International Immunology, Vol. 17, No. 3, pp. 279–288
doi:10.1093/intimm/dxh207 Advance Access publication 31 January 2005
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$CD25^+CD4^+$ T cells compete with naive $CD4^+$ T cells for IL-2 and exploit it for the induction of IL-10 production

Thomas Barthlott[1], Halima Moncrieffe[1], Marc Veldhoen[1], Christopher J. Atkins[2], Jillian Christensen[3], Anne O'Garra[3] and **Brigitta Stockinger[1]**

[1]Division of Molecular Immunology, [2]Division of Immune Cell Biology and [3]Division of Immune Regulation, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Keywords: CD25 regulation, homeostasis, IL-10, regulatory T cells, suppression

Abstract

Maintenance of homeostasis in the immune system involves competition for resources between T lymphocytes, which avoids the development of immune pathology seen in lymphopenic mice. $CD25^+CD4^+$ T cells are important for homeostasis, but there is as yet no consensus on their mechanisms of action. Although $CD25^+CD4^+$ T cells cause substantial down-regulation of IL-2 mRNA in responder T cells in an *in vitro* co-culture system, the presence of IL- protein can be demonstrated by intracellular staining. As a consequence of competition for IL-2, $CD25^+CD4^+$ T cells further up-regulate the IL-2R α chain (CD25), a process that is strictly dependent on IL-2, whereas responder T cells fail to up-regulate CD25. Similarly, adoptive transfer into lymphopenic mice showed that $CD25^+CD4^+$ T cells interfere with CD25 up-regulation on co-transferred naive T cells, while increasing their own CD25 levels. IL-2 sequestration by $CD25^+CD4^+$ T cells is not a passive phenomenon but instead initiates—in conjunction with signals through the TCR—their differentiation to IL-10 production. Although IL-10 is not required for *in vitro* suppression, it is vital for the *in vivo* function of regulatory T cells. Our data provide a link explaining the apparent difference in regulatory mechanisms *in vitro* and *in vivo*.

Introduction

An essential feature of the immune system is the control of cell numbers in peripheral lymphoid compartments. T cell homeostasis in naive and memory pools is maintained by competition for shared resources, such as access to antigen-presenting cells (APC), cytokines, chemokines or ligands for co-stimulatory molecules (1, 2). In the past 15 years, the concept of immune regulation by a dedicated sub-population of T cells has gained considerable support and influence. $CD25^+CD4^+$ T cells, a sub-population representing 5–10% of the total CD4 T cell pool, have been attributed with an important role in maintaining peripheral self-tolerance and control of immune pathology (3, 4). Discrepancies exist concerning the putative mechanisms underlying the regulatory effect of $CD25^+CD4^+$ T cells *in vivo* and *in vitro*. While *in vivo* regulatory activity of $CD25^+CD4^+$ T cells is linked to cytokines such as IL-10 and transforming growth factor-β (TGF-β) (3), their action *in vitro* is reported to be contact dependent, but cytokine independent (4–6), and the mechanism of suppression is thought to be inhibition of IL-2 production in the responder T cell population (5).

The protective effect of $CD25^+CD4^+$ T cells in a number of experimental models of organ-specific immune responses provided the basis on which the concept of active immune regulation by this T cell sub-population was established (7, 8) and their role in maintaining T cell homeostasis was established subsequently (9, 10).

$CD25^+CD4^+$ T cells have been shown to regulate CD4 T cell responses *in vivo* and *in vitro*. The basis for *in vitro* suppression is thought to be distinct from that of *in vivo* regulation since blocking of cytokines such as IL-10 and TGF-β does not affect suppression *in vitro*, but interferes with regulation of some inflammatory processes *in vivo* (3), and $CD25^+CD4^+$ T cells are thought to suppress IL-2 transcription *in vitro* (5). $CD25^+CD4^+$ T cells co-express all three subunits required for a functional high-affinity IL-2R (11). Their dependence on IL-2 signaling for their generation, maintenance and function is well documented (9, 12, 13). In this paper, we show that despite down-regulation of IL-2 mRNA, responder T cells produce some IL-2 protein, which is scavenged by $CD25^+CD4^+$ T cells

Correspondence to: B. Stockinger; E-mail: bstocki@nimr.mrc.ac.uk

Transmitting editor: E. Simpson

Received 13 December 2004, *accepted* 16 December 2004

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and contributes to their regulatory activity. Competition for IL-2 by $CD25^+CD4^+$ T cells causes failure in responder T cells to up-regulate CD25 and thereby interferes with the autocrine feedback loop that drives responder T cells to full effector function. In contrast, CD25 levels on $CD25^+CD4^+$ T cells increase even further in co-culture or following co-transfer *in vivo* with responder T cells. Importantly, however, competition for IL-2 is not a passive process, since $CD25^+CD4^+$ T cells exposed to IL-2 and TCR signals initiate cell division and IL-10 cytokine production. Thus, competition for an important lymphocyte growth factor and its exploitation for the induction of the immunoregulatory cytokine IL-10 contributes to the regulatory activity of $CD25^+CD4^+$ T cells *in vitro* and may provide the explanatory link for the seemingly unrelated mechanisms of suppression *in vitro* and *in vivo*.

Methods

Mice

Mice of strains C57BL/10, C57BL/6 CD45.1, BALB/c and C57BL/10 $Rag^{-/-}$ were obtained from the specific pathogen free (SPF) facilities at the National Institute for Medical Research (NIMR). Mice were bred and kept according to the institute's guidelines.

Antibodies and flow cytometry

Anti-CD4 APC (RM4-5), anti-CD25 PE (PC61), anti-CD44 biotin (IM7), anti-CD45RB FITC (30-F11), anti-CD45.1 biotin (A20), anti-CD45.2 biotin (104), anti-IL-2 PE (JES6-5H4), anti-IL-10 APC (JES5-16E3), anti-IFNγ-PE (XMG1.2), anti-IL-4 PE (11B11) and streptavidin PerCP were purchased from BD Pharmingen (San Diego, CA, USA). Anti-CD3 (145-2C11) and anti-CD28 (37.51) mAb were produced and purified in our laboratory using standard procedures. Cells were analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA) using Cellquest software (Becton Dickinson). Dead cells were excluded using forward and side scatter.

T cell purification

Single-cell suspensions from spleens and lymph nodes were stained with CD4 APC, CD25 PE and CD45RB FITC followed by anti-PE microbeads (Miltenyi Biotec, Surrey, UK) and positively selected on an AutoMACS (Miltenyi Biotec) according to the manufacturer's procedures. The positive fraction was then sorted on a Moflo cytometer (Cytomation, Fort Collins, CO, USA) to obtain pure populations of $CD25^+CD45RB^{lo}CD4$ T cells, the negative fraction was sorted into $CD25^-CD45RB^{lo}CD4$ T cells and into $CD25^-CD45RB^{hi}CD4$ T cells.

Cell lines, in vitro assays and cytokine production assays

The B cell myeloma X63-Ag8 transfected with IL-2 cDNA (X63-IL-2) was used as an IL-2-producing cell line. X63-IL-2 cells (1×10^2) were cultured with sorted CD4 T cell subpopulations. Supernatants were taken after overnight culture and assessed for IL-2 content using an Alamar Blue-based (Biosource, Camarillo, CA, USA) CTLL-2 assay. Consumption of exogenous IL-2 (X63-IL-2 supernatant used at 0.5 U ml^{-1}) was assessed in flat-bottom 96-well plates with 5×10^4 accessory cells (irradiated C57BL/10 $Rag^{-/-}$ splenocytes) in the absence or presence of 0.5 μg ml^{-1} soluble anti-CD3 mAb. Supernatants were assessed for remaining IL-2 content after 48 h as described above. To assess the roles of TCR and IL-2 signals, 1×10^5 $CD25^+CD45RB^{lo}CD4$ T cells were cultured in flat-bottom 96-well plates with 5×10^4 accessory cells in the presence or absence of 0.5 μg ml^{-1} soluble anti-CD3 and 5 U ml^{-1} exogenous IL-2. For standard T cell activation assays, 5×10^4 naive $CD25^-CD45RB^{hi}CD4$ T cells were activated with or without equal numbers of regulatory $CD25^+CD45RB^{lo}CD4$ T cells in flat-bottom 96-well plates with 0.5 μg ml^{-1} soluble anti-CD3 and 5×10^4 accessory cells. Unless mentioned otherwise, supernatants were tested 2 days later for IL-2 content as described above and cultures were pulsed for 8 h with 5 μCi [^{3}H]thymidine to assess T cell proliferation.

Quantitative mRNA analysis for IL-2

Cultures were set up in 24-well plates containing 1×10^6 $CD25^-CD45RB^{hi}CD4$ T cells with or without equal numbers of 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled $CD25^+CD45RB^{lo}CD4$ T cells or 1×10^6 $CD25^+$-$CD45RB^{lo}CD4$ T cells together with 1×10^6 accessory cells and anti-CD3 as above. RNA was extracted using RNeasy Kit (Qiagen, Hilden, Germany), DNase treated (Roche, East Sussex, UK) and reverse transcribed with oligo poly deoxythymine (pdT) 12–18 (Amersham, Bucks, UK) according to the manufacturers' protocol. cDNA was analyzed for the expression of IL-2 and 36B4 by real-time PCR using the ABIPRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). Quantification of target gene expression was by comparison with the housekeeping gene 36B4 (14) using SYBR Green (Applied Biosystems). The primer sequences were as follows: IL-2 forward CAG GAT GGA GAA TTA CAG GAA CCT, IL-2 reverse TTT CAA TTC TGT GGC CTG CTT, 36B4 forward GGA CCT GAC GGA CTA CCT CAT G and 36B4 reverse TCT TTG ATG TCA CGC ACG ATT T.

Cytokine detection by intracellular staining and ELISA

For *in vitro* intracellular IL-2 detection, cultures were set up in 96-well plates. During the last 6 h of culture, Brefeldin A (10 μg ml^{-1}, Sigma, Poole, UK) was added. For *in vitro* intracellular IL-10 detection, cultures were set up in 24-well plates as described above. Exogenous IL-2 was added at 20 U ml^{-1} at the beginning and on day 3 of culture. During the last 4 h of culture, cells were re-stimulated with phorbol 12,13 dibutyra (PdBU) and ionomycin (both at 50 ng ml^{-1}, Sigma) in the presence of Brefeldin A (10 μg ml^{-1}). Intracellular stainings were performed as described (15). To determine IL-10 in the supernatant, 1×10^6 $CD25^+CD45RB^{lo}CD4$ T cells were activated in 96-well plates in the absence of accessory cells by plate-bound anti-CD3 (0.5 μg ml^{-1}) with or without plate-bound anti-CD28 (5 μg ml^{-1}) in the presence of 20 U ml^{-1} IL-2. IL-10 ELISA was done with ready-SET-Go kit (eBioscience, San Diego, CA, USA) according to the manufacturer's procedure.

Adoptive transfers

C57BL/10 $Rag^{-/-}$ mice were injected intravenously with 2×10^6 $CD25^-CD45RB^{hi}CD4$ T cells (CD45.1), 2×10^6 $CD25^+CD45RB^{lo}CD4$ T cells (CD45.2) or a mixture of both (CD45.2). Mesenteric lymph nodes were isolated 2–3 weeks after transfer and analyzed by flow cytometry. For intracellular

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IL-2 detection, cells were re-stimulated with PdBU and ionomycin in the presence of Brefeldin A as described.

Results

CD25⁺CD4⁺ T cells consume IL-2 in vitro

Since regulatory T cells express the IL-2R α chain in addition to the β and γc chains (11), they are well equipped to bind and consume IL-2. In order to test this, we cultured CD4 T cells sorted on the basis of their CD25 and CD45RB expression patterns (Fig. 1a) with a B cell myeloma (X63-Ag8) cell line transfected with IL-2 cDNA (X63-IL-2) (16). In these cells, IL-2 is expressed under the heterologous cytomegalovirus promoter and endogenous regulatory sequences are missing. Any reduction in IL-2 levels following co-culture with $CD25^+CD4^+$ T cells is therefore unlikely to be due to inhibition of transcription by $CD25^+CD4^+$ T cells but more likely to indicate IL-2 consumption by these cells. Co-culture of X63-IL-2 myeloma cells, which constitutively secrete IL-2, with $CD25^+CD4^+$ T cells decreased the amount of IL-2 measurable in the cell culture supernatants; no changes in IL-2 were observed in co-cultures with $CD25^-CD45RB^{hi}$ naive T cells or activated $CD45RB^{lo}CD4$ T cells lacking CD25 expression compared with control wells containing X63-IL-2 myeloma cells in medium (Fig. 1b). Similarly, addition of exogenous IL-2 to graded numbers of $CD25^+CD4^+$ T cells resulted in the reduction of IL-2 amounts over a 3-day culture period, an effect that was not enhanced by stimulation of $CD25^+CD4^+$ T cells by anti-CD3 (Fig. 1c).

To assess if IL-2 consumption by $CD25^+CD4^+$ T cells is taking place in standard *in vitro* assays used to determine regulatory activity, naive CD4 T cells were stimulated with anti-CD3 antibody in the presence or absence of $CD25^+CD4^+$ T cells. After 48 h, supernatants were harvested and analyzed for IL-2 content (Fig. 1d, white bars). As expected, co-culture of naive and regulatory T cells led to a pronounced decrease of IL-2 production and strongly suppressed proliferation (data not shown). Supernatant collected from cultures which only had the naive CD4 T cell input was re-cultured with freshly sorted $CD25^+CD4^+$ T cells or accessory cells only. Forty-eight hours later, culture supernatants were again assessed for IL-2 content (Fig. 1d, black bars). Additional culture with accessory cells alone did not lead to a decrease in IL-2 levels compared with the first culture period, indicating that IL-2 was not degraded during the second culture step (left black bar). However, addition of $CD25^+CD4^+$ T cells reduced the IL-2 content to levels similar to those observed in the primary co-culture of naive and regulatory T cells (right black bar).

Responder T cells continue to make IL-2 protein in the presence of CD25⁺CD4 T cells despite reduced IL-2 mRNA levels

If $CD25^+CD4^+$ T cells indeed exert their suppressive effect through IL-2 consumption, it follows that the naive CD4 T cell population, when activated, produces IL-2 even in the presence of the regulatory population, since $CD25^+CD4^+$ T cells cannot make IL-2 themselves. However, the mechanism of *in vitro* suppression is thought to be inhibition of IL-2 transcription in the responder population (5) which might preclude any production of IL-2 protein. We assessed mRNA for IL-2 at intervals up to 20 h after the onset of culture by real-time PCR. Responder CD4 T cells were cultured with APC and anti-CD3 on their own or in the presence of $CD25^+CD4^+$

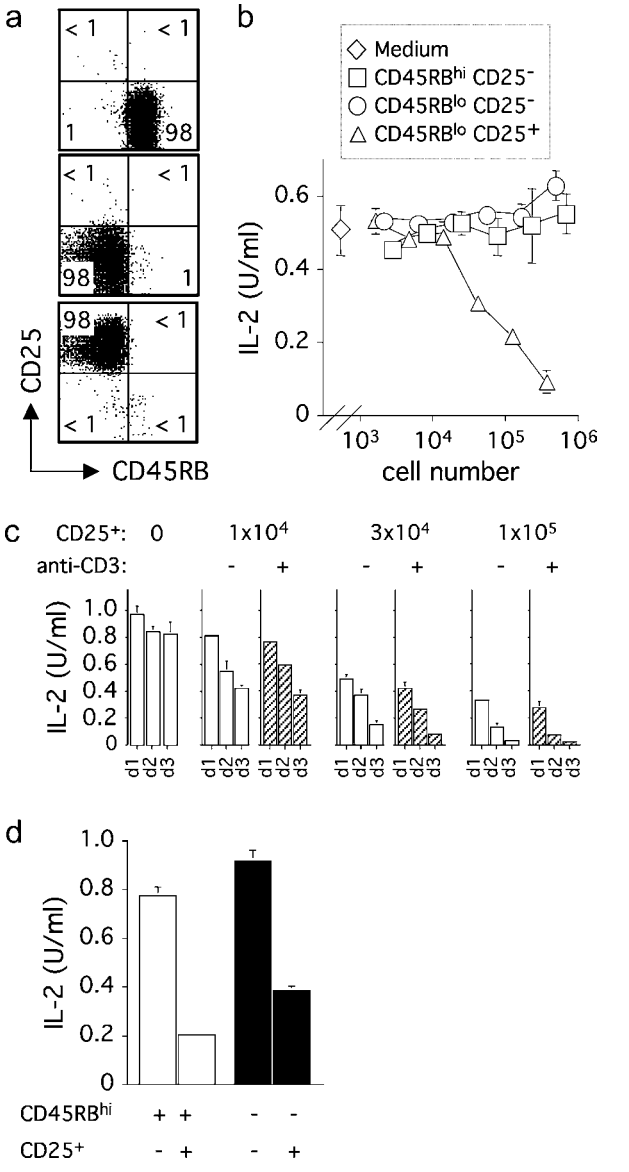

Fig. 1. $CD25^+CD4$ T cells consume IL-2 *in vitro*. (a) Purity of CD4 T cells sorted either for $CD45RB^{hi}CD25^-$, $CD45RB^{lo}CD25^-$ or $CD45RB^{lo}CD25^+$ expression. (b) X63-IL-2 cells were cultured overnight in either medium alone or in the presence of increasing numbers of sorted CD4 T cells with the indicated phenotype and the supernatants were assessed for IL-2 content. (c) Graded numbers of $CD25^+CD4^+$ T cells were cultured in the presence of accessory cells with or without anti-CD3 in medium containing 1 U ml^{-1} exogenous IL-2. Culture supernatants were assessed for IL-2 content over a 3-day culture period. (d) $CD45RB^{hi}CD4^+$ T cells were activated in the absence or presence of equal numbers of $CD25^+CD4^+$ T cells and IL-2 content was determined after 48 h (white bars). Supernatants from stimulated $CD45RB^{hi}CD4^+$ T cells were removed after 48 h and added to accessory cells in the absence or presence of freshly sorted $CD25^+CD4^+$ T cells (at the same concentrations and absolute numbers as in the first culture). After 48 h, the remaining IL-2 content in the supernatants was determined (black bars).

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T cells. At intervals of 1–2 h following *in vitro* activation, the cells were subjected to quantitative PCR (Fig. 2a). IL-2 mRNA is induced by ligation of TCR and co-stimulatory molecules within 2 h in naive T cells, peaks around 5 h and is rapidly diminished thereafter. The presence of $CD25^+CD4^+$ T cells did not interfere with the early onset of IL-2 transcription but drastically reduced peak expressions of IL-2 mRNA compared with mRNA levels in CD4 T cells cultured on their own. Despite the strong reduction, levels of IL-2 mRNA in the co-cultures were clearly above those of $CD25^+CD4^+$ T cells on their own, which showed no IL-2 mRNA activity.

To directly assess whether responder T cells make IL-2 protein despite the reduction in IL-2 mRNA levels, we activated naive CD4 T cells in the presence or absence of CFSE-labeled $CD25^+CD4$ T cells and determined their IL-2 production over a 3-day period of culture by measuring IL-2 content of supernatants as well as intracellular protein. While the amount of IL-2 detectable in the supernatant of cultures containing $CD25^+CD4$ T cells was negligible at all time points (Fig. 2b, left), the proportion of responder T cells expressing intracellular IL-2 protein, while reduced to some extent in the presence of $CD25^+CD4$ T cells, gradually increased throughout the 3-day culture period (Fig. 2b, right).

Effect of IL-2 competition on responder and regulatory T cell phenotype

In order to determine phenotypic consequences of the supposed effects of IL-2 competition on responder as well as regulatory T cells, we took advantage of the fact that $CD25^+CD4^+$ T cells behave like anergic cells *in vitro* and do

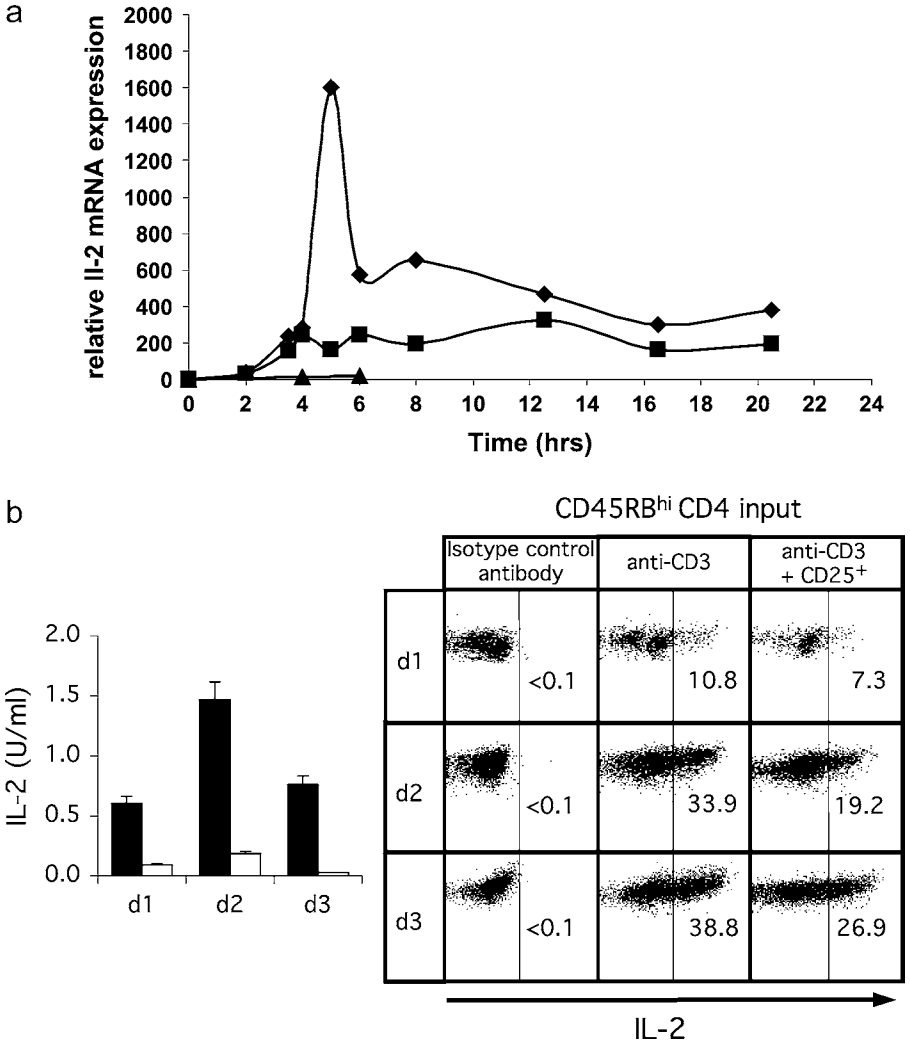

Fig. 2. IL-2 production and transcription by $CD45RB^{hi}CD4$ T cells. (a) $CD45RB^{hi}CD4^+$ T cells were activated as above in the absence or presence of $CD25^+CD4^+$ T cells. IL-2 transcription was analyzed by real-time PCR at 1–2 h intervals after the onset of culture. The graph shows relative IL-2 mRNA expression of responder CD4 T cells cultured on their own (diamonds), in the presence of $CD25^+CD4^+$ T cells (squares) or IL-2 mRNA in $CD25^+CD4^+$ T cells (triangles). Mean values of duplicate cultures normalized to the housekeeping gene 36B4 are shown. (b) $CD45RB^{hi}CD4^+$ T cells were activated with anti-CD3 and accessory cells in the absence or presence of $CD25^+CD4$ T cells. On d1, d2 and d3 of culture, supernatants were assessed for IL-2 content and cells were stained for intracellular IL-2 after a 4-h pulse with PdBU/ionomycin. Staining with isotype control antibody is shown for comparison. Dot plots are gated on CFSE-negative CD4 T cells (naive $CD45RB^{hi}$ input). Percentages are indicated in the respective dot plots.

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not divide in response to anti-CD3 stimulation (6, 17). $CD25^+CD4^+$ T cells were labeled with CFSE and added into a standard regulatory *in vitro* assay as described above. Non-labeled responder CD4 T cells and CFSE-labeled $CD25^+CD4^+$ T cells are readily distinguishable based on their CFSE profile (Fig. 3a). Assessment of suppression by measuring thymidine incorporation as well as IL-2 content of supernatants showed pronounced suppression throughout the 3-day assay period (data not shown).

When gating on the CFSE-negative responder T cells during a 3-day time course, there were fewer blast cells in the responder CD4 T cell population in the presence of $CD25^+CD4^+$ T cells. Initial increase in cell size was similar on day 1 and day 2, but responder T cells failed to differentiate into full blasts in the presence of $CD25^+$CD4 T cells on day 3 of culture (Fig. 3b, left panel). CD44 levels, however, were similarly up-regulated in the absence or presence of $CD25^+$CD4 T cells, indicating that responder T cells did get activated (Fig. 3b, middle panel). The most prominent difference was the absence of CD25 up-regulation in the responder CD4 T cell population, when co-cultured with $CD25^+CD4^+$ T cells (Fig. 3b, right panel). Since up-regulation of the IL-2 α chain is strictly dependent on IL-2 (18, 19), this suggests that responder T cells were lacking this crucial signal. Analysis of the CFSE-positive $CD25^+CD4^+$ T cells showed firstly that they increased in size when co-cultured in the presence of responder T cells but not when cultured on their own (Fig. 3c, left panel). Furthermore, cell division became visible after 3 days of co-culture with responder CD4 T cells (Fig. 3c, middle panel). CD25 levels on the $CD25^+CD4^+$ T cells (Fig. 3c, right panel) increased further when the cells were activated in the presence of naive CD4 T cells but not when they were activated on their own. Given the IL-2 dependency of CD25 expression on the one hand and the fact that $CD25^+CD4^+$ T cells do not themselves produce IL-2 (12, 20), this strongly suggests that the responder T cell population produced IL-2, which was sequestered by $CD25^+CD4^+$ T cells at the expense of the responder population.

In order to test what effect the presence or withdrawal of IL-2 has on CD25 expression by both responder T cells and $CD25^+CD4^+$ T cells, naive CD4 T cells were cultured with or without regulatory CD4 T cells in the presence or absence of recombinant exogenous IL-2 and CD25 levels were assessed 48 h later. Exogenous IL-2 can overcome the inhibitory effect regulatory cells exert on CD25 expression by responder CD4 T cells (Fig. 3d, left) and $CD25^+CD4^+$ T cells up-regulate CD25 even further if additional exogenous IL-2 is present (Fig. 3d, right). Taken together, these results strongly suggest that naive CD4 T cells, cultured in the presence of $CD25^+CD4^+$ T cells are producing some IL-2 and that the seeming absence of IL-2 detectable in culture supernatants is due to its consumption by the regulatory population.

Reciprocal regulation of CD25 expression in vivo

In order to test whether similar reciprocal regulation of CD25 expression on naive and regulatory CD4 T cells can be observed *in vivo*, naive $CD45RB^{hi}$CD4 T cells and $CD25^+$CD4 T cells were adoptively transferred either alone or in a 1 : 1 ratio into $Rag^{-/-}$ mice. After 3 weeks, CD4 T cells were isolated and

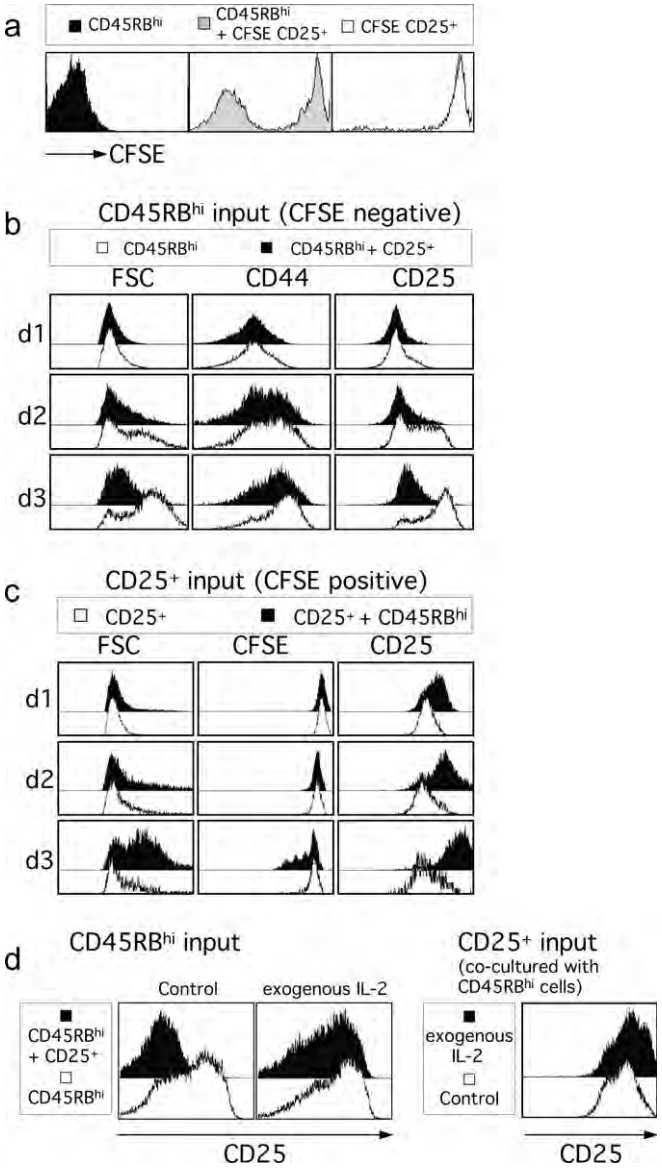

Fig. 3. Phenotype of $CD45RB^{hi}$ and $CD25^+$CD4 T cells after activation. (a) $CD45RB^{hi}CD4^+$ T cells (black histograms), $CD45RB^{hi}$ and CFSE-labeled $CD25^+CD4^+$ T cells (gray histograms) or CFSE-labeled $CD25^+CD4^+$ T cells (white histograms) were activated with anti-CD3 in the presence of accessory cells and analyzed after 48 h. CFSE profiles on CD4 T cells are shown. (b) $CD45RB^{hi}CD4^+$ T cells were activated in the absence (white histograms) or presence (black histograms) of equal numbers of CFSE-labeled $CD25^+CD4^+$ T cells. Activation markers were assessed on the CFSE-negative CD4 T cell population over a 3-day period. (c) Assessment of activation markers on the corresponding CFSE-labeled $CD25^+$ population cultured on its own (white histograms) or with equal numbers of unlabeled $CD45RB^{hi}CD4^+$ T cells (black histograms). (d) $CD45RB^{hi}CD4^+$ T cells were activated with anti-CD3 and accessory cells in the absence (white histograms) or presence (black histograms) of equal numbers of CFSE-labeled $CD25^+CD4^+$ T cells. Cultures were set up in medium alone or medium containing exogenous IL-2 (20 U ml^{-1}). After 48 h CD25 expression on the naive CD4 T cell input (CFSE-negative) was determined by flow cytometry. CD25 expression on the $CD25^+CD4^+$ T cell input (CFSE-positive) cultured with $CD45RB^{hi}$CD4 T cells in medium alone (white histogram) or medium containing exogenous IL-2 (black histogram) is shown in the right panel.

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analyzed for CD25 expression using the allotypic CD45 marker to distinguish the naive CD4 T cell input from the regulatory CD4 T cell input. As expected, transfer of naive CD4 T cells alone led to a proportion of activated $CD25^+$ T cells which fails to develop when regulatory CD4 T cells are co-transferred (Fig. 4a, left panel). The majority of regulatory T cells lose CD25 expression when transferred on their own into lymphopenic hosts. However, CD25 expression is maintained on the majority of regulatory T cells when naive CD4 T cells are co-transferred (Fig. 4a, right panel), an observation also noted by others (21, 22). As previously seen *in vitro*, IL-2-producing CD4 T cells derived from the naive input can be found by intracellular staining despite the presence of regulatory CD4 T cells *in vivo* (Fig. 4b).

TCR signals and IL-2 drive $CD25^+CD4^+$ T cells to division and IL-10 production

We next tested the effect of exogenous IL-2 on viability and division potential of $CD25^+CD4^+$ T cells. IL-2 has a profound effect on the viability of $CD25^+CD4^+$ T cells (Fig. 5a, left panel), but it is not sufficient to induce their proliferation in the absence of a TCR stimulus (Fig. 5a, right panel). Interestingly, in conjunction with a TCR stimulus, the presence of IL-2 not only promotes cell division as previously shown (6, 8) but also the secretion of IL-10 after a 6-day culture period (Fig. 5b). These effects are seen with sorted $CD25^+CD4^+$ T cells in the absence of any APC, but addition of co-stimulatory signals in the form of anti-CD28 mAb led to earlier IL-10 production detectable on day 3 of culture. Figure 5c illustrates the differentiation of $CD25^+CD4^+$ T cells to IL-10 production and the link to cell division in response to IL-2 and TCR signals. $CD25^+CD4^+$ T cells were cultured either with $CD45RB^{hi}CD4$ T cells or anti-CD3 antibody and recombinant IL-2 over 5 days. Substantial division was seen in co-culture with $CD45RB^{hi}CD4$ T cells or anti-$CD3^+$ IL-2 and with increasing cell division a proportion of the $CD25^+CD4^+$ T cells produced IL-10. In contrast, very little cell division was seen in response to IL-2 alone and no IL-10 production was observed. Furthermore, the expanding IL-10-producing cells were not recently activated T_h1 or T_h2 cells, since the majority made neither IFNγ nor IL-4 (Fig. 5d).

Discussion

Regulation of size is a universally important problem in biology and a number of principles defined in ecology can be applied to a wide variety of biological systems including the immune system. Cells compete for limited resources with members of the same population, which have different efficiencies in making use of the resource. An example for this is the well-documented difference of naive T cells for homeostatic expansion in response to peptide/MHC epitopes and IL-7 when transferred into lymphopenic hosts which results in drastic repertoire shifts (23–26). Furthermore, interference competition may limit access to a resource. An example in the immune system is competition for access to APCs during maturation of the immune response (27). Interference strategies common in the immune system are secretion of cytokines

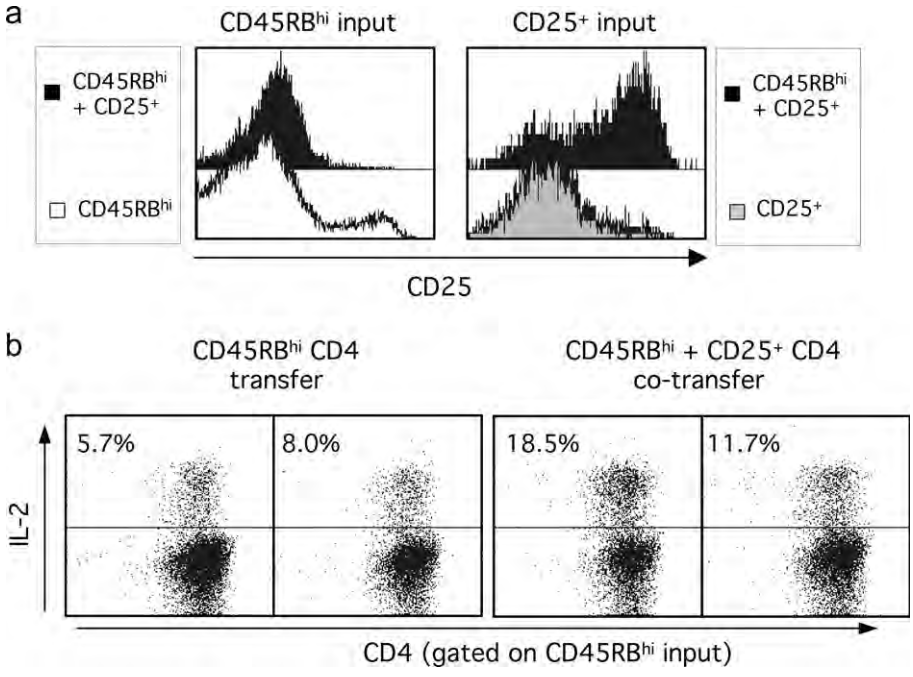

Fig. 4. IL-2 dependence of CD25 expression on regulatory T cells. (a) $CD45RB^{hi}CD4^+$ T cells (CD45.1, white histograms) and $CD25^+CD4^+$ T cells (CD45.2, gray histograms) were adoptively transferred either alone or together (black histograms) into C57BL/10 $Rag^{-/-}$ hosts. Mesenteric lymph nodes were taken 3 weeks later and CD4 T cells were analyzed for CD25 expression using CD45.1 and CD45.2 markers to distinguish $CD45RB^{hi}CD4$ input from $CD25^+CD4^+$ input. Data are representative for three mice per group. (b) $CD45RB^{hi}CD4$ T cells (CD45.1) and $CD25^+CD4^+$ T cells (CD45.2) were adoptively transferred either alone or together into C57BL/10 $Rag^{-/-}$ hosts. Mesenteric lymph node cells were taken 2 weeks later and re-stimulated with PdBU/ionomycin. CD4 T cells were analyzed for intracellular IL-2 using the CD45.1 marker to distinguish $CD45RB^{hi}CD4^+$ input from $CD25^+CD4^+$ input. Two mice per group are shown.

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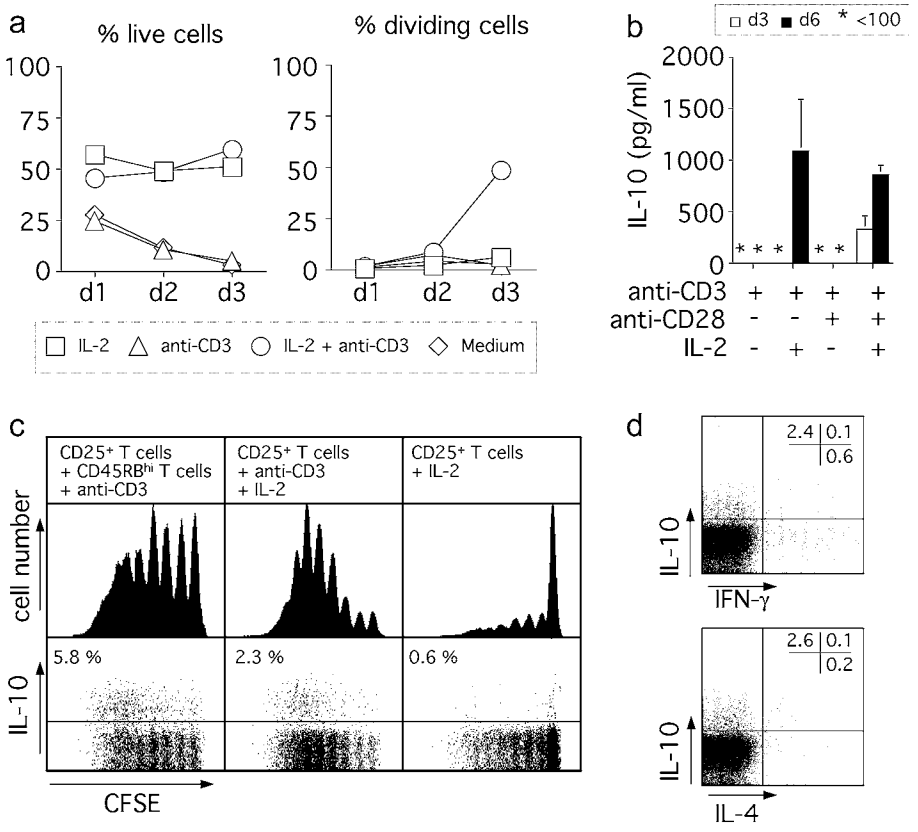

Fig. 5. TCR-mediated signals and IL-2 drive CD25$^+$CD4 T cells to division and IL-10 production. (a) CFSE-labeled CD25$^+$CD4$^+$ T cells were cultured with accessory cells in medium alone, medium containing 20 U ml^{-1} exogenous IL-2, medium containing anti-CD3 or medium containing both IL-2 and anti-CD3. The percentage of live cells within the CFSE-positive CD4 T cell population and the percentage of dividing CD4 T cells (determined by CFSE dilution) are shown. (b) CD25$^+$CD4$^+$ T cells were cultured in the absence of accessory cells and activated by plate-bound anti-CD3 alone or by plate-bound anti-CD3 and anti-CD28 with or without exogenous recombinant IL-2 (20 U ml^{-1}). On day 3 and day 6, the supernatant was analyzed for IL-10 content by ELISA. (c) CFSE-labeled CD25$^+$CD4$^+$ T cells (CD45.2) were either activated by anti-CD3 and accessory cells in the presence of CD45RB^{hi}CD4$^+$ T cells (CD45.1) or in the presence of IL-2. A control culture contained CFSE-labeled CD25$^+$CD4$^+$ T cells maintained with IL-2 in the absence of anti-CD3 stimulation. CFSE profiles and intracellular IL-10 staining on CD45.2-positive CD4 T cells at day 5 of culture are shown (after 4 h PdBU/ionomycin re-stimulation). (d) CD25$^+$CD4$^+$ T cells were activated for 5 days in the presence of IL-2 and accessory cells and analyzed for IL-10 and IFNγ or IL-10 and IL-4 by intracellular staining after a brief re-stimulation.

such as IL-10 and TGF-β, which play an important role in the down-regulation of inflammatory immune responses and curtail the expansion or functional properties of other cells (28, 29). The latter two molecules are important mediators in the regulatory function of CD25$^+$CD4$^+$ T cells *in vivo* (3). On the other hand, their mechanism of action *in vitro* is considered a contact-dependent phenomenon which does not involve cytokines such as IL-4, IL-10 or TGF-β (5, 6) but functions by down-regulation of IL-2 mRNA, thus suppressing the production of IL-2.

Our data confirm that CD25$^+$CD4$^+$ T cells suppress IL-2 mRNA, although levels remain above the background levels in CD25$^+$CD4$^+$ T cells. Importantly, however, our data also show that production of IL-2 protein is not fully abolished irrespective of the reduction in IL-2 mRNA. This was evident firstly by intracellular staining for IL-2 protein, which showed increasing proportions of responder T cells expressing the protein over the 3-day culture period irrespective of the presence of CD25$^+$CD4$^+$ T cells, even if the proportion of IL-2-positive responder cells was somewhat lower in the co-cultures. Furthermore, the phenotypic alterations in CD25 expression, an IL-2-dependent feature, in both responder and regulatory T cells implicated IL-2, which could only have been derived from the source of responder cells given the inability of CD25$^+$CD4$^+$ T cells to produce IL-2 themselves. CD25$^+$CD4$^+$ T cells constitutively express CTL associated antigen (CTLA)-4 (30–32) which has higher avidity for its ligands CD80 and CD86 than CD28 (33, 34) so that they might compete for co-stimulatory molecules on APC. Given that co-stimulatory signals through CD28 stabilize IL-2 mRNA (35, 36), one could envisage competition via CTLA-4 to compromise the positive signal for stabilization of IL-2 mRNA in the responder population. Recent data, however, provided evidence that while IL-2 appears critical for the *in vitro* activation of regulatory T cells, addition of exogenous IL-2 to the cultures does not correct the reduction in IL-2 mRNA in the responder population (37) even though it overcomes *in vitro* suppression.

The implication of cytokine consumption in the suppressive activity *in vitro* seems to contradict earlier data that showed suppression was contact dependent but not cytokine dependent. However, we would stress that most cytokines will act over short distances between closely interacting cell types

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under physiological conditions. Given that regulatory cells express high-affinity IL-2R while the responder population only up-regulates CD25 in the course of activation, one can envisage that close contact of the two cell populations will favor sequestering of IL-2 by $CD25^+CD4^+$ T cells. In contrast, physical separation by a membrane will inevitably delay access of IL-2 to $CD25^+CD4^+$ T cells, thereby favoring the autocrine feedback loop (18) leading to the up-regulation of CD25 in the responder population. Our data as well as those recently described by de la Rosa *et al.* (38) clearly show that IL-2 in conjunction with a TCR trigger drives further differentiation of $CD25^+CD4^+$ T cells into IL-10 production. APC do not seem involved in this process, since differentiation to IL-10 was seen in response to anti-CD3 alone without any APC. The effect of IL-2 on further differentiation of $CD25^+CD4^+$ T cells is compatible with the notion that IL-2 improves the regulatory function (37). The fact that anti-IL-10 antibodies do not abrogate *in vitro* suppressor function in our opinion indicates that the effects of IL-2 mRNA suppression and IL-2 protein sequestration are overriding additional suppressive mediators such as IL-10 *in vitro*, whereas these are essential for the suppressive function *in vivo*. Our data therefore provide a unifying basis for the suppressive mechanisms of regulatory T cells *in vivo* and *in vitro*.

While the importance of IL-10 for the *in vivo* function of $CD25^+CD4^+$ T cells is well recognized, it is as yet less clear whether sequestration of IL-2 is involved in the differentiation to IL-10 production. Adoptive transfer of naive and regulatory T cells confirmed our *in vitro* observation of reciprocal regulation of CD25 expression, suggesting that IL-2 is indeed involved also *in vivo*.

IL-2 is a major growth and differentiation factor regulating the duration and magnitude of T cell responses following antigenic stimulation (39) but is also critical for the down-regulation of immune responses through programming T cells for activation-induced cell death (40, 41). Although it is clear that T cells *in vivo* are not absolutely dependent on IL-2 for proliferation (42–44), there is substantial evidence that IL-2 plays a role for the regulatory action of $CD25^+CD4^+$ T cells *in vivo*. $CD25^+CD4^+$ T cells are unable to engraft and prevent disease after transfer into neonatal IL-$2^{-/-}$ hosts, but proved very efficient in neonatal IL-2R$\beta^{-/-}$ hosts, indicating that IL-2 is necessary for the expansion and function of adoptively transferred $CD25^+CD4^+$ T cells (11). The importance of expression of the IL-2Rα and β chains for survival and function of regulatory T cells is highlighted in numerous studies (9, 11–13) and the link to IL-2 is conspicuous.

Other studies showed suppression of both antigen-driven expansion (45) as well as homeostatic expansion (9, 10) in co-transfer of IL-$2^{-/-}$ T cells and $CD25^+CD4^+$ T cells from wild-type mice into T cell-deficient hosts. While this seemingly contradicts a role for IL-2 in the function of regulatory T cells, it is worth considering that the source of IL-2 need not obligatorily be T cells and dendritic cells that transiently make IL-2 (46) could have been present in the experimental models mentioned above. Furthermore, whether or not IL-2 is found to be involved in regulatory T cell function may depend on the experimental readout. *In vivo* regulation is complicated by the fact that the majority of experimental models employs lymphopenic mice in which differential homeostatic expansion of transferred T cell populations influences the outcome of the physiological immune balance. Thus, homeostatic or antigen-driven expansion might be controlled by $CD25^+CD4^+$ T cells irrespective of whether they have access to IL-2, possibly via competition for APC and co-stimulation. On the other hand, if the readout is immune pathology, which develops relatively late after transfer, the absence of IL-2 may more severely compromise the regulatory function. It is interesting to note that IL-10 is crucially important for control of the wasting disease induced by the transferred naive T cells (22), but not for regulation of autoimmmune gastritis (47), and both IL-10 and TGF-β are implicated in the control of inflammatory bowel disease (48–50). $CD25^+CD4^+$ T cells appear to make use of IL-2 generated by co-transferred CD4 T cells as they retain their CD25 expression after transfer only when other CD4 T cells are present (22). Similar observations were made with antigen-specific regulatory T cells transferred into normal mice (21). Sequestering of IL-2 from activated CD4 T cells may contribute to regulation of their expansion, but more importantly this interaction may set in motion the program of IL-10 production, probably linked to cell division, and thus supplant exploitation competition with interference competition.

We have not been able to detect IL-10 in the $CD25^+CD4^+$ T cell population after transfer, but given the low proportion of cells progressing to IL-10 production *in vitro*, it is conceivable that the number of IL-10 producers was below the detection limit. Alternatively IL-10-producing $CD25^+CD4^+$ T cells might have been located at peripheral sites such as the skin or intestine in keeping with their expression of the integrin α_E β_7 (CD103) (51–53). Interestingly, systemic application of recombinant IL-2 in clinical trials, while clinically ineffective, stimulated a 3-fold increase in serum levels of IL-10 (54).

In conclusion, it is conceivable that IL-2 is one component of a multi-step process involved in the regulatory function of $CD25^+CD4^+$ T cells that involves both competition for resources (IL-2, APC and co-stimulatory signals) as well as further differentiation to a functional state that allows interference competition via suppressive cytokines.

Acknowledgements

We thank André Boonstra for help with the IL-10 ELISA and Richard Hocking for technical support.

Abbreviations

APC	antigen-presenting cells
CFSE	5, 6 carboxyfluorescein diacetate succinimidyl ester
CTLA	CTL associated antigen 4
PdBu	phorbol 12, 13 dibutyrate
TGF-β	transforming growth factor-β

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