

Homeostasis and anergy of CD4⁺CD25⁺ suppressor T cells *in vivo*

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CD4⁺CD25⁺ suppressor T (T_s) cells play a critical role in the maintenance of peripheral tolerance. We examined here proliferative and functional responses as well as differential gene expression in T_s cells. We found that T_s cells were hypo-responsive to antigenic stimuli *in vivo* and unable to flux Ca²⁺ upon T cell receptor (TCR) engagement. However, T_s cells were not impaired in their proliferative response to lymphopenia, which was dependent on major histocompatibility complex class II expression. Homeostatic proliferation did not abolish T_s cell anergy; rather, it substantially augmented T_s cell function. DNA array analyses identified genes that may inhibit responsiveness at a number of levels in multiple signaling cascades in T_s cells, as well as several anti-apoptotic genes that may mediate their survival.

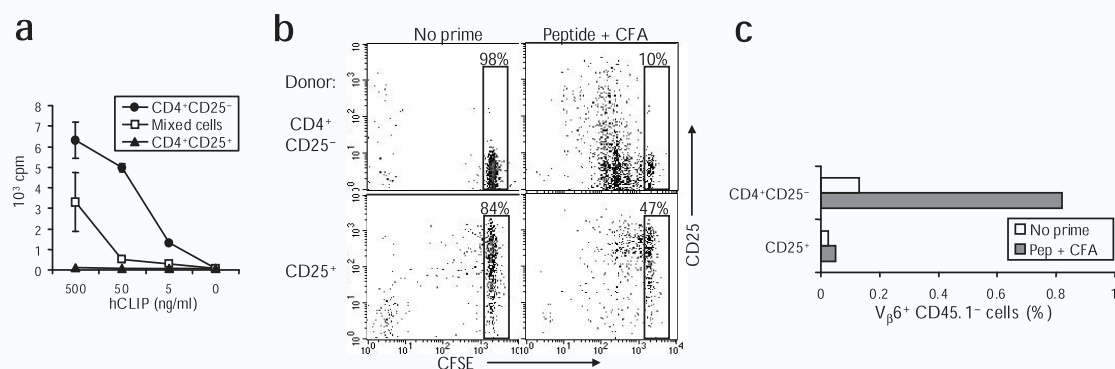
CD4⁺CD25⁺ suppressor T (T_s) cells are essential for the maintenance of self-tolerance. Mice that are rendered deficient for T_s cells—by *in vivo* depletion of CD25⁺ cells, reconstitution of T cell-deficient mice with CD25⁺ T cells or thymectomy on day 3 of life, before T_s cells leave the thymus—develop severe and fatal autoimmunity that is characterized by wasting disease and autoimmune inflammation in multiple organs¹. In these models, transfer of CD4⁺CD25⁺ T cells prevents disease². The mechanisms that mediate T_s cell suppression of autoimmunity are largely unknown and are likely multifaceted. Cytokines produced by T_s cells that inhibit autoimmunity include interleukin 10 (IL-10), transforming growth factor-β (TGF-β) and IL-4³. Other mechanisms may include suppression by unknown cell surface molecules or short-acting cytokines operative during T suppressor–T effector cell contact, or by T_s cell-mediated depletion of residual IL-2 from lymphoid microenvironments.

T_s cells are also capable of inhibiting naïve T cell activation *in vitro*. Contact between T_s and responder T cells is required for this suppression,

which also occurs in an antigen-presenting cell (APC)-free system^{4,5}. Although stimulation through the T cell receptor (TCR) of T_s cells is required to induce its suppressor activity, CD4⁺CD25⁺ cells are themselves anergic, that is, they are unresponsive to TCR stimulation, as detected by the lack of DNA replication and IL-2 secretion *in vitro*. This anergic status of freshly isolated T_s cells is somehow linked to their suppressor activity, in that exposing T_s cells to large amounts of IL-2 concomitantly removes the block on proliferation and abrogates T_s cell suppressor activity^{6,7}.

Very little is known about the nature of the signaling blockade in naturally occurring polyclonal T_s cell populations. Here, we examined the extent to which unperturbed T_s cells are unresponsive *in vivo* and investigated, with differential gene expression analyses, mechanisms that may contribute to T_s cell functional phenotypes. Our results indicate that T_s cells have a genetic program that maintains suppressor function. They also suggest that T_s cells navigate a delicate balance between activation and apoptosis.

Figure 1. T_s cells are hypo-responsive to strong antigenic stimuli *in vitro* and *in vivo*. (a) A total of 2×10⁴ CD4⁺CD25⁺ and 8×10³ CD4⁺CD25⁺ T cells from TCR-Tg mice were cultured alone or mixed with 5×10⁴ irradiated B6 splenocytes in the presence of varying amounts of the hCLIP peptide. (b,c) CD25⁺ and CD4⁺CD25⁺ T cells were MACS-purified from TCR-Tg mice, labeled with CFSE and 1.4×10⁶ were transferred separately into (CD45.1 congenic × B6)F₁ mice. The next day, mice were immunized with hCLIP in CFA (Peptide + CFA) or left unprimed (No prime). Two days after priming (b) draining (inguinal) lymph node (gated for CD4⁺CD45.1⁺ expression) and (c) spleen cells were analyzed by flow cytometry. The percentage events in the undivided CFSE^{hi} population are shown (b). The donor cell populations were also tested *in vitro* as in a; the T_s cells were suppressive and anergic (data not shown). Data are representative of three independent experiments.



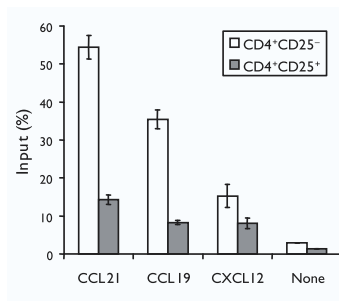


Figure 2. T_S cells are refractory to lymphoid chemokine-induced migration. Total B6 lymph node cells (10⁶) were tested for their ability to migrate towards CCL21 (500 ng/ml), CCL19 (500 ng/ml) and CXCL12 (200 ng/ml) in transwell chambers. The input and migrated cell populations were analyzed by flow cytometry and, to determine the percentage input value, the number of CD4⁺CD25⁻ or CD4⁺CD25⁺ T cells in the migrated population was divided by the number of the same cell type in the input population. Data are mean ± s.d. from triplicate wells.

Results

T_S cells are hyporesponsive *in vivo*

Using mice transgenic (Tg) for the TCR that recognizes the human invariant chain peptide hCLIP, which is presented by I-A^b (TCLi)⁸, we confirmed that freshly isolated CD4⁺CD25⁺ cells are anergic, that is, they do not proliferate or produce IL-2 upon TCR engagement but can suppress the proliferative response of CD4⁺CD25⁻ cells *in vitro*^{6,7}. Purified CD4⁺CD25⁺ and CD4⁺CD25⁻ lymph node and spleen cells from TCLi TCR-Tg mice were stimulated alone or in combination with titration of hCLIP. T_S cells suppressed the CD4⁺CD25⁻ T cell response in the presence of limiting peptide (≤ 50 ng/ml), although this suppression was reduced at increasing peptide concentrations (>500 ng/ml). IL-2 production by the responder T cells was also suppressed by T_S cells at limiting peptide concentrations (data not shown). In contrast, the T_S cells by themselves were unresponsive to even the highest peptide dose (Fig. 1a).

Whereas *in vitro* anergy is well documented, the responsiveness of T_S cells to strong TCR stimuli *in vivo* has not been addressed. To test whether T_S cells were anergic *in vivo*, CD25⁺ and CD4⁺CD25⁻ T cells with the TCLi TCR were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred separately into CD45.1 congenic mice. The recipient mice were immunized the following day with hCLIP in complete Freund's adjuvant (CFA) or left unimmunized. In a separate experiment, no proliferation of either cell type was observed in mice immunized with CFA-PBS alone (data not shown). Two days after antigenic challenge, the CD4⁺CD25⁻ donor population had proliferated greatly, with only 10% of the total cell population remaining undivided in the draining lymph nodes and with a sixfold expansion of donor cells in the spleen (Fig. 1b,c). In contrast, the CD4⁺CD25⁺ T cells were hyporesponsive: 47% of donor cells did not divide and there was a reduction in donor cell numbers in the draining lymph nodes (data not shown). In addition, a modest expansion of donor CD4⁺CD25⁺ T cells was observed in the spleen. The few proliferating T_S cells in the draining lymph node contained no more annexin V⁺ cells than the responding CD4⁺CD25⁻ donor cells, which suggested that the observed hyporesponsiveness of CD4⁺CD25⁺ cells was not due to proliferation followed by increased apoptosis (data not shown).

Assuming that the rates of apoptosis and emigration from the lymph node were comparable for the two proliferating cell populations, it was possible to estimate the percentage of donor cells that entered the cell cycle. Dividing the number of cells in each CFSE peak by 2ⁿ (n=number cell divisions), we calculated that 20% and >60% of the CD4⁺CD25⁺ and CD4⁺CD25⁻ V_β6⁺ donor T cells, respectively, proliferated in the draining lymph node after stimulation with peptide + CFA by day 2. Thus, T_S cells were hyporesponsive *in vivo*, even when confronted with a high-affinity peptide ligand-major histocompatibility complex (MHC) and CFA-elicited proinflammatory cytokines and costimulation.

T_S cells are hyporesponsive to lymphoid chemokines

Poor accumulation of donor T_S cells, compared to CD4⁺CD25⁻ T cells, in the lymphoid organs of host animals after adoptive transfer⁹ (Fig. 1c, unimmunized controls) suggested that the CD4⁺CD25⁺ T cells were prone to apoptosis after transfer or that their homing ability to lymphoid organs was limited. CCL19, CCL21 and CXCL12 are potent attractants for naïve T cell migration into lymph nodes¹⁰. To assess the homing properties of T_S cells, we analyzed the responsiveness of C57BL/6 (B6) lymph node cells to several chemokines in chemotaxis assays. Of the migrated CD4⁺ T cells, the CD25⁺ subset was underrepresented (Fig. 2).

For CCL21 and CCL19, CD4⁺CD25⁺ T cells showed only 25% the migratory capacity that CD4⁺CD25⁻ T cells had. For CXCL12, a weaker chemoattractant used in this assay, T_S cells migrated 50% as efficiently as CD4⁺CD25⁻ T cells. No chemotaxis of either subset was observed towards CCL2, CCL4 or CCL22 (data not shown). Thus, one likely reason for the paucity of T_S cells in peripheral lymphoid tissues after transfer is that they do not respond well to chemokine-induced migration. Increased expression of regulator of G protein signaling 1 (RGS-1) (Table 1), an inhibitor of chemokine responsiveness¹¹, may be responsible for this phenotype. Increased expression of T lymphoma invasion and metastasis factor 1 (Tiam-1) and CD44 may also promote T_S cell migration into peripheral tissues rather than secondary lymphoid organs^{12,13} (Table 1).

TCR-proximal signaling is impaired in T_S cells

To further characterize the signaling blockade involved in maintaining T_S cell anergy, we analyzed the ability of T_S cells to mobilize intracellular Ca²⁺ stores after TCR ligation. Indo-1-loaded lymph node cells from TCLi TCR-Tg mice were stained with fluorescent antibodies to CD4 and CD25 and stimulated with hCLIP-pulsed splenic APCs. The

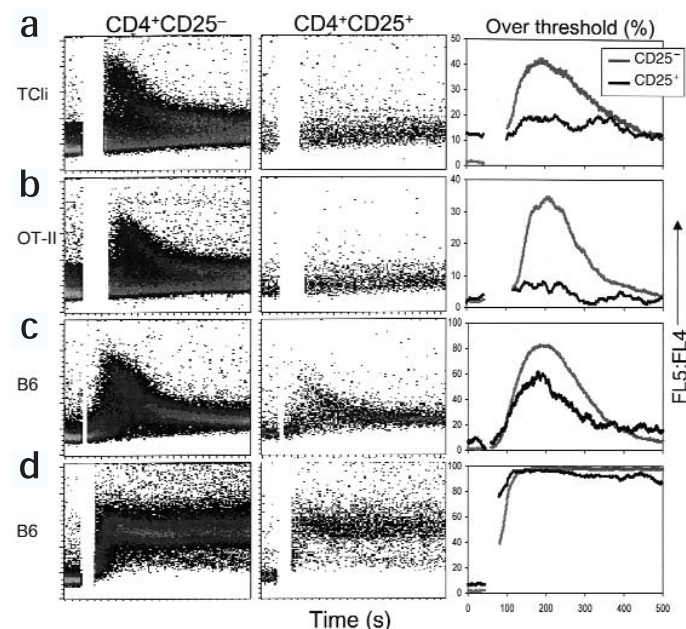


Figure 3. T_S cells do not flux calcium. Indo-1-loaded lymph node cells from (a) TCLi or (b) OT-II TCR-Tg mice or (c,d) B6 mice were stimulated with (a,b) peptide-coated splenocytes (c) soluble anti-CD3 or (d) PMA + ionomycin and analyzed by flow cytometry. Cells were also stained and gated for (a,c,d) CD4⁺CD25^{or-} or (b) I-A^bCD8-CD25^{or-}. Data were analyzed and plotted as percentage events over a FL5:FL4 ratio threshold value, which was selected as 97–98% of background events (before stimulation) for the CD25⁻ population and was then applied to the CD25⁺ population. This was necessary because the background FL5:FL4 value for the two populations differed.

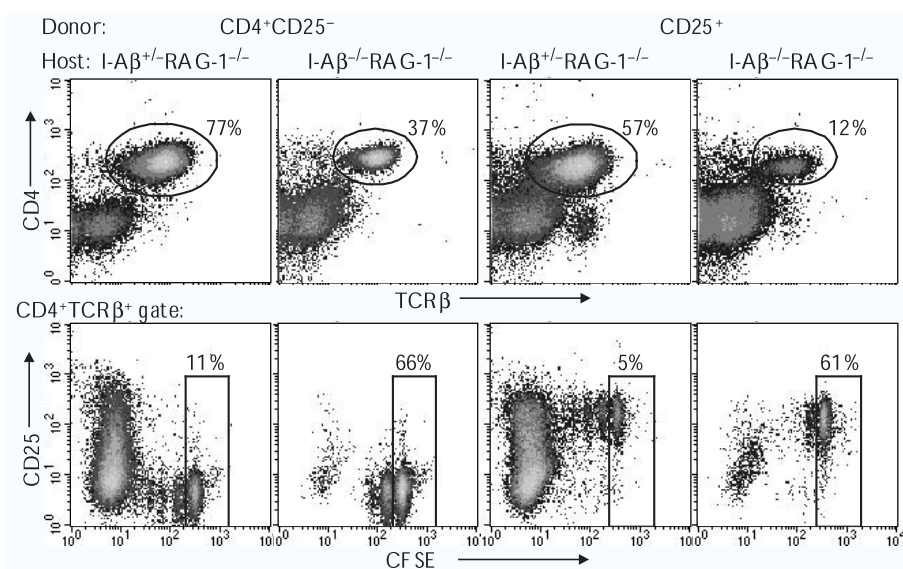


Figure 4. CD4⁺CD25⁻ T cells require MHC class II for homeostatic expansion. CD25⁺ and CD4⁺CD25⁻ B6 spleen and lymph node cells were purified by MACS followed by BP107 (anti-MHC class II) panning to deplete residual APCs. CFSE labeled cells (2.2×10^6) were transferred into I-Ab^{+/+}RAG-1^{-/-}, I-Ab^{-/-}RAG-1^{-/-} or B6 mice. Seven days later, cell suspensions from recipient lymph nodes were analyzed by flow cytometry. The percentage events in the corresponding regions are shown. No proliferation of either cell type was observed in B6 hosts (data not shown).

CD4⁺CD25⁻ T cell population responded well to the antigenic peptide, whereas the CD4⁺CD25⁺ T cell population was refractory (**Fig. 3a**).

We did similar experiments with T cells from OT-II TCR-Tg mice, which have a TCR specific for ovalbumin amino acids 323 through 339 (OVA(323–339)) presented by I-A^b. Unlike the TCR, the OT-II TCR is dependent on CD4–MHC class II interactions for T cell activation. Thus, in these experiments, the cells were stained for MHC class II and CD8, rather than CD4, so as not to disrupt normal TCR signaling. As with the TCR, the OT-II T_S cell population was unresponsive (**Fig. 3b**). In some experiments, magnetic-activated cell sorting (MACS)-purified CD25⁺ and CD4⁺CD25⁻ OT-II cells were tested separately, and the same results were obtained (data not shown).

We also followed the response of polyclonal CD4⁺CD25⁺ and CD4⁺CD25⁻ B6 T cells to anti-CD3, concanavalin A (Con A) and phorbol 12-myristate 13-acetate (PMA)-ionomycin. Although both the CD4⁺CD25⁻ and CD4⁺CD25⁺ T cell populations fluxed calcium in response to PMA-ionomycin (**Fig. 3d**), the CD4⁺CD25⁺ population responded weakly to anti-CD3 in comparison to CD4⁺CD25⁻ T cells (**Fig. 3c**) and did not respond at all to Con A stimulation (50 µg/ml, data not shown). Thus, the T_S cells were not only anergic in their ability to produce IL-2 and proliferate, they also contained a blockade to proximal signaling events immediately after TCR stimulation. In this regard, they resembled T cells in *in vivo* models of anergy^{14,15}.

MHC class II-dependent homeostatic proliferation

In addition to antigen- and mitogen-driven T cell proliferation, T cells also divide when placed in a lymphopenic environment. The exact mechanism responsible for this so called “homeostatic” proliferation is unknown; however, it likely results from a combination of cytokine signaling and low-affinity TCR interactions with self-MHC. Naïve CD4⁺ and CD8⁺ T cells require self-MHC to proliferate, whereas memory CD8⁺ T cell proliferation in a lymphopenic environment is independent of TCR interactions with self-MHC^{16–19}. Like memory CD8⁺ T cells, homeostatic expansion of memory CD4⁺ T cells is unaffected by MHC deficiency (C. Surh, personal communication).

Because T_S cells were hyporesponsive to antigenic stimuli *in vivo*, we wondered whether they were also unable to undergo homeostatic proliferation. The cell surface phenotype of T_S cells is somewhat similar to that of antigen-experienced T cells, therefore, we also examined

the role of MHC in driving homeostatic expansion of CD4⁺CD25⁺ T cells. B6 CD4⁺CD25⁻ and CD4⁺CD25⁺ T cell populations (98% and 95% purity, with 0.3% and 0.9% contaminating MHC class II⁺ cells, respectively) were labeled with CFSE and transferred into MHC class II I-Ab^{+/+}RAG-1^{-/-} (recombination activating gene 1-deficient) and I-Ab^{-/-}RAG-1^{-/-} mice. On day 7, host lymph node and spleen cells were analyzed by flow cytometry. Marked proliferation and accumulation of both CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells was observed in the lymph nodes of I-Ab^{+/+}RAG-1^{-/-} animals compared to the I-Ab^{-/-}RAG-1^{-/-} hosts (**Fig. 4**). CD25 was no longer expressed on the majority of the donor T_S cells that had divided more than five times, and CD25 expression was acquired by a subset of the CD4⁺CD25⁻ donor population (**Fig. 4, bottom**). Thus, the T_S cells were capable of homeostasis-driven proliferation, despite their anergic phenotype.

Unlike that of memory T cells, however, this response was largely dependent on interactions with MHC class II. This emphasizes the idea that T_S cells are a unique lineage of T cells that—like normal naïve CD4⁺CD25⁻CD45RB^{hi} cells—have not been activated by a high-affinity peptide ligand–MHC in the periphery and—unlike antigen-experienced cells—require interactions with self-MHC–peptide complexes for *in vivo* homeostasis.

Effect of homeostatic proliferation on T_S cells

The induction of T_S cell proliferation *in vitro* by TCR stimuli and exogenous IL-2 coincides with the loss of suppressor function^{6,7}. We therefore considered whether T_S cells that proliferate in the lymphopenic hosts might have also lost their suppressive ability. To examine this possibility, CD4⁺CD25⁻ and CD25⁺ T cells isolated from Thy-1 heterozygous B6 mice were labeled with CFSE and transferred into TCRα^{-/-} recipients. On day 6, lymph node and spleen cells were pooled. A small portion of these cells were analyzed by flow cytometry (**Fig. 5a,b**), and CD4⁺ T cells were enriched with anti-CD4 microbeads and sorted by fluorescence-activated cell sorting (FACS) for CD4 and Thy1.1 expression as well as CFSE loss or retention.

As with the RAG-1^{-/-} hosts, proliferation of both cell populations was observed, with 15% and 18% of the expanded CD4⁺CD25⁻ and CD4⁺CD25⁺ donor populations, respectively, remaining in the undivided CFSE^{hi} subset (**Fig. 5a**). In addition, the cell surface phenotype of the two populations became nearly identical after several rounds of cell



Table 1. Differential expression analysis reveals genes that maintain energy and inhibit apoptosis

TIGR ^a or Genbank designation	Gene symbol	Fold change				Gene product description
		DNA arrays ^b		Real-time PCR ^c		
		Expt. 1	Expt. 2	CD25 ⁺ versus CD25 ⁻	T _{Act} versus CD25 ⁻	
Cell surface						
TC29600	<i>Il2ra</i>	25.0 (I)	28.0 (I)	183.0	128.0	CD25; T cell activation marker; T _S cells marker.
TC38230	<i>Itgae</i>	6.6 (I)	4.5 (I)	20.0	0.13	Integrin α _E ; pairs with β ₇ to form the E-cadherin receptor. Confirmed by flow cytometry to be expressed on 18% of CD4 ⁺ CD25 ⁺ cells versus 1% of CD4 ⁺ CD25 ⁻ cells
X05719	<i>Cd152</i>	13.0 (I)	52.0 (I)	19.0	7.3	Inhibitor of T cell activation; associates with SHP-2
X85214	<i>Tnfrsf4</i>	4.7 (I)	4.3 (I)	14.0	9.8	OX40; promotes longevity of CD4 ⁺ T cell responses
TC28811	<i>Tnfrsf9</i>	4.0 (I)	15.0 (I)	9.4	6.6	4-1BB; inhibitor of apoptosis
U82534	<i>Tnfrsf18</i>	3.0 (I)	4.8 (I)	4.8	2.2	GITR; inhibitor of apoptosis
TC35849	<i>Tnfrsf1b</i>	3.7 (I)	20.0 (I)	4.0	0.72	TNFR2; p75 chain of TNFR; promotes inflammatory and anti-apoptotic mechanisms
TC31804	<i>Tgfb1</i>	6.4 (I)	14.0 (I)	3.0	0.24	Signal-transducing receptor forTGF-β; an inhibitor of cell growth and inflammation
	<i>Pd1</i> [*]	–	–	2.7	0.3	Phosphatase-associated inhibitor of T cell activation
	<i>Cd7</i> [*]	–	–	0.65	0.05	Receptor for T cell costimulation
X03151	<i>Cd90</i>	-1.7 (D)	-2.7 (D)	0.64	0.33	GPI-linked receptor for T cell costimulation; associated with lipid rafts; reduced expression in T _S cells confirmed by flow cytometry
X04836	<i>Cd4</i>	-2 (D)	-2.6 (D)	0.63	0.15	T cell coreceptor for MHC class II; associates with Lck; reduced expression in T _S cells observed by flow cytometry
TC25435	<i>Il7ra</i>	-3.2 (D)	-2.3 (D)	0.54	0.04	Receptor for IL-7; critical for T cell growth and homeostasis; reduced expression confirmed by flow cytometry
	<i>Tnfsf6</i> [*]	–	–	0.28	0.77	FasL; induces apoptosis on Fas-expressing cells
TC28211	<i>Tgfb1</i>	-5 (D)	-3.2 (D)	0.15	0.19	Betaglycan; coreceptor for TGF-β family antagonists
U02298	<i>Scya5</i>	-4.8 (D)	-8.0 (D)	NT ^d	NT	RANTES; proinflammatory chemokine induced with T cell activation ⁷³
Signaling						
TC29515	<i>Socs2</i>	41.0 (I)	13.0 (I)	34.0	20.0	Suppresses signaling through the receptors for growth hormone, insulin-like growth factor, IL-6 and LIF, but not IL-2
TC20983	<i>Rgs1</i>	15.0 (I)	84.0 (I)	22.0	2.1	Inhibits chemokine-induced signaling through heterotrimeric G proteins
U05245	<i>Tiam1</i>	5.0 (I)	17.0 (I)	10.0	0.34	A guanine nucleotide-exchange factor for Rac, which promotes cytoskeletal reorganization and cell migration; associates with CD44
X61940	<i>Mkp1</i>	3.4 (I)	2.6 (I)	6.8	0.75	MAPK phosphatase-1; inhibits Erk, Jnk and p38
	<i>Fap1</i> [*]	–	–	5.2	0.19	An inhibitor of Fas-mediated apoptosis
TC17088	<i>Socs1</i>	18.6 (I)	1.7 (NC)	2.5	1.34	Suppresses signaling through multiple cytokine receptors
	<i>Fyn</i> [*]	–	–	1.0	0.33	TCR-proximal Src family kinase; increased in many experimentally anergized T cells
	<i>Lck</i>	–	–	0.61	0.24	TCR-proximal Src family kinase; phosphorylates TCR-ζ
TC39954	<i>Pde3b</i>	-10.0 (D)	-20.0 (D)	0.030	0.19	Phosphodiesterase 3b; PDE3 inhibitors diminish T helper type 1 immune responses ⁷⁴
Nuclear						
TC36054	<i>Irf4</i>	3.2 (I)	9.4 (I)	16.0	1.5	Through its peptidyl-prolyl isomerase activity, FKBP52 binds to and induces a conformational change in IRF-4, inhibiting its transcription-promoting activity
TC15783	<i>Fkbp52</i>	5.4 (I)	7.7 (I)	1.9	0.1	Plays a role in regulating growth arrest and apoptosis
	<i>Rarg</i> [*]	–	–	5.2	0.19	GATA-1; transcriptional repressor; negatively regulates IL-5 and IFN-γ promoters
TC14626	<i>Yy1</i>	4.4 (MI)	7.0 (I)	4.6	0.69	Inhibitor of DNA replication
TC26904	<i>Geminin</i>	4.0 (I)	3.9 (I)	2.7	0.62	

TIGR ^a or Genbank designation	Gene symbol	Fold change				Gene product description
		DNA arrays ^b		Real-time PCR ^c		
		Expt. 1	Expt. 2	CD25 ⁺ versus CD25 ⁻	T _{Act} versus CD25 ⁻	
TC14662	<i>Mcm5</i>	5.7 (MI)	3.2 (MI)	2.1	3.1	Cdc46; regulator of DNA replication
TC19211	<i>Tgif</i>	5.1 (I)	4.0 (I)	2.1	0.24	TG-interacting factor; a transcriptional corepressor that suppresses TGF-β-induced transcription; also increased in anergic B cells
TC32149	<i>Satb1</i>	-2.9 (D)	-2.6 (D)	0.40	0.29	A matrix attachment region binding protein; an important regulator of gene expression during thymocyte development
X58636	<i>Lef1</i>	-3.4 (D)	-2.8 (D)	0.39	0.13	Redundant transcription factors that promote gene expression and T cell differentiation
TC24094	<i>Tcf1</i>	-2.9 (D)	-2.8 (D)	0.33	0.082	

^aSome TC (Tentative Consensus entries of the TIGR Mouse Gene Index) sequences were identified as 3' untranslated regions of known genes by homology to the Celera mouse gene database (<http://www.celera.com>). ^bAffymetrix fold-change values were calculated with GeneChip software: negative and positive fold change values indicate lower and higher expression in freshly isolated B6 CD4⁺CD25⁺ versus CD4⁺CD25⁻ cells, respectively. GeneChip significance scores were as follows: I, increase; D, decrease; NC, no change; MI, mild increase. ^cGenes identified by Affymetrix DNA array analysis, or by a previous array analysis of *in vivo*-anergized T cells²² (indicated by asterisks), were quantified by real-time PCR in cDNA from freshly isolated B6 CD4⁺CD25⁺, CD4⁺CD25⁻ and Con A-activated CD4⁺CD25⁻ T cells (T_{Act}). The fold change in mRNA levels was calculated as 2ⁿ, where n = the difference in PCR cycle number at which the signals were first detected above background (Ct) (n = Ct^{CD25⁻} - Ct^{CD25⁺} or n = Ct^{CD25⁻} - Ct^{T_{Act}}). All Ct values were first normalized to those of HPRT. The fold change of discordantly regulated genes in T_S cells and T_{Act} as compared to naive CD4⁺CD25⁻ cells are shown in bold. ^dNT, not tested. (For additional DNA array results for known genes see Web Fig. 1 on the supplementary information page of *Nature Immunology* online.)

division (Fig. 5b). Similar to naïve CD4⁺ T cells that have undergone homeostatic expansion, the CD4⁺CD25⁻ donor cells acquired a memory-like CD44^{hi}CD62L^{lo}CD45RB^{lo} expression pattern¹⁷. The CD4⁺CD25⁺ T cells also converted to this phenotype and maintained their normal CD44^{hi}CD45RB^{lo} expression, whereas they lost CD62L expression. The CD4⁺CD25⁺ T cells lost CD25 expression, whereas the CD4⁺CD25⁻ T cells gained expression. Both cell populations showed a nearly identical unimodal Gaussian distribution of CD25 fluorescence, with slightly, yet reproducibly, higher expression being acquired by the CD4⁺CD25⁻ donor cells (Fig. 5b).

In a similar experiment, the anergic status of the expanding donor cells was assessed. The proliferating (CFSE^{lo}) CD4⁺CD25⁺ donor T cells were reisolated from the TCR α ^{-/-} hosts and stimulated *in vitro* with ConA and APCs. The T_S cells that had undergone homeostatic proliferation were as anergic (stimulation index (SI)=2.2±0.8) as freshly isolated

CD4⁺CD25⁺ T cells (SI=2.6±1.1). However, the expanded CD4⁺CD25⁻ donor T cells showed decreased responsiveness (SI=15±4.8) in comparison to freshly isolated CD4⁺CD25⁻ T cells (SI=164±14) (Data were representative of three independent experiments.) Mechanisms that may contribute to CD4⁺CD25⁻ hyporesponsiveness after homeostatic proliferation include unresponsiveness of previously activated cell cycle machinery and activation-induced cell death²⁰. Taken together, these results suggested that TCR- and cytokine-derived signals that result in homeostatic proliferation may be distinct from those that are downstream of mitogenic and antigenic stimuli, and which are blocked in T_S cells. However, these data do not formally rule out the possibility that the blockade is transiently removed or bypassed upon transfer into lymphopenic hosts, and is recovered immediately upon *in vitro* culture.

Because the CD4⁺CD25⁻ T cells became hyporesponsive to TCR stimulation during homeostatic proliferation (see above), and because

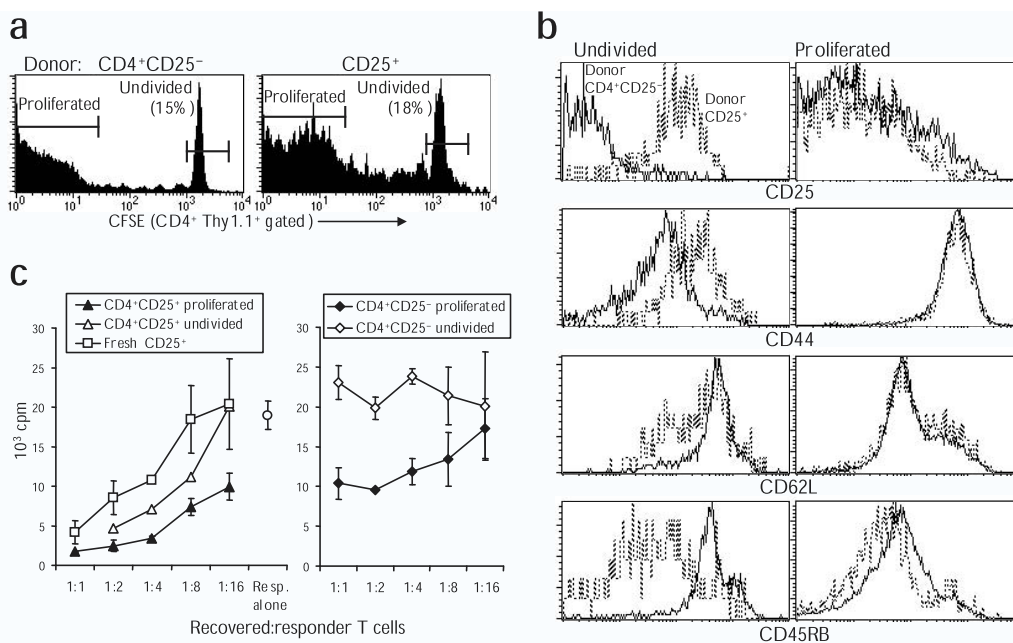
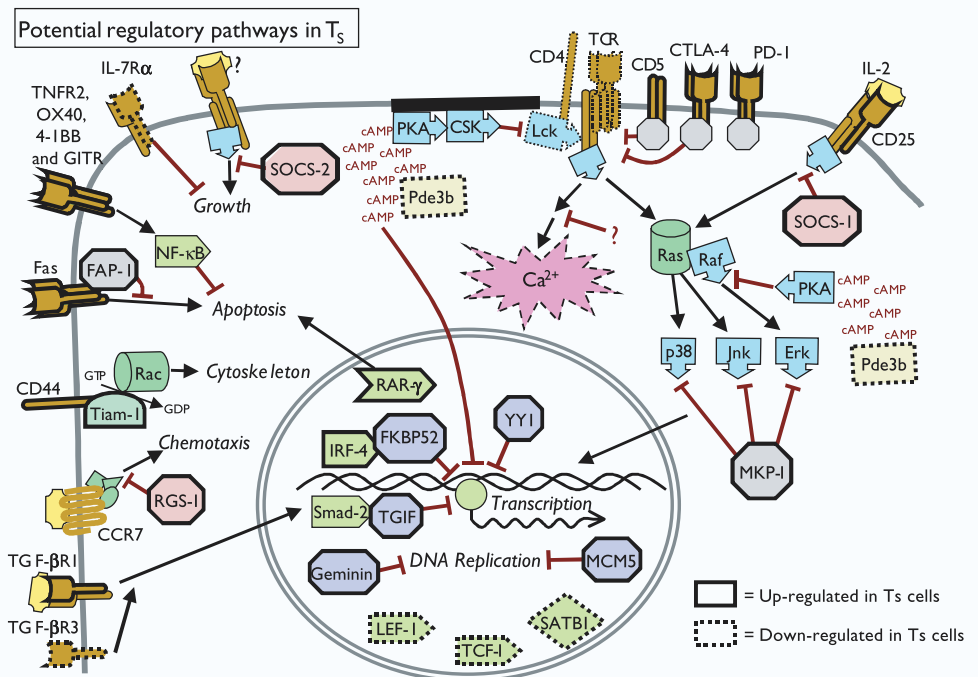


Figure 5. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells acquire a similar cell surface phenotype after homeostatic proliferation, whereas T_S cell suppressor function is enhanced. CD25⁺ and CD4⁺CD25⁻ T cells from (B6xB6.PL)_{F1} donors were CFSE labeled and transferred separately (5×10⁵ per recipient) into TCR α ^{-/-} hosts. Six days after transfer, spleen and lymph node cells were analyzed by (a,b) flow cytometry (dotted lines, donor CD25⁺ cells; solid lines, donor CD4⁺CD25⁻ cells) or donor cells were isolated by FACS for CFSE retention (undivided) or loss (proliferated). (c) The suppressor activity of sorted cell populations and freshly isolated CD25⁺ T cells was analyzed in the presence of Con A (1 µg/ml) and 5×10⁴ irradiated LPS blasts, for APCs. CD4⁺CD25⁻ T cells (2.5×10⁴) were the responders. Data are representative of four independent experiments.

Figure 6. Regulation of multiple signaling pathways in CD4⁺CD25⁺ T_S cells. TCR signaling pathways: increased expression of the phosphatase-associated cell surface molecules CTLA-4⁴⁶, PD-1^{47,48} and CD5⁴⁹ may contribute to the observed inhibition of TCR-proximal signaling events. Reduction of PDE3B, which likely results in increased cAMP and protein kinase A (PKA) activation, may affect various pathways. These include Lck inhibition through PKA phosphorylation of c-Src tyrosine kinase (Csk)⁵⁰; blockade of the MAPK-Erk pathway through Raf-1 phosphorylation⁵¹; and inhibition of TCR-induced transcription through cAMP responsive element binding protein (CREB), cAMP responsive element modulator (CREM)⁵² and the inducible cAMP early repressor (ICER)⁵³. MAPK signaling pathways in T_S cells may also be attenuated by MKP-1—an inhibitor of Erk, Jnk and p38 signaling⁵⁴—and TCR induced IL-2 production⁵⁵. Cytokine signaling: multiple cytokine-responsive pathways may be attenuated by SOCS-1, SOCS-2 and MKP-1. Signals from low, but not high, doses of IL-2 may be inhibited by the increased SOCS-1 and MKP-1 expression. High doses of IL-2, which abrogate T_S cell anergy *in vitro*⁶⁷, overwhelm this blockade, and some IL-2R signaling is required for T_S cell development *in vivo*⁶⁶. The considerable increase in SOCS-2 mRNA suggested hyporesponsiveness to other factors, including insulin-like growth factor-1 (IGF-1)⁶⁷, growth hormone (GH)⁶⁸, IL-6⁶⁹ and leukemia inhibitory factor⁶⁹. Gigantism observed in SOCS-2-deficient mice⁶¹ suggests T_S cells may constitutively maintain unresponsiveness to these growth factors *in vivo*. Effects of TGF-β may be influenced by changes in expression of the gene for its coreceptor, TGF-βR1, as well as genes for two proteins that effect TGF-β signaling: TG-interacting factor (TGIF)⁶² and TGF-βR3⁶³. Decreased IL-7Rα (confirmed by FACS) suggested that T_S cells are also less responsive to this key factor for T cell growth and homeostasis^{64,65}. Homing and migration: homing of transferred T_S cell populations may be influenced by increased expression of genes encoding RGS-1¹¹ and Tiam-1¹² as well as the α₆β₇ integrin (Table 1). Transcription and DNA replication: transcriptional repression may be facilitated in T_S cells by increased FKBP52⁶⁶ and Yin-yang 1 (YY1)^{67,68}, as well as decreased lymphoid enhancer-binding factor 1 (LEF-1) and T cell factor 1 (TCF-1)⁶⁹. T_S cell DNA synthesis may be controlled by increased geminin and minichromosome maintenance protein 5 (MCM5)^{70,71}. Survival and apoptosis: increased cell surface expression of Fas⁷² (M. A. Gavin, unpublished data) and decreased mRNA for FasL⁶ indicated that T_S cells may be prone to apoptosis⁷². Pro-apoptotic signals may also derive from elevated RAR-γ⁶⁶. Increased expression of several other genes may counteract this tendency. Fas-mediated apoptosis may be blocked by FAP-1⁷⁴. T_S cell survival may also be facilitated by several linked genes of the TNFR-NGFR superfamily, including those encoding TNFR2, OX40, 4-1BB and GITR.



the two cell populations converged to a similar cell surface phenotype (Fig. 5b), evaluating their suppressive function *in vitro* was of interest. CD4⁺Thy1.1⁺CFSE^{hi} and CD4⁺Thy1.1⁺CFSE^{lo} T cells sorted from the TCRα^{-/-} hosts were analyzed for their ability to suppress B6 CD4⁺CD25⁺ T cells. Freshly isolated B6 CD4⁺CD25⁺ cells were also analyzed as a control. Despite their decreased CD25 expression, the divided donor T_S cells were four times more suppressive on a per cell basis than freshly isolated T_S cells (Fig. 5c). The undivided CFSE^{hi} T_S cells were also slightly more potent suppressor cells compared to fresh T_S cells. In addition, we found that the expanded CD4⁺CD25⁺ T cells also contained some moderate suppressor activity (Fig. 5c). Whereas T_S cells mediate suppression by a mechanism that requires suppressor-responder cell contact and that does not rely on IL-2 depletion, IL-2 is an integral factor in promoting the burst of proliferation observed on day 3 of mitogen-activated T cell cultures. Thus, the suppressive capacity of the expanded CD4⁺CD25⁺ T cells could be attributed to the increased CD25 expression (Figs. 4 and 5b), which enabled them to consume IL-2 produced by the responder T cells.

Pathways for control of homeostasis

The molecular mechanisms responsible for the functional differences between CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells are unknown. To determine which genes mediate their disparate phenotypes, RNA from FACS-purified normal B6 CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells was analyzed by global gene expression profiling with the Affymetrix mu11K and mu19K oligonucleotide arrays. Two screens were done. In

the first experiment, biotinylated cRNA was amplified directly from cDNA. In the second experiment, two sequential rounds of *in vitro* transcription were used to amplify enough biotinylated cRNA for analysis²¹. With a few exceptions, only transcripts that were differentially expressed in both screens, as scored by the Affymetrix GeneChip software, were considered for confirmation by real-time polymerase chain reaction (PCR) (Table 1, see CD25⁺ versus CD25⁺). (For additional DNA array results for known genes see Web Fig. 1 on the supplementary information page of *Nature Immunology* online.) Seventy-seven of 79 transcripts analyzed by PCR gave results consistent with the differences determined by the Affymetrix arrays. Of all the genes that were present in both array analyses and confirmed by PCR, *Il2ra* itself was up-regulated the most in CD4⁺CD25⁺ T cells (Table 1). The mRNAs for OX40 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which are also highly expressed on T_S cells, were also among the most abundant transcripts in T_S compared to CD4⁺CD25⁺ T cells.

Gene expression analysis of *in vivo*-anergized cells derived from TCR-Tg mice that expressed cognate antigen identified several genes that may promote anergy²². In our DNA array analysis of CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells, some of these genes showed similar changes in expression, whereas others were not scored as differentially expressed. Thus, to establish a comparison between polyclonal T_S cells and the *in vivo*-anergized cells previously described²², we analyzed genes that were specific to those anergized cells (Table 1, see asterisks). We found that the three genes for Fyn, CD7 and FasL were down-regulated or unchanged in T_S cells unlike in the anergized cells.

A similar discrepancy was found for *Cd4* expression. Thus, anergic and suppressive T cells derived from experimental Tg models do not necessarily mimic naturally occurring polyclonal CD4⁺CD25⁺ T_S cells.

To determine the differences between CD4⁺CD25⁺ T_S cells and CD4⁺ T cells that expressed CD25 *via* TCR-mediated activation, we compared mRNA levels for Con A-activated CD4⁺CD25⁺ T cells with naïve CD4⁺CD25⁺ T cells (**Table 1**, see T_{Act} versus CD25⁺). Our results were consistent with published data on array analyses of activated T cells^{22,23}, but they also identified differences between T_S cells and recently activated T cells: 15 of 34 genes were regulated differently between T_S cells and activated CD4⁺CD25⁺ T cells (**Table 1**, see values in bold). These included genes encoding key regulators of T cell survival, such as Fas-associated phosphatase 1 (FAP-1)²⁴, glucocorticoid-induced tumor necrosis factor receptor (TNFR) family-related gene (GITR)²⁵ and retinoic acid receptor- γ (RAR- γ)²⁶. Rather than present all the DNA array and real-time PCR results here, we have focused on described genes that may play a role in mediating the T_S cell characteristics discussed here. How these genes may contribute to T_S cell behavior is summarized in **Fig. 6**.

Discussion

Cell surface expression of various T cell markers on T_S cells—such as decreased TCR and CD4, as well as increased CD5—suggests that, of all T cells found in the periphery, T_S cells have the highest affinity for self-MHC ligands. This cell surface phenotype is also observed on CD8⁺CD4⁺CD25⁺ thymocytes, which suggests that T_S cells escape deletion by decreasing the strength of TCR-derived signals, a model that is supported by published findings in a TCR-Tg system^{1,27}. Thus, the affinity that T_S cells have for self-MHC-peptide complexes may represent the boundary between that of a useful naïve T cell interacting with self and the high affinity an effector T cell shows for nonself ligands.

We found that, in addition to modulations in these cell surface molecules, T_S cell survival mechanisms could also be maintained by the altered expression of gene products involved in multiple pathways. As well as genes with markedly altered expression, such as those encoding phosphodiesterase 3b (PDE3B), suppressor of cytokine signaling 2 (SOCS-2), CTLA-4 and CD25, the expression of many genes, although reproducible, was not markedly different between CD4⁺CD25⁺ and CD4⁺CD25⁺ T cells; several fold-change values ranged between 0.2–5. This suggested that the key regulators of T_S cell responsiveness and survival were present at limiting concentrations and that they had to work in concert to effect T_S cell anergy and homeostasis. For example, survival may have been facilitated by the moderate up-regulation of several linked genes from the TNFR-nerve growth factor receptor (NGFR) superfamily, including those encoding TNFR2, OX40, 4-1BB and GITR. Signals derived from each of these receptors inhibit TCR-induced apoptosis and prolong survival^{25,28–30}. A chromosomal locus that segregates with susceptibility to neonatal thymectomy-induced autoimmune gastritis maps to a relatively narrow interval that contains these members of the TNFR-NGFR superfamily³¹. T_S cell survival may also have been enhanced by increased FAP-1 or by mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1)—and PDE3B-attenuated signals derived from the TCR. Such a system of multiple checks and balances would allow T_S cells to maintain functionality and flexibility in the face of both inflammatory immune responses and evolving antigenic landscapes.

Whichever the mechanism of T_S cell anergy, stimulation through the TCR was required to induce T_S cell suppressor function *in vitro* and homeostatic proliferation *in vivo*. The profound block in TCR signaling of T_S cells does not inhibit homeostatic proliferation³². T_S cell homeostatic proliferation—like that of naïve CD4⁺CD25⁺ cells, but unlike that of memory T cells (C. Surh, personal communication)—is dependent on interactions

with MHC. Nevertheless, T_S cells remained anergic upon TCR stimulation, immediately after homeostatic proliferation. Thus, homeostatic proliferation may have been partly driven by the TCR-responsive Ras-MAPK cascade, as occurs in positive selection^{33–36}, and may have been independent of Ca²⁺ flux. This model is consistent with the similarity in TCR affinity for self-ligands in driving both homeostatic proliferation and positive selection^{18,37}. After transfer into lymphopenic hosts, T cells do not enter the cell cycle until day 2–3³² (S. R. Clarke, unpublished data). Similarly, *in vitro*, T cell suppression by T_S cells is most potent on day 3 of culture⁷. Thus, in place of or in addition to bypassing TCR blockades, T_S cells may have responded during this lag period by temporarily removing their signaling block, for example, by up-regulating PDE3B and IL-7R α .

The augmented suppressor function that we observed in T_S cells after homeostatic proliferation suggested that T_S cells behave as a lineage of professional suppressor cells^{38,39}. We found that enhanced T_S cell suppressor function occurred despite the loss of CD25 expression on the majority of expanding cells; this indicated that a mechanism other than IL-2 depletion dominated this activity. A mechanism involving contact between the suppressor and responder T cells, as described *in vitro* for both human- and mouse-derived T_S cells, was likely involved. This enhanced activity was reminiscent of the finding that CD8⁺ T cell effector functions are also augmented by homeostatic proliferation^{40–42}. After expansion in lymphopenic hosts, CD8⁺ T cells are cytolytic and elaborated interferon- γ (IFN- γ) immediately upon TCR engagement *ex vivo*. Similarly, T_S cell functionality was amplified with the metabolic activation induced by homeostatic proliferation, which suggested that CD4⁺CD25⁺ T_S cells are genetically programmed to function as a T_S cell lineage.

In conclusion, we examined the anergic properties of CD4⁺CD25⁺ T_S cells and found that they respond poorly to TCR ligands and lymphoid chemokines, but not to stimuli that originate from lymphopenia. T_S cell homeostatic proliferation does not abrogate their anergic status, which involves a TCR-proximal blockade of Ca²⁺ mobilization. In addition, homeostatic proliferation enhances T_S cell suppressor function. The results of global gene expression analyses suggested several previously characterized signaling mechanisms—including pathways that promote T cell survival and block TCR and cytokine signaling—that may be responsible for the unique characteristics of T_S cells.

Methods

Mice. Mice aged 6–20 weeks old were used for all experiments. MHC class II (I-A β)-, TCR α - and RAG-1-deficient mice on a B6 background were from the Jackson Laboratory (Bar Harbor, ME). Tc1 TCR-Tg mice were as described⁸ and OT-II TCR-Tg mice were a gift of F. Carbone⁴³. Thy1.1 (B6.PL) and CD45.1 (B6.SJL) congenic mice were from Jackson Laboratory and B6 mice were from the Charles River Breeding Laboratories (Wilmington, MA). Mice were immunized subcutaneously with 0.2 mg of peptide emulsified in CFA (Sigma, St. Louis, MO). Mice were housed in specific pathogen-free conditions and used according to the guidelines of the Institutional Animal Care Committee at the University of Washington.

Cell isolations. Cell populations were isolated with an AutoMACS magnetic cell sorter (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocols. Briefly, red blood cell-depleted splenocytes were combined with lymph node cell suspensions in PBS, 0.5% bovine serum albumin (BSA) and incubated with the Fc receptor-blocking monoclonal antibody (mAb) 2.4G2 (PharMingen, San Diego, CA) for 10 min at 4 °C after which biotinylated anti-CD25 (mAb 7D4, PharMingen) was added for 30 min at 4 °C. Cells were washed twice and incubated with a 1:10 dilution of streptavidin-conjugated magnetic beads (Miltenyi) at a concentration of 10⁸ cells/ml in PBS, 0.5% BSA, 1 mM EDTA for 20 min at 8 °C. The cells were washed once, passed through a nytex filter to remove clumps, pelleted and resuspended at 2 $\times$ 10⁶/ml. CD25⁺ T cells were isolated with the AutoMACS, and CD4⁺ T cells were isolated from the CD25⁺ fraction with anti-CD4 microbeads (Miltenyi). Contaminating MHC class II⁺ cells were removed from the CD25⁺ and CD4⁺CD25⁺ fractions by panning with BP107 (anti-I-A β)-coated plates. CD25⁺ populations were >93% CD4⁺CD25⁺ and CD4⁺CD25⁺ populations were 98% pure. When necessary, cells were labeled with 1 ml of PBS-0.1% BSA-5 μ M CFSE for 10 min at 37 °C and washed three times in RPMI-10% fetal calf serum (FCS). For analysis, axillary, brachial, inguinal and mesenteric lymph nodes were pooled, except where noted. To recover cells from the TCR α ⁺ hosts, spleen and lymph node cells were pooled and CD4⁺ T cells were isolated by

AutoMACS. During incubation with anti-CD4 microbeads, the cells were also stained with phycoerythrin (PE)-anti-Thy1.1 and allophycocyanin-anti-CD4, and the CD4⁺ fraction was sorted for CD4⁺ Thy1.1⁺ expression and CFSE retention or loss, which excluded the brightest three peaks of CFSE fluorescence, on a FACS Vantage cell sorter. For real-time PCR analysis, *in vitro*-activated T cells were generated by culturing purified B6 CD4⁺CD25⁺ T cells with irradiated (2700 rad) lipopolysaccharide (LPS)-activated spleen cells (LPS blasts: 2 days at 10 µg/ml of LPS) and 1 µg/ml of Con A for 46 h. Activated T cells were 79% CD25⁺ and 55% CD69⁺ CD25⁺ and were purified from dead APCs by density-gradient centrifugation (Lympholyte-M, Cedarlane Laboratories, Ontario, Canada).

Proliferation assays. Indicated numbers of CD4⁺CD25⁺ T cells, CD4⁺CD25⁺ T cells or both populations together were stimulated for 72 h with 5×10⁴ APCs + Con A or peptide in 96-well round-bottomed plates, and pulsed with 1 µCi/well of [³H]thymidine for the final 8 h. Irradiated LPS blasts (2700 rad) or fresh splenocytes (1200 rad) were used as APCs. To examine the responsiveness of T cells that had undergone homeostatic proliferation, 10⁴ freshly isolated cells or donor T cells recovered by FACS from TCRα⁺ hosts were stimulated with 2×10⁴ irradiated LPS blasts and 0.5 µg/ml of Con A. The SI was calculated as a fold-change above background thymidine incorporation in freshly isolated CD4⁺CD25⁺ T cells incubated with APCs without Con A.

Calcium flux. Ca²⁺ flux in lymph node cells from TClI or OT-II TCR-Tg or B6 mice in response to TCR stimulation was analyzed as described⁴⁴. For APCs, B6 splenocytes were pulsed with 10 µg/ml of hCLIP or 25 µg/ml of OVA for 3 h at 37 °C. Anti-CD3 (mAb 2C11) or Con A was added at 50 µg/ml, PMA at 100 nM and ionomycin at 1 µM. Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Chemotaxis assay. Transwell chambers (Costar, Corning, NY) were precoated with the assay media (RPMI-1640, 1% BSA, 1% FCS) and aspirated. Prewarmed lymph node cells (10⁶ in 100 µl) were added to the top chamber, chemokines were added to the wells (600 µl final volume, prewarmed) and the top chambers were placed into the wells and incubated at 37 °C. After 4 h, cells were collected from the bottom chamber, counted and analyzed by flow cytometry.

Array analysis. RNA was generated (RNeasy, Qiagen, Valencia, CA) from FACS-sorted cells from young B6 mice (>97% purity). Preparation of the biotinylated RNA probe was done as described (Affymetrix GeneChip Expression Analysis Manual, Santa Clara, CA), except Thermoscript reverse transcriptase (Life Technologies, Gaithersburg, PA) was used for first-strand cDNA synthesis (48 °C 10 min, 52 °C 40 min). In some experiments, the first *in vitro* transcription was done with normal rNTPs with high concentration (2,500 U/µl) T7 RNA polymerase (Epicentre Technologies, Madison, WI), according to the manufacturer's protocol. The second round of cDNA synthesis and *in vitro* transcription was done as described⁴¹. Biotinylated RNA was generated with BioArray High Yield reagents (Enzo Diagnostics, Farmingdale, NY), according to the manufacturer's protocol. Samples were hybridized to the Affymetrix mul1K and mul19K chips and read at the HHMI facility at the University of Texas Southwestern Medical Center.

Real-Time PCR. cDNA (0.2 µl) from a 20 µl reaction (Thermoscript, Life Technologies) starting with 1 µg of total RNA (pretreated with DNase I, 15 min 25°C) was used for each 25 µl PCR reaction. Primers were generated for 3' ends of transcripts as found in Genbank and the TIGR Mouse Gene Index with the Primer3 website (20 bp, 59 °C annealing temperature, ~100 bp amplicon)⁴⁵. PCR amplicons were detected with SIBR green and were generated according to manufacturers protocols (PE Biosystems, Warrington, UK) with a PE 7700 Sequence Detector. All PCR reactions were analyzed by PAGE and contained one amplicon of the correct size. PCR primer pairs were as follows. CD4: GCCCTCGGTATACACACA-CA, GGAGGGATTGAAACACGAA; CD7: GGTGGGATTCTCTCTCTGTA, CCC-CAAGCTTTGTTTCTGAT; CTLA-4: ATGTACCACCGCCATACCT, GGATCCAAAGGAGGAAGTCA; FAP-1: CACTCTTGAGAGCAGCAAGG, ACAGCATCTTGACGTCCGTA; Fkbp-52: CCCGTTACACACAAGGATA, CCAGTGAAAATGGAGACTAA; Fyn: GAATGCTGAAGGGTTCAGGT, GGTGGCCATGACTACAATGC; Geminin: CTTC TGGACCAACCTTGAA, TGGATCTCTTGTTGGCAGAG; HPRT: TGGAAGAAGATGTCTTGATTGTTGAA, AGCTTGCAACCTTAACCATTTTG; IL-2Rα: TGGAAGGTTGA GGGGTAAG, ACACCTGTAAGCCGAGCTCT; IL-7Rα: CTCGTAAGAGAGCCCCAC AT, TGGAAGTGGATGGAAGTCAA; IRF-4: TCAGCTCTTTCACGAGGATG, TGAGC-CAAGCAAGTAAAGTCTG; Lck: AATCCGGGAAAAGTGATACG, ACCAGAACCAGG GAGAAGTG; ITGAE: CTTTGGCATACAGATGTGC, GCAGTCCAAACAGGAATA-CA; LEF-1: GGAGCCCAAAAAGACCTCAT, CTCCTTTAGCGTGCACACTCAG; MCM5: CATTTGCTAAAGCTGGGATCA, TCCTCCCTTTTGTCTCATC; MKP-1: CATGGGAG CTGGTCCTTAIT, CTTCGGGTGCAAGTCAATTGT; PD-1: CTGTTGGGACGCTGTAT-GAT, CCAGCAACCAGACTGAAAAA; Pde3b: GCGAGCTTCATTTAGCACTG, AGAT-GATCCTCTGGGACTGG; RAR-γ: GGGCCCTCTACACTACAGA, AAGTGGGATCA GCTAAGCA; RGS-1: TTTTGCTGACTGCCAAAGGAT, TTTTCACTGCTTCACTCAAAA; SATB-1: TTTGTGAAAACCTTGCTGCTC, CTCTTAGCCAGCAGTCCCT; SOCS-1: CCTGGTTGTAGCAGCTTGTG, CCCTGGTTTGTGCAAAGATA; SOCS-2: AAGT-CAACGGGTACCCCAAA, CCGGAATTTGGAGAAAAAGA; TCF-1: TCCGATGACAG TGCTTAGG, GGCTAGCAAGCAATTTCTGTG; TGF-βRI: GATCTGCAAAATTTGGG TAGG, CCAACTTCCACTGCCACTTA; TGIF: AAATCTGGTTGAGGTCTGG, TGTA CCCAGAGGACACTTGC; Thy-1: CCACACTGCTACAGCATCT, GCCAGGACACAG AAGAGACA; Tiam-1: GCAGAGGTGTCGGATAGTCA, ATGGCACTCTCTTAAGC AC; TNFRSF-1β: CCACTTGTAGCCTGTGGATG, CCTTCCCAACAACAATGAG;

TNFRSF-4: AAGGCTTGGAGATTGCCTAA, GAGCTATGGCTCATCGTGAA; TNFRSF-9: CCTCTCTTCTCTCTCTGTGG, ATTCCTCCACATATTCGAAGC; TNFRSF-18: TGA-GAGCGGTGAGTTACGAT, CTTGTGCTAAACGTTGGTGTCT; FasL: TGTCTCATTGGCA CCATCTT, TGCTCTCAAACATCCCTCTT; YY1: GAGGCCAGAGAGTGTGTGAA, TGATACAAGCCACAGGCATT.

Note: Supplementary information can be found on the Nature Immunology website (http://immunology.nature.com/supp_info/).

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