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Distinct roles of CTLA-4 and TGF-β in $CD4^+$ $CD25^+$ regulatory T cell function

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Running title: CTLA-4 function on $CD4^+CD25^+$ regulatory T cells

Abbreviation used in this paper: Treg, regulatory T cells; WT, wild-type; CFSE, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester.

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Summary

Both CTLA-4 and TGF-β have been implicated in suppression by $CD4^{+}CD25^{+}$ regulatory T cells (Tregs). In this study, the relationship between CTLA-4 and TGF-β in Treg function was examined. Blocking CTLA-4 on wild-type Tregs abrogated their suppressive activity *in vitro* whereas neutralizing TGF-β had no effect, supporting a TGF-β-independent role for CTLA-4 in Treg-mediated suppression *in vitro*. In CTLA-4-deficient mice, Treg development and homeostasis was normal. Moreover, Tregs from CTLA-4-deficient mice exhibited uncompromised suppressive activity *in vitro*. These CTLA-4-deficient Tregs expressed increased levels of suppressive cytokines IL-10 and TGF-β, and *in vitro* suppression mediated by CTLA-$4^{-/-}$ Tregs was markedly reduced by neutralizing TGF-β, suggesting that CTLA-4-deficient Tregs develop a compensatory suppressive mechanism through CTLA-4-independent production of TGF-β. Together, these data suggest that CTLA-4 regulates Treg function by two distinct mechanisms; one during functional development of Tregs and the other during the effector phase, when the CTLA-4 signaling pathway is required for suppression. These results help explain contradictions in the literature and support the existence of functionally distinct Tregs.

Introduction

$CD4^+CD25^+$ regulatory T cells (Tregs) are potent inhibitors of T cell activation *in vitro* and *in vivo*. The suppressive mechanism utilized by Tregs is not well understood. It appears that *in vitro* the suppression is cell contact dependent, likely mediated through direct T:T interactions [1]. In most of these *in vitro* systems and some *in vivo* systems, suppression is not inhibited by antibodies to cytokines such as TGF-β or IL-10, and Tregs from TGF-β deficient, or IL-10 deficient mice exhibit uncompromised suppressive activity [2-8]. However, in many other *in vivo* experimental models, Treg-mediated suppression requires TGF-β or IL-10, and in certain instances, Tregs themselves produce these cytokines [9-12], suggesting that the suppression is achieved through these soluble inhibitors.

CTLA-4-deficient mice develop a fatal lymphoproliferative disease [13, 14]. Although the exact cause of the disease in these mice is not clear, the prevailing view is that the lymphoproliferation develops as a consequence of a lack of intrinsic control of T cell activation in the absence of CTLA-4. However, bone marrow chimera and co-transfer studies show that mice with both wild type (WT) and CTLA-$4^{-/-}$ cells fail to develop lymphoproliferation. This suggests that the uncontrolled T cell activation in CTLA-$4^{-/-}$ may be due to a lack of extrinsic regulation of T cells [15, 16]. Since Tregs constitutively express CTLA-4, it has been postulated that CTLA-4 is critical to Treg functions. In this regard, it has been shown that anti-CTLA-4 treatment blocks Treg function *in vitro* and that Tregs from CTLA-4 deficient mice display impaired suppressive activity [17]. Moreover, studies have demonstrated that CTLA-4 blockade *in vivo* abrogates Treg-mediated protection against autoimmune colitis and transplant rejection [10, 11]. However, since T cells in CTLA-4-deficient mice undergo polyclonal

activation (as evidenced in part by CD25 expression) and proliferation soon after birth, the reduced Treg function may be a result of contamination of Tregs with activated T cells. In addition, increased autoimmunity after *in vivo* CTLA-4 blockade may be a consequence of enhanced pathogenicity of effector T cells instead of inhibition of Treg function. In this study, we investigated the roles of CTLA-4 and TGF-β in Treg-mediated suppression *in vitro*. We found that although CTLA-4 blockade abrogated WT Treg function, CTLA-4-deficient Tregs exhibited uncompromised suppressive activity. However, suppression mediated by CTLA-4-deficient Tregs was qualitatively different from that mediated by WT Tregs in that it is at least partially TGF-β-dependent.

Results

Normal Treg development and homeostasis in CTLA-4 deficient mice

In initial sets of experiments, we determined the role of CTLA-4 in $CD4^+CD25^+$ regulatory T cell development, and peripheral homeostasis. One of the difficulties in analyzing Tregs from CTLA-4-deficient mice is distinguishing Tregs from activated T cells, which also express CD25. In this study, we used two approaches to minimize contamination of Tregs with activated T cells. First, mice expressing a CTLA-4Ig transgene under the control of the keratin 14 promoter were used. The CTLA-4Ig fusion protein is constantly secreted by the epithelial cells, resulting in an average serum concentration of approximately 20 ng of CTLA-4Ig/ml. CTLA-4Ig binds to B7-1 and B7-2 and blocks their engagement with CD28, thereby delaying T cell activation and lymphoproliferation in CTLA-4-deficient mice [18]. Generally, CTLA-$4^{-/-}$ mice die within 3-4 weeks of age. In contrast, 4-week-old CTLA-4Ig transgenic CTLA-$4^{-/-}$ mice are healthy and the cellularity of their secondary lymphoid organs is normal (data not shown). Second, in several experiments CTLA-$4^{-/-}$ mice were treated with a mixture of

anti-B7-1 and anti-B7-2 mAbs beginning from one week of age to prevent lymphoproliferation. Finally, to ensure that we were working with true regulatory T cells, $CD4^+CD25^+$ T cells were further separated based on expression of CD62L. CD62L is expressed highly on resting T cells and its expression is down regulated on recently activated cells. Most $CD25^+$ Tregs in normal young unimmunized mice are $CD62L^+$, and $CD62L^+$ cells have been shown to exhibit regulatory activity [19]. Therefore, using CD62L as an additional marker helps to exclude the recently activated T cells from the Treg population. This is especially important when T cells from CTLA-4-deficient mice were analyzed. Even with the CTLA-4Ig transgene, CTLA-$4^{-/-}$ mice start to show signs of T cell activation and lymphadenopathy by six weeks of age. By using additional CD62L marker on young CTLA-4Ig transgenic CTLA-$4^{-/-}$ mice, we were able to identify a distinct population of Tregs that are present in CTLA-$4^{-/-}$ mice at a percentage similar to that seen in their WT CTLA-4Ig transgenic littermates (Fig. 1A). It has been shown recently that Tregs express high levels of the transcription factor FoxP3, which is critical for Treg development and function [20-22]. Because FoxP3 protein is not expressed in $CD4^+CD25^-$ cells after activation, it is the only marker identified so far that is uniquely expressed at high levels in Tregs. To determine if $CD4^+CD62L^+CD25^+$ cells from CTLA-$4^{-/-}$ mice were of the same lineage as Tregs from WT mice, we examined FoxP3 expression in CTLA-$4^{-/-}$ Tregs by western blot analysis. CTLA-$4^{-/-}$ Tregs express 24-fold higher FoxP3 protein than the $CD4^+CD62L^+CD25^-$ cells (Fig. 1B) similar to that observed in WT mice, suggesting that the Tregs developed in the absence of CTLA-4 were of the same lineage as those in WT mice.

We have demonstrated previously that development and peripheral homeostasis of Tregs is dependent on CD28 interaction with B7 molecules [23, 24]. To determine if

this effect was mediated indirectly through CTLA-4, we treated CTLA-$4^{-/-}$ mice on 7, 11, and 13 day after birth with a combination of anti-B7-1 and anti-B7-2 mAbs and examined the percentage of Tregs at various times afterwards. The anti-B7 treatment led to a marked reduction of Tregs as seen in WT mice (Fig.1C middle panel, [23, 24]). Moreover, the CTLA-$4^{-/-}$ Tregs recovered with similar kinetics as their WT counterparts after the anti-B7 treatment was stopped, such that the percentage of Tregs returned to normal level in CTLA-$4^{-/-}$ mice by 28 days after the last anti-B7 treatment (Fig.1C right panel). Thus, the homeostasis of the Tregs was B7-dependent and CTLA-4-independent, and CTLA-4 deficiency did not affect the development or peripheral homeostasis of the Tregs.

In vitro suppression by WT Tregs is CTLA-4-dependent and TGF-β-independent

Although Tregs are present in CTLA-$4^{-/-}$ mice in normal numbers, the disease in CTLA-4-deficient mice could result from a functional defect in the resident Tregs. It has been proposed that Treg-mediated suppression is dependent on costimulation through CTLA-4 [17], which may induce TGF-β production by T cells [25] and Tregs in particular [26], although these later findings remain controversial [27]. We therefore examined the effect of blocking CTLA-4 or TGF-β in our *in vitro* suppression assays. The mAbs we used have been shown to function as antagonists in a variety of systems both *in* vitro and *in vivo*. $CD4^+CD62L^+CD25^-$ cells from CTLA-4Ig transgenic CTLA-$4^{-/-}$ mice were used as responder cells to distinguish effects of the anti-CTLA-4 mAb treatment on the Treg versus responder populations. Responder cells were labeled with CFSE and their proliferation was assessed by the dilution of the intracellular dye. Proliferation of the CTLA-4-deficient responder cells was markedly inhibited by the WT Tregs (Fig. 2A, left panel). Moreover, the addition of anti-CTLA-4 Fab fragments completely abolished the

suppression by the WT Tregs (Fig. 2A, right panel). The effect of the anti-CTLA-4 antibody was specific as it had no effect on Treg activity of CTLA-4 deficient T cells (see below). In contrast, suppression by WT Tregs was not affected in the presence of high concentration of TGF-β neutralizing antibodies (Fig. 2B). Thus, our results are in agreement with previous reports [3, 17] which suggested that CTLA-4 expression on Tregs is critical for their function. However, this function of CTLA-4 is not mediated through TGF-β, since the in vitro function of Tregs was TGF-β independent.

CTLA-4$^{-/-}$ Tregs exhibited normal suppressive activity in vitro

In an attempt to substantiate the results obtained using anti-CTLA-4 antibodies, we next compared the suppression activity of Tregs from CTLA-4$^{-/-}$ and WT mice *in vitro*. Surprisingly, CTLA-4-deficient Tregs were able to suppress proliferation of WT responders (Fig. 3A, left panel). Addition of anti-CTLA-4 Fabs to the suppression assay enhanced proliferation of the WT responders, as demonstrated by the increased fraction of cells diluting CFSE in responder alone cultures that received the antibody in (Fig. 3A, right panel shaded histograms). The same treatment did not affect the proliferation of the CTLA-4$^{-/-}$ effectors as illustrated in Figure 2A by the similarity of CFSE dilution of the shaded histograms between right and left panels. Moreover, the anti-CTLA-4 Fabs did not change the suppression of the CTLA-4$^{-/-}$ Tregs (Fig. 3A, compare CFSE dilution between lined histograms in the right and left panels). These results demonstrate that, at the concentration used in experiments presented in Figure 2A and 3A, the anti-CTLA-4 Fabs were efficacious and its activity was specific. A more quantitative comparison of suppressor activity of WT and CTLA-4$^{-/-}$ Tregs at various Tregs to responders ratios showed that the suppression by the two populations of Tregs was indistinguishable (Fig. 3B), demonstrating that loss of CTLA-4 expression did not affect the suppressive activity

of the Tregs in these knockout mice. In addition, CTLA-$4^{-/-}$ Tregs were equally efficient at suppressing proliferation of CTLA-$4^{-/-}$ responders (data not shown). These findings were different from previous reports showing CTLA-$4^{-/-}$ Tregs have 50% reduction in regulatory activity [17]. It is likely that the reduction observed previously was due to contamination of activated cells with Tregs. By using young CTLA-4Ig transgenic CTLA-$4^{-/-}$ mice combined with sorting for $CD62^{high}$ Tregs, we were able to assess CTLA-deficient Treg activity more accurately than previously reported. Taken together, our results demonstrate that although anti-CTLA-4 mAb affects suppressor function in WT Tregs, Tregs can develop and function normally in the CTLA-$4^{-/-}$ mice.

Lack of contribution of IL-10 to the functions of CTLA-$4^{-/-}$ Tregs

The conflicting results presented thus far suggested that there may be compensatory changes in CTLA-$4^{-/-}$ Tregs that enabled them to suppress in a CTLA-4-independent manner. Therefore, we compared the expression of the immunosuppressive cytokine IL-10 in Tregs from WT and CTLA-$4^{-/-}$ mice. The mRNA levels for IL-10 were five fold higher in CTLA-$4^{-/-}$ Tregs than in WT Tregs as assessed by quantitative real-time PCR analysis (Fig. 4A). Consistently, CTLA-$4^{-/-}$ Tregs secreted 10 fold more IL-10 upon *in vitro* stimulation with anti-CD3 and splenic APCs (Fig. 4B). However, anti-IL-10 antibody had no effect on in vitro suppression mediated by either WT (data not shown) or CTLA-$4^{-/-}$ Tregs (Fig. 4C). Thus, our results show that although CTLA-$4^{-/-}$ Tregs expressed significantly higher level of IL-10, IL-10 did not contribute to the suppression function of the CTLA-$4^{-/-}$ Tregs.

In vitro suppression by CTLA-$4^{-/-}$ Tregs is partially TGF-β-dependent

We next examined the expression TGF-β by CTLA-$4^{-/-}$ Tregs. No increase in TGF-β message (Fig. 5A) or protein (Fig. 5B) was observed when compared to that expressed by

WT Tregs. TGF-β and TGF-β receptors are widely expressed by normal tissues, and the biological activity of TGF-β is mainly controlled after the secretion of the cytokine (reviewed in [28]). Most of the TGF-β is secreted in its latent inactive form due to its linkage to the latency-associated peptide (LAP), which has to be cleaved to release the active TGF-β. The LAP has two mannose-6-phosphate (M6P) containing carbohydrate modifications that mediate its binding to the cell surface through M6P/insulin-like growth factor II receptor. Moreover, the cell surface anchoring of latent TGF-β has been shown to be important to its conversion to biologically active form [29-31]. The ELISA we used to quantify secreted TGF-β involved chemical activation of TGF-β by denaturing LAP with acid, therefore it could not distinguish between latent and active TGF-β. In addition, since culture supernatant was analyzed, the level of membrane-bound TGF-β was not assessed by the assay. Thus, we examined the levels of membrane-bound TGF-β on various cells using flow cytometry. Tregs from WT mice did not show any TGF-β or LAP staining even after *in vitro* activation, whereas LAP was readily detectable on Tregs freshly isolated from CTLA-4$^{-/-}$ mice, and TGF-β expression was detected after *in vitro* activation (Fig. 5C). Thus, Tregs from CTLA-4KO mice express more TGF-β, especially after activation.

To determine if the higher level of TGF-β expression by CTLA-4$^{-/-}$ Tregs contributed to their suppression *in vitro*, we examined the suppressive activity of CTLA-4$^{-/-}$ Tregs in the presence of TGF-β neutralizing antibodies *in vitro*. Anti-TGF-β treatment led to a significant reduction of suppression by these cells especially at lower Treg to responder ratios (Fig. 5D). Addition of anti-IL-10 mAb did not have any effect on the partial reversion of suppression by anti-TGF-β antibodies (data not shown). Similar results were

observed using WT T effector cells in the suppression assays (data not shown). Taken together, these results suggest that the membrane-bound TGF-β on the surface of CTLA-$4^{-/-}$ Tregs contribute to the suppressive activity.

Discussion

In this study, we have shown that suppression by WT Tregs is CTLA-4-dependent and TGF-β independent. However, CTLA-$4^{-/-}$ Tregs exhibited uncompromised suppressive activity and expressed higher levels of suppressive cytokines IL-10 and TGF-β. Moreover, the *in vitro* suppression by CTLA-$4^{-/-}$ Tregs was partially dependent on TGF-β.

The role of CTLA-4 in Treg-mediated suppression has been controversial. It has been reported that suppressive activity of T cell clones derived from $CD4^+CD25^+$ cells in human PBL strongly correlate with high level of CTLA-4 expression [8] consistent with a role of CTLA-4 in Treg function. Similarly, we have observed by comparing gene expression in Tregs and $CD4^+CD25^-$ cells that CTLA-4 is one of the few genes that are consistently highly over-expressed in mouse and human Treg under various activation conditions (data not shown). However, the ability to directly demonstrate a functional role of CTLA-4 *in vitro* has been controversial. Several findings in the present study help to explain these discrepancies. First, we observed that CTLA-4 blockade abrogated WT Treg suppressor activity, but found that a relatively high concentration of anti-CTLA-4 Fab fragments (100 μg/ml) was required to completely abrogate suppression by WT Tregs *in vitro*. Previous studies used much less antibody in their suppression assays and often used whole antibodies [2] which might have agonist effects in the culture [32]. To avoid the confounding issues of agonist effect of the whole anti-CTLA-4 antibodies, we

only used the Fab fragments in all the experiments presented in this study. We have shown previously [33] that Fab fragments of anti-CTLA-4 are 10 times less efficient in binding to CTLA-4 when compared with whole anti-CTLA-4 mAbs, therefore, we used relatively high concentration of Fabs in our cultures. In addition, it is important to distinguish the effects of CTLA-4 blockade on the $CD25^-$ responders from that on the Tregs. In the current study, we took advantage of T cells isolated from CTLA4Ig transgenic CTLA-$4^{-/-}$ mice to ensure that the antibody effects were limited to the Treg populations. Interestingly, even at these high concentrations the anti-CTLA-4 antibody did not break the anergic phenotype of the Tregs (like engagement of GITR [34, 35]) or induce death of Tregs (data not shown). Thus, the basis for CTLA-4-mediated regulation of Tregs is most likely due to a direct role on their suppressive function. First, CTLA-4 engagement of B7 may result in inhibition of responder proliferation. This may occur directly or via the production of a secondary inhibitor. In accordance with this notion, it has been demonstrated that CTLA-4 can engage B7 on antigen presenting cells to induce indeolamine 2,3-dioxygenase expression resulting in the degradation of tryptophan and resultant inhibition of T cell proliferation [36, 37]. Alternatively, it is possible that CTLA-4 sends a signal to Tregs to activate a suppressor mechanism. Tregs must be activated through their T cell receptors to induce their suppressive activity. Thus, CTLA-4 may contribute to the induction of suppression by sending an independent signal or by altering TCR signaling.

We have shown in this study that CTLA-$4^{-/-}$ Tregs have normal suppressive activity, whereas it has been previously reported that CTLA-$4^{-/-}$ Tregs were less effective suppressors [17]. This was likely due to contamination of Tregs with activated T cells in previous studies. T cells from CTLA-$4^{-/-}$ mice express activation markers such as CD25

as early as 7 days after birth. Even in the presence of a CTLA-4Ig transgene, a concomitant increase in CD25 and decrease in CD62L markers became apparent in CTLA-4$^{-/-}$ mice at 6 weeks of age (data not shown). In mice that exhibited overt signs of T cell activation, the separation between CD62L^{high} and CD62L^{low} Tregs became less distinctive, and even CD62L^{high} Tregs from these mice showed less regulatory activity when compared to Tregs from young CTLA-4$^{-/-}$ mice with minimal *in vivo* T cell activation (data not shown).

Our observation that CTLA-4$^{-/-}$ Tregs express higher levels of the suppressive cytokines IL-10 and TGF-β than WT Tregs, suggests that in addition to its role in Treg effector function, CTLA-4 also controls the functional development of Tregs. Two functionally distinct populations of Tregs have been described, the natural and adaptive Tregs (reviewed in [38]). The natural Tregs develop in the thymus, express CD4^{+}CD25^{+} markers, are naturally suppressive, and suppress in a cell-cell contact dependent manner. In contrast, adaptive Tregs are not naturally suppressive, but acquire the function through IL-10 and/or TGF-β induction and depend on these cytokines for their suppressive activity. Interestingly, most *in vivo* evidence support a requirement for suppressive cytokines in the function by either population of Tregs. While the adaptive Tregs can be generated *in vitro* and from either CD25^{+} or CD25^{-} cells with various treatments (reviewed in [39-41]), how these cells arise *in vivo* is not clear. Our data suggest that the adaptive Tregs can derive from natural Tregs in the absence of CTLA-4 signal. Consistent with this idea, we have observed that chronic *in vitro* stimulation of WT Tregs with anti-CD3 and anti-CD28 in the absence of APC (therefore no B7 to engage CTLA-4) leads to high levels of IL-10 and increased TGF-β expression (data not shown). These results suggest that CTLA-4 expression in Treg controls the functional differentiation of

Tregs *in vivo*. We and others have recently reported that Tregs proliferate vigorously *in vivo* in normal hosts likely due to their higher sensitivity to self antigens [24, 42]. It is possible that CTLA-4 controls the functional differentiation of Tregs during this homeostatic process to prevent the emergence of adaptive Tregs and nonspecific immunosuppression. In the setting of autoimmunity and transplant rejection, chronic and strong activation of the Tregs may override CTLA-4 function, thus permitting their differentiation into adaptive Tregs. Although we did not detect LAP or TGF-β expression on $CD4^+CD62L^+CD25^+$ WT Tregs even after activation, the expression of LAP was readily seen on the $CD62L^-CD25^+CD4^+$ cells in WT mice (data not shown). It has been suggested that Tregs lose CD62L expression after extensive *in vivo* activation and proliferation [42]. Therefore, it is possible that these cells represent chronically activated adaptive Tregs. Our observations may also help reconcile the difference between our finding no TGF-β expression by $CD4^+CD62L^+CD25^+$ Tregs and previous reports that showed TGF-β expression by $CD4^+CD25^+$ Tregs [26, 43]. It is conceivable that the TGF-β-expressing cells in WT mice, detected in previous studies, were mostly $CD62L^-$ Tregs, and the prevalence of this population may vary greatly dependent on the age, strain, and antigen experience of the mice used in the studies.

Although CTLA-$4^{-/-}$ Tregs expressed both IL-10 and TGF-β upon *in vitro* reactivation, neutralizing alone TGF-β abrogated the suppression whereas IL-10 neutralization had no effect. It has been suggested in a chemical-induced colitis model that IL-10 facilitates the differentiation of TGF-β producing cells, but itself has little effect during the effector phase of the suppression [44]. Indeed, it has been shown that Th1 cytokines suppress TGF-β expression by naïve T cells [45]. We have shown

recently that naïve CTLA-$4^{-/-}$ T cells skew to Th2 type upon activation likely due to higher TCR signaling strength in the absence of CTLA-4 [46]. CTLA-4-deficient cells produce very little IFNγ cytokines and high level of Th2 cytokines, IL-4 and IL-5, which may indirectly favor the generation of IL-10 and TGF-β expressing cells. This result also suggests that TGF-β expression can be induced independently of CTLA-4 and the lymphoproliferation in CTLA-$4^{-/-}$ mice is not due to the lack of TGF-β expression in the absence of CTLA-4 signaling as previously proposed [25].

In summary, the results presented in this study support a role of CTLA-4 in both function and development of regulatory T cells. CTLA-4 is functional during the effector phase of Treg activity and controls the development IL-10 and TGF-β producing adaptive Tregs. We propose that CTLA-4 and TGF-β maintain tolerance by controlling T cell activation at two distinct stages. Future experiments will be focused on identifying the contributions of CTLA-4 and TGF-β in in vivo suppression by Tregs. Understanding the actions of these molecules may help us to design more effective therapeutics for autoimmune disease and transplantation.

Material and methods:

Mice:

WT C57BL/6 mice, CTLA-4-deficient, or CTLA-4 heterozygous mice on the C57BL/6 background were generated by breeding CTLA-4 heterozygous mice. The genotypes of the progenies were determined by PCR typing within the first week of age. CTLA-4-deficient mice were treated with a mixture of anti-B7.1 and anti-B7.2 mAbs (100 μg each) by ip injection on day 8, 11, 14 after birth to delay the generalized lymphoproliferation. In some experiments, WT, CTLA-4 heterozygous or CTLA-4-

deficient mice carrying a CTLA-4Ig transgene under the control of keratin-14 promoter were used [18] as the expression of CTLA-4Ig in the CTLA-4-deficient mice delays the onset of lymphoproliferation until six to eight weeks of age. These mice are on a mix of C57BL/6 and 129 backgrounds, and all are used for experiments between four and six weeks of age. All the mice were housed in the specific pathogen-free animal facilities at UCSF.

Antibodies and other reagents:

Anti-B7.1 (16-10A1), anti-B7.2 (GL-1), anti-CD3 (145-2C11), anti-CD28 (PV-1), and anti-CTLA-4 (4F10) mAbs were purified from hybridoma culture supernatants in our laboratory. Purified anti-TGF-β (2G7) mAb was a kind gift from Dr. Chatenoud (Inserm, France). Purified anti-IL-10 mAb (JES5-16E3) were purchased from BD-Pharmingen (San Diego, CA) FITC, RPE, and SpectroRed conjugated anti-CD25, anti-CD4, and anti-CD62L mAbs were purchased from Southern Biotechnology Associates (Birmingham, AL). Allophycocyanin (APC) conjugated anti-CD62L, anti-CD4, and streptavidin were purchased from BD-PharMingen. Biotinylated rabbit anti-human TGF-β and LAP antibodies were purchased from R & D Systems (Minneapolis, MN). Anti-FcR (2.4G2) mAb were used as culture supernatant to block FcR-mediated binding before staining cells for analysis by flow cytometry or FACS.

Purification of Tregs using FACS:

Splenocytes were enriched for $CD4^+$ cells by negative selection on AutoMACS (Miltenyi, Germany). The CD4-enriched splenocytes were then combined with lymph node cells, incubated with anti-FcR culture supernatant for 5 minutes, and stained with fluorochrome conjugated anti-CD25, anti-CD4, and anti-CD62L mAb.

$CD4^+CD25^+CD62L^+$ Tregs were separated on a Moflo cell sorter (Cytomation, Fort Collins, CO). The sorted Tregs were between 92% and 98% pure.

Suppression assay:

Suppressor assays were set up in 96 well U-bottom plates with 20,000 to 50,000 $CD4^+$ $CD62L^+$ $CD25^-$ cells, equal numbers of T-depleted irradiated splenocytes, graded numbers of Tregs, and anti-CD3 mAb (3 μg/ml). In some experiments, Fab fragments of anti-CTLA-4 mAb (100 μg/ml), anti-TGF-β mAb (50 μg/ml), or anti-IL-10 (20 μg/ml) was added to the wells at the beginning of the culture. The cultures were incubated at 37°C for 64-72 hours and pulsed with ^{3}H-Thymidine (Perkin Elmer Biosciences, Shelton, CT) during the last 6-14 hours.

5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling and proliferation assay:

Sorter purified T cells were washed twice in PBS and resuspended in PBS at 5 x 10^6 cells/ml. Cells were labeled in 1.5 μM CFSE for five minutes and quenched with heat-inactivated FCS. Cells were washed twice in before they were plated for suppression assay as described above. Proliferation of the labeled cells were determined at 72 hour after stimulation using flow cytometry.

Analysis of membrane TGF-β expression by flow cytometry:

The level of membrane-bound TGF-β or LAP expression on freshly isolated T cells was determined as follows. Unfractionated whole lymph node cells were incubated with anti-FcR mAb and then with biotinylated anti-TGF-β or anti-LAP antibodies on ice for 30 minutes. The cells were then washed before labeling with fluorochrome-conjugated anti-CD4, anti-CD25, anti-CD62L, and streptavidin. Expression of these markers was

determined using a FACS Calibur (Becton Dickenson, San Jose, CA). To assess the expression of TGF-β and LAP on activated Tregs and $CD4^{+}CD62L^{+}CD25^{-}$ cells, the cells were purified first on Moflo, and then activated with plate bound anti-CD3 (10 μg/ml), soluble anti-CD28 (2 μg/ml), and 100 U/ml IL-2 as described [26]. After 24 h, the cells were harvested and stained as described above for fresh cells.

ELISA:

The level of IL-10 and TGF-β in the culture supernatant was determined by ELISA using antibody pairs purchased from BD-PharMingen (San Diego, CA). For TGF-β ELISA, the culture supernatant was first treated with acid to lower the pH to 2.0 to denature latency-associated peptide to allow the detection of active TGF-β. The supernatant was then brought back to neutral pH before ELISA.

Real time RT-PCR:

Total RNA was extracted from cell sorter purified Tregs using RNAeasy columns (Qiagen, Valencia, CA). The cDNA was synthesized from 0.5 μg total RNA using Superscript II RNase H- reverse transcriptase and oligo dT as primers (Invitrogen, Carlsbad, CA). Primer and probe pairs for IL-2, IL-10, TGF-β1, and HPRT were purchased from Applied Biosystems (Foster City, CA). The real time PCR was performed on ABI 7700 using TaqMan Universal PCR master mix (Applied Biosystems) in duplicates and the average threshold cycles (Ct) of the duplicate were used to compare the relative abundance of the mRNA. Ct of HPRT was used to normalize all samples.

Western blot:

Tregs and $CD4^{+}CD62L^{+}CD25^{-}$ cells were purified using a Moflo cell sorter. Two x 10^{5} of each cell type were lysed in sample buffer (62.5 mM Tris, pH 6.8, 12.5% glycerol, 2%

SDS, 30 ng/ml bromophenoblue), sonicated and passed through 28 gauge needles. The lysates were clarified by centrifugation and boiled for 5 minutes before separating on a 10% SDS PAGE gel. The samples were transferred to PVDF membrane after electrophoresis and incubated with rabbit anti-FoxP3 antisera (gift from Dr. Steven Ziegler) followed by HRP-conjugated anti-rabbit Ig. The blot was developed with SuperSignal® Chemiluminescent Substrate (Pierce, Rockford, IL) and visualized on a Kodak Image Station 440CF (Eastman Kodak) and quantified using Kodak Digital Science 1D Image Analysis software 3.0.

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Footnote:

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Figure legends:

Fig. 1. Normal development and homeostasis of regulatory T cells in CTLA-4$^{-/-}$ mice. A. Percentages of Tregs in CD4 gate in LN of 5-week-old CTLA-4$^{-/-}$ CTLA-4 Ig transgenic and CTLA-4$^{+/+}$ CTLA-4 Ig transgenic littermates were compared. The left two panels are representative dot plots of flow cytometry analysis of CD4+ cells and right panel is a chart of all mice examined in the experiment. Each circle represent one mouse and the bar represent the mean of the group. B. Western blot analysis of FoxP3 expression in Tregs purified from CTLA-4$^{-/-}$ CTLA-4Ig transgenic and CD4^{+}CD62L^{+}CD25^{-} and Tregs purified from CTLA-4$^{+/+}$ CTLA-4 Ig transgenic mice. The numbers below the bands are relative band intensity. C. CD4^{+} splenocytes from age matched CTLA-4 $^{+/-}$ untreated mice (left) or CTLA-4$^{-/-}$ mice treated with 100 μg anti-B7-1 and anti-B7-2 on day 9, 12 and 15 after birth were harvested on day 10 (middle) and day 28 (right) after the start of treatment and analyzed by FACS for expression of CD62L and CD25. Results are representative of five (A) and two (B and C) independent experiments.

Fig. 2. In vitro suppression by WT Tregs is CTLA-4-dependent and TGF-β-independent. A. Left panel, CFSE-labeled CD4^{+}CD62L^{+}CD25^{-} cells from CTLA-4$^{-/-}$ CTLA-4Ig transgenic mice were stimulated with WT T-depleted splenocytes and anti-CD3 alone (filled) or in the presence of equal number of unlabeled WT Tregs (solid line). Right panel, conditions are as in the left panel with the addition of anti-CTLA-4 Fab fragments (100μg/ml) at the initiation of the culture. Cells were harvested at 72 hours and analyzed by FACS for CFSE intensity. B. WT Tregs were co-cultured with CD4^{+}CD62L^{+}CD25^{-} cells from CTLA-4$^{+/-}$CTLA-4Ig transgenic mice at the indicated ratio with (filled) or without (open) 50μg/ml anti-TGF-β mAb. Proliferation was quantified by ^{3}H-thymidine

incorporation. These data are representative of two (A) and six (B) independent experiments.

Fig. 3. CTLA-4$^{-/-}$ Tregs exhibit uncompromised suppressive activity in vitro. A. Left panel, CFSE-labeled CD4^{+}CD62L^{+}CD25^{-} cells from WT CTLA-4Ig transgenic mice were stimulated with WT T-depleted splenocytes and anti-CD3 alone (filled) or in the presence of equal number of unlabeled CTLA-4$^{-/-}$ Tregs from CTLA-4Ig transgenic mice (solid line). Right panel, conditions are as in the left panel with the addition of anti-CTLA-4 Fab fragments (100μg/ml) at the initiation of the culture. Cells were harvested at 72 hours and analyzed by FACS for CFSE intensity. B. Tregs from WT CTLA-4Ig transgenic (filled) or CTLA-4$^{-/-}$ CTLA-4 transgenic mice (open) were co-cultured with CD4^{+}CD62L^{+}CD25^{-} cells from WT CTLA-4Ig transgenic mice at the indicated ratio. Proliferation was quantified by ^{3}H-thymidine incorporation. These data are representative of seven independent experiments.

Fig. 4. Function of CTLA-4$^{-/-}$ Tregs is not dependent on their enhanced IL-10 expression. A. Real-time PCR analysis of IL-10 mRNA level in freshly isolated Tregs from CTLA-4$^{-/-}$ CTLA-4Ig transgenic or CTLA-4$^{+/+ \text{ or} +/-}$ CTLA-4Ig transgenic mice. B. Tregs purified from CTLA-4$^{-/-}$ CTLA-4Ig transgenic or CTLA-4$^{+/+ \text{ or } +/-}$ CTLA-4Ig transgenic mice were stimulated in vitro for 48 hours, and the culture supernatant was analyzed for IL-10 secretion by ELISA. C. CTLA-4$^{-/-}$ Tregs were co-cultured with CD4^{+}CD62L^{+}CD25^{-} cells from CTLA-4$^{+/-}$CTLA-4Ig transgenic mice at the indicated ratio with (filled) or without (open) 20μg/ml anti-IL-10 mAb. Cells were incubated at 37°C for total of 68 hours and ^{3}H-thymidine was added during the last 14 hours of the culture. Proliferation was quantified by ^{3}H-thymidine incorporation. Results are representative of two independent experiments.

Fig. 5. In vitro suppression by CTLA-$4^{-/-}$ Tregs is partially dependent on TGF-β. A. Real-time PCR analysis of TGF-β, mRNA level in freshly isolated Tregs from CTLA-$4^{-/-}$ CTLA-4Ig transgenic or CTLA-$4^{+/+\ or +/-}$ CTLA-4Ig transgenic mice. B. Tregs purified from CTLA-$4^{-/-}$ CTLA-4Ig transgenic or CTLA-$4^{+/+\ or\ +/-}$ CTLA-4Ig transgenic mice were stimulated in vitro for 48 hours, and the culture supernatant was analyzed for TGF-β, secretion by ELISA. C. Left four panels, LN cells from CTLA-$4^{-/-}$ CTLA-4Ig transgenic or CTLA-$4^{+/+\ or\ +/-}$ CTLA-4Ig transgenic mice were stained with CD4, CD62L, CD25, and cell surface LAP or TGF-β, and analyzed by flow cytometry. Histograms of LAP or TGF-β (shaded) and background (bold line) staining on Tregs is shown. Right two panels, Purified Tregs cells were stimulated as described in B for 24 hours. Cell surface TGF-β expression was determined by flow cytometry. Results are representative of three independent experiments. D. CTLA-$4^{-/-}$ Tregs were co-cultured with $CD4^{+}CD62L^{+}CD25^{-}$ cells from CTLA-$4^{+/-}$CTLA-4Ig transgenic mice at the indicated ratio with (filled) or without (open) 50μg/ml anti-TGF-β mAb. Cells were incubated at $37^{o}C$ for total of 68 hours and ^{3}H-thymidine was added during the last 14 hours of the culture. Proliferation was quantified by ^{3}H-thymidine incorporation. These data are representative of six independent experiments.

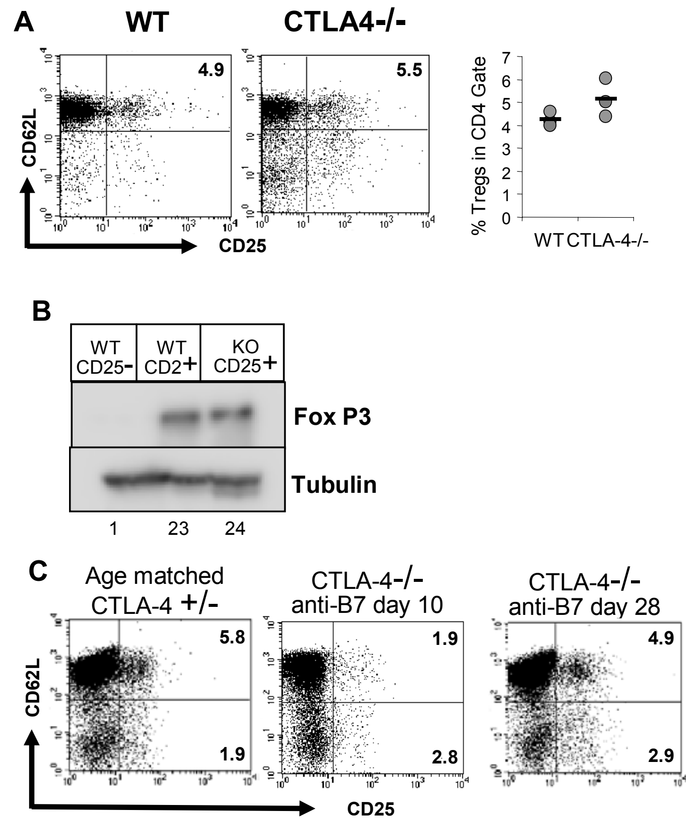
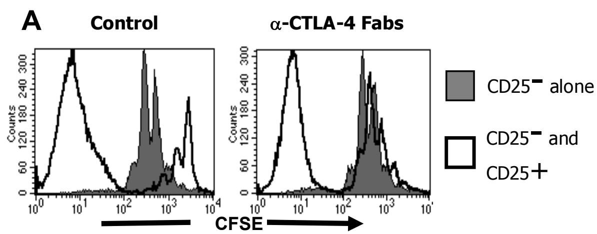


Figure 1 Tang et al



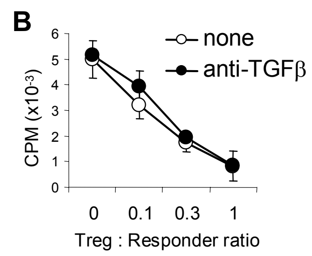


Figure 2 Tang et al

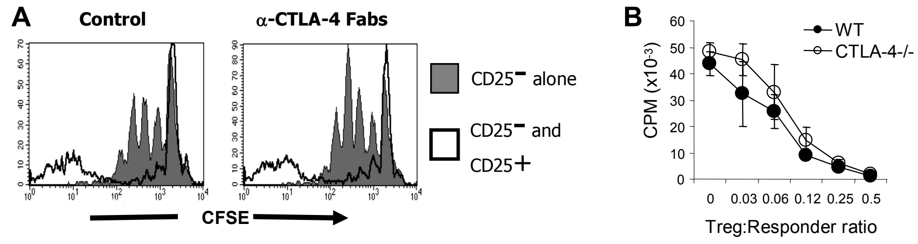


Figure 3 Tang et al

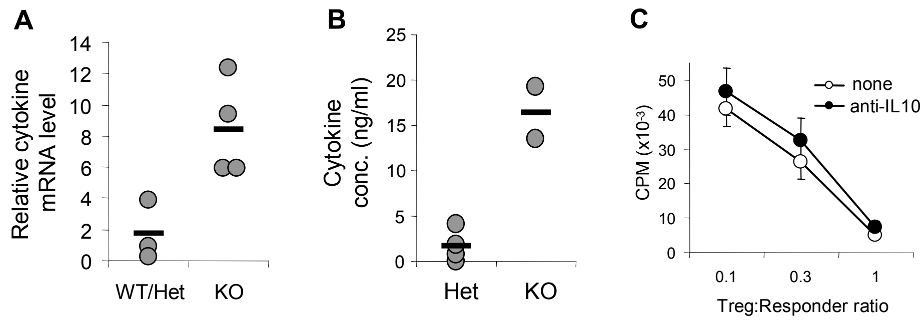


Figure 4 Tang et al

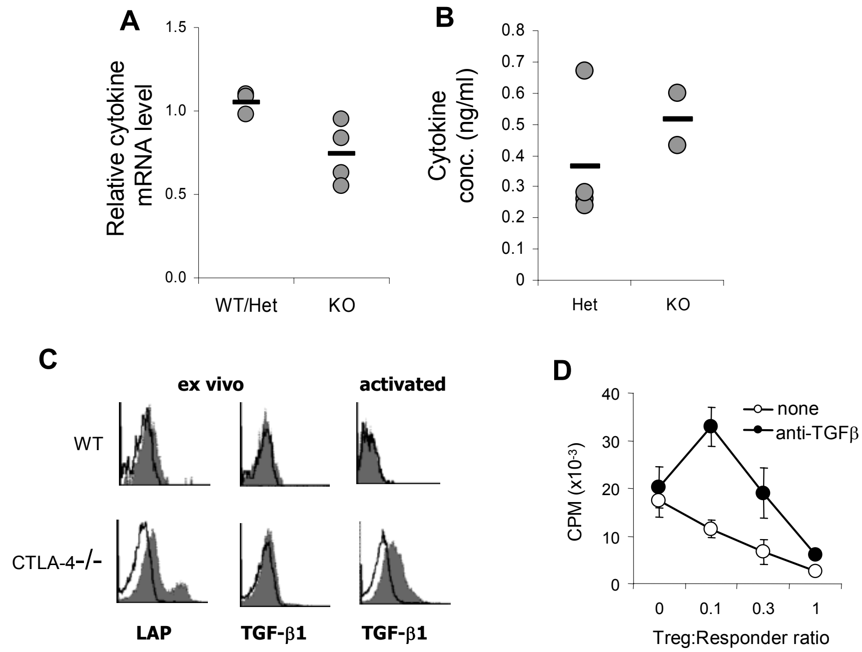


Figure 5 Tang et al