IL-2 coordinates IL-2–producing and regulatory T cell interplay

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Many species of bacteria use quorum sensing to sense the amount of secreted metabolites and to adapt their growth according to their population density. We asked whether similar mechanisms would operate in lymphocyte homeostasis. We investigated the regulation of the size of interleukin-2 (IL-2)–producing CD4+ T cell (IL-2p) pool using different IL-2 reporter mice. We found that in the absence of either IL-2 or regulatory CD4+ T (T reg) cells, the number of IL-2p cells increases. Administration of IL-2 decreases the number of cells of the IL-2p cell subset and, pertinently, abrogates their ability to produce IL-2 upon in vivo cognate stimulation, while increasing T reg cell numbers. We propose that control of the IL-2p cell numbers occurs via a quorum sensing–like feedback loop where the produced IL-2 is sensed by both the activated CD4+ T cell pool and by T reg cells, which reciprocally regulate cells of the IL-2p cell subset. In conclusion, IL-2 acts as a self-regulatory circuit integrating the homeostasis of activated and T reg cells as CD4+ T cells restrain their growth by monitoring IL-2 levels, thereby preventing uncontrolled responses and autoimmunity.

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Abbreviations used: T reg, regulatory T.

The central role of regulatory CD4+FOXP3+ T (T reg) cells in self-tolerance and in the control of autoimmune diseases is well established (Shevach, 2000; Malek and Castro, 2010; Josefowicz et al., 2012). It has also been demonstrated that IL-2–IL-2R signaling pathways play a major role in T reg cell biology. Mice genetically deficient for IL-2 (Schorle et al., 1991; Sadlack et al., 1995; Wolf et al., 2001), IL-2Rα (Willerford et al., 1995), IL-2Rβ (Suzuki et al., 1995; Malek et al., 2000), or STAT5 (the transcription factor downstream of the IL-2R signaling; Snow et al., 2003; Burchill et al., 2007; Yao et al., 2007) lack or have reduced numbers of T reg cells and develop lethal lymphoid hyperplasia and autoimmune diseases. In fact, IL-2 is required for the survival and expansion of T reg cells; T reg cells from IL-2–deficient donors fail to survive in IL-$2^{-/-}$ hosts (Almeida et al., 2006) or to expand in the absence of IL-2R signals (Almeida et al., 2002, 2006; Fontenot et al., 2005b). Blocking IL-2R (Bayer et al., 2005) or neutralizing IL-2 (Setoguchi et al., 2005) reduces T reg cell numbers. IL-2 also plays a role in the stability of FOXP3 expression and FOXP3-dependent gene signature (Gavin et al., 2002; Hill et al., 2007; Yu et al., 2009). Although these studies demonstrated that IL-2 is an essential resource for T reg cells, the mechanisms regulating the critical cell source providing IL-2 remained to be identified.

Previous observations indicated that αβ T cells represent the major source of the IL-2 required for maintaining normal population size of T reg cells and for the fulfillment of their regulatory role (Almeida et al., 2006). Using a strategy of mixed BM chimeras where IL-2–deficient

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hosts ($Rag2^{-/-}IL\text{-}2^{-/-}$) were reconstituted with precursor cells from IL-2–deficient ($IL\text{-}2^{-/-}$) donors together with precursor cells from either $TCR\alpha^{-/-}$ (providing a non-T cell hematopoietic source of IL-2) or $CD25^{-/-}$ IL-2-sufficient donors (providing a T cell source of IL-2), it was shown that only the chimeras comprising a population of IL-2-sufficient T cells showed relative frequencies of T reg cells similar to those of normal mice and were protected from death (Almeida et al., 2006). The mixed BM chimeras that received precursor cells from the $TCR\alpha^{-/-}IL\text{-}2^{+}$ donors and whose T cells were IL-2–deficient, contained a minor population of T reg cells, but were not rescued from death. Moreover, BM chimeras obtained by rescuing IL-2–competent hosts ($Rag2^{-/-}IL\text{-}2^{+}$) with similar mixes of IL-2–deficient and IL-2-sufficient hematopoietic precursors only survived if they contained populations of IL-2–sufficient T cells (Almeida et al., 2006). Thus, IL-2 produced by the host's nonhematopoietic cells or by non-T, BM-derived cells was not sufficient to generate/maintain a fully functional cohort of T reg cells able to prevent autoimmune disease and death (Almeida et al., 2006).

At steady state, IL-2 is produced mainly by $CD4^{+}$ T cells and, to a lesser extent, by $CD8^{+}$ T, NK, and dendritic cells (Setoguchi et al., 2005; Almeida et al., 2006; Malek, 2008). Because $CD4^{+}$ T reg cells themselves are unable to produce IL-2 because of FOXP3-dependent repression of the *il2* gene (Wu et al., 2006; Ono et al., 2007), the corollary is that T reg cells rely mainly on IL-2 produced by other T cells. Of note, IL-2–deficient T reg cells expanded when co-transferred with $IL\text{-}2^{+}CD4^{+}$ T cells but not when alone or together with $IL\text{-}2^{-/-}CD4^{+}$ T cells (Almeida et al., 2006). Of relevance, in chimeras containing a mix of IL-2–competent and IL-2–deficient BM cells, there was a direct correlation between the fraction of IL-2–competent hematopoietic cells and the fraction of $CD4^{+}$ T reg cells lately generated in the chimeras (Almeida et al., 2006). On the whole, these findings indicate that IL-2–producing (IL-2p) T cells must play a master role in the immune system homeostasis as they are essential for the maintenance of T reg cell populations (Almeida et al., 2006, 2012).

Here, we investigated the mechanisms underlying the control of IL-2p T cells. We used lines of IL-2 reporter mice and studied the behavior of IL-2 reporter–expressing $CD4^{+}$ T cells in different experimental conditions: in the absence or in the presence of excess of IL-2, in mouse chimeras bearing different proportions of IL-2–competent precursors, and in the absence of T reg cells. Our findings suggest that the subset of IL-2p T cells is regulated by quorum sensing–like mechanisms dependent on both IL-2 and T reg cell numbers.

RESULTS

IL-2 reporter mice

In this study, we used different lines of IL-2 reporter mice, generated either by replacing the *il2* gene with a single copy of a cDNA coding for green fluorescent protein (Ki/Ko.GFP/IL-2; Naramura et al., 1998) or containing 33 copies of a transgene encoding for GFP under the control of an 8.4-kb IL-2 promoter (Tg IL-2-GFP; Yui et al., 2001). We also used a transgenic IL-2.BAC-inThy1.1 (2BiT) reporter mice expressing Thy1.1 under the control of the IL-2 promoter (Luther, 2009). In all lines, reporter expression (GFP or Thy1.1) has been shown to correlate with T cell activation coupled to IL-2 production; naive T cells required stimulation to become reporter positive (Naramura et al., 1998; Yui et al., 2001; Luther, 2009; Fig. S1). It should be pointed out that the two lines of GFP reporter mice show distinct but complementary characteristics and phenotypes due to the different properties of IL-2 and GFP and the different number of GFP gene copies present. Indeed, IL-2 is only transiently transcribed and because its mRNA is swiftly degraded and the protein secreted, it is rapidly lost from the cells. In contrast GFP synthesis is longer but has a half-life of 36–54 h (McCaughtry et al., 2007), allowing it to accumulate in the cell with detectable expression lasting up to 2–3 wk, depending on the initial protein levels. In the Ki/Ko.GFP/IL-2 mice containing a single gene copy, GFP expression was low and short lasting, with ∼1–2% of the $CD4^{+}$ T cells being GFP^{+} (Fig. 1 a); in contrast, in the Tg IL-2-GFP mouse, transcription of 33 copies lead to higher levels of GFP produced/accumulated labeling ∼10–15% of the $CD4^{+}$ T cells as GFP^{+} (Fig. 1 b). In the BAC transgenic 2BiT mice, expression of the Thy1.1 reporter was similar to that observed in Ki/Ko.GFP/IL-2 mice (Fig. 1 c). Reporter expression by $CD8^{+}$ T cells and other non-T cells (dendritic cells) in the Ki/Ko mice was <0.1%, and ∼1–3% in the TgIL-2-GFP mice (not depicted). In conclusion, depending on the mouse strain, *il2* reporter expression acts either as a marker of recent IL-2 gene activation (in Ki/Ko and 2BiT mice) or as a tracer (in Tg IL-2-GFP mice) of a subset of cells having activated the IL-2 locus within the last 2–3 wk (IL-2p cells), which might prompt the cells to engage in future IL-2 production.

Interestingly, in wild-type B6 mice, after in vivo administration of anti-CD3 or in vitro stimulation with PMA and ionomycin, 13–32% of the mouse spleen $CD4^{+}$ T cells were readily induced to IL-2 secretion (Fig. 1, d and e). Multiple lines of evidence suggest that in Tg IL-2-GFP mice, IL-2-GFP^{+} and GFP^{-} $CD4^{+}$ T cells may represent distinct functional cell subsets. We compared the fate of sort-purified $CD4^{+}$IL-2-GFP^{+} and $CD4^{+}GFP^{-}$ cells from TgIL-2-GFP. $Ly5^{b}$ donors upon transfer into either intact $B6.Ly5^{a}$ or T cell deficient hosts. 1 wk after transfer into intact $B6.Ly5^{a}$ hosts cell division tracer labeled $CD4^{+}GFP^{-}$ and $CD4^{+}GFP^{+}$ T cells did not proliferate and maintained a relatively stable GFP-expression phenotype with a slight decay of GFP expression among the GFP^{+} cells and acquisition of GFP-expression by some of the original GFP^{-} cells (Fig. 2 a). In immune-deficient $CD3\varepsilon^{-/-}$ hosts both cell subsets proliferated and modified reporter-expression due both to activation, inducing GFP expression among a fraction of the GFP^{-} cells, with cell division/selection causing dilution/loss of GFP among the injected GFP^{+} population (Fig. 2 b). Most importantly, $CD4^{+}$ GFP^{-} and $CD4^{+}GFP^{+}$ T cells showed distinct expansion/accumulation capacities. Thus, 1 wk after transfer the fraction of fast diving cells was higher among GFP^{+} T cells in agreement with their recent activation state and corresponding IL-2

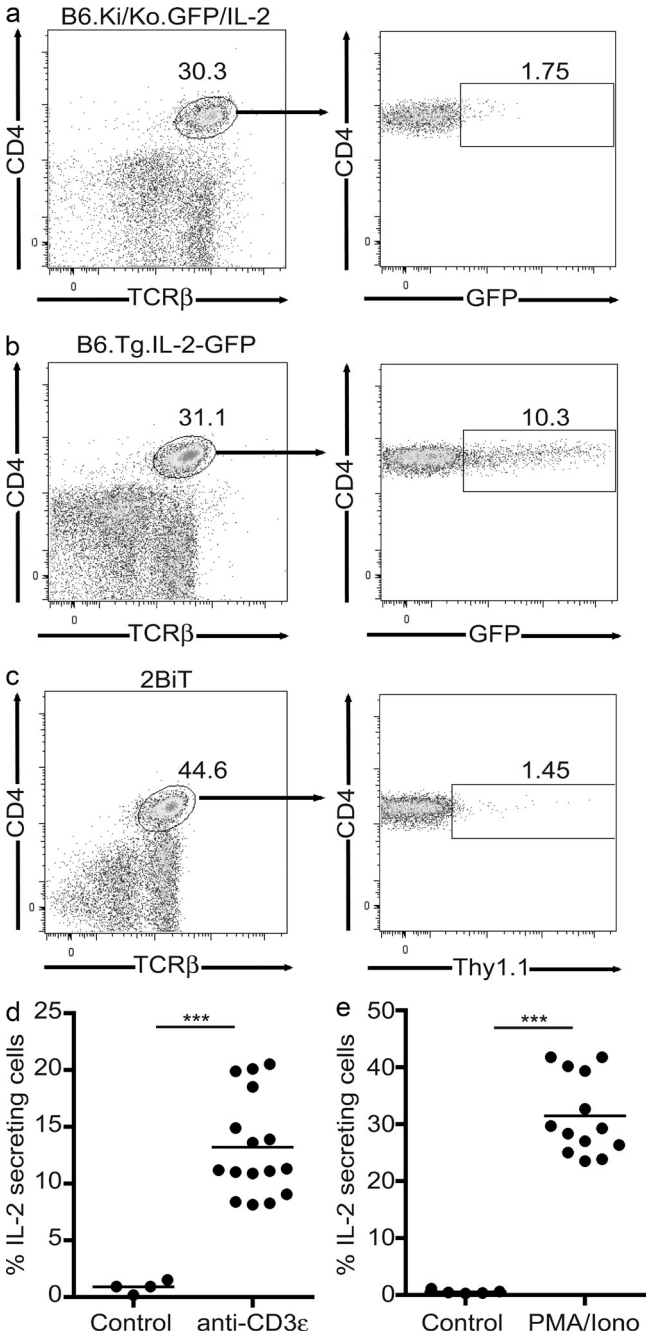

Figure 1. Reporter mice. Representative flow cytometry plots show the expression of GFP by gated αβCD4+ T cells in the lymph nodes (LN) from B6.Ki/Ko.GFP/IL-2 (a), B6.Tg.IL-2-GFP (b), and 2BiT reporter mice (c). (d) Fraction of IL-2–secreting cells recovered from the spleen of WT B6 mice 3 h after in vivo injection with 50 µg/per mouse of anti-CD3ε mAb. (e) Fraction of IL-2–secreting cells recovered from the spleen of WT B6 mice 4 h after in vitro culture with PMA and ionomycin. Each point represents a single mouse. The bar represents the mean value.

reporter expression. This observation suggests that the subset of GFP+ T cells may be enriched cells in a different functional state that rapidly proliferate in a lymphopenic environment. 6 wk later, CD4+ T cell recovery was 10-fold higher in the mice injected with GFP− cells than in those receiving GFP+ cells (Fig. 2 c) suggesting that the progeny of the GFP+ cell subset has lower accrual capacity and fails to survive in the host. Finally, transcriptome analysis showed that GFP− and GFP+ CD4+ T cells from Tg IL-2-GFP mice display distinct gene expression patterns confirming their different functional state (Fig. 2 d). Indeed, GFP+ cells express higher levels of protein kinases transcripts, in particular those associated with the MAPK pathway, confirming the different activation state (Fig. S2).

Increased number of CD4+IL-2-GFP+ reporter cells in the absence of IL-2

We investigated the parameters controlling the number of the CD4+IL-2-GFP+ cells at steady state. CD4+IL-2-GFP+ cells could either be an autonomous cell population representing a constant fraction of the total number of CD4+ T cells or they could be modulated by environmental factors, e.g., IL-2, fluctuating independently of the number of CD4+ T cells. To investigate the possible role of IL-2 in the control of the IL-2p CD4+ T cell subset, we derived IL-2–deficient B6.Ki/Ko.GFP/IL-2 and B6.TgIL-2-GFP mice. In both IL-2–deficient reporter strains, the fraction of CD4+GFP+ T cells was significantly increased both in the peripheral T cell pool and in the thymus. Thus, the fraction of IL-2 reporter (GFP+) in the peripheral CD4+ T cell pool of B6.Ki/Ko.GFP/IL-2 mice augments from <2% in the presence of IL-2 to ∼10–15% in the absence of IL-2 (Fig. 3 a), and in the B6.TgIL-2-GFP strain it increases from 3–10% to ∼35–40% (Fig. 3 b). In the thymus SP CD4+ T cell compartment, the rise of the fraction of IL-2p cells were of lower magnitude (Fig. 3 c). These observations suggest that the lack of IL-2 leashes an increased "activation" of CD4+ T cells, which was translated in an increased fraction of IL-2p GFP+ cells. To exclude the possible contribution of sublatent inflammatory manifestations that may occur in IL-2–deficient mice, we conducted experiments where the fraction of precursor cells competent to produce IL-2 and GFP was varied at the precommitted stage. Lethally irradiated T cell–deficient CD3$\varepsilon^{-/-}$ hosts were reconstituted with combinations of BM precursor cells from IL-2–sufficient Ly5^b B6.Ki/Ko.GFP/IL-2 or Ly5^b B6.TgIL-2-GFP donors mixed at different ratios with IL-2–deficient Ly5^a B6.IL-2$^{-/-}$ BM donors (100:0, 50:50, 20:80, and 10:90, respectively). Control chimeras were reconstituted with a mix of progenitor cells from Ly5^b B6.TgIL-2-GFP and Ly5^a IL-2$^{wt/wt}$ mice. We studied the correlation between the numbers of IL-2-GFP–competent Ly5^b CD4+ T cells and the actual number of GFP+ cells recovered. In the control chimeras, reconstituted with mixes of IL-2–competent donors only, we observed a strong direct linear correlation ($r^2 = 0.91$) between the numbers of GFP+ and Ly5^b IL-2-GFP–sufficient CD4 T cells (Fig. 3 d, left). In contrast, in the chimeras that received different ratios of Ly5^b B6.Ki/Ko.GFP/IL-2 or Ly5^b B6.TgIL-2-GFP cells diluted among IL-2–deficient cells the direct correlation between the total number of CD4+Ly5^b IL-2–competent and IL-2p GFP+ T cells was lower ($r^2 = 0.59$ and 0.54, respectively; Fig. 3 d, middle) than that observed in the control chimeras (Fig. 3 d,

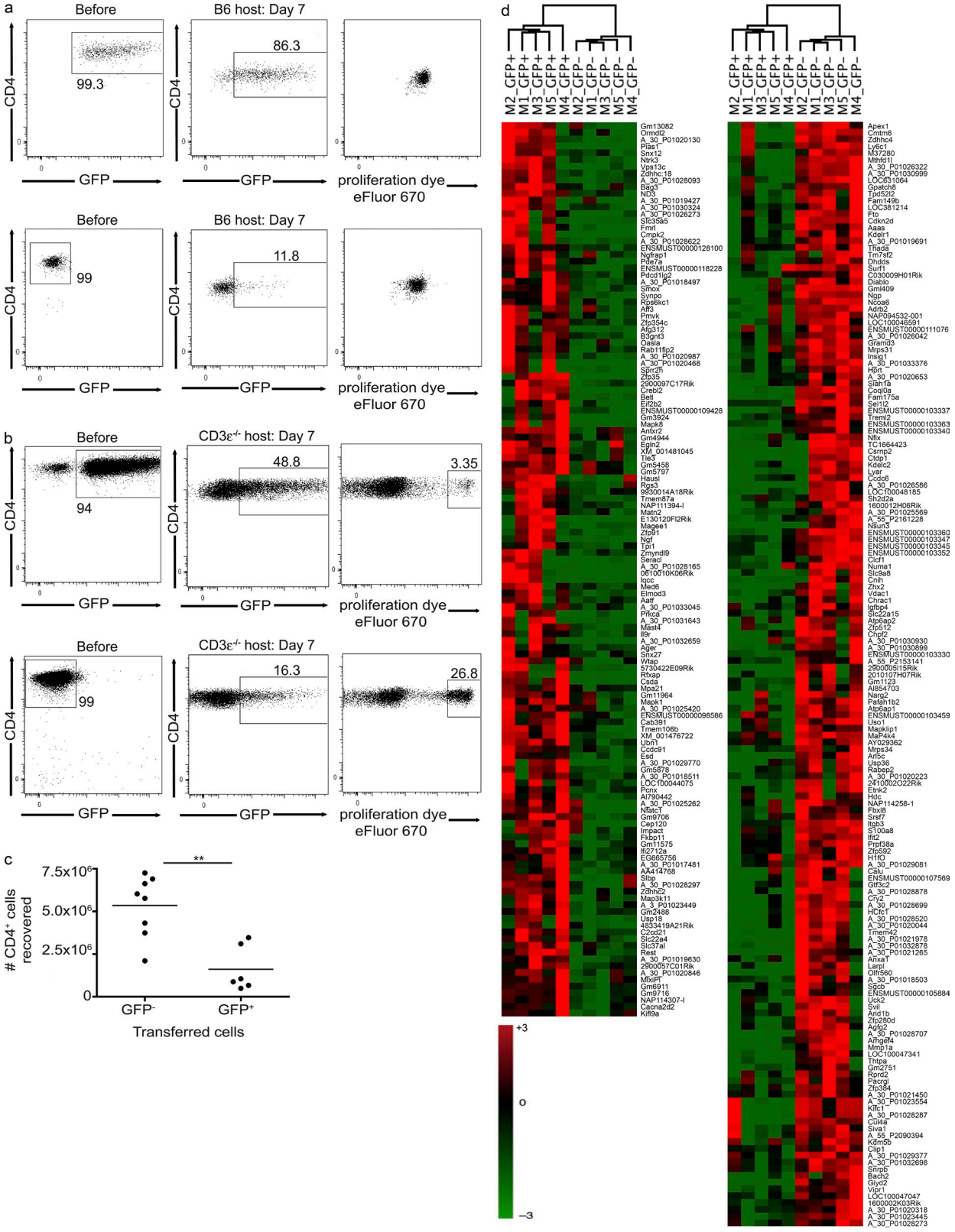

Figure 2. Properties of GFP⁻ versus GFP⁺ CD4⁺ T cells. (a, left) Representative dot plots for the GFP expression by sorted GFP⁺ (top) and GFP⁻ (bottom) CD4⁺ T cells from Ly5ᵇ Tg.IL-2-GFP mice before cell transfer. Middle panels show representative dot plots for the GFP expression by the same cells 7 d after transfer of 10^6 cells into WT Ly5ᵃ intact B6 hosts. Right panels show dilution of the proliferation dye eFluor 670 by the transferred cells 7 d after transfer. Differences in CD4 intensities before and after transfer were due to the use of different antibodies for staining (Phycoerythrin-cyanine7

left), suggesting that when diluted in an IL-2–deficient environment, the fraction of CD4$^+$IL-2-GFP–sufficient cells that activate the IL-2 locus increases (Fig. 3 d, right). We should point out that in the experimental chimeras receiving Ly5^b WT BM cells diluted among IL-2–deficient BM cells, peripheral reconstitution was in general poorer when compared with the control chimeras receiving WT BM cells only, probably due to a lower reconstitution capacity of the BM precursors from the IL-2–deficient donors. In general, we observed that the relative representation of GFP$^+$ cells increased when the number of CD4$^+$Ly5^b IL-2–competent cells decreased, diluted among IL-2–deficient cells (Fig. 3 d, right). Overall, these results obtained using two independent strategies suggest that the regulation of the number of cells of the IL-2p subset is not cell autonomous, i.e., was independent of the number of cells capable of producing IL-2, and that the IL-2p CD4$^+$ T cell subset can "adapt" to IL-2 deficiency by the progressive recruitment and accumulation of new IL-2p cells.

Increased numbers of CD4$^+$IL-2-GFP$^+$ reporter cells in mice with reduced T reg cells

The previous findings suggested the existence of IL-2–mediated feedback loops controlling the IL-2p subset, which could act either directly inhibiting T cell activation (Villarino et al., 2007) or indirectly as faulty T reg cell number and function were described in IL-2–deficient mice (Schorle et al., 1991; Almeida et al., 2002). We observed that in the BM chimeras containing variable numbers of IL-2-GFP–competent cells (Ly5^b B6.TgIL-2-GFP) diluted among IL-2–deficient (Ly5^a B6.IL-2$^{-/-}$) cells (Fig. 3 d) there was a strong linear correlation ($r^2 = 0.84$) between numbers of IL-2-GFP$^+$ cells and that of T reg cells (Fig. 3 e), suggesting a possible role for T reg cells in IL-2p cell homeostasis. We studied IL-2 reporter GFP expression by CD4$^+$ T cells in mice that have reduced T reg cell numbers, but where the ability to produce IL-2 is intact. In IL-2Rα–deficient strains with reduced numbers of T reg cells (Almeida et al., 2002), the fraction of GFP$^+$ cells in the peripheral CD4$^+$ T cell pool rises from 2 to 15–25% in B6.Ki/Ko.GFP/IL-2 (Fig. 4 a) and from ∼5–10 to 30–50% in B6.TgIL-2-GFP mice (Fig. 4 b). The relative representation of the GFP$^+$ cells was also slightly increased in the mature SP CD4$^+$ cell pool in the thymus (Fig. 4 c). It could be claimed that the increase in IL-2$^+$ reporter cells observed in these IL-2Rα–deficient mice was not caused by the reduced number of T reg cells, but caused by the inability of the cells that lack CD25 to respond to changes in IL-2 levels. It should be pointed out, however, that the vast majority of the CD4$^+$GFP$^+$ cells in both GFP reporter mice did not express CD25 (unpublished data) and remained under control.

We have previously shown that in chimeras reconstituted with BM cells from IL-2Rα–deficient donors, restitution of a population of T reg cells prevented the chaotic accumulation of lymphoid cells, and rescued the mice from death (Almeida et al., 2002). We examined whether mature T reg cells could also alter the fraction of CD4$^+$GFP$^+$ IL-2 reporter cells in B6.CD3ε$^{-/-}$ chimeras reconstituted with progenitor cells from B6.TgIL-2-GFP.IL-2Rα$^{-/-}$ donors. We found that the transfer of 2.5×10^5 purified Foxp3$^+$ T reg cells from FOXP3-mRFP donors sufficed to significantly reduce the fraction of CD25-deficient GFP$^+$ cells among the BM-derived CD4 T cells (Fig. 4, d and e). More important, in BM chimeras reconstituted with a 50:50 mixture of Ly5^a B6.TgIL-2-GFP.IL-2Rα$^{-/-}$ and Ly5^b B6.TgIL-2-GFP cells the representation of GFP$^+$ cells between the two Ly5^a CD25-deficient and Ly5^b CD25-sufficient CD4$^+$ T cells was identical (Fig. 4, d and f). Generally, these observations indicate that control of the IL-2p cell subset may occur independently of the ability of the cells to express IL-2Rα and suggest that T reg cells may have a direct role in their regulation.

We next investigated the effects of the conditional ablation of T reg cells in the number of IL-2p cells. We crossed FOXP3^{DTR} (Kim et al., 2007) with IL-2.BAC-inThy1.1 (2BiT) reporter mice expressing Thy1.1 under the control of IL-2 (Luther, 2009). The administration of diphtheria toxin (DT)–depleted T reg cells from male mice (Fig. 5 a) and provoked a significant increase in the number of Thy1.1$^+$CD4$^+$ reporter T cells (Fig. 5 a). To prevent the inflammatory response that follows strong T reg cell depletion, we titrated the dose of DT administered and varied the levels of T reg cell depletion (Fig. 5 b). In these conditions we obtained a negative linear relationship ($r^2 = 0.89$) between the fraction of IL-2p (Thy1.1$^+$) and T reg (FOXP3$^+$) cells, i.e., decreases in T reg cells correlated with significant increases of the fraction of IL-2 reporter$^+$ cells (Fig. 5 c). It should be pointed out that with low doses of DT, increases in the fraction of IL-2p cells was observed in the absence of any clinical signals of developing inflammatory disease.

Conclusively, in 2-wk-old B6.TgIL-2-GFP.FOXP3$^{sf/Y}$/J *scurfy* mice genetically deficient for *foxp3*, the fraction of CD4$^+$ GFP$^+$ rises from 8–10 to 15–25% in the peripheral T cell pool (Fig. 6 a) and from ∼1 to 3–4% among SP CD4$^+$ thymus cells (Fig. 6 b). Overall, these findings indicate that the T reg cell

coupled before transfer and Alexa Fluor 700 coupled after transfer). Similar results were obtained from a second independent experiment. (b) Representative dot plots for the GFP expression by sorted GFP$^+$ (top) and GFP$^-$ (bottom) CD4$^+$ T cells from Ly5^b Tg.IL-2-GFP mice 7 d after transfer of 10^6 cells into CD3ε$^{-/-}$ hosts (left). Right panels show dilution of the proliferation dye eFluor 670 by the transferred cells 7 d after transfer into CD3ε$^{-/-}$ hosts. Similar results were obtained from a second independent experiment. (c) The number of cells recovered in CD3ε$^{-/-}$ hosts 6 wk after the transfer of 5×10^4 purified GFP$^-$ and GFP$^+$ CD4$^+$ T cells from Ly5^b Tg.IL-2-GFP mice. Similar results were obtained from a second independent experiment. (d) Gene expression patterns of GFP$^-$ and GFP$^+$ CD4$^+$ T cells. The transcripts differentially expressed by GFP$^-$ and GFP$^+$ CD4$^+$ T cells from five independent cell preparations are displayed. Up- and down-regulated transcripts are indicated in red and green, respectively. The magnitude of expression is depicted by the color bar. Full raw data is available from the NCBI Gene Expression Omnibus under accession no. GSE50149.

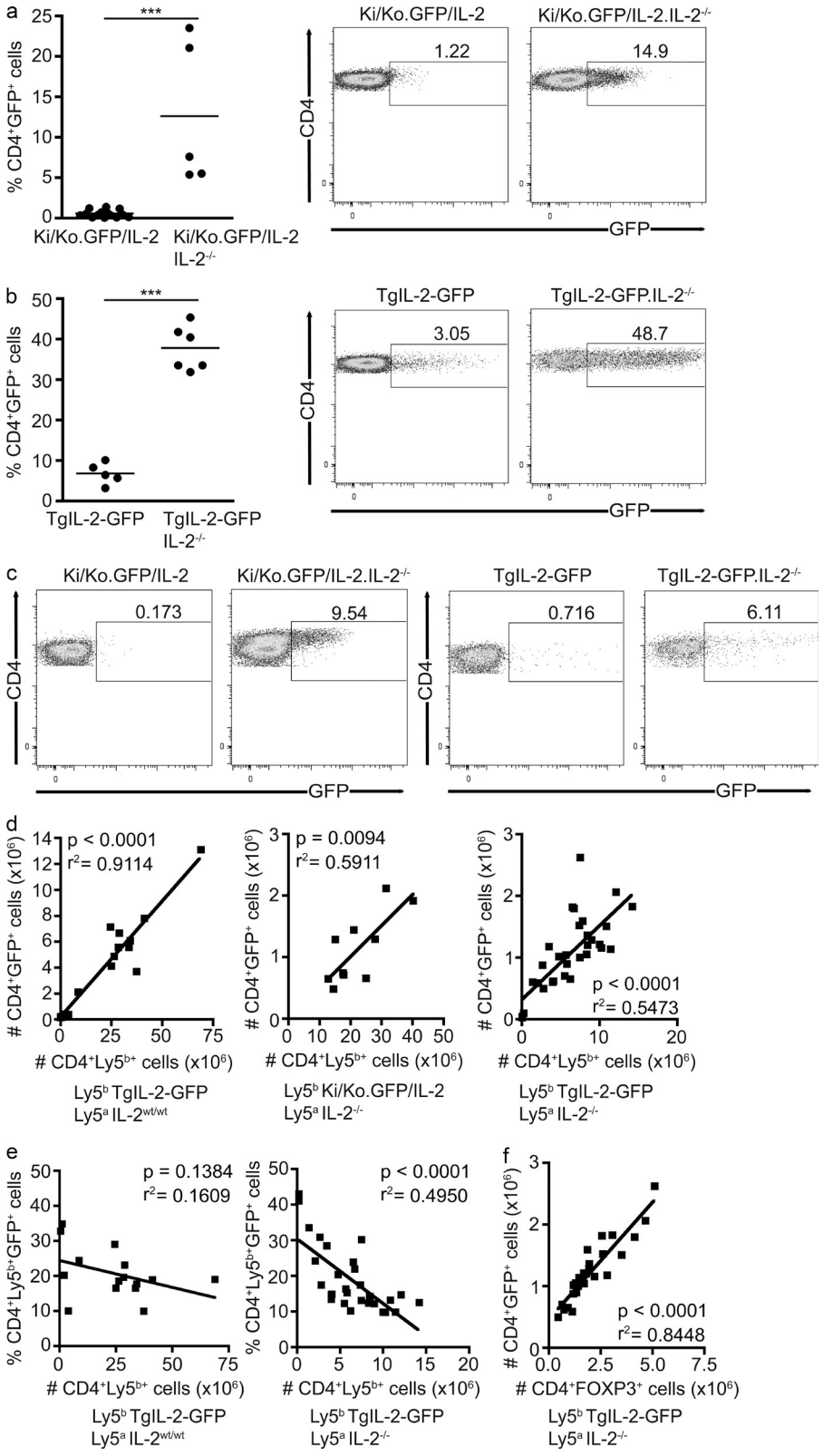

Figure 3. IL-2p cells in IL-2–deficient mice. IL-2–deficient mice were studied at 6 wk of age before the onset of signs of inflammatory disease. (a) The percentage of $CD4^+GFP^+$ T cell observed in the LN/spleen of heterozygous ($IL\text{-}2^{+/-}$) and homozygous ($IL\text{-}2^{-/-}$) B6.Ki/Ko.GFP/IL-2 reporter mice. (b) The percentage of $CD4^+GFP^+$ T cells observed in the LN/spleen of control $IL\text{-}2^{+/+}$ and $IL\text{-}2^{-/-}$ B6.Tg.IL-2-GFP reporter mice. For a and b, each point corresponds to different individual mice. The bar represents the mean value of the mice studied. Representative flow cytometry plots for the GFP expression on gated $\alpha\beta CD4^+$ T cells from the spleen of the different control and mutant mice are shown (right). (c) Shows representative dot plots for the GFP expression on gated $\alpha\beta CD4^+$ SP cells from the thymus of the different control and IL-2–deficient reporter mice studied in a and b. (d) Lethally irradiated $CD3^{-/-}$ mice were reconstituted with BM precursor cells from allotype different B6.$IL\text{-}2^{-/-}$ ($CD45.1-\ Ly5^a$) and $IL\text{-}2^+GFP^+$ ($CD45.2-\ Ly5^b$) reporter mice mixed in different proportions (0:100, 50:50, 80:20, 90:10, and 100:0). Mice were sacrificed 8 wk after reconstitution and the peripheral T cell subsets from the different donors analyzed. (left) The correlation between the number of $CD4^+GFP^+Ly5^b$ and total $CD4^+Ly5^b$ cells in control chimeras reconstituted with precursor cells from WT B6.$Ly5^a$ and Tg IL-2/GFP. $Ly5^b$; (middle left) the correlation between the number of $CD4^+GFP^+Ly5^b$ and total $CD4^+Ly5^b$ cells in the BM chimeras receiving different percentages of IL-2–competent precursor cells from Ki/Ko.GFP/IL-2.$Ly5^b$ donors diluted among precursors from IL-2 $Ly5^a$ deficient donors; (middle right) the correlation between the number of $CD4^+GFP^+Ly5^b$ and total $CD4^+Ly5^b$ cells in the BM chimeras receiving different percentages of IL-2–competent precursor cells from B6.Tg.IL-2-GFP.$Ly5^b$ donors diluted among precursors from IL-2 $Ly5^a$ deficient donors; (right panel) the correlation between the percentage of $CD4^+GFP^+Ly5^b$ and the number of $CD4^+Ly5^b$ cells in the BM chimeras receiving different percentages of IL-2–competent precursor cells from B6.Tg.IL-2-GFP. $Ly5^b$ donors diluted among precursors from IL-2 $Ly5^a$ deficient donors. (e) The correlation between the numbers of $CD4^+FOXP3^+$ and $CD4^+GFP^+$ cells present in the in the BM chimeras receiving different percentages of IL-2–competent precursor cells from Tg.IL-2-GFP. $Ly5^b$ donors diluted among precursors from IL-2 $Ly5^a$–deficient donors. Data are gathered from three independent experiments.

population can control the number of cells of the IL-2p subset. The existence of a mutually dependent regulation of T reg and IL-2p cell populations has been previously suggested (Almeida et al., 2006). These findings support the notion that the regulation of the two cell populations is tightly linked (Almeida et al., 2006, 2012): T reg cell numbers are dependent on the paracrine production of IL-2 by other T cells, which are themselves kept under control by T reg cells (Almeida et al., 2012).

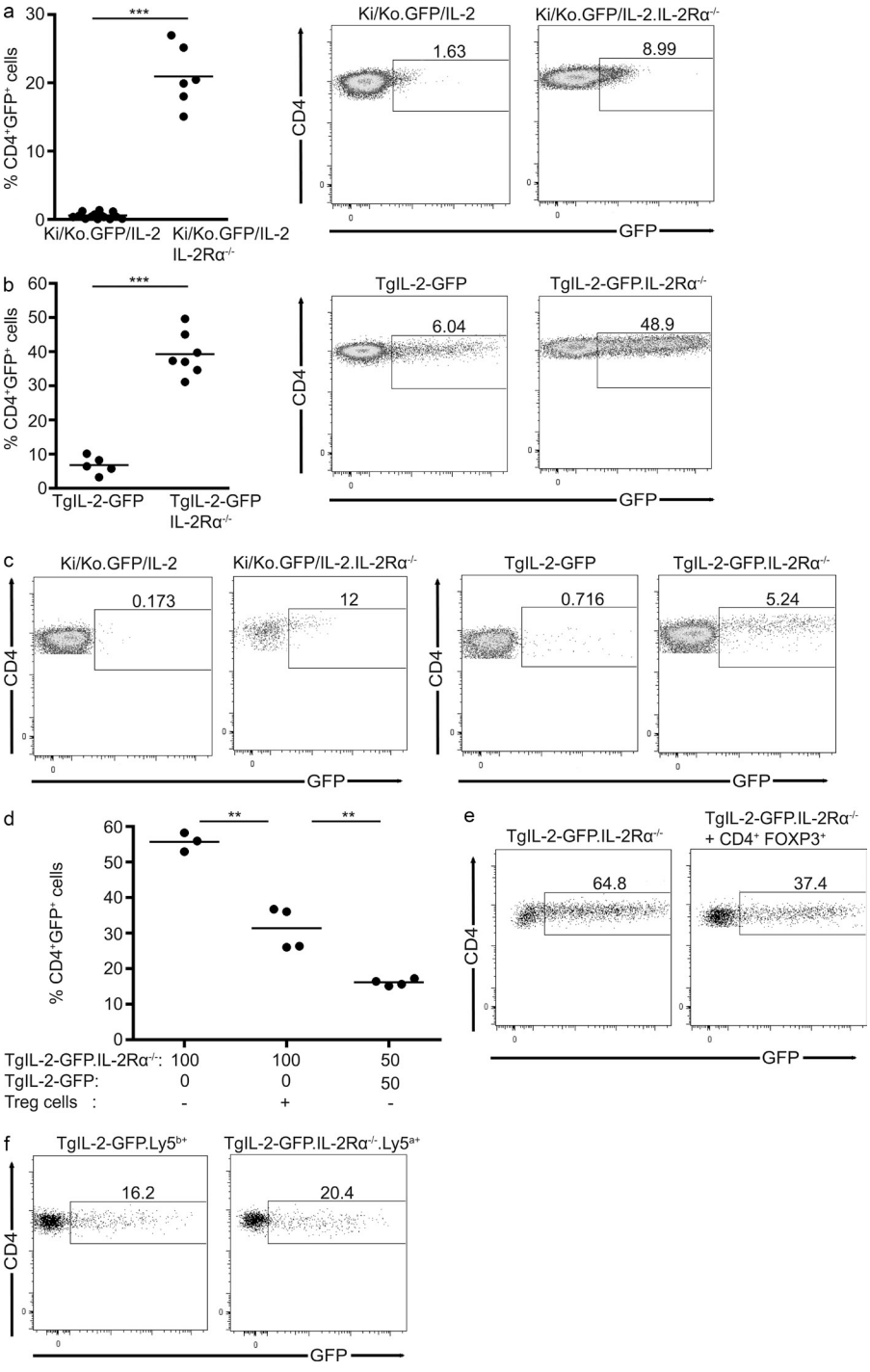

Figure 4. IL-2p cells in Il-2Rα–deficient mice. IL-2Rα-deficient mice were studied at 6 wk of age before the onset of signs of inflammatory disease. (a) The percentage of $CD4^+GFP^+$ T cell observed in the LN/spleen of control and $IL2R\alpha^{-/-}$ B6.Ki/Ko.GFP/IL-2 reporter mice (left). (b) The percentage of $CD4^+GFP^+$ T cell observed in the LN/spleen of control and $IL2R\alpha^{-/-}$ B6.Tg.IL-2-GFP reporter mice (left). For a and b each point corresponds to different individual mice. The bar represents the mean value of the mice studied. Representative flow cytometry plots for the GFP expression on gated $\alpha\beta CD4^+$ T cells from the spleen of the different control and mutant mice are shown (right). (c) Representative dot plots for the GFP expression on gated $\alpha\beta CD4^+$ SP cells from the thymus of the different control and IL-2Rα–deficient reporter mice studied in a and b. (d) Lethally irradiated $CD3^{-/-}$ mice were reconstituted with BM precursor cells from $IL2R\alpha^{-/-}$B6.Tg.IL-2-GFP.$Ly5^a$ donors. The following week, these chimeras received 2.5×10^5 purified $FOXP3^+$ T reg cells from FOXP3-mRFP donors. Alternatively, lethally irradiated $CD3^{-/-}$ mice were reconstituted with 50:50 mixtures of BM precursor cells from $IL2R\alpha^{-/-}$B6.Tg.IL-2-GFP.$Ly5^a$ donors and B6.Tg.IL-2-GFP.$Ly5^b$ donors. Mice were sacrificed 8 wk after reconstitution, and the peripheral T cell subsets from the different donors analyzed. Panel shows the percentage of $CD4^+GFP^+$ T cell observed in the different chimeras. Similar results were obtained in a second independent experiment. (e) Dot plots show the fraction of $CD4^+GFP^+$ T cells in chimeras which were either reconstituted with $IL2R\alpha^{-/-}$B6.Tg.IL-2-GFP.$Ly5^a$ cells alone (left) or received an additional transfer of T reg cells (right). Similar results were obtained in two independent experiments comprising groups of three to four mice each. (f) A representative dot plot with the percentage of $CD4^+$ GFP^+ T cells between the IL2Rα-sufficient (left) and the IL2Rα-deficient (right) cells present in the BM chimeras reconstituted with mixtures of progenitor cells from $IL2R\alpha^{-/-}$B6.Tg.IL-2-GFP.$Ly5^a$ donors and B6.Tg.IL-2-GFP.$Ly5^b$ donors. Similar results were obtained in two independent experiments comprising groups of 3–4 mice each.

Administration of IL-2 alters the number of $CD4^+$IL-2-GFP^+ reporter cells

We next investigated whether we could manipulate the number of IL-2p cells through IL-2 administration. B6.Ki/Ko.GFP/IL-2 and 2BiT IL-2 reporter mice were injected with either soluble recombinant mouse IL-2 or with cytokine antibody immune complexes (Boyman et al., 2006; Grinberg-Bleyer et al., 2010). Administration of both strategies of IL-2 increased the frequency and number of $CD4^+FOXP3^+$ T reg cells in the peripheral pools of both IL-2 reporter lines (Fig. 7, a and b) without affecting thymus cellularity (not depicted). IL-2 treatment increased T reg cell number was caused by enhanced survival rather than by promoting their proliferation. As shown in Fig. 7, after the administration of IL-2/anti–IL-2 JES6-1A12 immune complexes, the expression of $Caspase3^+$ by the peripheral $FOXP3^+CD4^+$ cells from the IL-2 2BiT–treated

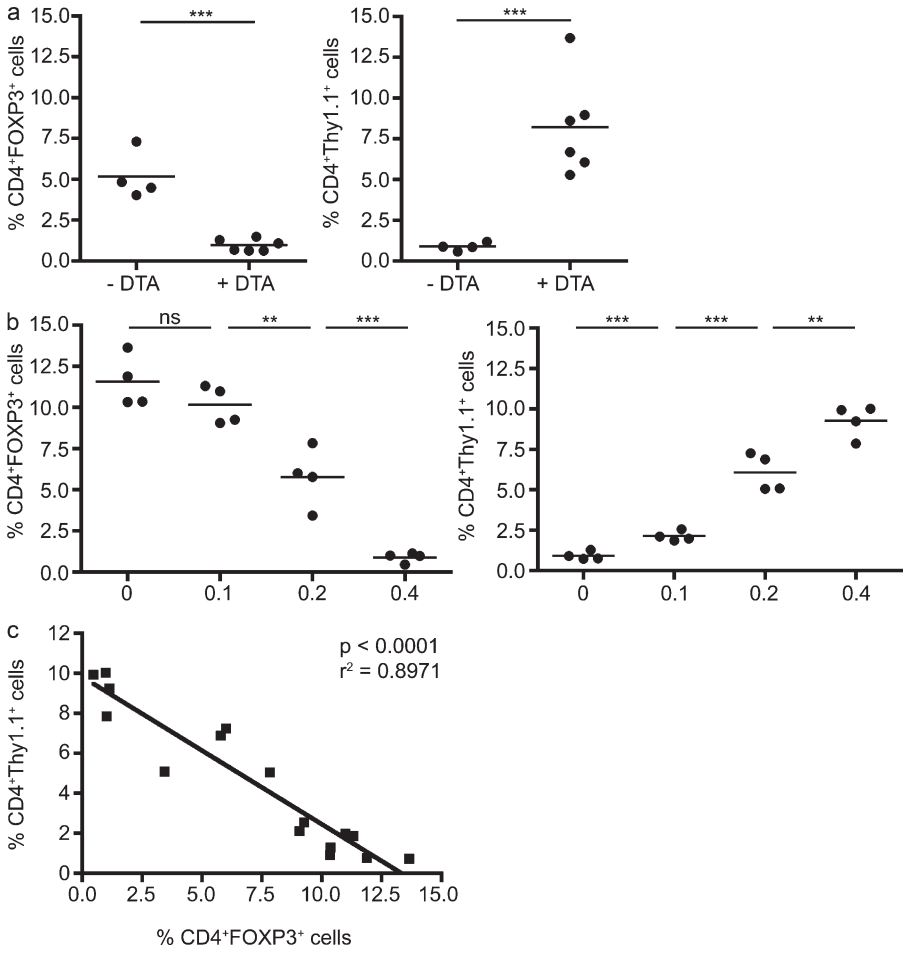

Figure 5. Conditional ablation of T reg cells. (a) 6-wk-old FOXP3^{DTR}Thy1.1 mice were injected i.p. at days 0, 1, 3, 6, 9, with 0.4 µg DT. Mice were sacrificed and cells analyzed by flow cytometry at day 10. (left) The percentage of CD4$^+$FOXP3$^+$ T cells; (right) the fraction of cells expressing the IL-2-Thy1.1 reporter recovered in the same untreated and DT-treated mice. Note that DT treatment significantly reduced the fraction of FOXP3$^+$ cells while increasing the number of Thy1.1$^+$ cells. (b) 11-wk-old FOXP3^{DTR}Thy1.1 mice were injected i.p. at days 0, 1, 3, 6, and 9 with 0.1, 0.2, or 0.4 µg DT. Mice were sacrificed and cells analyzed by flow cytometry at day 10. (left) The percentage of CD4$^+$FOXP3$^+$ T cells; (right) the fraction of cells expressing the IL-2-Thy1.1 reporter recovered in the untreated and DT-treated mice. Note the dose response effects of DT treatment. (c) The correlation between the fraction of CD4$^+$FOXP3$^+$ and CD4$^+$Thy1.1$^+$ cells recovered in the mice treated with different doses of DT. The different percentages of T reg cells observed in the control mice from a and b are related to age differences. Similar results were obtained in two to three independent experiments.

mice was significantly reduced, whereas the fraction of proliferating Ki67$^+$ cells remained unaltered (Fig. 7, c and d). Although T reg cells increased after IL-2 administration, the frequencies of GFP$^+$ and Thy1.1$^+$ IL-2 reporter cells were significantly reduced in both strains of mice (Fig. 7, e and f; and not depicted). Notably, IL-2 treatment was also accompanied by a marked decrease in the percentage of CD4$^+$ T cells committed to IL-2 secretion after in vitro stimulation with PMA and ionomycin in the absence of IL-2 (Fig. 7 g). This in vitro effect was not caused by the suppressive effects of the augmented representation of T reg cells in the populations studied, as it was also observed when the stimulated CD4$^+$ T cells were previously depleted of the CD25$^+$ T reg cell fraction (Fig. 7 h). As naive CD4 T cell lack expression of CD25 and CD122, these results suggest an indirect effect of the IL-2 treatment. Most importantly, we have also found that the injection of exogenous IL-2,

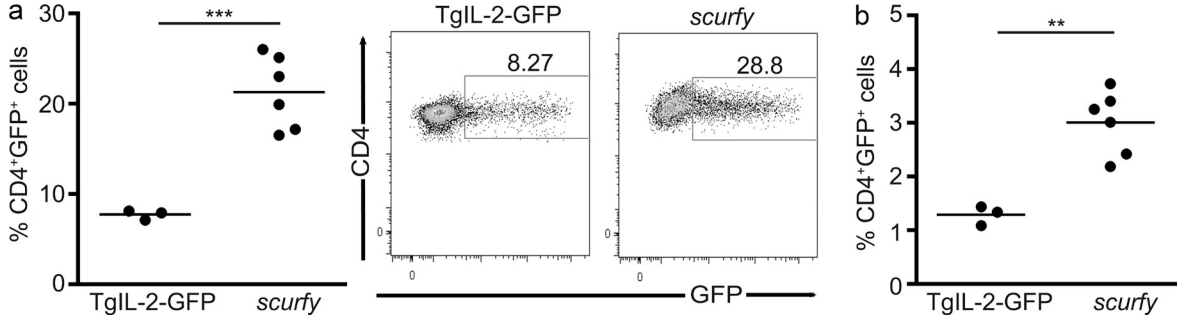

Figure 6. IL-2p cells in FOXP3-deficient *scurfy* mice. (a) Shows the percentage of CD4$^+$GFP$^+$ T cells observed in the spleen of 2-wk-old control littermates and B6.TgIL-2-GFP.FOXP3$^{sf/x}$/J *scurfy* mice, genetically deficient for *foxp3* (left). Representative dot plots of control littermate (middle) and scurfy mouse (right). (b) The percentage of CD4$^+$GFP$^+$ T cell observed in the among SP CD4$^+$ thymus cells of 2-wk-old control littermates and B6.TgIL-2-GFP.FOXP3$^{sf/x}$/J *scurfy* mice.

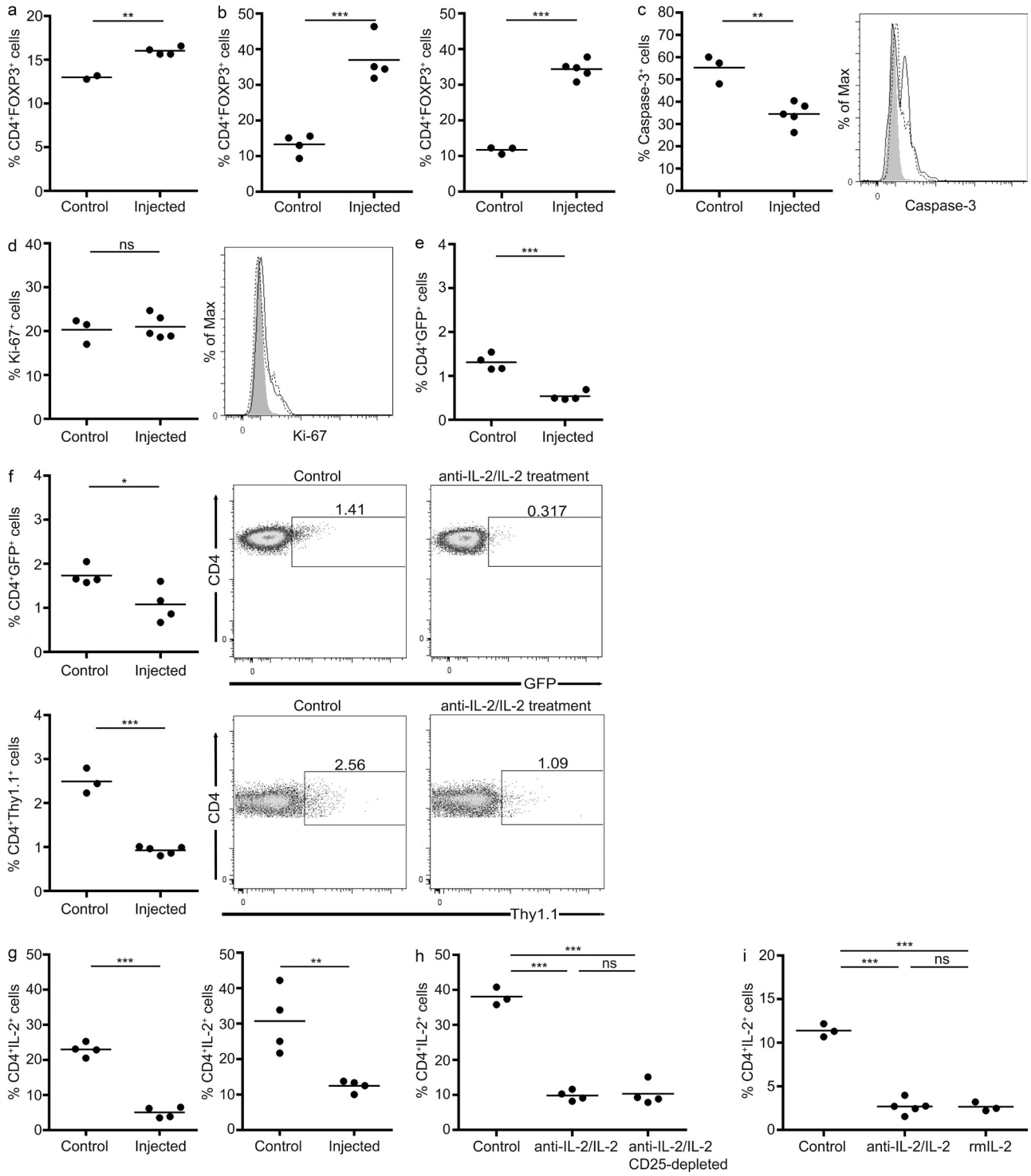

Figure 7. IL-2 treatment. Mice either received daily injections of 1 µg recombinant murine IL-2 i.p. for 5 consecutive days and were sacrificed 4 h after the last injection (a) or were injected i.p. for 3 consecutive days with anti–IL-2–IL-2 immune complexes containing 1 µg recombinant murine IL-2 and 5 µg anti–IL-2 antibody from the JES6-1A12 clone and sacrificed 3 d after the last injection (b– d). (a) The fraction of $CD4^+FOXP3^+$ cells recovered from the spleen of control and rIL-2–treated B6.Ki/Ko.GFP/IL-2 reporter mice. Each point represents a single mouse. The bar represents the mean value. (b) The fraction of $CD4^+FOXP3^+$ cells recovered from the spleen of control and aIL-2/IL-2–treated B6.Ki/Ko.GFP/IL-2 (left) or 2BiT (right) reporter mice. Each point represents a single mouse. The bar represents the mean value. (c) Shows the fraction of Caspase3$^+$ cells among $CD4^+FOXP3^+$ cells from the spleen of control and anti–IL-2/IL-2 2BiT–treated mice. A representative histogram is shown (right). (d) The fraction of Ki67$^+$ cells among $CD4^+FOXP3^+$ cells from the spleen of control and anti–IL-2/IL-2 2BiT treated mice. A representative histogram is shown (right). (e) The fraction of $CD4^+GFP^+$ cells recovered from the spleen of control and rIL-2–treated B6.Ki/Ko.GFP/IL-2 reporter mice. (f) Shows the fraction of $CD4^+$ IL-2 reporter$^+$ (GFP$^+$ or Thy1.1$^+$) cells

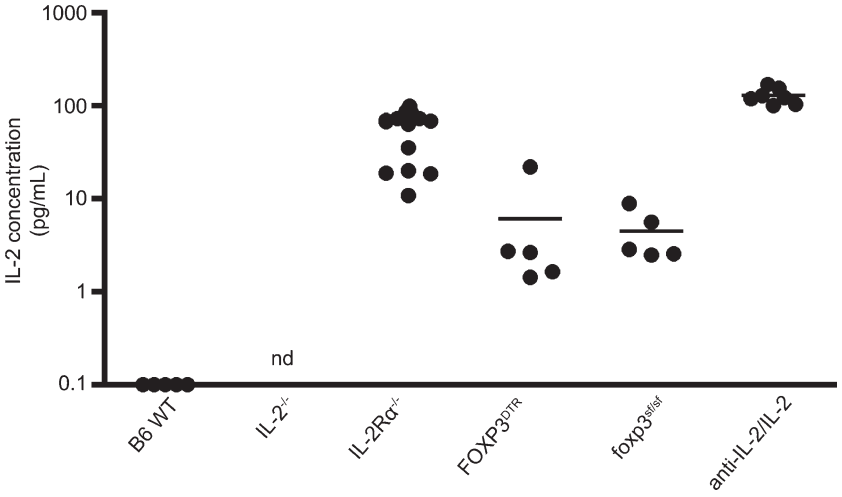

Figure 8. Plasma levels of IL-2. Shows serum IL-2 levels in WT, IL2$^{-/-}$, IL-2R$\alpha^{-/-}$, and FOXP3^{DTR} mice after T reg cell depletion upon DT treatment, foxp3$^{sf/Y}$ B6 mice, and B6 after treatment with anti–IL-2–IL-2 immune complexes. All mice were 6–8-wk-old except the scurfy mouse (2 wk old). Each dot represents an individual mouse. ND, not detected.

either soluble or in complexes, into WT B6 mice abridged the ability of the CD4$^+$ T cells to secrete IL-2 upon subsequent anti-CD3 challenge in vivo (Fig. 7 i). Overall, these observations suggest that IL-2 treatment renders naive CD4 T cells anergic and unable to respond to further stimulation by the production of either IL-2 or other cytokines (unpublished data) by an indirect effect that is likely mediated by T reg cells.

On the whole, these findings corroborate that IL-2 plays a critical role in the feedback loops that regulate IL-2p cell numbers either directly preventing IL-2 production or by boosting T reg cell numbers. These observations lead us to predict changes in the systemic levels of IL-2 in situations with disrupted equilibrium between T reg and IL-2p effector cells. We therefore measured circulating IL-2 levels in different experimental settings. Although IL-2 levels were increased in 2-wk-old *scurfy* mice and in 6–8 wk old mice with reduced numbers of T reg cells and in FOXP3^{DTR} mice after DT treatment, they were very low or undetectable in age-matched WT mice (Fig. 8), suggesting that, in steady-state conditions, IL-2 must operate at close range by modulating near vicinity cell-to-cell interactions. It should be pointed out that the increased IL-2 levels observed in the *scurfy* mice did not prevent an increased fraction of their CD4$^+$ T cells from activating the IL-2 locus as indicated by the expression of the IL-2 reporter.

DISCUSSION

Parsimony suggests that IL-2–producing $\alpha\beta$CD4$^+$ T cells are at the core of the immune system homeostasis because they are the main providers of the IL-2 essential for the survival and function of T reg cells (Almeida et al., 2002; Gavin et al., 2002; Bayer et al., 2005; Fontenot et al., 2005a; Setoguchi et al., 2005; Almeida et al., 2006; Hill et al., 2007; Yu et al., 2009). However, the mechanisms underlying the in vivo regulation of this population of IL-2p cells remain largely unknown. Here, we used different lines of reporter mice to identify a subset of cells having been committed to IL-2 synthesis (IL-2p). In the mice studied, reporter genes act either as short-term (Ki/Ko.GFP/IL-2, 2BiT; Naramura et al., 1998; Luther, 2009) or long-term tracers (TgIL-2-GFP; Yui et al., 2001) of the cells having activated the *il-2* locus. In the Ki/Ko.GFP/IL-2 and 2BiT mice only about ≤2% of the CD4$^+$ T cells were reporter positive, indicating that, at steady state, few cells are actually synthetizing IL-2 because IL-2 synthesis is transient and rapidly lost after T cell activation (Sojka et al., 2004). In contrast, in the TgIL-2-GFP mice (Yui et al., 2001), where GFP expression acts as a long-term subset tracer, ∼10% of the CD4$^+$ T cells were reporter positive. This observation suggests that in normal mice a sizeable fraction of the peripheral pool of CD4 T cells belongs to a clade of IL-2p cells that has recently been engaged in IL-2 synthesis. Interestingly, in unmanipulated mice a significant fraction of splenic CD4$^+$ T cells could be promptly induced to IL-2 secretion after either in vivo short-term challenge cognate stimulation with anti-CD3 antibodies or in vitro stimulation with PMA and ionomycin. The distinct gene expression patterns and expansion/accrual abilities of the CD4$^+$GFP$^-$ and CD4$^+$GFP$^+$ cells after lymphopenia driven proliferation (LDP), suggest that they may represent distinct functional T cell subsets. We postulate that maintenance of a stable pool of the IL-2p cell subset may be vital to preserve the immune system's homeostasis by controlling

recovered from the spleen of control and anti–IL-2/IL-2–treated B6.Ki/Ko.GFP/IL-2 (top) or 2BiT (bottom) reporter mice. Representative dot plots are also shown. (g) Shows the fraction of IL-2–secreting cells 4 h after in vitro culture with PMA and ionomycin recovered from the spleen of B6.Ki/Ko.GFP/IL-2 reporter mice untreated and treated with either rIL-2 (left) anti–IL-2–IL-2 immune complexes (right). (h) The fraction of IL-2–secreting cells 4 h after in vitro culture with PMA and ionomycin of purified total CD4$^+$ and CD4$^+$ depleted of CD25$^+$ (CD4$^+$CD25$^-$) T cells recovered from the spleen of 2BiT reporter mice untreated and treated with anti–IL-2–IL-2 immune complexes. (i) The fraction of IL-2–secreting cells recovered from the spleen of WT B6 mice 3 h after in vivo injection with 50 µg/per mouse of anti-CD3ε mAb. WT B6 mice were treated with either soluble mouse IL-2 or cytokine antibody immune complexes or left untreated. Each point represents a single mouse. The bar represents the mean value. Representative histograms of IL-2–secreting cells are also shown. Controls are untreated mice. Similar results were obtained in two to three independent experiments.

T reg cell function while ensuring prompt immune responses by helping effector T cell expansion. In our studies we learned that the number of IL-2p cells is not cell autonomous, but tightly controlled by mechanisms that implicate IL-2 and T reg cells.

Our findings suggest the existence of an environmentally dependent feedback loop where IL-2 can regulate its own production in two ways. In IL-2–deficient mice, there was a marked increase of the number of IL-2 reporter–positive $CD4^+$ T cells, suggesting that in the absence of IL-2 the immune system attempts to restore physiological levels of IL-2. Furthermore, in the BM chimeras where we varied the fraction of cells capable of IL-2 synthesis, we found that the number of T cells expressing GFP was regulated independently of the number of IL-2–sufficient cells, indicating the existence of compensatory mechanisms that tend to maintain constant the number of cells of the IL-2p subset. Conversely, administration of mouse rIL-2 (either soluble or in cytokine antibody complexes) reduced the fraction of IL-2 reporter–positive cells. Remarkably, the injection of IL-2 strongly abridged the ability of $CD4^+$ T cells to secrete IL-2 (or other cytokines; not depicted) after either in vivo cognate triggering or in vitro strong PMA and ionomycin stimulation. These observations suggest that IL-2 could directly inhibit activation and IL-2 (cytokine) synthesis by making $CD4^+$ T cells refractory to further stimulation, as it has been shown to occur in vitro (Villarino et al., 2007). However, we have observed that control of the IL-2p $CD4^+$ T cell subset happened independently of the ability of the cells to fully express CD25 (or CD122) and perceive IL-2 (Fig. 3). Alternatively, the state of $CD4^+$ T cell anergy could be an indirect effect mediated by T reg cells, as IL-2 administration raised T reg cell numbers and IL-2 favors T reg cell function. Most likely, by inducing a state of overall T cell paralysis T reg cells also affect IL-2 production.

Several lines of evidence support the role of T reg cells in the regulation of the IL-2p cell subset. In the mouse BM chimeras containing different numbers of IL-2–sufficient $CD4^+$ T cells diluted among IL-2–deficient $CD4^+$ T cells, there was a strong positive linear correlation between the numbers of IL-2 reporter$^+$ and $CD4^+FOXP3^+$ T reg cells. In IL-$2^{-/-}$ and $CD25^{-/-}$ IL-2 reporter mice with reduced T reg cell numbers, the number of IL-2 reporter–positive $CD4^+$ T cells augmented. An increase that, in the case of the CD25-deficiency, could be corrected by the transfer of purified populations of $FOXP3^+$ T reg cells. Furthermore, the controlled in vivo ablation/reduction of T reg cells resulted in corresponding increases of the number of IL-2 reporter $CD4^+$ T cells, whereas the rise in T reg cell numbers, induced upon IL-2 administration, reduced the fraction of IL-2p cells and the ability of the $CD4^+$ T cells to secrete IL-2 after stimulation. Convincingly, $FOXP3^{sf/x}$ mice, genetically deficient in T reg cells, showed marked increases in the number of IL-2 reporter positive $CD4^+$ T cells. The activity of T reg cells may occur through two independent mechanisms not mutually exclusive. First, by influencing T cell responses through IL-2 consumption (Barthlott et al., 2003; Malek and Castro, 2010), thereby affecting IL-2 levels, and, second, by directly inhibiting naive T cell activation and IL-2 production. The observation that the raised levels of circulating IL-2 observed in B6.TgIL-2-GFP.$FOXP3^{sf/x}$/J *scurfy* mice (Fig. 8) did not prevent the increase of the fraction of $CD4^+GFP^+$ IL-2p cells supports the existence of a direct suppressive effect of T reg in the regulation of the IL-2p cell subset. Previous reports that a FOXP3 transgene restored T reg cell function and protected against the onset of autoimmunity in IL-2R$\beta^{-/-}$ mice show that the T reg cell activity can occur in the absence of IL-2 signals (Soper et al., 2007) and contradict the IL-2 consumption hypothesis as the sole mechanism of action for T reg cells. A direct suppressive effect of T reg must exist to also control IL-2 independent responses and populations (Zaragoza et al., 2011). The observation that circulating concentrations of IL-2 in the serum of WT mice are below detection levels (<1 pg/ml; Fig. 8) indicates that the IL-2 mediated regulation must act at close range, modulating interactions between different intervening cell populations (Rudensky, 2011). Because the observed changes in the number of IL-2p cells were less evident in the thymus this homeostatic regulation is likely to occur mainly at peripheral sites. T reg cells have been shown to repress the capacity of antigen-presenting cells to activate other T cells by inhibiting DC maturation and activation via LAG-3 (Huang et al., 2004; Wing et al., 2008) or CTLA-4 dependent mechanisms, which down-regulate CD80 and CD86 expression by dendritic cells (Onishi et al., 2008; Wing et al., 2008; Qureshi et al., 2011). In vitro studies have demonstrated that T reg cells inhibit *il2* transcription in naive $CD4^+$ T cells (Thornton and Shevach, 1998; Luther, 2009) probably by promoting the inducible cAMP early repressor (ICER) nuclear translocation and suppressing nuclear factor of activated T cell c1 (Nfatc1; Vaeth et al., 2011). Although the T reg cells are able to suppress all cytokine synthesis by effector T cells the critical finding reported here is that they also inhibit IL-2 production. In conclusion, regulation of the number of T reg and IL-2p cell populations is reciprocal: the number of T reg cells is dependent on the amounts of IL-2 produced by other T cells, whereas T reg cells regulate the number of IL-2p cells. The two populations exist in tight equilibrium.

It is generally believed that control of lymphocyte numbers may be determined by cellular competition for trophic survival factors (Freitas and Rocha, 2000). However, it is not clear whether additional mechanisms exist to control proliferating lymphocyte populations in situations where resources are not limiting, e.g., during immune responses. It remains unanswered if lymphocyte populations count the number of their individuals and know when to stop expanding. Our present findings suggest that the control of lymphocyte numbers could also be achieved by the ability of lymphocytes to perceive their own population density.

We propose that the homeostasis of the IL-2p subset occurs via a quorum sensing–like mechanism (Almeida et al., 2012), where $CD4^+$ T cells can sense the levels of IL-2 they produce. $CD4^+$ T cells may either be directly inhibited by IL-2, an inhibitory loop mediated by STAT family transcription factors that has been shown to occur in vitro (Long and Adler, 2006;

Villarino et al., 2007) or indirectly, via a subpopulation of $CD4^+$ T reg cells expressing the high-affinity IL-2Rα-chain. These two independent pathways may operate in a complementary manner to provide robustness and safeguard control of T cell activation. We could postulate that below local threshold levels of IL-2 and in the absence of T reg cells, naive T cells upregulate IL-2 production to compensate for its absence, whereas under activating conditions, IL-2 excesses triggers T reg cells to repress CD4 T cell activation and proliferation, bringing IL-2 to physiological levels. The quorum-sensing hypothesis (de Freitas, 2009) implies a feedback control of T cell activation that still allows immune responses to occur; this likely plays a part in the contraction phase of the $CD4^+$ T cell responses (Fehérvari et al., 2006). In the initial stages of an immune response, the presence of antigen modifies the steady-state equilibrium and provokes proliferation of antigen-specific cells. Consequently, the number of IL-2p cells and the IL-2 concentrations increase: in response T reg cells proliferate to reestablish a steady state by suppressing further T cell activation and decreasing the number of IL-2p cells. This mechanism definitively enables $CD4^+$ T cell populations to restrain their growth by monitoring the number of IL-2p cells, thus preventing uncontrolled lymphocyte proliferation during immune responses (Fehérvari et al., 2006; Almeida et al., 2012). Failure of the quorum-sensing mechanism results in uncontrolled $CD4^+$ T cell activation and autoimmune disease. The inability of $CD4^+$ T cells to detect IL-2, due to defects of IL-2R expression (Willerford et al., 1995; Malek et al., 2000) or IL-2 signaling (Burchill et al., 2007), or due to failure to produce IL-2 (Schorle et al., 1991), leads to lymphoid hyperplasia and lethal autoimmune disease. Moreover, other autoimmune diseases have linked defects in the IL-2–IL-2R signal pathways. Autoimmunity in NOD mice seems to be dependent on lower IL-2 production resulting from genetic defects that map to the *il-2* region (Yamanouchi et al., 2007) and can be partially prevented by exogenous IL-2 administration (Grinberg-Bleyer et al., 2010). Progression of atherosclerosis (Dinh et al., 2012) and autoimmune vasculitis (Saadoun et al., 2011) can also be thwarted by IL-2 therapy. Polymorphisms linked to IL-2 receptors, IL-2Rα and IL-2Rβ, are associated with several autoimmune diseases, such as type 1 diabetes, multiple sclerosis, celiac diseases, and rheumatoid arthritis (Gregersen and Olsson, 2009; Todd, 2010).

Our results demonstrate that IL-2, rather than being a mere growth factor required for the proliferation of some T cells, represents a key molecule playing a master role in $CD4^+$ T cell homeostasis. IL-2 may act as an indicator (alarm signal) of excessive T cell activation. Using IL-2, it should be possible to down-modulate overall $CD4^+$ T cell activation, increase T reg cell numbers, and control autoreactive immune responses or, alternatively, to boost effector T cell responses. By modifying IL-2 levels, we were able to alter the IL-2p/T reg cell equilibrium. While increasing T reg cell numbers/function, administration of IL-2 abridged the capacity of T cells to produce cytokines upon in vivo or in vitro stimulation. The strong feedback effect of IL-2 abrogating in particular natural IL-2 production should be considered, as its repeated therapeutic usage might imbalance the immune system's steadiness, preclude ongoing or new immune responses, and prevent natural T reg cell restoration late after interruption of therapy. Finally, these studies suggest that monitoring systemic IL-2 levels might be a relevant indicator in the follow up of situations of disrupted equilibrium between T reg and IL-2p effector cells.

MATERIALS AND METHODS

Mice. C57BL/6.Ly^a and C57BL/6.Ly5^b mice (Charles River); B6.Rag2$^{-/-}$, B6.CD3ε$^{-/-}$, B6.IL-2$^{-/-}$ (Schorle et al., 1991), and B6.IL-2Rα$^{-/-}$ (Willerford et al., 1995) mice (Centre de Développement des Techniques Avancées–Centre National de la Recherche Scientifique, Orléans, France); B6.TgIL-2-GFP (Yui et al., 2001), B6.Ki/Ko.GFP/IL-2 (Naramura et al., 1998), B6.FOXP3^{mRFP} (Wan and Flavell, 2005), B6.FOXP3^{DTR} (Kim et al., 2007), and B6.IL-2.BAC-inThy1.1 (2BiT; Luther, 2009) mice; B6.TgIL-2-GFP.IL-2$^{-/-}$, B6.TgIL-2-GFP.IL-2Rα$^{-/-}$, B6.Ki/KoGFP/IL-2.IL-2$^{-/-}$, B6.Ki/KoGFP/IL-2.IL2Rα$^{-/-}$ and FOXP3^{DTR}.IL2.BAC-inThy1.1, B6.FOXP3$^{sf/x}$/J, B6.TgIL-2-GFP.FOXP3$^{sf/x}$/J, and B6.IL-2.BAC-Thy1.1.FOXP3$^{sf/x}$/J mice were kept in our animal facilities at the Pasteur Institute. All mice were matched for age (6–12 wk) and sex. Mice were cared for in accordance with Pasteur Institute guidelines in compliance with European animal welfare regulations, and all animal studies were approved by the Pasteur Institute Safety Committee in accordance with French and European guidelines and by the ethics Committee of Paris 1 (permits 2010–0002, 2010–0003, and 2010–0004).

BM chimeras. 6–8-wk-old B6.CD3ε$^{-/-}$ mice were lethally irradiated (900 rad) with a ^{137}Ce source and received i.v. ∼1.5 × 10^6 T cell–depleted (<0.5%) BM cells from different donor mice mixed at different ratios. T cell depletion was performed by auto-MACS (Miltenyi Biotec) magnetic sorting after incubating the BM cells with anti–CD3-Biotin (145-2C11) antibody from BD followed by anti-Biotin microbeads (Miltenyi Biotec). The use of mice with different Ly5 allotype markers allowed us to discriminate the T cells originating from the different IL-2$^+$ (Fehérvari et al., 2006) and IL-2$^{-/-}$ donors. Host mice were sacrificed at 8-wk after transfer. Spleen, inguinal, and mesenteric lymph node (LN) cell numbers and phenotype were analyzed being the total peripheral T cell number representative of cells recovered in the host's spleen added to twice the number of cells recovered from the host's inguinal and mesenteric LN.

Cell transfers and in vivo proliferation analysis. LN and spleen cell suspensions were obtained from B6.TgIL-2-GFP mice and $CD4^+$ cells enriched by negative selection using the $CD4^+$ T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Singe-cell suspensions of ≥94% pure $CD4^+GFP^+$ and $CD4^+GFP^-$ cells sorted on a MoFlo Legacy (Beckman Coulter), were labeled for 10 min at 37°C in the dark with 5 μM cell proliferation dye eFluor 670 (eBioscience) and injected i.v. into nonirradiated B6.CD3ε$^{-/-}$ B6, Rag2$^{-/-}$ or B6.Ly5^a hosts. Host mice were sacrificed at 1 or 6 wk after transfer, and the dilution of the fluorescent dye in the donor $CD4^+$ T cells was analyzed by flow cytometry.

Flow cytometry analysis. Thymus, spleen, and inguinal and mesenteric LN single-cell suspensions were stained for cell surface and intracellular proteins with appropriate combinations of the following monoclonal antibodies conjugated to pacific blue, BD Horizon V500, Qdot-655, Alexa Fluor 700, allophycocyanin, allophycocyanin-eFluor 780 conjugate, fluorescein isothiocyanate, peridinin chlorophyll protein–cyanine 5.5, phycoerythrin, and phycoerythrin-cyanine7: anti-CD4 (RM4-5), anti-TCRβ (H57-597), anti-CD25 (PC61), anti-CD8α (53–6,7), anti-CD3ε (500A2), anti-CD19 (6D5), anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD62L (MEL-14), anti-CD44 (IM7), anti-B220 (RA3-6B2), anti–IL-2, anti-Helios (C22F6), anti-FOXP3 (FJK-16s), anti-active Caspase3 (01715), and anti–Ki-67 (mm1) purchased from BD, Invitrogen, and eBioscience. Biotinylated antibodies were visualized with Qdot-605- and allophycocyanin-cyanine7-streptavidin conjugates

(BD and Invitrogen). Before staining, cells were treated with Fc-Block (CD16/CD32; BD). Dead cells were excluded during analysis according to their light-scattering characteristics and/or 7AAD staining. All data acquisitions and analyses were performed with FACSCanto and LSRFortessa (BD) interfaced with FACSDiva (BD) and FlowJo (Tree Star) software.

Administration of IL-2. Mice were injected i.p. for 3 consecutive days with anti–IL-2–IL-2 immune complexes containing 1 µg recombinant murine IL-2 (eBioscience) and 5 µg anti–IL-2 antibody from the JES6-1A12 clone (eBioscience) diluted in 50 µl (Boyman et al., 2006). Mice were sacrificed 3 d after the last injection, and cells were analyzed by flow cytometry. Alternatively, mice received daily injections of 1 µg recombinant murine IL-2 (eBioscience) i.p. for 5 consecutive days and were sacrificed 4 h after the last injection (Grinberg-Bleyer et al., 2010).

In vivo treatment with anti-CD3. Mice were injected i.p with 50 µg/per mouse of anti-CD3ε mAb (145-2C11; BD; Ferran et al., 1991). 3 h after injection, spleen cells were labeled with mouse IL-2 Catch Reagent from IL-2 detection kit (Miltenyi Biotec). The IL-2 secretion assay was performed according to the manufacturer's protocol and cells were analyzed by flow cytometry.

Detection of IL-2–secreting cells. $CD4^+$ T cells isolated from mouse spleen by using a CD4 T isolation kit II (Miltenyi Biotec) followed by auto-MACS (Miltenyi Biotec) magnetic sorting were cultured in 96-well plates at a 1.10^7 cells/ml concentration with 500 ng/ml of PMA (Sigma-Aldrich) and 50 ng/ml of ionomycin (Sigma Aldrich) in RPMI medium and for 4h at 37°C 5% CO_2. After in vivo injection with anti-CD3 antibodies or in vitro stimulation with PMA and ionomycin, IL-2–secreting cells were detected using a mouse IL-2 secretion assay detection kit (Miltenyi Biotech) and analyzed by flow cytometry.

Depletion of FOXP3$^+$ T reg cells. $FOXP3^{DTR}$Thy1.1 male mice were injected i.p. at days 0, 1, 3, 6, 9, with 0.1, 0.2, or 0.4 µg of DT (EMD Millipore) reconstituted according to the manufacturer's protocol. Mice were sacrificed and cells analyzed by flow cytometry at day 10.

ELISA for IL-2. Circulating IL-2 levels were detected in mouse serum using a BD IL-2 Elisa kit following the manufacturer's protocol.

Gene expression analysis. Spleen and inguinal and mesenteric LN cells from B6.TgIL-2-GFP were enriched for $CD4^+$ T cells by auto-MACS (Miltenyi Biotec) magnetic sorting using a $CD4^+$ T cell isolation kit (Miltenyi Biotec), and then further purified into >95% pure conventional GFP^+ and GFP^- $CD4^+$ T cell populations by sorting on FACS Aria II cytometer. Cells were lysed, RNA was extracted, and cDNA was prepared using Miltenyi's SuperAmp protocol. Microarray experiments were performed using single-color hybridization of murine RNA to Agilent Whole Mouse Genome Oligo Microarrays. Results were analyzed by discriminatory genes analysis by ANOVA, and functional annotation. Experiments were performed on five individual mice.

Statistical analysis. Sample means were compared using the Student's *t* test. Sample means were considered significantly different at $P < 0.05$.

Online supplemental material. Fig. S1 shows IL-2 versus IL-2 reporter expression. Fig. S2 shows Gene expression patterns of GFP^- and GFP^+ $CD4^+$ T cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20122759/DC1.

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Author contributions: I.F. Amado and J. Berges performed research and analyzed data. R.J. Luther and C. Weaver contributed critical new reagents or analytical tools. M.-P. Mailhé provided vital technical assistance. I.F. Amado, S. Garcia, A. Bandeira, and A. Liston discussed data and helped to write the paper. All of the authors analyzed the results and commented on the manuscript. A.A. Freitas designed research, analyzed data, and wrote the paper.

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