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$CD25^+$ $CD4^+$ T Cells Regulate the Expansion of Peripheral CD4 T Cells Through the Production of IL-10[1]

Oliver Annacker,[2] Ricardo Pimenta-Araujo, Odile Burlen-Defranoux, Theolis C. Barbosa,[3] Ana Cumano, and Antonio Bandeira

The mechanisms by which the immune system achieves constant T cell numbers throughout life, thereby controlling autoaggressive cell expansions, are to date not completely understood. Here, we show that the $CD25^+$ subpopulation of naturally activated ($CD45RB^{low}$) CD4 T cells, but not $CD25^-$ $CD45RB^{low}$ CD4 T cells, inhibits the accumulation of cotransferred $CD45RB^{high}$ CD4 T cells in lymphocyte-deficient mice. However, both $CD25^+$ and $CD25^-$ $CD45RB^{low}$ CD4 T cell subpopulations contain regulatory cells, since they can prevent naive CD4 T cell-induced wasting disease. In the absence of a correlation between disease and the number of recovered $CD4^+$ cells, we conclude that expansion control and disease prevention are largely independent processes. $CD25^+$ $CD45RB^{low}$ CD4 T cells from IL-10-deficient mice do not protect from disease. They accumulate to a higher cell number and cannot prevent the expansion of $CD45RB^{high}$ CD4 T cells upon transfer compared with their wild-type counterparts. Although $CD25^+$ $CD45RB^{low}$ CD4 T cells are capable of expanding when transferred in vivo, they reach a homeostatic equilibrium at lower cell numbers than $CD25^-$ $CD45RB^{low}$ or $CD45RB^{high}$ CD4 T cells. We conclude that $CD25^+$ $CD45RB^{low}$ CD4 T cells from nonmanipulated mice control the number of peripheral CD4 T cells through a mechanism involving the production of IL-10 by regulatory T cells. *The Journal of Immunology,* **2001, 166: 3008–3018.**

The regulation of the magnitude of protective immunity to foreign Ags as well as the control of autoaggressive immune reactions are ensured by regulatory T cells. Regulatory CD4 T cells have been described in a variety of experimental systems to protect from autoimmune diseases (1–6) as well as from inflammatory bowel disease (IBD)[4] (7, 8) and allograft rejection (9). Furthermore, regulatory CD4 T cells play a key role in the homeostasis of the peripheral CD4 T cell pool (10).

Useful surface markers for the discrimination between functional subsets of CD4 T cells are CD25 and CD45RB. CD25 is a component of the IL-2R and is transiently expressed on CD4 T cells after activation (11), and $CD25^+$ T cells make up approximately 10% of the peripheral CD4 T cell pool. Transfer of CD25-depleted splenic cells into nude mice of susceptible strains leads to the development of organ-specific autoimmune diseases, which can be prevented by the cotransfer of purified $CD25^+$ CD4 T cells (2, 3). Moreover, the lack of $CD25^+$ cells allows for efficient clearance of tumors, demonstrating the active suppression of anti-self immune responses by regulatory T cells (12). $CD25^+$ CD4 T cells were found to produce high levels of TGF-β and IL-10 compared with $CD25^-$ CD4 T cells (3). Recently, it was shown that the protective effect of $CD25^+$ CD4 T cells is dependent on CTLA-4 (13).

CD45RB is another marker of activation used for the discrimination of different CD4 T cell subsets. This surface molecule is up-regulated during thymic development (14), and its expression on naive CD4 T cells decreases upon activation (15). According to this marker, roughly one-third of CD4 T cells are activated in unmanipulated mice.

In an experimental system of IBD several groups showed that after transfer into immunodeficient recipients, naive $CD45RB^{high}$ CD4 T cells cause a wasting disease characterized by intestinal inflammation (7, 8). High levels of IFN-γ and TNF-α are found in both the spleen and the intestine of the recipients (8, 16–18), and injected T cells can expand at least 200-fold under specific pathogen-free (SPF) conditions (10). In contrast, naturally activated $CD45RB^{low}$ CD4 T cells usually do not induce disease, expand less, and protect the recipients from naive T cell-induced IBD (7, 8, 10, 16, 17). This protective effect is dependent on TGF-β and IL-10 (17, 19–22). The $CD45RB^{low}$ CD4 T cell pool contains most of the $CD25^+$ CD4 T cells; the latter contribute one-third to the pool of $CD45RB^{low}$ CD4 T cells. In a recent study Read et al. showed that the protection from IBD in this experimental system is enriched in the $CD25^+$ T cell population within the $CD45RB^{low}$ CD4 T cell pool and is also CTLA-4 dependent (23).

The general mechanisms regulating the expansion and survival of peripheral CD4 T cells are to date not well understood (for review, see Ref. 24). The peripheral T cell pool is divided into a naive and an activated/memory compartment, which are apparently independently regulated (25, 26). The size of the activated/memory CD4 T cell pool is controlled by regulatory T cells within this pool (10). The administration of recombinant murine IL-10 protected recipients reconstituted with $CD45RB^{high}$ CD4 T cells from disease and decreased the number of recovered splenic CD4

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[4] Abbreviations used in this paper: IBD, inflammatory bowel disease; SPF, specific pathogen-free; RAG-2°, recombination activating gene-2 deficient; IL-10°, IL-10 deficient; wt, wild type.

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T cells (17), suggesting a role for IL-10 in the regulation of the expansion of peripheral CD4 T cells.

Here, we investigated the survival, the dynamics of expansion, and the homeostatic equilibrium of different peripheral CD4 T cell subpopulations from normal donors upon transfer into T and B cell-deficient mice, their regulatory properties, and the role of IL-10 in the expansion process. Our results show that although both $CD25^+$ and $CD25^-$ $CD45RB^{low}$ CD4 T cell pools contain regulatory T cells, only the $CD25^+$ population can efficiently regulate the size of the activated/memory CD4 T cell compartment via a mechanism involving the production of IL-10. Furthermore, our results show that control of CD4 T cell peripheral expansion and disease prevention are largely independent processes. Finally, the data demonstrate that although the $CD25^+$ CD4 T cell population reaches a homeostatic equilibrium at low cell numbers, a fraction of these cells has a high potential of expansion upon transfer into alymphoid recipients.

Materials and Methods

Mice

C57BL/6-Ly5.2 mice were obtained from Janvier (Le Genest-St-Isle, France). C57BL/6-Ly5.1, C57BL/6 recombination-activating gene-2-deficient (RAG-2°) and C57.BA (Thy1.1) mice were purchased from CDTA (Orleans, France). All animals were kept under SPF conditions in the animal facilities of the Institut Pasteur (Paris, France). C57BL/6-IL-10-deficient (IL-10°) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred under SPF conditions in our animal facilities. Donors and recipients were sex-matched and were used at 6–12 wk of age. IL-10° donors were used at 5–8 wk of age.

Antibodies

The following mAbs were used: anti-Ly5.1-FITC or -PE (clone A.20); anti-CD4-biotin, -FITC, -PE, -TriColor, or -allophycocyanin (L3T4); anti-CD8-FITC or -PE (CT-CD8a); anti-CD45RB-PE (23G2); anti-CD25-FITC (7D4) or -PE (PC61); anti-CD38-FITC (90); anti-CD69-PE (H1.2F3); anti-CD44-PE (IM7.8.1); and anti-Thy1.1-PE (Ox-7). All Abs were purchased from PharMingen (San Diego, CA) or Caltag (Burlingame, CA).

T cell preparations

Before sorting, splenic single-cell suspensions were first enriched for $CD4^+$ or $CD25^+$ cells by positive selection on midiMACS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. In brief, cells were first incubated with biotinylated anti-CD4 Abs for 20 min on ice in PBS supplemented with 0.5% FCS, then incubated in the same buffer with streptavidin-microbeads for 15 min. The magnetically labeled positive fraction was retained on a midiMACS column. Alternatively, for the enrichment of $CD25^+$ cells, FITC-labeled anti-CD25 Abs and anti-FITC-microbeads were used. In all cases, after enrichment the cells were labeled with anti-CD45RB-PE, anti-CD4-Tricolor, and anti-CD25-FITC Abs for 20 min on ice and then sorted on a FACStar^{Plus} (Becton Dickinson, Mountain View, CA). For CD45RB, the brightest 40–50% and the dimest 20–30% of $CD4^+$ cells were sorted as high and low, respectively. $CD25^+$ and $CD25^-$ CD4 T cells were sorted out of the $CD45RB^{low}$ population. The purity of the sorted populations was routinely >96%.

Labeling with CFSE

Labeling of cells with CFSE was performed as previously described (27). In brief, after washing FACS-sorted CD4 T cells twice in PBS, the cells were resuspended at 1–2 $\times$ 10^7/ml PBS and incubated 10 min at room temperature with CFSE at a final concentration of 6 μM. This solution was incubated with an equal volume of FCS for an additional 2 min before washing twice with PBS.

Cell transfers

RAG-2° mice were injected iv. or i.p. with 3 $\times$ 10^5 CD4 T cells from FACS-sorted subpopulations. When cells were coinjected (at a ratio of 1:1), $Ly5.1^+$ and $Ly5.2^+$ donor cells were used. For CFSE-labeled cells, 0.5–1.5 $\times$ 10^6 CD4 T cells from $Ly5.1^+$ origin were injected i.v. Here, coinjections were made with $Ly5.1^+$ and $Thy1.1^+$ donors at a ratio of 1:1.

Preparation of intestinal cells

Whole intestines were first flushed extensively to eliminate the lumen content, then were longitudinally opened and cut into 1- to 2-cm pieces. These were incubated twice in OptiMEM medium (Life Technologies, Gaithersburg, MD) containing 5% FCS and 450 U of collagenase (Sigma, St. Louis, MO) for 20 min at 37°C. After filtering through gauze, lymphoid cells were isolated on a 40% Percoll gradient. The cells were then washed and stained for fluorocytometric analysis.

Flow cytometric analysis

Single-cell suspensions from spleen; axillary, inguinal, and mesenteric lymph nodes; or intestine were incubated for 20 min at 4°C in microtiter plates with 50 μl of the appropriate Ab preparations in PBS supplemented with 3% FCS and 0.01% azide. When possible, one million cells were stained. Alternatively, the whole organ cell suspension was used. The Ab concentrations used were tested for optimal stainings of splenic control samples before experimental use. Dead cells were excluded from the analysis by propidium iodide. Blood samples were first stained with appropriate Abs before lysing erythrocytes with FACS Lysing Solution (Becton Dickinson). Flow cytometric analysis was performed on a FACScan (Becton Dickinson) using CellQuest software (Becton Dickinson).

Statistical analysis

Unless otherwise indicated, analysis was performed using the unpaired *t* test. In cases where the variances between compared groups were significantly different, the unpaired *t* test was modified with Welch's correction. The data were considered significantly different at $p < 0.05$.

Results

The incidence of CD4 T cell-induced wasting in alymphoid mice correlates with the frequency of $CD25^+$ cells in the transferred population

Splenic CD4 T cells from normal unmanipulated mice were sorted into four subpopulations according to the expression of CD45RB and CD25 markers: 1) $CD45RB^{high}$ cells, hereafter denoted RB^{high}; 2) $CD45RB^{low}$ cells (of which about one-third is $CD25^+$), denoted RB^{low}; 3) $CD45RB^{low}$ cells, which were depleted of $CD25^+$ cells, denoted 25^- RB^{low}; and 4) $CD45RB^{low}$ cells expressing the CD25 marker, hereafter referred to as 25^+ RB^{low} CD4 T cells (Fig. 1*A*).

Syngenic RAG-2° mice, kept under SPF conditions, received 3 $\times$ 10^5 of either sorted CD4 T cell subset. The weight of the recipients was scored twice a week, and animals were sacrificed after 12–14 wk or when they lost at least 20% of their initial weight.

Mice injected with (naive) RB^{high} T cells invariably developed signs of wasting. Only one of eight animals survived for 12 wk after transfer (Fig. 1*B*), and all recipients lost weight (Fig. 1*C*), developed diarrhea, and had a markedly enlarged colon upon analysis. Noninjected control RAG-2° mice kept under the same conditions never developed signs of wasting or diarrhea (data not shown). In the group of animals that received the total pool of RB^{low} cells, one of seven mice became sick (Fig. 1*C*) and was sacrificed 8 wk after transfer (Fig. 1*B*). Half of the recipients of the 25^- RB^{low} pool remained healthy throughout the experimental period of 3.5 mo (Fig. 1*C*). The other half suffered from wasting, but the disease progressed more slowly compared with that in recipients of RB^{high} T cells, and only two of them lost >20% of their initial body weight within 12 wk after transfer (Fig. 1*B*). The only experimental group in which all recipients invariably gained weight and did not develop signs of wasting was the one injected with 25^+ RB^{low} T cells (Fig. 1*C*). Thus, the incidence of a wasting disease in alymphoid recipients after transfer of different CD4 T cell populations appears to correlate with the frequency of $CD25^+$ cells in the transferred population.

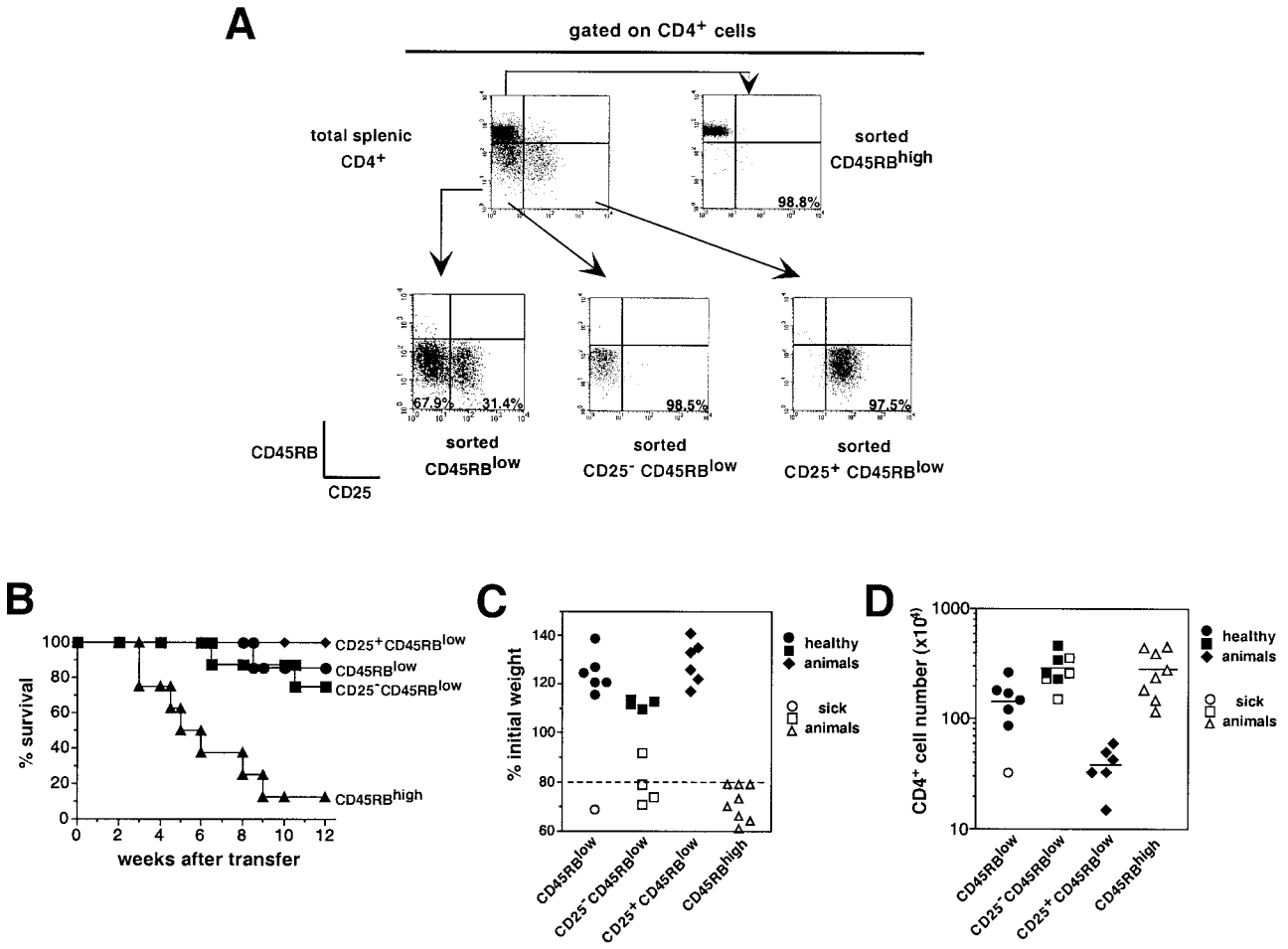

FIGURE 1. Incidence of wasting disease and T cell numbers at equilibrium in alymphoid recipients reconstituted with different CD4 T cell subsets. Sorted normal CD4 T cells of the indicated phenotype (3×10^5) were transferred into syngenic RAG-2° hosts. *A*, FACS profiles of sorted CD4 populations before injection. *B*, The time of sacrifice for each recipient (six to eight recipients per group), either 12–14 wk after transfer or when they lost at least 20% of their original weight. *C*, Body weight of the recipients at the time of sacrifice. Sick animals were defined by weight loss below their initial weight, which was associated with diarrhea and a markedly enlarged colon. *D*, Sum of the number of $CD4^+$ cells in spleen; axillary, inguinal, and mesenteric lymph nodes; blood (assuming 3 ml of blood per animal); and intestine. The background of noninjected control RAG-2° mice was 1.3×10^5 $CD4^+$ cells ($n = 4$). The data are pooled from three or four independent experiments per group.

Peripheral expansion of CD4 T cell subsets: $CD25^+$ $CD45RB^{low}$ CD4 T cells reach homeostatic equilibrium at low cell numbers

To assess the accumulation and the respective homeostatic equilibrium of the injected T cell populations, at the time of sacrifice the number of $CD4^+$ cells was scored in the spleen; axillary, inguinal, and mesenteric lymph nodes; as well as blood and intestine of all the recipients described in the previous section. In this series of transfers, on the average, 2.9×10^6 CD4 T cells were recovered from mice injected with RB^{high} T cells (Fig. 1*D*). In animals injected with the total pool of naturally activated/memory RB^{low} T cells, we could only score half the number of cells found in the previous group (on the average, 1.4×10^6; $p < 0.03$), as shown previously (10). Interestingly, the number of cells (on the average, 2.9×10^6) obtained from mice reconstituted with 25^- RB^{low} T cells was similar to that scored in recipients of RB^{high} T cells regardless of the state of health of the recipients (Fig. 1*D*) and the time point of sacrifice. Finally, recipients of 25^+ RB^{low} T cells only yielded approximately the number of cells injected (on the average, 3.9×10^5), that is, 7-fold less compared with the 25^- RB^{low} ($p < 0.0001$) or the RB^{high} ($p < 0.001$) population and about 4-fold less compared with recipients of unfractionated RB^{low} T cells ($p < 0.01$; Fig. 1*D*).

The organ distribution of the $CD4^+$ cells in all groups of mice is shown in Table I. The majority of T cells were found in the spleen, accounting for roughly half of the recovered CD4 T cells. In the intestine, with the exception of recipients of the 25^+ RB^{low} fraction, similar numbers of CD4 T cells were recovered in both healthy and sick animals in all other groups (Table I). However, it cannot be formally excluded that the observed differences in cell numbers between different CD4 T cell subsets are due not to different expansions of these cells, but to a differential migration pattern predominantly into other organs, such as liver, lung, or bone marrow, which have not been investigated here.

In conclusion, disease is directly correlated neither with the total number of T cells recovered from the recipients nor with the number of T cells present in the intestine. In addition, the 25^+ RB^{low} T cell population accumulates to low cell numbers after transfer into RAG-2° hosts and is barely detectable in the intestine.

Both $CD25^+$ and $CD25^-$ $CD45RB^{low}$ CD4 T cells contain regulatory cells capable of preventing a wasting disease induced by naive CD4 T cells

In the experiments described above (see Fig. 1), half of the recipients of the 25^- RB^{low} T cell population became sick, whereas the other half remained healthy for at least 3 mo. This differential

Table I. *Organ distribution of CD4$^+$ cells in RAG-2° mice reconstituted with different CD4 subpopulations*[a]

Phenotype of Injected CD4$^+$ Subpopulations	No. of Animals	No. of Recovered CD4$^+$ Cells ($\times 10^4$)[b]			
		Spleen	Lymph Nodes[c]	Blood[d]	Intestine
CD45RB^{low}	7	75.7 (±21.5)	20.2 (±5.1)	7.6 (±1.6)	29.1 (±5.9)
CD25$^-$CD45RB^{low}	8	140.3 (±26.7)	48.0 (±12.4)	27.1 (±8.1)	19.9 (±7.6)
CD25$^+$ CD45RB^{low}	6	20.9 (±4.4)	11.0 (±1.8)	1.9 (±0.4)	1.1 (±0.4)
CD45RB^{high}	8	105.1 (±33.4)	31.5 (±11.0)	38.3 (±10.6)	35.1 (±8.3)

[a] RAG-2° recipients were injected with 3×10^5 FACS-sorted CD4 T cells. The animals were analyzed for the presence of CD4$^+$ cells in the indicated organs after 12–14 wk or when they dropped below 80% of their starting weight.
[b] Average ± SEM.
[c] Axillary, inguinal, and mesenteric lymph nodes.
[d] Data are expressed as cell number per milliliter of blood.

outcome could be the result of differences in the frequency of potentially pathogenic or, alternatively, of regulatory T cells in the individual inoculums of this CD4 T cell subset.

To directly assess the presence of regulatory activity in the two CD45RB^{low} subpopulations, RAG-2° recipients were coinjected with 3×10^5 RB^{high} T cells and 3×10^5 CD4 T cells of either the 25$^+$ or 25$^-$ RB^{low} T cell subset (Fig. 2*A*). The majority of the animals injected with these mixtures were protected from disease (Fig. 2*B*), and a similar fraction of animals in both groups developed a wasting disease with similar kinetics (Fig. 2*C*). In conclusion, the CD25$^-$ CD45RB^{low} T cell subpopulation contained sufficient regulatory activity to prevent CD4 T cell-induced wasting in 40% of the recipients.

CD25$^+$ CD45RB^{low} CD4 T cells control the size of the activated/memory CD4 T cell pool

The cell recovery from recipients of the total RB^{low} T cell pool was significantly different from that of either 25$^-$ or 25$^+$ subfractions (see Fig. 1). This could be interpreted to indicate that the restricted expansion of total RB^{low} T cells after transfer into RAG-2° hosts was due to control of the accumulation of 25$^-$ RB^{low} T cells exerted by 25$^+$ RB^{low} T cells. To investigate whether the

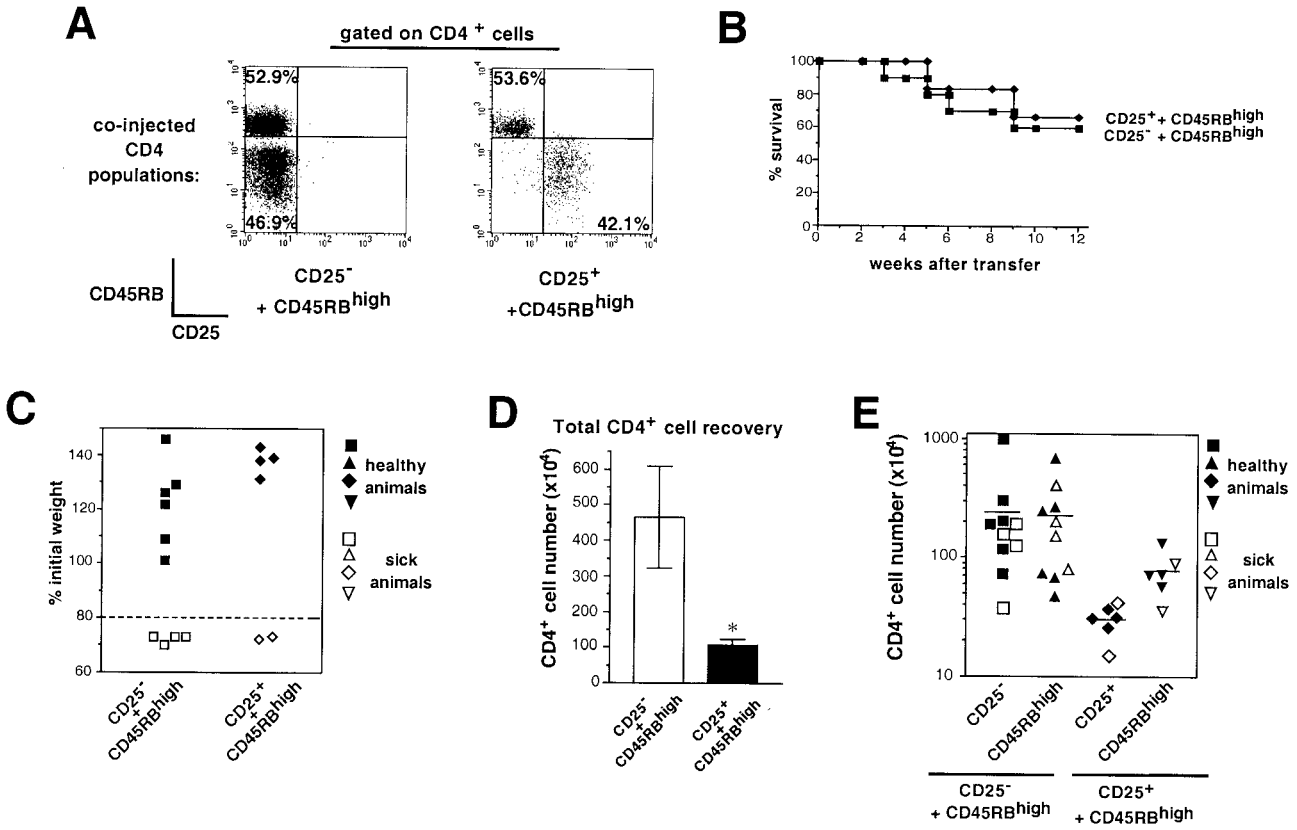

FIGURE 2. CD25$^+$, but not CD25$^-$ CD45RB^{low}, CD4 T cells inhibit the accumulation of CD45RB^{high} CD4 T cells transferred into RAG-2° hosts. Sorted CD25$^-$CD45RB^{low} (CD25$^-$) or CD25$^+$CD45RB^{low} (CD25$^+$) CD4 T cells (3×10^5) were coinjected with 3×10^5 CD45RB^{high} CD4 T cells into RAG-2° hosts. *A*, FACS profiles of the coinjected populations. *B*, Recipients were sacrificed at 12–14 wk after transfer or when they dropped below 80% of their starting weight as indicated by the survival curve. *C*, Weight of the recipients at sacrifice (time points of sacrifice as indicated in *B*). Sick animals were defined as described in Fig. 1. *D*, Sum of the total CD4$^+$ cell numbers scored in spleen; axillary, inguinal, and mesenteric lymph nodes; blood (assuming 3 ml of blood per animal); and intestine. The mean (± SEM) is shown. ∗, The difference is statistically significant ($p < 0.04$, by unpaired *t* test with Welch's correction). *E*, CD4$^+$ cell number for each donor population in the two groups of recipients. CD4$^+$ cells from different origins were identified by the expression of different Ly5 isoforms. The data are pooled from three independent experiments. The difference between recovered CD45RB^{high} T cell numbers in the two groups is statistically significant ($p < 0.05$, by unpaired *t* test with Welch's correction; see also text).

$CD25^+$ T cell pool is indeed responsible for control of the size of the activated/memory CD4 T cell compartment, we analyzed the coinjected recipients (see Fig. 2) for the level of T cell reconstitution either 12–14 wk after transfer or when the recipients dropped to <80% of their initial weight. The identification of the origin of each donor population was based on the expression of the Ly5.1 and Ly5.2 markers.

The total CD4 T cell recovery from mice injected with mixed 25^- RB^{low} and RB^{high} T cells was >4-fold higher compared with that in animals that received mixed 25^+ RB^{low} and RB^{high} cells ($p < 0.04$, by unpaired *t* test with Welch's correction; Fig. 2*D*). In the group cotransferred with 25^- RB^{low} and RB^{high} T cells both populations expanded to similar numbers as those scored in animals injected with either population alone (Figs. 2*E* and 1*D*), with a similar distribution of each subset in all organs (Table II). Again, no significant difference was observed between healthy and sick animals (Fig. 2*E*).

In the group of recipients injected with 25^+ RB^{low} and RB^{high} cells, similar numbers of 25^+ RB^{low} T cells were recovered as from animals injected with these cells alone regardless of the presence of RB^{high} T cells in the injected animals (Fig. 2*E*). However, approximately 4-fold less T cells of originally RB^{high} T cells were scored in these animals compared with recipients of RB^{high} T cells injected alone ($p < 0.004$) or coinjected with 25^- RB^{low} T cells ($p < 0.05$, by unpaired *t* test with Welch's correction). T cell numbers were mostly reduced in spleen, blood, and intestine (Table II). Interestingly, the relative frequency of originally 25^+ RB^{low} to RB^{high} T cells in the intestine was 1:10 to 1:20, since 25^+ RB^{low} T cells were again barely detected in the intestine (Table II). This ratio was independent of the health state of the mice, and the presence of 25^+ RB^{low} T cells did not inhibit the activation of the original RB^{high} T cells, as assessed by the low density of the CD45RB marker (Fig. 3).

Taken together, these results show that the 25^+, but not the 25^-, RB^{low} T cell population has the potential to efficiently inhibit the accumulation of other CD4 T cells. Here again, no direct correlation was found between the magnitude of T cell accumulation and the incidence of wasting disease in any of the experimental groups.

$CD25^+$ $CD45RB^{low}$ CD4 T cells can expand in vivo

$CD25^+$ T cells do not expand upon stimulation with anti-CD3 Abs in vitro (28, 29), which was interpreted to reflect an anergic state of these cells (28). The reconstitution of RAG-2° mice with 25^+ RB^{low} cells yielded approximately the number of cells injected (see above). However, because in transfer experiments only a minority of the injected cells survives in the host 24–48 h after transfer (10), this suggested that the $CD25^+$ T cell population could nevertheless expand to a certain extent in the recipients.

To more accurately address this issue, CD4 T cells from normal $Ly5.1^+$ donors were sorted into three subpopulations according to the expression of CD25 and CD45RB and were labeled with CFSE. Then, 0.5–1.5 × 10^6 cells of each subset were separately injected into congenic $Ly5.2^+$ RAG-2° hosts. Thirty-six to 48 h after transfer, donor-derived CD4 T cells were analyzed in the peripheral lymphoid organs, the blood, and the gut. While no donor cells were recovered from the intestine, the bulk of the populations were found in the spleen. Naive RB^{high} T cells survived much better than naturally activated 25^- or 25^+ RB^{low} T cells (Fig. 4*C*). On the average 2.6% of the RB^{high} T cell population could be recovered at this early time point, whereas only 1.1 and 0.3% of injected 25^- and 25^+ RB^{low} T cells, respectively, were recovered. At this time point, very few of the injected cells had divided, as assessed by CFSE staining (data not shown).

In the following days all populations expanded, and 12 days after transfer very few cells from any of the injected populations remained CFSE positive, indicating that in all cases the bulk of the cells present at this time point went through a minimum of six rounds of division. As shown in Fig. 4*C*, 4- and 6.8-fold the number of injected cells were recovered from recipients of 25^- RB^{low}

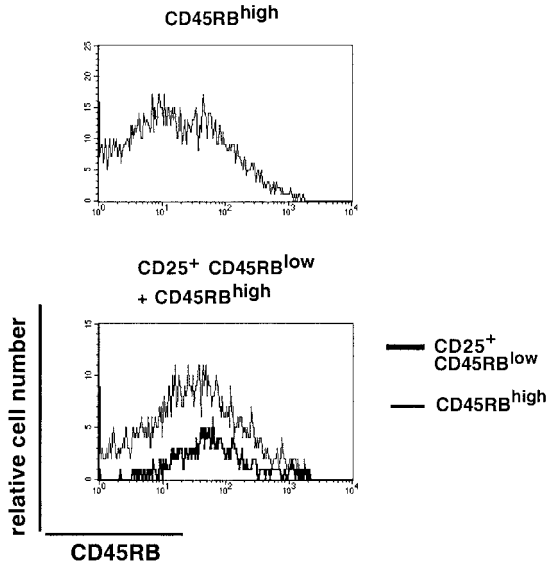

FIGURE 3. Originally $CD45RB^{high}$ CD4 T cells acquire an activated/memory ($CD45RB^{low}$) phenotype after transfer into RAG-2° animals, which is not inhibited by the presence of $CD25^+$ $CD45RB^{low}$ T cells. At sacrifice, mice injected with sorted CD4 T cells, as shown in Figs. 1 and 2, were analyzed for the expression of CD45RB. Shown are representative stainings for CD45RB on gated $CD4^+$ cells in mesenteric lymph nodes. $CD4^+$ cells from different origins were identified by the expression of different Ly5 isoforms. Note the density of CD45RB on sorted populations shown in Figs. 1*A* and 2*A*.

Table II. *Organ distribution of $CD4^+$ cells in RAG-2° mice reconstituted with coinjected CD4 subpopulations*[a]

Coinjected Populations	No. of Animals	Phenotype of Injected $CD4^+$ Subpopulations	No. of Recovered $CD4^+$ Cells ($\times 10^4$)[b]			
			Spleen	Lymph Nodes[c]	Blood[d]	Intestine
$CD25^-$	10	$CD25^-$	139.0 (±68.1)	28.2 (±7.7)	16.0 (±4.0)	26.0 (±10.8)
+ $CD45B^{high}$		$CD45RB^{high}$	129.0 (±54.5)	18.3 (±4.2)	16.5 (±4.6)	26.1 (±8.8)
$CD25^+$	6	$CD25^+$	16.7 (±2.7)	8.5 (±1.9)	1.3 (±0.3)	1.2 (±0.8)
+ $CD45RB^{high}$		$CD45RB^{high}$	32.1 (±5.4)	16.4 (±2.9)	5.9 (±1.8)	10.9 (±4.2)

[a] RAG-2° recipients were injected with 3 × 10^5 FACS-sorted CD4 T cells of either population as indicated. Differentiation of the origin of the cells was achieved by using Ly5-congenic donors. The animals were analyzed for the presence of $CD4^+$ cells in the indicated organs after 12–14 wk or when they dropped below 80% of their starting weight.
[b] Average ± SEM.
[c] Axillary, inguinal, and mesenteric lymph nodes.
[d] Data are expressed as cell number per milliliter of blood.

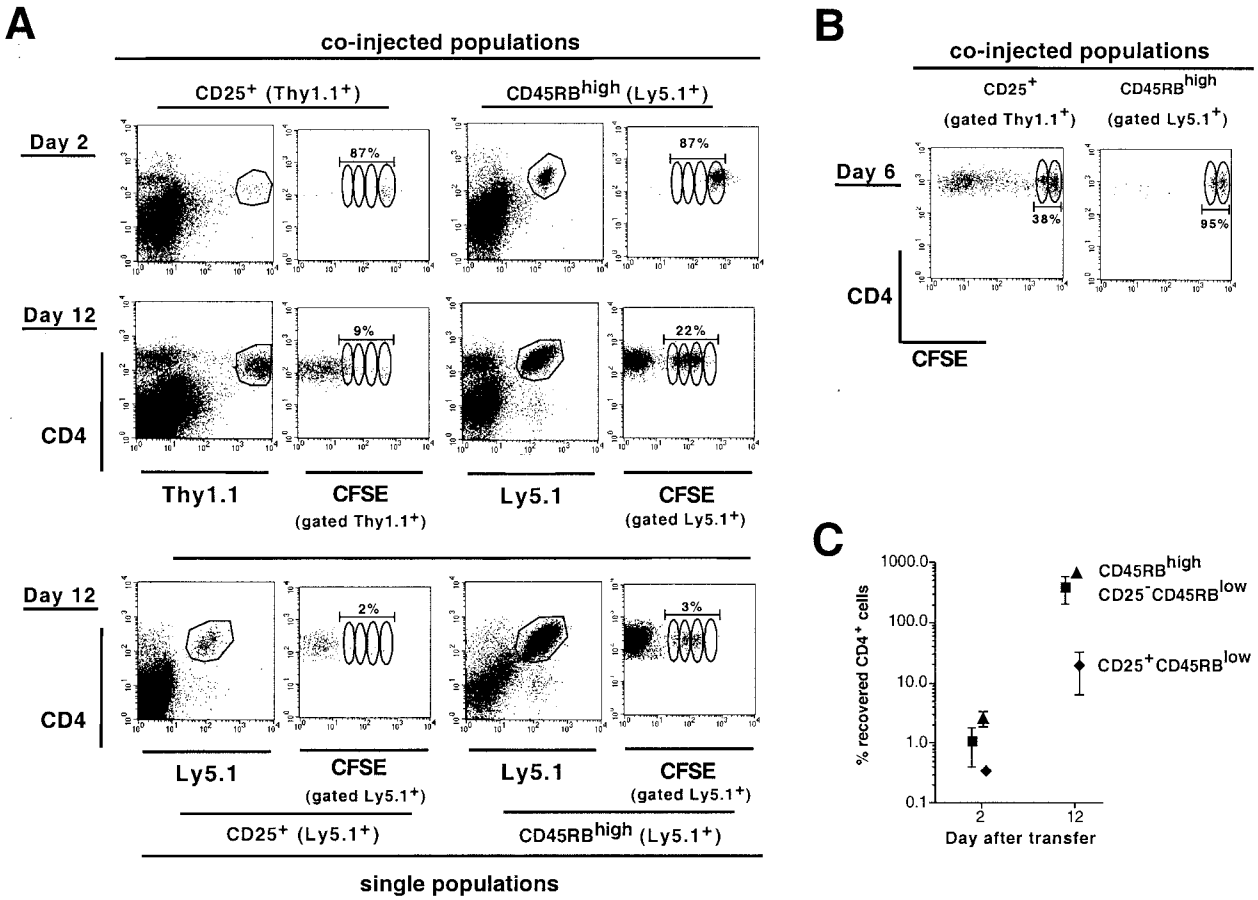

FIGURE 4. Regulatory CD4 T cells can expand in vivo. C57BL/6 RAG-2° hosts were injected with 0.5–1.5 × 10^6 FACS-sorted and CFSE-labeled $CD4^+$ cell subsets of donor mice congenic for the Thy1.1 or Ly5 allotype marker. *A*, Representative staining pattern for the presence of donor cells and the corresponding CFSE pattern of these cells in the spleen on different days after reconstitution as indicated. Coinjections were performed at a ratio of 1:1. The survival and the recovery of $CD25^+$ cells were similar in recipients of single or mixed injections. The number of $CD45RB^{high}$ T cells in coinjected mice was similar to that in recipients of these cells alone on day 2, but the recovery after 12 days was strongly reduced (see text). *B*, Expansion of CD4 T cells of the indicated donor phenotype 6 days after transfer in coinjected recipients. The data are from an experiment performed independently of those shown in *B*. *C*, Total CD4 T cell numbers in spleen; axillary, inguinal, and mesenteric lymph nodes; blood (assuming 3 ml of blood per animal); and intestine in the recipients of the indicated populations (two to five mice per group). Data represent the CD4 T cells recoveries as a percentage of the number of injected cells (average ± SEM). The lack of error bars indicates that the deviation is smaller than the symbols.

and of RB^{high} T cells, respectively. In contrast, recipients of 25^+ RB^{low} T cells contained 20% of the number of injected cells. However, taking into account the differential survival of the injected populations in the three groups of recipients, the increase in cell numbers over the surviving populations on day 2 was 60-fold for the 25^+ RB^{low} T cell population (corresponding to five rounds of division of the surviving T cells), compared with 360- and 260-fold for the 25^- RB^{low} and the RB^{high} T cell populations (equivalent to seven or eight rounds of division), respectively (Fig. 4*C*).

In conclusion, these results show that a fraction of the 25^+ RB^{low} cells has an expansion capacity not very different from that of the 25^- RB^{low} or RB^{high} T cells. The lower reconstitution capacity of $CD25^+$ cells 3.5 mo after transfer probably reflects a difference in the homeostatic regulation of steady state numbers in these populations.

$CD25^+$ $CD45RB^{low}$ CD4 T cells regulate the size of the $CD45RB^{high}$ CD4 T cell compartment early after transfer

In these series of experiments CFSE-labeled 25^+ RB^{low} and RB^{high} T cells were also coinjected into RAG-2° recipients, and the mice were analyzed 2, 6, and 12 days later. On day 2 after transfer no significant cell division was detected (Fig. 4*A*). On day 12 after transfer the 25^+ RB^{low} population had expanded as much as the same population injected alone (Fig. 4*A*). In contrast, the RB^{high} population in the coinjected animals showed a higher frequency (22%) of cells that were CFSE positive after 12 days compared with the ones isolated from hosts receiving RB^{high} T cells alone (3%; Fig. 4*A*). Moreover, at this time point the absolute number of RB^{high} T cells recovered from mice injected with this population alone was 10-fold higher than that in the mice coinjected with 25^+ RB^{low} T cells. Interestingly, 6 days after transfer 60–80% of the 25^+ CD4 T cells found in the spleen of the hosts had lost the CFSE staining, indicating that they had already gone through at least six or seven rounds of division. In contrast, at this time point >90% of the RB^{high} T cells did not divide more than once (Fig. 4*B*). The same result was observed when the two populations were independently injected (data not shown).

These experiments show that a fraction of 25^+ RB^{low} T cells have a quite remarkable potential of expansion, and that by day 6 they were already engaged in cell division. In addition, they show that the homeostatic activity of 25^+ RB^{low} on RB^{high} T cells operates shortly after transfer.

The expression of the CD25 molecule on $CD25^+$ $CD45RB^{low}$ CD4 T lymphocytes in vivo is not stable and is influenced by the presence of other CD4 T cells

The frequency of $CD25^+$ T cells is constant throughout the adult life of normal mice (30), which led to the hypothesis that these

cells constitutively express this molecule (29, 31). Since T cells express high levels of CD25 upon activation (11), it was also suggested that $CD25^+$ T cells are continuously activated in vivo (32). Here we analyzed whether the reconstitution of the CD25 compartment is dependent on the origin of the injected cells.

At sacrifice, the peripheral lymphoid organs of RAG-2° animals injected with different CD4 T cell subsets, as shown in Figs. 1 and 2, were analyzed for the expression of CD25 on the recovered CD4 T cells as well. Interestingly, the frequency of lymph node T cells that stained positively for CD25 was similar in the recipients of 25^+ and 25^- RB^{low} cells (Fig. 5). In the spleen, the frequency of $CD25^+$ cells was 13.9% (SEM = 3.0; $n = 8$) within the group that received 25^- RB^{low} cells compared with 19.4% (±1.1; $n = 4$) in the recipients of 25^+ RB^{low} cells. The values observed were independent of the state of health of the mice. In contrast, transfers of naive RB^{high} cells reconstituted the CD25 compartment to a lower extent (in the spleen 5.6% (±2.0); $n = 5$).

To assess whether the expression of CD25 on originally $CD25^+$ cells is autonomously regulated within the population or requires the presence of other T cells in the recipient, we also analyzed the recipients coinjected with mixed CD4 T cell subsets, described in Fig. 2, for expression of the CD25 marker. Originally RB^{high} T cells showed similar frequencies of CD25-expressing cells in mesenteric lymph nodes regardless of the presence of other T cells in the injected recipients (5.8% (±1.2; $n = 7$) in the presence of 25^- RB^{low} cells and 8.5% (±3.0; $n = 6$) in the presence of 25^+ RB^{low} T cells). The frequency of CD25-expressing cells in the originally 25^- RB^{low} population was modestly increased by the presence of RB^{high} cells in mesenteric lymph nodes, but was within the same range as in animals injected with 25^- RB^{low} cells alone (25^- RB^{low} alone, 18.9% (±2.0; $n = 8$); 25^- RB^{low} in coinjections, 22.5% (±3.2; $n = 7$); Fig. 5). In the presence of RB^{high} cells, the donor $CD25^+$ population consisted mostly of $CD25^+$ cells in the mesenteric lymph nodes (70.3% (±3.6; $n = 6$) compared with 21.9% (±4.1; $n = 4$) in recipients of $CD25^+$ cells alone). These findings were similar in spleen and pooled axillary and inguinal lymph nodes.

We conclude that for the majority of transferred $CD25^+$ CD4 T cells, the expression of the CD25 molecule requires the presence of other CD4 T cells. In addition, the 25^- RB^{low} population can generate higher frequencies of $CD25^+$ cells than RB^{high} T cells upon transfer.

$CD25^+$ $CD45RB^{low}$ CD4 T cells from IL-10° mice cannot efficiently regulate peripheral CD4 T cell numbers

Administration of rIL-10 leads to decreased numbers of splenic T cells recovered from mice injected with RB^{high} cells (17), and IL-10° mice develop, apart from IBD, splenomegaly (33). Given these data, we hypothesized that IL-10 plays a role not only in the protection from disease, but also in regulation of the expansion of CD4 T cells. Earlier studies have shown that IL-10° mice at 6 wk of age contain normal numbers of thymocytes and splenic T cells (19). To confirm and extend these findings, we analyzed the thymus, spleen, lymph nodes, blood, and intestine of 6-wk-old IL-10° and wild-type (wt) mice for CD4 and CD8 T cells. Indeed, IL-10° T cells were indistinguishable from wt mice with regard to numbers and expression of CD45RB, CD25, CD38, CD69, and CD44, including the presence of $CD25^+$ $CD4^+CD8^-$ thymocytes (data not shown), suggesting that the development of both CD4 and CD8 T cells is not strongly affected by the lack of IL-10.

To address the question of whether IL-10 is necessary for efficient control of the size of the activated T cell pool, we performed the same transfer experiments described above with donor cells from healthy IL-10° mice bred onto the C57BL/6 background. Transfer of 3×10^5 sorted RB^{high} or 25^- RB^{low} cells from IL-10° donors induced wasting in all RAG-2° recipients, suggesting that the regulatory T cells in the 25^- RB^{low} population are IL-10 dependent. The wasting in mice injected with IL-10° 25^- RB^{low} cells developed somewhat faster than that in hosts of IL-10° RB^{high} T cells (Fig. 6*A*). Both populations expanded to a similar level (Figs. 6*C* and 1*D*), with a comparable organ distribution of the recovered T cells (Table III) as the corresponding populations from wt animals.

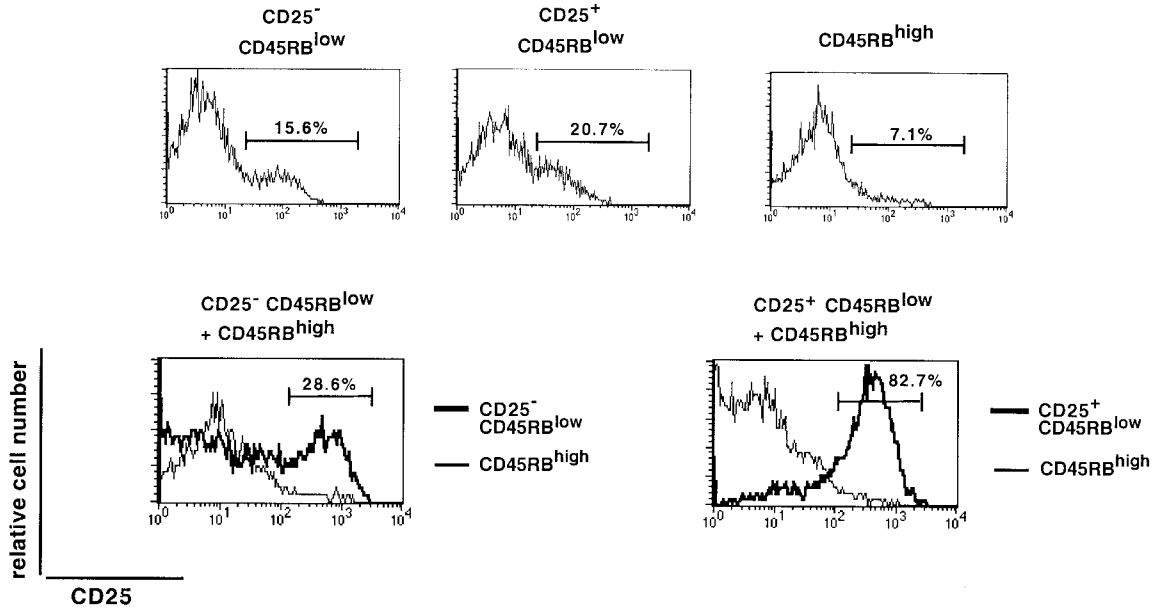

FIGURE 5. Frequency of CD25-expressing cells in recipients of distinct CD4 T cell subpopulations. At sacrifice, the mice described in Figs. 1 and 2 were analyzed for expression of CD25. Flow cytometric analysis for the expression of CD25 on gated donor $CD4^+$ cells from mesenteric lymph nodes in the indicated groups of recipients is shown. $CD4^+$ cells from different origins were identified by the expression of different Ly5 isoforms. Analysis of spleen and that of pooled axillary and inguinal lymph nodes yielded similar profiles.

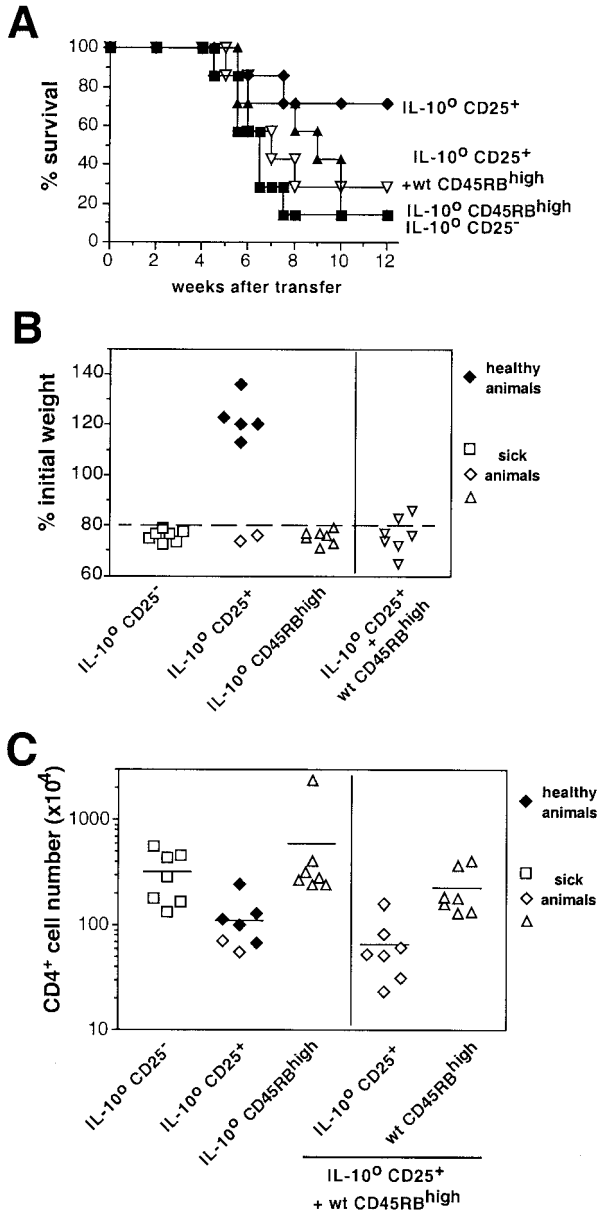

FIGURE 6. IL-10°CD25$^+$ CD45RB^{low} CD4 T cells do not efficiently regulate peripheral CD4 T cell numbers. C57BL/6 RAG-2° recipients were independently injected with 3×10^5 sorted CD4$^+$ cell subsets from IL-10°C57BL/6 donors as described in Fig. 1. As indicated, another group of recipients was injected with 3×10^5 CD25$^+$ T cells together with 3×10^5 wt CD45RB^{high} T cells. CD25$^+$ and CD25$^-$ CD4 cells were sorted from the CD45RB^{low} population. *A*, The time point of sacrifice for each recipient (seven recipients per group), either 12–14 wk after transfer or when they lost at least 20% of their original weight. *B*, Body weight of the recipients at the time of sacrifice. Sick animals were defined by weight loss below their initial weight, which was associated with diarrhea and a markedly enlarged colon. *C*, Sum of the number of CD4$^+$ cells in spleen; axillary, inguinal, and mesenteric lymph nodes; blood (assuming 3 ml of blood per animal); and intestine. Differentiation of the origin of the cells in coinjected recipients was achieved by using Ly5.1 congenic markers. The data are pooled from two or three independent experiments per group.

In the group of mice transferred with 3×10^5 IL-10° 25$^+$ RB^{low} cells, five of seven animals remained healthy for 12–14 wk (Fig. 6*B*). From these mice, on the average, 1.1×10^6 CD4 T cells could be recovered, representing 3-fold more than from animals that received wt 25$^+$ T cells ($p < 0.02$; Figs. 6*C* and 1*D*). This was largely due to increased cell numbers in spleen and blood of the recipients (Table III), and IL-10° 25$^+$ RB^{low} cells were found in the intestine only in very low numbers. The difference in cell recovery was not due to an increased survival of IL-10°CD4 T cells, since similar numbers were obtained 2 days after transfer into RAG-2° recipients compared with the corresponding wt population (data not shown). Furthermore, the frequency of CD25-expressing cells among the recovered IL-10°CD4 T cells was for all populations indistinguishable from that of the corresponding wt CD4 T cells (data not shown).

Consistent with previous results (22), when 3×10^5 IL-10° 25$^+$ RB^{low} cells were injected together with 3×10^5 wt RB^{high} cells, the IL-10°CD25$^+$ T cells could not prevent wasting in the RAG-2° hosts (Fig. 6, *A* and *B*). The numbers of cells recovered from mice coinjected with IL-10° 25$^+$ RB^{low} T cells and wt RB^{high} cells were not significantly different from those recovered from recipients of either population alone (Figs. 6*C* and 1*D*). Interestingly, the expression of CD25 as well as the organ distribution of the coinjected populations were very similar to those in the recipients of wt T cells (data not shown and Table III).

As shown above, regulation of the peripheral expansion of RB^{high} by 25$^+$ RB^{low} cells was already effective by 12 days after transfer. We ascertained the lack of regulatory activity of IL-10° T cells by coinjecting normal CD25$^-$ cells with IL-10°CD25$^+$ T cells at different ratios into RAG-2° recipients. As shown in Fig. 7, IL-10°, but not wt CD25$^+$, cells showed a complete absence of regulatory effect on the expansion of CD25-depleted CD4 T cells. Even at a ratio of six IL-10°CD25$^+$ T cells to one wt CD25$^-$ cells, no signs of inhibitory activity were detected.

Taken together, the data demonstrate that 25$^+$ RB^{low} cells from IL-10° mice are unable to control the accumulation of peripheral CD4 T cells. Moreover, it was shown previously that normal animals contain potentially harmful T cells in the CD45RB^{low} population (10). Thus, although it is possible that IL-10° animals contain higher frequencies of potentially disease-inducing activated CD4 T cells, the data allow the possibility that the disease inhibitory activity of 25$^-$ RB^{low} T cells is also IL-10 dependent.

Discussion

Regulatory T cells protect from autoimmune diseases and IBD. Also, T cells contained in the CD45RB^{low} CD4 population control the size of the peripheral CD4 T cell compartment (10). In the present study we characterize two different subpopulations of CD4 T cells in their capacity to both prevent T cell-induced wasting disease and control peripheral CD4 T cell homeostasis. We show, first, that CD25$^+$ CD45RB^{low} CD4 T cells, which prevent the onset of wasting, contribute to the regulation of peripheral T cell numbers. In contrast, CD25$^-$ CD45RB^{low} CD4 T cells, which also prevent wasting, do not contribute significantly to the regulation of CD4 T cell homeostasis.

We conclude that control of CD4 T cell peripheral expansion and disease prevention are largely independent processes. Furthermore, we show that the mechanism underlying the regulation of the size of the peripheral T cell compartment is IL-10 dependent. Our results also provide the first description of the population dynamics of CD25$^+$ T cells upon in vivo transfer. They establish that although CD25$^+$ T cells reach a homeostatic equilibrium at low cell numbers, a fraction of these cells has a high potential of expansion.

CD25$^+$ CD4 T cells were described to contain regulatory CD4 T cells in several experimental systems, including the one used here (2, 3, 12, 23, 30, 34). However, there is increasing evidence that regulatory CD4 T cells exist in the CD25$^-$ compartment as well (35, 36). Our data showing the capacity of the CD25$^-$

Table III. *Organ distribution of CD4$^+$ cells in RAG-2° mice reconstituted with CD4 subpopulations from IL-10° donors*[a]

Coinjected Populations	Phenotype of Injected CD4$^+$ Subpopulations	No. of Animals	No. of Recovered CD4$^+$ Cells ($\times 10^4$)[b]			
			Spleen	Lymph Nodes[c]	Blood[d]	Intestine
	IL-10° CD25$^-$ CD45RB^{low}	7	138.0 (±49.6)	24.5 (±6.3)	27.4 (±9.8)	79.6 (±36.2)
	IL-10° CD25$^+$ CD45RB^{low}	7	78.0 (±23.5)	8.8 (±2.5)	8.1 (±2.2)	1.5 (±0.3)
	IL-10° CD45RB^{high}	7	102.2 (±13.5)	39.7 (±8.3)	34.4 (±2.7)	60.3 (±14.1)[e]
IL-10° CD25$^+$ + wt CD45RB^{high}	IL-10° CD25$^+$ CD45RB^{low}	7	41.0 (±12.8)	19.8 (±6.3)	1.3 (±0.2)	1.9 (±1.4)
	wt CD45RB^{high}		85.3 (±36.8)	30.4 (±7.3)	20.9 (±5.4)	48.1 (±21.7)

[a] RAG-2° recipients were injected with 3×10^5 FACS-sorted CD4 T cells from IL-10° donors. In addition, 3×10^5 IL-10° CD25$^+$ CD4 T cells were also injected with 3×10^5 wt CD45RB^{high} CD4 T cells. Differentiation of the origin of the cells was achieved by using Ly5-congenic donors. The animals were analyzed for the presence of CD4$^+$ cells in the indicated organs after 12–14 wk or when they dropped below 80% of their starting weight.

[b] Average ± SEM.

[c] Axillary, inguinal, and mesenteric lymph nodes.

[d] Data are expressed as cell number per milliliter of blood.

[e] $n = 6$; one animal, excluded from these data, contained more than 2000×10^4 CD4$^+$ cells in the intestine. Including this animal results in an average CD4$^+$ cell number of 352.1 (±292) $\times 10^4$ in the intestine in this group.

CD45RB^{low} CD4 T cells to protect from wasting disease are well in line with a recent report (23) in which a similar reduction in the incidence of CD4 T cell-induced IBD was observed at a comparable CD25$^-$/RB^{high} T cell ratio.

This observation raises the question of the relationship between CD25$^+$ and CD25$^-$ regulatory CD4 T cells. At this point we cannot exclude that the two subsets differ in their development, function, and/or specificity, although this is not very likely. First, the expression of CD25 on different CD4 T cell subsets is not stable after transfer into alymphoid hosts, and in the case of CD25$^+$ T cells it is dependent on the presence of other CD4 T cells. It is thus possible that in normal animals CD25 expression is dynamic and, therefore, the marker is not identifying the entire pool of regulatory T cells. Secondly, both CD25$^+$ and CD25$^-$ regulatory T cells depend on IL-10 for efficient disease protection. Furthermore, CD25$^-$ regulatory T cells do not compensate for a block of the function of CD25$^+$ regulatory T cells when the protective activity of total CD45RB^{low} T cells is inhibited by anti-TGF-β or anti-CTLA-4 Ab treatment (20, 23).

The dissociation between protection of disease and systemic (and local) regulation of CD4 T cell numbers observed in our studies indicates that both processes are to a large extent independently regulated. This may be the result of a quantitative difference in the number of regulatory CD4 T cells required to control both processes. Disease protection may require lower numbers of regulatory T cells and/or rely on the presence of appropriate TCR specificities in the pool of regulatory T cells. Thus, it can be effective even in the absence of efficient growth control. The lack of appropriate specificities would also explain why the CD25$^+$ CD4 population did not confer protection from wasting in some cases, while showing a quite efficient growth inhibitory activity on other CD4 T cells in the sick recipients. Similar observations were described in other systems: tolerance can be ensured when T cells expand (37), and differentiation can take place in the absence of overt proliferation of T cells (38, 39).

Taken together, it is possible that regulatory CD25$^-$ T cells are descendants of thymic regulatory CD25$^+$ T cells (34, 35) and represent an alternative state of the same functional pool of peripheral regulatory T cells. CD25$^+$ CD4 T cells might be enriched for regulatory T cells simply because they are activated (effector state), but regulatory T cells might become CD25$^-$ T cells in the absence of the appropriate stimuli (memory state).

In contrast to our previous report (10), we could now recover sizable numbers of CD4 T cells from the intestines of healthy recipients. Moreover, similar numbers of intestinal T cells were observed between healthy and sick recipients within the same experimental group. However, this appears to represent a different organ distribution of the cells rather than a higher level of T cell expansion, because the differences in total cell numbers from recipients of CD45RB^{low} T cells compared with recipients of CD45RB^{high} T cells was in this study very similar to what we reported previously. This argues for a systemic regulation of peripheral CD4 T cell numbers and not for a compartmentalized control in individual organs. The increased frequency of intestinal CD4 T cells reported here could perhaps reflect a subclinical state of inflammation in these overall healthy mice due to an unbalanced ratio of regulatory to target CD4 T cells. Nevertheless, a >3-fold reduction in the number of intestinal T cells belonging to the transferred CD45RB^{high} CD4 T cell population was observed in coinjections with the CD25$^+$ CD45RB^{low} T cells. This is consistent with our previous report, namely that regulatory CD4 T cells inhibit the accumulation of CD4 T cells in the intestine. The observation that similar T cell numbers are scored in the intestines of sick and healthy animals reinforces the conclusion that T cell expansion and incidence of disease are not directly linked.

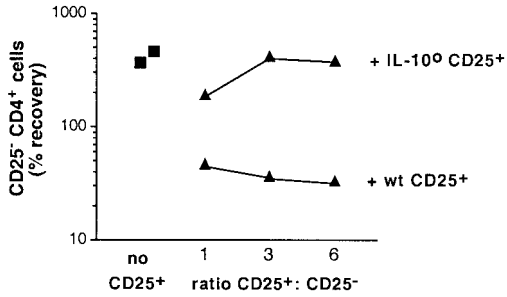

FIGURE 7. High numbers of IL-10°CD25$^+$ CD4 T cells do not show growth inhibitory activity. CD4 T cells from wt or IL-10° donors were separated by two consecutive rounds of magnetic bead purification into CD25$^+$ and CD25$^-$ subsets. CD25$^-$ CD4 T cells (9×10^5; <1% CD25$^+$ cells) from wt animals were then injected into RAG-2° hosts, either alone or together with wt CD25$^+$ (>93% pure) or IL-10°CD25$^+$ CD4 T cells (>92% pure) at the indicated ratios. Eleven days after transfer the recipients were analyzed for CD4$^+$ cells in spleen; axillary, inguinal, and mesenteric lymph nodes; blood (assuming 3 ml of blood per animal); and intestine. The resulting numbers are expressed as a percentage of the injected cell numbers. The symbols show individual mice from the same experiment. ■, wt CD25$^-$ CD4 T cells injected alone; ▲, CD25$^-$ CD4 T cells injected with the indicated CD25$^+$ CD4 T cell population. Differentiation of the origin of the cells was achieved using Thy1.1 and/or Ly5.1 congenic markers.

Our studies also assessed the proliferative potential and the homeostatic equilibrium of peripheral $CD25^+$ CD4 T cells. The idea has been that regulatory T cells have a limited capacity of expansion, perhaps as a result of their own growth inhibitory activity. This is in line with the inability of these cells to proliferate in vitro upon stimulation unless exogenous IL-2 is added. Here we provide evidence that a fraction of $CD25^+$ $CD45RB^{low}$ cells is capable of considerable in vivo proliferation despite the fact that the population reaches a homeostatic equilibrium at low cell numbers. The present data do not provide information on the rate of apoptosis occurring after each round of division, but extensive apoptosis during the expansion process will only increase the number of cell divisions required to account for the observed cell numbers.

The reasons why the homeostatic equilibrium of $CD25^+$ $CD45RB^{low}$ T cells is reached at low cell numbers are nevertheless unclear at this point. It could be that these cells are driven and/or regulated by different growth factors or have limited functional niches compared with the other CD4 T cell populations.

Asseman et al. (22) demonstrated that the IBD protective function of regulatory CD4 T cells is IL-10 dependent. The lack of efficient growth inhibitory activity of $CD25^+$ T cells from IL-10° mice reveals a role for this IL in peripheral T cell homeostasis. In the results presented here $CD25^+$ T cells from IL-10° mice showed many characteristics of wt $CD25^+$ CD4 T cells, and most recipients of IL-10°$CD25^+$ T cells remained healthy, although this subset contained potentially aggressive T cells. This suggests that the $CD25^+$ pool of IL-10°CD4 T cells, although not homogeneous, is highly enriched for cells of the regulatory lineage, which, in the absence of IL-10, have a higher potential of expansion.

Other groups reported a linkage between the susceptibility to autoimmune diseases and the balance between IL-12 and IL-10 as well as a role for IL-12 in CD4 T cell expansion (40, 41). It is thus possible to envisage that IL-10 produced by regulatory T cells leads to down-regulation of IL-12 production by APC, resulting in decreased levels of IL-2 and, in turn, restricted CD4 T cell expansion. Our data show that regulatory $CD25^+$ T cells prevent extensive T cell expansion and do not seem to interfere significantly with the activation of naive T cells in the recipient.

The onset of IBD and splenomegaly in IL-10° mice occurs relatively late in life compared with other situations in which deregulation of peripheral T cell homeostasis is already apparent 3–4 wk after birth. This strongly suggests that factors other than IL-10 are involved in the regulation of peripheral T cell numbers. Indeed, spontaneous autoimmune disease and disruption of T cell homeostasis were recently described in mice transgenic for a T cell-targeted dominant negative TGF-β receptor (42, 43). It is worth pointing out, however, that whatever the cellular interactions or mechanisms that delay the development of disease in IL-10° mice, they are disrupted in the transfer experiments presented here. Thus, further studies are needed to dissect the dependence of T cell homeostasis from cytokines produced by regulatory T cells.

Acknowledgments

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