Major Histocompatibility Complex Class II–positive Cortical Epithelium Mediates the Selection of $CD4^+25^+$ Immunoregulatory T Cells

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Abstract

$CD4^+25^+$ T cells are a unique population of immunoregulatory T cells which are critical for the prevention of autoimmunity. To address the thymic selection of these cells we have used two models of attenuated thymic deletion. In K14-A_β^b mice, major histocompatibility complex (MHC) class II I-A^b expression is limited to thymic cortical epithelium and deletion by hematopoietic antigen-presenting cells does not occur. In H2-DMα–deficient mice, MHC class II molecules contain a limited array of self-peptides resulting in inefficient clonal deletion. We find that $CD4^+25^+$ T cells are present in the thymus and periphery of K14-A_β^b and H2-DMα–deficient mice and, like their wild-type counterparts, suppress the proliferation of cocultured $CD4^+25^-$ effector T cells. In contrast, $CD4^+25^+$ T cells from MHC class II–deficient mice do not suppress responder $CD4^+$ T cells in vitro or in vivo. Thus, development of regulatory $CD4^+25^+$ T cells is dependent on MHC class II-positive thymic cortical epithelium. Furthermore, analysis of the specificities of $CD4^+25^+$ T cells in K14-A_β^b and H2-DMα–deficient mice suggests that a subset of $CD4^+25^+$ T cells is subject to negative selection on hematopoietic antigen-presenting cells.

Key words: autoimmune disease • self-tolerance • thymic development • IL-2 receptor α chain (CD25) • suppressor T cells

Introduction

The thymic development of T cells results in the acquisition of both immunologic tolerance to self and reactivity to foreign antigenic stimuli. The developmental processes of positive selection or self-restriction and negative selection or self-tolerance are compartmentalized within the thymus. The critical and unique function of thymic cortical epithelium in supporting positive selection of $CD4^+$ T cells is well accepted (1, 2). Conversely, multiple tissue types can mediate the opposing process of negative selection. Interactions between immature T cells and bone marrow–derived APCs, particularly dendritic cells, lead to tolerance via clonal deletion (3–5). In addition to APCs, thymic medullary epithelium can mediate incomplete deletion of high affinity thymocytes reactive to both endogenous mouse mammary tumor virus superantigens and MHC–peptide ligands (6, 7). The question of whether clonal deletion or tolerance induction can occur in the cortex remains controversial and the accepted view is that the thymic medulla is the primary site of negative selection.

Interactions between immature T cells and thymic epithelium can also result in tolerance via nondeletional mechanisms. Early studies of murine radiation chimeras showed that functional unresponsiveness, rather than clonal deletion, was induced to self-antigens expressed by radioresistant thymic epithelium (8). Similarly, nude mice grafted with allogeneic fetal thymic epithelium devoid of hematopoietic precursors acquired donor-specific tolerance to heart and skin transplants but rejected both third party grafts and donor-specific hematopoietic cells (9–11). In this system, transplant tolerance could be transferred by $CD4^+$ cells to naive nude mice and was "dominant" over the alloreactive effector cells. These experiments suggested that

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donor-specific tolerance to allografts generated by thymic epithelium was mediated by "regulatory" $CD4^+$ T cells.

Separate experiments have implicated $CD4^+$ regulatory cells generated in the thymus in the prevention of autoimmunity. Studies in two different systems have identified a population of $CD4^+$ T cells constitutively expressing the IL-2 receptor α chain, CD25, which function as immunoregulatory cells. In the first model, peripheral mature $CD4^+$ T cells depleted of the $CD4^+CD25^+$ population induced organ-specific autoimmunity when inoculated into histocompatible T cell–deficient *nu/nu* mice (12, 13). In the second model, genetically susceptible mice thymectomized on day 3 of life (3dTx)* developed similar organ-specific autoimmune disease (14, 15). The disease process in 3dTx mice was induced by $CD4^+$ cells but could be inhibited by normal adult $CD4^+$ cells; the inhibitory activity was again contained in the subset of $CD4^+$ cells coexpressing CD25 (16).

There is strong evidence that regulatory $CD4^+CD25^+$ T cells develop and acquire their suppressive phenotype in the thymus (17–19). Clearly, the development of autoimmunity in 3dTx mice suggests a requirement for an intact thymus in the preservation of peripheral tolerance. Closer examination of $CD4^+CD25^+$ thymocytes demonstrate that they comprise ~5% of the CD4 single positive (SP) pool and have a regulatory phenotype similar to their peripheral counterparts (18, 19). They can prevent autoimmunity when adoptively transferred into 3dTx mice or, with $CD4^+CD25^-$ effector cells, into *nu/nu* hosts (18). Intrathymic transfer experiments suggest that $CD4^+25^+8^-$ thymocytes arise from double negative thymocyte precursors, and fluorochrome labeling has demonstrated that $CD4^+CD25^+$ cells emigrate from the thymus to populate the periphery (17, 18).

In several experimental systems, the expression of a regulatory cell phenotype does not occur until cells have entered the CD4 SP thymocyte pool (17–19). Recently, two of us (M.S. Jordan and A.J. Caton, Wistar Institute) have shown that an agonist peptide reexpressed as self on thymic epithelium is sufficient for the generation of functionally suppressive $CD4^+CD25^+$ T cells (19). Given that acquisition of a cell surface phenotype characteristic of regulatory $CD4^+$ T cells (such as CD25) occurs "relatively late" during CD4-SP development, it has been suggested that the generation of regulatory $CD4^+CD25^+$ T cells is the result of high affinity interactions with medullary epithelium (19, 20). While this model system demonstrated that thymic epithelium is sufficient for the development of these cells, it was unable to place the generation of $CD4^+CD25^+$ T cells in the context of positive and negative selection.

We have delineated the thymic development of $CD4^+CD25^+$ cells in two different models of attenuated negative selection. In K14-A_β^b mice, an MHC class II I-A^b transgene is expressed only on thymic cortical epithelium; both medullary epithelium and bone marrow–derived APCs are class II-negative (21). Clonal deletion of $CD4^+$ T cells cannot be documented, and $CD4^+$ T cells react in MLRs to I-A^b–positive hematopoietic APCs. H2-DMα–deficient mice have H-2A^b molecules loaded almost exclusively with the class II–associated invariant chain peptide (22–24). The $CD4^+$ T cells selected in H2-DMα–deficient mice are not tolerant to the broad array of peptides expressed by wild-type APCs and these cells also react vigorously in MLRs against B6 hematopoietic APCs (22, 23). In both K14-A_β^b and H2-DMα–deficient mice, the introduction of I-A^b-positive hematopoietic APCs eliminates these autoreactive cells (25, 26). Thus, clonal deletion is deficient in both K14-A_β^b and H2-DMα–deficient mice. The nondeletional mechanisms of T cell tolerance have not been examined in these models.

The data presented herein demonstrate that $CD4^+25^+$ T cells are selected on cortical epithelium and that these cognate interactions are sufficient for the phenotypic and functional development of $CD4^+CD25^+$ immunoregulatory T cells. Furthermore, analysis of the specificities of $CD4^+25^+$ T cells in K14-A_β^b and H2-DMα–deficient mice suggests that a subset of $CD4^+25^+$ T cells is subject to negative selection on hematopoietic APCs.

Materials and Methods

Mice. C57BL/6 (designated WT or B6 in text) and C57Bl/6 recombination activating gene 2-deficient (Rag-2°) were purchased from The Jackson Laboratory or Centre de Dévelopment des Techniques Avancées pour l'experimentation animale (Orleans, France). The MHC class II–deficient mice (27), K14-A_β^b mice (21), and H2-DMα–deficient mice (24) have been described previously. H2-DMα–deficient mice were a gift from Luc van Kaer (Vanderbilt University, Nashville, TN) and are seven generations backcrossed onto a C57BL/6 background. Both K14-A_β^b and MHC class II–deficient mice are >20 generations. All mice were maintained under SPF conditions in the animal facilities of the University of Pennsylvania or the Institut Pasteur.

Media, Reagents, Antibodies, and Flow Cytometry. All cells were grown in RPMI 1640 (Mediatech) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mm L-glutamine, 10 mm Hepes (all from GIBCO BRL), and 50 μm 2-ME (Sigma-Aldrich). FITC and biotin–anti-CD25 (7D4), PE, and APC–anti-CD4 (RM4-5), and streptavidin–PE were purchased from BD PharMingen. Purified anti-CD3ε (2C11) was purchased from Accurate. Murine rIL-2 was purchased from Peprotech. Cells were analyzed on a Becton Dickinson FACSCalibur™ using CELLQuest™ software (Becton Dickinson).

Cell Purification. Thymocytes, LN, and spleen cells were initially prepared by lysing erythrocytes with ACK lysis buffer. Cell preps were then enriched for $CD4^+$ T cells by negative selection. Single cell suspensions were incubated with anti-CD8 (2.43), anti-B220 (RA3), anti-MAC-1(M1/70.15), F4/80, and anti-MHC class II (M/5114) culture supernatants for 30 min at 4°C followed by incubation with microbead-conjugated goat anti–rat IgG (PolySciences, Inc.) for 30 min at 4°C. Cell preps were placed on a Bio-Mag magnetic stand (PolySciences, Inc.) and the negative fraction was harvested. $CD4^+25^+$ T cells were subsequently labeled with biotin–anti-CD25 (7D4), APC-anti-CD4 (RM4-5), and streptavidin–PE and purified by flow cytometry

*Abbreviations used in this paper: 3dTx, thymectomized on day 3 of life; DP, double positive; IBD, inflammatory bowel syndrome; Rag-2°, recombination activating gene 2–deficient; SP, single positive.

on a FACSVantage™ Cell Sorter (Becton Dickinson). The purity of $CD4^+25^+$ T cells was >95%.

Proliferation Assays. In anti-CD3ϵ stimulated proliferation assays, $CD4^+25^-$ cells (5×10^4) were cultured in 96-well plates with MHC class II–deficient APCs (10^5) or B6 APCs as indicated, 1 μg/ml anti-CD3ϵ, and the indicated numbers of $CD4^+25^+$ cells for 72 h at 37°C/5% CO_2. In the K14-A_β^b anti-B6 MLR or H2-DMα–deficient anti-B6 MLR, either K14-A_β^b or H2-DMα–deficient $CD4^+25^-$ cells (5×10^4) were cultured in 96-well plates with 1–2 $\times 10^5$ irradiated, T cell–depleted B6 or H2-DMα–deficient spleen cells as indicated. B6, MHC class II–deficient, or H2-DMα–deficient stimulators (APCs) were initially prepared by erythrocyte lysis with ACK buffer. APCs were incubated with anti-Thy 1.2 (MMT1), anti-CD4 (172.4), and anti-CD8 (TIB-211) for 45 min on ice. APCs were subsequently incubated with rabbit complement (Accurate Antibodies) for 45 min at 37°C. APCs were then irradiated at 2,500 rad of γ-irradiation. MLRs were cultured for 96 h at 37°C/5% CO_2, pulsed with ^{3}[H]thymidine for the final 16 h, and harvested on an automatic harvester (Wallac). All proliferation experiments were set up in triplicate and results are expressed as a mean of triplicates.

CFSE Labeling and Adoptive Transfers. Donor $CD4^+$ T cells were purified and enriched from spleen and LNs by negative selection

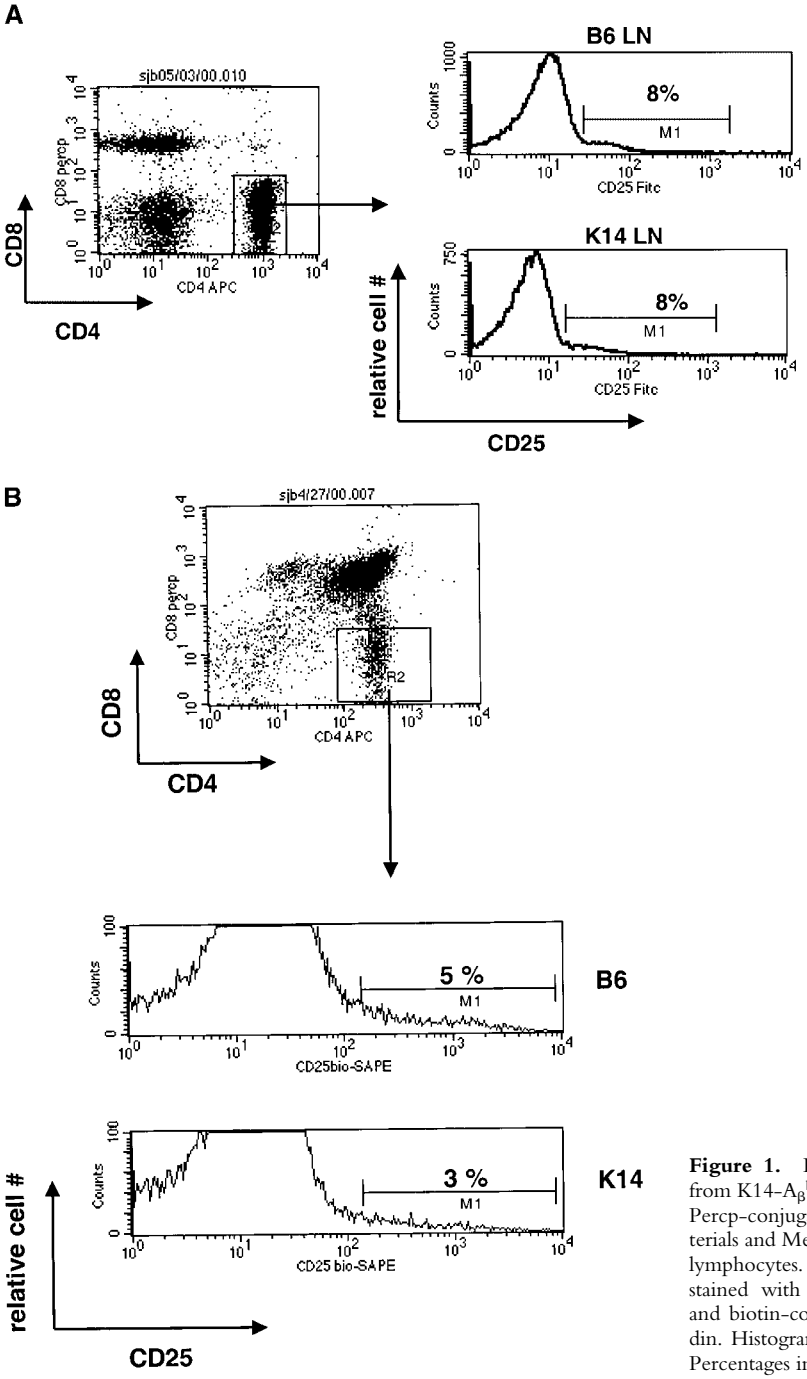

Figure 1. K14-A_β^b mice have $CD4^+25^+$ T cells. (A) Total LN cells from K14-A_β^b or B6 (WT) were stained with APC-conjugated anti-CD4, Percp-conjugated anti-CD8, and FITC-conjugated anti-CD25 as in Materials and Methods. Percentages indicated in histograms are of live $CD4^+$ lymphocytes. (B) Total thymocytes from K14-A_β^b or B6 (WT) mice were stained with APC-conjugated anti-CD4, Percp-conjugated anti-CD8, and biotin-conjugated anti-CD25 followed by PE-conjugated streptavidin. Histograms are gated through live CD4-single positive thymocytes. Percentages indicated are of live CD4-single positive thymocytes.

as indicated above. Single cell suspensions were labeled with CFSE (Molecular Probes) as described previously (28). A total of 1–3 × 10^7 CFSE-labeled C57BL/6 $CD4^+$ T cells were injected intravenously into indicated mice in a total volume of 0.2 mL sterile PBS. Recipients were killed at 72 h and spleen and LNs were harvested. Total spleen and LN cells were purified, labeled with APC–anti-CD4 (RM4-5) and analyzed by flow cytometry as above.

Induction of Inflammatory Bowel Disease. For the preparation of $CD45RB^{high}$ CD4 T cells, C57Bl/6-Thy1.1 splenic single cell suspensions were first enriched for $CD4^+$ by positive selection on midiMacs™ columns (Miltenyi Biotec) according to the manufacturer's instructions. In brief, cells were first incubated with anti-CD4–FITC antibodies for 20 min on ice in PBS supplemented with 0.5% FCS, then incubated in the same buffer with anti-FITC microbeads for 15 min. The magnetically labeled positive fraction was retained on a midiMacs column. After enrichment, the cells were then labeled with anti-CD45RB-PE antibodies for 20 min on ice and then sorted on a $FACStar^{Plus}$™ (Becton Dickinson). For CD45RB, the brightest 40–50% of $CD4^+$ cells were sorted as "high." The purity of the population was >96%. For the preparation of $CD25^+$ CD4 T cells, splenic single cell suspensions were enriched by positive selection on midiMacs columns with anti-CD25–FITC or biotin-labeled antibodies followed by anti-FITC or streptavidin–PE magnetic microbeads. Given that class II° mice contain very low numbers of $CD25^+$ CD4 T cells, instead of sorting, one or two additional rounds of magnetic sorting was performed. For all populations, >95% of the CD4 T cells were $CD25^+$.

6–8-wk-old C57Bl/6 RAG-2° mice were injected intravenously with 3 × 10^5 $CD4^+CD45RB^{high}$ T cells either alone or with 3 × 10^5 purified $CD4^+25^+$ T cells. Mice were monitored and weighed every other day and killed if they lost 20% of their initial body weight, had extensive diarrhea, or appeared morbidly ill. Whole intestines were first flushed extensively to eliminate the lumen content, then longitudinally opened and cut into 0.5 cm pieces for fixation in PBS-buffered formalin before sectioning. Paraffin sections were stained with H&E.

Results

$CD4^+CD25^+$ Regulatory T Cells Are Present in K14-A_β^b Mice. To understand the thymic requirements for the development of $CD4^+25^+$ T cells, we compared the phenotype and function of these cells in K14-A_β^b and wild-type C57BL/6 (B6) mice. Flow cytometric analysis of LNs and spleen revealed that peripheral K14-A_β^b $CD4^+$ T cells expressed CD25 (Fig. 1 A). Like B6 mice, K14-A_β^b $CD4^+CD25^+$ cells are also found in the mature CD4 SP thymocyte pool (Fig. 1 B).

To determine if these cells have immunoregulatory characteristic similar to their wild-type counterparts, purified $CD4^+CD25^+$ T cells from K14-A_β^b or B6 animals were cultured for 3 d with T cell–depleted, MHC class II–deficient APCs and soluble anti-CD3ε antibody, with or without exogenous rIL-2. In this and the following assay, MHC class II–deficient APCs were used as K14-A_β^b $CD4^+$ cells respond to I-A^b-positive (B6) APCs (21). $CD4^+CD25^+$ T cells from both B6 mice and K14-A_β^b mice did not proliferate to anti-CD3ε stimulation unless provided with exogenous rIL-2 (Fig. 2 A). Thus, these data demonstrate that both K14-A_β^b and B6 $CD4^+CD25^+$ T cells are anergic in vitro.

To examine suppressor function (29, 30), a fixed number (5 × 10^4 cells) of K14-A_β^b or wild- type B6 $CD4^+CD25^-$ effector T cells were cultured with T cell–depleted, MHC class II–deficient APCs, and soluble anti-CD3ε in the presence of increasing numbers of $CD4^+CD25^+$ lymphocytes from the same strain. Proliferation of responder K14-A_β^b and B6 $CD4^+25^-$ T cells was inhibited by the addition of $CD4^+25^+$ T cells to cultures in a dose-dependent fashion (Fig. 2 B). The ratios of K14-A_β^b $CD4^+25^+$ cells required to suppress the proliferation of a fixed number of effector cells were equivalent to wild-type regulatory cells. For example, a final effector to responder ratio of 1:1 resulted in

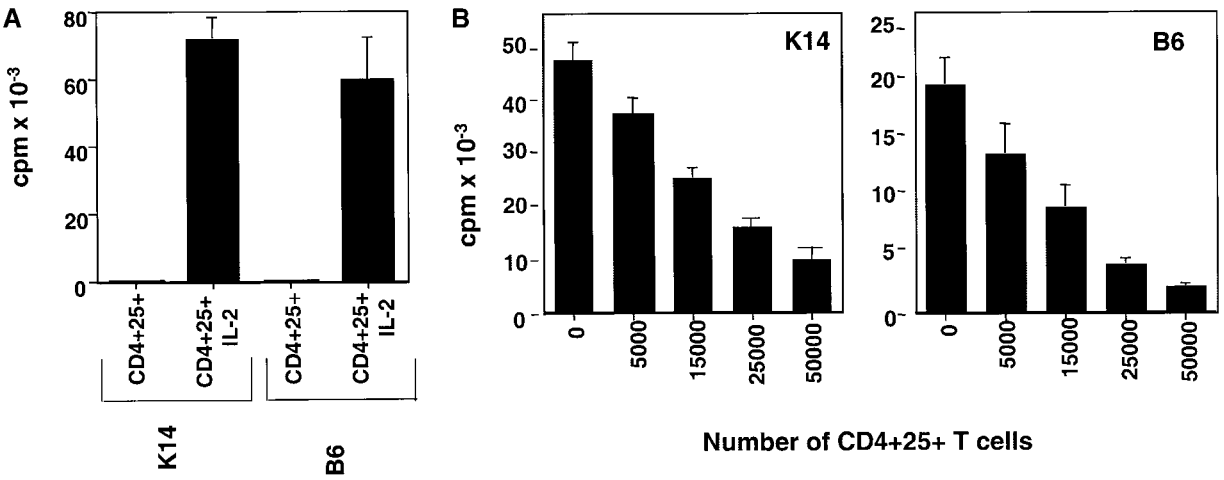

Figure 2. K14-A_β^b $CD4^+25^+$ cells are anergic and suppressive. (A) $CD4^+25^+$ cells (5 × 10^4) from K14-A_β^b or B6 (WT) were cultured with MHC class II-negative APCs (10^5), soluble anti-CD3ε (1 μg/ml), and rIL-2 (10 U/ml) for 72 h, pulsed with [^{3}H]thymidine for 16 h and harvested. (B) $CD4^+25^-$ cells (5 × 10^4) from K14-A_β^b or B6 mice were incubated with MHC class II-negative APCs (10^5), soluble anti-CD3ε (1 μg/ml), and the indicated number of $CD4^+25^+$ cells from K14-A_β^b or B6 mice for 72 h, pulsed with [^{3}H]thymidine for the final 16 h and harvested. Results are representative of three experiments.

an 80% inhibition of $CD4^+25^-$ proliferation in both K14-A_β^b and B6 cultures. Thus, K14-A_β^b $CD4^+25^+$ T cells appear functionally indistinguishable from wild-type regulatory $CD4^+25^+$ T cells.

Development of $CD4^+25^+$ Regulatory T Cells Requires MHC Class II Expression. K14-A_β^b mice were generated on a MHC class II–deficient background and MHC class II–deficient mice contain $CD4^+$ T cells which are restricted to classical and nonclassical MHC class I antigens (31). Thus, it was possible that the $CD4^+25^+$ T cells present in K14-A_β^b mice were selected on MHC class I antigens. We addressed this possibility by (i) examining the phenotype and function of $CD4^+25^+$ T cells in MHC class II–deficient mice and (ii) generating K14-A_β^b mice lacking β2-microglobulin (β_2m) and MHC class I antigens (designated K14-$A_\beta^b/\beta_2m^{-/-}$ in this text).

A significant fraction of the residual $CD4^+$ T cells present in LNs and the spleen of MHC class II–deficient

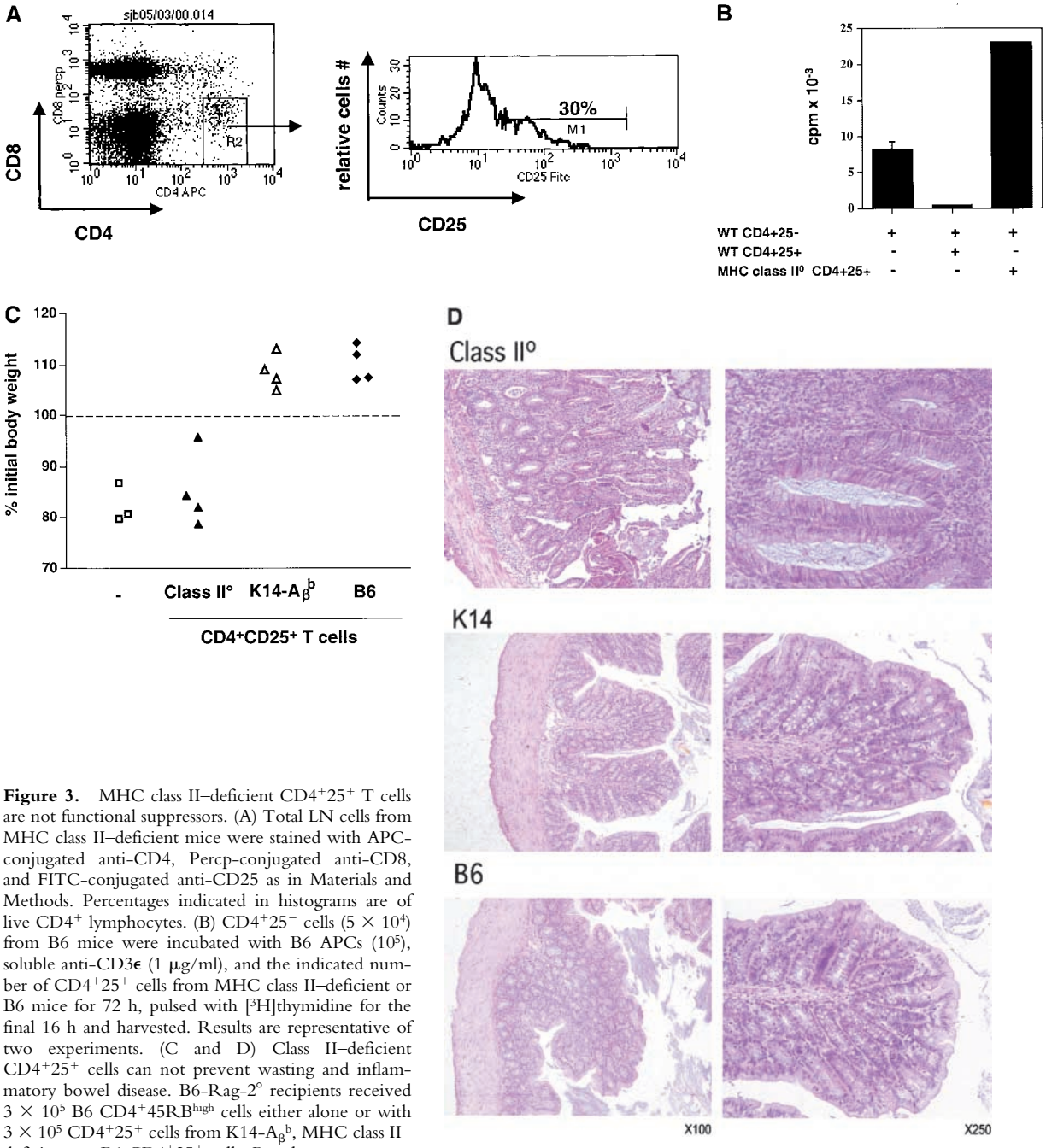

Figure 3. MHC class II–deficient $CD4^+25^+$ T cells are not functional suppressors. (A) Total LN cells from MHC class II–deficient mice were stained with APC-conjugated anti-CD4, Percp-conjugated anti-CD8, and FITC-conjugated anti-CD25 as in Materials and Methods. Percentages indicated in histograms are of live $CD4^+$ lymphocytes. (B) $CD4^+25^-$ cells (5×10^4) from B6 mice were incubated with B6 APCs (10^5), soluble anti-CD3ε (1 μg/ml), and the indicated number of $CD4^+25^+$ cells from MHC class II–deficient or B6 mice for 72 h, pulsed with [^{3}H]thymidine for the final 16 h and harvested. Results are representative of two experiments. (C and D) Class II–deficient $CD4^+25^+$ cells can not prevent wasting and inflammatory bowel disease. B6-Rag-2° recipients received 3×10^5 B6 $CD4^+45RB^{high}$ cells either alone or with 3×10^5 $CD4^+25^+$ cells from K14-A_β^b, MHC class II–deficient, or B6 $CD4^+25^+$ cells. Results are representative of two experiments. (C) Shown are the percentages of initial body weight for individual mice on the day of killing. (D) H&E histology of colonic sections from individual animals which received effector cells and either MHC class II–deficient, K14-A_β^b, or B6 $CD4^+25^+$ cells. Note the extensive mononuclear cell infiltrates, mucosal hyperplasia, and crypt abscesses in the recipients of MHC class II–deficient $CD4^+25^+$ cells.

mice express CD25 (Fig. 3 A, and data not shown). To examine the suppressor function of these cells, a fixed number of B6 $CD4^+CD25^-$ effector T cells were cultured with T cell–depleted, B6 APCs, and soluble anti-CD3ϵ. In addition, indicated cultures (Fig. 3 B) received an equal number of FACS®-sorted B6 or MHC class II–deficient $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ T cells from MHC class II–deficient mice did not suppress the proliferation of wild-type effector T cells. Rather, the addition of these cells to B6 $CD4^+CD25^-$ effector T cells resulted in a two to threefold increase in the proliferation of cultured cells (Fig. 3 B). Thus, MHC class II–deficient $CD4^+CD25^+$ T cells are functionally distinct from B6 $CD4^+CD25^+$ T cells as they do not have regulatory activity.

FACS®-sorted $CD4^+25^+$ lymphocytes from K14-A_β^b/$\beta_2m^{-/-}$ (MHC class I–deficient) mice cultured with responders at a final effector to responder ratio of 1:1 resulted in an 85% inhibition of proliferation (data not shown). Thus, these cells have the same suppressive capabilities as those from MHC class I–sufficient K14-A_β^b mice, suggesting again that selection of these cells is MHC class II dependent. Taken together, these data suggest that the immunoregulatory $CD4^+25^+$ T cells which are present in K14-A_β^b mice require MHC class II expression on cortical epithelium for their selection.

Thymic Expression of MHC Class II Is Sufficient for the Generation of Regulatory $CD4^+25^+$ Cells. The suppressive function of immunoregulatory $CD4^+25^+$ T cells is acquired in the thymus (18, 19) and purified K14-A_β^b $CD4^+25^+$ SP thymocytes did not proliferate to TCR signals unless exogenous rIL-2 was added to cultures (Fig. 4 A). Furthermore, K14-A_β^b $CD4^+25^+$ SP thymocytes suppressed proliferation of anti–CD3ϵ-treated K14-A_β^b $CD4^+25^-$ effector cells similarly to peripheral K14-A_β^b $CD4^+25^+$ T cells examined in parallel (Fig. 4 A). Thus, K14-A_β^b $CD4^+25^+$ thymocytes are anergic and suppressive. These data confirm that selection on MHC class II-positive cortical epithelium is sufficient for the

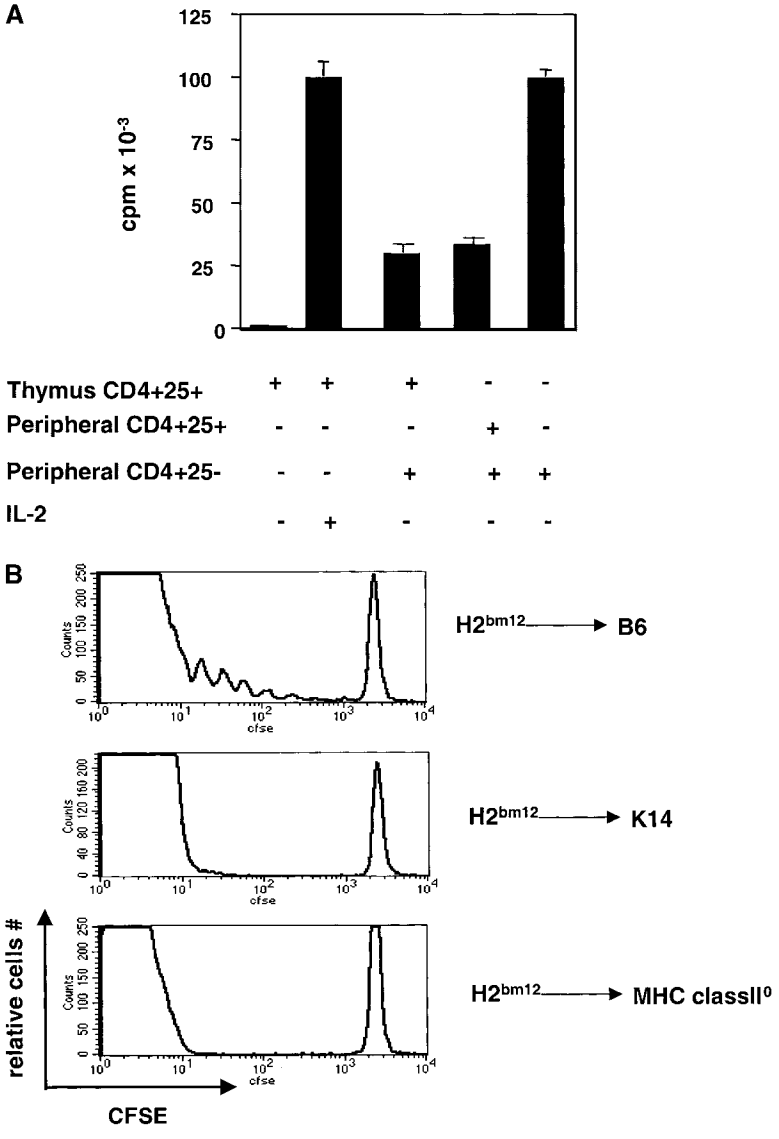

Figure 4. K14-A_β^b $CD4^+25^+$ T cells are generated in the thymus. (A) $CD4^+25^-$ cells (5×10^4) from K14-A_β^b mice were incubated with MHC class II-negative APCs (10^5), soluble anti-CD3ϵ (1 μg/ml), and the indicated number of K14-A_β^b $CD4^+25^+$ thymocytes or peripheral K14-A_β^b $CD4^+25^+$ cells for 72 h, pulsed with [^{3}H]thymidine for 16 h, and harvested. Results are representative of three experiments. (B) CFSE-labeled, alloreactive H-$2^{bm12}CD4^+$ cells (10^7) were intravenously injected into indicated hosts. After 72 h animals were killed. LN and spleen cells were stained with APC-conjugated anti-CD4, and analyzed by FACS®. Histograms are gated on live $CD4^+$ splenocytes. This figure is representative of four independent experiments.

complete development of $CD4^+25^+$ immunoregulatory T cells.

The suppressive ability of K14-A_β^b $CD4^+25^+$ cells suggests that peripheral expression of MHC class II molecules is not required for the functional and phenotypic development of these cells. However, it remained possible that these immunoregulatory thymocytes could derive from activated recirculating cells (32). Therefore, it was important to establish that K14-A_β^b mice do not express functional MHC class II antigens in the periphery.

The division of transferred CFSE-labeled alloreactive $CD4^+$ T cells is a very sensitive measure of functional MHC class II expression in host mice (33, 34). We transferred alloreactive CFSE-labeled H-2^{bm12} $CD4^+$ T cells into K14-A_β^b, MHC class II–deficient, or B6 mice and examined their proliferation after 72 h. Alloreactive H-2^{bm12} $CD4^+$ T cells extensively divide 72 h after transfer into B6 hosts and localize in the spleen (Fig. 4 B) and LNs (data not shown). In contrast, no cells undergoing division were identified 72 h after H-2^{bm12} $CD4^+$ T cells were transferred into either K14-A_β^b or class II–deficient mice. Similar results were obtained when host mice were sublethally irradiated with 450 rads of whole body γ-irradiation 48 h before adoptive transfer (data not shown). These results do not directly address the requirement for peripheral antigens in the development of regulatory T cells which can suppress organ-specific autoimmune disease. However, they do supply functional corollaries to our earlier immunohistochemical and FACS® data that MHC class II antigens are not available in the periphery of K14-A_β^b mice (21), and suggest that peripheral cognate T cell–MHC interactions are not required for the development of $CD4^+25^+$ regulatory cells.

K14-A_β^b $CD4^+25^+$ T Cells Prevent Inflammatory Colitis. To examine their in vivo function, $CD4^+25^+$ fractions were sorted from B6, K14-A_β^b, or MHC class II–deficient mice and tested for their ability to inhibit the colitis induced by transfer of $CD4^+45RB^{high}$ cells into Rag-2° recipients. It has been reported that severe combined immunodeficient mice reconstituted with 3×10^5 $CD4^+45RB^{hi}$ B6 effector T cells develop an inflammatory bowel syndrome (IBD) which results in severe wasting and histologically resembles Crohn's disease (35, 36). In this model, the wasting and IBD can be controlled by the cotransfer of $CD4^+25^+$ T cells (37, 38).

B6 $CD4^+45RB^{high}$ effector CD4 cells were transferred into B6 Rag-2° mice either alone or with $CD4^+CD25^+$ cells purified from K14-A_β^b, class II°, or B6 mice. Animal weight was monitored every other day and animals were killed either when they had lost 20% of their body weight, had significant diarrhea, or between days 40 and 44.

Effector $CD4^+45RB^{high}$ cells alone induced an overt wasting disease with diarrhea and weight loss (Fig. 3 C) associated with colonic thickening and inflammatory infiltration of the colon (data not shown). Cotransfer of either B6 or K14-A_β^b $CD4^+CD25^+$ cells prevented intestinal inflammation; recipient mice gained weight over the course of the experiment and had normal colonic histology (Fig. 3, C and D). In contrast, recipients of cotransfered $CD4^+45RB^{high}$ effector T cells and MHC class II–deficient $CD4^+CD25^+$ T cells developed severe wasting diarrhea and weight loss. These symptoms were associated with chronic colitis with hyperplasia of the glandular mucosae, increased numbers of crypts, and diffuse mononuclear cell infiltration. These results support the in vitro observations that K14-A_β^b $CD4^+25^+$ regulatory T cells are functionally similar to the regulatory T cells present in wild-type mice. Secondly, the population of $CD4^+$ cells present in MHC class II–deficient mice which expresses CD25 (presumably as an activation marker) do not have this regulatory function.

The K14-A_β^b $CD4^+25^+$ Repertoire Contains Autoreactive Cells. The experiments presented above show that regulatory cells which developed in either B6 or K14-A_β^b mice could inhibit an inflammatory process mediated by B6 effector cells. However, the effector T cell responses in this model of IBD are promoted by bacterial antigens, rather than self-antigens (37). Therefore, we asked if $CD4^+25^+$ T cells could control the activation of a polyclonal self-reactive repertoire. K14-A_β^b $CD4^+$ T cells proliferate vigorously in vitro to B6 APCs (21). These in vitro MLRs were performed using unfractionated K14-A_β^b $CD4^+$ T cells in which the ratio of $CD25^-$ to $CD25^+$ cells is ~10–15:1. Ratios of K14-A_β^b $CD25^+$ regulatory to $CD25^-$ effector cells of 1:1 are required to inhibit 80% of anti-CD3ε-induced proliferation (Fig. 2 B). Therefore, we examined the regulatory function of $CD4^+25^+$ cells in an MLR at a responder to suppressor ratio of 1:1. Purified K14-A_β^b $CD4^+25^-$ effector proliferated significantly to γ-irradiated, T cell–depleted B6 hematopoietic APCs in an MLR. The addition of an equal number of K14-A_β^b $CD4^+25^+$ T cells to this MLR suppressed the proliferation of B6-reactive K14-A_β^b responder cells in this MLR to 10% of the unsuppressed level (Fig. 5 A). Strikingly, $CD4^+25^+$ cells purified from B6 mice were unable to significantly suppress the anti-B6 proliferation of K14-A_β^b $CD4^+25^-$ responders at similar ratios (Fig. 5 A).

It was possible that B6 $CD4^+25^+$ T cells failed to inhibit the K14-A_β^b anti-B6 MLR because regulatory T cells only suppressed the activity of syngeneic cells. However, after stimulation via anti-CD3ε added to the culture, B6 $CD4^+25^+$ T cells could suppress the K14-A_β^b $CD4^+25^-$ anti-B6 MLR (Fig. 5 A). $CD4^+25^+$ T cells require TCR signals to become active suppressors (29, 30) and these results suggest that B6 $CD4^+25^+$ T cells were not activated by B6 APCs, and therefore could not suppress a K14-A_β^b anti-B6 MLR. In contrast, K14-A_β^b $CD4^+25^+$ cells received activation signals from B6 hematopoietic APCs to block an I-A^b reactive MLR.

This pattern of restriction of the $CD25^+$ fraction of $CD4^+$ cells mirrors total $CD4^+$ T cells from these respective mice: K14-A_β^b $CD4^+$ T cells proliferate in MLR to syngeneic B6 APCs; B6 $CD4^+$ T cells do not (21). To directly test if K14-A_β^b $CD4^+25^+$ T cells were reactive to I-A^b-positive APCs, we cultured FACS®-purified K14-A_β^b or B6 $CD4^+25^+$ lymphocytes (from LNs and spleen) with

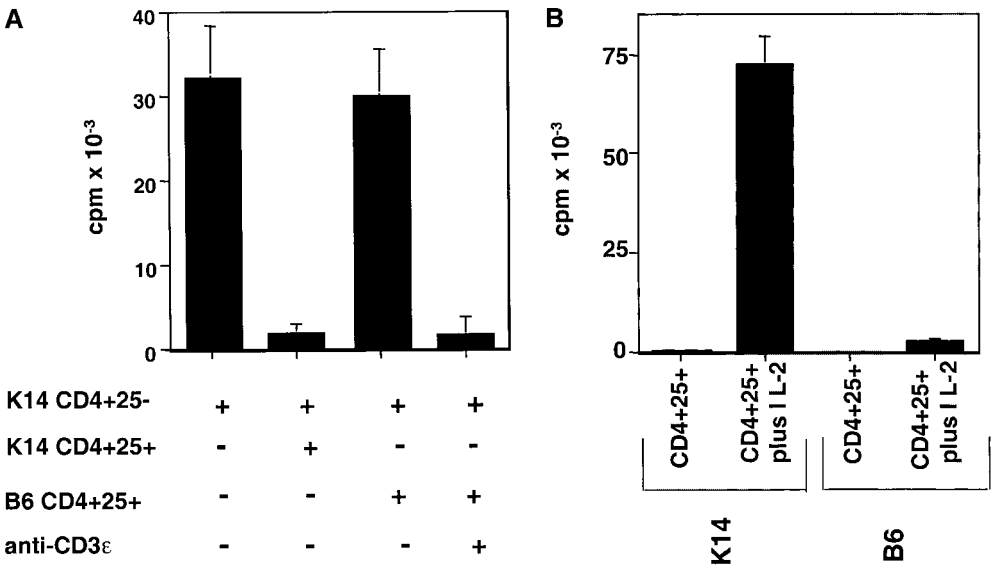

Figure 5. K14-A_β^b $CD4^+25^+$ cells are activated by I-A^b-positive APCs. (A) $CD4^+25^-$ T cells (5×10^4) from K14-A_β^b mice were incubated with T cell–depleted B6 splenocytes. The indicated wells also received either K14-A_β^b $CD4^+25^+$ cells (5×10^4), B6 $CD4^+25^+$ cells (5×10^4), or WT $CD4^+25^+$ cells (5×10^4), and anti-CD3ε (1 μg/ml). Wells were cultured for 96 h and pulsed with [^{3}H]thymidine for 16 h and harvested. (B) $CD4^+25^+$ cells (5×10^4) from K14-A_β^b or B6 mice were cultured with B6 splenocytes (10^5) and rIL-2 (10 U/ml) in the indicated wells for 96 h, pulsed with [^{3}H]thymidine for the final 16 h, and harvested. Results are representative of three experiments.

γ-irradiated, T cell–depleted B6 splenocytes and measured the proliferative response. We previously observed that $CD4^+25^+$ T cells were unresponsive to stimulation with anti-CD3ε Ab. Consistent with these results, neither B6 nor K14-A_β^b $CD4^+25^+$ cells proliferated in the presence of B6 APCs. However, when exogenous rIL-2 was added to cultures, K14-A_β^b $CD4^+25^+$ T cells proliferated to the same extent as K14-A_β^b $CD4^+25^-$ when stimulated by B6 APCs. In contrast, B6 $CD4^+25^+$ T cells proliferated only minimally (Fig. 5 B). Furthermore, the addition of Y-3P (39), an anti-I-A^b–blocking antibody, to cultures significantly inhibited the proliferation of K14-A_β^b $CD4^+25^+$ T cells (data not shown) confirming that the reactivity of these cells is I-A^b dependent. Thus, the specificity of K14-A_β^b $CD4^+25^+$ cells is significantly different from B6 mice but similar to the $CD4^+25^-$ effector cells from K14 mice.

The H-2DMα–deficient $CD4^+25^+$ Repertoire also Contains I-A^b Reactive Suppressor Cells. Both $CD4^+25^-$ effector cells and $CD4^+25^+$ regulatory T cells in K14-A_β^b mice have significant reactivity to syngeneic I-A^b-positive B6 APCs. Based on our observation that K14-A_β^b $CD4^+25^+$ T cells contain an autoreactive repertoire we hypothesized that, similarly to $CD4^+$ effector cells, $CD4^+25^+$ T cells which react to MHC class II-positive hematopoietic thymic APCs undergo clonal deletion. To extend this hypothesis, we examined the function of $CD4^+25^+$ T cells in another model of attenuated negative selection, the H2-DMα–deficient mouse (22–24).

Flow cytometric analysis demonstrated that $CD4^+25^+$ cells were present in the thymus and periphery of H-2DMα-deficient mice (Fig. 6 A, and data not shown). H2-DMα–deficient $CD4^+$ cells react in MLRs against wild-type (B6) APCs loaded with a broad array of I-A^b–associated peptides (22–24). Therefore, we asked if, similar to K14-A_β^b regulatory cells, H-2DMα–deficient $CD4^+25^+$ T cells could be activated by B6 hematopoietic APCs to suppress this proliferation, confirming a role for negative selection in shaping the $CD4^+25^+$ repertoire. The addition of purified H-2DMα–deficient $CD4^+25^+$ suppressor cells to H-2DMα–deficient $CD4^+25^-$ T cells culture at a ratio of 1:1 inhibited this proliferation to <1% of effector T cell proliferation. Again, FACS®-sorted $CD4^+25^+$ cells from B6 mice had no significant effect on this anti–I-A^b proliferation, unless TCR signals were provided by the addition of antibodies to CD3ε (Fig. 6 B).

To determine if, similar to K14-A_β^b regulatory cells, H2-DMα–deficient $CD4^+25^+$ T cells were activated by B6 I-A^b molecules expressing a broad array of peptides, $CD4^+25^+$ cells were cocultured with either self H2-DMα–deficient APCs or B6 APCs with or without exogenous rIL-2. As shown in Fig. 6 C, H2-DMα–deficient regulatory cells did not proliferate significantly to self H2-DMα–deficient APCs in the presence of rIL-2. In contrast, H2-DMα–deficient $CD4^+25^+$ T cells proliferated vigorously when stimulated with B6 APCs in the presence of exogenous rIL-2. Thus, $CD4^+25^+$ T cells are tolerant to the repertoire of self-peptides on hematopoietic APCs and reactive to foreign antigen suggesting that the repertoire of these cells grossly mirrors that of the effector $CD4^+$ T cell populations which develop in the same thymus.

Discussion

It is generally accepted that $CD4^+25^+$ T cells are generated in the thymus, however, the selection events involved in the development of these cells remain poorly defined. In this report, we have taken a reductionist approach to determine the cognate interactions which are sufficient for the complete development of $CD4^+25^+$ immunoregulatory T cells. We find that $CD4^+25^+$ T cells are present in the thymus and periphery of K14-A_β^b and DMα-deficient mice. Like their wild-type counterparts, these cells do not proliferate to TCR-mediated stimulation unless exogenous IL-2 is provided, and their activation dominantly suppresses the proliferation of cocultured responder $CD4^+$ T cells. We

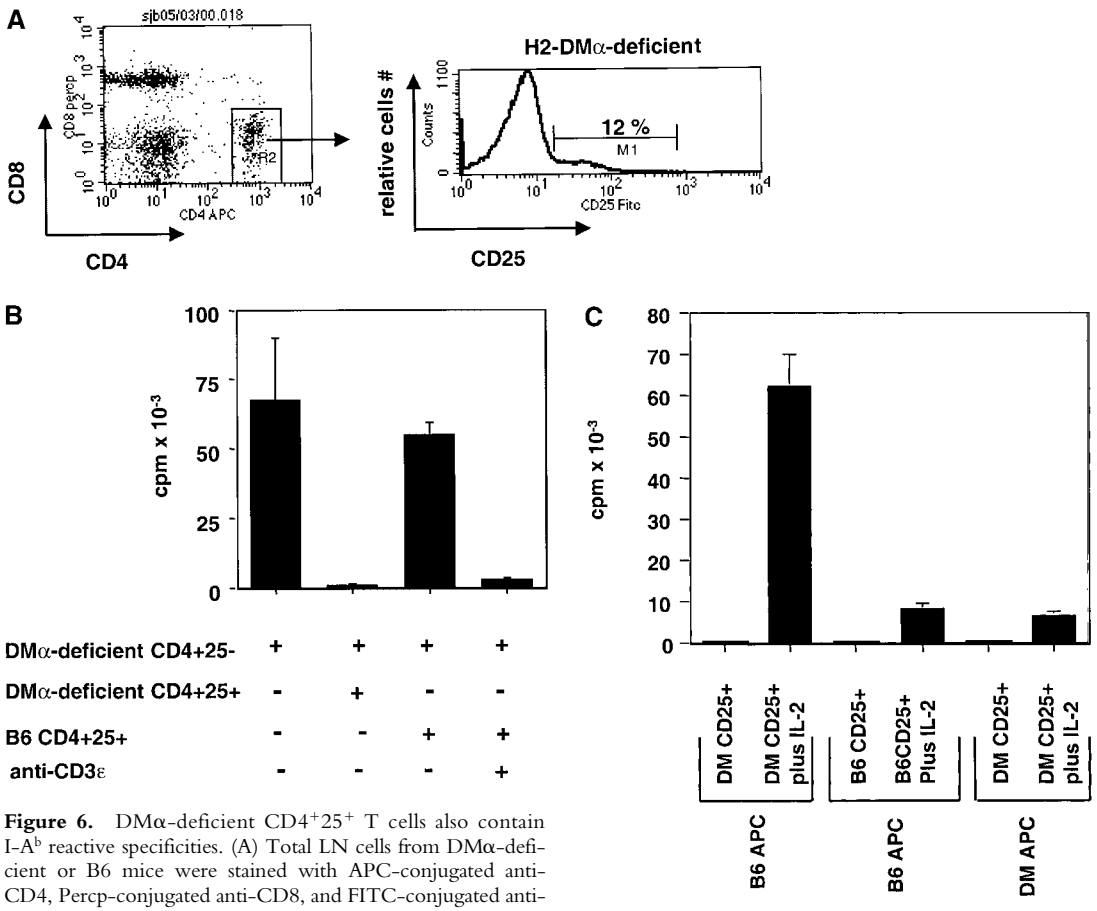

Figure 6. DMα-deficient $CD4^+25^+$ T cells also contain I-A^b reactive specificities. (A) Total LN cells from DMα-deficient or B6 mice were stained with APC-conjugated anti-CD4, Percp-conjugated anti-CD8, and FITC-conjugated anti-CD25. Percentages in histogram are live $CD4^+$ lymphocytes. (B) $CD4^+25^-$ cells (5×10^4) from DMα-deficient or B6 mice were incubated with T cell–depleted B6 splenocytes (2×10^5). Additionally, indicated wells received either DMα-deficient $CD4^+25^+$ cells (5×10^4), B6 $CD4^+25^+$ cells (5×10^4), or B6 $CD4^+25^+$ cells (5×10^4) and anti-CD3ε (1 μg/ml). Wells were cultured for 96 h and pulsed with [^{3}H]thymidine for the final 16 h and harvested. (C) $CD4^+25^+$ cells (5×10^4) from DMα-deficient or B6 mice were cultured with either DMα-deficient or B6, T cell–depleted splenocytes (2×10^5), and rIL-2 (10 U/ml) as indicated, for 96 h. Wells were pulsed with [^{3}H]thymidine for the final 16 h and harvested. Results are representative of three experiments.

also demonstrate that the repertoire of $CD4^+25^+$ T cells from two distinct models of attenuated negative selection, K14-$A_\beta{}^b$ and DMα-deficient mice, contain I-A^b reactive specificities, whereas B6 mice do not. Taken together, these data indicate that $CD4^+25^+$ T cells are selected on thymic cortical epithelium and that cognate interactions with MHC class II on cortical epithelium are sufficient for the development of $CD4^+25^+$ regulatory T cells. Our data also suggest that a subset of developing $CD4^+25^+$ T cell population are subject to negative selection on hematopoietic APCs.

Thymic Generation of $CD4^+25^+$ Cells. To prove that the regulatory population of $CD4^+25^+$ cells was class II dependent, we examined the phenotype and function of $CD4^+25^+$ T cells in MHC class II-negative mice. As we noted above, a significant percentage of the $CD4^+$ T cells remaining in MHC class II–deficient mice are CD25-positive (Fig. 3 A). However, we found that $CD4^+25^+$ T cells purified from MHC class II-negative mice do not suppress the proliferation of cocultured effector $CD4^+$ T cells in vitro (Fig. 3 B). Hence, $CD4^+25^+$ T cells in MHC class II-negative mice are functionally distinct from $CD4^+25^+$ immunoregulatory T cells. Therefore, the development of regulatory $CD4^+25^+$ T cells requires the thymic expression of MHC class II molecules.

Previous examination of $CD4^+25^+$ T cell development suggested that thymic differentiation of these immunoregulatory T cells occurred at a late stage of $CD4^+$ T cell development. These conclusions were based on BrdU labeling studies which demonstrated that these cells were not derived from $CD25^+$ $CD4^+8^+$ double positive (DP) thymocytes and, separately, that expression of the CD25 antigen was not apparent until the $CD4^+$ SP stage (17, 19). Early models proposed that the differentiation of immunoregulatory CD4 $^+$T cells resulted from cognate interactions with self-antigens on hematopoietic APCs of sufficient affinity for the induction of anergy and the acquisition of suppressive characteristics but of insufficient affinity to induce clonal deletion (18, 40). In contrast, a recent report has used transgenic/bone marrow chimeras to show that immunoregulatory $CD4^+25^+$ T cells are generated by high affinity interactions with self-peptide on thymic epithelium rather than hematopoietic APCs (19). This developmental pathway is reminiscent of the tissue requirements for the induction of nondeletional transplant tolerance to epithelial

antigens which is also mediated by regulatory $CD4^+$ cells (10). Indeed, Coutinho and his colleagues first proposed that regulatory cells developed after high avidity interactions with thymic epithelium. However, in both of these model systems the individual role of cortical and medullary epithelium could not be distinguished. Our observation that immunoregulatory $CD4^+25^+$ T cells are present in K14-A_β^b (Fig. 1 A) suggests that, similar to other $CD4^+$ T cells, positive selection on cortical epithelium is necessary and sufficient for their differentiation from DP precursors.

Our experiments do not rule out the possibility that medullary epithelium and bone marrow–derived APCs play an inductive role that does not require MHC–TCR interactions. Punt, Singer, and colleagues have suggested that such a two-step strategy of clonal deletion operates to purge autoreactive DP thymocytes which encountered cognate self-antigen on cortical epithelium and the development of regulatory T cells could have a similar requirement (41). The current systems do not permit us to distinguish these possibilities.

$CD4^+25^+$ T Cells Do Not Require Peripheral MHC Expression for Development. It has been suggested in an animal model of thyroiditis that antigen-specific regulatory T cells require the relevant autoantigen in the periphery for complete function (42). However, this study monitored the onset of autoimmune disease as a marker of the presence of functional regulatory cells and did not distinguish development of suppressor cells from either persistence of these cells in the peripheral T cell pool or activation to suppress autoreactivity. Additionally, recent examination by Tung and his colleagues of autoimmune oophoritis showed that persistence of a relevant self-antigen in the periphery is required for the maintenance of T cell tolerance (43). Our results indicate that cognate recognition of MHC class II molecules in the periphery is not an absolute requirement for development of the regulatory population of $CD4^+$ cells in that we have clearly shown that there is a significant number of functional $CD4^+25^+$ T cells in the periphery of K14-A_β^b mice (Fig. 1 A and 2 B). While it remains possible that K14-A_β^b mice express MHC class II on squamous epithelium (expression can be induced on keratinocytes during inflammation, unpublished data), the expression levels in disease-free mice are below our ability to detect by immunohistochemistry or FACS® (21) and insufficient to drive allogeneic $CD4^+$ T cell proliferation (Fig. 3 C). As K14-A_β^b $CD4^+25^+$ T cells can be activated by B6 APCs to suppress a K14-A_β^b anti-B6 MLR, these cells recognize self-antigens on I-A^b-positive hematopoietic APCs which are not present in either the thymus or the periphery of K14-A_β^b mice. We have not directly examined suppression of organ-specific autoimmunity and it remains possible that activation of suppressor cells in the periphery requires the presence of the inciting autoantigen. Nevertheless, our data indicate that peripheral MHC class II is not required for the development of functional $CD4^+25^+$ immunoregulatory T cells.

$CD4^+25^+$ T Cells Can Be Subject to Deletion. In K14-A_β^b mice, the thymic cell types which can mediate clonal deletion (hematopoietic APCs and medullary epithelium) are MHC class II-negative. In this setting, we have previously shown that ~15% of the K14-A_β^b $CD4^+$ T cell repertoire responds to B6 hematopoietic APCs (21). Therefore, it is striking that the MHC class II antigen reactivity of K14-A_β^b $CD4^+25^+$ T subset mirrors this autoreactivity and is significantly different from that of B6 $CD4^+25^+$ T cells.

Activation of regulatory $CD4^+25^+$ T cells by self-antigens suppresses autoimmunity. It is, thus, quite surprising that the peripheral $CD4^+25^+$ T cell population of B6 mice neither proliferates to nor suppresses an anti-B6 MLR response. These data indicate that the repertoire of B6 $CD4^+25^+$ T cells differs significantly from K14-A_β^b $CD4^+25^+$ T cells in that it has been purged of cells specific for self-hematopoietic APCs. The restriction pattern of $CD4^+25^+$ T cells seems to mirror the repertoire of the effector $CD4^+$ T cell populations selected in the same thymus. It could be argued that the $CD4^+$ T cell specificities generated in K14-A_β^b mice represent the positively selected $CD4^+$ T cell repertoire before efficient clonal deletion. Thus, our observation that K14-A_β^b $CD4^+25^+$ T cells, but not B6 $CD4^+25^+$ T cells, are activated by syngeneic APCs suggests that expression of MHC class II on hematopoietic APCs results in the deletion of a subset of $CD4^+25^+$ thymocytes that are reactive to self-antigens expressed on hematopoietic APCs.

We turned to H2-DMα–deficient mice to extend these observations about the repertoire of $CD4^+25^+$ T cells (22–24). Similar to K14-A_β^b mice, these mice also have attenuated negative selection. Similar to K14-A_β^b $CD4^+25^+$ T cells, the repertoire of H2-DMα–deficient $CD4^+25^+$ T cells also parallels that of the effector $CD4^+$ T cell population. A simple explanation for these data would be that restriction of $CD4^+25^+$ regulatory T cells occurs at the level of the T cell, that regulatory cells suppress autoreactive T cells which arise in the same thymus. However, wild-type (B6) $CD4^+25^+$ T cells can be activated by TCR signals supplied by anti-CD3ε to suppress both K14-A_β^b and H2-DMα–deficient effector cells, arguing that there is a deficiency in the B6 regulatory T cell repertoire rather than its function. These data argue that the repertoire differences noted for K14-A_β^b $CD4^+25^+$ T cells are not unique to the K14-A_β^b system and that our conclusions that $CD4^+25^+$ T cells are subject to clonal deletion on APCs may be generalized. These results also suggest that regulatory $CD4^+25^+$ T cells don't function in vivo in the periphery to prevent autoreactivity to self dendritic cells, presumably because tolerance to self-peptides present on hematopoietic APCs is mediated by clonal deletion. Rather, we would propose that $CD4^+25^+$ T cells routinely function to suppress reactivity to peripheral antigens which cannot induce deletion.

A Model for Development of $CD4^+25^+$ T Cells. Recently, two of us have reported that development of regulatory $CD4^+25^+$ cells in a TCR transgenic system occurs after high affinity interactions with agonist self-peptide on thymic epithelium (19). In this model, it was proposed that generation of these cells was a high avidity event on medullary epithelium (19, 20). Analysis of the K14-A_β^b system

demonstrates that cognate interactions with MHC class II-positive cortical epithelium results in the development of functional $CD4^+25^+$ regulatory T cells. We also hypothesize that positively selected $CD4^+25^+$ thymocytes which recognize ubiquitous self-antigens with high affinity are subject to negative selection on hematopoietic APCs. Therefore, a subset of $CD4^+25^+$ T cells which are negatively selected in wild-type mice remain present in K14-A_β^b and H2-DMα–deficient thymi and can function to suppress an anti-B6 MLR. These selection events would restrict the repertoire of developing $CD4^+25^+$ T cells to tissue (epithelial)-specific antigens. This scenario is consistent with the previous observations that depletion and or manipulation of $CD4^+25^+$ T cells in wild-type animals results in organ-specific autoimmune disease rather than systemic autoimmunity.

We suggest that high affinity cognate interactions between developing $CD4^+$ T cells and self-peptides on thymic cortical epithelium leads to self-tolerance through the induction of regulatory T cells. Recently, Hogquist and colleagues used the K14 promoter to target an MHC class I–restricted high affinity peptide to thymic cortical epithelium (44). In this system, they noted the reexpression of RAG proteins and receptor editing, rather than clonal deletion of self-reactive cells. Taken together with the data presented in this report, these results suggest that thymic cortical epithelium induces tolerance to self-antigens through nondeletional mechanisms.

In conclusion, our results indicate that anergic/suppressive $CD4^+25^+$ T cells are selected through cognate interactions on thymic cortical epithelium and are subject to clonal deletion on hematopoietic APCs.

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