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IL-2 and IL-7 Determine the Homeostatic Balance between the Regulatory and Conventional CD4⁺ T Cell Compartments during Peripheral T Cell Reconstitution

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Work over the last decades has led to the identification of the factors that influence the survival and homeostasis of conventional T cells. IL-7 and TCR signaling promote the survival of naive CD4⁺ and CD8⁺ T cells in lymphoreplete mice and their proliferation in a lymphopenic environment, whereas survival and homeostatic proliferation of memory CD4⁺ and CD8⁺ T cells crucially depend on a combination of IL-7 and IL-15. In contrast, there is little information regarding the factors driving the proliferation of regulatory CD4⁺ T cells in response to lymphopenia. In this study, we investigated whether regulatory CD4⁺ T cell proliferation in response to lymphopenia was guided by classical homeostatic resources, such as IL-2, IL-7, or TCR–MHC interactions. Altogether, our data suggest that, although homeostatic proliferation of conventional naive CD4⁺ T cells is closely related to IL-7 levels, the proliferation of regulatory CD4⁺ T cells in response to lymphopenia appears to be primarily controlled by IL-2. The capacity of IL-7 to augment conventional T cell proliferation with minimal concomitant regulatory T cell expansion may be clinically exploitable in the treatment of patients with lymphopenia, especially in the case of chronic viral diseases or cancer immunotherapy. The Journal of Immunology, 2012, 189: 3339–3346.

The size of the peripheral T cell pool is notably constant, despite continuous output from the thymus, turnover of existing cells, and clonal expansion of Ag-specific cells in the course of an immune response (1). This process is achieved through several homeostatic mechanisms that regulate both cell survival and proliferation. Environmental factors that regulate these responses vary, depending on the T cell subset and on the nature, naive or memory, of the T cell (2). The precise identification of these factors regulating T cell homeostasis appears to be crucial for the development of new strategies and clinical trials for future immunotherapy, such as cancer or antiviral therapies, or in the context of autoimmunity and lymphoproliferative diseases.

Work over the last decades established a critical role for cytokines in the maintenance and homeostatic proliferation of memory T cells (2). More precisely, it was shown that survival and homeostatic proliferation of memory CD8⁺ and CD4⁺ T cells crucially depend on a combination of IL-7 and IL-15 (3-7). However, optimum memory CD4⁺ T cell function may depend on interactions with MHC II molecules (8). In contrast, it was shown that disruption of MHC class I molecule–TCR interactions did not affect self-renewal, function, or survival of memory CD8⁺ T cells (9).

Numerous studies have led to the identification of the factors that influence the survival and homeostasis of naive T cells (10). In physiological settings, IL-7 and TCR signaling promote the survival and normal function of naive CD4⁺ and CD8⁺ T cells (11–13). In a lymphopenic environment, the same cues promote the proliferation of these cells (14, 15). More precisely, naive CD4⁺ T cells can be divided into two subsets as a function of their behavior after transfer into lymphopenic mice (16, 17). On the one hand, a small proportion of the initially injected CD4⁺ T cells expands strongly in response to interactions with self-peptides or commensal bacterium-derived peptides presented by MHC class II molecules. IL-7 is not required for this process, which is called “spontaneous proliferation” (18). On the other hand, the vast majority of injected naive T cells cycles slowly in response to the great availability of IL-7 in lymphopenic environments (19). TCR signaling can also synergize with IL-7 to enhance this latter process, which is called “homeostatic T cell proliferation” (18).

It is well established that IL-2 is essential for regulatory CD4⁺ T cell survival in the periphery (20–22). Accordingly, defective IL-2 signaling leads to spontaneous lymphoproliferative and autoimmune diseases in mice and humans because of the impaired development and function of these cells (23). Surprisingly, there is little information regarding the factors driving the proliferation of regulatory CD4⁺ T cells in response to lymphopenia. Cozzo et al. (24) and Hsieh et al. (25) proposed that this process is dependent on interactions with self-peptide/MHC complexes, but the role of ILs has not been clearly addressed.

In the present study, we investigated whether regulatory CD4⁺ T cell proliferation in response to lymphopenia was guided by “classical” homeostatic resources, such as IL-2, IL-7, or TCR–MHC interactions. We show that proliferation of regulatory CD4⁺ T cells in response to lymphopenia is not controlled by IL-7 levels but rather requires IL-2 production by conventional CD4⁺ T cells.

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Abbreviations used in this article: CTv, CellTrace violet; LN, lymph node; MFI, mean fluorescence intensity.

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This result is of importance, because several clinical trials of recombimant human IL-7 are ongoing in the settings of acquired immunodeficiency, cancer, and chronic viral infection.

**Materials and Methods**

**Mice**

C57BL/6 mice (CD45.2) were obtained from Harlan Laboratories. C57BL/6 CD45.1 mices and C57BL/J6 CD3e−/− mice were maintained in our own animal facilities under specific pathogen-free conditions. C57BL/6 CD3−/− mice (26) were crossed with MHC Ih2−/− mice (27) to obtain C3dε/MHC II double-deficient mice (CD3ε−/− Il2−/− mice) (13). C57BL/6 Foxp3-GFP reporter mice were initially provided by Dr. Bernard Malissen (Centre d’Immunologie de Marseille-Luminy, Marseille, France) (28, 29) and maintained in our own animal facilities. Experiments were carried out in accordance with the guidelines of the French Veterinary Department.

**Cell suspensions**

Peripheral and mesenteric lymph nodes (LN) and spleen tissue were homogenized and passed through a nylon cell strainer (BD Falcon) in RPMI 1640 Glutamax (Life Technologies), supplemented with 10% FCS (Biochrom) for adoptive transfer (LNs only), or in 5% FCS, 0.1% Na3 (Sigma-Aldrich) in PBS for flow cytometry (pooled LN and spleen cells - periphery).

**Adoptive transfer of CD4+ T cells**

LN cells (pooled superficial cervical, axillary, brachial, inguinal, and mesenteric LNs) were incubated on ice for 20 min with a mixture of anti-CD3 (53-6.7), anti-CD11b (Mac-1), anti-GR1 (8C5), and anti-CD19 (1D3) Abs, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat lgs (Dynal Biotech). Purified T cell subsets were generally 95–97% pure. When indicated, CD4+ T cells were labeled with 5 μM CFSE (Molecular Probes) before injection. In some experiments (Fig. 6), regulatory GFP-Foxp3+ CD4+ T cells from C57BL/6 Foxp3-GFP reporter mice were FACs sorted in a MoFlo XDP flow cytometer (Beckman Coulter) and labeled with 5 μM Cell Trace violet proliferation kit (Invitrogen), according to the manufacturer’s guidelines, before injection. Purified CD4+ T cells (5 × 10^5 cells) and/or regulatory CD4+ T cells (1 × 10^5 cells) were injected i.v. into sex-matched lymphopenic recipient mice.

**In vivo treatment with anti–IL-7R and/or anti–IL-2–blocking Abs and IL-2 immune complexes**

In the experiments depicted in Figs. 4 and 6, mice were injected i.p. every 2 d, beginning at the time of cell transfer, with anti–IL-2 Abs (SB46 and JES6-1A12, 200 μg each/mouse; Bio X Cell) and/or anti–IL-7R Ab (A7R34, 200 μg/mouse) obtained from hybridoma supernatants. In some experiments (Fig. 5), mice were injected i.p. every 2 d with IL-2/anti–IL-2 complexes, beginning at the time of cell transfer. IL-2/anti–IL-2 complexes were made, as previously described (30), by mixing 2 μg recombinant mouse IL-2 (0.5 μg/mouse; PeproTech) with 10 μg anti–IL-2 Ab (clone SB46, 2.5 μg/mouse; Bio X Cell).

**Cell surface staining and flow cytometry**

Cell suspensions were collected and dispensed into 96-well round-bottom microtiter plates (Greiner Bioscience; 6 × 10^5 cells/well). Surface staining was performed by incubating the cells on ice for 15 min each step, with Abs in 5% FCS (Biochrom), 0.1% Na3 (Sigma-Aldrich) in PBS. Each cell-staining reaction was preceded by a 15-min incubation with purified anti-CD16/32 Abs (FcγRIII/II block, 2.4G2) obtained from hybridoma supernatants.

Peridin chlorophyll protein-conjugated anti-CD4 Ab (RM4-5), FITC-conjugated anti-CD25 Ab (7D4), PE cyanin 7-conjugated anti-CD3 Ab (145-2C11), biotinylated anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61), allophycocyanin-H7-conjugated anti-CD8ε (53-6.7), Pacific Blue-conjugated anti-CD4 (RM4-5), allophycocyanin-conjugated anti-CD45.1 (A20), and allophycocyanin-conjugated streptavidin were obtained from BD Biosciences. Allophycocyanin-Alexa Fluor 750-conjugated anti-CD8ε Ab (53-6.7) and biotinylated anti-CD127 (ATK34) Ab were obtained from eBioscience. Pacific Blue-conjugated streptavidin was obtained from Invitrogen.

For intranuclear Foxp3 staining, cells were fixed and permeabilized with the eBioscience Foxp3 staining buffer set and then stained with PE-conjugated anti-Foxp3 Ab (FJK-16s). Four- and seven-color immunofluorescence analyses were carried out with a FACSCalibur flow cytometer and a BDLSRII flow cytometer, respectively (BD Biosciences). List-mode data files were analyzed with CellQuest and Diva software (BD Biosciences).

**In vitro culture assay**

LN cells were incubated on ice for 20 min with anti-CD8 (53-6.7), anti-CD11b (Mac-1), anti-GR1 (8C5), and anti-CD19 (1D3) Abs, obtained from hybridoma supernatants, and then with magnetic beads coupled to anti-rat Ig (Dynal Biotech). Purified CD4+ T cells were labeled with biotinylated anti-CD25 (clone PC61) Ab. Then, CD25+ and CD25− T cells were separated using MACS streptavidin MicroBeads (Miltenyi Biotec). CD25+ CD4+ T cells (50 × 10^3) and CD25− CD4+ T cells (50 × 10^3) were cultured separately in the presence or absence of recombinant human IL-2 (10 ng/ml) or recombinant mouse IL-7 (10 ng/ml) (both from R&D Systems).

**Calculations**

The average number of cell cycles was calculated as follows. First, we estimated the CFSE dilution factor (f): CFSE mean fluorescence intensity (MFI) of nondivided cells (cycle 0) divided by CFSE MFI of the entire corresponding to the vast majority of transferred CD4+ T cells, which had undergone a limited number of divisions, whereas the cytoplasmic dye had totally disappeared from others. Thus, as previously described by us and other investigators (13, 16–18), we confirmed that CD4+ T cells from normal C57BL/6 mice can be divided into two subsets with respect to their behavior after transfer into lymphopenic mice. The first subset (CFSE+ CD4+ T cells), corresponding to the vast majority of transferred CD4+ T cells, cycles very slowly; this process is called “homeostatic T cell proliferation.” The second subset (CFSE− CD4+ T cells) is generated by the strong expansion of a small proportion of injected CD4+ T cells (31). This process is termed “spontaneous proliferation” (17, 18) and requires interactions with MHC molecules (13).

To study the homeostatic proliferation of conventional and regulatory CD4+ T cells, we focused on CFSE+ CD4+ T cells. As previously reported (13), we observed a similar proliferation pattern of conventional CD4+ T cells (CFSE+ Foxp3− CD4+) in both recipient mice (Fig. 1A, 1B). This slow and limited proliferation of naive CD4+ T cells is known to result directly from the greater availability of IL-7 in lymphopenic environments (19, 32, 33). In contrast, although regulatory CD4+ T cells (CFSE− Foxp3+ CD4+) were able to proliferate even more strongly than were their conventional CD4+ T cell counterparts after transfer into lymphopenic MHC class II-expressing recipient mice, this proliferating capacity appeared to be strongly compromised in mice lacking the expression of MHC class II molecules (Figs. 1A, 1B). Consequently, the proportion of regulatory CD4+ T cells among CFSE+ CD4+ T cells decreased with time after transfer into CD3ε−/− mice lacking the expression of MHC class II molecules (Fig. 1C), reflecting, in this setting, a progressive dysregulation of the homeostatic balance between regulatory and conventional CD4+ T cells.
Lymphopenia-induced T cell spontaneous proliferation is thought to strictly depend on interactions with MHC molecules (13). Accordingly, very few CFSE\(^2\) cells were generated at early time points after transfer into mice lacking MHC class II molecules (Fig. 1A, Supplemental Fig. 1). We showed previously that these cells are, in fact, responding to MHC class I molecules (13). Interestingly, these rare cells included, in proportion, less regulatory CD4\(^+\) T cells than when recipient mice were expressing MHC class II molecules (Fig. 1D).

Thus, the greater availability of IL-7 found in lymphopenic environments is not able to induce homeostatic proliferation of regulatory CD4\(^+\) T cells by itself. As proposed by Cozzo et al. (24) and Hsieh et al. (25), our data suggest that, in contrast with their conventional CD4\(^+\) T cell counterparts, homeostatic T cell proliferation of regulatory CD4\(^+\) T cells in response to lymphopenia requires both IL-7 and TCR signaling.

We then compared the expression level of IL-7R\(^\alpha\) (CD127) on conventional and regulatory CFSE\(^+\) CD4\(^+\) T cells (Fig. 2). Park et al. (34) clearly demonstrated that high consumption of IL-7 led to a marked downregulation of its receptor, CD127. Thus, the study of CD127 expression on T cells in the course of peripheral T cell reconstitution may help us to evaluate whether IL-7 is consumed by these cells. Two days after their transfer into both CD3\(^\varepsilon\)/2 and CD3\(^\varepsilon\)/2 MHC II\(^D/D\) recipient mice, CD127 expression was strongly decreased in conventional CD4\(^+\) T cells (Fig. 2A).

This downregulation remained stable for 1 mo in MHC class II-expressing CD3\(^\varepsilon\)/2 recipient mice, whereas it rapidly returned to control levels in CD3\(^\varepsilon\)/2 MHC II\(^D/D\) recipient mice (Fig. 2B). This latter result may indicate that TCR signaling boosts IL-7 consumption and subsequent IL-7R downregulation. In contrast, at all studied time points, CD127 was not downregulated at the cell surface of regulatory CD4\(^+\) T cells in either group of recipient mice, indicating a weak consumption of IL-7 by these cells.

Taken together, these data suggest that, in contrast to their conventional CD4\(^+\) T cell counterparts, homeostatic proliferation of the regulatory CD4\(^+\) T cell pool in response to lymphopenia is independent of IL-7.

The extent of both homeostatic and spontaneous proliferations of regulatory CD4\(^+\) T cells in response to lymphopenia requires IL-2 rather than IL-7

IL-2 was shown to be crucial for regulatory CD4\(^+\) T cell homeostasis in the periphery. Moreover, IL-2 allows regulatory CD4\(^+\) T cells to proliferate in vitro in response to anti-CD3 Ab stimulation. Thus, we decided to compare, in vitro and in vivo, the effect of IL-2 and IL-7 on the survival and lymphopenia-induced proliferation of conventional and regulatory CD4\(^+\) T cells.

First, to ascertain whether IL-2 and/or IL-7 promote, in vitro, the survival of conventional and/or regulatory CD4\(^+\) T cells, CD25\(^+\) and CD25\(^+\) CD4\(^+\) T cell subsets from C57BL/6 mice were cul-
proliferation of regulatory CD4+ T cells observed in MHC class II-

expressing lymphopenic recipient mice. To address this question,
transfer. CD127 fluorescence graphs of CD4+ T cells from control C57BL/6
mice are also shown. The open graphs represent isotype-stained cells.
Number in each graph represents CD127 MFI index (CD127 MFI of the indicated recovered CD4+
T cells/CD127 MFI of conventional CD4+ T cells [Foxp3+ ε/ε] from control C57BL/6 mice.

FIGURE 2. CD127 expression on conventional and regulatory CD4+
T cells undergoing homeostatic proliferation in response to lymphopenia. CFSE-labeled LN CD4+ T cells (5 x 10^6) from C57BL/6 mice were injected i.v. into C57BL/6 CD3ε+/ε− mice and C57BL/6 CD3ε+/ε− MHC II^+/− mice. At various times after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (A) CD127 fluorescence graphs (filled graphs) of CFSE^− Foxp3+ CD4^+ CD3+ CD8− T cells and CFSE^− Foxp3− CD4^+ CD3+ CD8− T cells recovered 2 d after transfer. CD127 fluorescence graphs of CD4^+ T cells from control C57BL/6 mice are also shown. The open graphs represent isotype-stained cells. Number in each graph represents CD127 MFI. (B) CD127 expression of the indicated transferred cells at various times after transfer. Results are expressed as MFI index (CD127 MFI of the indicated recovered CD4^+ T cells/CD127 MFI of conventional CD4^+ T cells [Foxp3− ε/ε] from control C57BL/6 mice x 100). The dashed line represents the MFI index of Foxp3^− (left panel) and Foxp3+ (right panel) CD4^+ T cells from control C57BL/6 mice. Results are expressed as means ± SEM for two independent experiments with at least three mice per group and per day.

FIGURE 3. In vitro survival of regulatory CD4^+ T cells depends mainly
on IL-2 in the absence of TCR stimulation. Purified LN CD25^− and CD25^+ CD4^+ T cells from C57BL/6 mice were cultured separately for 3 d with medium alone or in the presence of IL-2 or IL-7. (A) Absolute numbers of recovered CD4^+ T cells 1, 2, and 3 d after the onset of culture. (B) Proportion of CD4^+ T cells expressing Foxp3 1, 2, and 3 d after the onset of culture. Black-, gray-, and white-filled symbols represent individual experiments. *p < 0.05, **p < 0.01.

As previously reported by many groups, IL-7 deprivation had only a modest and nonsignificant effect on lymphopenia-induced T cell spontaneous proliferation (Fig. 4D). In contrast, IL-2 deprivation resulted in a strong decrease in the proportion of regulatory CD4^+ T cells among CFSE^− CD4^+ T cells (Fig. 4C). Anti–IL-2R Abs preferentially affected the homeostatic proliferation of conventional CD4^+ T cells (Fig. 4A, 4B). Consequently, this latter treatment resulted in a strong increase in the proportion of regulatory CD4^+ T cells among CFSE^− CD4^+ T cells (Fig. 4C). Anti–IL-7R Abs have a weak, although significant, effect on the homeostatic proliferation of regulatory CD4^+ T cells.

IL-2 alone is sufficient to drive regulatory T cell homeostatic proliferation

A total of 5 x 10^6 CFSE-labeled CD4^+ T cells was injected into CD3ε+/ε− II^+/− mice treated or not with anti–IL-7R, anti–IL-2, or both blocking Abs (Fig. 4). Fourteen days after transfer, homeostatic proliferation of regulatory CD4^+ T cells was clearly reduced in mice treated with anti–IL-2 Ab (Fig. 4A, 4B). Accordingly, anti–IL-2/Ab treatment resulted in a decrease of the proportion of regulatory CD4^+ T cells among CFSE^− CD4^+ T cells (Fig. 4C).

Accordingly, anti-IL-2 Ab treatment resulted in a decrease of the proportion of regulatory CD4^+ T cells among CFSE^− CD4^+ T cells (Fig. 4C). In contrast, we observed that administration of anti–IL-7R Abs preferentially affected the homeostatic proliferation of conventional CD4^+ T cells (Fig. 4A, 4B). Consequently, this latter treatment resulted in a strong increase in the proportion of regulatory CD4^+ T cells among CFSE^− CD4^+ T cells (Fig. 4C). Anti–IL-7R Abs have a weak, although significant, effect on the homeostatic proliferation of regulatory CD4^+ T cells.

Altogether, our results strongly suggest that, during immune reconstitution, the balance between the regulatory and the conventional CD4^+ T cell compartments is closely related to IL-2 and IL-7 levels in vivo.
agonist complexes partially restored the homeostatic proliferation of regulatory CD4+ T cells injected into lymphopenic mice lacking expression of MHC class II molecules, whereas the homeostatic proliferation of conventional CD4+ T cells remained unchanged (Fig. 5A). More precisely, we observed a 6-fold increase in the average number of divisions of regulatory CD4+ T cells when CD3ε−/− IIα−/− mice were treated with IL-2/anti–IL-2 complexes (Fig. 5B). Consequently, this treatment resulted in a significant increase in the proportion of regulatory CD4+ T cells among CFSE+ CD4+ T cells (Fig. 5C). Thus, ILs are able to drive the homeostatic proliferation of both conventional and regulatory CD4+ T cells independently of TCR signaling. However, although the homeostatic proliferation of conventional CD4+ T cells is closely related to IL-7 levels, the proliferation of regulatory CD4+ T cells appears to be primarily controlled by IL-2.

As noted above (Fig. 1), in the absence of MHC class II molecules, very few regulatory T cells were contained within CFSE− CD4+ T cells recovered from the periphery of recipient mice 14 d after transfer. IL-2 treatment led to a significant increase in the proportion (Fig. 5D) and absolute number (Supplemental Fig. 2) of regulatory T cells among CFSE− CD4+ T cells, confirming that IL-2 plays a role in the extent of regulatory CD4+ T cell spontaneous expansion in response to lymphopenia.

Regulatory CD4+ T cells receive help from conventional CD4+ T cells to undergo homeostatic proliferation in response to lymphopenia

Because we noticed that IL-2 was a crucial factor in driving the homeostatic proliferation of regulatory CD4+ T cells in response to lymphopenia, we speculated that such a process requires assistance from conventional T cells. To address this hypothesis directly, 1 × 10^6 FACS-sorted, regulatory CD4+ T cells from C57BL/6 Foxp3-GFP CD45.2 mice, labeled with CellTrace violet (CTv), were injected alone or with 5 × 10^6 conventional CD4+ T cells from C57BL/6 CD3ε−/− IIα−/− mice into CD45.1 CD3ε−/− mice. Proliferation of transferred regulatory CD45.2+ GFP+ CD4+ T cells was analyzed 14 d after transfer (Fig. 6). When injected alone, regulatory CD4+ T cells underwent homeostatic proliferation. This proliferation required MHC class II molecule expression and was strongly decreased by IL-2 deprivation (Fig. 6A, 6B). Interestingly, spontaneous proliferation of regulatory CD4+ T cells in response to lymphopenia was completely abolished in the absence of MHC class II molecule expression. As described by Duarte et al. (36), when injected alone, some regulatory CD4+ T cells lost Foxp3 expression and underwent spontaneous proliferation in response to lymphopenia that induced CTv complete dilution and their accumulation as CTv− GFP− CD45.2+ CD4+ T cells. These converted cells may provide the IL-2 required for the homeostatic proliferation of regulatory CD4+ T cells. Interestingly, regulatory CD4+ T cell homeostatic proliferation was significantly increased when regulatory CD4+ T cells were coinfected with conventional T cells (Fig. 6C, 6D). Thus, the presence of conventional T cells at the moment of the transfer significantly increased the extent of regulatory T cell homeostatic proliferation, strongly suggesting that conventional CD4+ T cells are providing the resources (IL-2) required for this process.

Discussion

Several situations lead to T cell lymphopenia, such as chemotherapy, radiotherapy, and viral infections. In the last decade,
several groups, including our own, have tried to decipher the mechanisms that could help to replenish the peripheral T cell pool after a lymphopenic episode. On one hand, it was shown that the bulk of naive T cells proliferate slowly in response to lymphopenia. Such a process has been termed “homeostatic proliferation,” and it relies on both TCR signaling and the greater availability of IL-7 found in lymphopenic environments (13, 37, 38); these are the two main factors ensuring naive T cell survival in a nonlymphopenic environment (10). On the other hand, in the case of profound lymphopenia, some naive CD4+ T cells are able to proliferate and expand strongly, a process known as spontaneous proliferation, which relies primarily on interactions with MHC class II molecules (18, 31).

**FIGURE 5.** IL-2 is able to induce the proliferation of regulatory CD4+ T cells independently of self-recognition. CFSE-labeled LN CD4+ T cells (5 x 10⁶) from C57BL/6 mice were injected into C57BL/6 CD3ε⁻⁻ mice and C57BL/6 CD3ε⁻⁻/L2⁻⁻ mice treated or not treated with IL-2/anti-IL-2 complexes. Fourteen days after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (A) Foxp3/CFSE dot plots of CD4+ CD3+ CD8⁻ T cells recovered from representative recipient mice 14 d after transfer. (B) Average number of cell cycles of CFSE⁻ Foxp3⁻ CD4+ CD3+ CD8⁻ T cells and CFSE⁺ Foxp3⁺ CD4+ CD3+ CD8⁻ T cells 14 d after transfer. (C) Proportion of CFSE⁺ CD3+ CD4+ CD8⁻ T cells expressing Foxp3. Results are shown as means ± SEM for two independent experiments with at least three mice/group. (D) Proportion of CFSE⁺ CD3+ CD4+ CD8⁻ T cells expressing Foxp3. Results are shown as means ± SEM for two independent experiments with at least three mice/group. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 6.** Regulatory CD4+ T cells need help from conventional CD4+ T cells to undergo homeostatic proliferation in response to lymphopenia. A total of 1 x 10⁶ Foxp3-GFP⁺ CD4+ T cells from the LNs of C57BL/6 Foxp3-GFP mice was FACS sorted, labeled with CTV, and injected i.v. into lymphopenic C57BL/6 CD3ε⁻⁻ mice and C57BL/6 CD3ε⁻⁻ mice treated or not treated with IL-2-anti-blocking Abs. (A) Foxp3-GFP/CTV dot plots of CD4+ CD3+ CD8⁻ T cells recovered from representative recipient mice 14 d after transfer. (B) Average number of cell cycles of recovered CTV⁺ Foxp3-GFP⁺ CD4+ CD3+ CD8⁻ T cells, 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group. A total of 1 x 10⁶ Foxp3-GFP⁺ CD4+ T cells from the LNs of C57BL/6 Foxp3-GFP CD45.2 mice was FACS sorted, labeled with CTV, and injected i.v. alone or with 5 x 10⁶ CD25⁻ CD4⁺ T cells from normal C57BL/6 CD45.1 mice into lymphopenic CD45.1 C57BL/6 CD3ε⁻⁻ mice. Fourteen days after transfer, LNs and spleen were recovered and pooled, and single-cell suspensions were prepared. (C) Foxp3-GFP/CTV dot plots of CD45.2⁺ CD4⁺ CD3+ CD8⁻ T cells recovered from representative recipient mice 14 d after transfer. (D) Average number of cell cycles of recovered CTV⁺ Foxp3-GFP⁺ CD45.2⁺ CD4⁺ CD3+ CD8⁻ T cells 14 d after transfer. Results are shown as means ± SEM for two independent experiments with at least three mice/group. **p < 0.01, ***p < 0.001.
In contrast, little is known about the factors mediating the proliferation of regulatory CD4+ T cells in response to lymphopenia. We show that IL-7 is not involved in the spontaneous proliferation of both regulatory and conventional CD4+ T cells in response to lymphopenia. Furthermore, although IL-7 is crucial for naive CD4+ T cell homeostatic proliferation in response to lymphopenia, it has minimal impact on the homeostatic proliferation of regulatory CD4+ T cells. These results agree with previous data showing that the maintenance of the regulatory CD4+ T cell pool in the periphery does not require IL-7 (39). Nevertheless, as shown by us (Fig. 3) and other investigators (40), IL-7 is able to significantly increase regulatory CD4+ T cell survival in vitro. Thus, although regulatory CD4+ T cells express very low surface levels of IL-7R, they are still able to integrate IL-7-mediated signals. However, the concentrations of IL-7 used in in vitro assays (10–50 ng/ml) are higher than the concentrations reached, in vivo, in a lymphopenic environment [=50 pg/ml in the serum of T cell-deficient mice (19)]. Interestingly, after injection into lymphopenic recipient mice expressing or not expressing MHC class II molecules, IL-7R surface levels on conventional CD4+ T cells decreased to the levels observed on regulatory CD4+ T cells but not to lower levels. Altogether, our results suggest that IL-7R expression on regulatory CD4+ T cells may be too low to enable them to respond to the IL-7 levels reached in a lymphopenic environment. In line with this hypothesis, it was shown that regulatory CD4+ T cells are able to proliferate in vivo in response to the high levels of IL-7 reached in IL-7-transgenic mice or in mice injected with high concentrations of exogenous IL-7/anti–IL-7 complexes (41, 42).

The data presented in this article demonstrate that IL-2, a key cytokine for the development of regulatory CD4+ T cells in the thymus and their homeostasis in the periphery, is also crucial to drive their proliferation in response to lymphopenia. More precisely, the extent of both spontaneous and homeostatic proliferation of regulatory CD4+ T cells in response to lymphopenia is strongly diminished by blocking IL-2 through injection of anti–IL-2 Abs. Such a conclusion may seem contradictory to previous data showing that IL-2 neutralization does not affect the lymphopenia-induced proliferation of regulatory CD4+ T cells (20). However, in this study, the investigators injected few CD4+ T cells (3 × 10^5) into lymphopenic recipients and analyzed their proliferation quite soon after transfer (4 d). In fact, they were only studying lymphopenia-induced spontaneous proliferation of regulatory T cells (homeostatic proliferation has not started after only 4 d) and, with such a protocol, the T cell compartment was far from being replenished at that time point. In the current study, by injecting 5 × 10^6 CD4+ T cells, reconstitution was already completed 1 wk after transfer (Supplemental Fig. 1), and we studied the effect of IL-2 neutralization 1 wk later (Fig. 4). It may be that IL-2 plays a role in increasing regulatory T cell half-life once the T cell compartment has been filled, thus allowing their accumulation with time rather than being required for the proliferation process itself. Such a hypothesis fits with our previous study suggesting that IL-2 is not absolutely required for regulatory T cell spontaneous proliferation in response to lymphopenia (31), as well as with previous data demonstrating that, after reconstitution of the peripheral T cell pool, the number of regulatory T cells is indexed to the number of IL-2–producing cells (43, 44).

Cozzo et al. (24) and Hsieh et al. (25), using TCR-transgenic regulatory CD4+ T cells, proposed that self-peptides drive the proliferation of regulatory CD4+ T cells in response to lymphopenia. Our data showing that regulatory T cells do not undergo homeostatic proliferation after transfer into T cell-deficient mice lacking the expression of MHC class II molecules support such a conclusion. However, as proposed by Carneiro et al. (45), our data may reflect, in part, an indirect role for interactions with MHC class II molecules. Indeed, such interactions could also be required to allow conventional CD4+ T cells to produce IL-2 that, in turn, drives the homeostatic proliferation of regulatory CD4+ T cells. Accordingly, our data strongly suggest that regulatory CD4+ T cells receive help from conventional CD4+ T cells to undergo homeostatic proliferation in response to lymphopenia. Moreover, IL-2 is able to drive regulatory CD4+ T cell homeostatic proliferation independently of interactions with MHC class II molecules. Indeed, injection of IL-2–agonist complexes partially restores the homeostatic proliferation of regulatory CD4+ T cells transferred into recipient mice deficient for the expression of MHC class II molecules. Thus, interactions with MHC class II molecules are required for the bulk of regulatory T cells to proliferate slowly in response to lymphopenia; however, it is difficult to determine whether such interactions act directly by stimulating regulatory T cells, indirectly by promoting IL-2 production by conventional T cells, or both.

Altogether, our data suggest that, although the homeostatic proliferation of conventional CD4+ T cells in response to lymphopenia is closely related to IL-7 levels, both the homeostatic and spontaneous proliferation of regulatory CD4+ T cells appear to be primarily controlled by IL-2. Accordingly, IL-2 therapy in lymphopenic patients leads to increases in regulatory-like CD4+ T cell counts at the expense of the conventional T cell compartment, leading to altered responses to pathogens (46–48). Conversely, administration of IL-7 in humans induces expansion of naive and memory T cell subsets (49–51) and, in some clinical trials, a relative decrease in the percentage of regulatory CD4+ T cells was observed (52). Thus, on one hand, the capacity of IL-7 to augment conventional T cell proliferation with minimal concomitant regulatory T cell expansion may be clinically exploitable in the treatment of patients with lymphopenia, especially in the case of chronic viral diseases (53) or cancer immunotherapy (54). On the other hand, increased systemic IL-7 levels during lymphopenia may lead to an imbalance between the conventional and regulatory T cell compartments at the expense of regulatory T cells and may exacerbate deleterious immune reactions, such as graft-versus-host disease (55) or autoimmunity (56–58).

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Disclosures
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References
IL-2 AND IL-7 IN LYMPHOPENIA-INDUCED T CELL PROLIFERATION


