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Direct Expansion of Functional CD25+ CD4+ Regulatory T Cells by Antigen-processing Dendritic Cells

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Abstract

An important pathway for immune tolerance is provided by thymic-derived CD25+ CD4+ T cells that suppress other CD25− autoimmune disease–inducing T cells. The antigen-presenting cell (APC) requirements for the control of CD25+ CD4+ suppressor T cells remain to be identified, hampering their study in experimental and clinical situations. CD25+ CD4+ T cells are classically anergic, unable to proliferate in response to mitogenic antibodies to the T cell receptor complex. We now find that CD25+ CD4+ T cells can proliferate in the absence of added cytokines in culture and in vivo when stimulated by antigen-loaded dendritic cells (DCs), especially mature DCs. With high doses of DCs in culture, CD25+ CD4+ and CD25− CD4+ populations initially proliferate to a comparable extent. With current methods, one third of the antigen-reactive T cell receptor transgenic T cells enter into cycle for an average of three divisions in 3 d. The expansion of CD25+ CD4+ T cells stops by day 5, in the absence or presence of exogenous interleukin (IL)-2, whereas CD25− CD4+ T cells continue to grow. CD25+ CD4+ T cell growth requires DC–T cell contact and is partially dependent upon the production of small amounts of IL-2 by the T cells and B7 costimulation by the DCs. After antigen-specific expansion, the CD25+ CD4+ T cells retain their known surface features and actively suppress CD25− CD4+ T cell proliferation to splenic APCs. DCs also can expand CD25+ CD4+ T cells in the absence of specific antigen but in the presence of exogenous IL-2. In vivo, both steady state and mature antigen-processing DCs induce proliferation of adoptively transferred CD25+ CD4+ T cells. The capacity to expand CD25+ CD4+ T cells provides DCs with an additional mechanism to regulate autoimmunity and other immune responses.

Key words: dendritic cells • CD25+ CD4+ regulatory T cells • anergy • IL-2 • CD86

Introduction

Evidence is accumulating that immunologic self-tolerance in the periphery can be maintained by CD25+ CD4+ regulatory T cells, which constitute 5–10% of CD4+ peripheral T cells (1–6). CD25+ CD4+ suppressors also play important roles during other immune responses, as in infection (7–9), tumors (10, 11), transplants (12), and graft versus host disease (13). A classical feature of CD25+ CD4+ regulatory T cells is that they are anergic upon TCR–mediated stimulation in vitro, failing to undergo proliferation, yet they suppress the activation and proliferation of other CD4+ and CD8+ T cells (14–16). CD25+ CD4+ T cells can inhibit IL-2 transcription by responder T cells and because of their anergy, it is unclear how the numbers of regulatory T cells are sustained and expanded (14–16). Nonetheless, CD25+ CD4+ T cells are able to undergo homeostatic expansion when transferred into lymphocyte-deficient hosts (17, 18).

Molecular mechanisms for the function of suppressive CD25+ CD4+ T cells are being identified. Cytotoxic T lymphocyte–associated antigen 4 (CTLA-4)* is expressed and required for the activation of suppression (19, 20),

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Abbreviations used in this paper: BM-DC, BM-derived DC; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTLA-4, cytotoxic T lymphocyte–associated antigen 4; GITR, glucocorticoid-induced TNF receptor; PEC, peritoneal exudate cell; TGC, thioglycollate.

whereas glucocorticoid-induced TNF receptor (GITR) signaling of $CD25^+$ $CD4^+$ T cells renders them nonsuppressive (21, 22). The transcription factor, Foxp3, programs the development of regulatory T cells (23–25). When DCs are stimulated through Toll–like receptors, they can produce IL-6 and unknown soluble factor(s), rendering the $CD25^-$ $CD4^+$ T cells resistant to suppression by $CD25^+$ $CD4^+$ T cells (26). However, the APCs that control the expansion of the "anergic" but suppressive $CD25^+$ $CD4^+$ T cells remain to be identified, hampering their experimental study and use in many of the important clinical conditions summarized above.

We have now investigated the interaction between different types of APCs and $CD25^+$ $CD4^+$ regulatory T cells. We find that $CD25^+$ $CD4^+$ T cells are not anergic, as long as the cells are stimulated with DCs. In fact, the $CD25^+$ $CD4^+$ T cells can proliferate in an antigen-specific manner in vitro and in vivo for several days at a rate that is comparable to that seen in $CD25^-$ $CD4^+$ T cells. $CD25^+$ $CD4^+$ T cells expanded by mature DCs, retain high expression of CTLA-4 and GITR, and are enriched in suppressor function. Importantly, $CD25^+$ $CD4^+$ T cells proliferate extensively in response to DCs, processing protein antigens in the steady state or after maturation in vivo in lymph nodes, thereby providing DCs with another mechanism for their emerging roles in the maintenance of peripheral immune tolerance (27).

Materials and Methods

Mice. BALB/c and C57BL/6 mice were purchased from Taconic Farms. OVA-specific, MHC class II–restricted, TCR transgenic mice were DO11.10 ($H-2^d$; provided by Dr. P. Marrack, National Jewish Medical and Research Center, Denver, CO) and OT-II ($H-2^b$; provided by F. Carbone, University of Melbourne, Parkville, Victoria/Australia). C57BL/6, $CD80^{-/-}$ $CD86^{-/-}$, and $IL-2^{-/-}$ mice were from The Jackson Laboratory, and BALB/c $IL-2^{-/-}$ mice were provided by Drs. M. and J. Lafaille (New York University, New York, NY). Specific pathogen-free mice of both sexes were used at 6–12 wk of age according to institutional guidelines.

Antibodies and Reagents. mAbs for MHC class II (M5/114, TIB120), B220 (RA3-6B2, TIB146), CD8 (3-155, TIB211), CD4 (GK1.5, TIB207), CD3 (145-2C11, CRL1975), and HSA (J11d, TIB183) were from American Type Culture Collection. FITC-conjugated anti-CD25 (7D4), $I-A^d$ (AMS-32), Gr1 (RB6-8C5), CD11c (HL3), and CD4 (H129.19), PE–anti-CD8a (53-6.7), B220 (RA3-6B2), CD86 (GL1), and CTLA-4 (UC10-4F10-11), biotinylated anti-CD25 (7D4), $I-A^b$ (AF6-120.1), $I-A^d$ (AMS-32), and mouse anti–human Vβ8 (BV8), APC–anti-CD11c (HL3), CD62L (MEL-14), CD25 (PC61), and CD4 (RM4-5), and PE-streptavidin, CyChrome-streptavidin, and PerCP streptavidin were from BD Biosciences. FITC- and biotin-KJ1.26 antibody to the TCR of DO11.10 T cells was from Caltag. Purified antibody to CD3 (145-2C11), CD25 (PC61), CD49b/Pan NK cells (DX5), CD16/CD32 (2.4G2), and control rat IgG were from BD Biosciences. We purchased biotin goat anti–GITR and IFN-γ from R&D Systems, rHu IL-2 from Chiron Corp., anti-CD11c, CD43, CD19, CD5, FITC, and PE microbeads from Miltenyi Biotec, carboxyfluorescein diacetate suc-

cinimidyl ester (CFSE) from Molecular Probes, and intracellular staining kit for CTLA-4 and OptEIA™ kits for mouse IL-2, IL-4, IL-10, and IFN-γ ELISA from BD Biosciences.

Proliferation Assays. Spleen and lymph node cell suspensions were depleted of $J11d^+$, $CD8^+$, and $DX5^+$ cells by panning. The remaining $CD4^+$-enriched cells were stained with antibodies to CD4 and CD25 (7D4) and sorted on a FACS Vantage™ (BD Biosciences) into $CD25^+$ and $CD25^-$ populations (>97% and >99% pure). 10^4 T cells were cultured for 3 d with APCs, either 10^3–10^4 DCs or 5–10 × 10^4 fresh spleen cells (irradiated with 15–20 Gy) in 96-well round-bottomed plates (Corning). 1 mg/ml OVA protein was pulsed into the BM cultures for 16 h before harvesting the DCs, or 1 μg/ml DO11.10 OVA 323–336 peptide was added continuously to the APC–T cell cocultures. To assess suppression by $CD25^+$ $CD4^+$ T cells, 5–10 × 10^4 whole spleen cells were used to stimulate mixtures of 1–2 × 10^4 $CD25^-$ and 1–2 × 10^4 $CD25^+$ $CD4^+$ T cells from DO11.10 or BALB/c mice (14–16, 21). 5% vol/vol supernatant of 2C11 hybridoma cells secreting anti-CD3 antibody or 1 μg/ml purified antibody was added for stimulation. [^{3}H]thymidine uptake (1 μCi/well; NEN Life Science Products) by proliferating lymphocytes was measured at 60–72 h. To assess the need for cell to cell contact, CFSE-labeled T cells were placed on both sides of a transwell chamber (Costar). The outer well contained DCs and T cells (3 × 10^5 each) and anti-CD3 antibody to stimulate cell growth, and the inner well had 5 × 10^4 T cells without or with either anti-CD3 or 5 × 10^4 DCs to determine if soluble factors from the outer well could drive T cell expansion.

BM-derived DCs (BM-DCs). These were prepared with GM-CSF (28). In brief, BM cells were grown in RPMI 1640 containing 5% FCS and the supernatant (3% vol/vol) from J558L cells was transduced with murine GM-CSF (provided by A. Lanzavecchia, Basel Institute, Basel, Switzerland). On day 5, OVA (Seikagaku), which contained <20 pg endotoxin/mg protein, was added in some wells at 1 mg/ml with or without LPS (Sigma-Aldrich) at 50 ng/ml for 16 h. On day 6, cells were collected and washed with HBSS. After Fc block, the cells were stained with FITC–anti-Gr1 mAb and PE–anti-CD86. After washing, the cells were incubated with anti-FITC microbeads and put onto MACS columns (Miltenyi Biotec) to eliminate residual $Gr1^+$ granulocytes. The negative cells were then incubated with anti–PE-MACS beads and put onto MACS columns to provide $CD86^{high}$ mature and $CD86^{low}$ immature DCs, which were irradiated with 15–20 Gy. In some experiments the $CD86^{high}$ and $CD86^{low}$ DCs were sorted by flow cytometry with similar results. For fixation, DCs were incubated with 0.75% paraformaldehyde for 30 min on ice. To measure IL-2 production, fixed or nonfixed DCs were cultured for 1 d with 0, 10, 100, or 1,000 ng/ml LPS and the concentration of IL-2 was measured by ELISA.

Other APCs. Spleen $CD8^-$ and $CD8^+$ DCs were prepared as previously described (29). Splenic B cells were prepared with $CD19^+$ MACS beads from spleen high density populations. Peritoneal exudate cells (PECs) were collected by washing the peritoneal cavity with PBS. 4 d earlier, some mice were given thioglycollate (TGC; Difco). In some instances, 2 d after injection of TGC, mice were given 100 U IFN-γ i.p. to up-regulate MHC class II on the macrophages. Lymph node $CD11c^+$ DCs were isolated with CD11c beads (29). For priming with CFA (Difco), a 1:1 emulsion of CFA and PBS was injected s.c. (50 μl/paw) and 5 d later, lymph node $CD11c^+$ DCs were prepared.

Proliferation of CFSE-labeled $CD25^+$ and $CD25^-$ $CD4^+$ T Cells. For in vitro studies, FACS®-purified $CD25^+$ or $CD25^-$ $CD4^+$ T cells were incubated with 1 μM CFSE for 10 min at

37°C and 10^4 T cells were cultured with OVA-pulsed or –unpulsed CD86$^+$ BM-DCs for 3 d before FACS® analysis for proliferation (progressive halving of the CFSE label). Dead cells were gated out with TOPRO-3 iodide (Molecular Probes) labeling. For in vivo proliferation, CD25$^+$ or CD25$^-$ CD4$^+$ T cells purified by flow cytometry or by MACS were labeled with 5 μM CFSE, and $0.7–1.0 \times 10^6$ T cells were injected i.v. into BALB/c recipients. 1 d later, 2×10^5 OVA-pulsed or –unpulsed, LPS-matured marrow DCs (depleted of macrophages by adherence to plastic for 2 h) were injected s.c. in each paw. Alternatively, the mice were given 25 μg soluble endotoxin-free OVA into the paw. It is known that DCs in the steady state are the major cell type presenting OVA to T cells in the steady state (30).

Results

BM-DCs Stimulate CD25$^+$ CD4$^+$ T Cell Proliferation– [^{3}H]thymidine Uptake Studies. To follow the antigen-dependent growth of CD25$^+$ and CD25$^-$ CD4$^+$ T cells,

we purified these populations from OVA-specific TCR transgenic DO11.10 mice by FACS® (Fig. 1 A, top). This step was the limiting one for the experiments, because only $2–3 \times 10^6$ purified CD25$^+$ CD4$^+$ T cells were obtained from eight mice. When standard bulk populations of spleen cells were tested as APCs, we found as expected from much earlier work (beginning with references 14 and 15) that the CD25$^+$ CD4$^+$ T cells were anergic or nonresponsive to stimulation with anti-CD3 mitogenic antibody, whereas the CD25$^-$ CD4$^+$ T cells responded (Fig. 1 A, bottom). Furthermore, mixtures of CD25$^+$ and CD25$^-$ CD4$^+$ T cells were suppressed, failing to proliferate to anti-CD3 when spleen cells were the APCs (Fig. 1 A). In contrast, when we tested DCs (even in low numbers) generated from BM progenitors with GM-CSF, the CD25$^+$ CD4$^+$ T cells were now responsive to anti-CD3, and suppression was no longer evident in mixtures of CD25$^+$ and CD25$^-$ CD4$^+$ T cells (Fig. 1 A). Strong responses were repeatedly
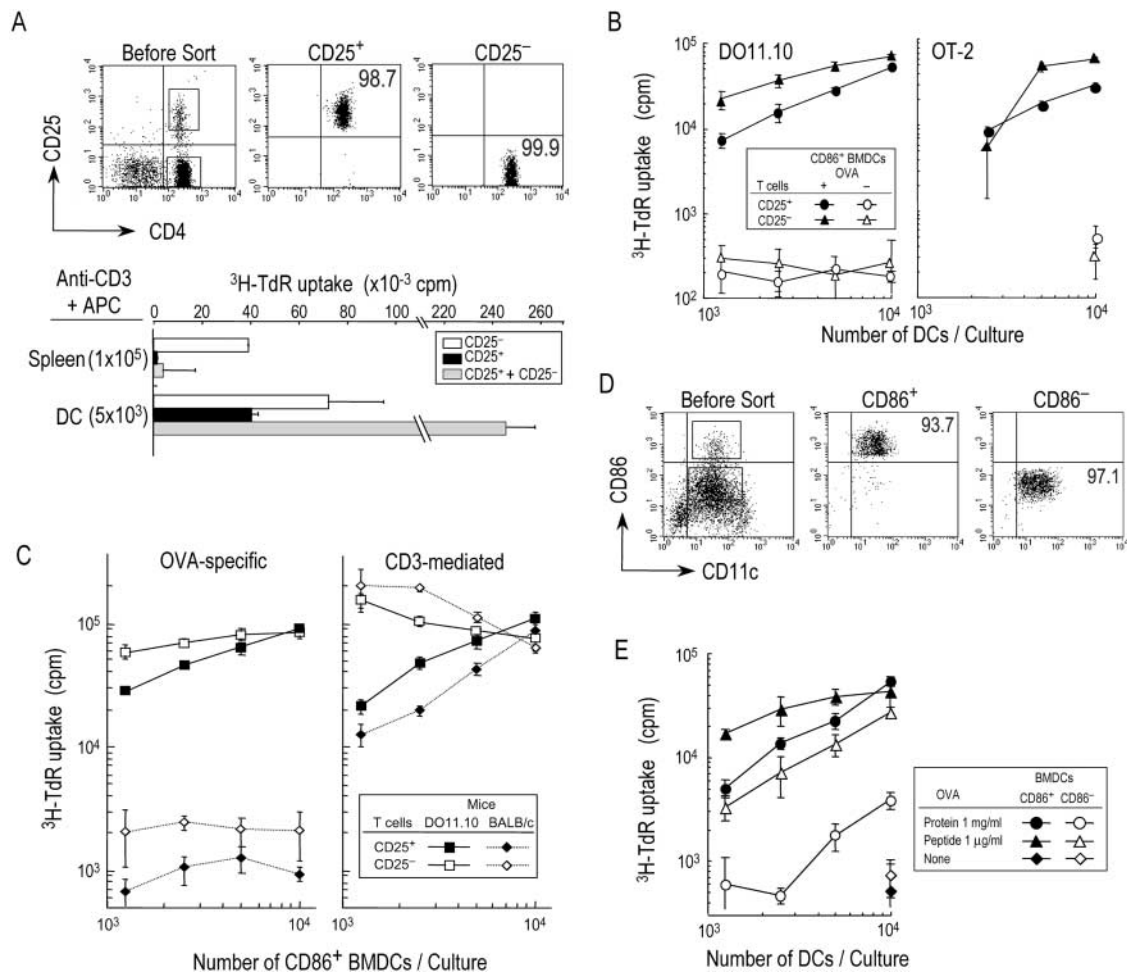

Figure 1. DCs stimulate CD25$^+$ CD4$^+$ T cell growth. (A) 10^4 CD25$^+$ or CD25$^-$ CD4$^+$ FACS®-purified (top) DO11.10 OVA-specific T cells were cultured for 3d with 10^5 spleen APCs or 5×10^3 CD86$^+$ mature DCs and anti–CD3 mAb. [^{3}H]thymidine uptake was assessed (60–72 h). (B) As in A, but T cells were from two OVA-specific TCR transgenic mice, DO11.10 and OT-2, and the DCs were pulsed or not pulsed with 1 mg/ml OVA protein. (C) CD25$^+$ CD4$^+$ T cells from wild-type BALB/c mice (◆) proliferate in response to DCs presenting anti-CD3 (right) but not OVA (left). (D and E) Day 6 marrow DCs were FACS® separated into mature CD86^{high} and immature CD86^{low} CD11c$^+$ subsets (D) and cultured with CD25$^+$ CD4$^+$ DO11.10 T cells (E) with OVA protein (1 mg/ml pulsed onto the DCs) or 1 μg/ml OVA 323–339 peptide continuously. One representative result of at least three experiments is shown.

observed with CD25$^+$ CD4$^+$ T cells from two different OVA-specific transgenics, DO11.10 and OT-II, and over a broad range of DC doses in the presence of OVA antigen (Fig. 1 B). Non-TCR transgenic BALB/c T cells also responded to DCs presenting anti-CD3 but did not respond to DCs presenting OVA, whereas DO11.10 T cells responded to both (Fig. 1 C), confirming that the responses by OVA-reactive CD25$^+$ CD4$^+$ transgenic T cells were antigen-specific.

To evaluate the effect of DC maturation on their capacity to stimulate CD25$^+$ CD4$^+$ T cells, we sorted the BM-DCs into mature and immature populations, expressing high and low levels of the CD86 T cell costimulatory molecule, respectively (Fig. 1 D). Both were active, but the mature CD86^{high} DCs were better stimulators for T cell proliferation when either OVA protein or preprocessed peptide was the source of antigen (Fig. 1 E). Dose response

studies indicated that as little as 0.01 μg/ml peptide could stimulate the proliferation of CD25$^+$ CD4$^+$ T cells significantly (unpublished data). Also, the DCs were equally active if they had been matured spontaneously (Fig. 1 D) or in the presence of LPS (not depicted), the latter to increase the yield of mature DCs. Therefore, CD25$^+$ CD4$^+$ T cells are not intrinsically unresponsive to TCR stimulation but are able to proliferate to anti-CD3 and to antigen when presented by DCs and in the absence of exogenous growth factors like IL-2.

BM-DCs Stimulate CD25$^+$ CD4$^+$ T Cell Proliferation–Extent of Cell Division. To certify the capacity of CD25$^+$ CD4$^+$ T cells to proliferate to antigen-presenting DCs, we documented their growth in two other ways. First, the number of CD25$^+$ CD4$^+$ cells expanded about fivefold in 3–5 d in the presence of OVA antigen (Fig. 2 A, right) at the same time that DNA synthesis was robust, 50–100 ×
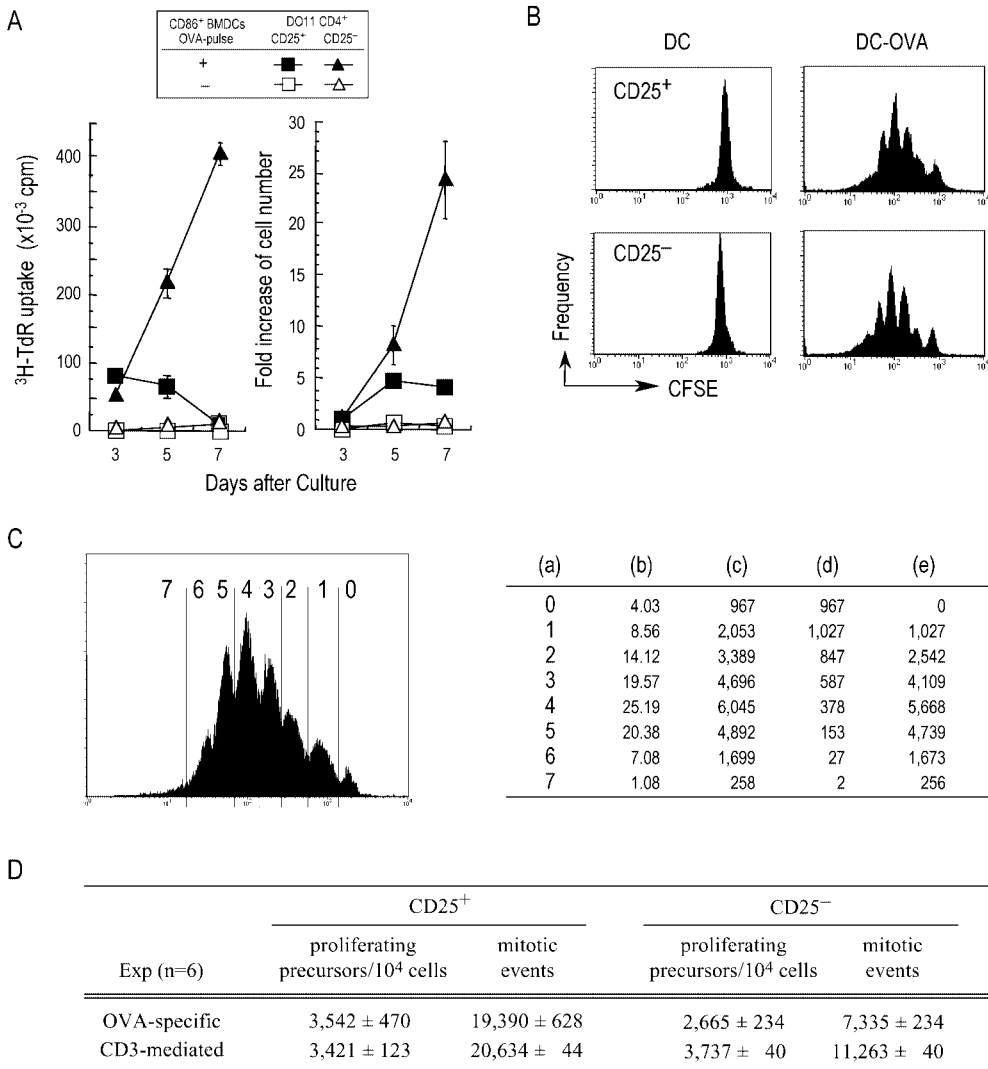

Figure 2. A large fraction of CD25$^+$ CD4$^+$ T cells are driven into multiple cell cycles by DCs. (A) As in Fig. 1, but the kinetics of proliferation ([^{3}H]thymidine and cell counts) were both followed. (B) 10^4 CFSE-labeled T cells were cultured for 3 d with 10^4 CD86$^+$ mature BM-DCs either OVA-pulsed (DC-OVA) or unpulsed (DC), before FACS® analysis. (C) Quantitative estimation of the number of T cells entering the cell cycle, and the number of mitotic events, was performed as follows. 10^4 CFSE-labeled CD25$^+$ CD4$^+$ T cells were cultured for 72 h with 1 mg/ml OVA-pulsed CD86$^+$ BMDCs (10^4), and analyzed for dilution of CFSE label (C). The percentage of total CD4$^+$ events under each division peak (a) was experimentally determined (b). In this experiment, 24,000 live T cells were recovered, from which the absolute T cell count in each division peak at the time of harvest could be calculated (c). The absolute number of original, or precursor, T cells required to have generated these daughters is extrapolated by dividing the numbers of cells in column c by the number of divisions, 2 n (d). The sum of the number of precursors giving rise to each peak represents the number of T cells at day 0 that entered cell cycle, which in this experiment was 3,020 (the sum of column d) from a starting number of 10,000 T cells, giving a precursor frequency of 30%.

(a)	(b)	(c)	(d)	(e)
0	4.03	967	967	0
1	8.56	2,053	1,027	1,027
2	14.12	3,389	847	2,542
3	19.57	4,696	587	4,109
4	25.19	6,045	378	5,668
5	20.38	4,892	153	4,739
6	7.08	1,699	27	1,673
7	1.08	258	2	256

Exp (n=6)	CD25$^+$		CD25$^-$	
	proliferating precursors/10^4 cells	mitotic events	proliferating precursors/10^4 cells	mitotic events
OVA-specific	3,542 ± 470	19,390 ± 628	2,665 ± 234	7,335 ± 234
CD3-mediated	3,421 ± 123	20,634 ± 44	3,737 ± 40	11,263 ± 40

The number of progeny in each peak (c) minus the number of precursors giving rise to the progeny (d) gives the number of mitotic events (e). The sum of these events represents the total number of cell divisions that occurred in the T cell subset by the time of harvest. (D) The experiment and calculation in C was performed in a total of six experiments where the TCR stimulus was specific OVA antigen (n = 3) or anti-CD3 antibody (n = 3).

10^3 cpm in cultures of 10^4 T cells (Fig. 2 A, left). However, the CD25$^+$ CD4$^+$ T cells did not expand beyond the initial 3–5 d of culture, whereas CD25$^-$ CD4$^+$ cells expanded in a sustained fashion (Fig. 2 A), the latter most likely because of the production of large amounts of IL-2 as will be shown below. We also added antigen-bearing DCs to CD25$^+$ CD4$^+$ T cells that had been expanded previously for 1 wk in culture, and again we observed a two- to three-fold expansion in T cell numbers (not depicted).

We then compared the proliferation of CFSE-labeled CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ T cells. Both populations underwent several cycles of cell division in 3 d (Fig. 2 B). Using this data and the approach of Wells et al. (31), in six experiments (three each using DCs to present anti-CD3 antibody or specific OVA antigen), we found that about one third of the cultured CD25$^+$ CD4$^+$ T cells underwent

at least one mitotic event during 3 d of culture (Fig. 2 D). During the same time period, a similar frequency of the CD25$^-$ CD4$^+$ T cells entered cell cycle, but the number of mitotic events was actually less (Fig. 2 D). We also verified that the major CD62L$^+$ and minor CD62L$^-$ subsets of CD25$^+$ CD4$^+$ T cells responded comparably to DC-OVA (not depicted). Therefore, in the first 3 d of culture, both CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ are stimulated by DCs to enter cell cycle and expand significantly.

Partial IL-2 Dependence of DC-induced CD25$^+$ CD4$^+$ T Cell Proliferation, Including IL-2–induced, Antigen-independent Proliferation. Because the CD25 marker for regulatory T cells is a component of the IL-2 receptor, we tested the role of IL-2 in our cultures. The addition of exogenous IL-2 only induced a minute response in the CD25$^+$ CD4$^+$ T cells themselves (Fig. 3 A, top; note the units on the y axis).
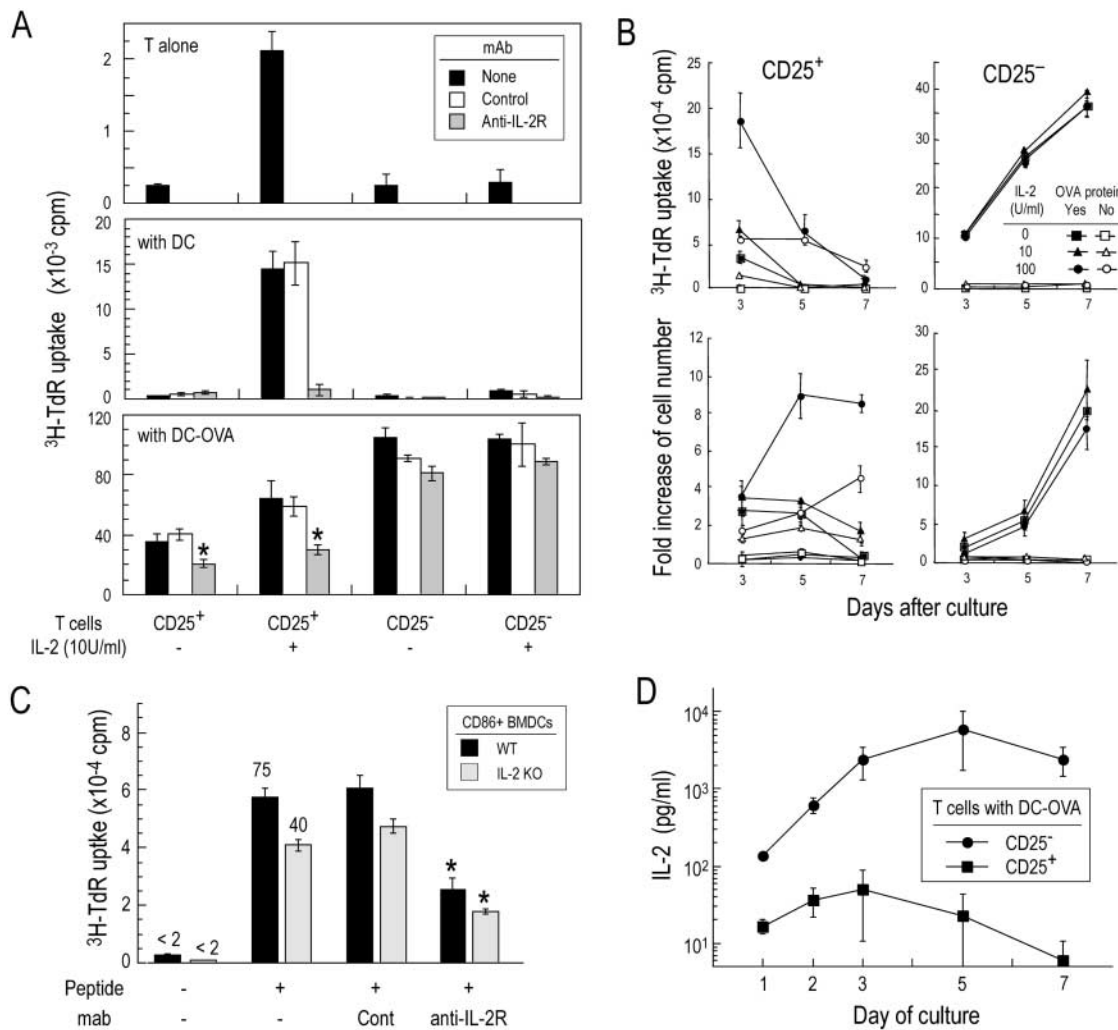
Figure 3. Role of IL-2 in CD25$^+$ CD4$^+$ T cell proliferation. (A) [^{3}H]thymidine uptake by CD25$^+$ or CD25$^+$ CD4$^+$ T cells alone (top), or T cells stimulated by CD86$^+$ DCs not pulsed (middle) or pulsed (bottom) with OVA protein ± IL-2 or PC61 anti–IL-2R mAb. (B) As in A, but IL-2 effects on [^{3}H]thymidine uptake and cell counts were assessed with time. (C) As in A, but anti–IL-2R mAb or control rat IgG was added to CD25$^+$ CD4$^+$ T cells stimulated with DCs from wild-type (WT) or IL-2$^{-/-}$ mice plus OVA peptide at 1 μg/ml for 3 d. The numbers above the bars indicate the amount of IL-2 detected by ELISA in the same culture. (D) IL-2 production (ELISA) after stimulation with DC-OVA or DCs. Statistical significance was determined using the unpaired Student's *t* test. *, P < 0.01.

However, IL-2 induced more significant proliferation of $CD25^+ CD4^+$, but not $CD25^- CD4^+$, T cells in the presence of DCs without OVA antigen, and this could be blocked by anti–IL-2R antibody completely (Fig. 3 A, middle). DCs with OVA stimulated $CD25^+ CD4^+$ T cell growth 5–10-fold more vigorously than in the absence of antigen (compare the y axes of Fig. 3 A, middle and bottom). The response of $CD25^+ CD4^+$ T cells was enhanced by low doses of exogenous IL-2 (Fig. 3 A). Proliferation in the absence of IL-2 was partially blocked (52.0 ± 9.3%, $n = 5$) by anti-CD25 antibody, whereas IL-2 and anti–IL-2R antibody had little or no effect on the responses of $CD25^- CD4^+$ T cells (Fig. 3 A, bottom). When the kinetics of the response to exogenous IL-2 was monitored, the stimulation of $CD25^+ CD4^+$ T cell growth was evident primarily in the first 3–5 d in culture (Fig. 3 B, left). In contrast, $CD25^- CD4^+$ T cells responded continuously for 1 wk to DCs, without any boost by exogenous IL-2 (Fig. 3 B, right). Thus, IL-2 enhances antigen-dependent and -independent proliferation of $CD25^+ CD4^+$ T cells in response to DCs.

$CD25^+ CD4^+$ T Cells Produce Low Levels of IL-2 in Response to DCs, and Their Proliferation Is Partially Dependent on B7 Costimulation. We first tested if the observed proliferative responses to DCs could be attributed to IL-2 made by the DCs themselves, using DCs from $IL-2^{-/-}$ mice and al-

dehyde-fixed DCs. We confirmed the findings of Granucci et al. (32) that DCs in the absence of T cells produced IL-2 upon stimulation and that this could be abolished by fixation of the DCs in paraformaldehyde (not depicted). DCs from $IL-2^{-/-}$ mice (Fig. 3 C) were active in stimulating $CD25^+ CD4^+$ T cells, and the growth was partially blocked with anti-CD25 antibody (Fig. 3 C). We then measured IL-2 production in the $IL-2^{-/-}$ DC–T cell cocultures because it is known that $CD25^+ CD4^+$ T cells do not produce detectable IL-2 in response to splenic APCs and anti-CD3 (14, 15). However, we found that the culture supernatants from $CD25^+ CD4^+$ T cells and OVA-DCs from wild-type mice did contain some IL-2 by ELISA (concentrations of IL-2 is shown above the bars in Fig. 3 C), but primarily in the first 3 d of the cultures and only at a small fraction of the levels induced by DCs from $CD25^- CD4^+$ T cells (Fig. 3 D). IL-10 was undetectable by ELISA in the culture supernatants of $CD25^+$ T cells stimulated by DC-OVA (<40 pg/ml), and other cytokines like IFN-γ (<40 pg/ml) and IL-4 (<10 pg/ml) were also absent (ELISA data is not depicted).

To assess the potential role of cell surface costimulators on DCs, we tested if formaldehyde-fixed DCs could induce T cell proliferation. Live DCs were more effective than fixed DCs (Fig. 4 A, threefold higher doses of DCs were used; Fig. 4 B) and anti–IL-2R antibody could par-
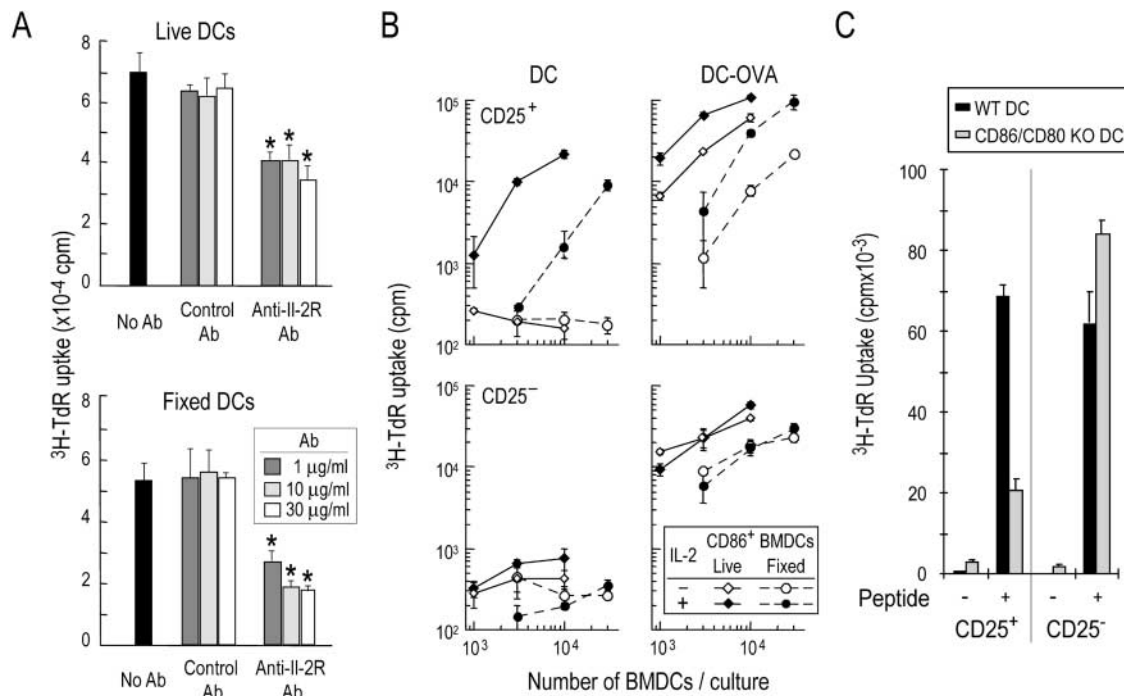

Figure 4. Membrane costimulation of $CD25^+ CD4^+$ T cells by DCs. (A) Comparison of T cell responses to live (top, T/DC ratio of 1:1) or formaldehyde-fixed (bottom, T/DC ratio of 1:3) $CD86^+$ mature marrow DCs plus DO11.10 peptide at 1 µg/ml for 3 d in the presence of the indicated concentrations of control and anti–IL-2R mAb. Statistical significance was determined using the unpaired Student's t test. *, P < 0.01. (B) Same as A, but the activity of aldehyde-fixed DCs were studied with DCs that were charged with OVA (DC-OVA) or not (DC), and then added to $CD25^+ CD4^+$ and $CD25^- CD4^+$ T cells in the presence or absence of IL-2, with only the former subset responding to IL-2 in the absence of OVA (top left). (C) 10^4 marrow DCs were generated from wild-type (WT) or CD80/CD86 knockout mice and matured in 50 ng/ml LPS before culture with $10^4 CD25^+$ or $CD25^- CD4^+$ T cells (purified from OT-II mice spleen and lymph node cells) for 3 d with or without 0.5 µg/ml OVA peptide. The degree of proliferation was assessed by incorporation of [³H]thymidine for the last 12 h. One representative result of three independent experiments is shown.

tially block T cell proliferation (Fig. 4 B). Nevertheless, aldehyde-fixed DCs stimulated the growth of CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ T cells in the presence of OVA antigen. In the absence of OVA but with IL-2, live and fixed DCs also stimulated the growth of some CD25$^+$ CD4$^+$, but not CD25$^-$ CD4$^+$, T cells (Fig. 4 B).

The activity of aldehyde-fixed DCs suggested that a membrane-bound costimulatory molecule was contributing to the T cell response. In fact, DCs prepared from mice genetically deleted of the CD80 and CD86 costimulatory molecules (also known as B7-1 and B7-2) were only one third as efficient at stimulating the proliferation of CD25$^+$ CD4$^+$ cells (Fig. 4 C). The proliferation of the transgenic CD25$^-$ CD4$^+$ T cells in parallel was actually maintained with B7-deficient DCs in this system in which the DC/T cell ratio was 1:1 (Fig. 4 C), but B7-deficient DCs were less active with lower DC/T cell ratios of 1:25 (not depicted). In sum, the response of CD25$^+$ CD4$^+$ T cells to antigen-bearing DCs is substantially blocked by anti-CD25 antibody. The requisite IL-2 is produced in small amounts by the responding T cells, and B7 costimulation contributes significantly to CD25$^+$ CD4$^+$ T cell proliferation.

The Proliferation of CD25$^+$ CD4$^+$ T Cells Induced by DCs Requires DC–T Cell Contact. Transwell experiments were then performed to show the need for DC–T cell contact in the proliferation of CD25$^+$ CD4$^+$ T cells.

These T cells, when cultured in the inner well with anti-CD3 or DC only, could undergo at most a single cell division whether or not the outer well was empty or contained mixtures of CD25$^+$ CD4$^+$ T cells with both DC and anti-CD3 antibody (Fig. 5, top). However, most CD25$^+$ CD4$^+$ T cells cultured together with DCs and anti-CD3 divided two to five times (Fig. 5), indicating that cell–cell contact with DCs was important for initiating their growth.

Retention of Suppressive Activity in CD25$^+$ CD4$^+$ T Cells Expanded by DCs. It was important to verify that the CD25$^+$ CD4$^+$ T cells retained their known phenotypic markers and suppressive properties after DC-induced expansion, which was 3–10-fold in the absence and presence of exogenous IL-2, respectively. In terms of phenotype, the expanded CD25$^+$ CD4$^+$ T cells maintained higher expression of CTLA-4 and GITR (19–22) relative to CD25$^-$ CD4$^+$ responders (Fig. 6 A). During expansion, expression of CD62L (the lymph node homing receptor) decreased on many of the CD25$^+$ CD4$^+$ T cells, but after 7 d of culture, most cells expressed CD62L (not depicted), as is the case for most regulatory T cells in lymphoid organs (33). CD25$^-$ CD4$^+$ T cells proliferating in response to DC-OVA up-regulated expression of CD25, CTLA-4, and GITR, and almost all cells had little or no CD62L at day 7 (Fig. 6 A and unpublished data). The percentage of CD25$^+$ CD4$^+$ T cells expressing the KJ1.26 clonotypic TCR
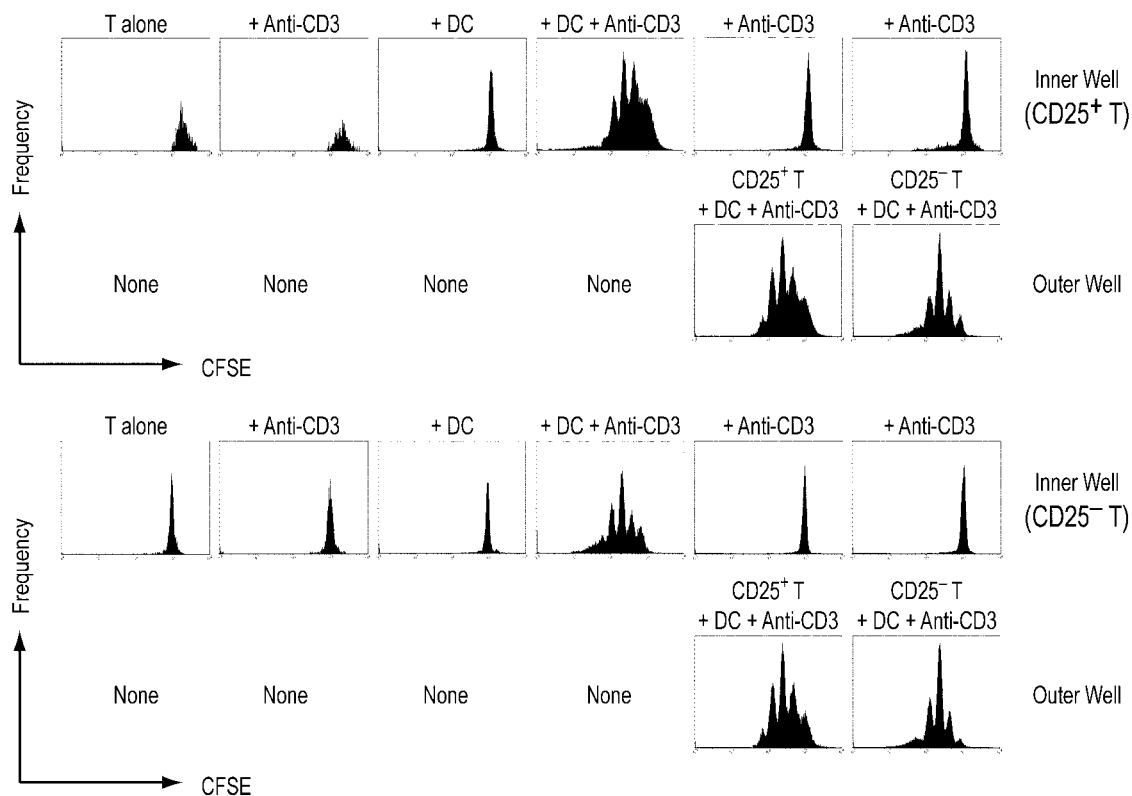

Figure 5. CD25$^+$ CD4$^+$ T cells must contact DCs to proliferate actively. CFSE-labeled CD25$^+$ CD4$^+$ T cells (top) or CD25$^-$ CD4$^+$ T cells (bottom) and the indicated stimuli were added to the inner and outer wells of transwell chambers, and the dilution of CFSE label per cell was followed by FACS® after 3 d of culture. Dead cells were gated out by TOPRO-3 staining. One representative result of three independent experiments is shown.
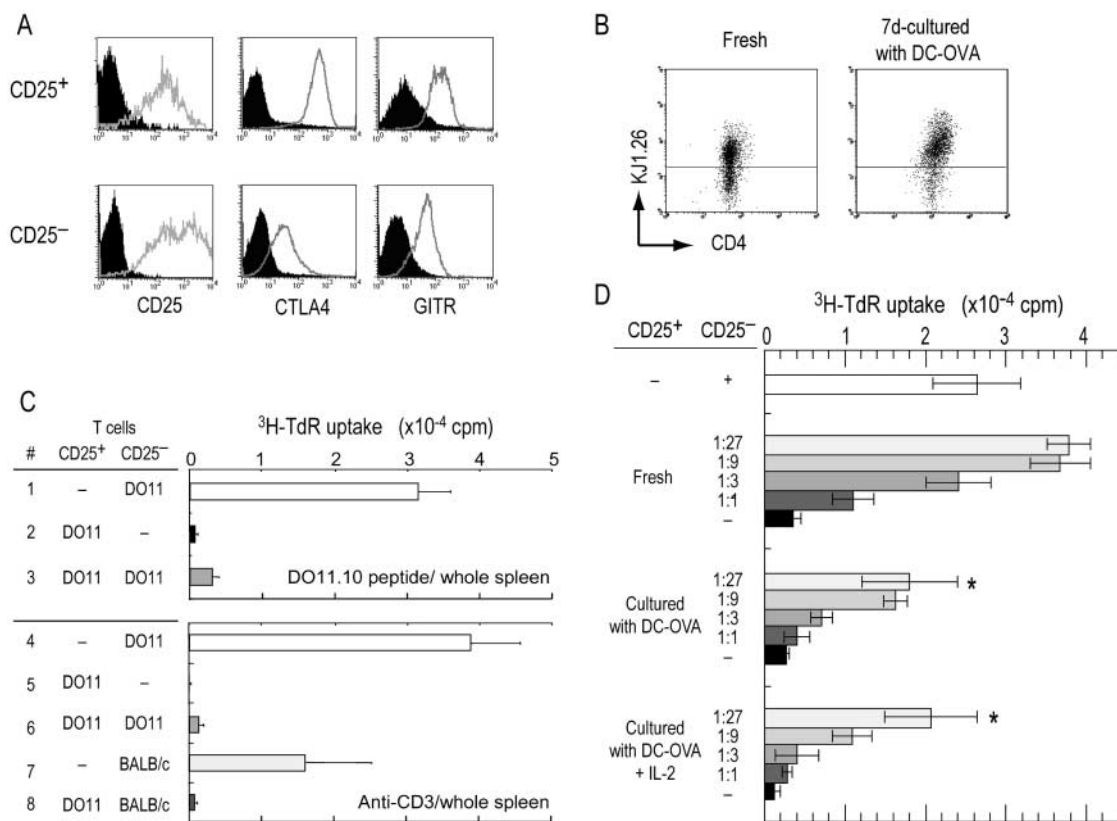
Figure 6. CD25⁺ CD4⁺ T cells expanded by mature BM-DCs retain phenotype and function. (A) Surface markers of CD25⁺ CD4⁺ and CD25⁻ CD4⁺ T cells after 7-d expansion by mature CD86⁺ DC-OVA (shaded histogram, isotype control). (B) As in A, but the expression of the KJ1.26 clonotypic receptor in CD25⁺ CD4⁺ T cells is shown before and after 7 d of culture with DC-OVA. (C) 10^4 DO11.10 T cells were cultured for 7 d with an equal number of OVA-pulsed CD86⁺ marrow DCs. CD11c⁺ DCs were eliminated by MACS, and then the recovered T cells were used to respond to 5×10^4 splenic APCs, or to suppress fresh CD25⁻ CD4⁺ T cells in the presence of 1 μg/ml OVA peptide (top) or anti-CD3 mAb (bottom). (D) CD25⁺ CD4⁺ T cells purified from DO11.10 mice were expanded with OVA-pulsed mature DCs for 7 d as in C, with or without exogenous 100 U/ml IL-2. Fresh or cultured CD25⁺ CD4⁺ T cells were then mixed with freshly isolated CD25⁻ CD4⁺ T cells from DO11.10 mice at the indicated ratios and cultured for 3 d. The degree of proliferation was assessed by incorporation of [³H]thymidine for the last 12 h. Representative results of three or more similar experiments. Statistical significance was determined using the unpaired Student's t test. *, P < 0.01.

marker was enriched after expansion, 80 versus 60% initially, and the mean fluorescence for KJ1.26 expression increased slightly (Fig. 6 B), indicating that DC-OVA were selectively expanding OVA-specific cells.

When the functions of the expanded CD25⁺ CD4⁺ cells were tested with whole spleen APCs, the T cells were indeed anergic upon challenge with OVA or anti-CD3 (Fig. 6 C, groups 2 and 5, respectively) in contrast to the robust responses of CD25⁻ CD4⁺ cells (Fig. 6 C, groups 1 and 4). Furthermore, the expanded CD25⁺ CD4⁺ cells could actively suppress the responses of CD25⁻ CD4⁺ cells to OVA or anti-CD3 (Fig. 6 C, groups 3, 6, and 8). The CD25⁺ CD4⁺ T cells expanded by DC-OVA were more active on a per cell basis than freshly isolated CD25⁺ CD4⁺ T cells when tested for their capacity to suppress OVA-specific T cell responses (Fig. 6 D). These findings on the retained phenotype and function of CD25⁺ CD4⁺ T cells also were noted after expansion with DC-OVA plus IL-2 (Fig. 6 D, bottom). In summary, after expansion by DCs, CD25⁺ CD4⁺ T cells express their characteristic markers and regulatory function.

Weak Stimulation of CD25⁺ CD4⁺ Suppressor T Cells by Other Types of APCs. To compare the responses of CD25⁺ CD4⁺ T cells to various sources of APCs, we first examined DCs from different sites. Splenic CD8⁺ and CD8⁻ DC subsets were tested immediately upon isolation or after maturation overnight with LPS. These stimulated CD25⁺ CD4⁺ T cells but to a much lesser degree than BM-DCs with either OVA protein or peptide as antigen (Fig. 7, A and B). These cultured splenic DCs had similar surface levels of CD80 and CD86 compared to the BM-DCs (not depicted). However, both splenic- and marrow-derived DCs were comparably potent in stimulating CD25⁻ CD4⁺ T cells (Fig. 7 A). CD19⁺ B cells stimulated with LPS overnight could elicit some T cell proliferative responses from CD25⁻ CD4⁺ but not from CD25⁺ CD4⁺ T cells (Fig. 7 A). Normal and TGC–elicited peritoneal macrophages were weak stimulators of both CD25⁺ and CD25⁻ CD4⁺ T cells, even when the macrophages were taken from mice given IFN-γ i.p. to enhance expression of antigen–presenting MHC class II products (Fig. 7 C). Because BM-DCs were generated in the presence of the in-

flammatory cytokine GM-CSF and in the presence of other phagocytes like neutrophils and macrophages, we turned our attention to DCs from lymph nodes expanded in the presence of an in vivo inflammatory stimulus, CFA. The CD11c^{+} DCs from CFA-stimulated lymph nodes were fourfold more numerous. On a per cell basis, CFA-elicited lymph node DCs were stronger stimulators of the growth of CD25^{+} CD4^{+} regulatory T cells, compared with lymph node DCs in the steady state (Fig. 7 D). Therefore, DCs seem to be the major APC capable of stimulating CD25^{+} CD4^{+} T cell growth, but DCs acquire greater activity under inflammatory conditions, either GM-CSF in vitro or CFA in vivo.

CD25^{+} CD4^{+} Regulatory T Cells Can Be Expanded In Vivo by Antigen-bearing Mature DCs. To extend the findings to the growth of CD25^{+} CD4^{+} T cells in vivo, we purified CD25^{+} and CD25^{−} CD4^{+} T cells from OVA-specific

TCR transgenic mice, labeled them with CFSE, injected the T cells into naive BALB/c mice, and followed their proliferation and distribution in response to challenge with OVA antigen. In each of three experiments, CD25^{+} CD4^{+} T cells proliferated in the draining but not distal lymph nodes (Fig. 8 A) and spleen (not depicted) of mice challenged with DC-OVA. As expected, DC-OVA also induced extensive proliferation of CD25^{−} CD4^{+} T cells in lymph nodes draining the DC injection site (Fig. 8 A). The proliferation was OVA antigen-dependent, being absent in CD25^{+} or CD25^{−} CD4^{+} T cells when animals received DCs that had not been exposed to OVA (Fig. 8 A). The total number of clonotype (KJ1.26)-positive T cells recovered upon stimulation with DC-OVA versus DC was increased 8–10-fold when either CD25^{+} or CD25^{−} CD4^{+} T cells were stimulated in vivo. However, the absolute numbers of clonotype-positive CD25^{+} CD4^{+} T cells in the
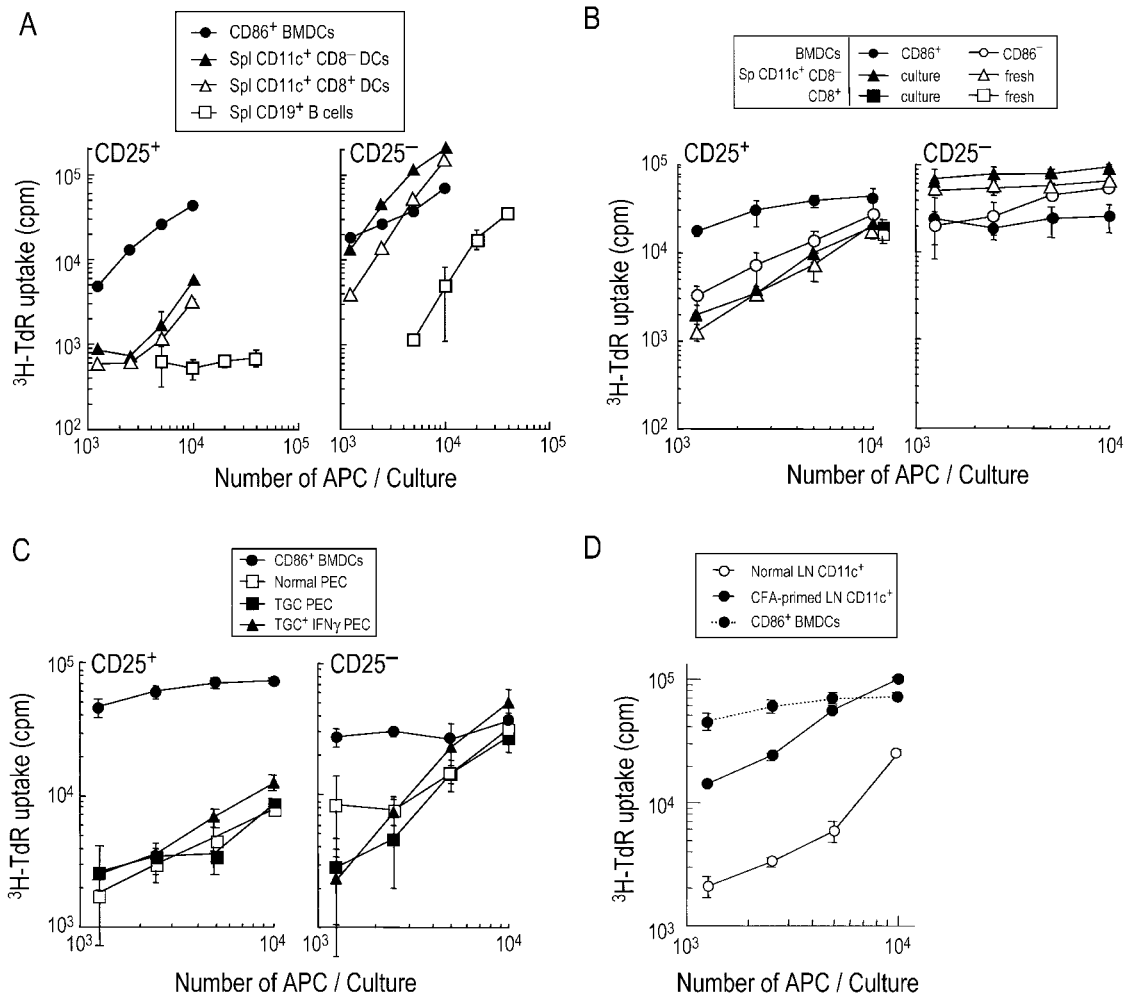
Figure 7. CD25^{+} CD4^{+} T cells primarily proliferate to DCs as APCs. Proliferation was assessed by incorporation of [^{3}H]thymidine for the last 12 h. (A) 10[4] T cells were cultured for 3 d with BM-DCs, spleen CD8^{+}, or CD8^{−} CD11c^{+} DCs matured by culture overnight in LPS, and CD19^{+} B cells matured in LPS. The APCs were exposed to 1 mg/ml OVA before use. Data with APCs lacking OVA were <10[3] cpm and are omitted. (B) As in A, but BM-DCs were compared with spleen CD8^{+} or CD8^{−} CD11c^{+} DCs, either fresh immature cells or matured by culture overnight, along with 1 μg/ml DO11.10 peptide. (C) As in A, but DCs were compared with macrophages, either PECs, TGC-elicited PEC, or IFN-γ–treated TGC-PEC. (D) CD25^{+} CD4^{+} T cells from DO11.10 mice were cultured for 3 d with lymph node CD11c^{+} DCs from untreated mice, or mice 5 d after CFA injection s.c. Representative results from three similar experiments.

lymphoid organs were always lower than expanded CD25$^-$ CD4$^+$ T cells (unpublished data). Interestingly, the levels of CD25 on the expanding CD25$^+$ CD4$^+$ regulatory T cells were increased during their growth in vivo and were much higher at day 3 than the CD25 expressed by responding CD25$^-$ CD4$^+$ T cells (Fig. 8). These results in mice replicate the findings in vitro that DCs are able to expand CD25$^+$ CD4$^+$ regulatory T cells.

To determine if DCs in vivo in the steady state could stimulate the expansion of CD25$^+$ CD4$^+$ T cells, the latter were adoptively transferred into mice followed by challenge with soluble OVA in the absence of any adjuvant or inflammatory stimulus. It is known that DCs are the main cell type that successfully captures and presents OVA for stimulation of T cells (30). Again, the adoptively transferred CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ T cells each underwent several cycles of cell division in vivo in the draining lymph nodes in response to OVA (Fig. 8 B). As in the case of proliferation stimulated by injected mature DCs, CD25$^+$ CD4$^+$ T cells stimulated in the steady state continued to express high levels of CD25, whereas their CD25$^-$ CD4$^+$ counterparts had not yet up-regulated CD25 expression at this time point (Fig. 8 B). Therefore, CD25$^+$ CD4$^+$ T cells, and not contaminants in the adoptively transferred populations, proliferate to antigen-bearing DCs in the steady state and after immigration from peripheral tissues.

Discussion

Our observations provide some new perspectives on the function of CD25$^+$ CD4$^+$ T cells and DCs. These T cells are known to suppress other T cells mediating autoimmunity, graft rejection, graft versus host disease, and resistance to tumors (for review see 1, 2, and 34), but CD25$^+$ CD4$^+$ T cells have always exhibited anergy in experimental studies, being unable to expand in response to mitogens (14, 15) and specific antigens (14, 16, 18, 35–37) and unable to produce IL-2 (14, 15). Previous work has used spleen cells as APCs. Spleen cells are a mixture of many cell types, with only ~1–1.5% being DCs, and the majority are in an immature state (30). In contrast, when DCs are added in relatively high numbers, CD25$^+$ CD4$^+$ T cells proliferate extensively in an antigen-dependent fashion, even in the absence of exogenous IL-2 (Figs. 1 and 2), which has had to be used at high doses in all previous work to expand these T cells in culture (14, 15). The expanded T cells, after several cycles of cell division, retain their suppressor cell phenotype and function (Fig. 6). The DCs are most active when generated under inflammatory conditions, e.g., when the DCs are derived from BM progenitors with GM-CSF in vitro or after challenge with an inflammatory adjuvant (CFA) in vivo, but other APCs like macrophages and B cells seem unable to expand CD25$^+$ CD4$^+$ T cells (Fig. 7).
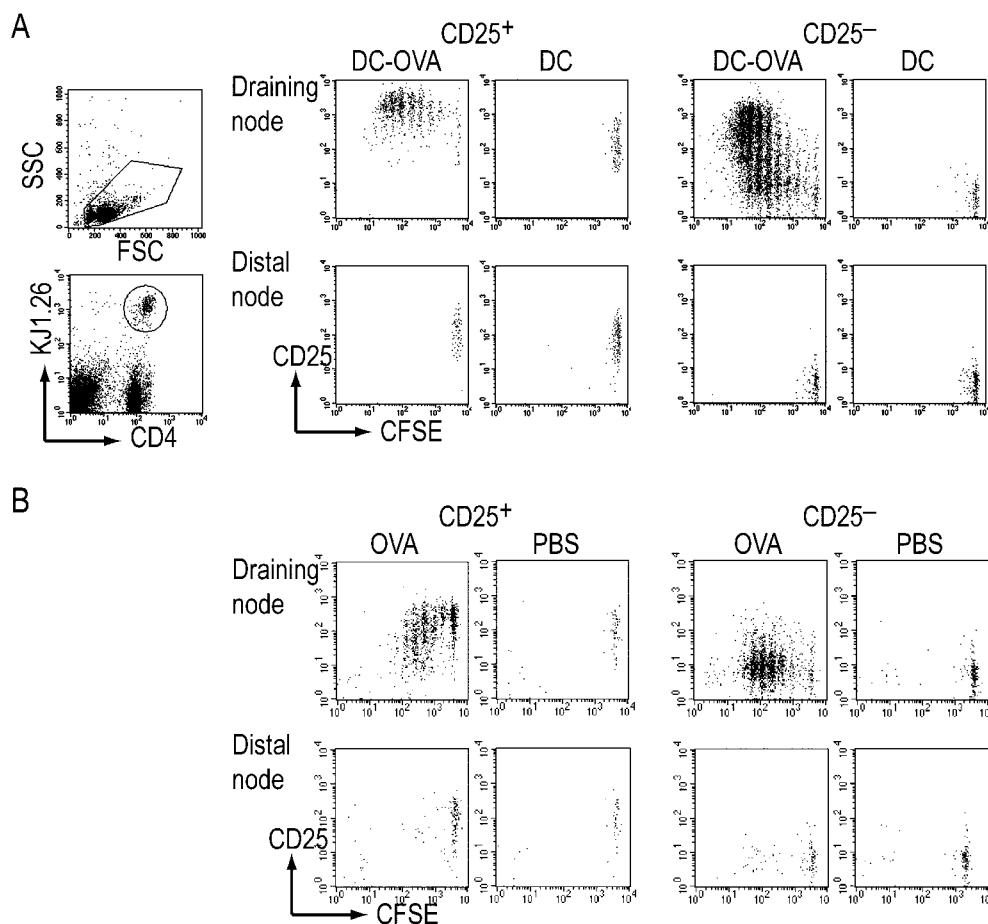

Figure 8. DCs stimulate CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ T cell proliferation in vivo. (A) 0.7 × 10^6 CFSE-labeled T cells were injected i.v. and stimulated with 2 × 10^5 marrow DCs or DC-OVA injected s.c. into the footpads 1 d later. Clonotype positive (KJ1.26$^+$) TCR transgenic T cells (top, circle) were analyzed for proliferation and expression of CD25 3 d later by dilution of the CFSE label in draining or distal (mesenteric) lymph nodes. (B) As in A, but OVA antigen was delivered by the injection of 25 μg soluble OVA into each footpad in the steady state. One representative result of three similar experiments.

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One mechanism for DC function involves cell surface molecule(s) on DCs because aldehyde-fixed DCs are active in expanding $CD25^+ CD4^+$ T cells and cell contact is essential (Figs. 4 and 5). We find that CD80 and/or CD86 contribute substantially as costimulatory molecules, though DCs that are deficient in both CD80 and CD86 still retain some stimulatory function (Fig. 4 C) implying the existence of additional costimulators on these cells. The need for CD80 and CD86 is consistent with the in vivo observation that blockade of these molecules in diabetes-prone nonobese diabetic mice reduces regulatory T cells and enhances autoimmune disease (38).

Another mechanism of DC function involves the production of soluble factors such as IL-2 (Fig. 3). We first found that DCs from $IL-2^{-/-}$ mice could stimulate the expansion of $CD25^+ CD4^+$ T cells in the absence of exogenous IL-2. This expansion could be blocked by ∼50% with anti-CD25 antibody and was accompanied by small amounts of IL-2 release into the medium, presumably from the T cells. These results are consistent with the in vivo findings that IL-2 and CD25 are important for the maintenance of peripheral $CD25^+ CD4^+$ T cells (39, 40). Again, IL-2 blockade did not fully suppress the response of $CD25^+ CD4^+$ T cells to DCs, so that additional growth factors are likely to be important after an initial cell contact–dependent activation by DCs (Fig. 5).

Although $CD25^+ CD4^+$ T cells are classically anergic to TCR stimulation, they are known to proliferate under select circumstances. For example, it has just been reported that LPS directly induces the proliferation of $CD25^+ CD4^+$ T cells through Toll-like receptor 4 (41), but high doses of LPS (10 μg/ml) are needed. In lymphopenic hosts, $CD25^+ CD4^+$ T cells can undergo division (17), and this depends upon MHC class II expressed by host cells (18), perhaps expressed by DCs capturing self-antigens in the steady state given our observations (Fig. 8 B). A 20-fold expansion of $CD25^+ CD4^+$ T cells takes place over 7–9 wk after transfer into newborn IL-2Rβ–deficient mice (42). Our results involve the transfer of $CD25^+ CD4^+$ T cells into intact adult mice, followed by a rapid proliferative response to stimulation by antigen processed by DCs (Fig. 8). These antigen-dependent responses are not due to contaminating $CD25^-$ $CD4^+$ T cells, which expressed much lower levels of CD25 when studied in parallel (Fig. 8).

We have used T cells from a TCR transgenic line that is specific for a foreign antigen, OVA. However, $CD25^+$ $CD4^+$ T cells with a high affinity for self-antigens can be selected during development in the thymus (36). In TCR transgenic mice, particularly the DO11.10 mice that we studied, $CD25^+ CD4^+$ T cells express an endogenous TCRα chain in addition to that expressed by the transgenic TCR (35, 43). We presume that this endogenous TCR allows for the selection of the $CD25^+ CD4^+$ T cells in the thymus because these cells are not found when the transgene is bred into a RAG knockout background (43). The OVA-specific TCR allows the $CD25^+ CD4^+$ T cells from these mice to suppress other T cells after stimulation with OVA-specific peptide as previously described (14, 16, 18,

35–37, 43), but a self-peptide acting on an endogenous TCRα might be required to positively select the $CD25^+$ $CD4^+$ fraction of DO11.10 T cells. Walker et al. (44) in this issue report the proliferation of $CD25^+ CD4^+$ T cells from DO11.10 mice in response to immunization with antigen in Freund's adjuvant in vivo. Interestingly, they find that $CD25^+ CD4^+$ T cells from DO11.10 mice also respond to OVA antigen expressed as a surrogate self-antigen in tissue cells, consistent with our data that immature steady-state DCs can capture antigens for stimulation of $CD25^+ CD4^+$ T cells.

Importantly, maturing DCs are not subject to suppression by $CD25^+ CD4^+$ T cells (Fig. 1 A). It has recently been reported that DCs stimulated via microbial ligands can produce IL-6 and block the suppressive activity of $CD25^+ CD4^+$ T cells (26). Our current data relate to the expansion of $CD25^+ CD4^+$ T cells that can suppress responses in standard assay systems involving splenic APCs rather than maturing DCs. The target for the action of the suppressor T cells is not yet apparent and may include a less stimulatory form of DC than the cells found in lymph nodes in the steady state or BM cultures expanded with GM-CSF. Nevertheless, in this issue, Oldenhove et al. (45) have now considered the suppression of mature DC function in vivo. After eliminating $CD25^+ CD4^+$ T cells, they find that mature DCs stimulate larger Th1 T cell and CTL responses in vivo.

Our studies deal with mouse $CD25^+ CD4^+$ regulatory T cells, but other reports have shown that their human counterparts respond poorly to mature monocyte-derived DCs in the allogeneic mixed leukocyte reaction (46–48). The work with human cells has used lower DC/T cell ratios (1:10) relative to our studies, as well as allogeneic responder T cells rather than highly enriched antigen-specific TCR transgenic T cells. In addition, the $CD25^+ CD4^+$ human T cell populations have the capacity to produce IL-10, whereas we do not detect this immunosuppressive cytokine in our cocultures of DCs and $CD25^+ CD4^+$ T cells.

The observation that mature DCs actively expand suppressive $CD25^+ CD4^+$ T cells provides a new mechanism for DCs to avoid the induction of autoimmunity (27, 49). Before infection, the thymus has already produced $CD25^+$ $CD4^+$ T cells capable of regulating autoimmunity (for review see 1, 2, and 34). We would like to suggest that these T cells are able to expand in the periphery under several circumstances. When DCs in lymph nodes are processing and presenting self-antigens in the steady state (50–52), the data in Fig. 8 B indicate that $CD25^+ CD4^+$ T cells would undergo expansion. Likewise, when mature DCs are inducing immune responses in the lymph node, with concomitant IL-2 production, bystander $CD25^+ CD4^+$ T cells would be expected to expand, as indicated by the data in Figs. 3, A and B, and 4 B. Additionally, when maturing DCs are themselves presenting cognate self-antigens, thymic-derived $CD25^+ CD4^+$ T cells would be expected to expand vigorously (Figs. 1–4 and 8 A), and serve to suppress autoreactive responses by other APCs. These potential roles for DCs in controlling $CD25^+ CD4^+$ T cells have

been identified here through the study of OVA-specific TCR transgenic T cells. The relevance of the findings to the control of bone fide autoimmunity and other immune responses will now need to be pursued directly.

References

1. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182:18–32.

2. Shevach, E.M. 2000. Regulatory T cells in autoimmunity. *Annu. Rev. Immunol.* 18:423–449.

3. Saoudi, A., B. Seddon, V. Heath, V. Fowell, and D. Mason. 1996. The physiological role of regulatory T cells in the prevention of autoimmunity: the function of the thymus in the generation of the regulatory T cell subset. *Immunol. Rev.* 149:195–216.

4. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J. Immunol.* 155:1151–1164.

5. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184:387–396.

6. Suri-Payer, E., A.Z. Amar, A.M. Thornton, and E.M. Shevach. 1998. CD4+ CD25+ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J. Immunol.* 160:1212–1218.

7. Kullberg, M.C., D. Jankovic, P.L. Gorelick, P. Caspar, J.J. Letterio, A.W. Cheever, and A. Sher. 2002. Bacteria-triggered CD4+ T regulatory cells suppress *Helicobacter hepaticus*–induced colitis. *J. Exp. Med.* 196:505–516.

8. Belkaid, Y., C.A. Piccirillo, S. Mendez, E.M. Shevach, and D.L. Sacks. 2002. CD4+ CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature.* 420:502–507.

9. Kursar, M., K. Bonhagen, J. Fensterle, A. Kohler, R. Hurwitz, T. Kamradt, S.H. Kaufmann, and H.-W. Mittrucker. 2002. Regulatory CD4+ CD25+ T cells restrict memory CD8+ T cell responses. *J. Exp. Med.* 196:1585–1592.

10. Shimizu, J., S. Yamazaki, and S. Sakaguchi. 1999. Induction of tumor immunity by removing CD25+ CD4+ T cells: a common basis between tumor immunity and autoimmunity. *J. Immunol.* 163:5211–5218.

11. Sutmuller, R.P., L.M. van Duivenvoorde, A. van Elsas, T.N. Schumacher, M.E. Wildenberg, J.P. Allison, R.E. Toes, R. Offringa, and C.J. Melief. 2001. Synergism of cytotoxic t lymphocyte–associated antigen 4 blockade and depletion of CD25+ regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. *J. Exp. Med.* 194:823–832.

12. Maurik Av, A., M. Herber, K.J. Wood, and N.D. Jones. 2002. CD4+ CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell–mediated graft rejection: implications for anti-CD154 immunotherapy. *J. Immunol.* 169:5401–5404.

13. Taylor, P.A., R.J. Noelle, and B.R. Blazar. 2001. CD4+ CD25+ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med.* 193:1311–1318.

14. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by CD25+ CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10:1969–1980.

15. Thornton, A.M., and E.M. Shevach. 1998. CD4+ CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287–296.

16. Thornton, A.M., and E.M. Shevach. 2000. Suppressor effector function of CD4+ CD25+ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164:183–190.

17. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T.C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166:3008–3018.

18. Gavin, M.A., S.R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4+ CD25+ suppressor T cells in vivo. *Nat. Immunol.* 3:33–41.

19. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T.W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25+ CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte–associated antigen 4. *J. Exp. Med.* 192:303–310.

20. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte–associated antigen 4 plays an essential role in the function of CD25+ CD4+ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192:295–302.

21. Shimizu, J., S. Yamazaki, T. Takahashi, Y. Ishida, and S. Sakaguchi. 2002. Stimulation of CD25+ CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 3:135–142.

22. McHugh, R.S., M.J. Whitters, C.A. Piccirillo, D.A. Young, E.M. Shevach, M. Collins, and M.C. Byrne. 2002. CD4+ CD25+ immunoregulatory T cells. Gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity.* 16:311–323.

23. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor *foxp3*. *Science.* 299:1057–1061.

24. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nat. Immunol.* 4:330–336.

25. Khattri, R., T. Cox, S.A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4+ CD25+ T regulatory

cells. *Nat. Immunol.* 4:337–342.

26. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of CD4+ CD25+ T cell–mediated suppression by dendritic cells. *Science.* 299:1033–1036.

27. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21:685–711.

28. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony–stimulating factor. *J. Exp. Med.* 176:1693–1702.

29. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Maeda, K. Takahara, Y. Akiyama, R.M. Steinman, and K. Inaba. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195:1289–1302.

30. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M.C. Nussenzweig, and R.M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196:1627–1638.

31. Wells, A.D., H. Gedmundsdottir, and L.A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction of a proliferative response. *J. Clin. Invest.* 100:3137–3183.

32. Granucci, F., C. Vizzardelli, N. Pavelka, S. Feau, M. Persico, E. Virzi, M. Rescigno, G. Moro, and P. Ricciardi-Castagnoli. 2001. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nat. Immunol.* 2:882–888.

33. Cohen, J.L., A. Trenado, D. Vasey, D. Klatzmann, and B.L. Salomon. 2002. CD4+ CD25+ immunoregulatory T cells: new therapeutics for graft-versus-host disease. *J. Exp. Med.* 196:401–406.

34. Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell.* 101:455–458.

35. Hori, S., M. Haury, A. Coutinho, and J. Demengeot. 2002. Specificity requirements for selection and effector functions of CD25+ CD4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc. Natl. Acad. Sci. USA.* 99:8213–8218.

36. Jordan, M.S., A. Boesteanu, A.J. Reed, A.L. Petrone, A.E. Holenbeck, M.A. Lerman, A. Naji, and A.J. Caton. 2001. Thymic selection of CD4+ CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2:301–306.

37. Apostolou, I., A. Sarukhan, L. Klein, and H. Von Boehmer. 2002. Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 3:756–763.

38. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4+ CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity.* 12:431–440.

39. Furtado, G.C., M.A. de Lafaille, N. Kutchukhidze, and J.J. Lafaille. 2002. Interleukin 2 signaling is required for CD4+ regulatory T cell function. *J. Exp. Med.* 196:851–857.

40. Murakami, M., A. Sakamoto, J. Bender, J. Kappler, and P. Marrack. 2002. CD25+ CD4+ T cells contribute to the control of memory CD8+ T cells. *Proc. Natl. Acad. Sci. USA.* 99:8832–8837.

41. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysaccharide. *J. Exp. Med.* 197:403–411.

42. Malek, T., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17:167.

43. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of CD25+ CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol. Methods.* 162:5317–5326.

44. Walker, L.S.K., A. Chodos, and A.K. Abbas. 2003. Antigen-dependent proliferation of CD4+ CD25+ regulatory T cells in vivo. *J. Exp. Med.* 198:249–258.

45. Oldenhove, G., M. de Heush, G. Urbain-Vansanten, J. Urbain, C. Maliszewski, O. Leo, and M. Moser. 2003. CD4+ CD25+ regulatory T cells control Th1 responses to foreign antigens induced by mature DCs in vivo. *J. Exp. Med.* 198:259–266.

46. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of CD4+ CD25+ T cells with regulatory properties from human blood. *J. Exp. Med.* 193:1303–1310.

47. Jonuleit, H., E. Schmitt, H. Kakirman, M. Stassen, J. Knop, and A.H. Enk. 2002. Infectious tolerance: human CD25+ regulatory T cells convey suppressor activity to conventional CD4+ T helper cells. *J. Exp. Med.* 196:255–260.

48. Dieckmann, D., C.H. Bruett, H. Ploettner, M.B. Lutz, and G. Schuler. 2002. Human CD4+ CD25+ regulatory, contact-dependent T cells induce interleukin 10–producing, contact-independent type 1–like regulatory T cells. *J. Exp. Med.* 196:247–253.

49. Steinman, R.M., and M.C. Nussenzweig. 2002. Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance. *Proc. Natl. Acad. Sci. USA.* 99:351–358.

50. Huang, F.-P., N. Platt, M. Wykes, J.R. Major, T.J. Powell, C.D. Jenkins, and G.G. MacPherson. 2000. A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J. Exp. Med.* 191:435–442.

51. Liu, K., T. Iyoda, M. Saternus, K. Kimura, K. Inaba, and R.M. Steinman. 2002. Immune tolerance after delivery of dying cells to dendritic cells in situ. *J. Exp. Med.* 196:1091–1097.

52. Scheinecker, C., R. McHugh, E.M. Shevach, and R.N. Germain. 2002. Constitutive presentation of a natural tissue autoantigen exclusively by dendritic cells in the draining lymph node. *J. Exp. Med.* 196:1079–1090.