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Peripheral CD8$^+$CD25$^+$ T Lymphocytes from MHC Class II-Deficient Mice Exhibit Regulatory Activity[1]

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We characterized CD8$^+$ T cells constitutively expressing CD25 in mice lacking the expression of MHC class II molecules. We showed that these cells are present not only in the periphery but also in the thymus. Like CD4$^+$CD25$^+$ T cells, CD8$^+$CD25$^+$ T cells appear late in the periphery during ontogeny. Peripheral CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice also share phenotypic and functional features with regulatory CD4$^+$CD25$^+$ T cells: in particular, they strongly express glucocorticoid-induced TNFR family-related gene, CTLA-4 and Foxp3, produce IL-10, and inhibit CD25$^-$ T cell responses to anti-CD3 stimulation through cell contacts with similar efficiency to CD4$^+$CD25$^+$ T cells. However, unlike CD4$^+$CD25$^+$ T cells CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice strongly proliferate and produce IFN-γ in vitro in response to stimulation in the absence of exogenous IL-2. ***The Journal of Immunology,*** **2005, 175: 246–253.**

In the early 1970s, R. K. Gershon suggested that T lymphocytes could act as regulatory cells, suppressing immune responses (1). However, these cells and the molecular mechanisms responsible for suppression proved difficult to characterize. Recently, advances in the identification of CD4$^+$ T cell subpopulations, together with the use of genetically modified mice, have led to a renaissance of this field, and regulatory T cells are now thought to be an additional mechanism by which peripheral self-tolerance is maintained, alongside T cell deletion and T cell anergy (2–4). The concept of regulatory T cells is attractive because it could explain how tolerance can be adoptively transferred by T cells, how pathological responses to self and harmless foreign Ags are prevented, and how bystander tissue insult is avoided during normal immune responses.

The existence of thymus-derived regulatory T cells was initially suggested by the onset of autoimmune diseases in mice after thymectomy on day 3 of life (5, 6). These disorders were found to be due to a loss of peripheral CD4$^+$ T cells that constitutively express IL-2Rα (CD25), which appear late in the periphery after birth (7). Physiologically generated CD4$^+$CD25$^+$ cells inhibit a wide range of autoimmune and inflammatory disorders such as gastritis, oophoritis, orchitis, thyroiditis, colitis, and spontaneous autoimmune diabetes (8–11).

Despite numerous studies, the mechanisms by which CD4$^+$CD25$^+$ T cells exert their regulatory function are unclear. Some studies have shown that regulation in vivo is dependent on the production of suppressive cytokines such as IL-10 and TGF-β, and cell-surface molecules such as CTLA-4 (12–15). In vitro experiments aimed at further dissecting the mechanisms by which T cells exert their regulatory function have given controversial results. Indeed, in contrast to findings in vivo, neither soluble cytokines nor CTLA-4 seem to be required for the suppressive effects of CD4$^+$CD25$^+$ cells in vitro (16–18). Taken together, in vitro studies of CD4$^+$CD25$^+$ T cells support a cell contact-dependent, cytokine-independent mechanism of suppression (18).

CD8$^+$ T cells have been reported to be essential in vivo to prevent experimental autoimmune encephalomyelitis and to participate in oral tolerance (19–22). Regulatory CD8$^+$ CD28$^-$ T cells can be generated/expanded in vitro by multiple rounds of stimulation with allogenic, xenogenic, or Ag-pulsed syngenic APCs (23). However, it is not known whether such cells exist in vivo. Indeed, no physiologically generated subset of regulatory CD8$^+$ T cells has yet been characterized in the periphery.

Here, we showed that a significant proportion of peripheral CD8$^+$ T cells constitutively express CD25 in mice lacking the expression of MHC class II molecules. The aim of this study was to analyze these lymphocytes in detail by comparison with regulatory CD4$^+$CD25$^+$ T cells. The overriding question was whether these two T cell subsets only share CD25 expression.

Materials and Methods

Mice

CD45.2 C57BL/6 mice were obtained from Centre d'élevage Janvier, and CD45.1 C57BL/6 mice were obtained from Centre de Développement des Techniques Avancées pour l'Expérimentation Animale. MHC II$^{\Delta/\Delta}$ mice were originally purchased from The Jackson Laboratory (24) and then maintained in our own animal facilities. C57BL/6 CD3ϵ-deficient mice were crossed with MHC II$^{\Delta/\Delta}$ mice to generate MHC II$^{\Delta/\Delta}$ CD3ϵ-deficient mice.

Staining and flow cytometry

Thymus and pooled lymph nodes and spleen were homogenized in PBS, 5% FCS, 0.2% NaN_3 with a nylon cell strainer (Falcon), and distributed in 96-well U-bottom microplates (4×10^6 cells per well). Staining was performed on ice for 30 min per step.

Abs were obtained from BD Pharmingen unless otherwise indicated. To prevent unspecific binding of mAb, all samples were preincubated with blocking anti-FcγRII/III mAb (2.4G2). The following Ab combinations were used: for surface phenotype analysis, PE anti-CD25, FITC anti-CD8α, PercP anti-CD4 and biotinylated anti-CD8β, anti-CD28, anti-CD44, anti-CD45.1, anti-CD45.2, anti-glucocorticoid-induced TNFR

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family-related gene (GITR)[4] (R&D Systems) or anti-TCRβ with allophycocyanin-streptavidin development (BD Pharmingen); for intracellular staining of CTLA-4, cells were first stained for surface expression of CD4 (PercP), CD8α (FITC), and CD25 (biotinylated mAb with allophycocyanin-streptavidin development). After surface staining, cells were fixed and permeabilized, and then intracytoplasmic staining was performed with PE anti-CTLA-4; for intracellular cytokine staining, cells were first stained for surface expression of CD4 or CD8α (PercP) and TCRβ (FITC). Cells were then fixed and permeabilized, and intracytoplasmic staining was performed with PE anti-IL-2 (JES6–5H4) or anti-IFN-γ (XMG1.2) and allophycocyanin anti-IL-4 (11B11) or anti-IL-10 (JES5–16E3).

Four-color immunofluorescence was analyzed with a FACSCalibur cytometer (BD Biosciences). List-mode data files were analyzed using Cell Quest software (BD Biosciences).

Preparation of purified T cell subsets

$CD4^+$ T cells and $CD8^+$ T cells were purified as previously described (25) according to whether or not they express CD25. Briefly, lymph node cells were depleted of macrophages, granulocytes and $CD4^+$ T cells or $CD8^+$ T cells by incubating them first with anti-CD11b (Mac-1) Ab, anti-GR1 (8C5) Ab, and anti-CD4 (GK1.5) Ab or anti-CD8 (Lyt-2) Ab, and then with magnetic beads coupled to anti-rat Ig (Dynal). B cells were removed using magnetic beads coupled to anti-mouse Ig (Dynal). Purified $CD4^+$ T cells and $CD8^+$ T cells were then labeled with biotinylated anti-CD25 (clone PC61). $CD25^+$ T cells were then positively selected using MACS streptavidin microbeads (Miltenyi Biotech). $CD25^+$ T cells were usually 90–95% pure.

For RT-PCR (Fig. 3*B*), all purified $CD25^-$ and $CD25^+$ subsets were stained for surface expression of CD4 or CD8α and CD25 (clone 7D4). $CD4^+$ $CD25^-$, $CD4^+CD25^+$, $CD8^+CD25^-$, and $CD8^+CD25^+$ T cells were then electronically sorted in a FACSVantage flow cytometer (BD Biosciences). $CD25^-$ and $CD25^+$ T cells subsets were in this case at least 99% pure.

In vitro culture assays

Coated anti-CD3 stimulation. $CD4^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of normal C57BL/6 mice. $CD8^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of MHC $II^{\Delta/\Delta}$ mice. Purified cells (5×10^4) were then cultured in the presence or absence of coated anti-CD3 mAb and human recombinant IL-2 (TEBU). Supernatants were collected 48 h after the beginning of culture. [^{3}H]Thymidine was added at the same time, and proliferation was measured 16 h later. IL-2, IL-4, IL-10, and IFN-γ production was measured with ELISA tests. In some experiments, PMA, ionomycin, and brefeldin A were added to the medium during the last 6 h of the 64-h culture period. Then, cells were stained for surface expression of CD4 or CD8α and TCRβ. After surface staining, cells were fixed and permeabilized, and intracytoplasmic staining was performed with PE anti-IL-2 or anti-IFN-γ and allophycocyanin anti-IL-4 or anti-IL-10.

Soluble anti-CD3 stimulation. $CD25^-$ T cells were purified from lymph nodes of CD45.1 mice. $CD25^+$ T cells were purified from lymph nodes of CD45.2 mice. Purified cells were labeled with CFSE (Molecular Probes). Labeled cells (5×10^4) were then cultured in the presence of soluble anti-CD3 Ab (0.2 μg/ml) and APCs (25×10^4 irradiated splenocytes from MHC $II^{\Delta/\Delta}$ CD3ϵ-deficient mice). At 12 h to 3 days after the beginning of culture, cells were stained for CD4 or CD8α and CD45.1 or CD45.2. CFSE dilution (Figs. 6–9) and CD25 expression (Fig. 7) or IL-2 production (Fig. 8) were measured. For measurements of IL-2 production, PMA, ionomycin, and brefeldin A were added to the medium during the last hour of the culture period. In some experiments (Fig. 9*A*), cells were cultured in the presence of 25 μg/ml anti-IL-10 mAb (JES2A5) or anti-IL-10 receptor mAb (1B1.2). Transwells with 0.2 μm Anopore membranes in 96-well plates (Nunc) were used in some other experiments (Fig. 9*B*). The average number of cell cycle in response to anti-CD3 stimulation was calculated as follows. First, we estimated the CFSE dilution factor (f) due to stimulation: f = CFSE mean fluorescence intensity (MFI) in absence of stimulation divided by CFSE MFI in presence of stimulation. Then, as the intracellular amount of CFSE is halved during each cell cycle, the average number of cell cycle (A) was calculated with the following formula: $A = \mathrm{LOG}_2(f)$.

RT-PCR

Total RNA was isolated from either 5×10^3 electronically sorted cells (Fig 3*B*) or cultured cells (Fig. 8*A*) using the Absolutely RNA Microprep kit (Stratagene) and oligo(dT) primers first strand cDNA prepared with the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). First strand cDNA (1.5 μl) was amplified in a 25 μl reaction using Amplitaq DNA polymerase (Roche). PCR was done at 94°C, 5 min; 20, 25, 30, 35, or 40 cycles at 94°C, 30 s; 56°C, 30 s; 72°C, 30 s followed by a 10-min final extension at 72°C with 0.2 μM primers.

PCR primer pairs (Invitrogen) were as follows: Foxp3, 5′-CCCAG GAAAGACAGCAACCTT-3′, 5′-CCTTGCCTTTCTCATCCAGGA-3′; IL-2, 5′-CAGGATGGAGAATTACAGGAACCT-3′, 5′-TTTCA ATTCTGTGGCCTGCTT-3′; hypoxanthine phosphoribosyltransferase (HPRT), 5′-GCGTCGTGATTAGCGATGATGA-3′, 5′-CCTGTCCAT AATCAGTCCATGAGGA-3′.

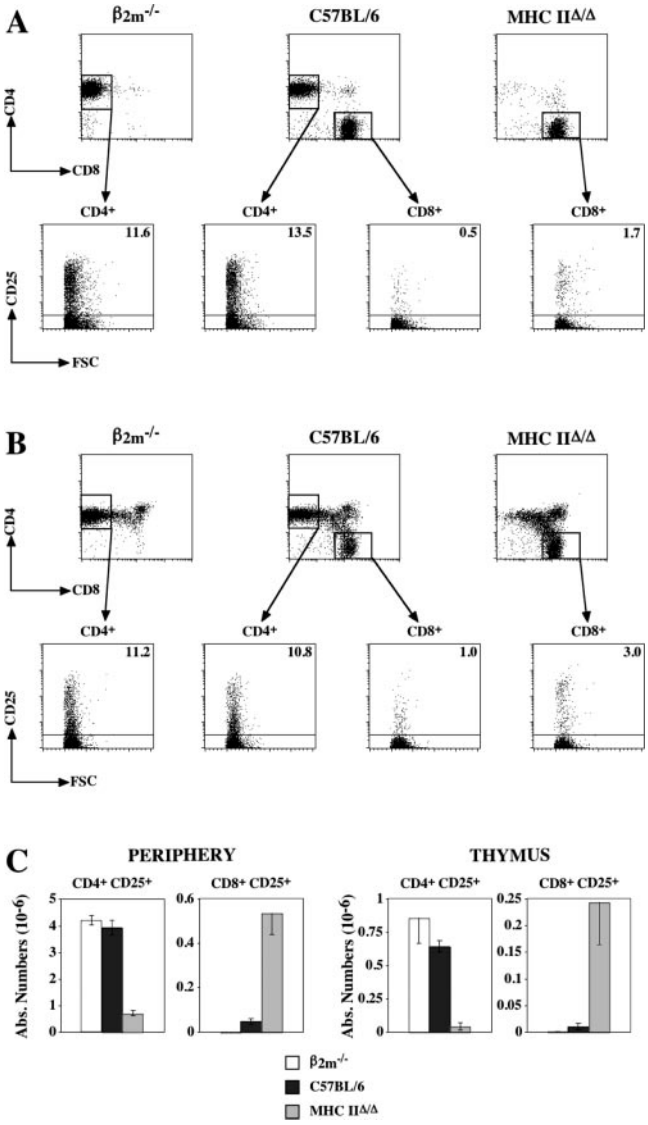

FIGURE 1. MHC restriction of $CD8^+CD25^+$ T cells. Thymus, lymph nodes, and spleen of normal C57BL/6 mice and C57BL/6 mice deficient for MHC class I ($\beta_2m^{-/-}$) or class II (MHC $II^{\Delta/\Delta}$) expression were recovered. Thymus and pooled lymph nodes and spleen were used to prepare single-cell suspensions that were stained for CD4, CD8α, TCRβ, and CD25. CD4/8 dot plots are shown for TCR^{high} peripheral cells (pooled lymph nodes and spleen (*A*) and TCR^{high} thymocytes (*B*). FSC/CD25 dot plots are shown for the indicated peripheral and thymic TCR^{high} cell subsets. The dot plots were derived from one mouse per group but were representative of five individual experiments with at least three mice per group. Absolute numbers of $CD4^+8^-$ TCR^{high} and $CD4^-8^+$ TCR^{high} cells expressing CD25 were calculated for the periphery and thymus (*C*). Data are mean ± SD values of at least fifteen mice in each group.

[4] Abbreviations used in this paper: GITR, glucocorticoid-induced TNFR family-related gene; MFI, mean fluorescence intensity; HPRT, hypoxanthine phosphoribosyltransferase.

Results

A significant proportion of CD8$^+$ T cell express CD25 in MHC class II-deficient mice

Thymus, lymph nodes, and spleen of normal C57BL/6 mice and C57BL/6 mice lacking MHC class I ($\beta_2m^{-/-}$) or class II (MHC II$^{\Delta/\Delta}$) expression were recovered and analyzed for the presence of CD4$^+$ and CD8$^+$ T cells expressing CD25 (Fig. 1, *A* and *B*). Independently of MHC class I expression, >10% of CD4$^+$ T cells expressed CD25 in both the periphery (spleen + lymph nodes) and the thymus. As a result, absolute numbers of recovered CD4$^+$CD25$^+$ T cells were not significantly different between normal C57BL/6 mice and C57BL/6 $\beta_2m^{-/-}$ mice (Fig. 1*C*). Surprisingly, some CD4$^+$CD25$^+$ T cells were detected in the periphery of MHC II$^{\Delta/\Delta}$ mice (Fig. 1*C*) suggesting that a fraction of regulatory CD4$^+$CD25$^+$ T cells might be MHC class I-restricted. Trobonjoca et al. (26) recently reported that CD4$^+$ T cells from MHC class II-deficient mice engrafted into congenic immunodeficient hosts induced aggressive inflammatory bowel disease and therefore contained few if any regulatory T cells. These results suggest that CD25 expression by CD4$^+$ T cells in these mice is activation-induced rather than constitutive.

As previously described (7), a small percentage of peripheral and thymic CD8$^+$ T cells from normal C57BL/6 mice expressed CD25 (thymus: 0.83 ± 0.30; periphery: 0.35 ± 0.05; Fig. 1*C*). Surprisingly, both the proportion and the absolute number of CD8$^+$CD25$^+$ T cells were strongly augmented in the absence of MHC class II molecule expression (thymus: 2.48 ± 0.21; periphery: 1.64 ± 0.51; Fig. 1*C*). By contrast, CD8$^+$CD25$^+$ T cells were not detected in mice lacking MHC class I expression. Thus, a significant subset of CD8$^+$ T cells constitutively expressing CD25 can be defined in both the thymus and the periphery of MHC class II-deficient mice.

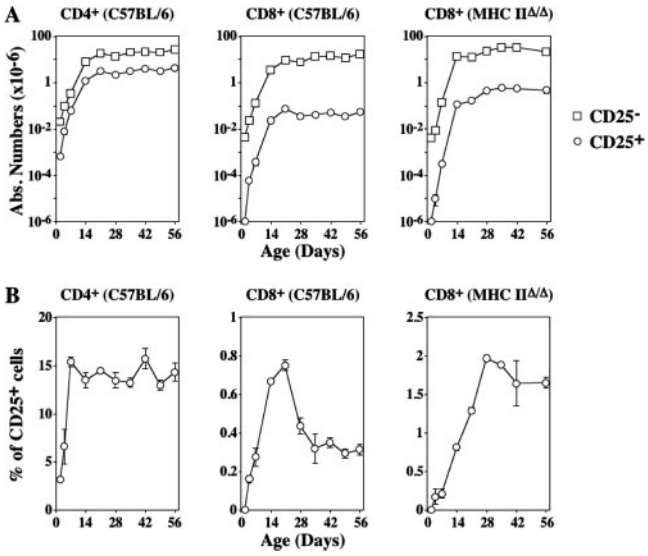

FIGURE 2. Ontogeny of CD8$^+$CD25$^+$ T cells. Pooled lymph nodes and spleen of normal C57BL/6 mice and C57BL/6 mice deficient for MHC class II expression (MHC II$^{\Delta/\Delta}$) were used to prepare single-cell suspensions that were stained for CD4, CD8α, TCRβ, and CD25, at various ages. *A*, Absolute numbers of peripheral CD4$^+$8$^-$ TCR^{high} and CD4$^-$8$^+$ TCR^{high} cells were calculated as a function of mouse age and CD25 expression. *B*, Percentages of CD25$^+$ cells among CD4$^+$8$^-$ and CD4$^-$8$^+$ TCR^{high} cells were calculated as a function of age. Data are mean ± SD values of at least three mice in each group per time point.

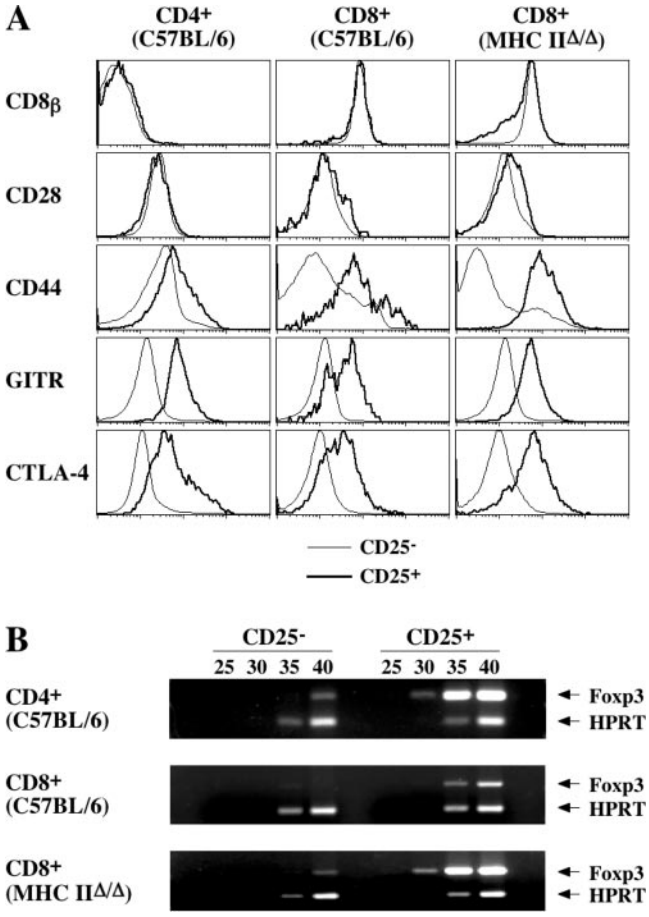

FIGURE 3. Phenotype of CD8$^+$CD25$^+$ T cells. Lymph nodes and spleen of normal C57BL/6 mice and C57BL/6 mice deficient for MHC class II expression (MHC II$^{\Delta/\Delta}$) were recovered, pooled, and used to prepare single-cell suspensions. *A*, CD8β, CD28, CD44, GITR (surface expression), and CTLA-4 (intracellular expression) fluorescence histograms of CD4$^+$8α^- and CD4$^-$8α^+ cells are shown as a function of CD25 expression. The histograms were derived from one mouse per group but were representative of three individual experiments with at least two mice per group. *B*, CD4$^+$ T cells were first purified by negative selection of non-CD4$^+$ T cells, CD8$^+$ T cells by negative selection of non-CD8$^+$ T cells. Purified CD4$^+$ T cells and CD8$^+$ T cells were then labeled with biotinylated anti-CD25 (clone PC61) and CD25$^+$ T cells were positively selected on the basis of their CD25 expression. All purified CD25$^-$ and CD25$^+$ subsets were stained for surface expression of CD4 or CD8α and CD25 (clone 7D4). CD4$^+$ CD25$^-$, CD4$^+$CD25$^+$, CD8$^+$CD25$^-$, and CD8$^+$ CD25$^+$ T cells were then electronically sorted. CD25$^-$ and CD25$^+$ T cells subsets were in this case at least 99% pure. Transcription of Foxp3 was then determined by RT-PCR with transcription of HPRT as control; 25, 30, 35, or 40 PCR cycles were done. Results are representative of two independent cell purifications.

CD8$^+$CD25$^+$ T cells appear late in the periphery during ontogeny

The percentage and absolute number of T cells expressing CD25 in the periphery were studied as a function of mouse age. Absolute numbers of peripheral CD4$^+$ and CD8$^+$ T cells increased with age, reaching a plateau 3–4 wk after birth (Fig. 2*A*). As previously described (7), few CD4$^+$CD25$^+$ were detected in the periphery of 2-day mice. Consequently, the proportion of peripheral CD4$^+$ T cells expressing CD25 was <4% 2 days after birth, which is far below the proportion observed in adult mice (Fig. 2*B*).

Interestingly, in both normal and MHC class II-deficient C57BL/6 mice, CD8 CD25$^+$ T cells began to appear in the periphery 4 days after birth (Fig. 2, *A* and *B*). In normal C57BL/6

mice, the proportion of peripheral $CD8^+$ T cells expressing CD25 peaked 3 wk after birth, then fell to the low level observed in adult mice. The kinetics of $CD8^+CD25^+$ T cell appearance in the periphery was different in mice lacking MHC class II molecules. The proportion of $CD25^+$ cells among $CD8^+$ T cells increased during the first 4 wk to reach a plateau. Thus, the ontogeny of $CD8^+CD25^+$ T cells in MHC class II-deficient mice runs parallel to that of $CD4^+CD25^+$ T cells in normal mice, suggesting that these two T cell subsets might share more than CD25 expression alone. We therefore compared the phenotypic and functional characteristics of $CD4^+$ and $CD8^+$ T cells expressing CD25.

$CD8^+CD25^+$ T cells from MHC class II-deficient mice have a similar phenotype to $CD4^+CD25^+$ T cells

We studied the expression of activation markers (CD44), costimulatory molecules (CD28, CTLA-4), adhesion molecules (LFA-1), and all molecules that might play a role in modulating the T cell response (TCR itself, CD5, CD8β, and GITR), by both peripheral $CD4^+$ and $CD8^+$ T cells, according to whether or not the cells expressed CD25 (Fig. 3*A* and data not shown).

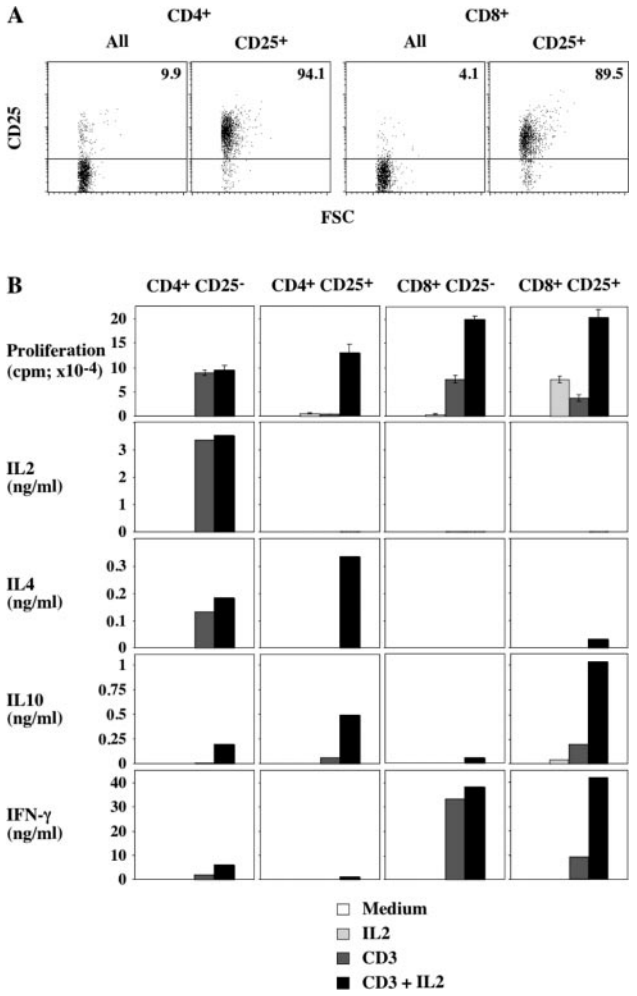

FIGURE 4. Proliferation and cytokine production by stimulated $CD8^+CD25^+$ T cells from MHC class II-deficient mice. $CD4^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of normal C57BL/6 mice. $CD8^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of MHC $II^{\Delta/\Delta}$ mice. *A*, FSC/CD25 dot plots are shown for purified $CD4^+$ T cells and $CD8^+$ T cells before (All) and after ($CD25^+$) positive selection of CD25-expressing cells. Numbers correspond to the percentage of cells expressing CD25. *B*, Purified cells were cultured in the presence or absence of coated anti-CD3 and IL-2. Supernatants were collected 48 h after the beginning of culture. [^{3}H]Thymidine was added at the same time, and proliferation was measured 16 h later. IL-2, IL-4, IL-10, and IFN-γ production was measured with ELISA tests. Data are mean $\pm$ SD values of triplicate wells. Results are representative of five independent experiments.

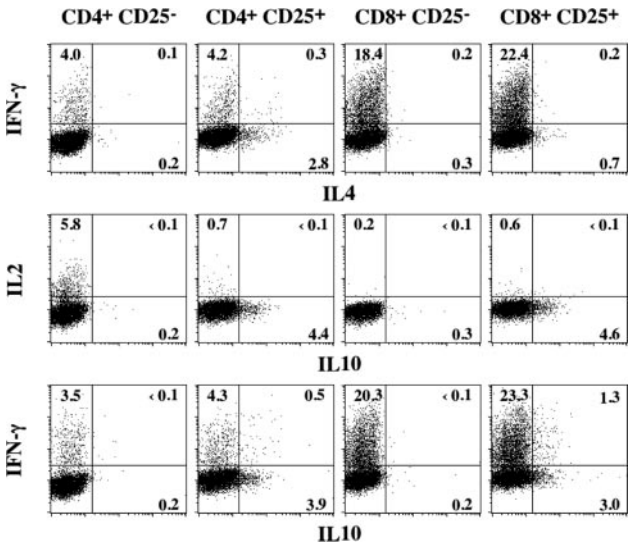

FIGURE 5. $CD8^+CD25^+$ T cells from MHC class II-deficient mice produce IL-10 independently of their capacity to produce IFN-γ. $CD4^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of normal C57BL/6 mice. $CD8^+$ T cells expressing and not expressing CD25 were purified from lymph nodes of MHC $II^{\Delta/\Delta}$ mice. Purified cells were cultured in the presence of coated anti-CD3 and IL-2 for 64 h. PMA, ionomycin, and brefeldin A were added for the last 6 h of culture. Then, cells were stained for surface expression of CD4 or CD8α, and TCRβ, followed by intracytoplasmic staining of IL-2, IL-4, IL-10, or IFN-γ. IFN-γ/IL-4, IL-2/IL-10, IFN-γ/IL-10 dot plots are shown for each studied T cell subset. Results are representative of three independent experiments.

First, it is important to note that $CD8^+CD25^+$ T cells from normal mice expressed lower densities of CD25 (Fig. 1*A*; MFI = 32.3 $\pm$ 7.6) than both $CD8^+CD25^+$ T cells from MHC class II-deficient mice (MFI = 70.6 $\pm$ 5.6) and $CD4^+CD25^+$ T cells from normal mice (MFI = 71.2 $\pm$ 7.1). Except the differential expression of CD25 per itself, the expression of all other tested markers was similar in $CD8^+CD25^+$ T cells from normal and MHC $II^{\Delta/\Delta}$ mice, and also in $CD8^+CD25^+$ and $CD4^+CD25^+$ T cells. Indeed, both $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells showed intermediate to high surface expression of CD44, GITR CD5, and LFA-1 (Fig. 3*A* and data not shown), and contained large amounts of intracellular CTLA-4 (Fig. 3*A*), when compared with their $CD25^-$ T cell counterparts. Interestingly, $CD8^+$ $CD25^+$ T cells co-expressed CD8 α and β chains (Fig. 3A), and all expressed the TCR β-chain (data not shown), suggesting that they were not related to resident gut TCR$\alpha\beta$ or TCR$\gamma\delta$ CD8$\alpha\alpha$ T cells. Finally, all $CD8^+CD25^+$ T cells expressed CD28, suggesting that they did not correspond to regulatory $CD8^+CD28^-$ T cells generated in vitro.

Finally, we examined the expression of Foxp3 in both $CD4^+$ and $CD8^+$ T cells as a function of CD25 expression (Fig. 3*B*). Foxp3, a transcription factor, acts as a key regulator of regulatory $CD4^+CD25^+$ T cell development and function. It has been suggested that Foxp3 expression would be restricted to $CD4^+CD25^+$ T cells (27, 28). We found that $CD4^+CD25^+$ T cells from normal mice and $CD8^+CD25^+$ T cells from MHC class II-deficient mice similarly expressed Foxp3 (Fig. 3*B*). However, a low but significant Foxp3 expression could be detected in their $CD25^-$ T cell counterparts suggesting that subsets other than $CD25^+$ T cells expressed this transcription factor. In normal mice, $CD8^+CD25^+$ T cells expressed

Foxp3 to a lesser extent than both CD4$^+$CD25$^+$ T cells from normal mice and CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice.

Proliferation and cytokine production by CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice in response to stimulation

Given the small percentage of CD8$^+$ T cells expressing CD25 in the periphery of normal C57BL/6 mice, and the impossibility of purifying enough of them for functional studies, the following experiments used CD8$^+$CD25$^-$ and CD25$^+$ T cells from MHC II$^{\Delta/\Delta}$ mice (Figs. 4 and 5). CD25$^+$ and CD25$^-$ CD4 T cells were purified from normal C57BL/6 mice. CD25$^+$ T cells were usually 90–95% pure (Fig. 4*A*).

First, we studied the response of CD4$^+$ and CD8$^+$ T cells to coated anti-CD3 as a function of their CD25 expression (Fig. 4*B*). This approach obviated the need for APCs and therefore ensured that cytokines measured in the supernatants were produced by T cells rather than by APCs. This was especially important for IL-10, which is strongly produced by macrophages and B cells (29).

CD4$^+$CD25$^-$ T cells proliferated to anti-CD3, and this response was not enhanced by exogenous IL-2. In fact, CD4$^+$CD25$^-$ T cells themselves produced large amounts of IL-2. They also produced IL-4 and a little IFN-γ (Fig. 4*B*). CD8$^+$CD25$^-$ T cells also proliferated to anti-CD3, but contrary to CD4$^+$CD25$^-$ T cells, their response was strongly enhanced by exogenous IL-2, in keeping with the observation that they mainly produced IFN-γ and no detectable IL-2.

Like other groups, we found that CD4$^+$CD25$^+$ T cells were anergic, in the sense that they did not cycle in response to anti-CD3 stimulation alone (10, 16, 30). This non-responsiveness was overcome by exogenous IL-2; in these conditions, IL-4, IL-10, and a little IFN-γ were detected in the supernatants. With regard to their proliferative capacities, CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice resembled CD8$^+$CD25$^-$ T cells. Indeed, they proliferated in response to anti-CD3 stimulation alone and their response was strongly increased in the presence of exogenous IL-2. Moreover, like CD8$^+$CD25$^-$ T cells, they produced large amounts of IFN-γ. Interestingly, CD8$^+$CD25$^+$ T cells also proliferated strongly in response to IL-2 alone, and this response was far stronger than the response of CD8$^+$ CD25$^-$ T cells and even CD4$^+$CD25$^+$ T cells in the same conditions. Finally, unlike CD8$^+$CD25$^-$ T cells, CD8$^+$CD25$^+$ T cells also produced large

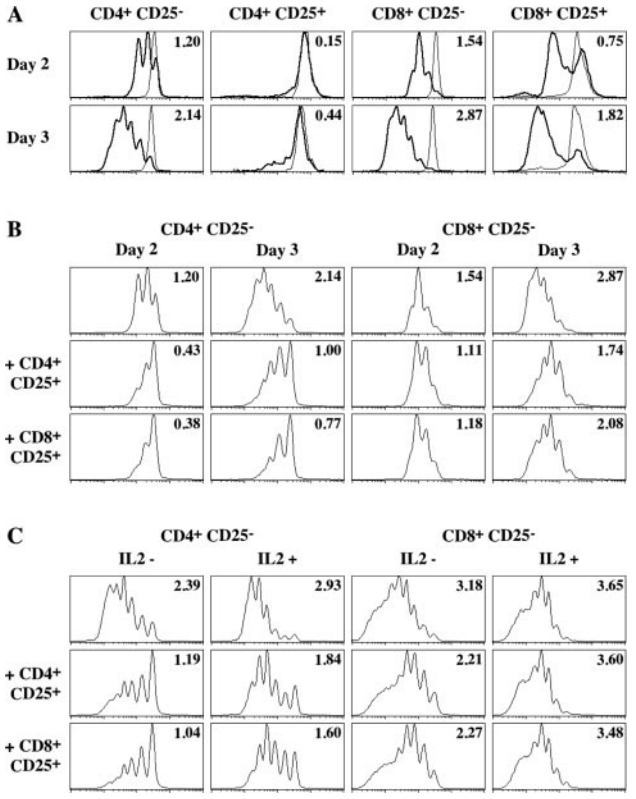

FIGURE 6. CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice regulate the response of both CD4$^+$ and CD8$^+$ CD25$^-$ T cells. CD4$^+$CD25$^-$ T cells and CD8$^+$CD25$^-$ T cells were purified from lymph nodes of CD45.1 C57BL/6 mice. CD4$^+$CD25$^+$ T cells were purified from lymph nodes of CD45.2 C57BL/6 mice, and CD8$^+$CD25$^+$ T cells from MHC II$^{\Delta/\Delta}$ mice (CD45.2). Cells were then labeled with CFSE and cultured in the presence of soluble anti-CD3 and APCs. *A*, CFSE fluorescence histograms of the indicated T cell subsets are shown 2 and 3 days after the beginning of culture (bold lines). CFSE fluorescence histograms in the absence of anti-CD3 stimulation are shown as controls (thin lines). *B*, CD4$^+$CD25$^-$ T cells and CD8$^+$CD25$^-$ T cells were cultured alone or together with the indicated CD25$^+$ T cells at a 1:1 ratio. CFSE fluorescence histograms of CD4$^+$ or CD8α^+ CD45.1$^+$ cells are shown 2 and 3 days after the beginning of culture. *C*, CD4$^+$CD25$^-$ T cells and CD8$^+$CD25$^-$ T cells were cultured alone or together with the indicated CD25$^+$ T cells at a 1:1 ratio in the presence or absence of exogenous IL-2. CFSE fluorescence histograms of CD4$^+$ or CD8α^+ CD45.1$^+$ cells are shown 3 days after the beginning of culture. Numbers correspond to the average number of cell cycles of the corresponding cell subset in response to anti-CD3 stimulation during the culture period (see *Materials and Methods*). Results are representative of at least three independent experiments.

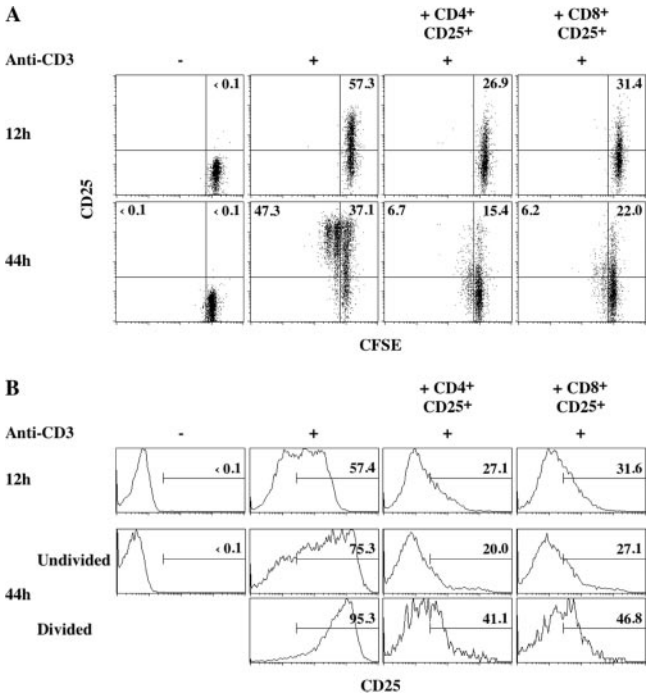

FIGURE 7. Like CD4$^+$CD25$^+$ T cells, CD8$^+$CD25$^+$ T cells inhibit the early activation of CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^-$ T cells were purified from lymph nodes of CD45.1 C57BL/6 mice. CD4$^+$CD25$^+$ T cells were purified from lymph nodes of CD45.2 C57BL/6 mice, and CD8$^+$CD25$^+$ T cells from MHC II$^{\Delta/\Delta}$ mice (CD45.2). CD45.1$^+$CD4$^+$CD25$^-$ T cells were labeled with CFSE and cultured for the indicated time alone or together with the indicated CD25$^+$ T cells at a 1:1 ratio in the presence or absence of soluble anti-CD3. Then, cells were stained for surface expression of CD4, CD45.1, and CD25. *A*, CFSE/CD25 dot plots are shown for CD4$^+$CD45.1$^+$ T cells as a function of time after transfer. *B*, CD25 fluorescence histograms of CD4$^+$CD45.1$^+$ T cells are shown as a function of time after transfer. For each condition, the percentage of CD4$^+$CD45.1$^+$ T cells expressing CD25 is indicated. Results are representative of at least two independent experiments.

amounts of IL-10 in response to anti-CD3 stimulation in the presence of IL-2, and produced detectable levels of IL-10 in response to anti-CD3 or IL-2 alone. With regard to their ability to produce IL-10, $CD8^+CD25^+$ T cells behaved like $CD4^+CD25^+$ regulatory T cells. However, unlike $CD4^+CD25^+$ T cells, $CD8^+CD25^+$ T cells cannot be considered naturally anergic.

IL-10 production by both $CD4^+CD25^+$ T cells from normal mice and $CD8^+CD25^+$ T cells from MHC class II-deficient mice was further confirmed by intracellular staining (Fig. 5). In response to anti-CD3 stimulation in the presence of IL-2, $CD8^+CD25^+$ T cells produced either IL-10 or IFN-γ or both cytokines. More precisely, $CD8^+CD25^+$ T cells produced IL-10 independently of their capacity to produce IFN-γ. Indeed, the proportion of $CD8^+CD25^+$ T cells producing IL-10 was similar among IFN-γ producing and IFN-γ non-producing cells (Fig. 5).

$CD8^+CD25^+$ T cells from MHC class II-deficient mice regulate the response of both $CD4^+$ and $CD8^+CD25^-$ T cells with similar efficiency to $CD4^+CD25^+$ T cells

We then studied the capacity of $CD8^+CD25^+$ T cells from MHC class II-deficient mice to inhibit the response of $CD25^-$ T cells to anti-CD3 stimulation. In these experiments (Figs. 6–8), stimulation was ensured by soluble anti-CD3 in the presence of irradiated APCs. Proliferation of $CD25^-$ T cells mixed with $CD8^+CD25^+$ T cells could not be assessed by measuring thymidine incorporation, as $CD8^+CD25^+$ T cells also strongly proliferated to anti-CD3 (Fig. 4). T cells were therefore labeled with CFSE before culture (the intracellular amount of this intracytoplasmic dye is halved during each cell cycle). $CD25^+CD4$ T cells were purified from normal C57BL/6 mice (CD45.2). $CD8^+CD25^+$ T cells from MHC $II^{\Delta/\Delta}$ mice (CD45.2). $CD25^-CD4^+$ and $CD8^+$ T cells were purified from CD45.1 C57BL/6 mice (Figs. 6–8).

We first verified the proliferative capacities of $CD4^+$ and $CD8^+$ T cells in response to soluble anti-CD3 as a function of their CD25 expression (Fig. 6*A*). We confirmed that $CD8^+CD25^+$ cells from MHC class II-deficient mice strongly cycled in response to anti-CD3, whereas $CD4^+CD25^+$ T cells did not. We then studied regulation by using a 1:1 ratio of $CD25^-$ and $CD25^+$ T cells. $CD25^-$ T cells were from CD45.1 mice, and $CD25^+$ T cells were from CD45.2 mice. Surprisingly, although they cycled, $CD8^+CD25^+$ T cells inhibited the proliferation of both $CD4^+$ and $CD8^+CD25^-$ T cells (Fig. 6*B*). At this ratio, $CD8^+CD25^+$ T cells inhibited the proliferation of $CD25^-$ T cells at least as efficiently as $CD4^+CD25^+$ T cells (Fig. 6*B*). Both $CD25^+$ T cell subsets regulated $CD4^+CD25^-$ T cells far more efficiently than $CD8^+CD25^-$ T cells, and their regulatory activity became increasingly evident with time.

When exogenous IL-2 was added to the cultures, $CD4^+CD25^+$ T cells cycled in response to anti-CD3 (Fig. 4 and data not shown). Furthermore, it has been shown that IL-2 almost completely abrogates the suppressive activities of $CD4^+CD25^+$ T cells (10, 16, 30). Here, we found that, in the presence of exogenous IL-2, both $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells lost their capacity to inhibit $CD8^+CD25^-$ T cell proliferation, and that both subsets regulated $CD4^+CD25^-$ T cells less efficiently (Fig. 6*C*).

IL-2 production and the up-regulation of CD25 surface expression are early events of naive $CD4^+$ T cell activation in response to anti-CD3 stimulation and can be observed as soon as 12 h after the beginning of culture (Figs. 7 and 8). Interestingly, like $CD4^+CD25^+$ T cells (31, 32), $CD8^+CD25^+$ T cells efficiently inhibited CD25 up-regulation on responder cells ($CD4^+CD45.1^+$ cells). CD25 surface expression was not delayed but was rather stably inhibited. Indeed, 44 h after the beginning of culture, in the presence of $CD25^+$ T cells, both non-proliferating and proliferating $CD4^+CD45.1^+$ T cells expressed lower amounts of CD25 than

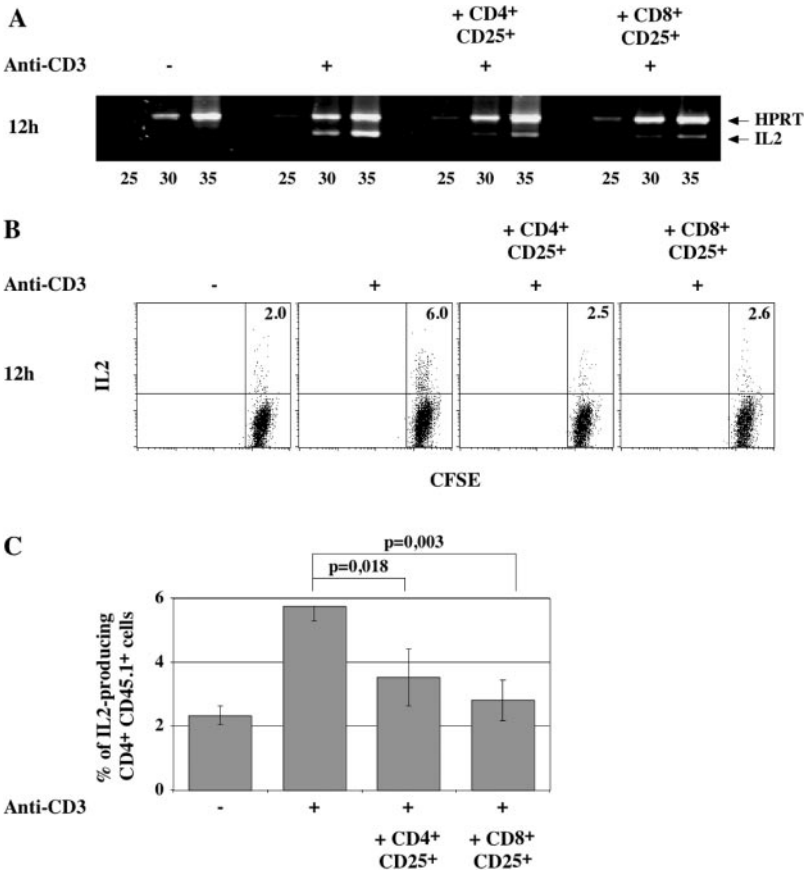

FIGURE 8. Like $CD4^+CD25^+$ T cells, $CD8^+CD25^+$ T cells inhibit IL-2 transcription and production by $CD4^+CD25^-$ T cells. $CD4^+CD25^-$ T cells were purified from lymph nodes of CD45.1 C57BL/6 mice. $CD4^+CD25^+$ T cells were purified from lymph nodes of CD45.2 C57BL/6 mice, and $CD8^+CD25^+$ T cells from MHC $II^{\Delta/\Delta}$ mice (CD45.2). $CD45.1^+CD4^+CD25^-$ T cells were labeled with CFSE and cultured for 12 h alone or together with the indicated $CD25^+$ T cells at a 1:1 ratio in the presence or absence of soluble anti-CD3. *A*, For each condition, transcription of IL-2 was determined by RT-PCR with transcription of HPRT as control; 25, 30, or 35 PCR cycles were done. When $CD45.1^+CD4^+CD25^-$ T cells were cultured alone, unstimulated $CD4^+CD25^-$ T cells (IL-2-nonproducing cells) were added to the culture wells to normalize final cell numbers before extracting RNA. *B* and *C*, PMA, ionomycin, and brefeldin A were added to the medium during the last hour of the 12-h culture period. Then, cells were stained for surface expression of CD4 and CD45.1. After surface staining, cells were fixed and permeabilized, and intracytoplasmic staining was performed with PE anti-IL-2. CFSE/CD25 dot plots are shown for $CD4^+CD45.1^+$ T cells (*B*). For each condition, the percentage of $CD4^+CD45.1^+$ T cells expressing CD25 was calculated and mean ± SD values of triplicate wells are shown (*C*). Results are representative of two independent experiments.

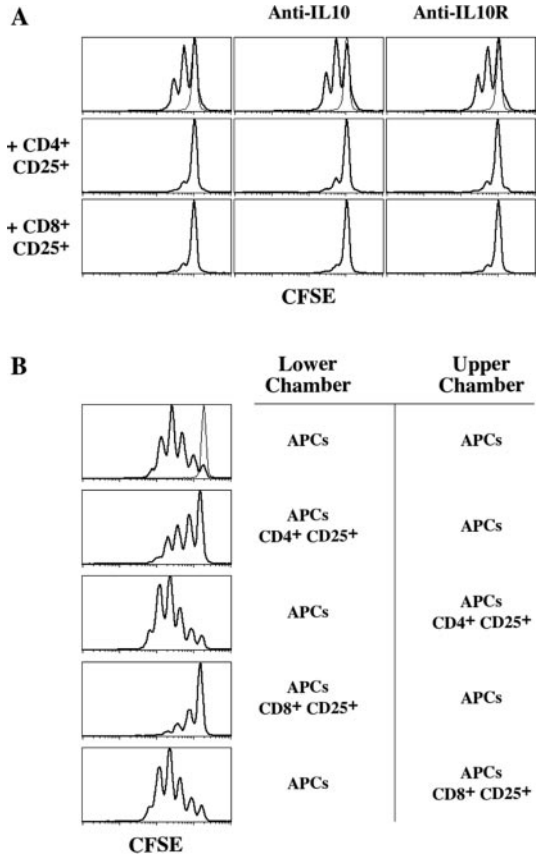

FIGURE 9. Like CD4$^+$CD25$^+$ T cells, CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice regulate CD4$^+$CD25$^-$ T cells through cell contacts. CD4$^+$CD25$^-$ T cells were purified from lymph nodes of CD45.1 C57BL/6 mice. CD4$^+$CD25$^+$ T cells were purified from lymph nodes of CD45.2 C57BL/6 mice, and CD8$^+$CD25$^+$ T cells from MHC II$^{\Delta/\Delta}$ mice (CD45.2). CD45.1$^+$CD4$^+$CD25$^-$ T cells were labeled with CFSE and cultured for 2 or 3 days in the presence (bold lines) or absence (thin lines) of soluble anti-CD3. *A*, CD4$^+$CD25$^-$ T cells were cultured alone or together with the indicated CD25$^+$ T cells at a 1:1 ratio in the presence or absence of anti-IL-10 mAb or anti-IL-10 receptor mAb. CFSE fluorescence histograms of CD4$^+$CD45.1$^+$ cells are shown 2 days after the beginning of culture. *B*, CD4$^+$CD25$^-$ T cells were cultured alone or together with CD25$^+$ T cells in the lower or upper chamber of Transwells. CFSE fluorescence histograms of CD4$^+$CD45.1$^+$ cells are shown 3 days after the beginning of culture. Results are representative of at least two independent experiments.

when cultured alone (Fig. 7*B*). Moreover, 12 h after the beginning of culture, we found that like CD4$^+$ CD25$^+$ T cells (16, 33, 34), CD8$^+$CD25$^+$ T cells inhibited both IL-2 transcription (Fig. 8*A*) and production (Fig. 8, *B* and *C*) by responder cells (CD4$^+$ CD45.1$^+$ cells). Thus, suppression of T cell proliferation by both CD4$^+$ and CD8$^+$CD25$^+$ T cells takes place during the early phase of T cell activation. However, it is interesting to note that 12 h after the beginning of culture, contrary to CD25, the up-regulation of CD69 on responder cells was not inhibited by CD25$^+$ T cell subsets (data not shown) showing that regulatory CD25$^+$ T cells do not completely abrogate T cell activation.

To determine whether IL-10 secretion by CD8$^+$CD25$^+$ T cells mediate their regulatory activity, we performed experiments in which anti-IL-10 or anti-IL-10 receptor mAb were added in the medium at the beginning of culture (Fig. 9*A*). We found that the addition of neutralizing Abs to IL-10 failed to abrogate suppression mediated by either CD4$^+$ or CD8$^+$CD25$^+$ T cells. Thus, like for CD4$^+$CD25$^+$ T cells from normal mice (16), IL-10 was not required for the regulatory activity of CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice in vitro. Finally, we performed Transwell experiments to examine the role of cell contacts in the immunosuppression mediated by CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice (Fig. 9*B*). Like CD4$^+$CD25$^+$ T cells, CD8$^+$CD25$^+$ T cells were inefficient in inhibiting the proliferation of CD4$^+$CD25$^-$ T cells when placed in the upper chamber of the Transwell. Thus, CD8$^+$CD25$^+$ T cells do not only act by sequestering the IL-2 from the responder cells. Like for CD4$^+$CD25$^+$ T cells, contacts between CD8$^+$CD25$^+$ T cells and CD25$^-$ T cells and/or their stimulating APCs were crucial for the immunosuppressive effect of CD8$^+$CD25$^+$ T cells

Discussion

We analyzed the absolute number, ontogeny, phenotype, and functional capacities of mouse CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice by comparison with regulatory CD4$^+$CD25$^+$ T cells. CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice resemble CD4$^+$CD25$^+$ T cells in several respects. 1) Both subsets were detected in the thymus and the periphery, where they appeared late during ontogeny. 2) Peripheral CD4$^+$ and CD8$^+$ CD25$^+$ T cells shared phenotypic features such as strong surface expression of CD44 and GITR, and strong intracellular expression of CTLA-4. 3) Both subsets strongly expressed the transcription factor Foxp3. 4) Both subsets secreted large amounts of IL-10 in response to TCR-mediated stimulation. 5) The two subsets inhibited CD25$^-$ T cell responses to anti-CD3 stimulation with similar efficiency through cell contacts. Thus, CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice share with regulatory CD4$^+$CD25$^+$ T cells quite more than the sole expression of CD25 and can therefore be considered as a subset of regulatory T lymphocytes.

One may wonder about peripheral CD8$^+$CD25$^+$ T cells from normal mice. In fact, whether they belong to the CD25$^+$ regulatory cell lineage remains unclear. On one hand, they express all surface markers characterizing regulatory CD25$^+$ T cells. On the other hand, these markers are also expressed by freshly activated T cells. Moreover, they differ from both CD8$^+$CD25$^+$ T cells from MHC class II-deficient mice and CD4$^+$CD25$^+$ T cells by their lower expression of Foxp3 and of CD25 by itself, the two most reliable markers of regulatory T cells. Interestingly, Cosmi et al. (35) reported recently the existence of human CD8$^+$CD25$^+$ thymocytes with regulatory activity but it is not known whether such a subset exists in the human periphery. Further studies will be thus required to clearly demonstrate the regulatory abilities of peripheral CD8$^+$CD25$^+$ T cells in normal mice.

The absolute number of CD25-expressing CD8$^+$ T cells is remarkably increased in the periphery (11-fold) of mice lacking MHC class II molecules relative to their wild-type counterparts. Such an increase could reflect adaptive compensation for the absence of regulatory CD4$^+$ CD25$^+$ T cells. Any such adjustment would have to take place early during thymic differentiation, because the absolute number of mature CD8$^+$CD25$^+$ T cells was also augmented (24-fold) in the thymus of mice lacking MHC class II molecules. Another possible explanation involves a particularity of MHC class II-deficient mice. Indeed, in the absence of MHC class II molecule expression, cell surface TCR expression increases strongly among immature double-positive thymocytes (36, 37), and this would in turn increase the average avidity of immature thymocytes for self-peptide/self-MHC class I complexes during positive selection. It has been suggested that, among positively selected thymocytes, CD4$^+$CD25$^+$ T cells have the highest avidity for self peptides presented by cortical epithelial cells in the thymus (38, 39). In MHC class II-deficient mice, increased avidity of cortical thymocytes for self-peptides could thus lead to increased generation of regulatory CD8$^+$CD25$^+$ T cells.

Unlike $CD4^+CD25^+$ T cells, $CD8^+CD25^+$ T cells from MHC class II-deficient mice cannot be considered as naturally anergic T cells. Indeed, in the absence of exogenous IL-2, they proliferated and produced IFN-γ in response to anti-CD3 stimulation. Thus, interestingly, although they were proliferating, $CD8^+CD25^+$ T cells from MHC class II-deficient mice regulated $CD25^-$ T cell responses to anti-CD3 stimulation with similar efficiency to regulatory $CD4^+CD25^+$ T cells. This ability of regulatory $CD8^+CD25^+$ T cells to cycle in response to anti-CD3 stimulation demonstrates that T lymphocytes do not need to be non-proliferative to exert their suppressive actions. Nevertheless, as previously described (10, 16, 30), both $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells regulated the proliferation of $CD25^-$ T cells less efficiently when exogenous IL-2 was added to the culture medium. Our observations suggest that IL-2 does not diminish regulatory efficiency by allowing $CD25^+$ T cells to cycle but rather by rendering $CD25^-$ T cells less receptive to suppressive signals. IL-2 could allow $CD25^-$ T cells to enter the cell cycle more rapidly, before $CD25^+$ T cells are able to exert their suppressive action. The lesser sensitivity to regulation of $CD8^+CD25^-$ T cells, which proliferate more rapidly than $CD4^+CD25^-$ T cells in response to anti-CD3, supports this hypothesis. Our results showing that cycling regulatory T cells still exert their suppressive activity help explaining recent data showing that regulatory $CD4^+CD25^+$ T cells are not anergic in vivo and proliferate as extensively as naive $CD4^+$ T cells after immunization, without losing their suppressive function (40, 41).

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Disclosures

The authors have no financial conflict of interest.

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