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Blockade of CTLA-4 on CD4+CD25+ Regulatory T Cells Abrogates Their Function In Vivo

Simon Read,* Rebecca Greenwald,† Ana Izcute,* Nicholas Robinson,* Didier Mandelbrot,† Loise Francisco,† Arlene H. Sharpe,† and Fiona Powrie‡*

Naturally occurring CD4+ regulatory T cells (T_\text{R}) that express CD25 and the transcription factor FoxP3 play a key role in immune homeostasis, preventing immune pathological responses to self and foreign Ags. CTLA-4 is expressed by a high percentage of these cells, and is often considered as a marker for T_\text{R} in experimental and clinical analysis. However, it has not yet been proven that CTLA-4 has a direct role in T_\text{R} function. In this study, using a T cell-mediated colitis model, we demonstrate that anti-CTLA-4 mAb treatment inhibits T_\text{R} function in vivo via direct effects on CTLA-4-expressing T_\text{R}, and not via hyperactivation of colitogenic effector T cells. Although anti-CTLA-4 mAb treatment completely inhibits T_\text{R} function, it does not reduce T_\text{R} numbers or their homing to the GALT, suggesting the Ab mediates its function by blockade of a signal required for T_\text{R} activity. In contrast to the striking effect of the Ab, CTLA-4-deficient mice can produce functional T_\text{R}, suggesting that under some circumstances other immune regulatory mechanisms, including the production of IL-10, are able to compensate for the loss of the CTLA-4-mediated pathway. This study provides direct evidence that CTLA-4 has a specific, nonredundant role in the function of normal T_\text{R}. This role has to be taken into account when targeting CTLA-4 for therapeutic purposes, as such a strategy will not only boost effector T cell responses, but might also break T_\text{R}-mediated self-tolerance. The Journal of Immunology, 2006, 177: 4376–4383.
function of this population in vitro (17). In the T cell transfer model of colitis, administration of anti-CTLA-4 mAb to mice that received both CD4^+ CD45RB<sup>high</sup> and CD4^+ CD25<sup>+</sup> populations led to development of colitis, suggesting a key role for CTLA-4 in T<sub>R</sub>-mediated control of intestinal homeostasis (6, 18). As CTLA-4 is induced on naive T cells following activation (31), anti-CTLA-4 mAb treatment may abrogate suppression indirectly via hyperactivation of colitogenic T cells or directly via effects on the CD4<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> population. In this report, we have used CTLA-4-deficient mice and anti-CTLA-4 mAb to dissect how CTLA-4 influences the balance between effector and T<sub>R</sub> cells in the intestine.

**Materials and Methods**

**Mice**

BALB/c wild-type (WT), B7<sup>-/-</sup>B7<sub>-/-</sub> (B7<sup>-/-</sup>B7<sub>-/-</sub> knockout (KO)), and B7<sup>-/-</sup>B7<sub>-/-</sub>-CTLA-4-deficient (B7<sup>-/-</sup>B7<sub>-/-</sub>-CTLA-4 KO) mice were maintained in accordance with the institutional guidelines of Brigham and Women’s Hospital and Harvard Medical School (Boston, MA; accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC)). C.B-17 scid mice were purchased from Taconic Farms. For some experiments, BALB/c, C.B-17 scid, BALB/c.C57B10D2, Ly<sub>9.2</sub> congenic, BALB/c-CTLA-4-deficient (CTLA-4 KO), and BALB/c-RAG2-deficient (RAG KO) mice were maintained in specific-pathogen-free conditions at the Sir William Dunn School of Pathology (University of Oxford, Oxford, U.K.) and were used at 6–10 wk of age. All procedures were conducted in accordance with the Animals (Scientific Procedures) Act 1986.

**Generation of mixed bone marrow chimeras**

Bone marrow isolated from 2- to 3-wk-old BALB/c:CTLA-4 KO was depleted of T cells using anti-CD4 and anti-CD8 Abs together with rat-anti coated Dynabeads (Dynal). CTLA-4 KO bone marrow was then mixed in a 1:1 ratio with bone marrow taken from BALB/c.C57B10D2, Ly<sub>9.2</sub> mice and injected i.v. into gamma-irradiated (5.5 Gy, 550 rad) BALB/c.C57B10D2, Ly<sub>9.2</sub> mice. Eight weeks later, T cell reconstitution was assessed by analysis of expression of the Ly<sub>9</sub> allele in peripheral blood. For additional experiments, CTLA-4<sup>-/-</sup> T<sub>R</sub> were sorted based on expression of CD4, CD25, and Ly<sub>9.1</sub>.

**Purification of CD4<sup>+</sup> T cells**

CD4<sup>+</sup> T cells were purified from spleens using anti-mouse CD4 (clone L3T4) coated MACS beads (Miltenyi Biotech) in accordance with the manufacturer’s instructions. Alternatively, non-CD4<sup>+</sup> cells were depleted using anti-CD8, anti-B220, anti-H-2, and anti-Mac-1 Abs, together with anti-rat coated Dynabeads (Dynal). Purified CD4<sup>+</sup> T cells were stained with anti-mouse CD4-CyChrome (clone RM4.5; BD Biosciences), and anti-mouse CD25-PE (clone BA2-1; BD Biosciences), anti-mouse CD28-Ig (clone PC61; BD Biosciences). Subpopulations of CD4<sup>+</sup> T cells were sorted using three-color sorting on a FACSVantage (BD Biosciences) or MoFlo (DakoCytoimmun) cell sorter. T cells were sorted into CD4<sup>+</sup> (CD45RB<sup>low</sup>)CD25<sup>+</sup> and CD4<sup>+</sup> (CD45RB<sup>high</sup>)CD25<sup>−</sup>) subpopulations. Sorted populations were >98.5% positive on reanalysis. FACs analysis of the sorted CD4<sup>+</sup> CD45RB<sup>high</sup> population showed <1% FoxP3<sup>+</sup> cells (anti-mouse FoxP3 staining set; eBioscience).

**Generation of mAb used in vivo**

Anti-mouse CTLA-4 mAb (clone UC10-4F10-11) (32) and anti-mouse IL-10 mAb (clone 1B1.2) (33) were purified from hybridoma supernatant by affinity chromatography and shown to contain <1.0 endotoxin units per milligram of protein. Purified hamster IgG was used as a control (Jackson ImmunoResearch Laboratories). Fab were generated using immobilized papain (Perbio) in accordance with the manufacturer’s instructions. HPLC analysis of purified Fab before use indicated that <0.5% of the material existed in a nonmonomeric form. Surface plasmon resonance was used to confirm the binding activity of the anti-CTLA-4 Fab using a BIACore1000 instrument (see Fig. 5A). Briefly, CTLA-4-Fab (24 ng, 1399RU) was captured onto a sensor chip (CM5) using a covalently bound anti-human Ig Ab. Anti-CTLA-4 or control Fab (20 ng/ml, 5 μl/min) was then passed through the cell and binding monitored. Nonspecific binding was assessed by measuring anti-CTLA-4 Fab binding to a control cell lacking CTLA-4 Igs.

**T cell reconstitution**

Immune-deficient mice, either C.B-17 scid or BALB/c.RAG2 KO mice, were injected i.p. with the sorted T cell populations. No differences were observed in the induction of colitis, or the protection from colitis between experiments using scid and RAG2 KO recipients (our unpublished observations). Mice received 4 × 10<sup>5</sup> CD4<sup>+</sup> CD45RB<sup>high</sup> cells alone or in combination with 1 × 10<sup>5</sup> CD4<sup>+</sup> CD25<sup>−</sup> cells. Control mice received 1 × 10<sup>5</sup> CD4<sup>+</sup> CD25<sup>−</sup> alone. In experiments where the pathogenic population came from B7-deficient mice, only 1 × 10<sup>5</sup> CD4<sup>+</sup> CD45RB<sup>high</sup> cells were transferred. Following T cell transfer, some mice received anti-CTLA-4 mAb (clone UC10-4F10-11) or a control hamster IgG; 200 μg of purified IgG were injected i.p. in PBS the day after T cell reconstitution and then on alternate days for 6–8 wk. Similarly, some mice received purified anti-CTLA-4 Fab or control Fab (100 μg) daily from the day after T cell transfer for 6–8 wk. In other experiments, mice were injected with 500 μg of anti-IL-10R mAb twice a week from the day after transfer until the end of the experiment. Mice were weighed weekly and monitored for clinical signs of colitis. Mice losing in excess of 20% of initial body weight or showing signs of severe disease were sacrificed.

**Histological examination**

Colons were removed from mice 6–8 wk after T cell reconstitution and fixed in buffered 10% formalin. Six-micrometer paraffin-embedded sections were cut and stained with H&E. Inflammation was scored in a blinded fashion, on a scale of 0–4 where a grade of 0 was given when there were no changes observed (34). Changes associated with other grades were as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with mild to moderate epithelial hyperplasia and mild to moderate mucin depletion from goblet cells; grade 3, marked inflammatory cell infiltrates that were sometimes transmural, with moderate to severe epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with crypt abscesses and occasional ulceration, with marked epithelial hyperplasia, mucin depletion and loss of intestinal glands.

**Immunofluorescence**

Tissue samples were snap-frozen, cryosectioned and fixed using acetone. Sections were blocked with donkey serum (Sigma-Aldrich) and then stained with biotinylated anti-mouse CD3 (clone 145-2C11; BD Biosciences) plus streptavidin-Cy5 (Jackson ImmunoResearch Laboratories). FoxP3 staining was performed using rabbit polyclonal anti-mouse FoxP3 Abs (generously provided by F. Ramsdell, Zymogenetics, Seattle, WA) and donkey anti-rabbit IgG FITC (Jackson ImmunoResearch Laboratories). The specificity of FoxP3 staining was confirmed by the absence of nuclear staining in organs from FoxP3<sup>−/−</sup> mice (62).

**Statistical analysis**

Colitis scores were compared using the Mann-Whitney U test and differences were considered statistically significant with p < 0.05.

**Results**

**CD4<sup>+</sup> CD25<sup>+</sup> T cells are present in mice lacking B7-1, B7-2, and CTLA-4**

It has been previously shown that administration of anti-CTLA-4 is able to abrogate suppression of colitis mediated by CD4<sup>+</sup> CD25<sup>+</sup> T<sub>R</sub> in the T cell transfer model of colitis (6, 18). To dissect the mechanism by which anti-CTLA-4 Ab administration results in a loss of immune regulation, CD4<sup>+</sup> T cell populations were isolated from CTLA-4-deficient mice and analyzed for their ability to inhibit colitis. Due to the aberrant T cell activation, lymphoproliferation and early mortality that occurs in CTLA-4-deficient mice, it was not possible to use these mice as a source of T cells for transfer experiments (35, 36). Therefore, the CTLA-4-deficient mice used in this study were maintained on a B7<sup>-/-</sup>B7<sub>-/-</sub> KO background. The absence of B7<sup>-/-</sup>B7<sub>-/-</sub> expression prevents ligiation of CD28, which has been shown to be critical for activation of naive T cells, and the lymphoproliferative phenotype is avoided (37). In this respect, the B7<sup>-/-</sup>B7<sub>-/-</sub> CTLA-4 KO mouse strain provides a...
CTLA-4-deficient T<sub>R</sub> prevent colitis

A more definitive marker of T<sub>R</sub> is expressed by CD4<sup>+</sup>FoxP3<sup>+</sup> cells (2). In the same way the frequency of FoxP3<sup>+</sup> cells) but again the majority of CD25<sup>+</sup> cells were present in both B7-1/B7-2 KO and B7-1/B7-2/CTLA-4 KO mice. The absence of B7.1/B7.2 has profound effects on peripheral T cell activation of effector T cells and in mediating T<sub>R</sub> activity.

**B7-sufficient CTLA-4 KO T<sub>R</sub> retain the ability to prevent colitis**

The absence of B7.1/B7.2 has profound effects on peripheral T cell homeostasis, and may alter the activity of the CD4<sup>+</sup>CD25<sup>+</sup> population taken from B7.1/B7.2/CTLA-4 KO mice. To rule out any effect related to the lack of B7 molecules, mixed bone marrow

**TABLE 1. Induction of, and protection from, colitis by CD4<sup>+</sup> T cell subsets lacking expression of CTLA-4<sup>a</sup>**

<table>
<thead>
<tr>
<th>Phenotype of Cells Injected</th>
<th>Incidence of Colitis (n)</th>
<th>Mean Colitis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD45RB&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;+&lt;/sup&gt;</td>
<td>WT</td>
</tr>
<tr>
<td>B7-1/B7-2 KO</td>
<td>None</td>
<td>9 (9)</td>
</tr>
<tr>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>None</td>
<td>4 (4)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2 KO</td>
<td>0 (5)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>0 (5)</td>
</tr>
<tr>
<td>None</td>
<td>WT</td>
<td>0 (8)</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>0 (5)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2 KO</td>
<td>0 (4)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>0 (7)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>0 (10)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2 KO</td>
<td>1 (5)</td>
</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>5 (6)</td>
</tr>
</tbody>
</table>

<sup>a</sup>C.B-17.scid mice received CD4<sup>+</sup> T cell subsets as described. Mice were sacrificed 6–8 wk after T cell transfer and analyzed by flow cytometry for the expression of CD4<sup>+</sup>, CD25<sup>+</sup>, and FoxP3. Mice were analyzed at 6–8 wk of age. Representative plots show log 0 fluorescence and are gated on CD4<sup>+</sup> lymphocytes.

**FIGURE 1.** CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells are present in both B7-1/B7-2 KO and B7-1/B7-2/CTLA-4 KO mice. Unfractionated splenocytes from WT, B7-1/B7-2 KO, and B7-1/B7-2/CTLA-4 KO mice were analyzed by flow cytometry for the expression of CD4<sup>+</sup>, CD25<sup>+</sup>, and FoxP3. Mice were analyzed at 6–8 wk of age. Representative plots show log 0 fluorescence and are gated on CD4<sup>+</sup> lymphocytes.
chimeras were generated using CTLA-4 KO (Ly9.1+) and BALB/c.C57B10D2.Ly9.2 congenic donors. As has been reported previously (41), these animals do not develop the lymphoproliferative pathology that is characteristic of intact CTLA-4 KO mice. CTLA-4 KO CD4+CD25+ Treg could be recovered from these mice using expression of the congenic marker Ly9.1. The sorted CTLA-4 KO CD4+CD25+ Treg contained a similar frequency of FoxP3+ cells as the counterpart WT CD4+CD25+ Treg and were also able to prevent colitis induced by transfer of WT CD4+CD45RBhigh cells to BALB/c.RAG2 KO mice (Fig. 3). This confirms our observation that Treg that cannot use CTLA-4 are still able to prevent colitis, irrespective of expression of B7.

It has been reported that CD4+CD25+ cells from CTLA-4 KO mice express increased levels of IL-10 (42). We have previously reported that protection from colitis by WT CD4+CD25+ Treg is largely independent of IL-10 (43), as IL-10-deficient Treg retained the ability to prevent disease and blockade of IL-10 signaling using an anti-IL-10R mAb resulted in only a marginal loss of protection mediated by WT CD25+ Treg (Fig. 3 and Ref. 43). By contrast, administration of an anti-IL-10R mAb completely abrogated suppression mediated by CTLA-4-deficient CD4+CD25+ Treg (Fig. 3).

So, although Treg that lack CTLA-4 can still prevent colitis, they appear to do so by using alternative immune suppressive pathways to those used by WT Treg.

Anti-CTLA-4 mAb treatment targets CD4+CD25+ Treg and not colitogenic T cells to abrogate suppression

We have previously reported that administration of anti-CTLA-4 mAb abrogates CD4+CD25+ Treg-mediated suppression of colitis (6, 18). However, whether the Ab functions via effects on effector T cells, Treg, or both is not known. To investigate this issue, the outcome of anti-CTLA-4 mAb administration was examined in transfer experiments where expression of CTLA-4 was restricted to the colitogenic or Treg cells. Suppression of colitis mediated by B7-1/B7-2-CTL A-4 KO CD4+CD25+ Treg was not affected by anti-CTLA-4 mAb treatment (Fig. 4A). In addition, while WT CD4+CD25+ Treg were able to prevent colitis induced by CD4+CD45RBhigh cells from B7-1/B7-2/CTL A-4 KO mice, the addition of anti-CTLA-4 mAb led to a loss of protection and the development of disease (Fig. 4B). Together, these data indicate that anti-CTLA-4 mAb abrogates Treg-mediated control of colitis via its effects on Treg and not colitogenic effector cells.

Anti-CTLA-4 Fab retains the ability to disrupt the function of WT Treg

Next, we investigated the possibility that the anti-CTLA-4 treatment was somehow eliminating the Treg population. Anti-CTLA-4
mAb bound to the surface of TR might lead to deletion of this population, or it might cross-link CTLA-4 providing an agonistic signal, inhibiting TR expansion. To explore these possibilities, anti-CTLA-4 Fab were generated and used for in vivo studies (Fig. 5A). Administration of anti-CTLA-4 Fab to SCID mice cotransferred with B7-1/B7-2/CTLA-4 KO CD4⁺CD45RB⁺ cells and WT CD4⁺CD25⁺ cells led to a loss of suppression of colitis similar to that seen in mice injected with intact anti-CTLA-4 mAb (Fig. 5B). Protection from colitis was not affected in similarly transferred mice that received a control Fab. These results indicate that the presence of anti-CTLA-4 was in some way able to disrupt the homing of CD4⁺CD25⁺ TR. To determine whether this was the case, the GALT from T cell-transferred SCID mice was analyzed for the presence of CD3⁺CD4⁺CD25⁺ or CD3⁺CD4⁻CD25⁻ T cells (Fig. 7A). In all mice analyzed, it was possible to identify FoxP3⁺CD3⁻ cells in the mesenteric lymph node (MLN), even though these mice had ongoing colitis. In addition, FACS analysis was performed to quantify FoxP3 expressing cells in spleen and MLN of these mice (Fig. 7B). FoxP3⁺CD4⁺ T cells could be found in both the spleen and MLN of T cell transferred mice, with no significant reduction in the frequency of FoxP3⁺ cells in anti-CTLA-4-treated mice compared with the controls (Fig. 7C). Together, these data demonstrate that FoxP3⁺CD4⁺ T cells are able to access the GALT in anti-CTLA-4-treated mice and yet fail to control the colitogenic T cell response.

**Discussion**

CTLA-4 has long been known to play an important role in controlling immune responses (19). Although many mechanisms have
to a loss of TR-mediated suppression, both in vitro and in vivo (6, 16). Administration of anti-CTLA-4 mAb has been linked to CTLA-4 expression, which is now widely used as a marker of TR. Despite the presence in vivo being contradictory, one of the reasons for this uncertainty is that CTLA-4 may fulfill a variety of functions, as it is expressed by different T cell subpopulations at various time points. CTLA-4 was first described to be up-regulated by naive CD4+ T cells upon activation (31). More recently, CTLA-4 has been shown to be specifically expressed on CD4+ CD25+ TR cells, and administration of anti-CTLA-4 mAb has been linked to a loss of TR-mediated suppression, both in vitro and in vivo (6, 11, 18). Polymorphisms in CTLA-4 have also been associated with autoimmune disorders in humans (44) and susceptibility to autoimmune disease in mice (45). Based on these and other reports, CTLA-4 expression is now widely used as a marker of TR. Despite the presence in vivo being contradictory, one of the reasons for this uncertainty is that CTLA-4 may fulfill a variety of functions, as it is expressed by different T cell subpopulations at various time points. CTLA-4 was first described to be up-regulated by naive CD4+ T cells upon activation (31). More recently, CTLA-4 has been shown to be specifically expressed on CD4+ CD25+ TR cells, and administration of anti-CTLA-4 mAb has been linked to a loss of TR-mediated suppression, both in vitro and in vivo (6, 11, 18). Polymorphisms in CTLA-4 have also been associated with autoimmune disorders in humans (44) and susceptibility to autoimmune disease in mice (45). Based on these and other reports, CTLA-4 expression is now widely used as a marker of TR. Despite this, its precise role in these systems remains controversial, in part due to conflicting results that have been obtained in vitro (46, 47). Here, using the T cell transfer model of colitis, we show that anti-CTLA-4 mAb disrupts TR activity in vivo by targeting TRx, not by exacerbating the activity of pathogenic T cells. This effect is mediated by blockade of the interaction between CTLA-4 and its ligands, and not depletion of CTLA-4 expressing regulatory cells, as it has been previously suggested (46). Our results demonstrate that CTLA-4 expression is not only a phenotypic characteristic of TR but that its presence on CD4+ CD25+ TR plays an important role in the functional activity of this population.

In this study, we have used T cells lacking CTLA-4 as a tool to investigate the role of CTLA-4 in CD4+ CD25+ TR function and to clarify the effects of the anti-CTLA-4 mAb. Mice deficient in B7-1 and B7-2 as well as CTLA-4 were used as donors (37), thus avoiding the problems associated with isolating CD4+ CD25+ T cells from CTLA-4-deficient mice (35, 36). Previous studies have shown that administration of anti-CTLA-4 mAb overcame the ability of CD4+ CD25+ T cells to protect from colitis (6, 11, 18). Here, we demonstrate that protection is dependent upon CTLA-4 expression by TRx but is independent of CTLA-4 expression by the colitogenic CD4+ CD45RBhi/pup population, indicating that the effects of the Ab are mediated through CTLA-4-expressing CD4+ CD25+ TRx.

The mode of action of the anti-CTLA-4 mAb was analyzed by comparing the activity of intact IgG and Fab. Fab were as effective as intact Ab, indicating that the Ab does not cause Fc-mediated deletion of the TRx; nor does it cross-link CTLA-4 providing an agonistic signal. Ligation of CTLA-4 has been reported to inhibit activation induced cell death in certain T cell populations (48, 49); it was thus possible that the effect of the mAb was due to the inhibition of a similar survival signal. However, anti-CTLA-4 did not reduce the accumulation of TR progeny following transfer in vivo. Instead, absolute numbers of CD25+ TRx were increased along with splenomegaly, although the frequency of TR progeny as a percentage of the transferred CD4+ cells remained similar. Comparable results have been observed in a clinical trial in humans, where anti-CTLA-4 treatment, despite inducing antitumor responses and autoimmunity, did not reduce the frequency of FoxP3+ T cells. Furthermore, in our system, it was possible to detect the FoxP3+ progeny of transferred CD4+ CD25+ TRx in the GALT of anti-CTLA-4-treated mice, consistent with these cells migrating to the lymphoid organs that drain the diseased tissue but being unable to prevent the development of disease.

B7-1/B7-2/CTLA-4 KO mice contained a reduced frequency of FoxP3+ T cells together with anti-CTLA-4 mAb. Eight weeks later, mice were sacrificed and tissues taken for analysis. A, Sections from mesenteric lymph nodes were stained for CD3 (red) and FoxP3 (green), or CD3 and a control rabbit Ig. Images are representative of the analysis of four mice from two independent experiments. Original magnification: ×630. B, Representative analysis of FoxP3 expression by transferred CD4+ T cells from anti-CTLA-4-treated mice. Single-cell suspensions from spleen and mesenteric lymphatic nodes were stained for flow cytometric analysis. Plots show log10 fluorescence and are gated on CD3+ CD45Rhi lymphocytes. C, Proportion of CD4+ T cells expressing FoxP3 in the spleen and mesenteric lymph nodules of anti-CTLA-4 or control mice. Each point represents a single mouse analyzed by flow cytometry as in B.
retained FoxP3 expression and T<sub>R</sub> function in the absence of CTLA-4, indicating that the receptor is not absolutely required for the development of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub>. Importantly, administration of the anti-CTLA-4 mAb did not overcome the regulatory activity of CTLA-4 deficient CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub>, showing that ligation of CTLA-4 on the colitogenic population had limited impact on the regulation of colitis in this system.

Recent reports have shown that the interaction of CTLA-4 with B7 ligands expressed by APCs may modulate immune responses. This raises the possibility that anti-CTLA-4 mAb may disrupt T<sub>R</sub> function by preventing a CTLA-4-mediated signal through B7-1/2 expressed on dendritic cells (DC). The data show that binding of a CTLA-4.Ig fusion protein to the surface of DC induces expression of indoleamine 2,3-dioxygenase, leading to the depletion of tryptophan and inhibition of T cell function (51). CTLA-4 expressed by CD25<sup>+</sup> T<sub>R</sub> has similar effects, suggesting that this interaction may be important for the suppressive activity of these cells (52). Our findings that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> are able to suppress colitis induced by CTLA-4-deficient CD4<sup>+</sup>CD45RB<sup>hi</sup> cells in a CTLA-4-dependent manner are in line with that data, raising the possibility that it is ligation of B7-1/B7-2 on DC by CTLA-4 expressed by T<sub>R</sub> that is crucial for T<sub>R</sub>-mediated control of colitis. Additional experiments are required to test this hypothesis.

As not only CTLA-4, but also B7 is up-regulated on T cells upon activation, the interaction of CTLA-4 with B7 expressed on effector T cells may also play a role in CD4<sup>+</sup>CD25<sup>+</sup> cell-mediated suppression. In a recent report, B7-deficient CD4<sup>+</sup>CD25<sup>+</sup> cells were found to be refractory to T<sub>R</sub>-mediated suppression in vitro and in vivo (53). Whether CTLA-4-expressed by T<sub>R</sub> was important in these interactions is not clear. Indeed, while this may represent an another mechanism by which CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> influence T cell responses, it does not appear to be essential, since in our model B7-1/B7-2/CTL A-4 KO CD4<sup>+</sup>CD45RB<sup>hi</sup> T cells remained susceptible to suppression by WT T<sub>R</sub> through a CTLA-4-dependent mechanism.

PD-1, another member of the CD28/CTLA-4 family, has also been linked to T<sub>R</sub>-mediated prevention of colitis. CD4<sup>+</sup>CD25<sup>+</sup>PD-1<sup>+</sup> T cells expressing high levels of FoxP3 and CTLA-4 have been shown to prevent colitis in the CD4<sup>+</sup>CD45RB<sup>hi</sup> transfer model (54). This protection, like that mediated by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells, was overcome by anti-CTLA-4 but not by anti-PD-1 Abs. Although the role of PD-1 in T<sub>R</sub> function remains elusive, this report further highlights the functional importance of CTLA-4 in protection from colitis in a different model.

The data presented here demonstrate that signaling through CTLA-4 is required for WT CD25<sup>+</sup> T<sub>R</sub> to exert their suppressive phenotype. However, T<sub>R</sub> generated in the absence of CTLA-4 retain the ability to suppress colitis, suggesting that they are able to compensate for the loss of this receptor. The functionality of CTLA-4-deficient CD25<sup>+</sup> T<sub>R</sub> cells in vitro has been linked to an increased production of the immune suppressive cytokines IL-10 and TGF-β (42). In vivo, CTLA-4-deficient T<sub>R</sub> cells rely on IL-10 more heavily than the WT T<sub>R</sub> population, as administration of an anti-IL-10R mAb abrogated the protection mediated by these cells. Although CTLA-4-deficient T<sub>R</sub> can compensate for the absence of CTLA-4, this compensation is not complete. Thus, CD4<sup>+</sup>CD25<sup>+</sup> T cells from B7-1/B7-2/CTL A-4 KO mice failed to suppress effector T cells of the same genotype, although they were effective in controlling both WT and B7-1/B7-2 KO CD4<sup>+</sup>CD45RB<sup>hi</sup> cells. By contrast, B7-1/B7-2/CTL A-4 KO CD4<sup>+</sup>CD45RB<sup>hi</sup> cells were susceptible to regulation by WT and B7-1/B7-2 KO CD4<sup>+</sup>CD25<sup>+</sup> cells. One explanation could be that the CD4<sup>+</sup>CD45RB<sup>hi</sup> population from B7<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> mice is less susceptible to regulation than that from WT or B7<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> mice. Thus, the combination of CTLA-4 deficiency in both effector and T<sub>R</sub> populations is sufficient to tip the balance away from a regulated immune response and toward the development of inflammatory pathology. These data are consistent with a growing acceptance that immune regulation is mediated by multiple mechanisms and that removal of one or other pathway may or may not result in a loss of suppression as a function of the particular assay.

In clinical studies, anti-CTLA-4 mAb has been developed as a reagent to enhance T cell immunity (55). Early results with anti-CTLA-4 mAb in humans have suggested that this reagent may be an effective means to enhance antitumor immunity; however, the treatment has also lead to transient negative side effects, including the development of enterocolitis (30). More recent trials with the same anti-CTLA-4 mAb have seem similar autoimmune and gastrointestinal perturbances (56–58). As FoxP3 expression was not perturbed by the therapy, the effects have been ascribed to the interaction of anti-CTLA-4 mAb with effector cells (50). The data presented herein offer an alternative interpretation, indicating that anti-CTLA-4 can target CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> function without changes in FoxP3 expression.

The clinical studies illustrate that the anti-CTLA-4 mAb treatment may also have an impact on T<sub>R</sub>-mediated suppression of T cell responses and alter the balance of immune regulation, especially in the gut. Although the studies show that manipulation of CTLA-4 signaling is useful as a mechanism to enhance immune responses in a therapeutic setting, they also highlight the need to separate its useful and harmful effects. This is particularly significant at this time, with the recent approval of CTLA-4 Ig therapy for the treatment of rheumatoid arthritis from the Food and Drug Administration (59). In this article, we present a model where CTLA-4 mediates two different effects. On one hand, it reduces the pathogenicity of effector cells (60, 61). On the other, it is required for T<sub>R</sub>-mediated control of immune responses. Both roles have to be taken into account when designing clinical trials, as the stimulation of antitumor effector cells by blocking CTLA-4 could be accompanied by the breakdown of T<sub>R</sub>-mediated self-tolerance.

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Disclosures
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References


