

# CD4<sup>+</sup> regulatory T cells require CTLA-4 for the maintenance of systemic tolerance

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Cytotoxic T lymphocyte antigen-4 (CTLA-4) plays a critical role in negatively regulating T cell responses and has also been implicated in the development and function of natural FOXP3<sup>+</sup> regulatory T cells. CTLA-4-deficient mice develop fatal, early onset lymphoproliferative disease. However, chimeric mice containing both CTLA-4-deficient and -sufficient bone marrow (BM)-derived cells do not develop disease, indicating that CTLA-4 can act in trans to maintain T cell self-tolerance. Using genetically mixed blastocyst and BM chimaeras as well as in vivo T cell transfer systems, we demonstrate that in vivo regulation of *Ctla4*<sup>-/-</sup> T cells in trans by CTLA-4-sufficient T cells is a reversible process that requires the persistent presence of FOXP3<sup>+</sup> regulatory T cells with a diverse TCR repertoire. Based on gene expression studies, the regulatory T cells do not appear to act directly on T cells, suggesting they may instead modulate the stimulatory activities of antigen-presenting cells. These results demonstrate that CTLA-4 is absolutely required for FOXP3<sup>+</sup> regulatory T cell function in vivo.

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Abbreviations used: Ab, antibody; CTLA4, cytotoxic T lymphocyte antigen-4; LCMV, lymphocytic choriomeningitis virus; *Mtb*, *Mycobacterium tuberculosis*.

Peripheral T cell tolerance is mediated in part by active processes that maintain a fine balance between stimulatory and inhibitory signals. Mature thymocytes exported from the thymus bear self-reactivity caused by positive selection on self-antigen–MHC complexes (1), and naive peripheral T cells require tonic interactions with self-MHC–antigen complexes to persist (2–6). In conjunction with T cells that can potentially recognize self-antigens not presented in the thymus, the inherent avidity of the T cell receptor repertoire to self-MHC molecules necessitates mechanisms that maintain peripheral self-tolerance. Of these, two processes have received the bulk of attention: (a) immune suppression by a distinct lineage of CD4<sup>+</sup> T cells called natural regulatory T (T reg) cells that function in trans to inhibit T cell activation (7), and (b) the modulation of T cell co-stimulatory molecules, especially those that transmit inhibitory signals in a cell-autonomous manner. Cytotoxic T lymphocyte antigen-4 (CTLA-4; CD152), a structural

homologue of the primary positive co-stimulatory molecule CD28, is the most extensively studied inhibitory T cell molecule essential for T cell homeostasis and tolerance induction (8, 9). Mice deficient in CTLA-4 develop a fatal lymphoproliferative disorder (10, 11) with aberrant T cell activation evident by 4 d after birth. Because development, selection, and peripheral TCR repertoire complexity appear grossly normal in *Ctla4*<sup>-/-</sup> mice (12–14), the aberrant activation is most likely initiated by unchecked recognition of self- or environmental antigens in peripheral tissues.

In conventional T cells, CTLA-4 can dampen stimulatory signals by at least three major mechanisms. First, it efficiently competes with the positive co-stimulatory molecule CD28 for their shared ligands, B7.1 and B7.2 (15, 16). Second, CTLA-4 transduces negative signals that induce

†C.A. Chambers died on December 19, 2004.

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cell cycle arrest and prevent IL-2 secretion (17–20). Third, CTLA-4 may limit the T cell dwell time with APCs (21). Additional complexity in understanding CTLA-4 function was revealed by the finding that *Ctla4*<sup>−/−</sup> T cells in mixed BM chimaeras remain quiescent in the presence of normal (CTLA-4-sufficient) BM-derived cells (22). How this regulation occurs has not been determined, but T reg cells have been suggested to be important, especially because T reg cells constitutively express high amounts of CTLA-4.

CD4<sup>+</sup> T reg cells arise in the thymus and have been shown in vitro and in vivo to dominantly inhibit conventional T cell responses to both self- and foreign antigens (7). These cells, initially characterized as CD25 (IL-2R $\alpha$ )<sup>+</sup>, are molecularly distinguished from other T cell subsets by expression of the transcription factor FOXP3 that endows T reg cells with their lineage and functional specificity (22, 23). As surface expression of CTLA-4 is generally only detected on T reg cells in unmanipulated mice, one interpretation of the mixed BM chimaera data were that CTLA-4 is primarily required for T reg cell function and/or maintenance and the lymphoproliferation observed in *Ctla4*<sup>−/−</sup> mice is primarily a consequence of defective T reg cells. Consistent with this interpretation, mice lacking functional T reg cells as a result of a mutation in the *Foxp3* gene (*scurfy*) exhibit pathology quite similar to *Ctla4*<sup>−/−</sup> mice (24). Though FOXP3<sup>+</sup> T reg cells with suppressive activity in vitro can be isolated from *Ctla4*<sup>−/−</sup> mice (unpublished data) (25), they are clearly incapable of regulating CTLA-4-deficient T cells in vivo.

Although the role of T reg cells in controlling autoreactive *Ctla4*<sup>−/−</sup> T cells in steady-state conditions has not been directly addressed, a few reports have investigated the in vivo relevance of CTLA-4 on T reg cells by means of an induced colitis model (26–28). Transfer of naive (CD25<sup>−</sup>) CD4<sup>+</sup> T cells into lymphopenic hosts rapidly leads to colitis unless FOXP3<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> T reg cells are also transferred. Protection from colitis is abrogated by injection of blocking antibody (Ab) against CTLA-4 suggesting that CTLA-4 is necessary for immune regulation in this model system (28, 29). Although the relevant target of Ab blockade (e.g., CTLA-4 on effector T cells or T reg) in this model was unknown, it has been shown that CTLA-4 blockade variably disrupts the control of colitogenic B7-deficient (*Cd80*<sup>−/−</sup>*Cd86*<sup>−/−</sup>) *Ctla4*<sup>−/−</sup> T cells by WT (CTLA-4<sup>+</sup>) T reg cells (30), suggesting that CTLA-4 on T reg cells is functionally relevant for initiating and/or maintaining regulation. However, the situation remained uncertain because experiments using Ab-mediated blockade of CTLA-4 have provided inconsistent results in the same model system, and T reg cells from *Ctla4*<sup>−/−</sup> mice have also been reported to prevent the progression of colitis (30). Given the contrasting observation in *Ctla4*<sup>−/−</sup> mice where endogenous T reg cells cannot regulate lymphoproliferation of *Ctla4*<sup>−/−</sup> T cells, it is clear that the requirements for regulation may be very distinct between the colitis model and CTLA-4 deficiency. Hence, the cellular requirements for maintaining tolerance of *Ctla4*<sup>−/−</sup> T cells in a steady-state condition in vivo that most closely models physiological peripheral T cell tolerance to self are not known.

In this study, we use mixed stem cell chimaeras and T cell transfer systems to define the cellular and molecular mechanisms involved in trans-regulation of CTLA-4-deficient T cells. We demonstrate that regulation: (a) is exclusively mediated by CTLA-4-sufficient T reg cells with a diverse TCR repertoire; (b) is reversible and depends on the continuous presence of T reg cells; (c) is not dependent on “reverse inside-out” B7 signaling (31, 32); and (d) is unlikely to involve direct and unique molecular alterations of *Ctla4*<sup>−/−</sup> T cells by T reg cells and their effector molecules. Collectively, these results demonstrate that T reg cells can dominantly control a large pool of self-reactive T cells in vivo and that CTLA-4 is essential for their cell function.

## RESULTS

### CTLA-4-sufficient blastocyst and BM-derived cells stably regulate *Ctla4*<sup>−/−</sup> T cells in vivo

*Ctla4*<sup>−/−</sup> mice develop massive lymphoproliferation and a multi-organ inflammatory response driven by self-antigen specific T cells (10, 11). Peripheral T cells are detectably activated by 4 d of age, and transfer of T cells from *Ctla4*<sup>−/−</sup> mice to lymphopenic hosts such as *Rag1*<sup>−/−</sup> mice recapitulates the disease (wasting and lymphoproliferation), highlighting the autoaggressive nature of *Ctla4*<sup>−/−</sup> T cells (unpublished data). As initially shown by Bachmann et al. (22), BM chimaeras generated using a 1:1 mixture of wild-type and *Ctla4*<sup>−/−</sup> (WT:*Ctla4*<sup>−/−</sup>) cells are protected from disease and remained healthy for extended periods of time (Fig. 1 A). This contrasts with *Ctla4*<sup>−/−</sup>-only BM chimaeras that succumb to a fatal lymphoproliferative and inflammatory disease. Similarly, mixed WT:*Ctla4*<sup>−/−</sup> blastocyst chimaeras generated without any manipulation of the lymphoid microenvironment (e.g.,  $\gamma$ -irradiation performed on BM recipients) were also disease resistant for their entire life span (Fig. 1, A and B). Trans-regulation of *Ctla4*<sup>−/−</sup> T cells appeared relatively insensitive to the ratio of WT:*Ctla4*<sup>−/−</sup> cells as both blastocyst and BM chimaeras with WT T cells comprising only 10–20% of the peripheral T cell compartment, albeit rare, were completely healthy. As initial experiments did not reveal any differences between mixed blastocyst and BM chimaeras, all subsequent analyses were performed with BM chimaeras.

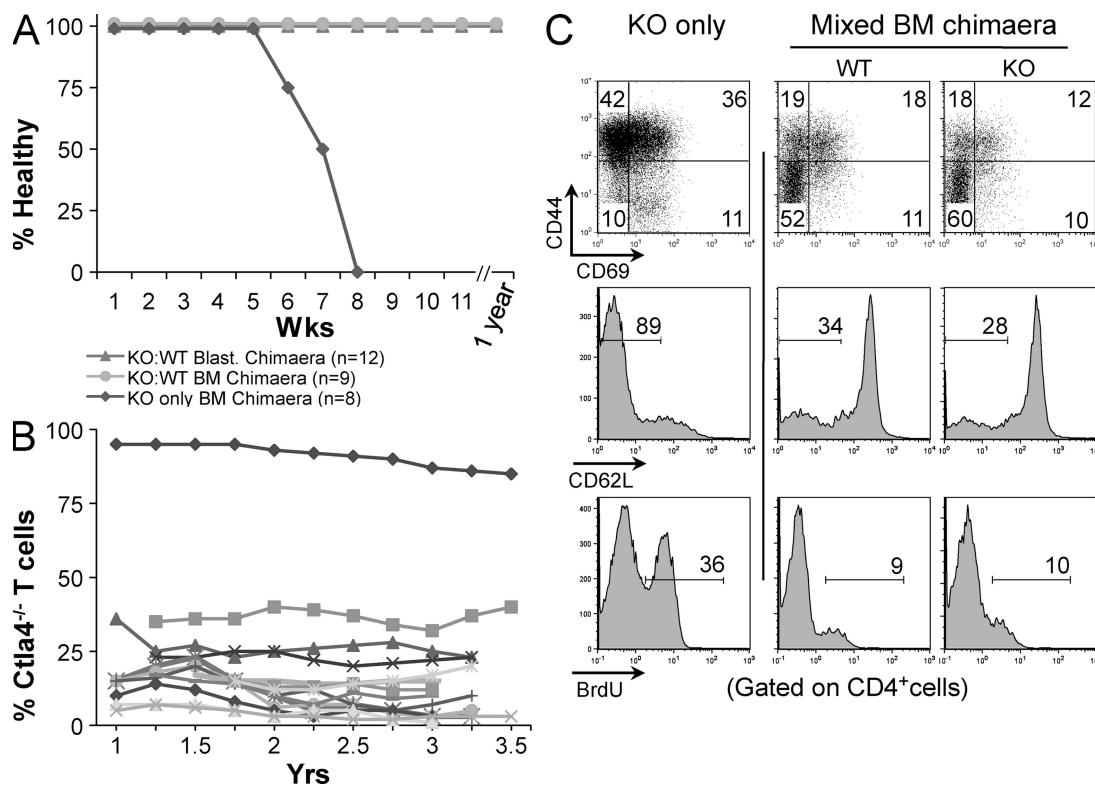
Phenotypically, WT and *Ctla4*<sup>−/−</sup> T cells in the mixed BM chimaeras were indistinguishable, with a majority of the cells expressing low levels of CD44 and CD69, and high levels of CD62L, which is indicative of a naive/unactivated state (Fig. 1 C). In contrast, recipients of *Ctla4*<sup>−/−</sup> BM alone developed a population of highly activated T cells, comparable to *Ctla4*<sup>−/−</sup> mice. The frequency of cycling *Ctla4*<sup>−/−</sup> T cells was dramatically reduced in the mixed BM chimaeras, as compared with chimaeras receiving *Ctla4*<sup>−/−</sup> BM only (Fig. 1 C and not depicted), and the naive *Ctla4*<sup>−/−</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells were as quiescent as WT T cells. Additionally, whereas *Ctla4*<sup>−/−</sup>-only BM chimaeras developed lymphoproliferative disease and obvious tissue infiltration by activated T cells, WT:*Ctla4*<sup>−/−</sup> mixed BM chimaeras remained healthy with no aberrant tissue infiltration or disruption in homeostasis (unpublished data).

The robustness of the trans-regulation in WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras was tested in both acute viral (lymphocytic choriomeningitis virus [LCMV]) and chronic *Mycobacterium tuberculosis* (*Mtb*) models, which primarily elicit CD8<sup>+</sup> or CD4<sup>+</sup> T cell responses, respectively (33–36). The frequency and magnitude of antiviral or antibacterial T cell responses in challenged WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras were essentially identical between WT and *Ctla4*<sup>-/-</sup> T cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20081811/DC1>, and not depicted). The results with LCMV infection confirmed previously published studies (37, 38). In the case of chronic *Mtb* infection, the WT:*Ctla4*<sup>-/-</sup> ratio of T cells in the lung and spleen upon analysis was similar to preinfection ratios in the blood (Fig. S1 A). Further, the proportions of naive, activated, or CD25<sup>+</sup>CD4<sup>+</sup> cells in the lung were comparable between WT and *Ctla4*<sup>-/-</sup> T cell compartments (Fig. S1 B). Finally, in vitro IFN- $\gamma$  responses to *Mtb* antigens by WT and *Ctla4*<sup>-/-</sup> T cells from infected mixed BM chimaeras showed no significant differences (unpublished data). This remarkable stability of the *Ctla4*<sup>-/-</sup> T

cell compartment upon robust activation and chronic antigen presentation was also observed in mixed BM chimaeras challenged with skin allografts (unpublished data).

### CTLA-4-sufficient $\alpha\beta$ T cells actively regulate *Ctla4*<sup>-/-</sup> T cells in the periphery

The WT BM-derived cell subset responsible for trans-regulating *Ctla4*<sup>-/-</sup> T cells has not been formally demonstrated. To this end, we first generated *Tcrb*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup> mixed BM chimaeras to confirm that  $\alpha\beta$  T cells are required. The absence of WT  $\alpha\beta$  T cells led to the expansion of activated *Ctla4*<sup>-/-</sup> T cells and death of the animals, demonstrating their requirement for regulation (Table I). In addition, CTLA-4-sufficient  $\alpha\beta$  T cells expressing MHC class I- or class II-restricted TCR transgenes in a RAG-1/2-sufficient background (that allows for the generation of FOXP3<sup>+</sup> T cells) were unable to prevent *Ctla4*<sup>-/-</sup> T cell expansion in mixed chimaeras (Fig. 2), suggesting that a diverse TCR repertoire is required for the regulation. Further, *Tcrd*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup> mixed chimaeras



**Figure 1. *Ctla4*<sup>+/+</sup> cells can regulate *Ctla4*<sup>-/-</sup> T cells in mixed BM and blastocyst chimaeras.** (A) Healthy *Rag1*<sup>-/-</sup> mice received a total of  $\sim 4 \times 10^6$  T cell-depleted BM cells from 2–3-wk-old *Ctla4*<sup>-/-</sup> mice (KO only) or a 1:1 mix from *Ctla4*<sup>-/-</sup> and WT mice (KO:WT). Blastocyst chimaeras were generated as per Materials and methods, and all chimaeras were monitored for disease incidence. Mice were considered healthy (% Healthy) if no evidence of prolonged weight loss (>2 wk), ruffled fur, hunched posture, or skin or eye inflammation were visible. Mice were also bled periodically to measure relative KO:WT T cell ratios. *Ctla4*<sup>-/-</sup> only recipients were killed for ethical reasons by 8 wk of age. (B) 12 blastocyst chimaeras, each line representing individual mice, were periodically bled over several years, and the frequency of *Ctla4*<sup>-/-</sup> T cells among total T cells enumerated via flow cytometry. (C) 6–8-wk-old KO only or KO:WT chimaera groups were killed, and LN cells were isolated and analyzed for expression of activation markers CD44, CD62L, CD69, and incorporation of the thymidine analogue BrdU, by flow cytometry. Results gated on *CD4*<sup>+</sup> LN T cells from representative chimaeras are shown. Data shown in A and C are representative of over a dozen experiments. Data in B are the cumulative results of at least three experiments.

**Table I.** Functional  $\alpha\beta^+$  T cells are required for regulating *Ctla4*<sup>-/-</sup> T cells in mixed BM chimaeras

Source of regulatory cells <sup>a</sup>	Rescue frequency <sup>b</sup>
TCRd <sup>-/-</sup> : <i>Ctla4</i> <sup>-/-</sup>	6/6
TCRb <sup>-/-</sup> : <i>Ctla4</i> <sup>-/-</sup>	0/5
Cd4 <sup>-/-</sup> : <i>Ctla4</i> <sup>-/-</sup>	7/8
Cd8 <sup>-/-</sup> : <i>Ctla4</i> <sup>-/-</sup>	4/5
Cd28 <sup>-/-</sup> : <i>Ctla4</i> <sup>-/-</sup>	0/15
wt: B7 <sup>-/-</sup> <i>Ctla4</i> <sup>-/-</sup>	4/4

<sup>a</sup>1:1 mixed BM chimaeras of indicated genotypes, except for those involving *Cd28*<sup>-/-</sup> BM cells where some were at a 3:1 ratio (n = 5). Control mixed chimaeras receiving *Ctla4*<sup>+/+</sup> instead of *Ctla4*<sup>-/-</sup> BM remained healthy (not depicted).

<sup>b</sup>Frequency of surviving mice with quiescent *Ctla4*<sup>-/-</sup> T cells.

remained healthy, indicating that  $\gamma\delta$  T cells are not necessary for regulation.

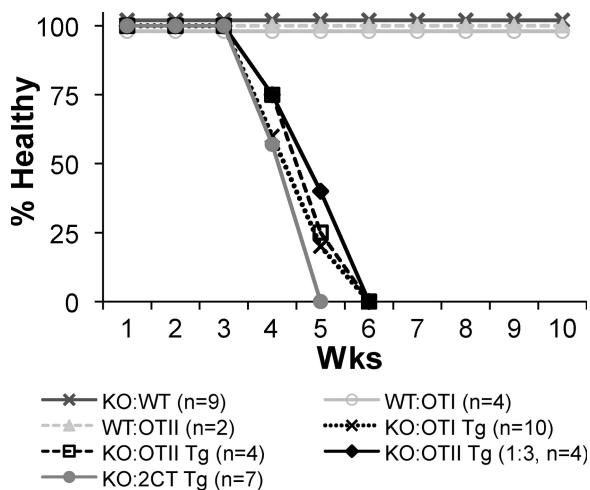
We next addressed whether regulation required the activity of a specific  $\alpha\beta$  T cell subset. As both CD4<sup>+</sup> (39–41) and CD8<sup>+</sup> (42, 43) regulatory T cells have been reported, we examined whether the absence of either of these WT T cell subsets affected tolerogenic potential in mixed BM chimaeras. For these experiments, mixed chimaeras consisting of CD4- or CD8-deficient along with *Ctla4*<sup>-/-</sup> BM-derived cells were generated. CTLA-4-sufficient *Cd4*<sup>-/-</sup> or *Cd8*<sup>-/-</sup> BM cells were able to regulate *Ctla4*<sup>-/-</sup> T cells in mixed BM chimaeras, as most recipient chimaeras remained healthy for > 6 mo (Table I). Also, no significant phenotypic differences were detected between the *Ctla4*<sup>-/-</sup> and CTLA-4<sup>+</sup> T cells in the respective chimaeras (not depicted). This data initially suggested either that CD4<sup>+</sup> or CD8<sup>+</sup> T cells were sufficient for

regulation or that CD4<sup>-</sup>CD8<sup>-</sup> T cells may be responsible. Interpretation of these results was hampered by the fact that lack of CD8 or CD4 coreceptors does not preclude the development of T cells positively selected on MHC class I or class II molecules, respectively (44, 45) (see below).

Given the requirement for TCR $\beta$ -expressing cells and a possible involvement of CD4<sup>-</sup>CD8<sup>-</sup>  $\alpha\beta$  TCR<sup>+</sup> T cells, it was formally possible that NKT cells were responsible for regulation. To test this, WT:*Ctla4*<sup>-/-</sup> mixed chimaeras were injected weekly with PK136 mAb that has been shown to deplete NK1.1-expressing cells (NKT and NK cells) in vivo (46). NK1.1<sup>+</sup> cell-depleted WT:*Ctla4*<sup>-/-</sup> chimaeras remained healthy for 2 mo, and analyses of lymphocytes in the treated mice confirmed that *Ctla4*<sup>-/-</sup> T cells remained quiescent (unpublished data). Thus, NKT and NK cells are not required for trans-regulation.

Depending on the model system, induction of T cell tolerance has been demonstrated to have short- or long-lived consequences on subsequent T cell responses. The question therefore arises whether tolerance in this model system is irreversible such that, once established, regulation no longer requires continuous suppression by WT T cells. To address this we first tested whether the established in vivo regulation of *Ctla4*<sup>-/-</sup> T cells in mixed BM chimaeras could be reversed by the selective depletion of WT T cells. To this end, WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras distinguished by the CD90 (Thy1) allele were generated. Upon establishing a stable peripheral T cell repertoire (>10 wk after reconstitution), WT T cells (CD90.1) were selectively depleted using an anti-CD90.1-specific Ab. To confirm effective depletion of WT T cells, mice were bled 2 wk after the initiation of Ab administration. By this time, >90% of T cells in both the CD4<sup>+</sup> and CD8<sup>+</sup> subsets were CD90.2<sup>+</sup>, indicating effective depletion of CD90.1<sup>+</sup> WT T cells (unpublished data).

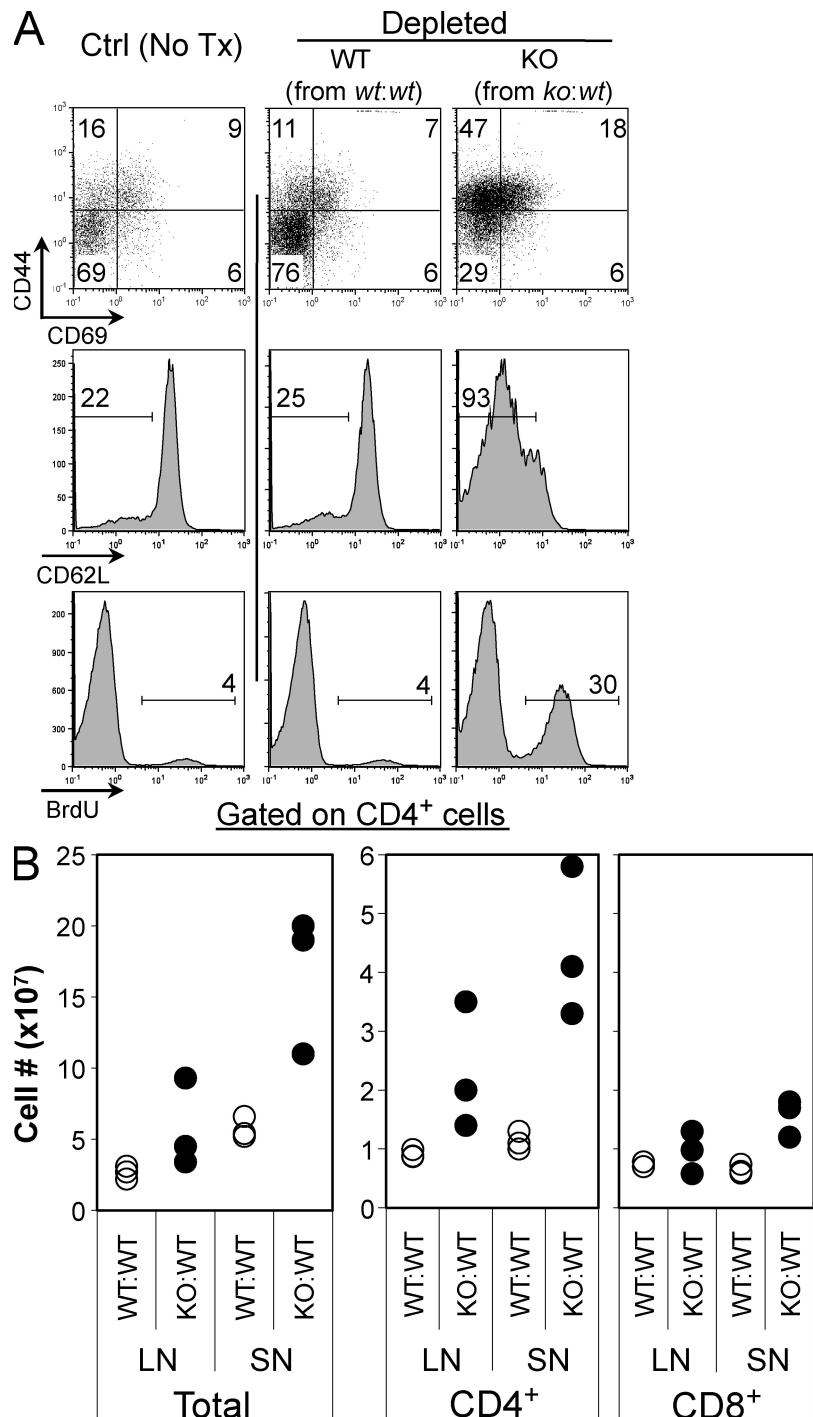
3–4 wk after initiating depletion, mice began to show symptoms of sickness similar to *Ctla4*<sup>-/-</sup>-only BM recipients (ruffled fur, inflammation around the ears and eyes, and loose stools indicative of enterocolitis). In contrast, CD90.1 WT:CD90.2 WT control chimaeras that were depleted of CD90.1<sup>+</sup> WT cells showed no visible signs of illness and appeared healthy throughout the study. Animals were killed 8 wk after WT T cell depletion, and lymphocytes were analyzed for activation status as shown in Fig. 3 A. Most T cells from the WT (CD90.1)-depleted WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras were activated (CD44<sup>hi</sup>CD69<sup>+</sup>CD62L<sup>lo</sup>) in contrast to the depleted WT:WT, which maintained a predominantly naive phenotype. Furthermore, almost a third of the *Ctla4*<sup>-/-</sup> cells were now cycling, as indicated by the threefold increase in total LN and spleen cell numbers, primarily in the CD4<sup>+</sup> T cell compartment (Fig. 3 B). This contrasts with WT T cells from depleted control chimaeras that had a relatively low frequency of cycling cells. Histological analyses confirmed that *Ctla4*<sup>-/-</sup> T cells had regained overt autoreactive characteristics as indicated by infiltration into various nonlymphoid organs (unpublished data). These data reveal that trans-regulation depends on the persistent presence of WT T cells and that the



**Figure 2.** A diverse repertoire of *Ctla4*<sup>+/+</sup> T cells are required to regulate *Ctla4*<sup>-/-</sup> T cells in vivo. *Rag1*<sup>-/-</sup> recipient mice received a total of  $\sim 4 \times 10^6$  T cell-depleted BM cells from a 1:1 mix of *Ctla4*<sup>-/-</sup> and WT mice (KO:WT), or *Ctla4*<sup>-/-</sup> and 2CT, OTI, or OTII TCR transgenic *Rag1*/*2*<sup>+/+</sup> mice. Some mice received a 1:3 ratio of *Ctla4*<sup>-/-</sup>:OTII BM cells. Mice were monitored for disease incidence (% Healthy), as described in Fig. 1 A. Data are pooled from two independent experiments.

regulated *Ctla4*<sup>-/-</sup> T cells are not permanently altered to prevent rampant autoreactivity. It is possible that disease induction is mediated solely by recent *Ctla4*<sup>-/-</sup> thymic emigrants not subject to peripheral regulatory processes caused

by the depletion of the WT T cells. However, this seems unlikely given the rapid kinetics of disease ( $\sim 3$  wk) and the high proportion of activated peripheral T cells. Moreover, transfer of regulated *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> T cells from mixed BM



**Figure 3. *Ctla4*<sup>-/-</sup> T cell quiescence depends on the persistent presence of WT T cells.** (A) WT:WT or *Ctla4*<sup>-/-</sup>:WT (KO:WT) mixed BM chimaeras received depleting anti-CD90.1 mAb (no treatment control, PBS injected only, and depleted) as indicated in Materials and methods. 8 wk after initiating depletion, chimaeras were killed and analyzed. LN cells were isolated and stained for CD44, CD62L, and CD69. Incorporation of the thymidine analogue BrdU as a measure of cell turnover was also assayed. Data are gated on CD90.2<sup>+</sup> CD4<sup>+</sup> T cells. (B) Enumeration of T cells from the LN and spleens (SN) of depleted mice. Each circle represents individual mice. Data are representative of two independent experiments with at least three chimaeras per group.

chimaeras into recipients without the appropriate WT T cells results in aberrant T cell activation as described below, which is consistent with a reversible autoreactive state of *Ctla4*<sup>-/-</sup> T cells held in check by CTLA-4-sufficient T cells.

#### Adoptive transfer of WT CD4<sup>+</sup> T cells maintains regulation

Although the BM chimaera system is useful in studying the overall development and/or regulation of hematopoietic cells, it is difficult to evaluate the importance of specific T cell subsets in the regulation of naive *Ctla4*<sup>-/-</sup> T cells. Instead, adoptive transfer of sorted peripheral T cell subsets into lymphopenic hosts was chosen to characterize the importance of various T cell subsets. Previously, Tivol et al. (47) showed that WT T cells rapidly eliminate cotransferred activated *Ctla4*<sup>-/-</sup> T cells in the transfer system, suggesting that distinct mechanisms of regulation operate on naive versus activated *Ctla4*<sup>-/-</sup> T cells *in vivo*. To avoid this “deletional” mechanism of tolerance, we sought to isolate naive *Ctla4*<sup>-/-</sup> T cells. Several potential *in vivo* sources of unactivated *Ctla4*<sup>-/-</sup> T cells exist, such as day 2 or 3 *Ctla4*<sup>-/-</sup> neonates or co-stimulation-impaired *Ctla4*<sup>-/-</sup> mice (e.g., *B7*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup> or *Cd28*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup>). However, we deemed these sources problematic because of the difficulty in obtaining sufficient numbers of naive T cells from *Ctla4*<sup>-/-</sup> neonates, and in the latter case, because of the possibility that T cells from these double-deficient mice may be abnormal because they are generated and maintained in a co-stimulation-deficient environment. We therefore chose to obtain naive *Ctla4*<sup>-/-</sup> T cells from regulated WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras.

We first tested the feasibility of the transfer assay for trans-regulation of naive T cells by performing bulk transfers of LN and/or spleen cells from healthy mixed BM chimaeras to *Rag1*<sup>-/-</sup> (or *Tcrb*<sup>-/-</sup>; similar data not depicted) recipients. Although the T cells rapidly up-regulated activation markers caused by homeostatic expansion, there was no significant alteration in the relative ratios of WT:*Ctla4*<sup>-/-</sup> T cells and no phenotypic difference between the transferred WT or *Ctla4*<sup>-/-</sup> T cells (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20081811/DC1>). The recipient mice remained healthy for several months and, unlike transfers using activated *Ctla4*<sup>-/-</sup> T cells, maintained *Ctla4*<sup>-/-</sup> T cells in the periphery, demonstrating efficient regulation upon transfer. In contrast, transfer of LN/spleen cells depleted of WT T cells (using CD90.1 congenic marker) resulted in lymphoproliferation and wasting, confirming the requirement for the continuous presence of WT T cells for regulation (unpublished data).

To determine whether regulation could be established by either CD4<sup>+</sup> or CD8<sup>+</sup>  $\alpha\beta$  T cells, sorted CD4<sup>+</sup> T cells from WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras were transferred alone (*Ctla4*<sup>-/-</sup> or WT) or together with either WT CD4<sup>+</sup> or CD8<sup>+</sup> T cells from C57BL/6 mice. Transfer of naive *Ctla4*<sup>-/-</sup> (but not WT) CD4<sup>+</sup> T cells alone resulted in lymphoproliferation and a wasting syndrome (data not shown). WT CD8<sup>+</sup> T cells were unable to regulate *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> T cells, as mice receiving sorted WT CD8<sup>+</sup> T cells and naive *Ctla4*<sup>-/-</sup>

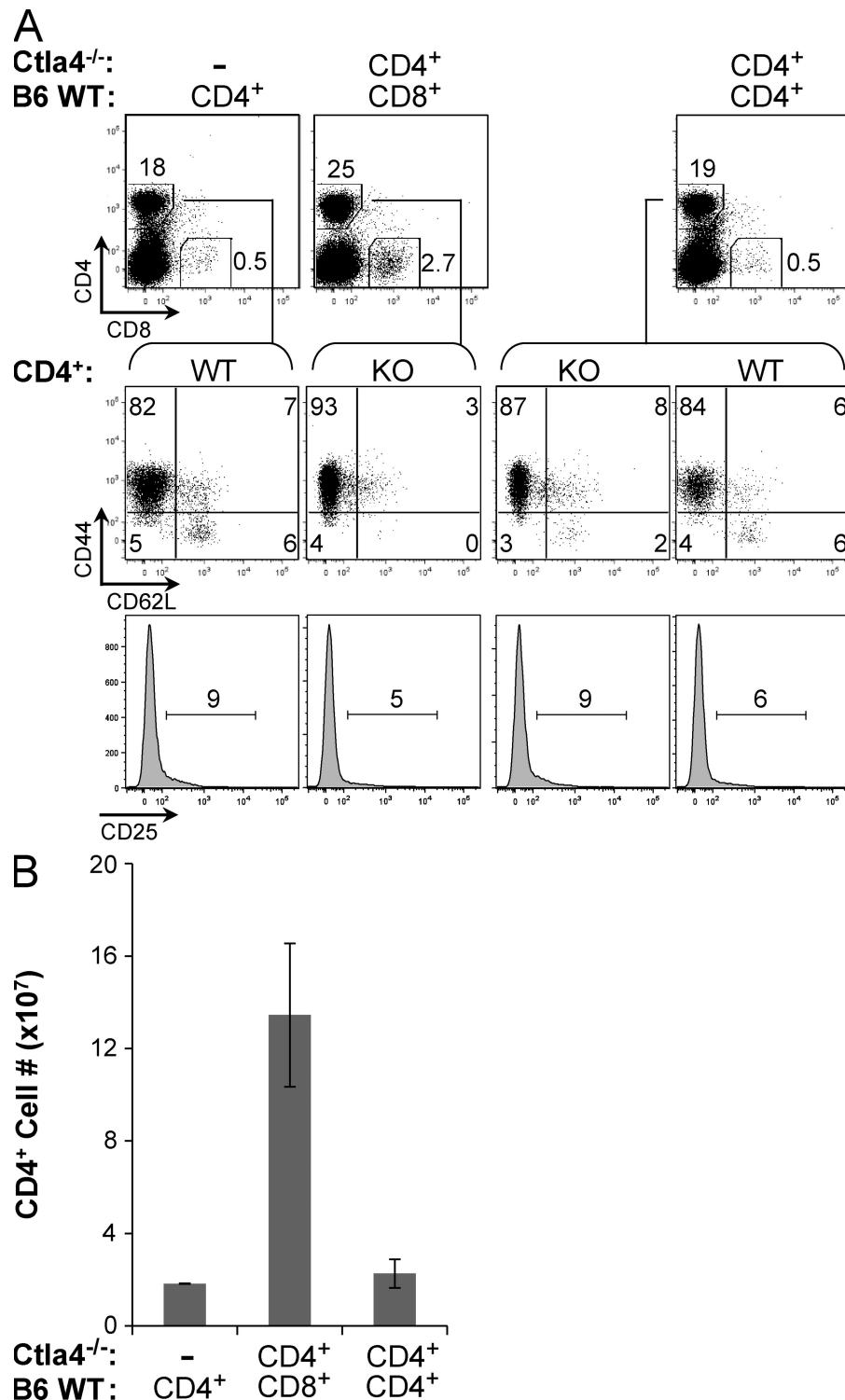
T cells became ill  $\sim$ 4 wk after transfer. In contrast, mice receiving WT CD4<sup>+</sup> T cells with *Ctla4*<sup>-/-</sup> T cells remained healthy, with no detectable signs of wasting. In all conditions, T cells were CD44<sup>hi</sup>CD62L<sup>lo</sup> because of homeostatic proliferation (Fig. 4 A). Upon sacrifice, mice receiving *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> and WT CD8<sup>+</sup> T cells displayed a marked increase in T cell number (approximately sixfold) compared with mice receiving *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> and WT CD4<sup>+</sup> T cells (Fig. 4 B). These data demonstrate that WT CD4<sup>+</sup>, but not CD8<sup>+</sup>, T cells are necessary and sufficient to regulate *Ctla4*<sup>-/-</sup> T cells. Our initial observation of regulation in *Cd4*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup> mixed chimaeras suggested that non-CD4<sup>+</sup> T cells can regulate *Ctla4*<sup>-/-</sup> T cells. However, a more likely interpretation attributes regulation to MHC class-II restricted T cells or functional FOXP3<sup>+</sup> T cells found in *Cd4*<sup>-/-</sup> mice (unpublished data) (44, 48).

#### CD4<sup>25</sup><sup>+</sup> T reg cells regulate *Ctla4*<sup>-/-</sup> T cells in trans

Among CD4<sup>+</sup> T cells, CTLA-4<sup>+</sup> FOXP3<sup>+</sup> T reg cells were the most likely population necessary for regulating *Ctla4*<sup>-/-</sup> T cells. In BM chimaeras consisting of *Cd28*<sup>-/-</sup>:*Ctla4*<sup>-/-</sup> cells, no trans-regulation was observed (Table I). *Cd28*<sup>-/-</sup> mice have approximately fivefold reduced number of T reg cells (49), but they do not develop autoimmune disease, reflecting the necessity of CD28 signaling for naive T cell activation. T cell composition in *Cd28*<sup>-/-</sup>:WT mixed chimaeras reflected the initial input ratios, demonstrating that *Cd28*<sup>-/-</sup> T cells are not significantly disadvantaged during reconstitution in the presence of WT T cells (unpublished data). These results suggested that either normal T reg cell numbers or CD4<sup>+</sup> T cell subsets other than T reg cells with normal CD28 signaling is required for the trans-regulation.

To directly test the functional relevance of T reg cells, we cotransferred WT CD4<sup>25</sup><sup>+</sup> T reg cells ( $\geq$ 90% FOXP3<sup>+</sup>) with *Ctla4*<sup>-/-</sup> T cells from healthy mixed BM chimaeras to *Rag1*<sup>-/-</sup> recipients in an  $\sim$ 1:10 ratio to mimic the normal frequency of T reg cells:conventional T cells. Mice receiving *Ctla4*<sup>-/-</sup> cells alone became visibly sick by  $\sim$ 3–4 wk of age despite the fact that  $\sim$ 10% of the CD4<sup>+</sup> T cells were FOXP3<sup>+</sup>; the same frequency as the starting donor population (Fig. 5 A). In contrast, mice infused with *Ctla4*<sup>-/-</sup> T cells and WT T reg cells remained healthy for several months showing no signs of lymphoproliferation or aberrant T cell infiltration into tissues.

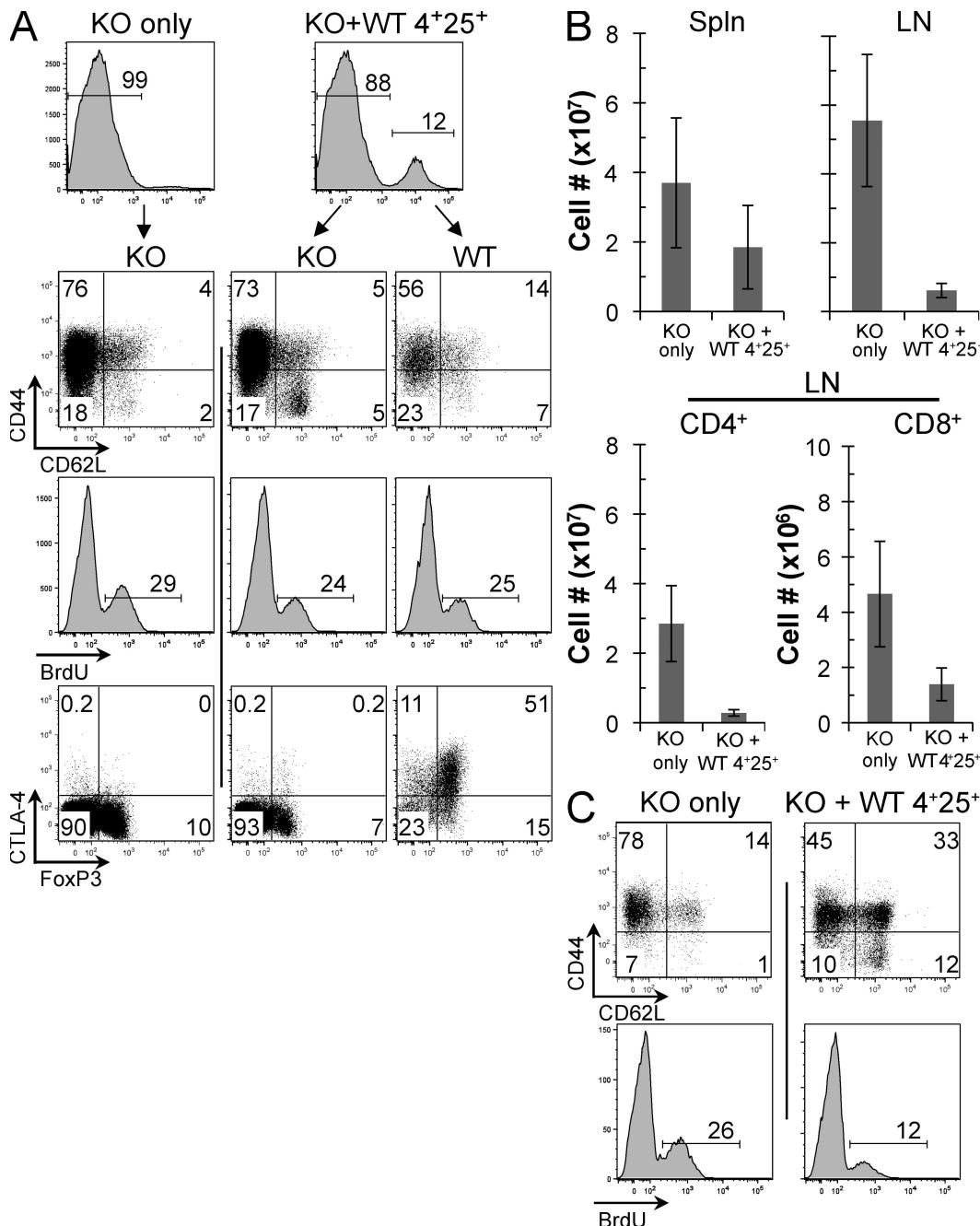
Comparative analyses of the regulated *Ctla4*<sup>-/-</sup> T cells in the transfer system at 3–4 wk post-transfer showed that the T reg cells suppressed the expansion of both CD4<sup>+</sup> and CD8<sup>+</sup> *Ctla4*<sup>-/-</sup> T cells. In the spleen and LNs, there was an approximately two- and sixfold increase in cell numbers, respectively, in mice receiving *Ctla4*<sup>-/-</sup> T cells only compared with those reconstituted with *Ctla4*<sup>-/-</sup> and WT T reg cells (Fig. 5 B). The suppression was more pronounced for CD4<sup>+</sup> T cells, with  $\sim$ 10-fold difference in number in the LNs. Given the varying degrees of T cell infiltration into nonlymphoid tissues in mice receiving only *Ctla4*<sup>-/-</sup> T cells (unpublished data), the extent of expansion noted in



**Figure 4. WT CD4<sup>+</sup> T cells regulate *Ctla4*<sup>-/-</sup> T cells *in vivo*.** CD4<sup>+</sup> or CD8<sup>+</sup> T cells from WT mice and CD4<sup>+</sup> *Ctla4*<sup>-/-</sup> T cells from mixed BM chimaeras were sorted and transferred (10<sup>7</sup> cells total) to *Rag1*<sup>-/-</sup> recipients. (A) Representative cytometric data showing the activation profile of CD4<sup>+</sup> T cells and the frequencies of CD25<sup>+</sup> CD4<sup>+</sup> T cells from each of the three recipient groups (WT CD4<sup>+</sup> T cells alone, *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> and WT CD8<sup>+</sup>, and *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> and WT CD4<sup>+</sup>) 3 wk after transfer. (B) Splenic CD4<sup>+</sup> T cell numbers from each group in A. Each group consisted of a minimum of two mice each and is representative of three experiments. Error bars denote the SD.

lymphoid tissues is most likely an underestimate of the overall number of *Ctla4*<sup>-/-</sup> T cells present in the mice. The activation status of *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> T cells with or without T reg cells revealed negligible differences in CD4<sup>+</sup> T cells, most likely as a result of homeostatic proliferation. In contrast, the CD8<sup>+</sup> T cell compartment displayed a significant

reduction in the frequency of “activated” cells (CD44<sup>hi</sup>CD62L<sup>-</sup>) and an approximately twofold reduction in BrdU uptake when CTLA-4-sufficient T reg cells were cotransferred (Fig. 5 C). Collectively, these data demonstrate that CTLA-4-sufficient, but not CTLA-4-deficient, T reg cells can completely regulate autopathogenic *Ctla4*<sup>-/-</sup> T cells in vivo.



**Figure 5. CD4<sup>+</sup>25<sup>+</sup> WT T cells are able to regulate *Ctla4*<sup>-/-</sup> T cells in vivo.** (A) WT T cell-depleted LN and spleen cells (KO) from healthy *Ctla4*<sup>-/-</sup>:WT mixed BM chimaeras were transferred (2 × 10<sup>7</sup> cells/mouse) either separately or with purified WT CD4<sup>+</sup>25<sup>+</sup> T cells (2 × 10<sup>6</sup> cells/mouse) into *Rag1*<sup>-/-</sup> recipients. Frequency and activation status of CD4<sup>+</sup> (A) and CD8<sup>+</sup> (C) T cells from the LNs were analyzed in recipients 12 wk after transfer. (B) Quantitation of total, CD4<sup>+</sup>, and CD8<sup>+</sup> T cell numbers in the spleen and LN of recipients at the time of analysis. Data are representative of three individual experiments with at least three mice per group. Error bars denote the SD.

## B7-mediated signaling in *Ctla4*<sup>-/-</sup> T cells is not required for the trans-regulation

We next investigated the potential mechanisms involved in regulating *Ctla4*<sup>-/-</sup> T cells by CTLA-4-sufficient T reg cells in vivo. Based on the expression of B7.1/2 on activated T cells, it has been suggested that CTLA-4 on T reg cells can engage its ligand B7 on T cells and directly inhibit their activation ("inside-out" signal) (31, 32). Although initial reports indicated that B7-deficient T cells were resistant to T reg cell-mediated suppression in vivo, more recent studies using different in vivo models have generated inconsistent data (30).

To test whether CTLA-4 sufficient T reg cells control *Ctla4*<sup>-/-</sup> T cells via B7, we generated mixed BM chimaeras using stem cells from WT and *Cd80*<sup>-/-</sup>*Cd86*<sup>-/-</sup>*Ctla4*<sup>-/-</sup> (B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup>) mice. 2 mo after reconstitution, both WT:*Ctla4*<sup>-/-</sup> and WT:B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> cohorts were healthy with no signs of inflammation or wasting (Table I). B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> T cells in the mice were maintained in a naive state, indistinguishable from WT cells, as assessed by expression of CD44 and CD62L (unpublished data). In conjunction with published data showing that B7<sup>-/-</sup> and B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> can be controlled in transfer models by WT T reg cells (30) (unpublished data), the regulation of *Ctla4*<sup>-/-</sup> T cells by CTLA-4-sufficient T reg cells in vivo does not require B7 signaling in the T cells.

## Trans-regulation does not involve significant molecular alterations in *Ctla4*<sup>-/-</sup> T cells

Although our data demonstrated that trans-regulation requires the continuous presence of CTLA-4-sufficient T reg cells, it was not known whether they were molecularly altering the target *Ctla4*<sup>-/-</sup> T cells or inhibiting activation by preventing access to antigens and/or cytokines. To distinguish between these possibilities, we performed global gene expression profiling of naive *Ctla4*<sup>-/-</sup> and WT T cells sorted from mixed BM chimaeras.

Overall, the differences were minimal (~1% of expressed genes, ~70/6000 assayed; unpublished data) and the gene expression signature of regulated naive *Ctla4*<sup>-/-</sup> T cells closely resembled naive WT T cells from the same animal. At the transcriptional level, there was no evidence of active suppression (50, 51) or altered TCR/CD28 signaling on *Ctla4*<sup>-/-</sup> T cells. Further analyses of select genes identified as altered in expression by microarray analyses (Fig. 6) revealed that *Gadd45β* (Myd118) was decreased in expression by approximately fourfold in *Ctla4*<sup>-/-</sup> T cells compared with the WT counterpart, whereas *Gadd45α* and *Gadd45γ* expression were not significantly altered (not depicted). *Gadd45β* is required for normal responses to TCR and inflammatory cytokine stimulations as indicated by the development of autoimmunity in its absence (52). In contrast, expression of other genes previously implicated in T cell suppression were not altered in naive *Ctla4*<sup>-/-</sup> T cells as illustrated by similar transcript levels for the representative genes in Fig. 6. These results suggest that CTLA-4-sufficient T reg cells indirectly prevent naive *Ctla4*<sup>-/-</sup> T cells from becoming fully activated, possibly by regulating the immune stimulatory activities of APCs.

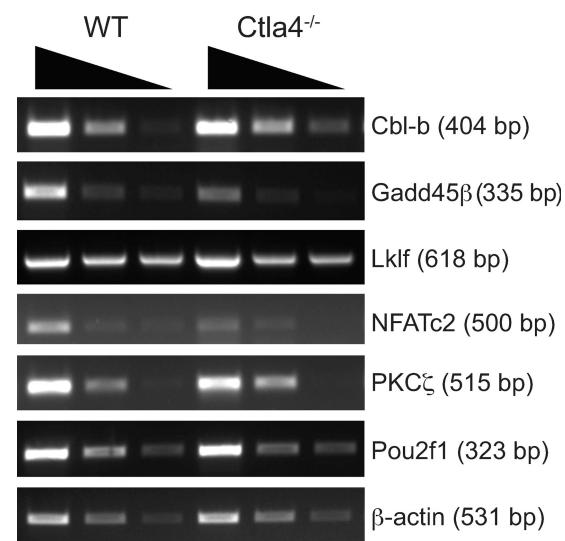
## TGF-β or IL-10 is not singularly essential for the establishment of trans-regulation

The two best-characterized effectors of T reg cells are the cytokines TGF-β and IL-10. Given the absence of alterations in TGF-β production by the T cells and candidate TGF-β target genes in regulated *Ctla4*<sup>-/-</sup> T cells, trans-regulation appeared not to extensively involve suppressive cytokines acting specifically on *Ctla4*<sup>-/-</sup> T cells. Consistent with this, WT:*Ctla4*<sup>-/-</sup> mixed chimaeras injected with blocking Abs to TGF-β had minimal alterations at 8 wk of treatment, and even at 3 mo only some of the mice showed increased frequency of activated T cells in the both WT and *Ctla4*<sup>-/-</sup> populations (unpublished data).

Similarly, regulation of *Ctla4*<sup>-/-</sup> T cells also deficient in IL-10 receptor-mediated signaling appeared largely intact as mixed WT:*Il10ra*<sup>-/-</sup>*Ctla4*<sup>-/-</sup> BM chimaeras survived and maintained stable T cell subset frequencies (Fig. 7). Interestingly, the *Il10ra*<sup>-/-</sup>*Ctla4*<sup>-/-</sup> subset had a lower frequency of naive T cells in the CD4<sup>+</sup> (Fig. 7 A), and especially in the CD8<sup>+</sup> (Fig. 7 B) T cell populations, but over time the differences became statistically insignificant (data not shown). Thus, IL-10R signaling in *Ctla4*<sup>-/-</sup> cells is dispensable for the establishment of trans-regulation. The functional redundancy of IL-10R signaling and TGF-β and the potential for long-term regulation of *Ctla4*<sup>-/-</sup>*Il10Ra*<sup>-/-</sup> T cells are currently under investigation.

## DISCUSSION

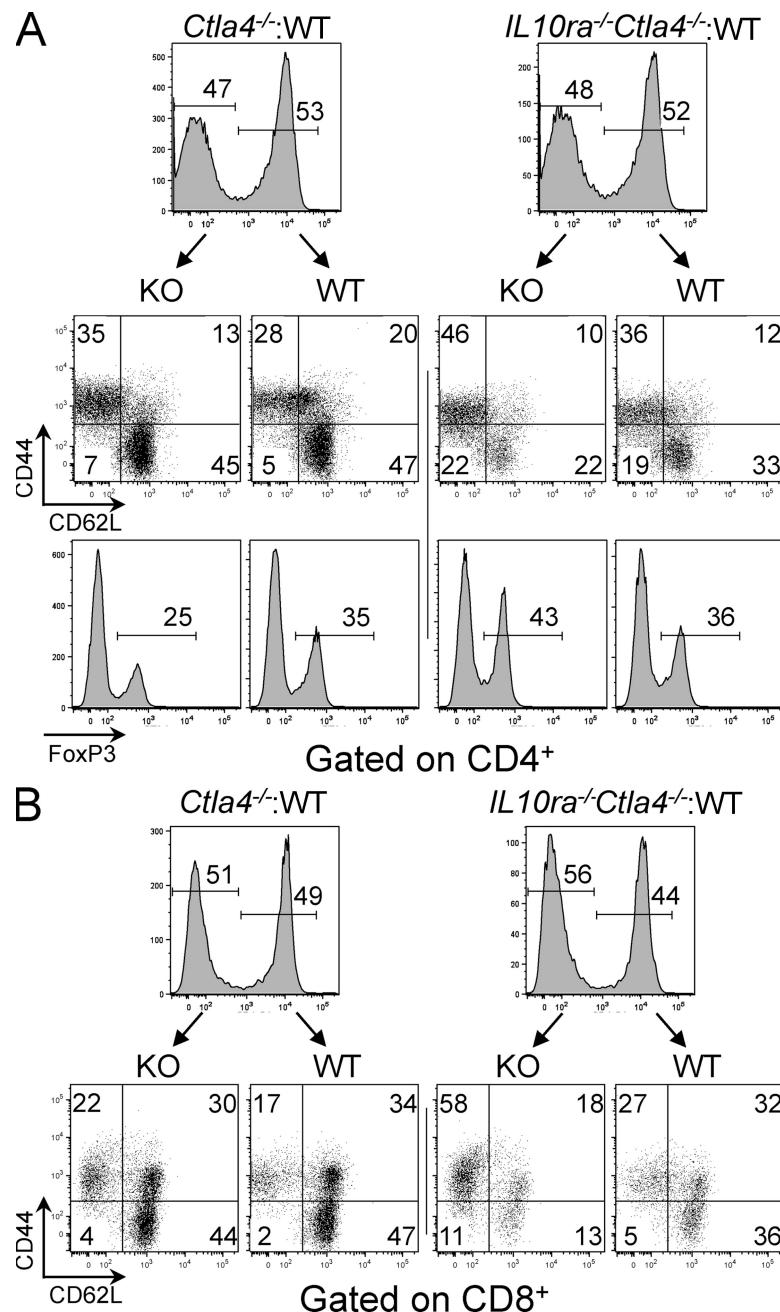
Using three in vivo model systems, mixed genotype blastocyst chimaeras, mixed BM cell chimaeras, and adoptive T cell



**Figure 6.** Expression of *Gadd45β* is decreased in naive *Ctla4*<sup>-/-</sup> T cells from mixed BM chimaeras relative to the WT T cells. Semi-quantitative RT-PCR analysis of the expression of select genes in regulated *Ctla4*<sup>-/-</sup> T cells from mixed BM chimaeras with inconsistent results from the array experiments. Threefold dilutions of cDNA prepared from naive (CD44<sup>+</sup>CD62L<sup>+</sup>) WT or *Ctla4*<sup>-/-</sup> T cells were analyzed for the genes indicated with documented functions in T cell activation and tolerance. Expression of β-actin was used as loading control. Data are representative of three independent experiments.

transfer into lymphopenic mice, we have conclusively demonstrated that naive *Ctla4*<sup>-/-</sup> T cells are regulated in trans by CTLA-4-sufficient T reg cells. The first model system does not involve whole body irradiation, ruling out experimental artifacts such as a dysregulated cytokine environment in irradiated mice as a caveat to the physiological relevance of the observed *Ctla4*<sup>-/-</sup> T cell homeostasis imposed in trans. Using

the latter two model systems we have identified CTLA-4-sufficient CD4<sup>+</sup>CD25<sup>+</sup> T reg cells as the subset necessary for trans-regulation of naive *Ctla4*<sup>-/-</sup> T cells. Because CTLA-4-deficient FOXP3<sup>+</sup> T reg cells in *Ctla4*<sup>-/-</sup> mice cannot prevent the lymphoproliferative disease, the results definitively show that CTLA-4 is essential for in vivo T reg cell-mediated regulation of naive T cell activation. This function of CTLA-4



**Figure 7. Regulation of *Ctla4*<sup>-/-</sup> T cells does not absolutely require signaling through the IL-10 receptor.** *Rag1*<sup>-/-</sup> mice received a 1:1 mix of  $4 \times 10^6$  BM cells from WT and either *Ctla4*<sup>-/-</sup> or *IL10ra*<sup>-/-</sup>-*Ctla4*<sup>-/-</sup> mice. Mice were killed at 12 wk after reconstitution, and CD4<sup>+</sup>FoxP3<sup>-</sup> (A) or CD8<sup>+</sup> (B) splenic T cells were analyzed for the activation markers CD44 and CD62L in WT and KO T cell populations. FoxP3<sup>+</sup> frequency was also assessed in the CD4<sup>+</sup> T cell subset (A, bottom). Data shown are representative flow cytometric results from the spleen of reconstituted mice (three mice per group) in two independent experiments.

is not simply restricted to controlling *Ctla4*<sup>-/-</sup> T cells, because a specific conditional loss of CTLA-4 in T reg cells in mice results in systemic lymphoproliferation whether or not naive T cells can express CTLA-4 (unpublished data).

Previous studies using mixed BM chimaeras had established that CTLA-4-sufficient cells can function in trans to maintain *Ctla4*<sup>-/-</sup> T cells in a naive state (22). Using the colitis model system, it was shown that naive B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> effector T cells were prevented from causing colitis by B7<sup>-/-</sup> CTLA-4-sufficient T reg cells, but not by B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> T reg cells (30). A major caveat to these studies was that the few T reg cells arising from B7-deficient mice may have altered properties caused by the loss of CD28 and CTLA-4 signaling during their development (53). More problematic was the observation that B7<sup>-/-</sup>*Ctla4*<sup>-/-</sup> T reg cells or sorted *Ctla4*<sup>-/-</sup> T reg cells from WT:*Ctla4*<sup>-/-</sup> mixed BM chimaeras were reported to be effective in regulating colitogenic CD4<sup>+</sup> T cells, indicating that CTLA-4 is in fact dispensable for T reg cell function. To account for this discrepancy, it was suggested that *Ctla4*<sup>-/-</sup> T reg cells can adapt to the loss of CTLA-4 and use alternate, compensatory mechanisms of immune suppression, such as the enhanced prominence of IL-10 as the suppressive factor (25, 30). The relevance of these findings to the regulation of *Ctla4*<sup>-/-</sup> T cells in vivo was unclear because even the proposed "adapted" *Ctla4*<sup>-/-</sup> T reg cells are incapable of regulating *Ctla4*<sup>-/-</sup> T cells in unmanipulated *Ctla4*<sup>-/-</sup> mice. Hence, the importance of T reg cells, or other immunoregulatory T cell subsets, in the control of *Ctla4*<sup>-/-</sup> T cells in trans and the potential regulatory mechanisms involved remained ambiguous.

Here, we have presented a systematic characterization of effector T cell-extrinsic CTLA-4 function to maintain T cell tolerance. Only T reg cells expressing CTLA-4 can restrain the autoreactive T cells in vivo. Regulation is long-lasting, and depending on the persistent presence of T reg cells, is stable in the face of infection or immunity against alloantigens. The equal participation of *Ctla4*<sup>-/-</sup> and WT T cells in immune responses against acute or chronic pathogen infection suggests that CTLA-4 is dispensable on effector T cells during the initiation and resolution of primary inflammatory T cell responses against foreign antigens.

The results, however, do not exclude T reg cell-independent functions of CTLA-4 and, conversely, CTLA-4-independent T reg cell effector mechanisms in the maintenance of T cell tolerance. There are three molecules whose absence in vivo causes early onset, lethal lymphoproliferative disease: CTLA-4, FOXP3, and TGF-β (54, 55). All three have immune-suppressive functions and, at this point, it is unclear whether they are components of a single pathway or parallel converging pathways. Current data would suggest that both hypotheses may be true. We have demonstrated that FOXP3<sup>+</sup> T reg cells require CTLA-4 for in vivo regulatory functions. However, CTLA-4 also appears to play a role in regulating effector T cell activation independent of T reg cells because CTLA-4 expression targeted specifically in activated conventional T cells (but not naive or T reg cells) can delay fatality of *Ctla4*<sup>-/-</sup> mice (unpublished data). TGF-β is required for the

maintenance and function of T reg cells (25, 56, 57). Although T reg cells produce TGF-β, studies have also shown that T reg cell-derived TGF-β is not essential for T cell homeostasis as long as other cells are capable of its production (58). Further, TGF-β has T reg cell-independent functions in regulating T cell development and homeostasis (59–61). Hence, although FOXP3<sup>+</sup> T reg cells use CTLA-4 and TGF-β as effectors of immune suppression, CTLA-4 and TGF-β also maintain T cell homeostasis and self-tolerance in a T reg cell-independent manner. Conversely, FOXP3<sup>+</sup> T reg cells use immune suppressive pathways other than CTLA-4 and TGF-β to regulate T cell activation both in vitro and in vivo (62).

Data presented here do not provide a definitive identification of the mechanism(s) responsible for CTLA-4<sup>+</sup> T reg cell-mediated trans-regulation of *Ctla4*<sup>-/-</sup> T cells. However, the fact that, (a) TGF-β, IL-10, and B7 signaling are not singularly required in *Ctla4*<sup>-/-</sup> T cells, (b) *Il35* expression (63) was normal in *Ctla4*<sup>-/-</sup> T reg cells (unpublished data), and (c) *Ctla4*<sup>-/-</sup> and WT T cells displayed highly similar global gene expression patterns, strongly suggests that T reg do not act directly on *Ctla4*<sup>-/-</sup> T cells. We thus favor the hypothesis that T reg cells control *Ctla4*<sup>-/-</sup> T cells by limiting immunogenicity of APCs in a noninflamed setting, a well-documented mechanism of T reg cell function in vitro (64, 65) and in vivo (66). In particular, several studies have suggested that the immunosuppressive enzyme IDO is produced by DCs upon encounter with T reg cells expressing functional CTLA-4, although the in vivo requirement for IDO in T reg cell-mediated immunosuppression has not been established (67, 68).

There are other possible mechanisms by which *Ctla4*<sup>-/-</sup> T cells could be maintained in a quiescent state by CTLA-4-sufficient T reg cells. It has been suggested that DCs can secrete exosomes containing MHC and co-stimulatory molecules that can be transferred intercellularly to other DCs to stimulate T cells (69). Hence, we have considered the possibility that CTLA-4-expressing T cells produce exosomes containing CTLA-4 that can be taken up by *Ctla4*<sup>-/-</sup> T cells and used to intrinsically maintain regulation. We have been able to both detect exosomes containing CTLA-4 and transfer CTLA-4 protein to *Ctla4*<sup>-/-</sup> CD4<sup>+</sup> T cells in vitro, but have yet to obtain any direct in vivo evidence for this mechanism in the mixed chimaera model (unpublished data).

Alternatively, because CTLA-4 has been estimated to have 50–100-fold higher affinity to B7 than CD28, it is conceivable that in the mixed chimaeras CTLA-4-sufficient T cells, especially T reg cells, are competitively engaging available B7 molecules on the APCs, thereby preventing CD28 signaling, and subsequent T cell activation in *Ctla4*<sup>-/-</sup> T cells. Physical competition alone appears unlikely given that as few as 10% of total T cells of WT origin can control the activation of the remaining 90% *Ctla4*<sup>-/-</sup> T cells. Nevertheless, we have observed that in B7.2:*Ctla4*<sup>-/-</sup> transgenic (overexpressing B7.2 on T and B cells) mixed BM chimaeras, tolerance was not established (unpublished data), consistent with a B7 "competition model" that predicts an increase in the probability of CD28 triggering in *Ctla4*<sup>-/-</sup> T cells that could result in a

breakdown in regulation. However, as B7.2 transgenic T cells are expanded and show signs of activation, the absence of regulation in these mixed chimaeras could alternatively be a direct consequence of aberrant T cell homeostasis in the tolerizing (*Ctla4*<sup>+/+</sup>) T cell population rather than increased access to B7 ligands. Ongoing studies with more refined cell type–dependent, temporal manipulation of relevant co-stimulatory ligands and receptors will help delineate the mechanism(s) of trans-regulation involving non-T accessory cells.

## MATERIALS AND METHODS

**Mice.** All mice were bred and maintained in our specific pathogen–free animal facilities. All animal experiments were approved by the University of Massachusetts Medical School Institutional Care and Use Committee. *Ctla4*<sup>−/−</sup> mice were backcrossed for 12 generations onto the C57BL/6 background. CD45.1<sup>+</sup> or CD90.1<sup>+</sup> congenic C57BL/6 mice were crossed with *Ctla4*<sup>−/−</sup> mice to generate a source of allelically distinguished *Ctla4*<sup>−/−</sup> cells in chimaeras and during transfer experiments. *Rag1*<sup>−/−</sup>, *Tcrb*<sup>−/−</sup>, *Tcrd*<sup>−/−</sup>, *Cd8a*<sup>−/−</sup>, and *Cd28*<sup>−/−</sup> mice were obtained from The Jackson Laboratory. *Cd80*<sup>−/−</sup>/*Cd86*<sup>−/−</sup> double-knockout mice (B7<sup>−/−</sup>) were obtained from A. Sharpe (Harvard Medical School, Boston, MA). *Cd4*<sup>−/−</sup> mice were obtained from N. Killeen (University of California, San Francisco, San Francisco, CA). 2CT, OTI, and OTII TCR transgenic mice were provided by K. Rock (University of Massachusetts Medical School, Worcester, MA). IL-10R $\alpha$  mutant mice were generated by ENU mutagenesis and contain a single base mutation that results in a stop codon at amino acid 220 of the IL-10R $\alpha$  chain (unpublished data). Similar to *Il10*<sup>−/−</sup> mice, *Il10ra*<sup>N/N</sup> mice (referred to as *Il10ra*<sup>−/−</sup> here for simplicity) suffer from severe inflammatory bowel syndrome. The mice were backcrossed >10 generations to C57BL/6 and then crossed to *Ctla4*<sup>−/−</sup> mice to generate *Il10ra*<sup>−/−</sup>/*Ctla4*<sup>−/−</sup> double-deficient mice.

**Generation of blastocyst chimaeras.** Blastocyst chimaeras were created by injecting *Ctla4*<sup>−/−</sup> embryonic stem cells (12) into C57BL/6 blastocysts and reimplanting the embryos into surrogate mothers. Resulting progeny were periodically bled over 3 yr, and the frequency of *Ctla4*<sup>−/−</sup> T cells monitored by enumerating the percentage of Ly9.1<sup>+</sup> T cells (*Ctla4*<sup>−/−</sup>) versus Ly9.1<sup>−</sup> T cells (*Ctla4*<sup>+/+</sup>) via flow cytometry.

**Preparation and transfer of BM.** The femurs and tibias of donor mice were flushed with RPMI-1640 supplemented with 5% FBS (HyClone; Invitrogen), sodium pyruvate, L-glutamine,  $\beta$ 2-mercaptoethanol, nonessential amino acids, and 100 U/ml penicillin/streptomycin (R5) using a 25-gauge needle. BM was mechanically separated by gentle pipetting, and RBCs were removed by a 1-min lysis step (0.15 M NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, and 0.1 mM EDTA, pH 7.3). After lysis and washing, BM cells were resuspended in 2.5 ml of R5 and depleted of T cells using anti-CD90.2 and/or a mixture of anti-CD4/CD8-coated magnetic beads (Invitrogen). BM was analyzed by flow cytometry for effective depletion of T cells (with anti-TCR- $\beta$ -FITC), washed 2 times in PBS, and adjusted to  $2 \times 10^7$  cells/ml. Mice were sublethally irradiated (300 rads) and injected with  $\sim 4 \times 10^6$  BM cells i.v. 4 wk after transfer, peripheral blood samples were obtained and analyzed for T cell reconstitution by flow cytometry.

**Cell preparation and adoptive transfer of peripheral cells.** LNs (pooled from superficial, deep cervical, axillary, brachial, inguinal, mesenteric, and lumbar) and spleen were isolated and homogenized via glass slides (or using a tissue homogenizer for sorting experiments), and RBCs were lysed. Cells were washed, filtered, and resuspended in R5. Transfers involving sorted CD4<sup>+</sup> or CD8<sup>+</sup> T cells were performed as follows: LN and spleen cells were isolated from WT mice or mixed BM chimaeras, pooled, labeled with fluorescently conjugated CD4 and CD8 Abs plus a congenic marker (to differentiate *Ctla4*<sup>−/−</sup> T cells in mixed chimaeras), and sorted on a MoFlo high-speed sorter (Dako). After sorting, cells were washed in PBS and  $\sim 1 \times 10^7$  total T cells were transferred to *Rag1*<sup>−/−</sup> recipients (subsequent 1:1 mix of WT CD4<sup>+</sup> or CD8<sup>+</sup> T cells and CD4<sup>+</sup> *Ctla4*<sup>−/−</sup> T cells injected on consecutive days after sorting).

For T reg cell cotransfer studies, LN and spleen cells from healthy mixed BM chimaeras (*Ctla4*<sup>−/−</sup>:CD90.1<sup>+</sup>WT) were labeled with CD90.1-biotin, followed by streptavidin magnetic beads, and then purified on an Auto-MACS System (Miltenyi Biotec) to deplete WT T cells. After purification, T cells were typically >96% CD90.1<sup>−</sup>. To obtain T reg cells, LN and spleen cells were isolated from C57BL/6 mice, labeled with Abs against CD4 and CD25, and sorted on a MoFlo high-speed sorter. Sorted purity was routinely >95%, of which  $\sim 90\%$  were FOXP3<sup>+</sup>, as determined by intracellular staining. Cells were washed in PBS, counted, and injected i.v. ( $2 \times 10^7$  cells/mouse) at approximately a 10:1 ratio of WT T cell–depleted cells from mixed chimaeras to T reg cells.

**In vivo mAb-mediated cell depletions.** Abs were purified from supernatants of the following hybridomas using protein G–Sepharose columns: anti-CD90.1 (clone OX7), anti-NK1.1 (PK136), and anti-TGF- $\beta$  (2G7). For WT T cell depletions, mice received three consecutive injections of OX7 mAb, followed by weekly injections to maintain depletion (0.25 mg i.p.). For NK depletions, mice received weekly injections of PK136 Ab (0.025 mg i.p.). For TGF- $\beta$  blockade, mice received 0.25 mg of 2G7 Ab every 4 d.

**Flow cytometric analysis.** Single-cell suspensions at  $10^6$  cells/sample were preincubated in 2.4G2 hybridoma supernatant to block Fc $\gamma$ R binding for 15 min, and then incubated with various combinations of the following conjugated Abs for 30 min: Ly9.1-FITC, CD25-FITC, FoxP3-FITC, CD69-FITC, CD8-PE, CD62L-PE, CD4-PECy5, CD4-PerCP-Cy5.5, CD25-PECy7, CD45.1-PECy7, CD8-APCCy7, CD44-APC, CD45.1-biotin, CD90.1-biotin, Streptavidin-ECD, Streptavidin-Pacific Blue. Abs used for flow cytometric analysis were purchased from BD or eBioscience. Cells were washed, fixed in 4% paraformaldehyde for 10 min, washed again, and then resuspended in FACS buffer (1X PBS/1.5% FCS/0.05% NaN<sub>3</sub>). Samples were analyzed on either an Epics XL-MCL (Beckman Coulter) or LSRII flow cytometer (BD). For intracellular FOXP3 staining, the manufacturer's protocol was followed (eBioscience). For peripheral blood analyses,  $\sim 50$ –100  $\mu$ l of blood from the tail vein was mixed with heparin, stained with fluorochrome-conjugated antibodies, briefly incubated in 0.25% saponin, fixed in 4% paraformaldehyde for 10 min, and resuspended in FACS buffer for analysis. At least 100,000 events were collected (20,000–50,000 events for blood) for each sample, and data were analyzed using FlowJo software (Tree Star, Inc.).

**BrdU analysis.** Animals received twice daily injections of 1 mg of BrdU i.p. for 2 d before sacrifice. After cell surface marker staining, cells were incubated in 50  $\mu$ l of 5 M NaCl, followed by the slow addition of 120  $\mu$ l of 95% EtOH. Cells were then stored at  $-20^{\circ}\text{C}$  overnight, washed, and incubated in 4% paraformaldehyde for 20 min on ice. Subsequently, cells were incubated in DNase solution (1X PBS/0.05% Tween-20/125 U DNase I/sample) for 15 min at room temperature, and then for 15 min on ice. Samples were washed once in PBS, once with labeling buffer (1X PBS/2% FBS/0.05% Tween-20) and incubated with anti-BrdU-FITC (BD) for 30 min. After being washed 2 times, cells were immediately analyzed by flow cytometry.

**Pathogen challenge.** Healthy mixed BM chimaeras 12 wk or more after reconstitution were used for these studies. For LCMV infections, chimaeras were challenged i.p. with  $5 \times 10^4$  PFU of strain Armstrong virus. Activation status, T cell ratios, and antigen-specific splenic T cell responses were analyzed at 7–9 d after infection. For *Mtb* infection, sonicated *Mtb* Erdman stock was titrated to deliver  $\sim 100$  CFU per mouse. Mice were exposed to infectious aerosol for 30 min in a Glass-Col inhalation Exposure System (Glass-Col, LLC). Upon sacrifice at 6 or 14 wk after infection, mice were immediately perfused with PBS. Lungs were minced, digested with collagenase and DNase for 30 min at  $37^{\circ}\text{C}$ , and strained, and RBCs were lysed. Spleens were also removed for analysis. Cells were then analyzed by flow cytometry.

**Semiquantitative RT-PCR analysis.** Naive (CD44<sup>lo</sup>CD62L<sup>hi</sup>) *Ctla4*<sup>−/−</sup> or WT CD4<sup>+</sup> T cells (>95% pure) from healthy mixed BM chimaeras

(pooled T cells from 3–5 mice/sort) were sorted on a MoFlo high-speed sorter using Abs to CD44, CD62L, and the congenic marker CD45.1. Cells were counted and resuspended in Trizol (Invitrogen). mRNA was isolated as per the manufacturer's protocol. cDNA preparation was performed with the OmniScript RT kit following the manufacturer's instructions (QIAGEN). Serial dilutions of cDNA were PCR amplified using the following primers: *Actb*, 5'-CTAGGCACCAGGGTGTGATGG-3' and 5'-TCTCTTGTGATGTCACGCACGA-3'; *Cblb*, 5'-TTCCAGAT-GGCAAATTCTATG-3' and 5'-TACATCCTTCTTCCTGCCTCT-TTA-3'; *Gadd45b*, 5'-TACGAGGCAGGCAAACGTGATGAAT-3' and 5'-ACCCATTGGTTATTGCCTCTGCTC-3'; *Lkif*, 5'-CTGGAGGC-CAAGCCAAACGCGGC-3' and 5'-CGTTGGGGACAGTAAACT-CAAAGGCA-3'; *Nfatc2*, 5'-TGCTGTTCTCATGGATGCCCTCAA-3' and 5'-TCCTCTCATGCTGTCATGAA-3'; *Pou2fl*, 5'-ACGCAC-CAGCATAGAGACCAACAT-3' and 5'-TCACAGCAGCACTGGTT-AAGGGA-3'; and *Pkcz*, 5'-TCCTTGATGCCGACGGACACATTA-3' and 5'-GCTGGTGAACACTGCGTGTCAAAGTT-3'.

**Histological analyses.** Tissues (pancreas, small intestine, liver, lung, heart, tongue, and ear) were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Two sections, 90  $\mu$ m apart, were cut from each block, and slides were viewed by light microscopy.

**Online supplemental material.** Fig. S1 demonstrates that challenge with the bacterial pathogen *Mtb* does not abrogate regulation of *Ctla4*<sup>−/−</sup> T cells in mixed BM chimaeras. Fig. S2 demonstrates that trans-regulation of *Ctla4*<sup>−/−</sup> T cells from mixed BM chimaeras is maintained in the presence of WT T cells upon adoptive transfer to lymphopenic hosts. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20081811/DC1>.

We would like to thank the UMMS Flow Cytometry Core Facility staff for their dedicated efforts in cell sorting and the Genomics Core for microarray data acquisition and analysis. We thank Airel Davis for excellent technical assistance. We thank the following investigators for providing essential mouse lines: Arlene Sharpe, Kenneth Rock and Nigel Killeen. The blastocyst chimaeras were generated in the laboratory of Dr. James Allison (at UC Berkeley, currently at Memorial Sloan-Kettering). We thank Dr. Tom Markees for performing skin graft experiments, Dr. Leslie Berg for discussion and critical reading of the manuscript, and Dr. Michael Brehm for performing LCMV infection studies. We also gratefully acknowledge the support of the UMMS Diabetes and Endocrinology Research Center and their staff, especially in providing purified Abs used for *in vivo* studies. This work was supported by grants from National Institutes of Health (AI054670, AI59880).

The authors declare that they have no conflicting financial interests.

Submitted: 13 August 2008

Accepted: 29 December 2008

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