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# Continuous Activation of Autoreactive CD4$^+$ CD25$^+$ Regulatory T Cells in the Steady State

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## Abstract

Despite a growing interest in CD4$^+$ CD25$^+$ regulatory T cells ($T_{reg}$) that play a major role in self-tolerance and immunoregulation, fundamental parameters of the biology and homeostasis of these cells are poorly known. Here, we show that this population is composed of two $T_{reg}$ subsets that have distinct phenotypes and homeostasis in normal unmanipulated mice. In the steady state, some $T_{reg}$ remain quiescent and have a long lifespan, in the order of months, whereas the other $T_{reg}$ are dividing extensively and express multiple activation markers. After adoptive transfer, tissue-specific $T_{reg}$ rapidly divide and expand preferentially in lymph nodes draining their target self-antigens. These results reveal the existence of a cycling $T_{reg}$ subset composed of autoreactive $T_{reg}$ that are continuously activated by tissue self-antigens.

Key words: CD4$^+$ T lymphocytes • suppressor cells • homeostasis • immune tolerance • self-antigens

## Introduction

CD4$^+$ CD25$^+$ regulatory T cells ($T_{reg}$) play a major role in the maintenance of self-tolerance and the control of autoimmune diseases (1, 2). They are also involved in the regulation of T cell homeostasis (3, 4) and in the modulation of immune responses to alloantigens, cancer cells, and pathogens (5–10). These findings have opened new prospects in immunotherapeutic interventions for several diseases. For instance, we and others have shown that transfer of $T_{reg}$ could be used to control autoimmune diabetes (11, 12), solid allograft rejection (13–15), or graft versus host disease (7, 9, 16) in mice. In addition, $T_{reg}$-specific depletion at the time of tumor inoculation dramatically increased immune rejection of some tumors (10). These recent data indicate that the $T_{reg}$ represent a master player in the immune system, and that their manipulation could be used in new therapeutics. It is therefore essential to gain more basic information on the biology of these cells.

The thymic origin of $T_{reg}$ has been established in mice from several observations. Thymectomy of day-3 neonates induced long-term depletion of $T_{reg}$ and a severe autoimmune syndrome (17). The thymus contains cells with similar phenotype and suppressor function as the $T_{reg}$ described in the spleen and LN (18). Finally, early thymocyte precursors have the potential to differentiate in mature $T_{reg}$ after adoptive transfer (18). Importantly, thymic selection of $T_{reg}$ precursors appears to favor the emergence of a repertoire of highly autoreactive $T_{reg}$. Indeed, $T_{reg}$ specific to self-Ag presented by the thymic epithelium are positively selected in the thymus and then colonize secondary lymphoid organs (19–22).

In the periphery, for a given age and genetic background, $T_{reg}$ represent a stable proportion of the CD4$^+$ T cells in the steady state, suggesting that the homeostasis of $T_{reg}$ is tightly regulated. In young adult mice not prone to autoimmune diseases, $T_{reg}$ constitute ~10% of CD4$^+$ T cells. This proportion seems be reduced in genetically autoimmune-prone individuals (11, 12). There is also some preliminary evidence in humans that type 1 diabetic patients may have decreased blood $T_{reg}$ cell numbers (23). Mice of the scurvy mutant strain, which have a deficit of $T_{reg}$, develop a severe autoimmune syndrome (24, 25). Thus, a decreased proportion of these cells could lead to an augmentation of the risk to

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*Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CFSE, 5,6-carboxy-fluorescein succinimidyl ester; GITR, glucocorticoid-induced TNF receptor; HA, hemagglutinin; $T_{reg}$, CD4$^+$ CD25$^+$ regulatory T cells.

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develop autoimmune diseases. On the opposite, an increased proportion of $T_{reg}$ may alter antitumor and antiinfectious immunity. Thus, homeostasis of $T_{reg}$ is likely an important process in the proper functioning of the immune system.

Several molecules are involved in regulation of the homeostasis of $T_{reg}$. IL-2 plays a critical role because IL-2 KO and IL-2Rβ KO mice have a profound deficit of $T_{reg}$ (26, 27). This cytokine might be involved in both their thymic production and their peripheral survival (3, 27, 28). B7/CD28 and CD40/CD40L costimulatory pathways are also involved in the regulation of homeostasis of $T_{reg}$. Indeed, B7-1 B7-2 double KO mice, CD28 KO mice, and CD40 KO mice all present a severe quantitative deficit of $T_{reg}$ (11, 29). Disruption of the B7/CD28 pathway in nonobese diabetic mice was associated with an exacerbation of autoimmune diabetes due to the defect of $T_{reg}$ (11).

Despite the emerging importance of $T_{reg}$ in the immune system, fundamental parameters of the biology and homeostasis of these cells, such as their lifespan, turnover, and recirculation properties remain poorly known. In this work, we addressed these points using a model of adoptive transfer of highly purified Thy-1 congenic $T_{reg}$ into unmanipulated normal hosts. Because donor cells represented <3% of the endogenous $T_{reg}$ population, their homeostasis should not be modified. Thus, studying the intrinsic behavior of donor cells provided data on the homeostasis of $T_{reg}$ in the steady state. We showed that $T_{reg}$ retain a stable expression of CD25 in vivo and that a $T_{reg}$ subset is composed of quiescent cells with long lifespan, whereas cells of the other $T_{reg}$ fraction have a rapid turnover and express multiple activation markers. This latter subset appears to be composed of autoreactive $T_{reg}$ that are continuously activated by tissue self-antigens.

## Materials and Methods

*Animals.* 6–8-wk-old female BALB/c mice were obtained from Charles River Laboratories. The ins-hemagglutinin (HA) transgenic mice expressing HA of influenza virus in islet β cells (30) were backcrossed >10 generations onto BALB/c genetic background and then intercrossed to generate mice homozygous for the ins-HA transgene. The TCR-HA transgenic mice (31) that express a TCR recognizing I-$E^d$-restricted HA epitope 110–120 (SFERFEIFPKE) were backcrossed >10 generations onto BALB/c genetic background and then bred with congenic Thy-1.1 BALB/c mice to generate [TCR-HA × Thy-1.1] F1 mice. Congenic Thy-1.1 BALB/c mice, ins-HA mice, and TCR-HA × Thy-1.1 mice were bred in our animal facility. Mice were housed in filter-topped cages under specific pathogen-free conditions. They were manipulated according to European Union guidelines.

*Cell Preparation and Adoptive Transfer.* $T_{reg}$ were purified as previously described (7). After a mechanical dissociation, spleen and peripheral LN (inguinal, brachial, axillary, and cervical) cells from Thy-1.1 BALB/c mice were first incubated in PBS 3% fetal calf serum with saturating amounts of biotin-labeled anti-CD25 mAb (7D4; BD Biosciences) and then with anti-biotin–coated microbeads (Miltenyi Biotec), and purified using magnetic cell separation LS columns (Miltenyi Biotec). The CD25-depleted cells (referred to as $CD25^-$ cells in the text), harvested from the flow through, were depleted of erythrocytes by ammonium chloride lysis. They contained <0.3% $CD4^+$ $CD25^+$ T cells. The positively selected cells (80% $CD25^+$) were stained with FITC-labeled anti-CD4 (GK1.5 or RM4-5; BD Biosciences), PE-labeled anti-CD62L (MEL-14; BD Biosciences), and CyChrome-streptavidin (BD Biosciences) that bound to free biotin-labeled CD25 molecules, uncoupled to beads. The $CD4^+$ $CD25^+$ $CD62L^{high}$ and $CD4^+$ $CD25^+$ $CD62L^{low}$ cells were sorted on a FACStar™ (Becton Dickinson), giving a purity of 98–99.5%. Then, purified cells were labeled with 5,6-carboxy-fluorescein succinimidyl ester (CFSE; Sigma-Aldrich) by incubation with 2.5 μM CFSE in protein-free PBS for 10 min at room temperature and 1 min with 1 vol serum. Cells were then washed twice in PBS and 0.55–0.7 × $10^6$ purified $T_{reg}$ or $10^7$ $CD25^-$ cells were intravenously transferred to congenic Thy-1.2 BALB/c mice. For the transfer experiments into ins-HA transgenic mice, we injected 0.65–1.25 × $10^6$ purified $T_{reg}$ or 2–3 × $10^6$ $CD25^-$ cells from TCR-HA transgenic mice.

*Antibodies and Flow Cytometric Analysis.* After a mechanical dissociation, cells from spleen or peripheral LNs were preincubated with 2.4G2 mAb (BD Biosciences) to block nonspecific binding to Fc receptors and then stained in PBS 3% fetal calf serum with saturating amounts of combinations of the following mAbs: FITC-conjugated anti-CD4 (clone GK1.5); CyChrome- and allophycocyanin-labeled anti-CD4 (RM4-5); allophycocyanin-labeled anti-CD25 (PC61); PE-labeled CD45RB (C363.16A); PE- and allophycocyanin-labeled anti-CD62L (MEL-14); and PE-labeled anti–Thy-1.1/CD90.1 (OX-7; all from BD Biosciences). We also used the following biotinylated antibodies: anti-CD5 (53-7.3; eBioscience); anti-CD25 (7D4; BD Biosciences); anti-CD38 (90; eBioscience); anti-CD44 (IM7; Caltag Laboratories); anti-CD54 (KAT-1; Caltag Laboratories); anti-CD69 (H1.2F3; BD Biosciences); anti-CD71 (R17 217.1.4; Caltag Laboratories); anti–Thy-1.1/CD90.1 (OX-7; BD Biosciences); anti-CD103 (M290; BD Biosciences); anti-CD122 (TM-b1; BD Biosciences); anti–OX-40/CD134 (OX-86; BD Biosciences); and anti–glucocorticoid-induced TNF receptor (GITR; goat polyclonal Ab; R&D Systems). The biotinylated mAbs were detected by CyChrome- or allophycocyanin-streptavidin (BD Biosciences). Labeling with the anti-clonotypic mAb (clone 6.5) specific to TCR-HA was revealed by a biotin anti–rat IgG2b Ab (BD Biosciences) and streptavidin-CyChrome (BD Biosciences). Isotype-irrelevant mAbs (BD Biosciences) were used as controls. Lymphocytes were gated according to their forward and side scatter characteristics and four-color FACSCalibur™ analyses were performed either with CELLQuest™ (Becton Dickinson) or FlowJo® (Tree Star) software.

After adoptive transfer in wild-type hosts, donor $T_{reg}$ represented <0.1% of splenocytes or LN cells. Therefore, we acquired 1–2 × $10^6$ events on a flow cytometer to detect significant numbers of donor cells. As controls, untransferred mice were systematically analyzed in the same time to evaluate the level of background. Within the $CD4^+$ Thy-$1.1^+$ gate, the number of events were typically of 500 for mice injected with $T_{reg}$ versus <10 for untransferred mice.

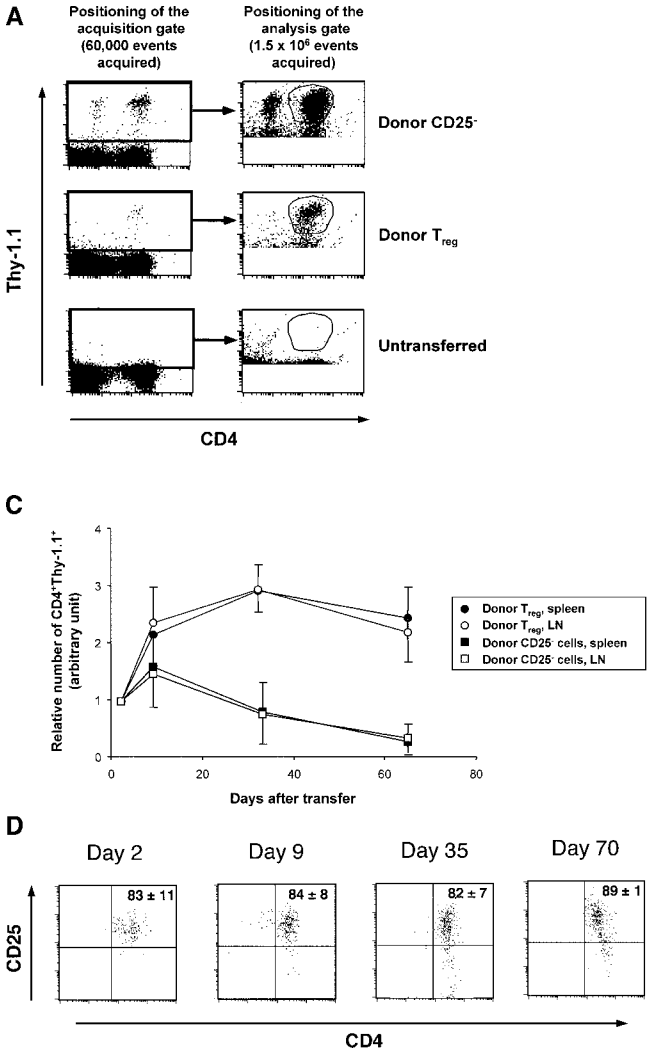
*Bromodeoxyuridine (BrdU) Labeling and Cell Cycling Analysis.* Mini osmotic pumps (ALZET2001; Durect Corporation), delivering 1.2 mg per day of BrdU (Sigma-Aldrich) for 7 d, were transplanted subcutaneously under ketamine/xylazine anesthesia to 7–8-wk-old mice (Charles River Laboratories). Then, LN cells and splenocytes were stained with anti-CD4 CyChrome (GK1.5; BD Biosciences), anti–CD25-PE (PC61; BD Biosciences), anti–CD44-biotin (IM7.8.1; Caltag), and streptavidin-allophycocyanin (BD Biosciences). For BrdU detection, triple-stained cells were fixed in 1% paraformaldehyde (Sigma-Aldrich) for 12 h in the

dark, permeabilized first in absolute ethanol for 4 min on ice, washed in PBS, incubated for 30 min at room temperature followed by 30 min on ice in PBS containing 1% paraformaldehyde and 0.01% Tween-20 (Sigma-Aldrich). After washing in PBS, cells were incubated for 30 min at room temperature with 1 mg/ml DNase I (Sigma-Aldrich) in a buffer containing 0.15 M NaCl, 4.2 mM $MgCl_2$, and 10 mM HCl, pH 5. After being washed in PBS, cells were incubated for 45 min at 4°C in PBS containing 5% fetal calf serum, 0.5% Tween-20, and FITC-labeled anti-BrdU mAb (B44; Becton Dickinson). Then, cells were washed twice in PBS and analyzed using a FACSCalibur™ flow cytometer and CELLQuest™ software (both from Becton Dickinson).

## Results

*Long-Term Survival and Stable CD25 Expression of $T_{reg}$ In Vivo.* To evaluate the recirculation properties, lifespan, turnover rate, and CD25 marker expression stability of the natural suppressor $T_{reg}$, we transferred $T_{reg}$ purified from normal mice, having a diversified T cell repertoire, into unmanipulated nonlymphopenic normal Thy-1 congenic mice. We focused this study on the transfer of the $CD62L^{high}$ $T_{reg}$ subset because we and others have previously demonstrated the high suppressive activity of this population in vivo (7, 32) and also because the $CD4^+$ $CD25^+$ $CD62L^{high}$ cell population should contain low, if any, contamination with activated conventional $CD4^+$ T lymphocytes, which display a $CD4^+$ $CD25^+$ $CD62L^{low}$ phenotype (33). Experiments described below and shown in Figs. 1, 2, and 3 were all performed in the BALB/c genetic background. For all of these experiments, similar findings were observed in the C57BL/6 genetic background (unpublished data).

2, 9, 35, or 70 d after transfer of $CD62L^{high}$ $T_{reg}$, low but reliable numbers of donor cells were found in both the



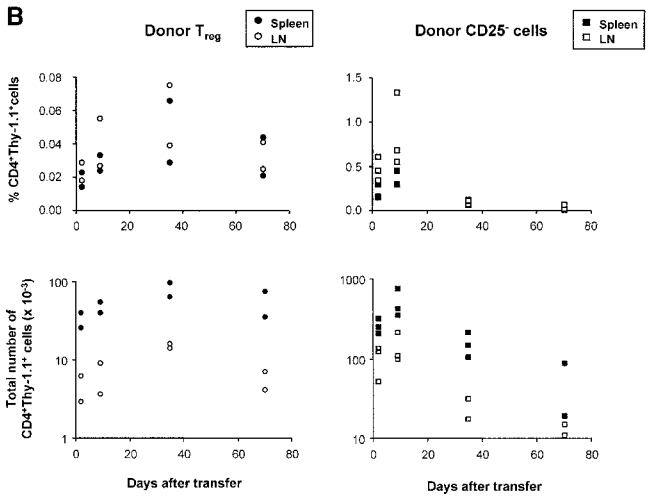




**Figure 1.** Long-term survival and stable CD25 expression of $T_{reg}$ after adoptive transfer. $CD62L^{high}$ $T_{reg}$ or $CD25^-$ cells purified from Thy-1.1 BALB/c mice were injected intravenously into 8–10-wk-old Thy-1.2 congenic BALB/c mice and analyzed by flow cytometry 2, 9, 35, and 70 d after transfer. (A) Reliable identification of very low percentages of donor $CD4^+$ Thy-$1.1^+$ cells in recipient mice is possible after acquisition of 1–2 million cells by flow cytometry. Left panels show dot plots on whole LN cells after acquisition of 60,000 events and right panels show dot plots after acquisition of $1.5 \times 10^6$ events (only Thy$1{-}1^+$ cells were saved to limit sizes of the files). Representative dot plots of mice injected with $CD25^-$ cells or $CD62L^{high}$ $T_{reg}$, as well as a dot plot of control noninjected mice, are shown. Noninjected mice were systemically included in these analyses to quantify background level. Depending on the transfer experiment and time point, the number of events in the $CD4^+$ Thy-$1.1^+$ gate was between 0 to 8 for noninjected mice versus 150 to 1,150 in mice injected with $T_{reg}$ in the spleen or LN. (B) Quantification of donor $CD4^+$ Thy-$1.1^+$ cells in the spleen (● and ■) and LNs (○ and □) is expressed in percentage (top) and total number (bottom) of $CD4^+$ Thy-$1.1^+$ cells per organ for one representative experiment out of four independent experiments. Each symbol represents one individual mouse. (C) The graph represents the relative average number of indicated donor cells from four independent experiments (total of 5–12 mice per time point). For each experiment, the mean number of donor cells at different time points were divided by the mean number of donor cells at day 2, giving relative mean values. The average of these mean values from the four experiments was represented in arbitrary units, the one at day 2 is by definition of 1. Error bars represent the SD. The increased proportion of $CD25^-$ T cells between days 2 and 9 was not statistically significant. (D) 2, 9, 35, and 70 d after transfer of $CD62L^{high}$ $T_{reg}$, donor cells were analyzed for expression of CD4 and CD25 in LNs. Each dot plot, gated on Thy-$1.1^+$, is representative of five to eight mice per time point from four independent experiments. Values indicate the mean percentage ± SD of $CD25^+$ cells among $CD4^+$ cells.

spleen and LN (Fig. 1, A–C), whereas in mice transferred with the CD62L$^{low}$ $T_{reg}$, donor cells were detected in the spleen but hardly in the LN (unpublished data). This result can be explained by the known important role of the CD62L molecule in the migration of lymphocytes from blood into LNs (33). A moderate increase of donor cell numbers was observed between days 2 and 35 after cell transfer, which could be due to a recirculation from the liver where we observed a sizeable proportion of donor cells at an early time point (unpublished data). Remarkably, in mice transferred with CD62L$^{high}$ $T_{reg}$, the numbers of donor cells at an early time point (day 9) or late time points (35 or 70 d) after transfer remained relatively stable (Fig. 1, B and C). We analyzed in parallel the survival of a population of conventional purified CD25$^-$ T cells, similarly injected into normal unmanipulated Thy-1 congenic mice. In contrast to the $T_{reg}$, the number of transferred CD25$^-$ T cells progressively decreased with time in the spleen and LN. 35 and 70 d after transfer their numbers were severely reduced (Fig. 1, B and C).

The CD25 molecule is the classical cell surface marker used to identify the natural suppressor CD4$^+$ T cells, but stability of its expression in the steady state has not been addressed. Up to 70 d after transfer, when the experiment was stopped, most of transferred cells maintained a stable phenotype with high level expression of CD4 and CD25 (Fig. 1 D). Similar findings were observed in the spleen (not depicted). This result shows that contrary to activated conventional T cells, which express CD25 for only a few days, the CD25 marker has a stable expression on $T_{reg}$ in vivo.

*Rapid Turnover of a $T_{reg}$ Subset.* The stable numbers of infused $T_{reg}$ observed at various times after cell transfer could be maintained by quiescent cells with long lifespan and/or by a more dynamic process involving a balance of divisions compensated by cell deaths. To explore their division rates, donor CD62L$^{high}$ $T_{reg}$ were labeled with CFSE before transfer. In LNs, cells had not yet divided 2 d after transfer. At day 9, a significant proportion of $T_{reg}$ had gone through at least one division, some having already divided extensively (more than six times). 35 and 70 d after transfer, a majority of the remaining cells had divided, most of them more than six times, whereas ~30% of the remaining cells had never divided. Similar findings were observed in the spleen at days 2 and 9, whereas over time the proportion of cells that had never undergone division dropped to only ±20 and 14% at days 35 and 70, respectively (Fig. 2 A). This result indicates that within the $T_{reg}$, two subsets exhibit very distinct fates. Some cells are quiescent and have long lifespan, in the order of months. On the opposite, another $T_{reg}$ subset appears to be constituted of cells with rapid turnover and short lifespan.

*$T_{reg}$ with a Rapid Turnover Acquire a Phenotype of Activated Cells.* T cell activation is followed by phenotypic changes with up-regulation of CD44, CD69, and OX40/CD134, and down-regulation of CD62L. Therefore, we analyzed these markers at various times after transfer of purified CD62L$^{high}$ $T_{reg}$. Quiescent $T_{reg}$ predominantly kept a stable CD62L$^{high}$ CD44$^{int}$ CD134$^{int}$ phenotype for at least 2 mo. In contrast, the cells that had divided extensively acquired an activated phenotype with up-regulated expression of CD44, CD69, and CD134, and down-regulated expression

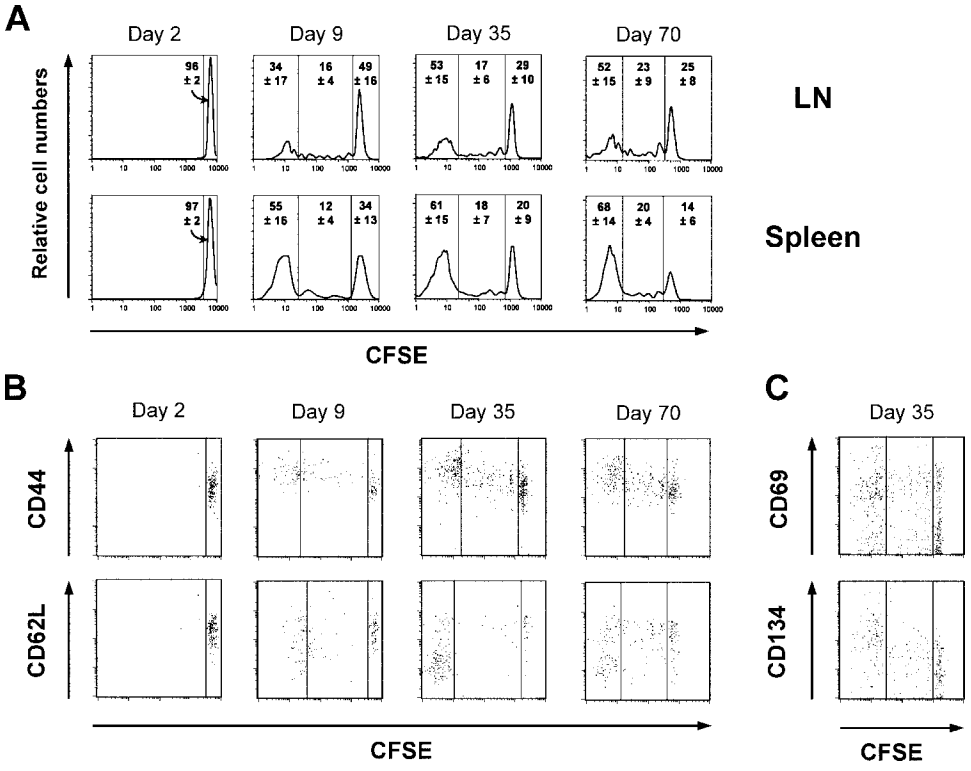



**Figure 2.** Rapid turnover and acquired activated phenotype of a $T_{reg}$ subset. 2, 9, 35, and 70 d after transfer of CFSE-labeled Thy-1.1 CD62L$^{high}$ $T_{reg}$ into Thy-1.2 BALB/c mice, donor cells (gated on CD4$^+$ Thy1.1$^+$ cells) were analyzed for cell division (A) and activation markers (B and C). The vertical bars delimit undivided cells from cells that had divided one to six times and cells that went through more than six divisions. (A) Each CFSE histogram is representative of four to six mice per time point. Values indicate the mean percentage ± SD of cells in the different quadrants. (B and C) Each dot plot is representative of four to six mice per time point.

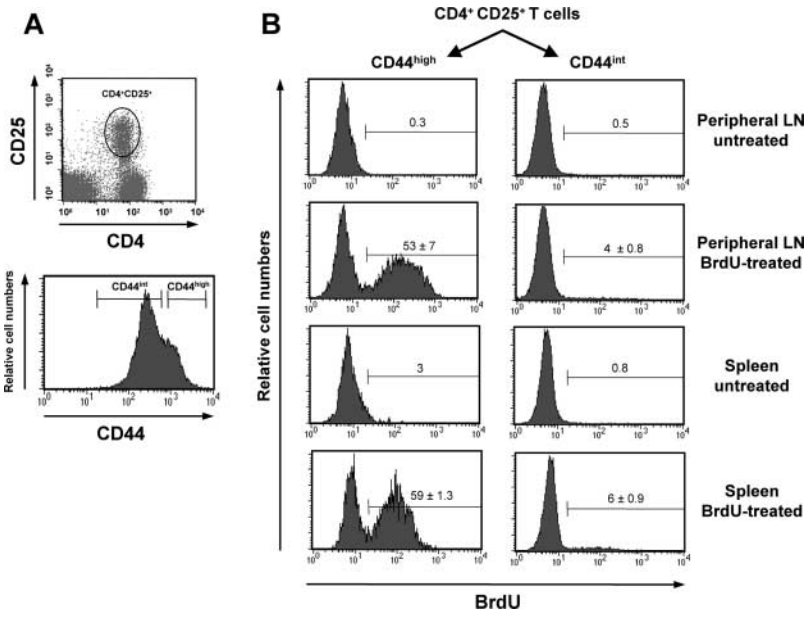



**Figure 3.** Analysis of turnover of $T_{reg}$ by BrdU incorporation. BALB/c mice were treated with BrdU administered continuously for 7 d using osmotic pumps. Then, peripheral LN cells and splenocytes were analyzed for cell surface expression of CD4, CD25, and CD44, and BrdU incorporated into DNA of divided cells. Levels of BrdU incorporations were quantified on gated $CD4^+$ $CD25^+$ $CD44^{high}$ cells and $CD4^+$ $CD25^+$ $CD44^{low}$ cells in LNs and the spleen as defined in A. Background BrdU staining levels were obtained from untreated mice (B). Representative results are shown and values indicate the mean ± SD of $BrdU^+$ cells from data from two independent experiments (seven mice). The percentage of $CD25^+$ $CD4^+$ T cells was not statistically different in mice receiving osmotic pump with BrdU, mice receiving osmotic pump with PBS, and unmanipulated mice. In A, $CD25^+$ $CD4^+$ T cells represented 8.2% of $CD4^+$ cells.

of CD62L (Fig. 2, B and C). CD62L down-regulation was observed predominantly after six divisions, suggesting a relative stability of this marker at high level expression even after activation, as previously described in vitro (7). Similar findings were observed in the spleen (not depicted).

*Rapid Turnover of $CD44^{high}$ Regulatory T Cells.* To confirm that a fraction of $T_{reg}$ has a rapid turnover at the steady state, we performed a BrdU incorporation assay. Normal mice received continuous administration of BrdU for 7 d. Cells that went through divisions during this period incorporated this nucleoside analogue in their DNA and were quantified by flow cytometry. Because up-regulation of CD44 was observed progressively from the first division of $T_{reg}$, whereas down-regulation of CD62L happened only after several rounds of divisions (Fig. 2 B), we quantified BrdU incorporation within the $CD44^{high}$ $T_{reg}$ and $CD44^{int}$ $T_{reg}$ subsets. After 1 wk of BrdU administration, 50–60% of $CD44^{high}$ $T_{reg}$ from peripheral LNs and spleen had incorporated the nucleoside analogue. This contrasts with data obtained for the $CD44^{int}$ $T_{reg}$ population, for which only ~5% of the cells had incorporated BrdU during the same period in these compartments (Fig. 3). Similar findings were observed in short-term BrdU incorporation experiments. After 24 h of BrdU treatment, $BrdU^+$ cells were contained within the $CD44^{high}$ $T_{reg}$ and not the $CD44^{int}$ $T_{reg}$ (not depicted). These experiments confirmed data obtained in the adoptive transfer experiments that a fraction of $T_{reg}$ population has a high turnover rate in the steady state.

*Two $T_{reg}$ Subsets with Different Phenotypes.* Our results indicate that $T_{reg}$ are constituted of two subsets with different phenotypes and turnover properties. To further define their phenotype, we performed flow cytometric analyses of these subsets in unmanipulated normal mice. Using cells

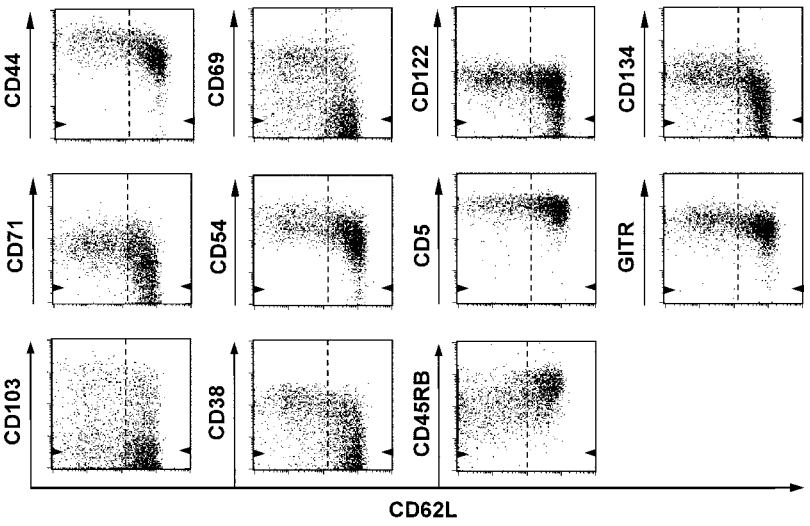



**Figure 4.** Cell surface phenotype reveals two $T_{reg}$ subsets. Peripheral LN cells of BALB/c adult mice were analyzed for cell surface expression of CD4, CD25, CD62L, and the indicated markers. Gated on $CD4^+$ $CD25^+$ cells, expression of the indicated markers was plotted with CD62L expression. The arrowheads on the left and right sides of the dot plots indicate the mean fluorescence intensity obtained with isotypic control mAb for the $CD62L^{high}$ and $CD62L^{low}$ $T_{reg}$ populations delineated by the dashed vertical line. Each dot plot is representative of three independent experiments (six mice).

**Table I.** *Phenotypic Characterization of the Two $T_{reg}$ Subsets and $CD4^+$ $CD25^-$ Cells*

| Cells | CD44 | CD69 | CD122 | CD134 | CD71 | CD54 | CD5 | GITR | CD103 | CD38 | CD45RB |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $CD4^+$ $CD25^+$ $CD62L^{high}$ | 476 | 73 | 40 | 48 | 34 | 183 | 931 | 235 | 48 | 23 | 472 |
| $CD4^+$ $CD25^+$ $CD62L^{low}$ | 1,248 | 197 | 72 | 107 | 79 | 468 | 1,158 | 408 | 170 | 116 | 189 |
| $CD4^+$ $CD25^-$ | 263 | 22 | 7 | 3 | 14 | 65 | 728 | 19 | 16 | 5 | 1,315 |

Peripheral LN cells of adult BALB/c mice were labeled with mAb specific to CD4, CD25, CD62L, and the indicated marker and analyzed by flow cytometry. Gated on $CD4^+$ $CD25^+$ $CD62L^{high}$, $CD4^+$ $CD25^+$ $CD62L^{low}$, and $CD4^+$ $CD25^-$ cells, results are expressed as mean fluorescence intensity of the indicated marker. Representative data from one out of six mice are shown.

from mice from clean animal facilities, the $CD4^+$ $CD25^+$ $CD62L^{low}$ cells are likely composed mostly of suppressor T cells because these cells have comparable suppressive activity in vitro as the $CD4^+$ $CD25^+$ $CD62L^{high}$ cells (34). Cells gated on the $CD4^+$ $CD25^+$ $T_{reg}$ phenotype were analyzed in LNs for CD62L expression and activation markers, such as CD44, OX40/CD134, GITR, CD69, and IL-2Rβ/CD122, all up-regulated upon activation of $T_{reg}$ (35 and unpublished data). Compared with $T_{reg}$ expressing high levels of CD62L, the $CD4^+$ $CD25^+$ $CD62L^{low}$ cells had increased expression of the activation markers CD44, OX40/CD134, GITR, CD69, and IL-2Rβ/CD122 (Fig. 4 and Table I). Importantly, these last two markers are up-regulated only for a few days upon $T_{reg}$ activation (35 and unpublished data), suggesting that most of the $CD4^+$ $CD25^+$ $CD62L^{low}$ cells are continuously activated in the steady state. The increased expression of the transferrin receptor CD71 in these cells, up-regulated in dividing cells (36), supports this hypothesis (Fig. 4 and Table I).

Besides its constitutive expression of CD25, natural suppressor $CD4^+$ T cells have been described by various phenotypes such as $CD5^{high}$ (37), $GITR^+$ (35, 38), $CD38^+$ (39), or $CD45RB^{low}$ (40) cells. Indeed, we observed that both subsets of $T_{reg}$ ($CD62L^{high}$ and $CD62L^{low}$) expressed higher levels of CD5, GITR, or CD38 than $CD4^+$ $CD25^-$ T cells, and lower levels of CD45RB than $CD4^+$ $CD25^-$ T cells (Table I). Again, CD38 and CD45RB were differentially expressed by the $CD62L^{high}$ $T_{reg}$ and $CD62L^{low}$ $T_{reg}$ subsets. This could be explained by the different activation status of the two subsets because CD38 is up-regulated whereas CD45RB is down-regulated upon T cell activation (39).

Based on data shown in Figs. 2, 3, and 4, it can be hypothesized that at a given time, a fraction of the $T_{reg}$ population is chronically activated. First, they up-regulate CD69 while remaining $CD62L^{high}$, and then, after several divisions they acquire a $CD69^+$ $CD62L^{low}$ phenotype. In support of this scenario, we found that after 24 h of BrdU incorporation, only 1% of the $CD69^-$ $CD62L^{high}$ cells were $BrdU^+$, whereas 6–10% of both $CD69^+$ $CD62L^{high}$ and $CD69^+$ $CD62L^{low}$ cells were $BrdU^+$ (not depicted). One $T_{reg}$ subset, subsequently referred to as activated $T_{reg}$, would predominantly have a $CD4^+$ $CD25^+$ $CD62L^{high/low}$ $CD44^{high}$ $CD69^+$ $CD122^{high}$ $CD134^{high}$ $CD71^{high}$ $CD54^{high}$ $CD5^{high}$ $GITR^{high}$ $CD38^{high}$ $CD45RB^{low}$ phenotype. The other subset, subsequently referred to as resting $T_{reg}$, would predominantly have a $CD4^+$ $CD25^+$ $CD62L^{high}$ $CD44^{int}$ $CD69^-$ $CD122^{low}$ $CD134^{int}$ $CD71^{low}$ $CD54^{int}$ $CD5^{int}$ $GITR^{int}$ $CD38^-$ $CD45RB^{int}$ phenotype. Interestingly, compared with the $CD62L^{low}$ $T_{reg}$, the $CD62L^{high}$ $T_{reg}$ also expressed higher levels of the CCR7 chemokine receptor (32). Importantly, it has been shown that among the $CD4^+$ $CD25^+$ $T_{reg}$, $CD62L^{high}$ and $CD62L^{low}$ cells have similar suppressive activity in vitro, as well as $CD45RB^{low}$ and $CD45RB^{high}$ cells, $CD69^+$ and $CD69^-$ cells, and $CD38^+$ and $CD38^-$ cells (32). From this, it can concluded that the two $T_{reg}$ subsets that we have defined have both potent suppressive activity in vitro. Thus, our data offer a model to explain the described phenotypic heterogeneity of the suppressor $T_{reg}$ for numerous markers, such as CD62L, CD44, CD69, CD38, or CD45RB (1, 2).

*Sustained Activation of Autoreactive $T_{reg}$ in the Steady State.* Highly autoreactive $T_{reg}$ precursors are positively selected in the thymus (19–21), suggesting that $T_{reg}$ are enriched in autoreactive cells in the periphery (22). Thus, we speculated that the activated $T_{reg}$ subset is constituted mostly of autoreactive cells that respond to self-antigens. To test this hypothesis, we used ins-HA transgenic mice expressing the model HA Ag in pancreatic islets and TCR-HA transgenic mice expressing a T cell receptor transgene specific to an HA peptide. In these mice, HA-specific $CD4^+$ T cells were identified using the anticlonotypic 6.5 mAb. Highly purified $CD62L^{high}$ $T_{reg}$ from TCR-HA transgenic mice were labeled with CFSE and transferred intravenously into ins-HA transgenic mice. At various times after transfer, donor $T_{reg}$ were analyzed by flow cytometry in pancreatic LNs and control peripheral LNs. In the pancreatic LN, the numbers of donor cells significantly increased, with a peak at days 5–7 depending on the experiment. Then, their numbers decreased dramatically by day 11. On the contrary, donor $T_{reg}$ did not expand in peripheral LNs and their numbers decreased progressively up to day 11 (Fig. 5 A). The increased proportion of donor $T_{reg}$ in pancreatic LNs was due to a significant proliferation of HA-specific $6.5^+$ cells, a phenomenon not observed in peripheral LNs (Fig. 5 B). The proliferating HA-specific $6.5^+$ $T_{reg}$ acquired a $CD44^{high}$ $CD62L^{high/low}$ activated phenotype (Fig. 5 C), as previously observed for the activated $T_{reg}$ subset derived from nontransgenic mice (Fig. 2 B). 11 d after transfer, we could hardly detect any $6.5^+$ cells that had divided in various LNs, spleens, or pancreas (not depicted), suggesting that most of

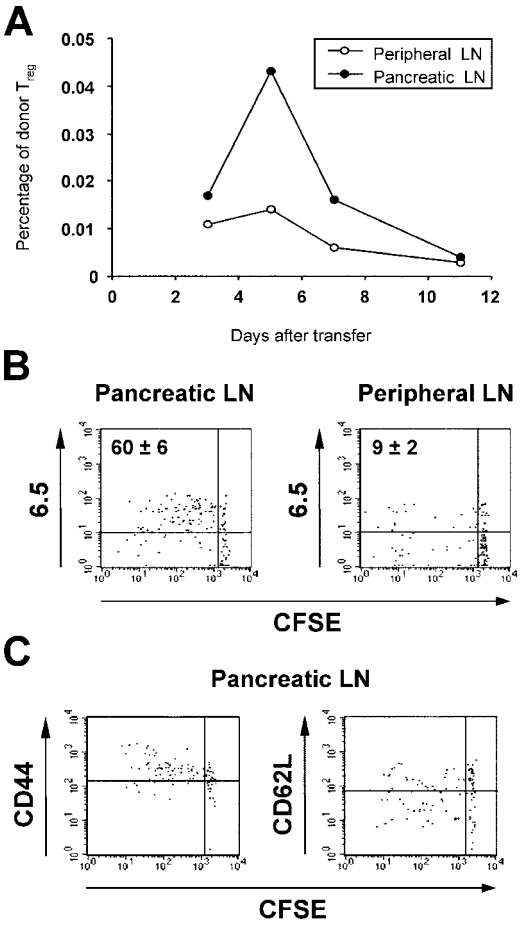



**Figure 5.** The activated $T_{reg}$ population contains autoreactive cells. CFSE-labeled CD62L$^{high}$ $T_{reg}$ purified from Thy-1.1 TCR-HA transgenic mice were intravenously injected into Thy-1.2 ins-HA transgenic mice. (A) The proportion of CD4$^+$ Thy-1.1$^+$ donor cells was determined by flow cytometry in pancreatic (●) and peripheral (○) LNs 3, 5, 7, and 11 d after transfer. The graph, which represents the percentage of donor $T_{reg}$ (CD4$^+$ Thy-1.1$^+$) per organ, shows the mean of two to three mice per time point and is representative of three independent experiments. (B and C) Representative phenotypic analysis of the donor cells on day 7 after transfer in the indicated LNs. Gated on CD4$^+$ Thy-1.1$^+$ cells, cells were analyzed for CFSE staining and expression of the anti-HA–specific TCR using the 6.5 mAb or of CD44 and CD62L. Values ± SD indicate the percentages of 6.5$^+$ divided cells.

the HA-specific cells were deleted shortly after expansion in pancreatic LNs. The preferential expansion of HA-specific $T_{reg}$ in pancreatic LNs of ins-HA transgenic mice indicates that activation of $T_{reg}$ is indeed auto-Ag driven. Thus, the low level physiological presentation of tissue self-Ag in draining LNs is sufficient to induce intense proliferation of specific autoreactive $T_{reg}$.

## Discussion

So far, CD25 has been the best cell surface marker used to identify the natural suppressor CD4$^+$ CD25$^+$ T cells. Recent studies showed that the in vitro suppressive activity of a polyclonal population (41) or even clones (42) of $T_{reg}$ was contained within cells expressing high but not intermediate levels of CD25, emphasizing the importance of this marker for this population. CD25 expression by $T_{reg}$ likely plays a critical role because their proliferation and survival is dependent on IL-2 (3, 26, 43, 44). However, conflicting data have been published on the stability of this marker on $T_{reg}$. We and others have shown a stable CD25 expression on $T_{reg}$ in vitro that maintained a suppressive activity, even after 4–6 wk of culture (7, 45). On the other side, after transfer of purified $T_{reg}$ into lymphopenic mice, injected cells expanded and most of them lost CD25 expression (28, 46). However, contrary to the cells that maintained CD25 expression, the CD25$^-$ cells had also lost their suppressive activity, suggesting that they resulted from the preferential expansion of contaminant cells (being either activated CD25$^+$ conventional CD4$^+$ T cells or CD25$^-$ cells) in the injected $T_{reg}$ population (28). To analyze the stability of CD25 expression on $T_{reg}$ in physiological steady-state conditions, we transferred, in nonlymphopenic congenic recipients, highly purified CD4$^+$ CD25$^+$ CD62L$^{high}$ cells (>98% pure), limiting the risk to inject CD25$^-$ cells or activated CD25$^+$ conventional CD4$^+$ T cells. Indeed, conventional activated CD4$^+$ T cells are prominently in the CD62L$^{low}$ population. Our data showed a remarkable in vivo stability of CD25 expression on $T_{reg}$ for at least 2 mo. These data reinforce the hypothesis that these cells belong to a specific lineage, distinct from conventional CD4$^+$ T cells or from other regulatory CD4$^+$ T cell populations secreting IL-10 or TGFβ immunosuppressive cytokines, which display unstable CD25 expression (42). In addition to the $T_{reg}$-specific transcription factor Foxp3 (47), stable CD25 expression is therefore a hallmark of the natural suppressor $T_{reg}$.

In vitro data showed that $T_{reg}$ do not proliferate to antigenic or anti-CD3 stimulation, except if IL-2, anti-CD28 antibody, or lipopolysaccharide is added to the culture (43, 44, 48). Their hyporesponsiveness has also been described after adoptive transfer of $T_{reg}$ that proliferated poorly to their cognate Ag administrated subcutaneously in complete Freund's adjuvant (46). Thus, $T_{reg}$ are usually considered anergic cells. This property has been challenged by data aimed at analyzing their turnover in steady-state conditions using BrdU incorporation experiments. After 3 d of BrdU administration, 10.5% of CD4$^+$ CD25$^+$ cells, versus only 4.5% of CD4$^+$ CD25$^-$ cells, were BrdU$^+$ (49, 50). However, without knowing the level of possible conversions from CD4$^+$ CD25$^+$ phenotype into CD4$^+$ CD25$^-$ phenotype and vice versa, a definitive point could not be made. Indeed, for example, some of the BrdU$^+$ CD4$^+$ CD25$^+$ cells could have incorporated BrdU while they were CD4$^+$ CD25$^-$ cells (for instance conventional activated CD4$^+$ T cells). Because our data showed few, if any, conversion from the CD4$^+$ CD25$^+$ phenotype into the CD4$^+$ CD25$^-$ phenotype and vice versa in steady-state conditions, we can now conclude from the BrdU incorporation experiments that $T_{reg}$ are cycling in vivo and therefore cannot be considered as "anergic" stricto-sensu. In support of this contention, after transferring CFSE$^+$ $T_{reg}$, a fraction of them rapidly lost CFSE staining, indicative of cell division.

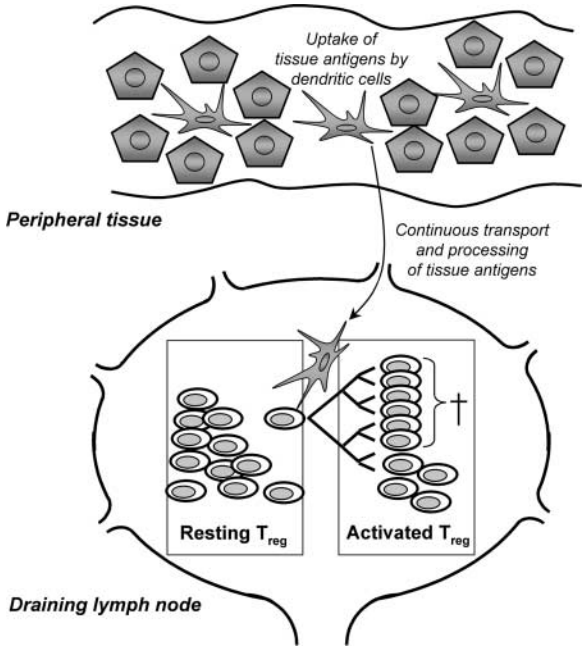



**Figure 6.** A model describing some of the features of the homeostasis of $T_{reg}$. In the steady state, tissue self-Ag, originated from dying cells, molecule shedding, or direct capture from live cells, are presented by Ag-presenting cells in draining LNs. This presentation of self-Ag induces activation of specific autoreactive cells derived from the pool of long-life resting $T_{reg}$. During rapid expansion, the cells enter the pool of activated $T_{reg}$, displaying an activated phenotype. Then, some of them die or recirculate in the spleen.

Importantly, only a subset, and not the whole population of $T_{reg}$, is cycling in the steady state. When analyzed 1 mo after transferring $CFSE^+$ $T_{reg}$, about one third in the LN and one fifth in the spleen of the remaining donor cells had not lost CFSE staining and thus had not divided for 1 mo. These cells were mostly $CD62L^{high}$, $CD44^{int}$, $CD69^-$, and $OX40^{int}$. In marked contrast, cells that had divided extensively (more than six divisions after 1 mo) displayed an activated phenotype because they were mostly $CD62L^{low}$, $CD44^{high}$, $CD69^+$, and $OX40^{high}$. BrdU experiments confirmed the existence of two subsets of $T_{reg}$, one with a rapid turnover, characterized by its $CD44^{high}$ phenotype, and a quiescent subset, characterized by its $CD44^{int}$ phenotype. Phenotypic analyses on nonmanipulated mice also support the existence of a resting $T_{reg}$ subpopulation and a subset displaying a phenotype of activated cells. Indeed, these latter cells expressed higher levels of markers up-regulated upon activation on $T_{reg}$ such as CD44, CD134, and GITR (35). Importantly, they also display increased expression of markers only transiently up-regulated upon cell activation, such as CD69 (unpublished data) and CD122 (35), as well as CD71, a molecule up-regulated in dividing cells (36). Altogether, these data strongly suggest that a $T_{reg}$ subset is composed of cells that are continuously activated.

By analogy with signals driving proliferation of conventional T cells, the signals that drive continuous activation of cycling $T_{reg}$ could be cytokine- and/or TCR-mediated. We show here that $T_{reg}$ expressing a TCR specific to islet-Ag proliferated extensively in pancreatic LNs but not in other LNs. Thus, in addition to the fact that a fraction of the repertoire of $T_{reg}$ is autoreactive (19–22), the activated $T_{reg}$ subset is likely composed for a large part of autoreactive $T_{reg}$. After expansion of activated $T_{reg}$ in the steady state, most of the cells probably die because no accumulation or significant migration into nonlymphoid tissues were observed (not depicted). Some of them recirculated preferentially in the spleen (not depicted), a property explained by their $CD62L^{low}$ $CCR7^{low}$ phenotype.

Thus, we propose a model that summarizes some of the features of the homeostasis of $T_{reg}$ in adults (Fig. 6). In permanence, tissue self-Ag, originated from dying cells, molecule shedding, or direct capture from live cells, are presented by Ag-presenting cells in draining LNs (51, 52). From the pool of resting $T_{reg}$, the highly autoreactive cells that encounter their cognate self-Ag rapidly expand and acquire a phenotype of activated $T_{reg}$, and then, most of them die or recirculate in the spleen.

These findings may shed new light on the mode of action of $T_{reg}$ and give plausible explanations on several previous findings. In vitro experiments have shown that switching on suppressor function on $T_{reg}$ is dependent on their activation via TCR engagement, but once activated, they exhibit a nonspecific bystander suppression on other T cells (34, 44). The existence of a pool of activated $T_{reg}$ in lymphoid tissues suggests that this subset exerts a basal and permanent suppression on T cell activation. This would explain why depletion of endogenous $T_{reg}$ in the steady state induced a rapid increased proliferation of memory CD8 T cells (3) and a rapid increase of antitumor responses (10). Our data also support a very dynamic process in which new clones, originated from the pool of resting $T_{reg}$, are activated when they encounter their cognate self-Ag, leading to their expansion followed by contraction in draining LNs. This phenomenon may have two important implications. In LNs, the pool of activated $T_{reg}$ would be enriched in cells specific to self-Ag originated from drained tissues. This would explain why $T_{reg}$ from pancreatic LNs, but not the ones from other LNs, efficiently regulated autoimmune diabetes (53). The second implication is related to the dynamism of this process. A removal of the source of tissue-Ag may lead to a rapid contraction of the pool of tissue-specific $T_{reg}$. Experimental findings support this hypothesis. The capacity of $T_{reg}$ from secondary lymphoid tissues to prevent autoimmune thyroiditis was lost in rats whose thyroids were ablated, while they maintained the capacity to prevent autoimmune diabetes (54). In addition, tolerance to ovarian Ag involved in prevention of autoimmune oophoritis was lost as soon as 1 wk after ablation of ovaries (55). Finally, cytotoxic treatments killing cycling cells, such as irradiation or chemotherapy, most likely delete activated $T_{reg}$. This could explain why these treatments may paradoxically increase severity of some autoimmune diseases (56).

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