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Abbreviations used in this paper: Treg, regulatory T; MHC-II, MHC class II; DC, dendritic cell; LN, lymph node; PLN, peripheral LN; EC, endothelial cell; BM, bone marrow.

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Conditional Ablation of MHC-II Suggests an Indirect Role for MHC-II in Regulatory CD4 T Cell Maintenance

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Although the importance of MHC class II (MHC-II) in acute homeostatic proliferation of regulatory T (Treg) cells has been established, we considered here the maintenance and state of Treg cells in mice that are almost completely devoid of MHC-II in their periphery but still make their own CD4 T cells and Treg cells. The latter was accomplished by conditional deletion of a loxP-flanked MHC-II β-chain allele using a TIE2Cre transgene, which causes a very high degree of deletion in hemopoietic/endothelial progenitor cells but without deletion among thymic epithelial cells. Such conditional MHC-II-deficient mice possess their own relatively stable levels of CD4+CD25+ cells, with a normal fraction of Foxp3+ Treg cells therein, but at a level ~2-fold lower than in control mice. Thus, both Foxp3low/− CD4+CD25+ cells, said to be a major source of IL-2, and IL-2-dependent Foxp3+ Treg cells are reduced in number. Furthermore, CD25 expression is marginally reduced among Foxp3+ Treg cells in conditional MHC-II-deficient mice, indicative of a lack of MHC-II-dependent TCR stimulation and/or IL-2 availability, and IL-2 administration in vivo caused greatly increased cell division among adoptively transferred Treg cells. This is not to say that IL-2 can cause Treg cell division in the complete absence of MHC-II as small numbers of MHC-II-bearing cells do remain in conditional MHC-II-deficient mice. Rather, this suggests only that IL-2 was limiting. Thus, our findings lend support to the proposal that Treg cell homeostasis depends on a delicate balance with a population of self-reactive IL-2-producing CD4+CD25+ cells which are themselves at least in part MHC-II-dependent. The Journal of Immunology, 2006, 176: 6503–6511.

There is great interest in deciphering the mechanisms of thymus-derived regulatory T (Treg) cell homeostasis (1–4). The ontogeny of CD4+CD25+ regulatory (Treg) cells is not fully understood, but they are characterized by expression of the transcriptional regulator, Foxp3 (for review, see Ref. 5). The latter is apparently essential for CD4+CD25+ Treg cell development and function, and its loss is associated with autoimmune disease (6–9). Having said this, there are a growing number of studies indicating that Foxp3-expressing Treg cells can also be generated in the periphery from CD4+CD25−Foxp3− T cells under certain conditions of suboptimal stimulation or otherwise tolerogenic conditions (10–12). Thus, while tools have recently been developed to allow the identification of live Foxp3-expressing cells at the single-cell level (13, 14), careful consideration needs to be given to the origin of such cells, whether any one cell was thymus-derived as a Treg cell or if in fact it was “converted” or “induced” into regulatory function in the periphery. Indeed, the magnitude and significance of the latter phenomenon under normal physiological conditions is of major current interest.

Treg cells have been shown to persist in the periphery for several weeks without cell division, maintaining their cell surface phenotype and ability to suppress proliferation of naive T cells in vitro (15). L-Selectinhigh Treg cells from normal mice also survived as quiescent cells for >70 days without changing their phenotype (16). However, transfer of purified CD4+CD25+ cells into lymphopenic (RAG1−/−), MHC class II (MHC-II) deficient vs sufficient hosts revealed that Treg cell “acute homeostatic expansion” requires MHC-II to an extent similar to that required by naive CD4+CD25− cells (17). Having said this, while acute homeostatic proliferation is most robust in the presence of MHC-II, it is also observed of naive CD4+CD25− cells (17, 18) and CD4+CD25− cells (17) to a certain extent even in the absence of MHC-II. In another study, however, Zap70/MHC-II double-deficient mice had ~7-fold fewer CD4+CD25− cells than did Zap70-deficient control mice, 2 mo after transfer of wild-type total CD4 T cells (19). It is not clear whether this apparent relative lack of CD4+CD25− cells is due to deficient competitiveness by Treg cells in the absence of MHC-II, due to a lack of generation of induced Treg cells, or both. Also, because these transfers were of total CD4 T cells, it is not known whether this MHC-II dependency is simply Treg cell intrinsic. That is, the relative lack of CD4+CD25− cells upon transfer into lymphopenic MHC-II deficient hosts might be, at least in part, an indirect consequence of a lack of help from other MHC-II dependent T cells and/or host-derived factors. Indeed, such a model has been postulated. For example, upon transfer into T cell-deficient hosts, CD4+CD25− T cells limit the expansion of naive CD4 T cells so that the latter reach a lower stable plateau (20). The CD4−CD25− T cells also reach a stable plateau. Thus, a feedback loop was envisaged in which expanding naive CD4 T cells contribute to their own regulation in that production of IL-2 by expanding naive CD4 T cells may contribute to the survival of CD4−CD25− T cells (20). The role of IL-2 in Treg cell survival and expansion has been well-established (2, 21–23). Furthermore, it has been suggested that a subset of preactivated self-reactive CD4+CD25− T cells is a major source
of IL-2 in vivo and that this might thereby serve to maintain Treg cells in the periphery (23).

Thus, it is still not entirely clear whether Treg cells are any more dependent on direct MHC-II/TCR interaction per se for survival and/or homeostatic proliferation than are naive CD4 T cells. Furthermore, a complicating factor in the above studies is that analyses were of CD4⁺ CD25⁺ cells as a surrogate of Treg cells. With the advent of appropriate tools, it is now possible to visualize Treg cells as Foxp3-expressing cells (13, 14).

We have previously described a loxP-flanked IA⁺ β-chain (ia⁺β⁻/⁻) allele that allows ablation of MHC-II by interbreeding with Cre recombinase-expressing transgenic mice (24). We also demonstrated that these mice express relatively normal levels of IA⁺ on B cells until after Cre recombinase-mediated deletion when MHC-II is lost (24, 25). We have now combined this demonstrated that these mice express relatively normal levels of IA⁺ on B cells until after Cre recombinase-mediated deletion when MHC-II is lost (24, 25). We have now combined this IA⁺β⁻/⁻ allele with the TIE2Cre transgene, which deletes loxP-flanked targets in early hematopoietic/endothelial progenitor cells (26).

The TIE2Cre mice have reduced numbers of preactivated CD4 T cells in their periphery but normal numbers of naive CD4 T cells. Thus, IA⁺β⁻/⁻ TIE2Cre⁺ mice lack MHC-II on the vast majority of all hematopoietic APC. Furthermore, IA⁺β⁻/⁻ TIE2Cre⁺ mice have reduced numbers of preactivated CD4 T cells in their peripheral but normal numbers of naive CD4 T cells. Thus, IA⁺β⁻/⁻ TIE2Cre⁺ mice represent a model in which there is virtually no MHC-II in the periphery and yet the mice produce their own naive CD4 T cells and Treg cells. We use these mice to examine the consequences of this hypomorphic MHC-II environment on Treg cell survival and functionality.

Materials and Methods

Mice

C57BL/6J, B6.PL-Thy1⁺/Cyl, and B6.129P2-H2⁺/⁻M11001/H11001 (IL-2-deficient) mice were from The Jackson Laboratory. The previously described TIE2Cre transgene (26) and the loxP-flanked MHC-II β-chain (ia⁺β⁻) allele (24) were crossed onto a C57BL/6J background for eight and two generations, respectively, before being intercrossed. Mice were screened for the presence of the TIE2Cre transgene by PCR using tail DNA (26). The IA⁺/⁻ genotype of mice was also determined by Southern blot analysis of tail DNA with BamHI and probe X described elsewhere (25). All breeding colonies were maintained in our animal facility and studies conducted according to institutional animal care and use guidelines.

Thymus immunohistology

A single thymic lobe from each 6-wk-old mouse was frozen in Tissue-Tek OCT Compound (VWR Scientific) using a dry-ice/methylbutane bath and stored at −80°C until sectioning. The other lobe may have been used for fluorocytometry to assess thymic dendritic cell (DC) MHC-II expression by flow cytometry (see later). Sections were cut onto silane-treated glass slides at 7-µm thickness and allowed to air dry before being fixed in cold acetone for 10 min and stored at −80°C until use. For staining, sections were hydrated in PBS for 10 min. Sections were then incubated with blocking buffer (PBS with 0.1% Tween 20 and 1% BSA, fraction V) for 30 min before incubation for 30 min with 2 µg/ml biotinylated anti-mouse IA⁺Ab (clone AF6-1201; BD Biosciences) in blocking buffer. The sections were then washed three times for 5 min in wash buffer (PBS with 0.1% Tween 20) and incubated for 30 min with 5 µg/ml streptavidin-Alexa 488 (Molecular Probes) and 2 µg/ml PE-conjugated anti-mouse CD11c (clone HL3; BD Biosciences) in blocking buffer. The sections were then washed three times as above and mounted using FluorSave (Calbiochem). Images were captured on a Zeiss confocal microscope.

Epidermal sheet immunohistology

Epidermal skin sheets were prepared essentially as described (27). Briefly, mouse ears were dissected, immersed in PBS, and separated into two dorsal ear halves. Epidermal sheets were prepared by incubating with 20 mM EDTA in PBS for 2 h at 37°C. Sheets were then filtered in acetone for 20 min at −20°C, washed with PBS and stored in 4°C until stained. All staining was performed in 1:5-mL microfuge tubes. Sheets were blocked with PBS/1% BSA for 1 h at room temperature and incubated with 1 µg/ml anti-CD45.2-PE (clone 104; BD Biosciences) and 0.5 µg/ml anti-mouse IA⁺β⁻/⁻PE (AF6-120.1; BD Biosciences) in PBS/1% BSA for 1 h at room temperature. After washing with PBS, the sheets were then mounted on glass slides and images captured on a Zeiss confocal microscope.

Flow cytometry

For the assessment of thymic DC MHC-II expression, one lobe of each thymus was diced with needles in 3 ml of HBSS with the added presence of 0.5 mg/ml collagenase type IV and 0.1 mg/ml DNase type I (both from Sigma-Aldrich), and then incubated at 37°C in a humidified incubator for 30 min. Tissue fragments were then further disrupted by gentle pipetting and the suspension transferred to a 15-ml tube containing 10 ml of PBS/10 mM EDTA. Otherwise, cells were recovered from thymus, spleen, Peyers’ patches, mesenteric lymph node (LN), and peripheral LN (PLN) (pooled inguinal, brachial and axillary LN) into 5 ml of PBS/1% FCS by crushing tissue through 0.1-mm pore-diameter nylon mesh (Millipore). Spleen cells were further treated by centrifugation at 1000 rpm for 5 min, resuspension into 2 ml of ACK erythrocyte cell lysis buffer (BioWhittaker), and then addition of 10 ml of PBS 1 min later. All cell suspensions were then centrifuged at 1000 rpm for 5 min, resuspended into PBS/1% FCS, and filtered through 0.1-mm pore-diameter nylon mesh. Aliquots of 10⁶ cells were made into 0.2 ml of PBS/1% FCS supplemented with 2 µg/ml FeBlock (BD Biosciences). Samples were left on ice for 30 min before Abs were added and left on ice in the dark for a further 1 h. Samples were washed by addition of 1 ml of PBS/1% FCS and centrifugation at 1000 rpm, 4°C for 5 min, and resuspended. The subsequent Abs were added, acquired, and analyzed as described above.

In vivo cell division assay

Erythroid cell-depleted spleen and LN pools were collected from B6.PL-Thy1⁺/Cyl mice were CFSE labeled for subsequent visualization of cell division by fluorescence dilution (29). Cells were placed in PBS at 2 × 10⁵ cells/ml, CFSE (Fluka) added to 1 µM, immediately mixed and placed in a 37°C/5% CO₂ incubator for 10 min. Cells were then diluted in medium (RPMI 1640 supplemented with 25 mM HEPEs, 2 mM glutamine, 50 µM 2-ME, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 1% FCS) and washed in the same medium and resuspended with anti-CD25 PE-conjugated anti-mouse CD25⁺ (clone PC91.2) and anti-CD8α-allophycocyanin (RA3-6B2), and anti-CD8α-allophycocyanin (53-6.7) (all from BD Biosciences). CFSE-labeled CD25⁺ B220⁺ CD8α⁺ cells were then enriched using a MoFlo cell sorter (DakoCytomation), washed in PBS and transferred to recipient mice i.v. Some recipient mice then immediately received 2 µg of (>20,000 IU) recombinant human IL-2 (PeproTech) in 0.1 ml of PBS by i.p. injection while others received PBS alone, and received repeat daily injections on the next 4 consecutive days. A day later, spleens were collected from recipient mice and CFSE dilution among the transferred Thy1⁺ CD25⁺ B220⁺ CD8α⁺ cells was assessed by flow cytometry after staining with anti-Thy1.1-PerCP (clone OX-7; BD Biosciences) and intracellular staining for FoxP3 using an anti-FoxP3-allophycocyanin staining kit (eBioscience). Ten million erythroid cell-depleted spleen cells were stained and data were collected on live (FL2-negative) Thy1⁺ cells from at least 3 million total events using a FACSCalibur (BD Biosciences).

T cell proliferation and suppression assay

Erythroid cell-depleted spleen cells were stained with anti-CD4-FTTC (clone RM4-5), anti-CD25-PE (PC61), and anti-CD8α-PerCP (53-6.7) (BD Biosciences) and various numbers (see Results) of CD4⁺ CD8α⁺ CD25⁺ T cells sorted directly into 0.1 ml of complete medium (RPMI 1640 supplemented with 25 mM HEPEs, 2 mM glutamine, 50 µM 2-ME, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% FCS) per well of a 96-well U-bottom plate, using a MoFlo cell sorter (DakoCytomation). For responder T cells in suppression assays, pooled erythroid cell-depleted spleen and PLN cells of 6- to 8-wk-old C57BL/6J mice were similarly stained and CD4⁺ CD8α⁺ CD25⁺ T cells collected by MoFlo sorting into complete medium (see above). Cells were then washed and dispensed into 96-well U-bottom plates at 5 × 10⁴ cells/well in complete medium (see above).

For stimulator APC, 5 × 10⁴ spleen-derived APC/well were used. Spleen APC were derived from erythroid cell-depleted spleen cells of 6- to 10-wk-old C57BL/6J mice by placing cells on ice for 30 min in complete medium at 2 × 10⁵ cells/ml with 10 µg/ml both anti-Thy1.2 (30-H12) and anti-NK1.1 (PK136) Abs. After washing with complete medium, rabbit complement (Cedarlane Laboratories) was added to 5% (v/v) in the same volume of complete medium and incubated at 37°C for 30 min. After washing three times, cells were filtered through 0.1-mm pore-diameter nylon mesh and irradiated (3000 rad).

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was conducted on thymus sections from 4-wk-old mice and revealed that MHC-II expression by thymic epithelial cells but not hemopoietic cells of ia\(^{\text{Bneo}}\) TIE2Cre\(^{\text{–}}\) mice. A. Thymus sections from 4-wk-old mice were stained for CD11c (red) and IA\(^{\beta}\) (green). Medullary areas of intense IA\(^{\beta}\) (i.e., MHC-II) staining are present, but arrows in the ia\(^{\text{Bneo}}\) TIE2Cre\(^{\text{–}}\) thymus images indicate CD11c\(^{+}\) DC that are negative for IA\(^{\beta}\). B. Flow cytometry of total thymocytes from 4-wk-old mice (left column) for CD11c\(^{\text{high}}\)/120G8\(^{\text{low}}\) thymic DC (unlike CD11c\(^{\text{high}}\) spleen DC, which are 120G8\(^{\text{–}}\); data not shown) with distinction from CD11c\(^{+}\)/120G8\(^{\text{high}}\) plasmacytoid DC. The boxed region is further analyzed (right column) with respect to MHC-II with anti-IA\(^{\beta}\) Ab (open plots) vs anti-Thy1.1 isotype control Ab (shaded plots).

To better quantify the degree of loss of MHC-II among thymic and other hemopoietic APC, flow cytometry was conducted using organs from mice at various ages. In a typical experiment, the vast majority of thymic CD11c\(^{\text{high}}\) DC in 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{\text{–}}\) mice lacked MHC-II whereas all thymic CD11c\(^{\text{high}}\) spleen DC from TIE2Cre\(^{\text{–}}\) littermates stained brightly for MHC-II (Fig. 1B). Similarly, in Peyer’s patches, mesenteric LN, PLN (data not shown), and spleen (Fig. 2), there was a very high degree of loss of MHC-II from various subsets of APC in ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice. However, approximately one in four of all ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice appeared to still have an unusually large fraction of MHC-II\(^{+}\) APC (10–20%) when compared with all other ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice (Fig. 2), for reasons that are not yet clear. All other 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice had lost MHC-II from 98.6 \pm 1.1\% (n = 9) of all CD11c\(^{+}\) spleen DC. Similarly, the majority of 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice had lost MHC-II from 97.2 \pm 1.8\% (n = 9) of all B cells. This degree of loss of MHC-II did not differ at 2,

All wells were in a final volume of 0.2 ml of medium with 1 \(\mu\)g/ml anti-CD3 Ab (2C11; BD Biosciences). Proliferation was determined as tritiated thymidine (1 \(\mu\)Ci/well; Amersham Biosciences) incorporation during the last 8 h of a 72-h culture period.

**Results**

**Generation of mice lacking MHC-II on hemopoietic cells**

Mice bearing Cre recombinase loxP sites of recombination in the MHC-II \(\beta\)-chain gene were generated by homologous recombination in 129/SvJ embryonic stem cells using conventional techniques as previously described (24). Conditional deletion of this ia\(^{\text{Bneo}}\) allele was achieved with a TIE2Cre Cre recombinase transgene (26). The latter causes a very high degree of deletion of loxP-flanked targets in hemopoietic progenitor cells and endothelial cells (EC) (26, 30, 31). However, also previously reported is the fact that the TIE2Cre transgene causes germine deletion in progeny mice when it is carried by the mother (26). We took advantage of this to generate an MHC-II\(^{\alpha\text{at}}\) allele (the ia\(^{\beta\Delta}\) allele) by crossing a homozygous ia\(^{\text{Bneo/neo}}\) male with a TIE2Cre female. All progeny were confirmed to be ia\(^{\beta\Delta/\Delta}\) and either TIE2Cre positive or negative. These mice were interbred to generate ia\(^{\beta\Delta/\Delta}\) TIE2Cre\(^{+}\) males, and the latter then bred with ia\(^{\text{Bneo/neo}}\) females to generate ia\(^{\text{Bneo/neo}}\) progeny that were either TIE2Cre positive or negative. Using this breeding scheme, of 289 progeny screened by Southern blot analysis, 267 had the expected ia\(^{\text{Bneo/neo}}\) genotype while 22 (8\%) had a ia\(^{\beta\Delta/\Delta}\) genotype. The latter was presumably due to a low level of early embryonic deletion of the ia\(^{\text{Bneo}}\) allele inherited from the mother by the TIE2Cre transgene inherited from the father, another feature of the TIE2Cre transgene previously reported (26).

Ia\(^{\beta\Delta/\Delta}\) TIE2Cre\(^{+}\) mice were anticipated to have a very high degree of loss of MHC-II from hemopoietic cells and EC but still possess their own CD4 T cells, because previous data had indicated that the TIE2Cre transgene does not delete loxP-flanked targets in thymic epithelial cells (26). Nonetheless, immunohistology was conducted on thymus sections from 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice and their TIE2Cre\(^{–}\) littermates to evaluate the presence of MHC-II. This clearly revealed that ia\(^{\text{Bneo}}\) TIE2Cre\(^{–}\) mice do indeed express MHC-II on both cortical and medullary epithelial cells of the thymus, and some CD11c\(^{+}\) DC could be discerned as being MHC-II deficient (Fig. 1A).

![FIGURE 1](http://www.jimmunol.org/) MHC-II expression by thymic epithelial cells but not hemopoietic cells of ia\(^{\text{Bneo}}\) TIE2Cre\(^{–}\) mice. A. Thymus sections from 4-wk-old mice were stained for CD11c (red) and IA\(^{\beta}\) (green). Medullary areas of intense IA\(^{\beta}\) (i.e., MHC-II) staining are present, but arrows in the ia\(^{\text{Bneo}}\) TIE2Cre\(^{–}\) thymus images indicate CD11c\(^{+}\) DC that are negative for IA\(^{\beta}\). B. Flow cytometry of total thymocytes from 4-wk-old mice (left column) for CD11c\(^{\text{high}}\)/120G8\(^{\text{low}}\) thymic DC (unlike CD11c\(^{\text{high}}\) spleen DC, which are 120G8\(^{\text{–}}\); data not shown) with distinction from CD11c\(^{+}\)/120G8\(^{\text{high}}\) plasmacytoid DC. The boxed region is further analyzed (right column) with respect to MHC-II with anti-IA\(^{\beta}\) Ab (open plots) vs anti-Thy1.1 isotype control Ab (shaded plots).

To better quantify the degree of loss of MHC-II among thymic and other hemopoietic APC, flow cytometry was conducted using organs from mice at various ages. In a typical experiment, the vast majority of thymic CD11c\(^{\text{high}}\) DC in 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{–}\) mice lacked MHC-II whereas all thymic CD11c\(^{\text{high}}\) spleen DC from TIE2Cre\(^{–}\) littermates stained brightly for MHC-II (Fig. 1B). Similarly, in Peyer’s patches, mesenteric LN, PLN (data not shown), and spleen (Fig. 2), there was a very high degree of loss of MHC-II from various subsets of APC in ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice. However, approximately one in four of all ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice appeared to still have an unusually large fraction of MHC-II\(^{+}\) APC (10–20%) when compared with all other ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice (Fig. 2), for reasons that are not yet clear. All other 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice had lost MHC-II from 98.6 \pm 1.1\% (n = 9) of all CD11c\(^{+}\) spleen DC. Similarly, the majority of 4-wk-old ia\(^{\text{Bneo}}\) TIE2Cre\(^{+}\) mice had lost MHC-II from 97.2 \pm 1.8\% (n = 9) of all B cells. This degree of loss of MHC-II did not differ at 2,
4, and 6 mo of age. Last but not least, immunohistology on epidermal sheets from 6-wk-old mice showed that 
\(\text{ia}^{+/\text{neo}}/\text{H9252}\) mice had also lost MHC-II from Langerhans’ cells (Fig. 3). Thus, 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice possess MHC-II on thymic epithelial cells but otherwise lack MHC-II from the vast majority of hemopoietic APC except for some examples of low-deletion mice still possessing MHC-II on as many as 20% of their APC. The latter mice where encountered were excluded from subsequent studies below (except where otherwise indicated) by analysis of either peripheral blood cells sampled before experimentation or spleen cells at the time of analysis.

**FIGURE 3.** Lack of MHC-II on Langerhans’ cells of 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice. Epidermal sheets from 6-wk-old mice were stained for MHC-II with anti-\(\text{IA}\beta\) Ab (red), as well as for CD45 (green). CD45 staining of hemopoietic cells including Langerhans’ cells is clearly visible in epidermal sheets of an 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mouse as well as a TIE2Cre– littermate, but MHC-II is only seen in the case of the latter. Results are representative of three mice per group.

**FIGURE 4.** Increased fraction of CD8–CD4+ but not CD8–CD4+CD25+ thymocytes in 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice. The latter mice have relatively lower levels of CD4+CD8a–CD25+ thymocytes in terms of percentage of total CD4+CD8a– thymocytes (A) and a smaller fraction of CD4+CD8a– thymocytes that have a Treg cell identity as judged by intracellular Foxp3 analysis (B). Profiles are representative of three littermates per group in at least three independent experiments.

Increased numbers of CD4+CD8− but not CD4+CD25+ thymocytes

Given the lack of MHC-II expression on thymic hemopoietic APC in 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice, one might have expected a relatively increased proportion of CD8–CD4+ cells in the thymus of 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice due to a lack of hemopoietic APC-mediated negative selection. Indeed, at both 4 and 6 wk of age, 
\(\text{ia}^{+/\text{neo}}/\text{TIE2Cre}\) mice had ~1.5- to 2.0-fold more CD8–CD4+ thymocytes in terms of both absolute numbers and relative proportion (Fig. 4), consistent with expectations based on bone
marrow (BM) chimeras carrying MHC-II-deficient hemopoietic RPC (32, 33). In a typical experiment with three 6-wk-old mice per group, CD8+ CD4+ thymocytes represented 7.82 ± 0.94% and 11.77 ± 0.90% of total thymocytes in *iaβneo/Δ* TIE2Cre− and *iaβneo/Δ* TIE2Cre+ mice, respectively. This translated to 11.05 ± 0.74 × 10^6 and 19.57 ± 4.40 × 10^6 CD8+ CD4+ thymocytes in the *iaβneo/Δ* TIE2Cre− and TIE2Cre+ littermates, respectively. However, CD8+ CD4+ thymocyte numbers were not significantly increased (*iaβneo/Δ* TIE2Cre+ mice, 5.93 ± 0.70 × 10^6; *iaβneo/Δ* TIE2Cre− mice, 7.11 ± 1.86 × 10^6, n = 3), but rather were at a relatively normal proportion in terms of total thymocytes (*iaβneo/Δ* TIE2Cre+ mice, 0.42 ± 0.07%; *iaβneo/Δ* TIE2Cre− mice, 0.43 ± 0.05%). As a result, CD8+ CD4+ thymocytes actually appeared to be a smaller fraction of total CD8+ CD4+ thymocytes (*iaβneo/Δ* TIE2Cre+ mice, 6.30 ± 0.29%; *iaβneo/Δ* TIE2Cre− mice, 4.16 ± 0.30%: Fig. 4A). We further analyzed thymocytes for expression of Foxp3, a transcriptional regulator considered to be a determining factor in CD4+ CD25+ Treg cell generation as well as in the maintenance of suppressive capacity (6–9). Consistent with the data regarding CD8+ CD4+ CD25+ thymocytes, intracellular staining for Foxp3 revealed *iaβneo/Δ* TIE2Cre+ mice to have a smaller fraction of total CD8+ CD4+ thymocytes that are Foxp3+ (Fig. 4B), but this does not translate to reduced absolute cell numbers.

**Reduced numbers of preactivated CD4 T cells and CD4+ CD25+ cells in *iaβneo/Δ* TIE2Cre+ mice**

Analysis of CD4 T cells in spleen and LN of *iaβneo/Δ* TIE2Cre+ mice revealed reduced numbers of CD44high preactivated cells compared with their TIE2Cre− littermates, as might be expected in the absence of MHC-II in the periphery. An example of this is shown with 4-mo-old mice in Fig. 5 and Table I. However, in terms of absolute cell numbers, there was no discernable defect in numbers of naive CD4 T cells (CD4+ CD25− L-Sellow/CD44low) between 4 wk and 6 mo of age (Table I and data not shown). Data from mice at various ages also reveals that CD4+ CD25− cell numbers remain fairly consistent throughout young adulthood of *iaβneo/Δ* TIE2Cre+ mice, albeit reduced compared with their TIE2Cre− littermates (Fig. 6). Of course, CD25 expression is not sufficient to conclude that such cells are Treg cells as this population is said to include self-reactive IL-2-secreting CD25high/- cells (23) as well as other activated T cells (34, 35). Thus, we further analyzed spleen cells by intracellular staining for Foxp3. At 2 mo of age, *iaβneo/Δ* TIE2Cre+ mice do indeed contain CD4 T cells that are Foxp3+ and CD25+ but at a reduced level compared with control littermates (Fig. 7). The same was true at 4 wk, 4 mo, and 6 mo of age (data not shown). Presumably the fraction of CD4+ CD25− cells that appears to be Foxp3low/- rather than Foxp3high (Fig. 7B) represents the fraction of CD4+ CD25− cells described by others as being CD25low/+ and Foxp3high cells (23), said to be a major source of IL-2 in vivo that might thereby maintain CD4+ CD25+ Foxp3high Treg cells in the periphery (23). Indeed, gating for CD25 on CD4+ CD25+ Foxp3low+ cells vs CD4+ CD25+ Foxp3high cells indicates that they are predominantly CD25low+ and CD25high, respectively (Fig. 7B).

**IL-2 deficiency results in loss of CD25 from Treg cells**

In the above analysis of Foxp3-expressing cells, there appeared to be a small but consistent reduction in CD25 expression among conventional CD4 T cells at least (36). We wondered therefore whether or not IL-2 deficiency would result in reduced CD25 expression among CD4+ CD25+ TIE2Cre− mice at 2 mo of age (Fig. 7A) as well as at 4 wk and 4 mo of age (data not shown). In a typical experiment with 4-mo-old mice, the mean fluorescence intensity of CD25 staining among CD4+ CD8− Foxp3− spleen cells of *iaβneo/Δ* TIE2Cre+ mice (n = 3) and their *iaβneo/Δ* TIE2Cre− littermates (n = 4) was 162 ± 10 and 224 ± 7 respectively (Student’s two-tailed *t* test, *p* = 0.03). This might be a reflection of the lack of MHC-II-dependent Treg cell TCR stimulation in *iaβneo/Δ* TIE2Cre+ mice and/or a consequence of a lack of IL-2-dependent stimulation. That is, CD4+ CD25+ Foxp3low/- cells, said to be a major source of IL-2 in vivo (23), would appear to be reduced in number ~2-fold in *iaβneo/Δ* TIE2Cre+ mice, and IL-2 is known to cause positive feedback up-regulation of CD25 on conventional CD4 T cells at least (36). We wondered therefore whether or not IL-2 deficiency would result in reduced CD25 expression on Treg cells, and, by extension, whether or not IL-2-deficient mice have Foxp3+ Treg cells that appear to be CD25−. Indeed, this proved to be the case (Fig. 8). Furthermore, while this manuscript was in preparation, others also demonstrated this to be the case (37, 38), and IL-2 administration in vivo caused up-regulation of CD25 on Treg cells (37). Also, IL-2-deficient mice appear to have CD4+ Foxp3high cell numbers only slightly lower than

### Table I. Normal numbers of naive CD4 T cells in 4-mo-old *iaβneo/Δ* TIE2Cre+ mice

<table>
<thead>
<tr>
<th>Absolute Cell Number ± SD (×10^6)</th>
<th><em>iaβneo/Δ</em> TIE2Cre+ mice</th>
<th><em>iaβneo/Δ</em> TIE2Cre− mice</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ CD25− L-Sel− CD44high</td>
<td>4.74 ± 2.57</td>
<td>0.81 ± 0.09</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>CD4+ CD25− L-Sel− CD44high</td>
<td>0.88 ± 0.28</td>
<td>0.30 ± 0.05</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Naive (CD4+ CD25− L-Sel− CD44high)</td>
<td>8.35 ± 2.25</td>
<td>8.66 ± 2.38</td>
<td></td>
</tr>
<tr>
<td>Active Treg cells and effector cells (CD4+ CD25− GITR− CD69low)</td>
<td>1.06 ± 0.52</td>
<td>0.35 ± 0.12</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Resting Treg cells and effector cells (CD4+ CD25− GITR+ CD69high)</td>
<td>1.20 ± 0.38</td>
<td>0.54 ± 0.09</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

*Absolute spleen cell numbers for various CD4 T cell subsets are from a representative flow cytometry analysis essentially as in Fig. 5, and in this case with three females per group. Statistical analysis of the probability of no significant difference between *iaβneo/Δ* TIE2Cre+ mice and *iaβneo/Δ* TIE2Cre− mice was done using the two-tailed Student *t* test.*
As stated earlier, the reduced levels of CD4<sup>+</sup> T cells in mice with the TIE2Cre transgene (26) to cause deletion of the former in early hemopoietic/endothelial progenitor cells. Most (but not all) of these iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice lack MHC-II on the vast majority of their T cell repertoire.

**FIGURE 6.** Marginally reduced but consistent levels of CD4<sup>+</sup>CD25<sup>+</sup> cells in iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice. Data points are average spleen cell numbers + or − SD from four to nine iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice (□) and their TIE2Cre<sup>-</sup> littermates (●) at each age shown. Also shown are data from germline MHC-II-deficient mice (iαβ<sup>−/−</sup>, △) and low-deletion mice (○), two mice each per data point where shown. Clearly, low-deletion mice are essentially identical to iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice where shown. The CD4<sup>+</sup>CD25<sup>+</sup> naïve CD4<sup>+</sup> ratio values for 1- and 6-mo-old iαβ<sup>−/−</sup> mice were ~1 and 3, respectively (data not shown). Naïve CD4<sup>+</sup> is defined as CD4<sup>+</sup>CD25<sup>−</sup>L-select<sup>−</sup>CD44<sup>low</sup> as in Fig. 5.

Those in wild-type control mice (Fig. 8). That is, while the frequency of Foxp<sup>+</sup> cells among total CD4<sup>+</sup> T cells in IL-2-deficient mice is ~4-fold lower than that in wild-type control mice, at 2 mo of age at least (Fig. 8), total CD4 T cell numbers are ~3-fold higher. Thus, the absolute numbers of spleen CD4<sup>+</sup>Foxp<sup>+</sup> cells in the IL-2-deficient and wild-type control mouse shown in Fig. 8 are 1.8 million and 2.4 million, respectively.

**IL-2 causes Foxp<sup>+</sup> Treg cell division even in the absence of MHC-II in iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice**

As stated earlier, the reduced levels of CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>3<sup>high</sup></sup> cells in iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice suggests the possibility of reduced IL-2 availability to support Treg cell maintenance, and that this may be the cause of reduced Treg cell levels rather than, or in addition to, the lack of TCR-dependent homeostatic stimulation of Treg cells in the absence of MHC-II in iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice. This led us to test whether Treg cells would undergo increased cell division in vivo upon IL-2 administration. Thus, CFSE-labeled CD8<sup>+</sup>B220<sup>+</sup>CD25<sup>+</sup> cells from B6.PL (Thy.1 congenic) donors were transferred to age- and sex-matched iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice. Some mice were then immediately given IL-2 while others were given PBS alone. Mice then received the same injection daily for the next 4 consecutive days and spleens harvested the day after the last injection. A representative experiment is depicted in Fig. 9, revealing that IL-2 administration to iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice causes a substantial increase in cell division among the transferred Thy.1<sup>+</sup>Foxp<sup>3<sup>high</sup></sup> Treg cells when compared with mice that received PBS alone, while there is no apparent effect of IL-2 on transferred Thy.1<sup>+</sup>Foxp<sup>3<sup>−</sup></sup> T cells.

**Suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> cells**

Having observed reduced CD25 expression on Treg cells of iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice, we next tested CD4<sup>+</sup>CD25<sup>+</sup> cells from iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice for suppressive capacity in vitro. CD4 T cell proliferation assays. Fig. 10 shows that CD4<sup>+</sup>CD25<sup>+</sup> cells from iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice are just as suppressive as CD4<sup>+</sup>CD25<sup>+</sup> cells from TIE2Cre<sup>-</sup> littermates, where the responder cells are CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> T cells from C57BL/6 mice. This indicates that the strong polyclonal stimulation of Treg cells by anti-CD3 Ab was sufficient to overcome whatever possible defect there may or may not have been in the Treg cells from iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice. Having said this, we have not yet investigated whether or not CD4<sup>+</sup>CD25<sup>+</sup> cells from iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice caused T cell suppression in this in vitro assay via the same mechanism or mechanisms as CD4<sup>+</sup>CD25<sup>+</sup> cells from their TIE2Cre<sup>-</sup> littermates. That is, it is quite possible that CD4<sup>+</sup>CD25<sup>+</sup> cells suppress T cell response via more than one mechanism, in ways that have not yet been fully elucidated, and that loss of any one mechanism of suppression is not sufficient to result in an apparent loss of suppressive capacity in the above in vitro assay.

**Discussion**

We have combined a loxP-flanked IA<sup>b</sup> β-chain (iαβ<sup>neo</sup>) allele (24) with the TIE2Cre transgene (26) to cause deletion of the former in early hemopoietic/endothelial progenitor cells. Most (but not all) of these iαβ<sup>neo</sup>TIE2Cre<sup>+</sup> mice lack MHC-II on the vast majority.
of all hemopoietic APC but they still have relatively normal thymic epithelial cell MHC-II expression. Thus, these mice represent a model in which the mice make their own CD4 T cells and Foxp3+ Treg cells but there is very little MHC-II expression in the periphery. This model is imperfect in that ~8% of all mice lose MHC-II expression completely by germline deletion of the \( \text{ia}^{\text{neo}} \) allele, and a further subset of mice (about one-quarter) proved to have an unusually low level of deletion (80–90%) for as yet unknown reasons. We are currently further backcrossing all lines to a C57BL/6d background to test whether this mosaicism is related to the genetic background of the mice or if it is simply an unavoidable feature of the TIE2Cre transgene or the \( \text{ia}^{\text{neo}} \) allele. Nonetheless, these minor obstacles were easily overcome here by genotyping and peripheral blood analysis for loss of MHC-II. These conditional MHC-II-deficient (\( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \)) mice otherwise had >95% of all hemopoietic APC lacking MHC-II expression. We cannot and do not suggest that \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice are completely devoid of MHC-II expression in their periphery. Rather, it is probably better to describe these mice as being MHC-II hypomorphic. Nonetheless, they provide a unique model to study the dependence of Treg cells on MHC-II and other factors in their survival and suppressive capacity. One might reasonably ask, however, why we did not conduct the above studies by simply generating BM-chimeric mice lacking MHC-II on hemopoietic cells rather than going to the trouble of generating conditional MHC-II-deficient mice. Besides the fact that BM-chimeric mice might not faithfully reproduce age-dependent processes (e.g., neonatal peripheral tolerance induction mechanisms), we would argue that the degree of loss of MHC-II observed in \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice is in fact as great as that which might be obtained when generating BM chimeras with MHC-II-deficient BM. For example, Merad et al. (39) showed the degree of host-derived spleen DC to still be ~3–10% of total spleen DC even 6 mo after BM transplantation. Second, some tissue APC reservoirs are not easily ablated and replaced by cells of donor origin when making BM chimeras. For example, Langerhans cells in BM chimeras are almost completely still of host origin even up to 18 mo after irradiation unless extraordinary steps are taken to cause their replacement by cells of donor origin (39). Perhaps as a consequence, 10–40% of inguinal LN DC in BM chimeras were still of host origin even 6 mo after BM transplantation (39). Other tissues such as the liver, kidney, and lung have also been reported to recruit donor BM-derived cells very slowly (e.g., Ref. 40). Third, the TIE2Cre transgene is known to delete \( \text{loxP} \)-flanked targets in EC (26, 31). Therefore, unlike BM chimeras carrying MHC-II-deficient hemopoietic cells, we fully expect that \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice will also be incapable of expressing MHC-II on EC.

![Figure 8](image1.png)

**FIGURE 8.** IL-2-deficient mice have Foxp3+ T cells. Spleen cells from a representative 9-wk-old IL-2-deficient mouse and an age-matched C57BL/6J mouse were analyzed for intracellular Foxp3 after pregating on CD4+CD8- cells. The total numbers indicate spleen cell numbers for each mouse in millions. Other numbers adjacent to gates indicate percentages of cells in their respective gate. CD25 staining is also shown for Foxp3high cells after gating as indicated.

![Figure 9](image2.png)

**FIGURE 9.** IL-2 increases Treg cell division in \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice. Two-month-old \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice received CFSE-labeled CD8a+ B220- CD25+ cells from B6.PL donors and five consecutive daily doses of recombinant human IL-2 or PBS as described in Materials and Methods. On day 6, recipient spleen cells were analyzed for intracellular Foxp3 after pregating on Thy1.1+ donor cells, and then CFSE among Thy1.1+ Foxp3+ vs Thy1.1+ Foxp3- cells. Numbers adjacent to gates indicate percentages of cells in their respective gate. Data are representative of three mice per group.

![Figure 10](image3.png)

**FIGURE 10.** Effective suppression by CD4+CD25+ cells of \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice. Both at 4 wk (A) and 2 mo of age (B), CD4+CD8-CD25+ spleen cells from \( \text{ia}^{\text{neo}} \) mice (●), \( \text{ia}^{\text{neo}} \) \( \text{TIE2Cre}^{-} \) mice (▲), and their \( \text{TIE2Cre}^{-} \) littermates (■) were sorted into U-bottom wells at various Treg:responder T cell ratios (shown on x-axis) in triplicate wells. Responders were \( 5 \times 10^6 \) C57BL/6J CD4+CD8+CD25- T cells/well and stimulators were \( 5 \times 10^5 \) irradiated spleen APC with \( 1 \mu\text{g/ml} \) anti-CD3. Proliferation was determined after 72 h by tritiated thymidine incorporation during the last 8 h. R-responders alone with no Treg cells.
As might be expected, \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice have reduced numbers of preactivated CD4 T cells in their periphery but normal numbers of naive CD4 T cells. Also, \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^-\) mice had greater numbers of CD4\(^+\)CD8\(^-\) thymocytes but normal numbers of Foxp3\(^-\) thymocytes, presumably a result of a lack of negative selection in the absence of MHC-II on thymic hemopoietic APC. It is tempting to speculate that the reduced level of Treg cells in the periphery of \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice is a direct reflection of the relatively lower thymic output in terms of percentage of total CD4 T cells. If the reduced Treg cell levels in the periphery were caused by relative thymic output, this would have a number of implications. First, because this Treg cell deficiency did not correct itself in the periphery, this would suggest that in the absence of MHC-II, Treg cells do not have their own microniche or homeostatic level independent of other CD4 T cells. By the same token, this would suggest that in the absence of MHC-II in \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice (or at least in this MHC-II hypomorphic state), Treg cells are able to compete for survival factors equally well with all other CD4 T cells. Having said this, we have not yet analyzed the turnover rate of Treg cells in \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice compared with that in wild-type control mice, i.e., survival or life span vs homeostatic proliferation. This will be addressed in future BrdU-labeling studies.

Another possible explanation for the relatively reduced Treg cell levels in \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice may lie in the fact that IL-2 is a significant factor in CD4\(^+\)CD25\(^+\) Treg cell survival and expansion (2, 21–23). Neutralization of IL-2 leads to loss of Treg cell numbers in vivo (21, 23). IL-2 is said to be critical for physiologic proliferation and maintenance of Treg cells much more so than for naive CD4 T cells (23). The dominant source of this IL-2 was suggested to be a subset of Foxp3lowCD4\(^+\)CD25low cells including self-reactive cells (23). Others, however, have shown that IL-2R\(^\alpha\)-deficient mice can be protected from lethal autoimmunity and show restored CD4\(^+\)CD25\(^+\) Treg cell numbers by reconstitution (after lethal irradiation) with BM from IL-2-deficient donors, indicating that some as yet unidentified non-T cell source can provide IL-2 for Treg cell restoration (20). Nonetheless, we clearly found \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice to have reduced numbers of Foxp3lowCD4\(^+\)CD25\(^+\) cells, suggesting a deficiency of the Treg cell generation and/or maintenance of Foxp3lowCD4\(^+\)CD25low cells described by others (23).

Thus, our findings suggest the possibility that in the almost complete absence of MHC-II in \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice, CD4\(^+\)CD25low/spleen cells are only reduced ~2-fold in number compared with TIE2Cre\(^+\) littermates. It is possible that these remaining cells are not MHC-II restricted. Indeed, the MHC-II and IL-2 requirements of the IL-2-secreting CD4\(^+\)CD25low cells described by others (23) remain to be determined. CD4 T cells bearing surface markers indicative of a state of activation are prominent in lymphoid organs draining mucosal sites even in MHC-II-deficient mice (49). Also, reports suggest the existence of CD8\(^+\)CD25\(^+\) Foxp3\(^+\) regulatory cells in MHC-II-deficient mice (49, 50). However, we also have to concede the possibility that both CD4\(^+\)CD25\(^+\) Foxp3low cells and CD4\(^+\)CD25\(^+\) Foxp3high Treg cells are found in \(\text{ia}\beta^{\text{neoA}}\) TIE2Cre\(^+\) mice, to the extent that they are seen, simply because of incomplete MHC-II absence. Along the same lines, the presence of Foxp3high Treg cells albeit reduced might be a result of the incomplete loss of IL-2-secreting CD4\(^+\)CD25\(^+\) Foxp3low cells.

In conclusion, we have generated mice that almost completely lack MHC-II in their periphery but still generate their own CD4 T cells and CD4\(^+\)CD25\(^+\) Treg cells. These mice make it possible to further decipher the role of MHC-II in Treg cell functionality in vivo.

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**Disclosures**

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**References**


