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Dendritic Cell–Dependent In Vivo Generation of Autoregulatory T Cells by Antidiabetogenic MHC Class II

Sue Tsai,* Pau Serra, † Xavier Clemente-Casares,* Robyn M. Slattery,‡ and Pere Santamaria*†‡

Several mechanisms have been proposed to explain how certain MHC class II molecules afford dominant resistance to autoimmune diseases like type 1 diabetes (T1D). However, it remains unclear how protective MHC types can blunt autoreactive T cell responses directed against a diverse repertoire of autoantigenic epitopes presented by disease-promoting MHCs. In this study, we show that expression of I-E on dendritic cells (DCs) of NOD mice promotes the differentiation of MHC promiscuous autoreactive CD4+ clonotypes into antidiabetogenic autoregulatory T cells. We expressed an I-Ea \textsuperscript{loxP} transgene in NOD mice and used cell type–specific I-E ablation to show that I-E–expressing DCs, but not B cells, promote the generation of autoreactive CD4+Foxp3\textsuperscript{+} regulatory T cells (Tregs) and their accumulation in the pancreas-draining lymph nodes. There, these Tregs suppress the presentation of β cell Ags to naive autoreactive CD4+ and CD8+ T cells restricted by diabetogenic MHC molecules in an I-E–independent manner. Whereas selective removal of I-E on DCs abrogated autoregulatory Treg formation and TID protection, selective removal of I-E on B cells was inconsequential. These results explain how certain MHC class II molecules can completely suppress antigenically complex autoimmune responses in an Ag-nonspecific manner. The Journal of Immunology, 2013, 191: 70–82.

The HLA gene region on human chromosome 6 accounts for >50% of the genetic risk for autoimmune diseases, including type 1 diabetes (T1D) (1–6). Genes encoding HLA-DQ and -DR or their murine counterparts (I-A and I-E, respectively) (7), as well as MHC class I genes (5, 8), play key roles, but the underlying mechanisms remain poorly understood. Human T1D is primarily associated with HLA-DQB1. Alleles encoding Ser, Ala, or Val at position 57 provide risk, whereas those encoding Asp at this position afford protection (reviewed in Refs. 9 and 10)). The NOD mouse is homozygous for a unique H-2 haplotype (H-2\textsuperscript{k}) that carries a nonproductive I-E\textsuperscript{o} gene and encodes an I-A\textsuperscript{b}/I-Ab\textsuperscript{a} heterodimer in which the Pro and Asp found at positions 56 and 57 in most I-A \textsuperscript{b} chains are replaced by His and Ser, respectively (11). Early studies of NOD mice expressing non-NOD MHC haplotypes or I-E\textsuperscript{o}, I-E\textsuperscript{k}, modified I-A\textsuperscript{b}\textsuperscript{a}, I-A\textsuperscript{b}\textsuperscript{b/I-Ab\textsuperscript{b}}, or I-A\textsuperscript{b}\textsuperscript{c} transgenes have proven that class II molecules play a direct role. However, whereas some authors suggested that protective alleles might afford resistance to TID by tolerizing autoreactive T cells (12–15), others argued against this (16–21).

Our studies of NOD mice expressing a diabetogenic, I-A\textsuperscript{87}–restricted TCR of unknown specificity (4.1-NOD mice (22)) documented an unexpected relationship between thymic tolerance of certain highly pathogenic I-A\textsuperscript{87}–restricted CD4+ T cells and the MHC-linked resistance to TID (23–25). We found that 4.1-TCR–transgenic thymocytes undergo deletion in H-2\textsuperscript{b}, H-2\textsuperscript{b/k}, H-2\textsuperscript{b/q}, and H-2\textsuperscript{b/b} NOD mice (TID-resistant strains) and that they do so by engaging protective MHC class II molecules on an hematopoietic cell type, upon undergoing thymic positive selection on peptide–I-A\textsuperscript{87} complexes expressed on thymic cortical epithelial cells (23, 24). Likewise, expression of protective class II transgenes, such as I-E\textsuperscript{o} (which restores expression of endogenous I-E\textsuperscript{87}), I-A\textsuperscript{b}\textsuperscript{a}, and I-A\textsuperscript{87}PD (I-A\textsuperscript{87} carrying Pro-56 and Asp-57) in 4.1-NOD mice, led to various degrees of 4.1-thymocyte tolerance and anergy and TID resistance (25). On the basis of these and other observations (26, 27), we proposed that protective MHC class II molecules afford TID resistance by tolerizing “4.1-like” (autoreactive, MHC-promiscuous, and diabetogenic) CD4+ T cells. This interpretation was consistent with the observation that the anti-diabetogenic activity of protective MHC class II molecules in both 4.1-NOD and NOD mice maps to residues around I-A\textsuperscript{b} chain position 57 (25), which are also implicated in the DQB1-linked resistance to human TID (9, 10). However, this interpretation could not explain how deletion of 4.1-like CD4+ T cells is able to prevent the development of an autoimmune disease that, in non–TCR-transgenic NOD mice, is driven by a diverse repertoire of autoreactive CD4+ and CD8+ T cell specificities.

Because a significant fraction of 4.1-NOD.I-E\textsuperscript{k} mice are resistant to insulins and diabetes despite exporting functional autoreactive 4.1-CD4+ T cells to the periphery (25), we reasoned that another mechanism by which protective MHC class II molecules might afford TID resistance would be to promote the differen-
tiation of 4.1-like MHC class II-promiscuous autoreactive CD4+ thymocytes into autoregulatory T cell types via one or more hematopoietic APC types. To investigate this, we expressed an I-E<sup>k</sup>loxP transgene in NOD mice, alone or in combination with highly diabeticogenic I-A<sup>β7</sup>– or K<sup>d</sup>-restricted TCRs and/or ubiquitous or cell type–specific Cre recombinase transgenes, and explored the effects of transgene expression on effector versus regulatory T cell (Treg) development, activation, and recruitment.

We report that I-E expression on CD11c+ dendritic cells (DCs), but not B cells or other I-E–expressing APCs, promotes the generation of autoregulatory CD4<sup>+</sup>Foxp3<sup>+</sup> Tregs from endogenous precursors and mobilizes their accumulation in the pancreatic draining lymph nodes (PLNs), where they suppress the presentation of cognate and noncognate β cell Ags to diabeticogenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells by local APCs. These results provide an explanation as to how certain MHC class II molecules can suppress antigenically complex autoimmune responses.

Materials and Methods

**Mice**

NOD/ShiLt, NOD.scid, NOD.mβ2m<sup>-/-</sup>, and BDC2.5-NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME), 4.1-NOD, 8.3-NOD, 4.1-NOD.Rag2<sup>-/-</sup>, and 8.3-NOD.Rag2<sup>-/-</sup> mice have been described previously (22, 23). 8.3-NOD.I-E<sup>a</sup>mβ2m<sup>-/-</sup>Rag2<sup>-/-</sup> mice were generated by intercrossing the offspring of 8.3-NOD.mβ2m<sup>-/-</sup>XI-E<sup>a</sup>Rag2<sup>-/-</sup>. All mice were followed for development of spontaneous diabetes by measuring urine glucose levels with Diastix strips (Miles, ON, Canada). Mice were considered overtly diabetic when their urine glucose levels were >16 mM blood glucose. NOD.I-E<sup>a</sup> mice were produced by backcrossing the I-E<sup>a</sup> transgene of C57BL/6.J-Eα16 mice (The Jackson Laboratory) (17) onto the NOD.Shij background for at least 10 generations. NOD.CD19P-Cre mice were produced by backcrossing a targeted CD19-Cre transgene (28) onto the NOD.Shij background for >10 generations. Briefly, a cassette encoding Cre recombinase was placed under the control of the CD19 promoter such that it replaced the second exon of the endogenous CD19 coding sequence. Mice that are hemizygous for the Cre recombinase insertion and thus carry only one copy of the CD19 allele are phenotypically normal. All NOD.CD11P-Cre–transgenic mice studied in this paper were hemizygous for the transgene. CD11c<sup>-</sup>DCs–transgenic C57BL/6 mice were from B. Reizis (Columbia University, New York, NY) (29). NOD.CD11P-Cre mice were produced by backcrossing this transgene onto the NOD.Shij background for at least 10 generations. NOD.ACTBP-Cre mice were produced by backcrossing the ACTBP-Cre transgene of C57BL/6.ACTBP-Cre mice (The Jackson Laboratory) onto the NOD.Shij background for >10 generations. NOD.I-E<sup>a</sup> mice were produced by microinjection of an 8.5-kb floxed I-E<sup>a</sup> (IE<sup>a</sup>loxP) transgene into NOD zygotes. The transgene was generated using the 8.2-kb HindIII fragment of the IE<sup>a</sup> gene as template. Dormant 120-bp loxP sites were inserted at the 854-bp XbaI site and 3573-bp Eco47III site. The animal studies were from eBioscience (San Diego, CA). CFSE was from Molecular Probes (Eugene, OR).

**Peptides**

The peptides NRP-A7 (KYNKANAFL) (30) and 2.5 mimotope (2.5mi) (31) were purchased from Mimotopes (Clayton, VIC, Australia).

**Flow cytometry**

Single-cell suspensions were stained with Abs diluted 1:100 in FACS buffer (PBS containing 1% FBS and 0.05% NaN<sub>3</sub>), washed, fixed in 1% paraformaldehyde in PBS, and analyzed with FACScan, FACSort, or BD LSRII flow cytometers. All Ab stainings of DCs were done in the presence of an anti-CD16/CD32 mAb (2.4G2; BD Pharmingen) to block FcRs. For intranuclear staining, cells were first stained for surface markers, followed by permeabilization using the Foxp3 staining buffer set (eBioscience), according to the manufacturer’s instructions. To enumerate Helios‘ Foxp3<sup>+</sup> cells in the CD4<sup>+</sup> subset, cells from the CD4<sup>+</sup>CD8<sup>+</sup> gate were subjected to gating on the Helios’Foxp3<sup>+</sup> subsets using cutoffs set for thymic Foxp3<sup>+</sup> (Helios<sup>+</sup>) and Foxp3<sup>+</sup> (Helios<sup>+</sup>) populations.

**Analysis of Treg TCRα and TCRβ repertoire by PCR**

CD4<sup>+</sup>CD25<sup>+</sup> Tregs and CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated from spleen and PLN cells by FACS and used as a source of mRNA using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD). cDNA was synthesized by oligo(dT)-primed reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen), followed by two rounds of PCR amplification using TCR-Cα– or Cβ- and Vα- and Vβ-chain–specific primers, as described previously (32).

**Histochemistry**

Scoring of insulitic lesions was performed on H&E-stained pancreas sections as described previously (33). Insulitis scores were determined using the following criteria: 0, no insulitis; 1, peri-insulitis; 2, insulitic lesion covering <25% of the islet surface area; and 3 and 4, insulitic lesion covering 25–50% and >50% of the total islet surface area, respectively.

**T cell isolation**

CD4<sup>+</sup> T cells were purified from the spleens of 8- to 12 wk-old NOD or 4.1-NOD mice using purification systems from Miltenyi Biotec (Auburn, CA) or BD IMAG following the manufacturer’s instructions. CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells were isolated using the EasySep Mouse Treg isolation kit from StemCell Technologies as per the manufacturer’s instructions. The purities of each population were confirmed to be >90% by flow cytometry. CD4<sup>+</sup> and CD8<sup>+</sup> T cells used in adoptive transfer experiments were isolated from the spleens of 6- to 8-wk-old NOD females using mAb-coated BD IMAG magnetic beads (BD Biosciences). Islet-associated CD4<sup>+</sup> T cells were drawn out from the islets by culturing islets in 0.5 U/ml Takeda rIL-2 (Genzyme, Cambridge, MA) and cultured (10<sup>5</sup> islets/mL) for 7–10 days in RPMI 1640 (Life Technologies) overnight. Total cells were collected the next day for flow cytometric analysis.

**DC isolation**

Spleens or thymi were immersed in HBSS (Life Technologies) containing collagenase D (0.25% w/v; Roche, Mannheim, Germany) and DNaseI (0.01% w/v; Roche) and shredded into small pieces with two syringe needles to facilitate digestion (in a 37°C water bath for 15 min, with shaking). Digested spleens were passed through a 40-μm cell strainer and subjected to DC isolation using anti-mouse CD11c-Ab–coated magnetic beads (Miltenyi Biotec). Purified splenic DCs were used immediately in flow cytometry analysis, or proliferation or coculture assays without further culturing. Bone marrow–derived DCs (BMDCs) were generated by culturing bone marrow cells in the presence of recombinant mouse IL-4 (5 ng/ml; R&amp;D Systems, Minneapolis, MN) and GM-CSF (5 ng/ml; R&amp;D Systems) for 7 d, followed by magnetic isolation using CD11c–Ab–coated magnetic beads (Miltenyi Biotec). LPS (1 μg/ml) were added to the culture for the last 24 h to generate mature BMDCs.

**In vivo CFSE dilution**

Purified CD8<sup>+</sup> T cells were stained with CFSE (0.5 μM) for 4 min at 37°C, washed twice with 15 ml RPMI 1640 medium (Life Technologies) containing 10% FBS (Sigma-Aldrich), and enumerated. Cells (5 × 10<sup>6</sup>) were injected i.v. into NOD or transgenic hosts, and spleens plus mesenteric lymph nodes (MLNs) and PLNs were collected for flow cytometric analysis 7 d after injection.

**Functional in vitro T cell assays/β<sup>k</sup> allorecognition assay**

For Ag-stimulated T cell proliferation, 8.3-CD8<sup>+</sup> T cells (2 × 10<sup>5</sup>) or BDC2.5-CD4<sup>+</sup> T cells (4 × 10<sup>5</sup>) were cocultured with DCs (10<sup>5</sup>) and various concentrations of NRP-A7 or 2.5 mimotope, respectively. To test I-E<sup>k</sup> allorecognition, 8.3-CD8<sup>+</sup> T cells (2 × 10<sup>5</sup>) were cocultured with NOD versus NOD.I-E<sup>a</sup> DCs (10<sup>5</sup>) and in the absence of NRP-A7 peptide. 10<sup>4</sup> DCs (4 × 10<sup>5</sup>) were cocultured with dissociated islet cells (10<sup>3</sup>) which contained autoregion-loaded APCs. Culture supernatants were collected at 48 h for cytokine determination by ELISA (R&amp;D Systems). Culture wells were pulsed with [3H]thymidine at this point (1 μCi/well; Molecular Probes) and harvested for measurement of [3H] incorpo-
ration at 72 h. For polyclonal T cell stimulation, Dynabeads Mouse T activator anti-CD3/CD28 beads (Invitrogen, Dynal, Oslo, Norway) were used accordingly with the manufacturer’s recommendations.

**In vivo Treg suppression assay**
CD4+CD25+ or CD4+CD25- T cells were activated with plate-bound anti-CD3 mAb (3 μg/ml) and Takeda rIL-2 (0.5 U/ml) and cocultured at different ratios with responder 8.3-CD8+ T cells (2 × 10^5) and gamma-irradiated (1200 rad) NOD splenocytes (10^5) in the presence of 1 μM IL-2 and Pantoquinone (10^-5) in the presence of 1 μg/ml NRP-A7 for 48 h. The supernatant was collected for IFN-γ determination by ELISA.

**In vitro thymocyte/DC coculture**
FACS-sorted CD4+CD8- single-positive (SP) thymocytes (4 × 10^5) were cultured with thymic DCs (10^5) in the presence or absence of murine thymic stromal lymphopoietin (TSLP) (20 ng/ml; R&D Systems) for 4 or 7 d, at which point the cultured cells were harvested and analyzed by flow cytometry for CD4 and Foxp3 expression.

**In vivo Treg suppression of DCs**
TCR-transgenic CD4+CD25+ Tregs (5 × 10^5) were injected i.v. into 15-wk-old NOD females, and the spleens and PLNs were collected 4 d later for analysis by flow cytometry. CD11c+ splenic DCs were isolated as described above; PLNs were subjected to collagenase-digestion and analyzed using the log-rank test, and comparison of final diabetes incidence was done using the χ^2 test.

**In vivo Treg recruitment**
To assess relative recruitment of NOD versus NOD.I-E^k Tregs, FACS-sorted CD4+CD25+ Tregs (10^5) from NOD and NOD.I-E^k mice were stained with 10 μM and 1 mM CFSE and injected 1:1 into NOD hosts. MLNs and PLNs were analyzed for presence of CFSE+ cells 4 d later. To assess relative recruitment by NOD versus NOD.I-E^k hosts, FACS-sorted CD4+CD25+ Tregs (10^5) from NOD.I-E^k mice were stained with 10 μM CFSE and injected into NOD and NOD.I-E^k hosts, and the frequency of CFSE+ cells in the MLNs and PLNs was quantified by flow cytometry 4 d later.

**In vivo Treg suppression of diabetes**
TCR-transgenic CD4+CD25+ Tregs (5 × 10^5) were injected i.v. into 5- to 6-wk-old 8.3-NOD.Rag2-/- or 8- to 12-wk-old NOD.scid females. Injected 8.3-NOD.Rag2-/- mice were monitored weekly for development of glycosuria using Diastix strips for at least 100 d. Treg-injected NOD.scid mice were infused with NOD splenic CD4+ and CD8+ T cells (2 × 10^5) 24 h later and followed for glycosuria for 100 d.

**Statistical analyses**
Data were compared by Student t, Mann–Whitney U, or two-way ANOVA tests using GraphPad software. Survival curve comparisons were performed using the log-rank test, and comparison of final diabetes incidence was done using χ^2 tests.

**Results**

**Diabetes resistance and enhanced development and recruitment of Tregs in oligoclonal but not monoclonal 4.1-NOD.I-E^k and 8.3-NOD.I-E^k mice**

To investigate whether protective MHC class II molecules might afford T1D resistance by promoting the differentiation of 4.1-like MHC class II promiscuous autoreactive CD4+ thymocytes into autoregulatory T cell types, we followed the fate of 4.1-CD4+ T cells in 4.1-NOD.I-E^k mice. As expected, 4.1-NOD.I-E^k mice were almost completely protected from T1D as compared with 4.1-NOD mice (Fig. 1A). The thymocyte profiles of both strains, including the absolute and relative numbers of the different thymocyte subsets and the levels of the transgenic TCR on CD4+CD8- (double-positive [DP]) and CD4+CD8+ (SP) thymocytes, were similar (Fig. 1B, 1C; data not shown). Likewise, the peripheral CD4+CD25+ T cells of 4.1-NOD.I-E^k and 4.1-NOD mice proliferated to a similar extent and secreted similar levels of IFN-γ in response to plate-bound anti-CD3 mAb or islet cell suspensions (Fig. 1D), indicating that the resistance of 4.1-NOD.I-E^k mice to T1D was not caused by deletional tolerance or functional inactivation. 4.1-NOD.I-E^k mice, however, harbored increased percentages of Foxp3+CD4+ T cells in the thymus and the pancreatic (but not mesenteric) lymph nodes (Fig. 1E). The absolute numbers of Foxp3+ CD4+ T cells were also significantly higher in the thymic CD4+CD8- subset of 4.1-NOD.I-E^k versus 4.1-NOD mice (Supplemental Fig. 1A), albeit not in the PLNs (owing to lower cellularity and absence of local inflammation in the former; data not shown). Helios staining confirmed that these differences in Treg frequencies originated in the thymus (Fig. 1F, Supplemental Fig. 1B, 1C).

To ascertain whether the increased peripheral frequency of Tregs in 4.1-NOD.I-E^k mice was associated with increased regulatory activity, we compared the ability of splenic Tregs purified from 4.1-NOD.I-E^k and 4.1-NOD donor mice to suppress the development of diabetes in two different adoptive transfer models. As shown in Fig. 1G and Supplemental Fig. 1D, the former had superior antidiabetogenic properties than the latter. Clearly, both phenotypes (diabetes resistance and increased Treg development and function) were 4.1-TCR transgene independent, because 4.1-NOD.I-E^k mice did not export Foxp3+4.1-CD4+ T cells (data not shown) and developed diabetes essentially like their RAG2-deficient counterparts (Fig. 1H). These observations indicated that the antidiabetogenic and pro-Treg developmental effects of 4-E expression in 4.1-NOD.I-E^k mice require an interaction between endogenous (nontransgenic) TCRs and I-E^k and are dissociated from epitope stealing (i.e., capture of the cognate I-A^k-binding epitope by I-E (34, 35) or blockade of the I-A^k peptide binding site by a peptide derived from I-E (36)).

To steer clear of potential effects of I-E^k on CD4+ effector T cell development and function altogether and to rule out the possibility that the observations described above were a peculiarity of the 4.1-TCR, we introduced the I-E^k transgene into 8.3-TCR–transgenic NOD (8.3-NOD) mice (22), expressing a diabetogenic H-2Kd–restricted TCR that is specific for the β cell epitope IGRP depleted (30, 37, 38). Similar to 4.1-NOD.I-E^k mice, 8.3-NOD.I-E^k mice showed near-complete resistance to T1D (Fig. 2A). Thymocyte flow cytometric profiles also showed no evidence of clonal deletion of 8.3-TCR–transgenic thymocytes; the absolute and relative numbers of CD4+CD8-, CD4+CD8+, CD4+CD8-, or CD4+CD8+ thymocytes in 8.3-NOD.I-E^k mice were similar to those seen in 8.3-NOD mice (Fig. 2B; data not shown).

Interestingly, however, the CD4+CD8+ and CD4+CD8- thymocyte subsets of 8.3-NOD.I-E^k mice expressed slightly lower levels of the transgenic TCRβ-chain (Fig. 2C), suggesting a potential cognate interaction between the transgenic MHC class I–restricted TCR on CD4+ thymocytes and I-E^kββ leading to 8.3-TCR downregulation. This is an intriguing observation, because one would not normally expect a MHC class I–restricted TCR to cross-react with a MHC class II molecule. However, we and others have previously observed peptide-MHC (pMHC)-binding promiscuity to be a feature of other pathogenic CD4+ and CD8+ T cell clonotypes (23, 24, 39), one that might presumably allow antidiabetogenic MHC class II molecules to effect disease resistance. Alternatively, this downregulation is due to the pairing of the transgenic TCRβ-chain with endogenous α-chains capable of enhancing this TCR’s intrinsic MHC promiscuity. Whatever the explanation, 8.3-TCR downregulation in thymocytes from 8.3-NOD.I-E^k was not inherited by peripheral 8.3-CD8+ T cells (data not shown). In addition, the peripheral CD8+ T cells of 8.3-NOD.I-E^k (and 8.3-NOD mice) did not cross-react with I-E^k expressed on splenic DCs from NOD.I-E^k (Supplemental Fig. 2A, top), and I-E^k expression on the DCs did not affect the in vitro responsiveness of the CD8+ T cells of either mouse strain to the cognate autoantigenic epitope (Supplemental Fig. 2A, bottom). Likewise, the splenic CD8+ T cells of 8.3-
NOD.I-E<sup>k</sup> and 8.3-NOD mice secreted similar levels of IFN-γ and proliferated equally well in response to NRP-V7 peptide-pulsed NOD DCs in vitro (Supplemental Fig. 2B), indicating that they are not hyporesponsive to antigenic stimulation.

Like 4.1-NOD.I-E<sup>a</sup> mice, 8.3-NOD.I-E<sup>a</sup> mice harbored increased percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in both the thymus and PLNs (Fig. 2D). As was the case with 4.1-TCR-transgenic mice, introduction of a RAG-2 deficiency into 8.3-NOD.I-E<sup>a</sup> mice abrogated both Foxp3<sup>+</sup>CD4<sup>+</sup> Treg development (Supplemental Fig. 2C) and their diabetes resistance (Fig. 2E), suggesting that these observations were not unique to the 4.1-TCR-transgenic mouse model.

Taken together, the above data suggested that, in both types of TCR-transgenic mice, expression of I-E<sup>a</sup> promoted Foxp3<sup>+</sup>CD4<sup>+</sup> Treg development in the thymus. To explore this further, we queried the ability of purified thymic DCs from NOD and NOD.I-E<sup>a</sup> mice to promote the development of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells from purified splenic CD4<sup>+</sup>CD25<sup>+</sup> Tregs. Although DCs from NOD.I-E<sup>a</sup> mice induced significantly higher yields of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells in vitro, these differences were not statistically significant (Fig. 2F). However, the ability of purified DCs from NOD and NOD.I-E<sup>a</sup> mice to promote Foxp3<sup>+</sup>CD4<sup>+</sup> Treg development in vivo was examined in a non-obese diabetic (NOD) strain of mouse, which is known to foster Treg development in human thymic cultures (40).

Collectively, these observations suggest that I-E<sup>a</sup>-expressing thymic DCs promote thymic Treg formation, either by promoting the conversion of uncommitted thymocytes into Tregs or by triggering the expansion of preformed Tregs. Because up to 20% of Foxp3<sup>+</sup>CD4<sup>+</sup> thymocytes lack CD25 expression (see representative example in Supplemental Fig. 2E), it is not possible to unambiguously determine which of these two alternative possibilities...
ties is at play by doing these experiments using CD25-depleted thymocytes.

Enhanced development and recruitment of Tregs in non–TCR-transgenic NOD.I-E<sub>αk</sub> mice

The I-E<sub>αk</sub>-associated increase in thymic Treg development seen in both 4.1- and 8.3-TCR–transgenic mice was not seen in thymi from non–TCR-transgenic NOD.I-E<sub>αk</sub> mice (Fig. 3A), suggesting that it is either driven by autoreactive TCRs or is only visible in TCR-transgenic animals owing to the oligoclonal nature of their repertoires. However, like the PLNs of their TCR-transgenic counterparts, the PLNs of non–TCR-transgenic NOD.I-E<sub>αk</sub> mice contained significantly increased percentages of Foxp3<sup>+</sup>CD4<sup>+</sup>T cells as compared with NOD mice (Fig. 3A). Furthermore, Tregs purified from pooled PLNs (but not spleen) of NOD.I-E<sub>αk</sub> mice exhibited different TCR usage than those isolated from the PLNs of NOD mice, as determined using a TCR<sub>α</sub>- or TCR<sub>β</sub>-specific nested PCR approach (Supplemental Fig. 3) (32), followed by sequencing to validate the specificity of the PCR approach (data not shown). These two separate lines of evidence...
suggested that the Foxp3^{+}CD4^{+} Treg subset developing in non-TCR-transgenic NOD.I-E^{a}k mice is also enriched for autoreactive Treg cell specificities, leading to enhanced recruitment and retention in the pancreas-draining LNs. To investigate this further, we compared the ability of equal numbers of purified splenic Tregs from NOD.I-E^{a}k versus NOD mice to protect 8.3.NOD.Rag2^{−/−} hosts from developing diabetes. As shown in Fig. 3B, Tregs from NOD.I-E^{a}k donors suppressed disease more efficiently than those derived from NOD mice. To substantiate this further, we compared the accumulation of splenic CFSE-labeled Tregs from NOD.I-E^{a}k and NOD donors (first labeled with low and high CFSE concentrations, respectively, and then pooled at 1:1 ratio)
into the MLNs and PLNs of wild-type NOD hosts. As shown in Fig. 3C, NOD-I-E\textsuperscript{kloxP}-derived Tregs accumulated in the PLNs more efficiently than NOD-derived Tregs, supporting the idea that I-E expression promotes the development of islet-Ag-autoreactive Tregs but is dispensable for enhanced recruitment of these T cells into the PLNs. That I-E expression on peripheral DCs plays a dispensable role was further supported by the observation that splenic Tregs purified from NOD-I-E\textsuperscript{kloxP} donors accumulate similarly in the MLNs and PLNs of NOD and NOD-I-E\textsuperscript{kloxP} hosts (data not shown).

We and others have previously shown that an important outcome of increased recruitment of autoreactive Tregs to the PLNs is the inhibition of autoantigen presentation by local DCs (41–44). Consistent with this, purified 4.1-NOD Tregs downregulated MHC class II (I-A\textsuperscript{\textgreek{g}}\textsuperscript{7}) and costimulatory molecule (i.e., CD80) expression specifically on PLN (but not MLN or splenic) DCs upon transfer into wild-type NOD hosts (Fig. 3D). Furthermore, the PLN DCs of unmanipulated NOD-I-E\textsuperscript{kloxP} mice expressed significantly lower levels of both I-A\textsuperscript{\textgreek{g}}\textsuperscript{7} and CD80 than the PLN DCs of unmanipulated NOD mice (Fig. 3E). In addition, the PLNs of NOD-I-E\textsuperscript{kloxP} mice exhibited a significantly reduced capacity to cross-present endogenous IGRP\textsubscript{206–214} to CFSE-labeled 8.3-CD8\textsuperscript{+} T cells, as compared with the PLNs of wild-type NOD hosts (Fig. 3D). Downregulation of I-A\textsuperscript{\textgreek{g}}\textsuperscript{7} and CD80 on the PLN DCs of NOD-I-E\textsuperscript{kloxP} versus NOD mice was specific for PLN DCs and not an artifact of I-E\textsuperscript{kloxP} transgenesis because neither splenic nor BMDCs exhibited this phenotype, even after LPS stimulation (data not shown).

Taken together, these data suggest that I-E\textsuperscript{kloxP} expression inhibits TID development by promoting the generation of autoreactive Foxp3\textsuperscript{+}CD4\textsuperscript{+} thymocytes and their recruitment to the PLNs.

**I-E\textsuperscript{kloxP} affords TID resistance exclusively via DCs**

Previous studies in bone marrow chimeras demonstrated that I-E expression affords diabetes resistance through a hematopoietic cell type (21, 45). Additional studies of NOD mice expressing promoter-modified I-E\textsuperscript{\textgreek{g}} transgenes to compartmentalize I-E expression on specific subsets of APCs provided additional supporting evidence in this regard (17, 46).

We have recently shown that the antidiabetogenic effects of another MHC class II molecule, I-A\textsuperscript{\textgreek{h}}, requires that it be expressed on DCs and macrophages but not B cells or thymic epithelial cells (47). This prompted us to ask whether the antidiabetogenic ability of I-E might also be mediated via DCs. To this end, we produced transgenic NOD mice expressing a loxP-flanked I-E\textsuperscript{\textgreek{g}} transgene (Fig. 4A) by direct microinjection of NOD oocytes (NOD-I-E\textsuperscript{kloxP}). This design resulted in lower levels of expression of I-E\textsuperscript{kloxP}β\textsuperscript{\textgreek{g}} as compared with those seen in the NOD-I-E\textsuperscript{kloxP}−transgenic line (Fig. 4B) but nonetheless afforded significant diabetes protection to NOD-I-E\textsuperscript{kloxP} 4.1-NOD-I-E\textsuperscript{kloxP} and 8.3-NOD-I-E\textsuperscript{kloxP} mice (Fig. 5). The diabetes resistance of these mice was mediated by I-E\textsuperscript{kloxP} expression rather than by a site-specific transgene integration effect because NOD-I-E\textsuperscript{kloxP} mice expressing a β-actin promoter-driven Cre recombinase transgene developed diabetes with the same incidence and tempo as wild-type NOD mice (Fig. 5A).

To be able to gauge the contribution of CD11c\textsuperscript{+} or CD19\textsuperscript{+} APCs to I-E\textsuperscript{kloxP}−induced TID resistance, we crossed NOD-I-E\textsuperscript{kloxP} mice with NOD mice expressing the Cre recombinase under the CD19 or CD11c promoters (CD19P-Cre and CD11cP-Cre, respectively) and compared the levels of I-E on the surface of splenic CD11c\textsuperscript{+} and CD19\textsuperscript{+} cells from NOD-I-E\textsuperscript{kloxP}, NOD-I-E\textsuperscript{kloxP}CD11cP-Cre, and NOD-I-E\textsuperscript{kloxP}CD19P-Cre mice (in this paper referred to as NOD-I-E\textsuperscript{kloxP}, NOD-I-E\textsuperscript{kloxP}CD11cP-Cre, and NOD-I-E\textsuperscript{kloxP}CD19P-Cre, respectively). As shown in Fig. 4C, the CD11cP-Cre and CD19P-Cre transgenes specifically reduced I-E expression by 10- to 20-fold on the CD11c\textsuperscript{+} and CD19\textsuperscript{+} cells of NOD-I-E\textsuperscript{kloxP} mice and I-E\textsuperscript{kloxP} mice, respectively, without affecting endogenous I-A\textsuperscript{\textgreek{g}} expression (Fig. 4D) or I-A\textsuperscript{\textgreek{g}}−restricted or K\textsuperscript{\textgreek{d}}−restricted Ag presentation (Fig. 4E, 4F). As expected, the expression of Cre in CD11c\textsuperscript{+} or CD19\textsuperscript{+} cells of non-I-E\textsuperscript{kloxP} transgenic NOD.CD11cP-Cre and NOD.CD19P-Cre mice did not alter local presentation of β cell autoantigens in the PLNs in vivo, as measured by dilution of CFSE in adoptively transferred naive 8.3-CD8\textsuperscript{+} T cells (Fig. 4G). Remarkably, however, selective removal of I-E\textsuperscript{kloxP} in CD11c\textsuperscript{+}, but not CD19\textsuperscript{+}, abrogated diabetes protection in non–TCR-transgenic NOD mice in terms of both disease incidence (Fig. 5B) and islet inflammation (Fig. 5C). Selective removal of I-E\textsuperscript{kloxP} in DCs also abrogated I-E−associated diabetes resistance in 4.1- and 8.3-TCR−transgenic animals (Fig. 5D, 5E). Thus, I-E affords TID resistance specifically via CD11c\textsuperscript{+} cells.

**I-E\textsuperscript{kloxP} expression in DCs does not impair the intrinsic diabetogenic activity of autoreactive T cells or the ability of DCs to prime naive autoreactive T cells**

We next investigated whether Cre and/or I-E\textsuperscript{kloxP} expression suppressed TID by impairing the development or intrinsic diabetogenic properties of autoreactive T cells by profiling thymic and peripheral T cells from 4.1-NOD mice expressing different combinations of Cre and I-E\textsuperscript{kloxP} transgenes. As shown in Supplementary Fig. 4, 4.1-NOD mice expressing I-E\textsuperscript{kloxP} in the presence or absence of CD19P-Cre or CD11cP-Cre transgenes did not exhibit altered T cell development profiles or TCR expression levels in the thymus (Supplementary Fig. 4A–C) or the spleen (Supplementary Fig. 4D, 4E). Furthermore, CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from each of these strains proliferated equally well and secreted similar levels of IFN-γ in response to stimulation with islet cell suspension in vitro (Supplemental Fig. 4F) and transferred diabetes to NOD.scid mice with an efficiency similar to that seen using CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from 4.1-NOD mice (Supplemental Fig. 4G). Taken together, these data indicated that I-E\textsuperscript{kloxP} expression in DCs impairs neither the intrinsic diabetogenic activity of autoreactive T cells nor the ability of DCs to prime naive autoreactive T cells.

**Selective removal of I-E on CD11c\textsuperscript{+} cells abrogated I-E−potentiating Treg development**

We next compared the frequency and function of Foxp3\textsuperscript{+}CD4\textsuperscript{+} Tregs in 4.1-NOD-I-E\textsuperscript{kloxP}, 4.1-NOD-I-E\textsuperscript{DC\textsuperscript{+}} and 4.1-NOD-I-E\textsuperscript{B\textsuperscript{+}} mice. Like 4.1-NOD-I-E\textsuperscript{kloxP} mice, 4.1-NOD-I-E\textsuperscript{kloxP} mice harbored increased percentages of Foxp3\textsuperscript{+}CD4\textsuperscript{+} in the PLNs, albeit not to the same extent and not in the thymus (compare Figs. 6A and 1E). The lower levels of I-E\textsuperscript{kloxP} transgene expression in NOD-I-E\textsuperscript{kloxP} versus NOD-I-E\textsuperscript{kloxP} mice (Fig. 4B) are likely responsible for both this observation as well as the lower diabetes resistance of 4.1-NOD-I-E\textsuperscript{kloxP} versus 8.3-NOD-I-E\textsuperscript{kloxP} versus 4.1-NOD-I-E\textsuperscript{kloxP} and 8.3-NOD-I-E\textsuperscript{kloxP} mice (compare Figs. 5D and 1A and Figs. 5E and 2A, respectively), as it would be expected based on previous observations (45). Importantly, selective abrogation of I-E\textsuperscript{kloxP}−induced TID resistance in 4.1-NOD-I-E\textsuperscript{DC\textsuperscript{+}} mice (Fig. 5D) correlated with normalization of the Foxp3\textsuperscript{+}CD4\textsuperscript{+} Treg content in the PLNs (Fig. 6A). Notably, this did not happen in 4.1-NOD-I-E\textsuperscript{B\textsuperscript{+}} mice (Fig. 6A), which develop TID essentially like 4.1-NOD-I-E\textsuperscript{kloxP} mice (Fig. 5D). Thus, I-E expression in TCR-transgenic NOD mice potentiates Treg development exclusively via CD11c\textsuperscript{+} cells.

**I-E\textsuperscript{kloxP}−induced increase in Treg development/recruitment seen in TCR-transgenic NOD-I-E\textsuperscript{kloxP} mice**

As noted above, the I-E\textsuperscript{kloxP}−induced increase in Treg development/recruitment seen in TCR-transgenic NOD-I-E\textsuperscript{kloxP} mice was less...
FIGURE 4.  Selective removal of an I-E<sub>kloxp</sub> transgene in CD11c<sup>+</sup> or CD19<sup>+</sup> cells of NOD.I-E<sub>kloxp</sub> by coexpression of CD11cP-Cre and CD19P-Cre transgenes. (A) An 8.2-kb HindIII fragment of the IEd<sup>a</sup>-gene was used to clone the final 8.5-kb floxed IEd<sup>k</sup> transgene, which was injected into NOD zygotes to generate I-E<sub>dloxp</sub>-transgenic founder mice. Dormant 120-bp loxP sites (triangles) were inserted at the 854-bp XbaI site and between exons 1 and 2 at the 3573-bp Eco47III site (thin lines: introns; black boxes: exons). (B) I-E expression on purified splenic DCs as determined by flow cytometric analysis of cells stained with an anti–I-E<sub>k</sub> Ab (n = 2 per strain type). Mean fluorescence intensities (MFIs) are normalized to those of unstained cells (n = 2 per strain type). (C) Top panels show representative I-E<sub>k</sub> staining profiles of splenic CD11c<sup>+</sup> DCs or splenic CD19<sup>+</sup> B cells from NOD, NOD.I-E<sub>d</sub>loxp<sup>+</sup>, NOD.I-E<sub>d</sub>loxp/CD11cP-Cre, and NOD.I-E<sub>d</sub>loxp/CD19P-Cre mice (in this paper referred to as NOD.I-E<sub>d</sub>loxp, NOD.I-E<sub>d</sub>floxed, and NOD.I-E<sub>d</sub>DC floxed, respectively) and the corresponding percentages of I-E<sup>k</sup> cells. Bottom panels show the levels of I-E on the DCs or B cells of the different strains, normalized to the corresponding values on cells from the positive control strain NOD.I-E<sub>d</sub>+, which reflect the extent of Cre-mediated removal of I-E<sub>kloxp</sub> in CD19<sup>+</sup> and CD11c<sup>+</sup> cells of NOD.I-E<sub>dDC</sub> floxed and NOD.I-E<sub>d</sub>B floxed mice. Data correspond to four to five mice per strain type. (D) I-A<sup>g7</sup> staining (Figure legend continues)
pronounced than that seen in their TCR-transgenic NOD.I-Env counterparts, suggesting that the magnitude of the I-EkloxP–induced phenotype is too small to be detected by flow cytometry in the polyclonal PLN repertoire of non–TCR-transgenic mice (data not shown). To ascertain whether NOD.I-EkloxP mice harbor increased frequencies of islet Ag-autoactive Tregs, as is the case for NOD.I-Env mice versus NOD Env mice, we compared the ability of purified splenic Tregs from NOD, NOD.I-EkloxP, NOD.I-EfloX, and NOD.I-EDC flox mice to suppress the development of spontaneous autoimmune diabetes in RAG2-deficient (Treg-free) 8.3-NOD mice in an I-E–independent manner. Remarkably, Tregs isolated from strains expressing I-EkloxP on DCs (I-EkloxP and NOD.I-EfloX) were significantly more potent at suppressing 8.3-CD8+ T cell–induced diabetes than those isolated from strains whose DCs do not express I-EkloxP (NOD and NOD.I-EDC flox mice) (Fig. 6B), confirming that I-E induces regulation by promoting, via DCs, the development of endogenous autoregulatory Foxp3+CD4+ Tregs.

Selective removal of I-E on CD11c+ cells of non–TCR-transgenic NOD mice abrogates I-E–potentiated suppression of autoantigen presentation in the PLNs

We have shown that Foxp3+CD4+ Tregs suppress T1D development, at least in part, by inhibiting the maturation of autoantigen-loaded DCs in vivo (41), a concept substantiated by the demonstration that PLN DCs are major cellular targets of autoreactive Tregs in vivo (42). We have also shown in this study that the Foxp3+CD4+ Tregs developing in NOD.I-EkloxP mice downregulate MHC class II and CD80 on PLN DCs (Fig. 3D) and that the PLNs of NOD.I-EkloxP mice have a reduced capacity to support the presentation of spontaneous autoantigen in the PLNs at least in part, by inhibiting the maturation of autoantigen-presenting DCs in vivo (41), a concept substantiated by the demonstration that PLN DCs are major cellular targets of autoreactive Tregs in vivo (42). We have also shown in this study that the Foxp3+CD4+ Tregs developing in transgenic NOD mice abrogated I-E–potentiated suppression of autoantigen presentation in the PLNs (Fig. 3D). To investigate whether the latter phenotype might be a consequence of the expression of I-E on CD11c+ cells versus other cell types, we compared the ability of the PLNs of NOD, NOD.I-EkloxP, NOD.I-EfloX, and NOD.I-EDC flox mice to cross-present endogenous IGRP206–214 to CFSE-labeled 8.3-CD8+ T cells.
FIGURE 6. DC expression of I-E in NOD.I-E<sup>kloxP</sup> mice potentiates Treg-dependent suppression of autoantigen presentation in the PLNs. (A) Percentages of Foxp<sup>3</sup> T cells in the MLNs, PLNs, and thymic (THY) CD4<sup>+</sup>CD8<sup>+</sup> T cells of 4.1-NOD (n = 9), 4.1-NOD.I-E<sup>kloxP</sup> (n = 12), 4.1-NOD.I-E<sup>B</sup> (n = 11), and 4.1-NOD.I-E<sup>DC</sup> (n = 6) mice. Data presented as means ± SEM. (B) Antidiabetogenic activity of splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells from NOD (n = 17), NOD.I-E<sup>kloxP</sup> (n = 12), NOD.I-E<sup>B</sup> (n = 7), and NOD.I-E<sup>DC</sup> (n = 7) mice in NOD.Rag2<sup>−/−</sup> hosts, followed for 100 d posttransfer. (C) In vivo proliferation of naive CFSE-labeled 8.3-CD8<sup>+</sup> T cells in the PLN and MLN of NOD (n = 5) versus NOD.I-E<sup>kloxP</sup> (n = 5), NOD.I-E<sup>B</sup> (n = 7), and NOD.I-E<sup>DC</sup> (n = 4) at day 7 after cell injection. Shown are representative flow cytometric profiles (left panel) and averaged percentages of CFSE-diluted cells ± SEM (right panel).

As shown in Fig. 6C, DC-specific removal of I-E<sup>kloxP</sup> in NOD.I-E<sup>B</sup> mice restored the capacity of the PLNs of NOD.I-E<sup>kloxP</sup> mice to support the proliferation of CFSE-labeled 8.3-CD8<sup>+</sup> T cells. In contrast, B cell–specific removal of I-E<sup>kloxP</sup> in NOD.I-E<sup>kloxP</sup> was inconsequential.

Taken together, these data demonstrate that I-E potentiates the development of autoregulatory Tregs via DCs, fueling a feed-forward negative feedback regulatory loop that keeps autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

**Discussion**

Several mechanisms have been previously proposed to explain how certain MHC class II molecules, such as I-E, afford resistance to certain autoimmune diseases including autoimmune diabetes. The finding that thymocytes expressing V<sub>b</sub>5, V<sub>b</sub>11, V<sub>b</sub>16, or V<sub>b</sub>17a elements undergo central and/or peripheral deletion in I-E<sup>−/−</sup> hosts (45, 52) provided evidence that I-E might afford T1D resistance by promoting the differentiation of MHC class II promiscuous T cell–transferable tolerance (i.e., capable of recognizing more than one MHC class II molecule) autoreactive T cell specificities into autoregulatory CD4<sup>+</sup> T cells capable of blunting diabetogenesis in a disease-specific manner, akin to the type of T cell–transferable tolerance proposed by Singer et al. (20, 55) in the 1990s.

Our initial studies of NOD mice expressing a diabetogenic, I-A<sup>β7</sup>–restricted TCR (4.1-NOD mice (22)) revealed an association between the MHC class II promiscuity of certain autoreactive CD4<sup>+</sup> T cells and the MHC-linked resistance to T1D (23–25). Because a significant fraction of 4.1-NOD.I-E<sup>α5</sup> mice are resistant to T1D despite exporting functional autoreactive 4.1-CD4<sup>+</sup> T cells to the periphery (25), we investigated whether I-E expression inhibits T1D by promoting the differentiation of other MHC class II promiscuous autoreactive CD4<sup>+</sup> thymocytes (4.1-like, but not 4.1) into autoregulatory T cell types. Furthermore, because previous studies had implicated different APC subsets as mediators of I-E–associated resistance to T1D (17, 25, 46, 53, 56, 57), we sought to address whether I-E expression afforded T1D resistance via one or more APC types. This was done by expressing an I-E<sup>kloxP</sup> transgene in NOD mice, alone and in combination with highly diabetogenic I-A<sup>β7</sup>– or K<sup>d</sup>–restricted TCRs and/or ubiquitous or cell type–specific Cre recombinase transgenes, and by exploring the effects of transgene expression on Treg development vis-à-vis autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cell development, activation, and
recruitment. Our data show that I-E affords dominant resistance to T1D to both TCR-transgenic and nontransgenic NOD mice in a DC-dependent manner by promoting the development of auto- regulatory CD4⁺Foxp3⁺ Tregs from endogenous MHC class II promiscuous precursors. Our data clearly exclude a role for professional APC types proposed in earlier studies, including B cells, CD11c⁺ macrophages, and thymic epithelial cells, although they cannot tell us whether the effects of I-E are mediated by a specific subset of DCs. Nevertheless, the observations that I-Eα expression is associated with enhanced development of thymic Tregs, and the increased ability of thymic DCs purified from NOD.I-Eα mice to promote thymocyte Treg development in vitro suggests that thymic DCs play a critical role.

Using two different, T1D-relevant TCR-transgenic NOD models, we show that I-E expression on DCs does not have intrinsic effects on DC function, on I-Aβ- or K⁺-restricted autoantigen presentation, or on the diabetogenic potential of effector CD8⁺ or 4.1-CD4⁺ CD25⁻ T cells. In fact, I-E- mediated Treg induction and diabetes resistance in these TCR-transgenic mice required the expression of endogenous TCR rearrangements. We note that this phenomenon differs from the one we have seen in I-Aβ- transgenic 4.1-NOD mice, where selective expression of I-Aβ on CD11c⁺ cells triggers the deletion of 4.1 thymocytes and their differentiation into Tregs in both RAG-sufficient and RAG-deficient 4.1-TCR–transgenic mice (47). The preferential accumulation of these I-E–induced Tregs in the PLNs support the idea that these Tregs are β cell–autoreactive, hence, retained by PLN-associated, autoantigen-loaded APCs. This would explain why these Tregs can suppress the presentation of both cognate and noncognate β cell Ags to diabetogenic CD4⁺ and CD8⁺ T cells by local APCs, as we and others have documented experimentally both in this study and elsewhere (41, 42, 44). In support of this interpretation of the data, polyclonal Tregs arising in NOD.I-Eα mice accumulate in the PLNs of wild-type NOD hosts significantly more efficiently than Tregs arising in NOD mice, suggesting that the former harbor increased frequencies of islet Ag-autoreactive Tregs. This interpretation is consistent with the observation that islet Ag-specific Tregs have superior ability to accumulate in the PLNs (58) and are superior at suppressing autoimmunity as compared with polyclonal, nonspecific Tregs (42, 59). Our data further suggest that these autoreactive Tregs need not have to engage I-E on local DCs to suppress autoantigen presentation and that the role of I-E is purely developmental in nature (i.e., promoting Treg development but not required for Treg function). Collectively, our data indicate that certain I-Aβ⁻/-restricted autoreactive CD4⁺ T cells (i.e., positively selected by peptide–I-Aβ complexes expressed on thymic cortical epithelial cells) differentiate into autoreactive regulatory (autoregulatory) CD4⁺ T cells upon engaging cross-reactive I-E complexes on DCs.

There is evidence suggesting that MHC promiscuity is not rare among autoreactive TCRs and certainly that it is not a peculiarity of the 41-TCR. The diabetogenic MHC class I-restricted H-2K⁺ TCR is thought to recognize I-E (39). Selective downregulation of the 8.3-TCR in thyocytes from 8.3-NOD.I-Eα mice suggests that the 8.3-TCR also crossreacts with I-E. This is not caused by an endogenous superantigen binding to the 8.3-TCR chain, because this phenotype has not been seen in transgenic NOD mice expressing a low affinity IGRP206–214-specific TCR that uses the same TCРβ chain and an almost identical TCRε rearrangement as the 8.3-TCR (S. Tsai and P. Santamaria, unpublished observations). Furthermore, an encephalitogenic, myelin proteolipid protein–specific TCR has been shown to cause autoimmune disease in the context of HLA-A3 but to undergo tolerance in the presence of HLA-A2 (60). In addition, structural studies have shown that, as compared with foreign Ag-specific TCRs, autoreactive T cells tend to either bind their cognate pMHC ligands with a unique topology or to recognize a peptide that occupies only part of the peptide binding site of the presenting MHC molecule (61). These TCRs make fewer contacts with peptide residues (61) and appear to catalyze the formation of unconventional synapse structures as compared with foreign Ag-specific TCRs (62). Such an altered mode of binding may account for the MHC promiscuity of certain autoreactive TCRs, whereby polymorphic MHC residues on other (nonselecting) MHC molecules would compensate for the paucity of molecular contacts with the selecting pMHC complex(es). Certain features of known T1D-relevant TCRs support the existence of such pMHC recognition anomalies. For example, nearly all IGRP206–214-reactive TCRs use identical TCРβ chains (including their CDR3α regions) but a highly diverse repertoire of TCРα chains (30, 63), suggesting that most contacts with peptide are made via the TCРα-chain. Likewise, B-9-23 autoreactive TCRs generally use a single Vα element in their TCРα-chain (64), and the BDC2.5-TCR recognizes several chromogranin A–derived peptides (64, 65), some of which bind to the I-Aβ⁻⁻ molecule in a very unusual fashion (64).

Whatever the structural mechanisms, we demonstrate that the diabetes-protective MHC class II I-E mediates disease protection by inducing immune regulation via autoreactive Tregs, a process in which DCs play a centric role. In addition, we propose a unifying concept for the phenomena of MHC-linked T1D resistance and susceptibility. That is, protective MHC class II molecules act by engaging autoreactive TCRs that are intrinsically MHC promiscuous. This built-in feature (MHC promiscuity) may contribute to the pathogenicity of these TCRs in genetic backgrounds that fail to censor their development but affords protective MHC class II molecules the opportunity to subvert their autoreactivity for the benefit of the host—by turning pathogenic autoreactivity into autoregulation. It is undisputed that genetic diversity at the MHC loci evolved to ensure survival by accommodating immune recognition of a diverse array of infectious agents; from a different perspective, the same MHC diversity may also be viewed as nature’s design against T cell–mediated autoimmunity.

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Disclosures
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References


