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Afonso R. M. Almeida; ... et. al

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# Homeostasis of Peripheral CD4<sup>+</sup> T Cells: IL-2R $\alpha$ and IL-2 Shape a Population of Regulatory Cells That Controls CD4<sup>+</sup> T Cell Numbers<sup>1</sup>

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We show that the lymphoid hyperplasia observed in IL-2R $\alpha$ - and IL-2-deficient mice is due to the lack of a population of regulatory cells essential for CD4 T cell homeostasis. In chimeras reconstituted with bone marrow cells from IL-2R $\alpha$ -deficient donors, restitution of a population of CD25<sup>+</sup>CD4<sup>+</sup> T cells prevents the chaotic accumulation of lymphoid cells, and rescues the mice from autoimmune disease and death. The reintroduction of IL-2-producing cells in IL-2-deficient chimeras establishes a population of CD25<sup>+</sup>CD4<sup>+</sup> T cells, and restores the peripheral lymphoid compartments to normal. The CD25<sup>+</sup>CD4<sup>+</sup> T cells regulated selectively the number of naive CD4<sup>+</sup> T cells transferred into T cell-deficient hosts. The CD25<sup>+</sup>CD4<sup>+</sup>/naive CD4 T cell ratio and the sequence of cell transfer determines the homeostatic plateau of CD4<sup>+</sup> T cells. Overall, our findings demonstrate that IL-2R $\alpha$  is an absolute requirement for the development of the regulatory CD25<sup>+</sup>CD4<sup>+</sup> T cells that control peripheral CD4 T cell homeostasis, while IL-2 is required for establishing a sizeable population of these cells in the peripheral pools. *The Journal of Immunology*, 2002, 169: 4850–4860.

Several different lines of evidence demonstrate that thymus T cell production does not determine the number of peripheral T cells. First, in the young adult mouse, T cell production in the thymus largely exceeds the number of cells required to replenish the peripheral T cell pools. Mice manipulated to have reduced rates of thymus T cell production can attain normal peripheral T cell numbers (1). Secondly, in mice grafted with multiple thymuses, the increased thymus mass and T lymphocyte production does not lead to the proportional increase of the peripheral T cell pool (2, 3). The number of T cells is also not limited by the peripheral T cell production capacity. Peripheral T cells in absence of the thymus in thymectomized hosts (4) or when transferred into T cell-deficient hosts are capable of considerable expansion (5–7). In a normal mouse, there are mechanisms that control both T cell survival and division in the peripheral pools and keep T cell numbers constant. It has been proposed that competition for resources or complex cell interactions play a role in lymphocyte homeostasis (8, 9). However, the mechanisms involved remain elusive.

Mutant IL-2R $\alpha$ <sup>-/-</sup> mice represent a paradigm for perturbed lymphocyte homeostasis (10). They develop massive enlargement of peripheral lymphoid organs associated with T and B cell ex-

pansion and autoimmune disease (10), indicating that IL-2R $\alpha$  is essential for the control of the size of the peripheral lymphoid compartment. It was generally believed that the defect in IL-2R $\alpha$ <sup>-/-</sup> mice was cell autonomous and that IL-2R $\alpha$  regulated the balance between clonal expansion and cell death following lymphocyte activation (10, 11). Thus, in the absence of the negative signals mediated by IL-2R $\alpha$ , T cells would undergo uncontrolled expansion (10). However, it was recently shown that when placed in a normal environment, TCR transgenic (Tg)<sup>3</sup> IL-2R $\alpha$ <sup>-/-</sup> cells exhibited normal clonal contraction after Ag-induced expansion (12), suggesting that activation-induced cell death (AICD) is kept and that IL-2R $\alpha$  signals could also control bystander T cell activation (12). Alternatively, IL-2R $\alpha$  could be required for the development and/or the function of a subpopulation of T cells capable of regulating peripheral T cell homeostasis. Different lines of evidence seem to support this latter alternative. First, recent findings showed that wild-type T cells could control the expansion of IL-2R $\beta$ -deficient T cells in mixed bone marrow (BM) chimeras, a property attributed to a population of cytotoxic CD8 T cells (13). Secondly, previous studies have also shown that Ag-induced expansion of TCR Tg IL-2-deficient T cells could be controlled by CD25<sup>+</sup> T cells (14). Finally, results indicate that regulatory CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells limit naive CD4 T cell expansion and suggest that they may play a role in the control of peripheral T cell numbers (15).

We decided to investigate if populations of regulatory CD25<sup>+</sup>CD4<sup>+</sup> T cells (16) could prevent the chaotic lymphocyte accumulation in IL-2R $\alpha$ <sup>-/-</sup> mice and control the expansion of peripheral naive CD4 T cells. We found that CD25<sup>+</sup>CD4<sup>+</sup> T cells could indeed control peripheral T cell accumulation and composition in mouse chimeras reconstituted with BM cells from IL-2R $\alpha$ <sup>-/-</sup> mice and rescued these mice from death. Similarly, recombination-activating gene (Rag)2<sup>-/-</sup> chimeras reconstituted with a mixture of BM cells from IL-2R $\alpha$  and IL-2-deficient donors

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<sup>3</sup> Abbreviations used in this paper: Tg, transgenic; AICD, activation-induced cell death; BM, bone marrow; Rag, recombination-activating gene; LN, lymph node; Treg, T regulatory; IBD, inflammatory bowel disease.

remained healthy, and the number and distribution of CD25<sup>+</sup> and CD25<sup>-</sup>CD4<sup>+</sup> T cells was as in normal mice. To relate these effects to homeostatic control of the CD4 peripheral T cell pool, we examined the selectivity and quantitative requirements ruling the expansion of naive CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup> and CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells transferred into CD3ε<sup>-/-</sup> T cell-deficient host mice (17). We found that the two cell populations show different homeostatic plateaus and that CD25<sup>+</sup>CD4<sup>+</sup> T cells can selectively inhibit the peripheral expansion of the naive CD4 T cells, but not CD8 T cells in a dose-dependent manner.

## Materials and Methods

### Mice

C57BL/6.Ly5.2 mice from Iffa-Credo (L'Arbresle, France), B6.CD3ε<sup>-/-</sup> (17), B6.IL-2Rα<sup>-/-</sup> (10), B6.TCRα<sup>-/-</sup> (18), and C57BL/6.Ly5.1 mice from the Centre de Développement des Techniques Avancées-Centre National de la Recherche Scientifique (Orléans, France), B6.IL-10<sup>-/-</sup> (19) from The Jackson Laboratory (Bar Harbor, ME) and B6.IL-2<sup>-/-</sup> (20) from our breeding facilities or from Dr. A. Schimpl (Institute for Virology and Immunobiology, University of Würzburg, Germany) were matched for age (6–12 wk) and sex.

### Cell sorting and cell transfers

Lymph node (LN) cells from the Ly5.2 and Ly5.1 donor mice were first enriched for CD4<sup>+</sup> T cells by negative selection using a Dynal MPC6 MACS (Dynal Biotech, Great Neck, NY). Briefly, cells were incubated with a mixture of rat Abs directed to mouse B220 (RA3-6B2), Mac1 (CD11b), and CD8α (53–6.7), all from BD PharMingen (San Diego, CA), followed by sheep anti-rat Ig-coated Dynabeads (Dynal Biotech). After removing the positive fraction, >90% of the remaining population was CD4<sup>+</sup>. These cells labeled with the appropriate combinations of anti-CD4 (L3T4/RM4-5), anti-CD45RB, and anti-CD25 (784), Abs were sorted on a FACStar<sup>Plus</sup> (BD Biosciences, Mountain View, CA). The purity of the sorted CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup> and CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> populations varied from 96–99.9%.

Intact nonirradiated B6.CD3ε<sup>-/-</sup> (17) hosts were injected i.v. with the purified CD4 T cell populations alone or mixed at different cell ratios. By using mice differing by Ly5 allotypes, we were able to discriminate the cells originating from the different donor mice. Host mice were sacrificed at different time intervals after cell transfer. Spleen, inguinal, and mesenteric LN cell suspensions were prepared and the number and phenotype of the cells from each donor population evaluated. The total peripheral T cells shown in the results represent the number of cells recovered in the host's spleen added to twice the number of cells recovered from the host's inguinal and mesenteric LNs.

### Labeling with CFSE

Cells were labeled with CFSE as described (21). Briefly, sorted CD4<sup>+</sup> T cells (10<sup>7</sup>/ml) were incubated for 10 min at 37°C with CFSE (10 μM).

### BM chimeras

Host 8-wk-old Rag2<sup>-/-</sup> B6 mice were lethally irradiated (900 rad) with a <sup>137</sup>Ce source and received i.v. 2–4 × 10<sup>6</sup> T cell-depleted BM cells from different donor mice, mixed at different ratios. T cell depletion (<0.1%) was done in a Dynal MPC6 MACS after incubating the BM cells with anti-CD4, anti-CD8, and anti-CD3 biotinylated Abs followed by streptavidin-coated Dynabeads. By using donor and host mice that differ according to Ly5 allotype markers, we were able to discriminate between the T cells originating from the different donors.

### Flow cytometry analysis

The following mAbs were used: anti-CD3ε (145-2C11), anti-CD4 (L3T4/RM4-5), anti-CD69 (H1.2F3), anti-CD25 (784), anti-CD45RB, anti-CD24/HSA (M1/69), and anti-TCRβ (H57) from BD PharMingen, and anti-CD44 (IM781) and anti-CD62L (MEL14) from Caltag Laboratories (San Francisco, CA). Cell surface four-color staining was performed with the appropriate combinations of FITC, PE, tricolor, PerCP, biotin, and APC-coupled Abs. Biotin-coupled Abs were secondary labeled with APC-, tricolor-, (Caltag Laboratories) or PerCP-coupled (BD Biosciences) streptavidin. Dead cells were excluded during analysis according to their light-scattering characteristics. All acquisitions and data analysis were performed with a FACSCalibur (BD Biosciences) interfaced to the Macintosh CellQuest software.

### Statistical analysis

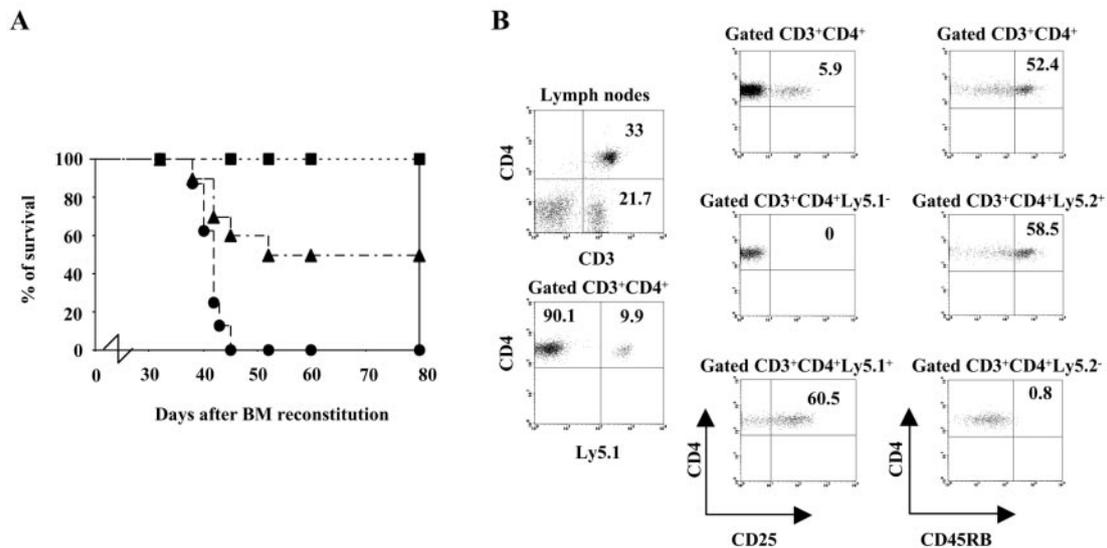
Sample means were compared using the unpaired Student's *t* test. In case the variances of the two samples were considerably different, the data were log-transformed to see if the variances become more similar. If so, the unpaired *t* test was applied to the log-transformed data. Otherwise, Satterthwaite's approximation was applied. Sample means were considered significantly different at *p* < 0.05.

## Results

### CD25<sup>+</sup>CD4<sup>+</sup> T cells inhibit CD4 T cells from IL-2Rα<sup>-/-</sup> mice and prevent death of chimeras reconstituted with BM cells from IL-2Rα<sup>-/-</sup> mice

Mutant IL-2Rα<sup>-/-</sup> mice develop massive lymphocyte accumulation and autoimmune disease (10). It was proposed that in the absence of negative signals mediated through IL-2Rα, T cells would undergo uncontrolled expansion suggesting that the IL-2Rα<sup>-/-</sup> defect was cell autonomous (10). We asked if a population of normal T cells could control the chaotic accumulation of T cells in IL-2Rα<sup>-/-</sup> mice. We examined whether mature CD25<sup>+</sup>CD4<sup>+</sup> T cells could alter the number and state of activation of CD4 T cells in B6.Rag2<sup>-/-</sup> chimeras reconstituted with 100% BM cells from B6.IL-2Rα<sup>-/-</sup> donors. Using these BM chimeras rather than intact B6.IL-2Rα<sup>-/-</sup> mice allowed us to increase the number of mice studied. We found that all chimeras injected exclusively with BM cells from B6.IL-2Rα<sup>-/-</sup> donors died within 40–50 days (Fig. 1A), with overt signs of runting, anemia, and in some mice, lymphoid hyperplasia (up to 300 × 10<sup>6</sup> lymphocytes). In contrast, the BM chimeras that received 10<sup>5</sup> CD25<sup>+</sup>CD4<sup>+</sup> T cells from normal B6.Ly5.1 donors 2 wk after BM reconstitution were all alive 6 mo later (Fig. 1A and data not shown). The effects of the CD25<sup>+</sup>CD4<sup>+</sup> T cells were time-dependent since only 50% of the chimeras survived if we delayed their transfer to 4 wk after BM injection (Fig. 1A). These differences suggest that either control of T cell numbers is more efficient earlier when the number of peripheral IL-2Rα<sup>-/-</sup> T cells is lower, or it may require the continuous presence of “regulatory” T cells at the onset of T cell production. The surviving chimeras remained healthy, the hematocrit levels were normal (40–45%), and the total number of T cells was similar (47.1 ± 7.7 and 81 ± 9.7 × 10<sup>6</sup> for the two groups of BM chimeras injected with CD25<sup>+</sup>CD4<sup>+</sup> T cells, respectively) to control mice (40–60 × 10<sup>6</sup> CD4 T cells). The composition of the peripheral LN T cells was as in normal mice comprising 5–10% of CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells, all of B6.Ly5.1 origin, and 50–60% of nonactivated CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells all from IL-2Rα<sup>-/-</sup> origin (Fig. 1B). This is in contrast to donor IL-2Rα<sup>-/-</sup> mice, where most (>80%) T cells have an activated phenotype (Ref. 10; data not shown). We should point out that the transfer of up to 2 × 10<sup>5</sup> CD25<sup>-</sup>CD4<sup>+</sup> T cells did not rescue the CD25<sup>-/-</sup> BM chimeras (data not shown).

To test if the production of regulatory T cells by the thymus could also control the chaotic peripheral accumulation of IL-2Rα<sup>-/-</sup> T cells, lethally irradiated lymphopenic B6.Rag2<sup>-/-</sup> mice were reconstituted with a mixture of T cell-depleted BM cells. In brief, 50% of the injected BM cells were from B6.Ly5.2.IL-2Rα<sup>-/-</sup> donors and the remaining 50% from B6.Ly5.2.TCRα<sup>-/-</sup> (unable to generate T cells) and normal B6.Ly5.1 donors mixed at different ratios. By keeping the fraction of cells from IL-2Rα<sup>-/-</sup> donors in the injected BM cohort at 50%, we fixed the rate of production of IL-2Rα<sup>-/-</sup> T cells in all chimeras studied. Thus, the resulting chimeras should all have the same number of peripheral IL-2Rα<sup>-/-</sup> T cells (1). By mixing BM cells from B6 normal and B6.TCRα<sup>-/-</sup> donors, we reduced the number of competent precursors available for thymus colonization and regeneration, as the normal competent precursor cells are diluted among incompetent



**FIGURE 1.** A, Lethally irradiated B6.Rag2<sup>-/-</sup> mice were reconstituted with  $4 \times 10^6$  BM cells from B6.Ly5.2IL-2R $\alpha^{-/-}$  and were left alone (10 mice) or received  $10^5$  CD25<sup>+</sup>CD4<sup>+</sup> T cells from normal B6.Ly5.1 donors 2 (9 mice) or 4 wk (8 mice) after BM reconstitution. Results show the time of survival of the chimeras reconstituted with BM cells from B6.Ly5.2IL-2R $\alpha^{-/-}$  alone (●), injected with CD25<sup>+</sup>CD4<sup>+</sup> T cells from normal B6.Ly5.1 donors 2 (■) or 4 wk (▲) later. B, Phenotypic characterization of the peripheral LN CD4 T cells in a chimera reconstituted with BM cells from B6.Ly5.2IL-2R $\alpha^{-/-}$  that received  $10^5$  CD25<sup>+</sup>CD4<sup>+</sup> T cells from normal B6.Ly5.1 donors 2 wk later. Chimeras were sacrificed for FACS analysis 16–20 wk after BM transfer. Similar results were obtained in the remaining eight mice from the same group.

precursors from the TCR $\alpha^{-/-}$  mutant donors (1). With this strategy, we could evaluate the role of different numbers of normal T cells in the control of a fixed number of IL-2R $\alpha^{-/-}$  T cells. We found that all the chimeras that could only generate IL-2R $\alpha^{-/-}$  T cells, i.e., which received 50% BM cells from B6.IL-2R $\alpha^{-/-}$  donors and 50% of BM cells from B6.TCR $\alpha^{-/-}$  donors, died 40–50 days after reconstitution (Fig. 2A). The presence of 5% cells from normal donors in the thymus of the chimeras was sufficient to rescue 80% of the mice (Fig. 2A). The presence of 10 or 50% of normal cells rescued 100% of the chimeras. In the 50/50 B6.Ly5.2IL-2R $\alpha^{-/-}$ /B6.Ly5.1 chimeras, the representation of the two types of donor cells remained unchanged both in the thymus and in the peripheral T cell pools (data not shown). In the surviving BM chimeras containing a fraction of cells from normal donors, the total number of peripheral T cells and the relative distribution of the CD25<sup>+</sup> and CD25<sup>-</sup> CD4 T cell subsets were as in normal mice (Fig. 2B). Thus, a developing population of normal CD4 T cells controlled the accumulation and state of activation of the IL-2R $\alpha^{-/-}$  T cells. These results show that the lethal accumulation of peripheral T cells in IL-2R $\alpha^{-/-}$  mice is not cell autonomous, but due to the lack of a population of CD4<sup>+</sup> T cells essential for peripheral T cell homeostasis. We demonstrated that expression of the IL-2R $\alpha$  chain is required for the generation of this population of regulatory CD4 T cells.

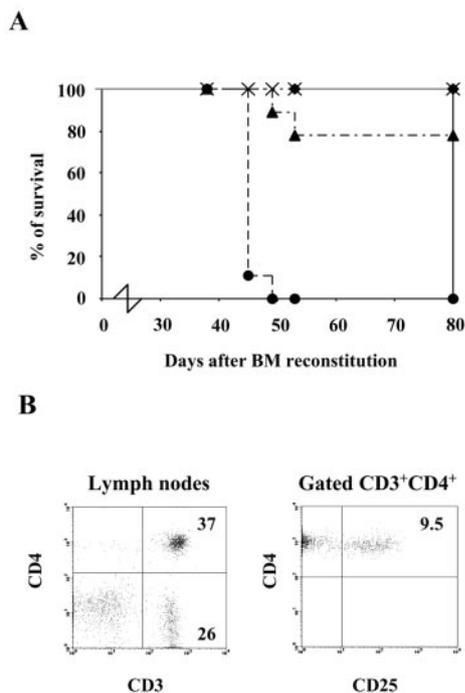
#### Control of T cell numbers in IL-2<sup>-/-</sup>/IL-2R $\alpha^{-/-}$ BM chimeras

With age, IL-2<sup>-/-</sup> mice develop fatal inflammatory bowel disease (IBD) and lymphocyte proliferation (20). The peripheral T cell compartments of these mice show overt signs of T cell activation and lack a well-defined population of CD25<sup>+</sup>, which never exceeds 1–2% of the CD4<sup>+</sup> T cells (14, 22). We investigated whether the CD25<sup>+</sup>CD4<sup>+</sup> T cells from IL-2<sup>-/-</sup> mice could rescue the defects of CD4 T cell homeostasis observed in IL-2R $\alpha^{-/-}$  mice. We reconstituted lethally irradiated lymphopenic B6.Rag2<sup>-/-</sup> mice with a 50/50 mixture of T cell-depleted BM cells from B6.IL-2R $\alpha^{-/-}$  and B6.IL-2<sup>-/-</sup> donors. Control mice received BM cells from either B6.IL-2R $\alpha^{-/-}$  or B6.IL-2<sup>-/-</sup> mice equally mixed at

50/50 with BM cells from B6.TCR $\alpha^{-/-}$  donors. The presence of 50% of BM cells from IL-2<sup>-/-</sup> donors rescued the totality of the IL-2R $\alpha^{-/-}$  BM chimeras (Fig. 3A) and restored the CD4 T cell populations to normal. In the peripheral T cell pools of these chimeras, the number and the distribution of CD25<sup>+</sup> and CD25<sup>-</sup> CD4 T cells was as in normal mice (Fig. 3B). Thus, in presence of IL-2, the hemopoietic precursors from IL-2-deficient donor mice generated a stable population of mature peripheral CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells able to control the homeostasis of the CD4 T cell compartment. Upon secondary transfer, this population of IL-2<sup>-/-</sup>CD25<sup>+</sup>CD4<sup>+</sup> cells was able to rescue B6.IL-2R $\alpha^{-/-}$  chimeras (data not shown). Chimeras injected with BM cells from B6.IL-2<sup>-/-</sup> donors alone show a normal number of CD25<sup>+</sup> and CD25<sup>-</sup> CD4 T cells (data not shown). This finding indicates that in Rag2<sup>-/-</sup> hosts, resident non-T cells can provide a source of endogenous IL-2 (23) sufficient to compensate for the lack of its production by the IL-2<sup>-/-</sup> hemopoietic cells. Overall, these results demonstrate that IL-2 is required for the establishment of a stable population of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells in the peripheral pools. In absence of this, population control of CD4 T cell numbers is lost and the mice develop lymphoid hyperplasia and autoimmune diseases. Overall, these findings indicate that populations of CD25<sup>+</sup> and CD25<sup>-</sup> CD4 T cells may have different homeostatic properties and that they may regulate each other. We decided to investigate this possibility using a cell transfer strategy.

#### Fate of naive CD4<sup>+</sup> and CD25<sup>+</sup>CD4<sup>+</sup> T cells transferred into T cell deficient hosts: different homeostatic plateaus

Peripheral T cells, when transferred into T cell-deficient hosts, are capable of considerable expansion (5–7), but their number is controlled at a homeostatic plateau. To investigate the homeostasis of peripheral CD4<sup>+</sup> T cell subpopulations, different numbers of purified CD4<sup>+</sup> cells, that is, CD45RB<sup>high</sup>CD25<sup>-</sup>, CD45RB<sup>low</sup>CD25<sup>-</sup>, and CD45RB<sup>low</sup>CD25<sup>+</sup> cells were i.v. transferred into syngeneic CD3 $\epsilon^{-/-}$  T cell-deficient hosts. In hosts receiving as few as  $5 \times 10^3$  and as many as  $10^5$  cells, CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells (from now on referred to as naive CD4) expanded to reach stable equilibrium at

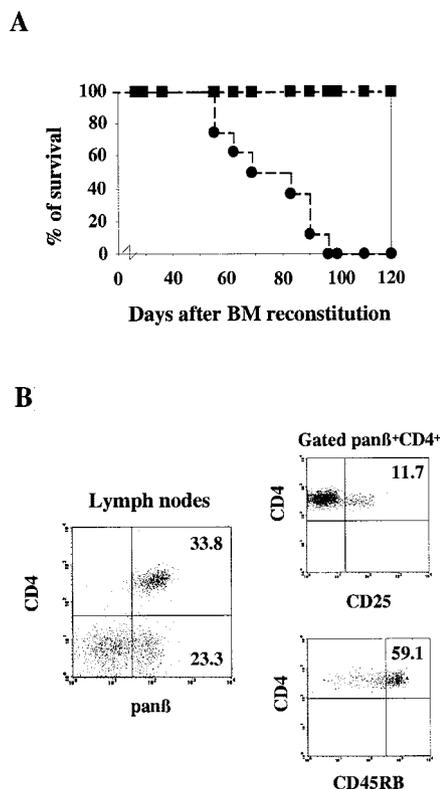


**FIGURE 2.** A, Lethally irradiated B6.Rag2<sup>-/-</sup> mice were reconstituted with  $4 \times 10^6$  cells from a mixture of 50% BM cells from B6.Ly5.2IL-2Rα<sup>-/-</sup> and 50% of BM cells from B6.Ly5.2.TCRα<sup>-/-</sup> and normal B6.Ly5.1 donors, the latter mixed at different ratios. Results show the time of survival of the chimeras reconstituted with 50% BM cells from B6.Ly5.2IL-2Rα<sup>-/-</sup> and 50% of BM cells from B6.Ly5.2.TCRα<sup>-/-</sup> (●), 50% BM from B6.Ly5.2IL-2Rα<sup>-/-</sup>, 45% BM from B6.Ly5.2.TCRα<sup>-/-</sup>, and 5% BM from normal B6.Ly5.1 (▲), 50% BM from B6.Ly5.2IL-2Rα<sup>-/-</sup>, 40% BM from B6.Ly5.2.TCRα<sup>-/-</sup>, and 10% BM from normal B6.Ly5.1 (×), 50% BM from B6.Ly5.2IL-2Rα<sup>-/-</sup>, and 50% BM from normal B6.Ly5.1 (■). Number of mice per group: nine. B, Dot plot shows the frequency of CD25<sup>+</sup>CD4<sup>+</sup> T cells in the LNs of chimeras reconstituted with 50% BM from B6.Ly5.2IL-2Rα<sup>-/-</sup> and 50% BM from normal B6.Ly5.1. Similar results were obtained in the other chimeras.

$\sim 1\text{--}2 \times 10^7$  cells, 10–12 wk after transfer (Fig. 4). In mice injected with the same number of CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells (from now on referred to as CD25<sup>+</sup>CD4<sup>+</sup>), these cells also expanded but reached equilibrium at 10-fold lower values, i.e., at  $1\text{--}2 \times 10^6$  cells/hosts (Fig. 4). Transfer of increasing numbers ( $>10^5$ ) of cells did not modify the final cell recovery (data not shown). These results indicate that naive CD4<sup>+</sup> and CD25<sup>+</sup>CD4<sup>+</sup> T cells are both able to expand and accumulate at the periphery, but their final number is regulated at different homeostatic plateau levels. It should be noted that at later times after transfer, mice injected with naive CD4<sup>+</sup> T cells developed a wasting autoimmune disease and eventually died ( $>15$  wk), while hosts of CD25<sup>+</sup>CD4<sup>+</sup> T cells remained healthy (data not shown). Total nonseparated LN CD4<sup>+</sup> T cells containing 10% CD25<sup>+</sup> cells expanded to a plateau of  $\sim 1\text{--}2 \times 10^7$  cells, but fail to develop signs of wasting disease (data not shown). Activated CD45RB<sup>low</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells expanded to similar plateaus as naive CD4<sup>+</sup> T cells (data not shown).

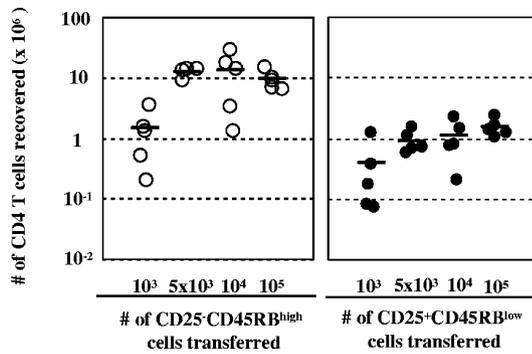
#### Sequential and secondary cell transfers

Cellular competition and the presence or absence of resident T cell populations can alter the peripheral fate of newly arriving thymus emigrants (8, 9, 24). To truly establish the homeostasis of the transferred peripheral T cells, we asked if the presence of a resident T cell population could interfere with the expansion of a sec-



**FIGURE 3.** Lethally irradiated B6.Rag2<sup>-/-</sup> mice were reconstituted with  $\sim 1 \times 10^6$  cells from a mixture of 50% BM cells from B6.IL-2Rα<sup>-/-</sup> and 50% of BM cells from B6.IL-2<sup>-/-</sup>. Control chimeras received a mixture of 50% BM cells from B6.IL-2Rα<sup>-/-</sup> or B6.IL-2<sup>-/-</sup> and 50% of BM cells from B6.Ly5.2.TCRα<sup>-/-</sup>. A, Results show the time of survival of the chimeras reconstituted with 50% BM cells from B6.IL-2Rα<sup>-/-</sup> and 50% of BM cells from B6.IL-2<sup>-/-</sup> (■), 50% BM cells from B6.IL-2Rα<sup>-/-</sup> and 50% of BM cells from B6.TCRα<sup>-/-</sup> (●), 50% BM cells from B6.IL-2<sup>-/-</sup> and 50% of BM cells from B6.TCRα<sup>-/-</sup> (data not shown). Note that the transfer of lower numbers of precursor cells delayed lymphoid reconstitution and death (compare to Fig. 2A) of the host mice injected with BM cells from B6.IL-2Rα<sup>-/-</sup> and from B6.TCRα<sup>-/-</sup> donors. Number of mice/group: nine. B, Phenotypic characterization of the peripheral LN CD4 T cells in a chimera reconstituted with BM cells from B6.IL-2Rα<sup>-/-</sup> and B6.IL-2<sup>-/-</sup> donors. Similar results were obtained in the remaining mice from the same group.

ond newly injected cell population or whether the injection of a new population could modify the fate of a resident population. We “parked”  $5 \times 10^4$  Ly5.1 naive CD4<sup>+</sup> T cells in different hosts. Seven weeks later, each host received the same number of a second population of Ly5.2 naive CD4<sup>+</sup> or CD25<sup>+</sup>CD4<sup>+</sup> T cells. Age-matched control mice received either the first or the second population alone. We sacrificed the mice at 7 (before the second injection) or 14 wk after the first injection. After transfer, naive T cells acquired a CD45RB<sup>low</sup> activated/memory phenotype, but only a few (1–2%) became CD25<sup>low</sup> (data not shown). In mice injected sequentially with two populations of naive CD4<sup>+</sup> T cells, the expansion of both populations was limited through competition and they shared the peripheral compartment of the host (Fig. 5A). The total T cell recovery was the same as in mice injected with either population alone ( $\sim 2 \times 10^7$ ). The transfer of  $5 \times 10^4$  CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> cells into mice injected 7 wk before with CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup> cells suppressed significantly ( $p < 0.001$ ) further expansion of the established  $5 \times 10^6$  CD45RB<sup>high</sup>CD25<sup>-</sup>CD4<sup>+</sup>-derived T cell population (Fig. 5B). The total T cell recovery diminishes accordingly. The number of



**FIGURE 4.** Expansion capacity of CD4 T cell populations of C57BL/6 mice. Different numbers of purified naive CD4<sup>+</sup> and CD25<sup>+</sup>CD4<sup>+</sup> T cells (1,000–100,000) were transferred into CD3ε<sup>-/-</sup> mice. The results show the number of CD4 T cells recovered 10–11 wk after transfer in the spleen and LN of each individual host (the mean value is also shown).

cells recovered from the second population of CD45RB<sup>low</sup> CD25<sup>+</sup>CD4<sup>+</sup> T cells did not change. These results show that a limited number ( $5 \times 10^4$ ) of newly transferred CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T can suppress the expansion of an abundant ( $5 \times 10^6$ ) population of resident CD4<sup>+</sup> T cells.

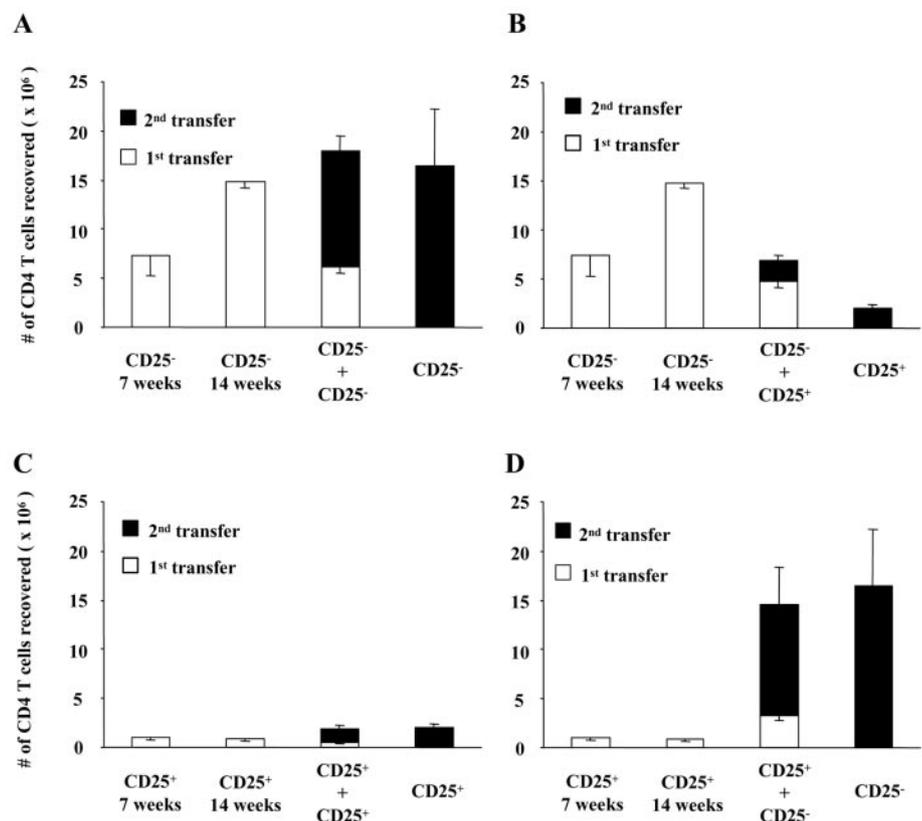
We also parked  $5 \times 10^4$  Ly5.1 CD25<sup>+</sup>CD4<sup>+</sup> T cells. Seven weeks later, each host received  $5 \times 10^4$  Ly5.2 naive CD4<sup>+</sup> or CD25<sup>+</sup>CD4<sup>+</sup> T cells. The transfer of a second population of CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells did not significantly modify the number of the established cells. The resident cells were able to persist and the new cells were able to accumulate as in noninjected hosts (Fig. 5C). In the mice hosting the first population of CD25<sup>+</sup>CD4<sup>+</sup> T cells, newly transferred naive CD4<sup>+</sup> T cells expanded and induced a 3- to 4-fold increase ( $p < 0.01$ ) in the number of resident CD4 T cells from CD25<sup>+</sup>CD4<sup>+</sup> origin (Fig.

5D). These results show that newly injected naive CD4<sup>+</sup> T cells helped the growth of the progeny of CD25<sup>+</sup>CD4<sup>+</sup> T cells ( $p < 0.01$ ). In contrast, the resident CD25<sup>+</sup>CD4<sup>+</sup> T cell progeny do not significantly ( $p = 0.5$ ) inhibit the growth of newly transferred naive CD4<sup>+</sup> T cells. In hosts that received CD25<sup>+</sup>CD4<sup>+</sup> cells, only ~30% of the recovered T cells remained CD25<sup>+</sup> (data not shown). This could represent true phenotypic changes or the expansion of a few contaminant CD4 T cells in the injected CD25<sup>+</sup> population. We investigated the suppressive capacities of the resident cells that express or not CD25. For this purpose, CD25<sup>+</sup>CD4<sup>+</sup> T cells were parked for 7 wk in host mice. At the end of this time period, CD25<sup>+</sup>CD4<sup>+</sup> T cells and CD45RB<sup>low</sup>CD25<sup>-</sup>CD4<sup>+</sup> T cells derived from the parked population were injected alone or coinjected with naive CD4<sup>+</sup> T cells from different Ly5 donors into secondary CD3ε<sup>-/-</sup> hosts. Although the resorted CD25<sup>+</sup> cells retained the capacity to suppress the growth of naive CD4<sup>+</sup> T cells ( $p < 0.001$ ), the suppressive capacity of the resorted CD25<sup>-</sup> cells was absent or reduced ( $p = 0.21$ ; Fig. 6). These results suggest that the suppressor effects correlate with the surface expression of CD25.

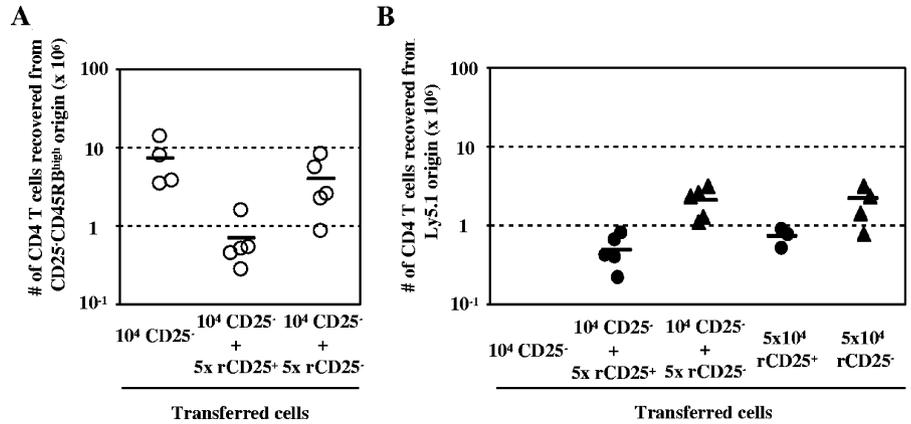
#### CD25<sup>+</sup>CD4<sup>+</sup> T cells inhibit peripheral expansion of naive CD4 T cells

We showed that CD25<sup>+</sup>CD4<sup>+</sup> T cells could control chaotic accumulation of IL-2Rα<sup>-/-</sup> T cells. We decided to investigate whether they could also control the homeostatic plateau of naive CD4 T cells transferred to T cell-deficient hosts. We quantified both the numbers of cells involved and the selectivity of the interactions. We transferred  $10^4$  purified naive Ly5.1 CD4<sup>+</sup> T cells or CD8 T cells alone or coinjected with variable numbers of Ly5.2 CD25<sup>+</sup>CD4<sup>+</sup> T cells, ranging from  $3 \times 10^3$ – $10^5$ , into CD3ε<sup>-/-</sup> hosts (Fig. 7, A and B). We found that the CD25<sup>+</sup>CD4<sup>+</sup> T cells limited the accumulation of naive CD4<sup>+</sup> T cells (15) and that their suppressive effects were dose-dependent (Fig. 7A). Increasing

**FIGURE 5.** Sequential cell transfers. *A*, T cell-deficient mice were injected with  $5 \times 10^4$  naive CD4<sup>+</sup>, and 7 wk later they received  $5 \times 10^4$  naive CD4<sup>+</sup> T cells, which differ in the Ly5 allotype. All mice were sacrificed 14 wk after the first injection. Control mice received either the first (left column in each quadrant) or the second (right column in each quadrant) population alone and were killed 7 wk after transfer. Note that the hosts were age matched, i.e., recipients were injected with the first population at 7 wk of age and received the second population at 14 wk of age, a difference which may explain the greater growth of the second cell population when transferred alone. *B*, As for *A*, except that the mice were injected first with  $5 \times 10^4$  naive CD4<sup>+</sup> and 7 wk later they received  $5 \times 10^4$  CD25<sup>+</sup>CD4<sup>+</sup> T cells. Differences between CD25<sup>-</sup>-derived cells at 14 wk in absence or in presence of V cells were highly significant ( $p < 0.001$ ). *C*, As for *A*, except that the mice were injected first with  $5 \times 10^4$  CD25<sup>+</sup>CD4<sup>+</sup> and later CD25<sup>+</sup>CD4<sup>+</sup> T cells. Results show the mean + SEM (four to five mice per group) of the number of cells recovered from the first (□) or the second (■) injected population. *D*, As for *A*, except that the mice were injected first with  $5 \times 10^4$  CD25<sup>+</sup>CD4<sup>+</sup> and later  $5 \times 10^4$  naive CD4<sup>+</sup> T cells. The use of Ly5 different T cells allows the easy identification of T cells from each donor population.



**FIGURE 6.** Secondary cell transfers:  $10^4$  naive  $CD4^+$  Ly5.2 T cells were transferred alone or coinjected with  $5 \times 10^4$  of either  $CD25^+$  and  $CD25^-$  Ly5.1 T cells recovered from mice injected 7 wk before with  $3 \times 10^4$   $CD25^+CD4^+$  Ly5.1 T cells. Control mice were injected with  $CD25^+$  and  $CD25^-$  Ly5.1 T cells alone. **A**, Results show the total number of  $CD4$  T cells from naive  $CD4^+$  Ly5.2 origin. The mean value is also shown. **B**, Shows the total number of Ly5.1 T cells recovered in mice injected with  $CD25^+$  (●) and  $CD25^-$  (▲) cells recovered from mice injected 7 wk before with  $10^4$   $CD25^+CD4^+$  Ly5.1 T cells alone or coinjected with naive  $CD4^+$  Ly5.2 (A). The mean value is also shown.



numbers of  $CD25^+CD4^+$  T cells progressively suppressed the expansion of the cotransferred naive  $CD4^+$  T cells, and at a 10:1 cell ratio, we recovered 10-fold less T cells from naive  $CD4^+$  origin ( $p < 0.005$ ). Total T cell recovery diminished according to the level of suppression, that is, overgrowth of the coinjected  $CD25^+CD4^+$  T cells did not compensate for the lack of expansion of the naive T cells ( $p$ -NS) (Fig. 7B). We also found that  $CD25^+CD4^+$  T cells did not affect the growth of coinjected total LN CD8 T cells ( $p = 0.4$ ; Fig. 7C), indicating that their inhibitory effects are lineage-specific. By varying either the number of T cells injected, or the ratio  $CD25^+/CD25^-$  T cells, we found that the number of T cells from naive  $CD25^-CD4$  origin recovered was not dependent on the number of cells transferred, but determined by the  $CD25^+/CD25^-$  ratio present in the inoculum (Fig. 7D). These results raised the possibility that the  $CD25^+CD4^+$  T cells might have blocked division of the naive  $CD4^+$  T cells. To test this possibility, we compared the fate of CFSE-labeled naive T cells transferred alone (Fig. 7E, top panel) or in the presence of an excess of  $CD25^+$  cells (Fig. 7E, bottom panel). Three days after transfer (Fig. 7E), the patterns of dilution of the CFSE labeling were similar in both groups of mice, and we recovered an identical number of cells in the two groups of host mice (data not shown). At day 10, the majority of the transferred cells were CFSE<sup>-</sup>, indicating that these cells underwent several rounds of division. However, the fraction of CFSE<sup>-</sup> cells was higher, and we recovered 27-fold more CD4 T cells in the mice injected with naive T cells in absence of  $CD25^+$  cells (Fig. 7E). The differences in total cell recovery could be due to either an increase in cell survival or to an increase in the rate of cell division of the  $CD4^+CD25^-$  naive T cells when transferred alone. Thus, the present results do not allow discriminating between these two possibilities or if the increase of the number of cells corresponds to an increased fraction of cells that enter cell cycle or to a reduced cell cycle time. Studies on the annexin V labeling of the transferred populations were not conclusive (data not shown). On the whole, these results indicate that the suppressive effects are not obtained through complete block of proliferation, but do not allow us to distinguish whether they affect the rate of cell expansion or the survival (accumulation) of the newly generated T cells. In contrast, we found that activated  $CD45R^{\beta low}CD25^-CD4^+$  T cells did not control expansion of naive CD4 T cells (data not shown).

#### *CD25<sup>+</sup>CD4<sup>+</sup> T cells inhibit peripheral expansion of IL2R $\alpha^{-/-}$ CD4 T cells*

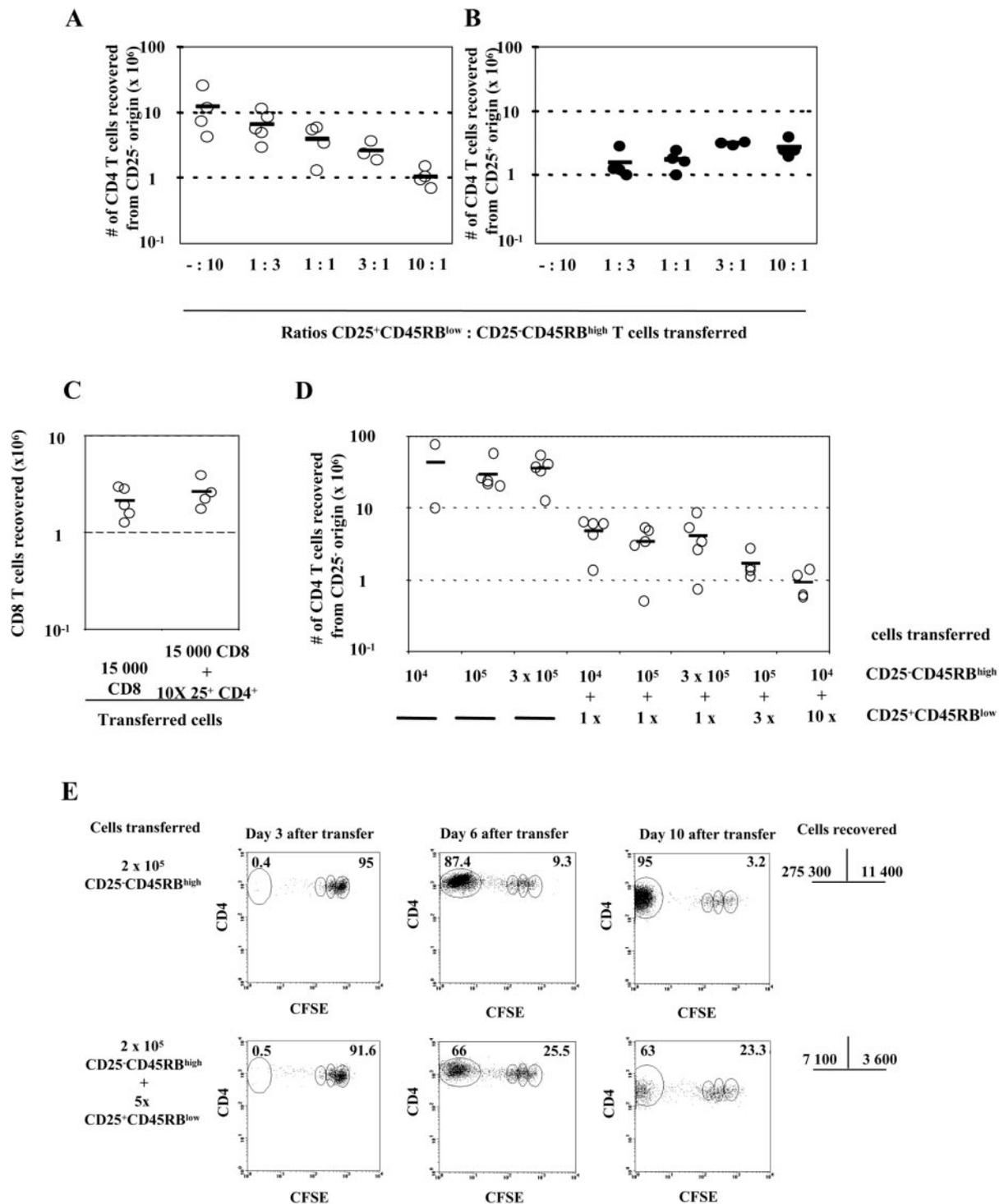
Finally, we studied whether the same forms of interaction also applied to populations of CD4 T cells from IL-2R $\alpha^{-/-}$  and IL-2<sup>-/-</sup> mice. We found that  $CD25^+CD4^+$  T cells from normal do-

nors inhibited the expansion of the  $CD4^+$  T cells from IL-2R $\alpha^{-/-}$  mice transferred into  $CD3\epsilon^{-/-}$  hosts ( $p < 0.05$ ; Fig. 8A). Similarly, naive  $CD4^+$  T cells from IL-2<sup>-/-</sup> origin expanded and were suppressed ( $p < 0.01$ ) while  $CD25^+CD4^+$  T cells from IL-2<sup>-/-</sup> mice slightly suppressed the expansion of naive  $CD4^+$  T cells from normal donors as well as  $CD25^+CD4^+$  T cells from normal donors at a 1:1 cell ratio (Fig. 8B). Altogether, these findings suggest that the control exerted by the  $CD25^+CD4^+$  T cells on the accumulation of peripheral CD4 T cells in the IL-2R $\alpha^{-/-}$  is due to their ability to regulate peripheral CD4 T cell homeostasis.

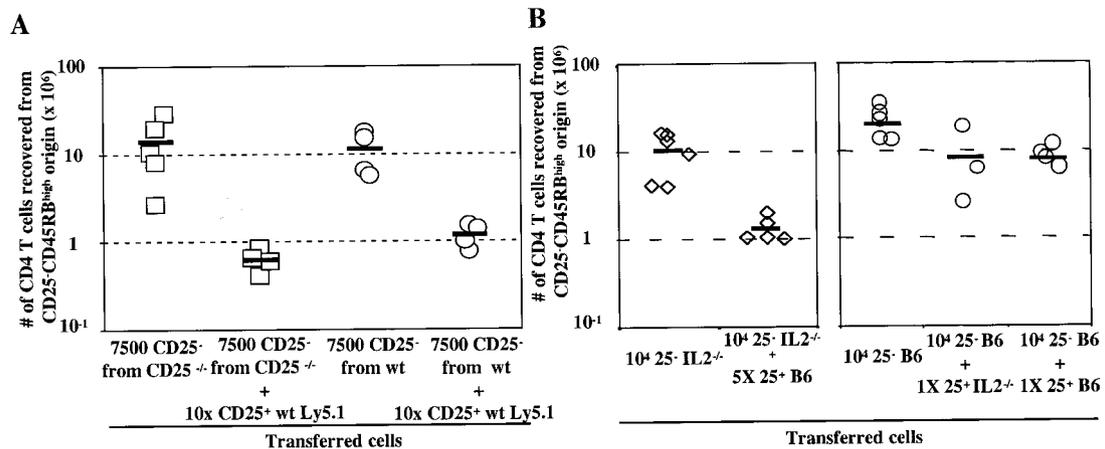
## Discussion

Peripheral T cells, in absence of a thymus (4, 25) or when transferred to T cell-deficient hosts (5, 7, 26), are capable of considerable expansion. The sequential transfer of a T cell population into successive hosts has shown that one T cell can generate up to  $10^{15}$  cells (7). This indicates that in a normal mouse, peripheral T cell division is limited by mechanisms that probably include resource competition and complex cell interactions (9). We studied the role of T cell interactions in the control of the number of peripheral  $CD4^+$  T cells. In particular, we investigated if  $CD25^+CD4^+$  T cells, which exert regulatory functions (27–32), could also govern peripheral  $CD4^+$  T cell homeostasis.

IL-2R $\alpha^{-/-}$  mutant mice are reported as a paradigm for perturbed lymphocyte homeostasis (10). The lack of the IL-2R $\alpha$  was believed to impair AICD in vivo (10), to modify the balance between clonal expansion and cell death, resulting in the deregulation of both the size and content of the peripheral lymphoid compartments. The primary uncontrolled T cell activation lead subsequently to secondary polyclonal B cell activation and autoantibody production. However, recent findings have shown that when placed in a normal environment, TCR Tg  $CD25^{-/-}$  T cells exhibited a significant reduction in Ag-induced expansion due to normal AICD (12). This observation was interpreted as indicating that the regulatory role of IL-2R $\alpha$  signals was mediated through the control of bystander T cell activation (12). We now demonstrate that the chaotic lymphocyte accumulation developed in adult IL-2R $\alpha^{-/-}$  mice is not cell autonomous: it is due to the lack of a T cell population essential for the homeostasis of peripheral T cell numbers. Two main lines of evidence support this conclusion. First, the presence of a limited number of  $CD25^+CD4^+$  T cells rescues mouse chimeras reconstituted with BM from IL-2R $\alpha^{-/-}$  mice from chaotic lymphocyte accumulation, polyclonal B and T cell activation, and death, and restores the peripheral lymphoid compartment to normal. Second, we show that  $CD25^+CD4^+$  T cells inhibit peripheral expansion of CD4 T cells from IL-2R $\alpha^{-/-}$  mice transferred into T cell-deficient adoptive hosts. Moreover, by



**FIGURE 7.** A, A population of 10,000 naive CD4<sup>+</sup> cells was transferred alone or mixed with increasing numbers of CD25<sup>+</sup>CD4<sup>+</sup> cells from different Ly5 allotype congenic donors into CD3ε<sup>-/-</sup> hosts. Results show the number of CD4 T cells from naive CD4<sup>+</sup> origin recovered 8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). B, The total number of CD4 T cells from CD25<sup>+</sup>CD4<sup>+</sup> origin recovered in the same hosts. C, A population of 15,000 purified CD8<sup>+</sup> LN cells was transferred alone or mixed 150,000 CD25<sup>+</sup>CD4<sup>+</sup> cells from different Ly5 allotype congenic donors into CD3ε<sup>-/-</sup> hosts. Results show the number of CD8 T cells recovered 8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). D, Different numbers of purified naive CD4<sup>+</sup> were cotransferred with CD25<sup>+</sup>CD4<sup>+</sup> T cells at different cell ratios. The results show the number of CD4 T cells from naive CD4<sup>+</sup> origin recovered 8–9 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). E, A total of  $2 \times 10^5$  CFSE-labeled CD45RB<sup>high</sup>CD25<sup>+</sup>CD4<sup>+</sup> T cells from B6.Ly5.1 donors were transferred alone (*top panels*) or in the presence (*bottom panels*) of  $1 \times 10^6$  CD45RB<sup>low</sup>CD25<sup>+</sup>CD4<sup>+</sup>Ly5.2 T cells into irradiated (400 rad) CD3ε<sup>-/-</sup> hosts. At days 3, 6, and 10, posttransfer mice were sacrificed and the expression of CFSE analyzed in gated Ly5.1<sup>+</sup>CD4<sup>+</sup> T cells. The figures show the relative fraction (percentage) of cells that have divided >8 times and in the first three rounds of division as well as the total number of CFSE<sup>-</sup> and CFSE<sup>+</sup> cells at day 10.



**FIGURE 8.** A,  $CD4^+ (CD25^-)$  T cells (7,500) from  $IL-2R\alpha^{-/-}$  or normal B6 donors were transferred alone or coinjected with 75,000  $CD25^+ CD4^+$  from normal mice. The results show the number of CD4 T cells recovered 7–8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). B, Naive  $CD4^+ (CD25^-)$  T cells (10,000) from  $IL-2^{-/-}$  mice were transferred alone or coinjected with 10,000  $CD25^+ CD4^+$  from B6.Ly5.1 mice, and naive  $CD4^+ (CD25^-)$  T cells (10,000) from normal B6.Ly5.1 donors were transferred alone or coinjected with 10,000  $CD25^+ CD4^+$  from normal B6.Ly5.2 or  $IL-2^{-/-}$  mice. The results show the number of CD4 T cells from  $CD25^-$  origin recovered 7–8 wk after transfer in the spleen and LN of each individual host (the mean value is also shown). Note that cells from  $IL-2^{-/-}$  mice inhibit expansion of naive T cells from normal donors and that the  $CD25^+ CD4^+$  T cells from normal B6 donors inhibited the expansion of the cells from  $IL-2^{-/-}$  mice.

using  $IL-2$ -deficient mice, which also develop lymphoid hyperplasia and autoimmunity late in life (20), we show that  $IL-2$  is required for the establishment of a stable and sizeable population of peripheral  $CD25^+ CD4^+$  regulatory cells. Thus,  $IL-2R\alpha/IL-2$  signals are not involved in control of bystander activation, but are instead required for the generation and peripheral expansion/survival of a population of regulatory CD4 T cells essential for peripheral CD4 T cell homeostasis.

We further dissected and quantified the type of cell interactions involved in peripheral homeostasis by following the fate of separated populations of CD4 T cells transferred into immune-deficient hosts (7). We found that after transfer into  $CD3\epsilon^{-/-}$  T cell-deficient hosts, purified naive  $CD4^+$  T cells expanded to reach a stable plateau at  $\sim 1-2 \times 10^7$  cells, independently of the number of injected cells. When a second population of naive  $CD4^+$  cells was transferred into the same hosts, the growth of each population was limited and the total T cell recovery was the same as in mice injected with only one population. We did not observe an overt advantage of either the tenant or the newcomer cells. These findings confirmed that cellular rivalry could alter the fate of T cells at the periphery (8, 9, 24), and attested that the expansion of the transferred naive  $CD4^+$  T cells is under homeostatic control (7). We found that the accumulation of the  $CD25^+ CD4^+$  T cells in T cell-deficient hosts is limited by a homeostatic plateau which singularly operates at values 10-fold lower than for total  $CD4^+$  or naive  $CD4^+$  T cells, i.e., at  $1-2 \times 10^6$  cells/host. We confirmed by the cotransfer of these two T cell populations that the presence of  $CD25^+ CD4^+$  T cells limited the expansion of the naive  $CD4^+$  T cells (15). Total T cell recovery diminished accordingly to the levels of suppression thus, excluding the presence of competition between the two populations.

We expanded these observations and showed that the inhibitory effects were dose-dependent and lineage-specific, as they did not affect naive  $CD8^+$  T cell expansion in vivo. However, lineage specificity seems dependent on the experimental conditions, as  $CD25^+ CD4^+$  T cells were shown to control memory but not naive  $CD8^+$  T cells (33), and to suppress both  $CD4^+$  and  $CD8^+$  T cell activation in vitro (34, 35). Suppression of naive  $CD4^+$  T cell growth was obvious when the number of  $CD25^+ CD4^+$  T cells exceeded the number of the naive  $CD4^+$  T cells by a factor of 10,

less noticeable and variable when the two populations were present at similar numbers. Thus, the physiological relevance of the  $CD25^+ CD4^+$  T cells could be arguable since in normal mice their number rarely exceeds 10% of the total  $CD4^+$  T cell pool. However, upon sequential cell transfers, we found that the injection of a limited number ( $5 \times 10^4$ ) of  $CD25^+ CD4^+$  T cells arrests the growth of an expanding population of  $5 \times 10^6$  resident naive  $CD4^+$ -derived T cells. More importantly, a limited number of  $CD25^+ CD4^+$  T cells rescues mice reconstituted with BM cells from  $IL-2R\alpha^{-/-}$  donors, controls the chaotic T cell accumulation, and reestablishes a peripheral T cell pool with a normal subpopulation composition. Thus, the suppressive effects mediated by the  $CD25^+ CD4^+$  T cells have physiological relevance since they were also obtained at physiological ratios of  $CD25^+$  to  $CD25^-$ . The changes in effectiveness observed between the different experimental protocols and schedules of injection may be due to the different fraction/number of activated cells to be suppressed. Immediately following transfer, most naive CD4 T cells are activated, while later, near steady state equilibrium, only a fraction proliferates (7). Alternatively, they could be due to the different capacity of the regulatory cells to suppress homeostatic proliferation, occurring in the cell transfer experiments, and natural T cell proliferation and reconstitution occurring in the BM chimeras. Finally, the homing of the  $CD25^+ CD4^+$  T cells could also differ between the different experimental protocols used. In experiments where the  $CD25^+ CD4^+$  T cells were transferred at the same time as the naive CD4 T cells, differential homing abilities of the two populations could explain the apparently too high  $CD25^+ : CD25^-$  ratio needed in the cotransfer experiments. The homing of the  $CD25^+ CD4^+$  T cells could be favored by the presence of activated T cells in the periphery of the host mice as it could happen in the sequential transfer experiments.

How do the  $CD25^+ CD4^+$  T cells regulate homeostasis of naive  $CD4^+$  T cells? To investigate the possible effects of  $CD25^+ CD4^+$  T cells in blocking the proliferation of  $CD25^-$  cells, we have transferred CFSE-labeled  $CD25^- CD4^+$  T cells alone or with an excess of  $CD25^+ CD4^+$  regulatory T cells (Fig. 7E). The dilution of CFSE labeling observed in both situations is similar, but the accumulation of cells observed in the CFSE-negative fraction of cells accounts for the differences in the number of cells recovered. Thus, homeostatic proliferation could be said to occur in both situations, but its extent could

be limited only when CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells are present. The possibility that CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells are capable of inhibiting the extent of homeostatic proliferation suggests the interesting possibility that the observed regulation of self-reactive responses is just a side effect of a broader function of these cells in the control of peripheral T cell numbers. If these cells control the magnitude of expansion of all naive CD25<sup>-</sup>CD4<sup>+</sup> T cells, this may also include expansion of self-reactive clones present within that population. However, the opposite can also be true, and the control of self-reactive responses may result in the control of total cell numbers recovered. In this study, we show that the presence of T regulatory (Treg) cells prevents the activation of CD4 T cells from CD25<sup>-/-</sup> origin, including self-reactive clones, and allows the establishment of a normal size naive peripheral T cell compartment; sequential cell transfer, the CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells suppress the expansion of activated T cells engaged in homeostatic proliferation, reducing the number of cells recovered.

It has been shown that IL-10 mediates the regulatory functions of the CD25<sup>+</sup>CD4<sup>+</sup> T cells (28, 36–39), but *in vitro* studies have excluded the role of IL-10 in CD25<sup>+</sup> T cell-mediated suppression (40). We examined the role of IL-10 in the suppression of T cell proliferation *in vivo*; in contrast to a previous report (15), we found that CD25<sup>+</sup>CD4<sup>+</sup> T cells from IL-10<sup>-/-</sup> mice inhibit the expansion of naive T cells as effectively as CD25<sup>+</sup> cells from wild-type mice (data not shown). This indicates that the effects of the CD25<sup>+</sup>CD4<sup>+</sup> T cells on T cell expansion are IL-10-independent. However, we confirmed that CD25<sup>+</sup> cells from IL-10<sup>-/-</sup> mice failed to protect against the wasting disease induced by the naive CD4<sup>+</sup> T cells (data not shown). TGFβ has also been implicated in IBD protection, and recent claims suggest that it may play a role in T cell homeostasis (41–43). However, we found that both naive and activated CD4 T cells expressed similar levels of mRNAs for the three subforms of TGFβ (data not shown). Moreover, CD25<sup>+</sup> T cells from TGFβ-deficient mice are referred to be suppressors (44). We also found that CD25<sup>+</sup>CD4<sup>+</sup> cells from TNF-α<sup>-/-</sup> and LTα<sup>-/-</sup> mice inhibit expansion of naive T cells (data not shown), excluding their role in this process. By using *lpr* and *gld* mutant mice, we excluded a possible role of Fas/Fas ligand interactions in these processes (data not shown). The possible involvement of CTLA-4 in T cell homeostasis is also unlikely, as it has also been shown that CD25<sup>+</sup>CD4<sup>+</sup> T cells from CTLA-4-deficient mice exhibit suppressor activity (45). Recent results implicating the glucocorticoid-induced TNFR (TNFRS18) in the regulatory activity of the CD25<sup>+</sup> T cells (46, 47) are not conclusive (44). Other possibilities are under investigation.

The suppressive capacity of the CD25<sup>+</sup>CD4<sup>+</sup> T cells, while maximal upon injection, was virtually lost when these cells were parked for 2 mo in the hosts. It is possible that regulation of existing and newly transferred cells differs, and/or that “parked” cells may evolve functionally. Upon secondary transfer, we showed that the parked CD25<sup>+</sup>CD4<sup>+</sup> T cell retained their suppressive abilities. Our *in vivo* observations contrast with recent *in vitro* data showing that T cells having lost CD25 expression suppress expansion of naive CD4 T cells (48). This apparent discrepancy may simply reflect differences in the *in vitro* and *in vivo* behavior of the Treg cells. Other reports have shown that the CD25<sup>+</sup> cells progeny of *in vivo* activated naive CD4<sup>+</sup> T cells were not able to confer effective protection of disease (49) or to control *in vitro* T cell proliferation (40). Moreover, CD25<sup>-</sup>CD4<sup>+</sup> thymocytes, when transferred to immune-deficient hosts, cannot generate a Treg cell population and induce autoimmune disease (50). In addition, the autoimmune manifestations that occur in neonatal thymectomized mice (day 3) correlate with the absence of CD25<sup>+</sup>CD4<sup>+</sup> T cells, and the reintroduction of CD25<sup>+</sup> cells generated in 3-day-old Tx mice was

unable to prevent disease (40), which could be avoided by CD25<sup>+</sup>CD4<sup>+</sup> T cells from normal donors (28). These observations indicate that the regulatory functions may be a property of a specific cell subpopulation, but that inside this subpopulation these functions correlate with the expression of the CD25 marker and may require continuous T cell stimulation (27, 32). The inhibitory effects may require direct T-T cell interactions (40) or act via a third party presenting cell. We found that the initial CD25<sup>+</sup>:CD25<sup>-</sup> ratio strictly determined the final number of CD4 T cells, suggesting a direct relationship between the two populations. Inhibition does not seem to require Ag specificity or mutual cognate recognition by the interacting cells, since *in vitro* the two populations do not need to recognize the same ligand (51). However, maintenance of the Ag-specific regulatory cells seems to require the continuous presence of the Ag (32).

To the question of whether the regulatory cells may represent a separate CD4<sup>+</sup> T cell lineage (52), the answer is yes. The IL-2Rα<sup>-/-</sup> mutant mice lack these cells. The transfer of a limited number of CD25<sup>+</sup>CD4<sup>+</sup> T cells in mouse chimeras reconstituted with BM cells from IL-2Rα<sup>-/-</sup> mice prevents lethal lymphocyte accumulation. However, delayed transfer of the CD25<sup>+</sup>CD4<sup>+</sup> cells was less effective in protection, stressing the importance of regulatory/naive cell ratios at the time of the initial peripheral seeding. The presence of as few as 5% of cells from a normal donor developing in the thymus of the chimeras suffices to reestablish full control of the number and state of activation of the peripheral CD4 IL-2Rα<sup>-/-</sup> T cells. It has been shown that CD25<sup>+</sup>CD4<sup>+</sup> T cells are generated in the thymus (38, 50, 53). Our findings support this claim. We also found that transferred CD25<sup>+</sup>CD4<sup>+</sup> T cells can persist for prolonged periods in absence of the thymus, as observed in the IL-2Rα<sup>-/-</sup> BM chimeras. Thus, these cells can be either long-lived or capable of self renewal at the periphery. Interestingly, in the different mixed IL-2Rα<sup>-/-</sup>/normal BM chimeras, the number of peripheral CD25<sup>+</sup>CD4<sup>+</sup> T cells was the same independently of the fraction of normal cells present in the BM inoculum. IL-2-deficient mice lack a significant number of CD25<sup>+</sup>CD4<sup>+</sup> cells at the periphery (38) and develop lymphoid hyperplasia and IBD (20). Recent studies suggested that disturbed peripheral homeostasis in IL-2-deficient mice resulted from either an IL-2-dependent AICD defect and/or the lack of CD25<sup>+</sup>CD4<sup>+</sup> regulatory cells (14). We found that IL-2<sup>-/-</sup>-derived cells rescue the IL-2Rα<sup>-/-</sup> BM chimeras from death, and that in these chimeras, the number and the distribution of CD25<sup>+</sup> and CD25<sup>-</sup>CD4 T cells in the peripheral T cell compartments was as in normal mice. Thus, in the presence of IL-2, the BM precursors from IL-2-deficient donor mice generated a well-defined population of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells capable to control the number of CD4 T cells in the peripheral compartments. These findings suggest that IL-2 is required for the peripheral survival and maintenance of the subset of the CD25<sup>+</sup> regulatory cells produced by the thymus. Production of IL-2 by proliferating CD4 T cells may also contribute to the survival of the regulatory cells. Thus, we may envisage a feedback loop in which expanding naive CD4 T cells contribute to their own regulation. The role of IL-2/IL-2R interactions in T cell homeostasis is further supported by results showing that IL-2Rβ<sup>-/-</sup> T cells in mice reconstituted with a mixture of IL-2Rβ<sup>-/-</sup> T and IL-2Rβ<sup>+</sup> BM cells did not expand or develop into an abnormally activated stage (13). However, in this last study, the cells responsible for the homeostatic control were believed to be CD8 T cells (13) and not the CD25<sup>+</sup>CD4<sup>+</sup> T cells that we now identified as capable of controlling IL-2Rα-deficient T cells. It is possible that other cell populations contribute to regulate peripheral T cell pools.

In summary, we demonstrate the role of T cell interactions in the control of the size of the peripheral CD4<sup>+</sup> T cell pool. We show that homeostasis of peripheral CD4<sup>+</sup> T cells follows subpopulation structure, CD25<sup>+</sup>CD4<sup>+</sup> T cells limiting the accumulation of dividing naive CD4<sup>+</sup> T cells. We found that IL-2R $\alpha$ -deficient mice lack a subpopulation of regulatory cells essential for CD4 T cell homeostasis. Adoptive replacement of the CD25<sup>+</sup>CD4<sup>+</sup> T cell population prevents the chaotic accumulation of lymphoid cells in the peripheral compartments of IL-2R $\alpha$ -deficient mice. It also prevents the subsequent polyclonal T and B cell activation, autoimmune hemolytic anemia, and IBD observed in IL-2R $\alpha$ <sup>-/-</sup> mice (10). We also show that IL-2-deficient mice also lack a sizeable population of CD25<sup>+</sup>CD4<sup>+</sup> T cells that expands in presence of IL-2 to control autoimmunity and lymphoid hyperplasia in IL-2R $\alpha$  chimeras. To conclude, we show that the mechanism by which IL-2R $\alpha$  and IL-2 play an essential role on T cell homeostasis is by shaping a population of CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells that control peripheral CD4 T cell numbers. We demonstrate that IL-2R $\alpha$  is an absolute requirement for the generation of the regulatory cells. These cells generate in the thymus in the absence of IL-2, but require IL-2 to establish a stable functional population in the peripheral compartments.

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