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Homeostasis of Peripheral CD4\(^+\) T Cells: IL-2R\(\alpha\) and IL-2 Shape a Population of Regulatory Cells That Controls CD4\(^+\) T Cell Numbers\(^1\)

Afonso R. M. Almeida,* Nicolas Legrand,* Martine Papiernik, † and António A. Freitas\(^2\)*

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\(^+\)CD4\(^+\) 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\(^+\)CD4\(^+\) T cells, and restores the peripheral lymphoid compartments to normal. The CD25\(^+\)CD4\(^+\) T cells regulated selectively the number of naive CD4\(^+\) T cells transferred into T cell-deficient hosts. The CD25\(^+\)CD4\(^+\)/naive CD4 T cell ratio and the sequence of cell transfer determines the homeostatic plateau of CD4\(^+\) T cells. Overall, our findings demonstrate that IL-2R\(\alpha\) is an absolute requirement for the development of the regulatory CD25\(^+\)CD4\(^+\) 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\(\alpha^-\) mice represent a paradigm for perturbed lymphocyte homeostasis (10). They develop massive enlargement of peripheral lymphoid organs associated with T and B cell expansion 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\(\alpha^-\) 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) IL-2R\(\alpha^-\)/ T 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\(^+\) T cells (14). Finally, results indicate that regulatory CD45RB\(^low\)/CD25 \(^+\)CD4\(^+\) 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\(^+\)CD4\(^+\) T cells (16) could prevent the chaotic lymphocyte accumulation in IL-2R\(\alpha^-\) mice and control the expansion of peripheral naive CD4 T cells. We found that CD25\(^+\)CD4\(^+\) T cells could indeed control peripheral T cell accumulation and composition in mouse chimeras reconstituted with BM cells from IL-2R\(\alpha^-\) mice and rescued these mice from death. Similarly, recombination-activating gene (Rag)\(^2\)\(-/-\) chimeras reconstituted with a mixture of BM cells from IL-2R\(\alpha\) and IL-2-deficient donors

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\(^*\)Abbreviations used in this paper: Tg, transgenic; AICD, activation-induced cell death; BM, bone marrow; Rag, recombination-activating gene; LN, lymph node; Treg, regulatory; IBID, inflammatory bowel disease.
remained healthy, and the number and distribution of CD25+ and CD25− CD4 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 CD45RBhighCD25+ CD4T cells and CD45RBlowCD25− CD4+ T cells transferred into CD3ε−/− T cell-deficient host mice (17). We found that the two cell populations showed different homeostatic plateaus and that CD25+ CD4+ 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 Ilf-a-Credo (L’Arbresle, France), B6.CD3ε−/− (17), B6.IL-2Rα−/− (10), B6.TCRα−− (18), and C57BL/6.Ly5.1 mice from the Centre de Development des Techniques Avancées-Centre National de la Recherche Scientifique (Orléans, France), B6.IL-10−/− (19) from The Jackson Laboratory (Bar Harbor, ME) and B6.IL-2−− (20) from our breeding facilities or from Dr. A. Schlümp (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+ T cells by negative selection using a Dynal MCC6 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+. These cells labeled with the appropriate combinations of anti-CD4 (L3T4/RM4-5), anti-CD45RB, and anti-CD25 (784), Abs were sorted on a CellQuest software.

Results

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.

Mutant IL-2Rα−− 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−− 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−− mice. We examined whether mature CD25− CD4+ T cells could alter the number and state of activation of CD4 T cells in B6. Rag2−− mice reconstituted with BM chimeras reconstituted with 100% BM cells from B6.IL-2Rα−− donors. Using these BM chimeras rather than intact B6.IL-2Rα−− mice allowed us to increase the number of mice studied. We found that all chimeras injected exclusively with BM cells from B6.IL-2Rα−− donors died within 40–50 days (Fig. 1A), with overt signs of runting, anemia, and in some mice, lymphoid hyperplasia (up to 300 × 106 lymphocytes). In contrast, the BM chimeras that received 106 CD25− CD4+ 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− CD4+ 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−− 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 × 106 for the two groups of BM chimeras injected with CD25− CD4+ T cells, respectively) to control mice (40–60 × 106 CD4 T cells). The composition of the peripheral LN T cells was as in normal mice comprising 5–10% of CD45RB−BMDCD25− CD4+ T cells, all of B6. Ly5.1 origin, and 50–60% of nonactivated CD45RB−BMDCD25− CD4+ T cells all from IL-2Rα−− origin (Fig. 1B). This is in contrast to donor IL-2Rα−− 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 × 106 CD25− CD4+ T cells did not rescue the CD25− 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−− T cells, lethally irradiated lymphopenic B6.Rag2−− 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−− donors and the remaining 50% from B6. Ly5.2.TCRα−− (unable to generate T cells) and normal B6. Ly5.1 donors mixed at different ratios. By keeping the fraction of cells from IL-2Rα−− donors in the injected BM cohort at 50%, we fixed the rate of production of IL-2Rα−− T cells in all chimeras studied. Thus, the resulting chimeras should all have the same number of peripheral IL-2Rα−− T cells (1). By mixing BM cells from B6 normal and B6.TCRα−− donors, we reduced the number of competent precursors available for thymus colonization and regeneration, as the normal competent precursor cells are diluted among incompetent
found that all the chimeras that could only generate IL-2R<sup>+</sup> BM 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<sup>−/−</sup> 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 chima reconstituted with BM cells from B6.Ly5.2IL-2R<sup>−/−</sup> that received 10<sup>5</sup> 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 TCRα<sup>−/−</sup> 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-2Rα<sup>−/−</sup> T cells. We found that all the chimeras that could only generate IL-2Rα<sup>−/−</sup> T cells, i.e., which received 50% BM cells from B6.IL-2Rα<sup>−/−</sup> donors and 50% of BM cells from B6.TCRα<sup>−/−</sup> 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-2Rα<sup>−/−</sup>/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<sup>+</sup> 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-2Rα<sup>−/−</sup> T cells. These results show that the lethal accumulation of peripheral T cells in IL-2Rα<sup>−/−</sup> mice is not cell autonomous, but due to the lack of a population of CD25<sup>+</sup> T cells essential for peripheral T cell homeostasis. We demonstrated that expression of the IL-2Rα 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-2Rα<sup>−/−</sup> 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-2Rα<sup>−/−</sup> 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-2Rα<sup>−/−</sup> and B6.IL-2<sup>−/−</sup> donors. Control mice received BM cells from the TCRα<sup>−/−</sup> donors or B6.IL-2<sup>−/−</sup> mice equally mixed at 50/50 with BM cells from B6.TCRα<sup>−/−</sup> donors. The presence of 50% of BM cells from IL-2<sup>−/−</sup> donors rescued the totality of the IL-2Rα<sup>−/−</sup> 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<sup>+</sup> 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 CD25<sup>−</sup> CD4<sup>+</sup> cells was able to rescue B6.IL-2Rα<sup>−/−</sup> 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<sup>+</sup> 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<sup>+</sup> 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> were i.v. transferred into syngeneic CD3<sup>−/−</sup> T cell-deficient hosts. In hosts receiving as few as 5 x 10<sup>3</sup> and as many as 10<sup>5</sup> 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
A lethally irradiated B6.Rag2−/− mice were reconstituted with 4 × 10⁶ cells from a mixture of 50% BM cells from B6.Ly5.2IL-2Rα−/− and 50% of BM cells from B6.Ly5.2.TCRα−/− 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−/− and 50% of BM cells from B6.Ly5.2.TCRα−/− (●), 50% BM from B6.Ly5.2IL-2Rα−/−, 45% BM from B6.Ly5.2.TCRα−/−, and 5% BM from normal B6.Ly5.1 (▲), 50% BM from B6.Ly5.2IL-2Rα−/−, 40% BM from B6.Ly5.2.TCRα−/−, and 10% BM from normal B6.Ly5.1 (×), 50% BM from B6.Ly5.2IL-2Rα−/−, and 50% BM from normal B6.Ly5.1 (■). Number of mice per group: nine. B, Dot plot shows the frequency of CD25+CD4+ T cells in the LNs of chimeras reconstituted with 50% BM from B6.Ly5.2IL-2Rα−/− and 50% BM from normal B6.Ly5.1. Similar results were obtained in the other chimeras.

~1–2 × 10⁷ cells, 10–12 wk after transfer (Fig. 4). In mice injected with the same number of CD45RBlowCD25⁺CD4⁺ T cells (from now on referred to as CD25⁺CD4⁺), these cells also expanded but reached equilibrium at 10-fold lower values, i.e., at 1–2 × 10⁶ cells/hosts (Fig. 4). Transfer of increasing numbers (>10⁵) of cells did not modify the final cell recovery (data not shown). These results indicate that naive CD4⁺ and CD25⁺CD4⁺ 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⁺ T cells developed a wasting autoimmune disease and eventually died (>15 wk), while hosts of CD25⁺CD4⁺ T cells remained healthy (data not shown). Total nonseparated LN CD4⁺ T cells containing 10% CD25⁺ cells expanded to a plateau of ~1–2 × 10⁷ cells, but fail to develop signs of wasting disease (data not shown). Activated CD45RBlowCD25⁺CD4⁺ T cells expanded to similar plateaus as naive CD4⁺ 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 second newly injected cell population or whether the injection of a new population could modify the fate of a resident population. We “parked” 5 × 10⁷ Ly5.1 naive CD4⁺ T cells in different hosts. Seven weeks later, each host received the same number of a second population of Ly5.2 naive CD4⁺ or CD25⁺CD4⁺ 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 CD45RBlow activated/memory phenotype, but only a few (1–2%) became CD25⁺ (data not shown). In mice injected sequentially with two populations of naive CD4⁺ 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 (~2 × 10⁷). The transfer of 5 × 10⁴ CD45RBlowCD25⁺CD4⁺ cells into mice injected 7 wk before with CD45RBhighCD25⁺CD4⁺ cells suppressed significantly (p < 0.001) further expansion of the established 5 × 10⁶ CD45RBhighCD25⁺CD4⁺-derived T cell population (Fig. 5B). The total T cell recovery diminishes accordingly. The number of
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 × 10<sup>5</sup>) of newly transferred CD45RB<sup>low</sup>CD25<sup>−</sup>CD4<sup>+</sup> T cells can suppress the expansion of an abundant (5 × 10<sup>6</sup>) population of resident CD4<sup>+</sup> T cells.

We also parked 5 × 10<sup>4</sup> Ly5.1 CD25<sup>−</sup>CD4<sup>+</sup> T cells. Seven weeks later, each host received 5 × 10<sup>5</sup> 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<sup>+</sup> 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 CD3<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.
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 cotransferred CD25⁺ CD4⁺ T cells did not compensate for the lack of expansion of the naive T cells (p < 0.05) (Fig. 7B). We also found that CD25⁺ CD4⁺ T cells did not affect the growth of cotransferred 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 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⁺, indicating that these cells underwent several rounds of division. However, the fraction of CFSE⁻ 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 CD45RBlowCD25⁻ CD4⁺ T cells did not control expansion of naive CD4 T cells (data not shown).

CD25⁺ CD4⁺ T cells inhibit peripheral expansion of IL-2Rα⁻/⁻ CD4⁺ T cells

Finally, we studied whether the same interactions also applied to populations of CD4 T cells from IL-2Rα⁻/⁻ and IL-2⁻/⁻ mice. We found that CD25⁺ CD4⁺ T cells from normal donors inhibited the expansion of the CD4⁺ T cells from IL-2Rα⁻/⁻ mice transferred into CD3e⁻/⁻ hosts (p < 0.05; Fig. 8A). Similarly, naive CD4⁺ T cells from IL-2⁻/⁻ origin expanded and were suppressed (p < 0.01) while CD25⁺ CD4⁺ T cells from IL-2⁻/⁻ 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-2Rα⁻/⁻ 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 10¹⁵ 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-2Rα⁻/⁻ mutant mice are reported as a paradigm for perturbed lymphocyte homeostasis (10). The lack of the IL-2Rα 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-2Rα signals was mediated through the control of bystander T cell activation (12). We now demonstrate that the chaotic lymphocyte accumulation developed in adult IL-2Rα⁻/⁻ 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-2Rα⁻/⁻ 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-2Rα⁻/⁻ 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 CD3<sup>-</sup>CD8<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 CD3<sup>-</sup>CD8<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 × 10<sup>5</sup> 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 × 10<sup>6</sup> CD45RB<sup>low</sup>CD25<sup>-</sup> Ly5.2 T cells into irradiated (400 rad) CD3<sup>-</sup>CD8<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.
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-2-Rα/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 CD3ε⁻/⁻ T cell-deficient hosts, purified naive CD4⁺ T cells expanded to reach a stable plateau at ~1–2 × 10⁷ 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 × 10⁶ 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 argued 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 × 10⁵) of CD25⁺ CD4⁺ T cells arrests the growth of an expanding population of 5 × 10⁶ resident naive CD4⁺-derived T cells. More importantly, a limited number of CD25⁺ CD4⁺ T cells rescues mice reconstituted with BM cells from IL-2-Rα⁻/⁻ 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⁺CD4⁺ regulatory T cells are present. The possibility that CD25⁺CD4 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⁺CD4⁺ 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⁻/⁻ origin, including self-reactive clones, and allows the establishment of a normal size naive peripheral T cell compartment; sequential cell transfer, the CD25⁺CD4⁺ 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⁺CD4⁺ T cells (28, 36–39), but in vitro studies have excluded the role of IL-10 in CD25⁺CD4⁺ 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⁺CD4⁺ T cells from IL-10⁻/⁻ mice inhibit the expansion of naive T cells as effectively as CD25⁺ cells form wild-type mice (data not shown). This indicates that the effects of the CD25⁺CD4⁺ T cells on T cell expansion are IL-10-independent. However, we confirmed that CD25⁺ cells from IL-10⁻/⁻ mice failed to protect against the wasting disease induced by the naive CD4⁺ 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⁺ T cells from TGFβ-deficient mice are referred to be suppressors (44). We also found that CD25⁺CD4⁺ cells from TNF-α⁻/⁻ and LTRα⁻/⁻ 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 been shown that CD25⁺CD4⁺ T cells from CTLA-4-deficient mice exhibit suppressor activity (45). Recent results implicating the glucocorticoid-induced TNFR (TNFR518) in the regulatory activity of the CD25⁺ T cells (46, 47) are not conclusive (44). Other possibilities are under investigation.

The suppressive capacity of the CD25⁺CD4⁺ T cells, while maximal upon injection, was virtually lost when these cells were packed 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⁺CD4⁺ 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⁺ cells progeny of in vivo activated naive CD4⁺ T cells were not able to confer effective protection of disease (49) or to control in vitro T cell proliferation (40). Moreover, CD25⁺CD4⁺ 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⁺CD4⁺ T cells, and the reintro-duction of CD25⁺ cells generated in 3-day-old Tx mice was unable to prevent disease (40), which could be avoided by CD25⁺CD4⁺ 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⁺:CD25⁻ 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⁺ T cell lineage (52), the answer is yes. The IL-2Rα⁻/⁻ mutant mice lack these cells. The transfer of a limited number of CD25⁺CD4⁺ T cells in mouse chimeras reconstituted with BM cells from IL-2Rα⁻/⁻ mice prevents lethal lymphocyte accumulation. However, delayed transfer of the CD25⁺CD4⁺ 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⁻/⁻ T cells. It has been shown that CD25⁺CD4⁺ T cells are generated in the thymus (38, 50, 53). Our findings support this claim. We also found that transferred CD25⁺CD4⁺ T cells can persist for prolonged periods in absence of the thymus, as observed in the IL-2Rα⁻/⁻ BM chimeras. Thus, these cells may be either long-lived or capable of self-renewal at the periphery. Interestingly, in the different mixed IL-2Rα⁻/⁻ normal BM chimeras, the number of peripheral CD25⁺CD4⁺ 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⁺CD4⁺ cells at the periphery (38) and develop lymphoid hyperplasia and IBD (20). Recent studies suggested that disturbed peripheral homeostasis in IL-2-deficient mice results from either an IL-2-dependent AICD defect and/or the lack of CD25⁺CD4⁺ regulatory cells (14). We found that IL-2⁻/⁻-derived cells rescue the IL-2Rα⁻/⁻ BM chimeras from death, and that in these chimeras, the number and the distribution of CD25⁺ and CD25⁻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⁺CD4⁺ 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⁺ 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⁻/⁻ T cells in mice reconstituted with a mixture of IL-2Rβ⁻/⁻ T cells and IL-2Rβ⁺ 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⁺CD4⁺ 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^+ T cell pool. We show that homeostasis of peripheral CD4^+ T cells follows a population structure, CD25^+CD4^+ T cells limiting the accumulation of dividing naive CD4^+ T cells. We found that IL-2Rα-deficient mice lack a subpopulation of regulatory cells essential for CD4 T cell homeostasis. Adoptive replacement of the CD25^+CD4^+ T cell population prevents the chaotic accumulation of lymphoid cells in the peripheral compartments of IL-2Rα-deficient mice. It also prevents the subsequent polyclonal T and B cell activation, autoimmune hemolytic anemia, and IBD observed in IL-2Rα^−/− mice (10). We also show that IL-2-deficient mice also lack a sizeable population of CD25^+CD4^+ T cells that expands in presence of IL-2 to control autoimmunity and lymphoid hyperplasia in IL-2Rα chimeras. To conclude, we show that the mechanism by which IL-2Rα and IL-2 play an essential role on T cell homeostasis is by shaping a population of CD25^+CD4^+ regulatory T cells that control peripheral CD4 T cell numbers. We demonstrate that IL-2Rα 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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References