-cells) (37). However, this is highly unlikely because 4.1-thymocyte tolerance is caused by both transgenic and wild-type MHC class II molecules. We cannot exclude the possibility that the different degrees of tolerance observed in this study reflect the effect of different levels of expression of the diverse transgenes in the relevant cell types. However, the fact that two different wild-type MHC class II haplotypes (H-2b and H-2k) have different degrees of tolerogenic activity on 4.1-thymocytes argues, in part, against this possibility.

The extensive MHC promiscuity of the 4.1-TCR during thymocyte development appears to result from its ability to recognize tolerogenic peptide/MHC class II complexes on dendritic cells and macrophages. The diabetogenic 4.1-TCR is not a classic alloreactive TCR, however, because it is only promiscuous for MHC molecules expressed on dendritic cells and macrophages, not on B-cells or thymic epithelial cells. It should also be pointed out that 4.1-thymocytes undergo neither positive nor negative selection in mice expressing deleting class II molecules (I-Ab [19], I-Ak, and I-E) exclusively on thymic epithelial cells. The only exception to this is the ability of the 4.1-TCR to undergo weak positive selection in chimeras whose thymic epithelial cells express I-Aγ7PD (but not I-Aγ7), as has been reported for another β-cell–reactive TCR (BDC-2.5) (20). It is also clear that the MHC promiscuity of 4.1-thymocytes is peptide-specific, because H-2Ma–deficient thymic and splenic dendritic cells, which can almost exclusively express I-Aγ molecules bound to CLIP, cannot trigger dulling of 4.1-CD4⁺CD8⁺ thymocytes. When taken together, these data suggest that the MHC-induced deletion of 4.1-thymocytes is mediated by one or more peptides that are selectively expressed in thymic bone marrow–derived APCs, as we had previously suggested for I-Ab (19).

Comparison of the amino acid sequences of the α- and β-chains of the nondeleting and deleting MHC class II molecules that we have tested so far did not reveal any obvious sequence homologies with the exception of I-Ab or I-Eb chain positions 55–67. Deleting MHC class II molecules (I-Aγ, I-Ak, I-Aγ7PD, and I-E) share a homologous 56–67 motif that is absent in weakly tolerogenic (I-Aγ and I-Aγ7PD) or nontolerogenic (I-Aγ7 and I-Aγ7PD) class II molecules. This sequence contains proline and aspartic acid at positions 56 and 57 (as opposed to histidine and serine in I-Aγ7) and two insertions at positions 65 and 67. Because I-Aγ, I-Ak, and I-Aγ7 have an identical 56–67 sequence but variable tolerogenic activity, the tolerogenicity of this motif may be modulated by other factors, such as other β-chain residues, the nature of the α-chains, or the timing and levels of expression of the different MHC molecules. This hypothetical structure/function association is tantalizing, as the MHC-linked resistance to type 1 diabetes in both humans and mice is associated with polymorphisms at and around position 57 (3,4). The hypothetical contribution of residues at β-chain positions 56–67 to 4.1-thymocyte deletion is compatible with the recently described influence of amino acid substitutions at positions 56, 57, and 61 (different in I-Aγ7 vs. I-Aγ, I-Ak, and I-Eγ) on structure (38,39). Our data are therefore compatible with a model in which structural differences conferred by polymorphisms between positions 56 and 67 would allow antidiabetogenic class II molecules to present tolerogenic peptides that cannot be presented at all by I-Aγ7 and only inefficiently by I-Ak and I-Aγ7PD (to certain highly diabetogenic T-cells).

In sum, our results lend support to the hypothesis that the MHC-associated resistance of non–TCR-transgenic mice (and perhaps humans, via DQB1*0602) to autoimmune diabetes might be mediated by thymocyte tolerance.
Because it is highly unlikely that all I-A\(^{\gamma}\)-restricted diabetogenic effector T-cells in NOD mice will also recognize the \(^4\,A\)L1-thymocyte–deleting peptide(s) in the context of antidiabetic MHC class II molecules, we propose that 4.1-CD4\(^+\) T-cells are representative of a group of highly diabetogenic and MHC-promiseus T-cells that, for as yet unknown reasons, would play a critical role in diabetogenesis. This would explain the presence of mildly insulinotic, but not diabetogenic, T-cells in congenic NOD.H-2\(^{g7}\), NOD.H-2\(^{g7q}\), or NOD.H-2\(^{g7mb1}\) mice (14,40,41); I-A\(^{\alpha}\)-transgenic NOD mice (16); NOD mice reconstituted with bone marrow from I-E\(^{\alpha}\)-transgenic NOD mice (11); and I-A\(^{\beta}\)-transgenic NOD mice (13,42). This hypothesis does not rule out the involvement of other mechanisms of MHC-associated protection from diabetes (i.e., immunoregulation), especially because the phenomena described here was observed with a single TCR. However, it is compelling because the geography and timing of negative selection of diabetogenic thymocytes in 4.1-NOD mice are in agreement with the widely accepted notion that the factors underlying the MHC-linked resistance to diabetes reside in the bone marrow and are linked to polymorphisms at around I-A1/E6 position 57 (3,4). Furthermore, it is attractive because it invokes a single mechanism by which multiple, structurally diverse MHC molecules can afford dominant resistance to a single autoimmune disease.

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