Foxp3+ CD25− CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion

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Expression of the IL-2 receptor α chain (CD25) by peripheral CD4 T cells follows cellular activation. However, CD25 expression by CD4 cells is widely used as a marker to identify regulatory T cells (TREG), although cells with regulatory properties are also found in the CD4+ CD25− subset. By using in vivo functional assays and Foxp3 expression as a faithful marker of TREG differentiation, we have evaluated the requirements for CD25 expression by peripheral TREG. We first show that in vivo depletion of CD25+ cells prevents the development of spontaneous encephalomyelitis in recombination-activating gene (RAG)-deficient anti-myelin basic protein T cell antigen receptor (TCR) transgenic mice, and allows disease induction in otherwise healthy RAG-competent transgenic mice. Similar treatment in normal thymectomized animals is followed by the fast recovery of a normal number of CD25+ TREG. Consistently, Foxp3-expressing TREG encompassed in the CD25− cell population convert to CD25+ after homeostatic expansion and are selectable by IL-2 in vitro. Surface expression of CD25 on TREG is controlled by the activity of conventional CD4 cells and is fully labile because it can be lost and regained without affecting the functional potential of the cells. These findings reveal that Foxp3-expressing CD25− cells constitute a peripheral reservoir of differentiated TREG, recruited to the CD25+ pool upon homeostatic expansion and/or activation. This analysis, together with the notion that physiological commitment of TREG takes place exclusively in the thymus should help for the interpretation of experiments assessing peripheral TREG differentiation from naïve CD4 T cells, defined as CD25−.

H ealthy unmanipulated mice bear a significant number of “naturally” activated B and T lymphocytes, which seem to represent physiological autoreactivity because they are equally represented in “germ-free” and “antigen-free” mice (1). Like other antigen-experienced CD4 cells, naturally activated CD4 cells are encompassed in the CD45RBlow pool (2). Like other antigen-experienced CD4 cells, naturally activated CD4 cells are encompassed in the CD45RBlow pool (2). As demonstrated in several experimental systems using adoptive transfers, CD4+ CD45RBlow cells (from now on denoted CD45RBlow) limit the pathological potential of the complementary CD45RBhigh naïve cells (3, 4). A subset of CD45RBlow cells expressing the CD25 marker is highly enriched in regulatory T cells (TREG) that limit both protective and pathological immune responses (5). Several reports, however, demonstrate that TREG are not exclusively contained within the CD25-expressing subset (6–9). Moreover, surface markers and genes that are highly represented or expressed in the CD25+ cells are also found in the CD45RBlowCD25− subpopulation although at a lower frequency or level, while being absent in the CD45RBhigh subset. This is the case for the surface molecules CD103 (8), glucocorticoid-induced tumor necrosis factor receptor (GITR) (10), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (11), Toll-like receptor (TLR)-4, -5, -7, and -8 (12), and the transcription factor Foxp3 (13). Expression of the Foxp3 gene is strictly required for TREG development and enough to confer conventional CD4 T cells with regulatory function (13–15). It is to date the only known TREG commitment/differentiation factor in mice.

For convenience of experimental design, most current studies use surface expression of CD25 to distinguish “conventional” T cells from TREG. This approach seems appropriate, because most in vivo studies successfully associated regulatory activity to this cellular subset, and >95% of the CD25+ cells in a normal mouse express Foxp3, as evaluated in GFP-Foxp3 fusion knock-in mice (16). Clearly, however, the reverse does not apply, because lack of CD25 expression in a cell population cannot be taken for absence of regulatory cells. This reservation is critical, in view of previous claims that regulatory cells can differentiate in the periphery from naïve CD4 cells, defined as CD25−.

The CD25 molecule is the α chain of the IL-2 receptor, and its expression results in higher affinity to IL-2 (17). Upon activation, conventional CD4 cells express CD25, while lacking memory cells or other phenotypical characteristics of TREG (18). Similar induction of CD25 expression upon activation may well occur on TREG, and it has been proposed that IL-2 promotes acquisition of this marker and functional activation (19). Several groups have reported that CD25+ cells lose CD25 expression upon adoptive transfer in lymphopenic mice, a phenomenon that is less marked if conventional CD4 cells, presumably serving as a source of IL-2, are present and undergoing homeostatic expansion (6, 20). Intriguingly, acquisition of CD25 expression by CD25− cells undergoing homeostatic expansion was also reported, although the nature of the cells contributing to this phenomenon was not assessed (6, 20, 21).

In this study, we investigated the relevance of CD25 surface expression for the definition of TREG and, thus, the possibility that they may arise from the naïve CD4 pool in the periphery. We show that administration of deleting anti-CD25 mAb in vivo targets both newly activated conventional cells and a limited subset of regulatory T cells. Furthermore, the bulk of Foxp3-expressing T cells encompassed in the CD45RBlowCD25− cell pool convert to a CD25+ phenotype in lymphopenic conditions, and these cells display functional characteristics of TREG. Finally, surface expression of CD25 on TREG is fully labile because it can be lost and regained without affecting the functional potential of the cells. Taken together, these analyses indicate that a reservoir of TREG is contained in the CD45RBlowCD25− population and that such cryptic TREG can rapidly be recruited to the CD25+ pool.

Materials and Methods

Mice. BALB/c, C57BL/6, C57BL/6-Thy1.1, Ighα, Gpilα, C57BL/6 RAG2−/−, B10-PL-MBP-TCR-Tg, and B10-PL-

Abbreviations: TREG, regulatory T cells; RAG, recombination-activating gene; TCR, T cell antigen receptor; TREG, regulatory T cell receptor; R−R− and R+/−, anti-myelin basic protein TCR transgenic mice homo- and heterozygous for a null mutation of the RAG1 gene; TREG, thymectomy-induced EAE, experimental autoimmune encephalomyelitis; LN, lymph node; PE, phycoerythrin.

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RAG1<sup>−/−</sup>-MBP-TCR-Tg mice were bred and maintained under specific pathogen-free conditions in our animal house. All animals were used between 4 and 10 weeks of age.

**Antibodies and Reagents.** Allophycocyanin (APC), CyChrome-, and phycoerythrin (PE)-conjugated anti-CD4 mAb (clone RM4-5), CD45RB-PE (clone 16A), and Thy1.2 biontin (CD90.2) were purchased from BD Biosciences. Thy1.1 (CD90.1) biontin and Alexa Fluor 488-CD25 (clone PC61) were home-made. Biotinylated antibodies were revealed with streptavidin-PE or -APC (BD Biosciences). CD25<sup>+</sup> cell depletion was performed with 200 μg of anti-CD25 mAb (clone PC61) injected i.p. As a control, mice received the same amount of rat IgG (Sigma–Aldrich). Depletion was evaluated by using the 7D4 anti-CD25 mAb (BD Biosciences). Pertussis toxin from *Bordetella pertussis* (Sigma–Aldrich) was injected i.v. (200 ng per mouse).

**Thymectomy and Disease Evaluation.** Four-week-old BALB/c mice were thymectomized (Tx), and absence of noticeable thymic remnants was confirmed at the end of the experiment. Experimental autoimmune encephalomyelitis (EAE) was scored every 3 days as described (22).

**Cell Purification and Transfer.** Pooled lymph nodes (LNs) stained with a mixture of anti-CD4-PE and CD25-Alexa mAbs, or with anti-CD4-CyChrome, CD25-Alexa, and CD45RB-PE were purified on a MoFlo High Speed Cell Sorter (Cytomation, Fort Collins, CO). Purity was routinely >98% for CD4<sup>+</sup>CD25<sup>+</sup> cells and >99% for the other CD4 subsets. Cells were suspended in PBS and injected in the retroorbital plexus (100 μl per mouse).

**Cell Recovery and Flow Cytometric Analysis.** Cell suspensions from spleen or mesenteric LNs were prepared, stained, and washed in PBS containing 2% FCS and 0.01% sodium azide. Propidium iodide was added to the final suspension. Analyses were performed inside a live lymphocyte gate on a FACSCalibur (Becton Dickinson) by using CELLQUEST software. Life lymphocyte counts were deduced from the acquisition of a fixed number of 10-μm latex beads (Coulter) mixed with a known volume of unstained cell suspension.

**Cell Cultures and Suppression Assays.** Cultures were set in RPMI medium 1640 containing 10% FCS, 100 μg/ml penicillin and streptomycin, 50 μM 2-mercaptoethanol (2-ME), 10 mM Hapes, and 1 mM sodium pyruvate (all purchased from Life Technologies, Grand Island, NY). IL-2 production was as follows: 2.5 × 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>+</sup> cells (target) mixed with 5 × 10<sup>5</sup> irradiated spleen cells and a saturating amount of IL-2. Cells were stimulated with 0.5 ml soluble anti-CD3 mAb and a saturating amount of IL-2. After the last 24 h of a 3-day culture (amplification). For pretreatments, the cells, and a saturating amount of IL-2 was added to the last 24 h of a 3-day culture (amplification). For pretreatments, the cells are self-reactive, "protected" by TR expressing endogenous TCR (22, 24). Nevertheless, in sick T<sub>R</sub> mice, the level was scored weekly, and plotted is the percentage of mice that developed EAE (score ≥2). (A) One-month-old T<sub>R</sub> animals (n = 7) received five weekly injections and were followed for 4.5 months. The percentage of mice that developed EAE was scored every 3 days as described (22).

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**Real-Time PCR.** Total RNA was extracted from 10<sup>6</sup> to 10<sup>7</sup> cells by using TriPure (Roche Diagnostics), treated with DNaseI and reverse transcribed by using SuperScript II RT and oligo(dt<sub>12-18</sub>) primer (all from Life Technologies). PCRs were performed by using the QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and the Light Cycler system (Roche), and consisted of 15 min at 95°C and 45 cycles of 15 s at 95°C, 20 s at 61°C (Foxp3), or 55°C [hypoxanthine phosphoribosyltransferase (HPRT)].

**Results**

**Depleting Anti-CD25 mAb Antibody Targets Both Activated Effector and Regulatory T Cells in Vivo.** To establish the contribution of CD25 surface expression to conventional, activated/effector cells and to the T<sub>R</sub> pool in the same model system, we treated anti-myelin basic protein (MBP) T cell antigen receptor (TCR) transgenic (Tg) mice (23) with a depleting anti-CD25 mAb. Recombination-activating gene (RAG)-deficient monoclonal mice (T/R<sup>−</sup>) spontaneously develop severe and progressing encephalomyelitis by 2 months of age. In contrast, RAG-competent T<sub>R</sub> mice (T/R<sup>+</sup>) remain healthy, although >90% of the cells are self-reactive, “protected” by T<sub>R</sub> expressing endogenously encoded TCR (22, 24). Nevertheless, in sick T/R<sup>−</sup> mice, <5% of all CD4 cells are CD25<sup>+</sup> (data not shown). Consistently with the respective phenotype often attributed to activated effector cells and T<sub>R</sub> (25, 26), the CD4<sup>+</sup>CD25<sup>+</sup> subset detected in T/R<sup>−</sup> mice expressed higher levels of CD4 and lower levels of CD25 than the CD4<sup>+</sup>CD25<sup>+</sup> subset detected in T/R<sup>+</sup> mice (data not shown). Depletion of CD25-expressing cells in T/R<sup>−</sup> animals prevented EAE when initiated before onset of encephalomyelitis, and interruption of the treatment restored the development of progressing EAE (Fig. 1A). Similar treatment administered to sick animals did not revert the disease.
were thymectomized and treated with the depleting anti-CD25 mAb in a first step, to exclude the potential contribution of responses to TCR triggering. As shown in Fig. 2A, CD4+ T cells in T/R+ animals encompass efficient T<sub>R</sub> confirms previous conclusions drawn from adoptive cell transfer experiments (22). More importantly for the present topic, in vivo depletion of all donor T cells by using mAbs to a Thy-1 allotype in T/R+ mice, protected by adoptive transfer of normal CD4 cells, was shown to lead to spontaneous severe-progressing EAE (27) whereas we evidence here that depletion of solely CD25<sup>+</sup> cells in T/R+ animals does not induce pathology (Fig. 1C). In additional, all T/R+ animals that developed disease upon combined injection of anti-CD25 antibody and pertussis stabilized at a stage of partial hind limbs paralysis (level 2) without progression of the peripheral CD4 cell pool after anti-CD25 mAb administration (22). More importantly for the present topic, depletion and functional analysis of these cells either Thy1.1<sup>+</sup> or Thy1.2<sup>+</sup> were controlled independently for this feature. Finally, the newly converted Thy1.2<sup>+</sup> cells were sort-purified from LNs of either CD25<sup>-</sup>-depleted (CD25<sup>-</sup>/Tx-depleted) or from rat IgG-injected (CD25<sup>-</sup>/Tx control) mice 3 months after depletion and tested for their capacity to suppress IL-2 production by stimulated CD4<sup>+</sup> CD25<sup>-</sup> cells. Primary cultures consisted in 2.5 x 10<sup>5</sup> CD4<sup>+</sup> CD25<sup>-</sup> cells isolated from normal mice, stimulated alone or in the presence of different numbers of CD25<sup>+</sup> cells isolated from Tx-depleted or Tx control mice. As a negative control, CD25<sup>-</sup> cells (CD25<sup>-</sup>) were also tested. Shown is the percentage of inhibition [(cpm in control) – (cpm in experiment)/cpm in control] plotted versus the ratio of the population tested/ CD4<sup>+</sup> CD25<sup>-</sup> cell number at the origin of the primary culture.

whereas the Thy1.1<sup>+</sup> cells (originally CD45RB<sup>B<sub>high</sub>/CD25<sup>-</sup>) expressing CD25 were barely detectable (Fig. 3A). More than 80% of the original CD45RB<sup>B<sub>high</sub></sup> cells became CD45RB<sup>low</sup> upon adoptive transfer (not shown); however, sequential transfer of total CD4<sup>+</sup> CD25<sup>-</sup> cells (Thy1.2<sup>+</sup>) in these mice did not increase the frequency of Thy1.1<sup>+</sup> cells that converted to a CD25<sup>+</sup> phenotype (Fig. 3A). Similar results were obtained in recipients of simultaneous cotransfer, confirming that the two cell subsets are controlled independently for this feature. Finally, Thy1.2<sup>+</sup> CD45RB<sup>low</sup>CD25<sup>-</sup> cells were mixed with 100-fold fewer Thy1.1<sup>+</sup> CD4<sup>+</sup> CD25<sup>-</sup> cells before adoptive transfer into RAG<sup>-/-</sup> animals. This deliberate contamination exceeded that detected in the blood and lymphoid tissues 2 days after administration of depleting anti-CD25 mAb in normal mice. At day 12 posttransfer, Thy1.1<sup>+</sup> cells were hardly detectable (average 0.2% and 0.1% of CD4 cells in spleen and mesenteric LN, respectively), whereas the percentage of Thy1.2<sup>+</sup> cells expressing the CD25 marker was the same as in single transfer (not shown). It is noteworthy that, inside the CD25<sup>-</sup> subset, the contribution of the newly converted Thy1.2<sup>+</sup> was therefore >95%. These last results indicate that CD45RB<sup>low</sup>CD25<sup>-</sup> cells undergo efficient conversion to a CD25<sup>+</sup> phenotype that largely dominates over the expansion of few contaminating CD4<sup>+</sup> CD25<sup>-</sup> cells. Together these findings demonstrate that acquisition of CD25 surface expression by CD45RB<sup>low</sup>CD25<sup>-</sup> cells in nonimmunized mice is the result of a specific phenotypic modification restricted to a subset of the naturally activated T cell pool.

We next assessed whether acquisition of CD25 upon homeostatic expansion is a signature of T<sub>R</sub> by monitoring the level of Foxp3 mRNA in each CD4 subset before and after adoptive
transfer (Fig. 3B). Strikingly, the originally CD45RB<sup>hi</sup>CD25<sup>-</sup> cells that converted to a CD25<sup+</sup> phenotype displayed levels of Foxp3 mRNA comparable with that of freshly isolated CD45RB<sup>hi</sup>CD25<sup+</sup> cells. In contrast, those that remained CD25<sup+</sup> expressed Foxp3 to a lower level than freshly isolated CD45RB<sup>hi</sup>CD25<sup+</sup> cells and rather similar to fresh CD45RB<sup>hi</sup>CD25<sup+</sup> cells. We interpret this result as evidence that CD25<sup+</sup> Foxp3-expressing cells inside the CD45RB<sup>low</sup> subpopulation selectively express CD25 upon homeostatic expansion. The amount of Foxp3 transcripts in the few CD45RB<sup>hi</sup>CD25<sup+</sup> cells that converted to a CD25<sup+</sup> phenotype remains low, indicating that this cell pool contains recently activated cells. Nevertheless, it is remarkable that the little increase of Foxp3 signal in this cellular subset correlates with a decreased signal (average value 0.2) in the remaining CD25<sup+</sup> cells, indicating again that Foxp3-expressing cells convert to a CD25<sup+</sup> phenotype upon homeostatic expansion.

Finally, we tested whether acquisition of CD25 expression by CD45RB<sup>low</sup>CD25<sup+</sup> cells reveals functional suppressor cells. As references, we used freshly isolated CD4<sup+</sup>CD25<sup+</sup> cells. Clearly, the CD25<sup+</sup> but not the CD25<sup+</sup> cell subset was unresponsive to TCR stimulation as measured by IL-2 production (Fig. 3C) and suppressed IL-2 production by conventional CD4 cells, to a level comparable with freshly isolated CD4<sup+</sup>CD25<sup+</sup> cells (Fig. 3D).

We conclude that the CD45RB<sup>low</sup>CD25<sup+</sup> cells that acquire in vivo CD25 expression display a regulatory phenotype similar to conventional CD4<sup+</sup>CD25<sup+</sup> T<sub>R</sub>.

**In Vitro Induction of CD4<sup+</sup>CD25<sup+</sup> Cells to Suppressor Activity Is Restricted to CD45RB<sup>low</sup> Cells.** The evidence that CD45RB<sup>low</sup>CD25<sup+</sup> cells contain Foxp3<sup+</sup> T cells that convert to a CD25<sup+</sup> T<sub>R</sub> phenotype upon homeostatic expansion prompted us to reevaluate their in vitro suppressor activity. As reported previously (12, 30), freshly isolated CD45RB<sup>low</sup>CD25<sup+</sup> cells do not show suppressive functions when tested in vitro. However, CD45RB<sup>low</sup>CD25<sup+</sup> but not CD25<sup+</sup>CD45RB<sup>hi</sup> cells treated with IL-2 and soluble anti-CD3 mAb for 6 days display suppressor function, comparable in efficiency with freshly isolated CD25<sup+</sup> cells (Fig. 4A). Similarly, using a protocol originally described to induce anergic (31) and suppressor (32) cells in vitro, we evidence that CD45RB<sup>low</sup>CD25<sup+</sup> but not CD25<sup+</sup>CD45RB<sup>hi</sup> cells treated with immobilized anti-CD3 mAb exhibit suppressor activity (Fig. 4B). We propose that these pretreatments selectively expand committed T<sub>R</sub> and thus mimic in part the homeostatic expansion/activation of the CD25<sup+</sup> T<sub>R</sub> we evidenced above.

**CD25<sup+</sup> Regulatory T Cell Expansion and CD25 Surface Expression Are Dissociated.** The findings that CD25<sup+</sup> T<sub>R</sub> acquire surface expression of CD25 during homeostatic expansion prompted us to reassess the phenotypic stability of the CD25<sup+</sup> T<sub>R</sub> isolated from normal mice. As reported before (6, 20, 33), upon adoptive transfer into lymphoid recipients, the vast majority of these cells lose CD25 expression (Fig. 5B). The number of Thy1.2<sup+</sup> cells (originally CD25<sup+</sup>) recovered in such transfer experiments is rather low (Fig. 5A), and any proliferative advantage to rare CD25<sup+</sup> contaminants in the original preparation may explain
this result. However, when the purified Thy1.2+CD4+CD25+ cells were voluntarily contaminated with 1% Thy1.1+CD4+CD25+ cells, <10% of the recovered CD4 cells were composed of Thy1.1+ cells, and the frequency of CD25-expressing cells was indistinguishable from that obtained in the pure Thy1.2+ cell transfer group (Fig. 5B). We next confirmed that cotransfer of CD4+CD25+ cells at a 1:1 ratio stabilizes to some extent the CD25+ phenotype (33), and further assessed whether the loss of CD25 expression in single transfer is reversible (Fig. 5C). At day 12 posttransfer of Thy1.2+CD25+ cells, a group of mice was analyzed to ensure ~80% of the CD4 cells stained negative for CD25 (Fig. 5B), whereas another group received 3 × 10^6 Thy1.1+CD25+ cells. After an additional 12 days, on average, 77% of the Thy1.2+ cells were CD25+. Because the Thy1.2+ cells did not expand significantly after this secondary transfer (Fig. 5A), we favor the interpretation that these cells underwent a reversion to a CD25+ phenotype. It is noteworthy that the Thy1.2+ cells that were overall CD25− at the time of the second transfer, while converting to a CD25+ phenotype, were also able to control the in vivo expansion of the infused Thy1.1+CD4+CD25− population (Fig. 5A), indicating that loss of CD25 expression does not correlate with loss of function.

Discussion
The present work establishes that, upon disruption of homeostasis, CD4 cell subsets contribute to the pool of T_R phenotypically defined as CD25+ and Foxp3+ by recruitment from a peripheral reservoir of differentiated CD25+ T_R. Membrane expression of CD25 appears therefore as a signature of activation equally for conventional T cells and T_R. This finding bears several consequences for the potential usage of CD25 as a therapeutic target and for our understanding of T_R origin and dynamics.

We show that in vivo depletion of CD25-expressing cells can induce both protection and susceptibility to the very same disease. Depleting anti-CD25 mAbs have already been used to target pathological cells in autoimmune mice and humans. For instance, similarly to what we observed in T/R− animals, clinical trials testing the sustained usage of anti-CD25 mAb in combined therapies for multiple sclerosis gave promising results (34). On the other hand, and similarly to what we report here for the T/R+ animals, a single anti-CD25 mAb administration targets T_R and sets a time window where immunization protocols gain in efficiency, an approach explored to improve tumor therapies (35). Short-lasting anti-CD25 mAb administration in mice does not induce or accelerate autoimmune diseases per se unless it is administered early in life or together with self-antigen and strong adjuvants (29, 36). The bulk of our analysis strongly suggests that this temporal limitation and the relative safety of these approaches must rely on the replenishment of the CD25+ T_R pool by recruitment of peripheral differentiated CD25+ T_R. Numerous efforts have been developed worldwide to identify molecular targets that would strictly distinguish all T_R from activated cells to improve the efficiency of T_R therapeutic depletion protocols. Our results, together with the indication that the number of CD25+ cells that can convert to a CD25+ T_R phenotype is limited (37), seem to predict that the use of these new tools may lead to an irreversible highly challenging state of tolerance because it may exhaust the pool of T_R.

We established that, upon homeostatic expansion, Foxp3-expressing cells encompassed in the CD45RBlowCD25− subset are the cells contributing to the pool of converted CD25+ T_R. We favor the idea that, under similar conditions, the few CD25− T_R cells encompassed in the CD45RBlow subpopulation are also those converting to a Foxp3+ CD25+ phenotype. In support of this proposition, it has been shown that Foxp3 expression, although rather low, is detectable in CD45RBlow cells but not in differenti-
ated helper cells (13). Whether acquisition of surface CD25 by Foxp3− Treg is necessary for their regulatory function remains to be formally established. However, we confirmed that CD25 is an activation marker and activated Treg are more efficient regulatory cells than untreated cells (12, 19). Our results therefore indicate that, at the steady state, a normal immune system maintains a reservoir of “inactive” Treg. Both the frequency of cells converting to a CD25+ phenotype upon homeostatic expansion and their level of Foxp3 mRNA expression indicate that Treg represent a unique differentiative pathway (13–15) that is adopted by CD4 conventional CD4 cells is limited because their repertoire is biased for low affinity against self-antigens, at least for those encountered in the thymus (41), and does not require exogenous IL-2 (42). The nature of the activating signals for Treg remains to be fully established; however, there is little doubt that IL-2 (19), produced mostly by conventional CD4 T cells, and TCR engagement by self-antigen/MHC complexes (16, 40, 41) are crucial. Additional signals are provided by inflammatory cytokines and co-stimulatory ligands binding to the Toll-like receptors expressed by Treg (12). Whether the CD25− and CD25+ Treg subsets cover different TCR activities in normal individuals or whether these cells are highly labile and therefore rapidly switch from an activating to a nonactivating environment remains to be clarified.

In addition, activated Treg may well limit CD25− Treg conversion to a CD25+ phenotype as they do for naive cells, a feature that would explain the remarkable stability of Treg CD25+ versus CD25− distribution at the steady state.

Finally, our findings that surface expression of CD25 is labile in differentiated Foxp3-expressing Treg may serve as a word of caution for the interpretation of experiments aiming at the induction of “naive” peripheral CD4+CD25− T cell differentiation to Foxp3+CD25+ Treg.

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