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Blockade of CTLA-4 on CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells Abrogates Their Function In Vivo<sup>1</sup>

Simon Read,* Rebecca Greenwald,† Ana Izcuc,*, Nicholas Robinson,*, Didier Mandelbrot,‡ Loise Francisco,† Arlene H. Sharpe,† and Fiona Powrie<sup>2*</sup>

Naturally occurring CD4<sup>+</sup> regulatory T cells (T<sub>R</sub>) 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<sub>R</sub> in experimental and clinical analysis. However, it has not yet been proven that CTLA-4 has a direct role in T<sub>R</sub> function. In this study, using a T cell-mediated colitis model, we demonstrate that anti-CTLA-4 mAb treatment inhibits T<sub>R</sub> function in vivo via direct effects on CTLA-4-expressing T<sub>R</sub>, and not via hyperactivation of colitogenic effector T cells. Although anti-CTLA-4 mAb treatment completely inhibits T<sub>R</sub> function, it does not reduce T<sub>R</sub> numbers or their homing to the GALT, suggesting the Ab mediates its function by blockade of a signal required for T<sub>R</sub> activity. In contrast to the striking effect of the Ab, CTLA-4-deficient mice can produce functional T<sub>R</sub>, 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<sub>R</sub>. 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<sub>R</sub>-mediated self-tolerance. The Journal of Immunology, 2006, 177: 4376–4383.

Immune responses to specific pathogens incorporate multiple regulatory mechanisms that have evolved to restrict reactivity to self-Ags, while allowing both the clearance of the pathogen and the development of long-term immunological memory (1, 2). This is especially critical in the intestine, where it is necessary to mount protective immune responses against pathogens while limiting responses to commensal bacteria and dietary and self-Ags, in the presence of a large antigenic load. Indeed, a breakdown in intestinal homeostasis and the development of aberrant inflammatory responses to intestinal bacteria have been associated with the pathogenesis of inflammatory bowel disease (IBD) in humans (3, 4).

Studies using mouse models of IBD have provided convincing evidence that functionally specialized populations of regulatory T (T<sub>R</sub>) cells play an important role in the control of intestinal inflammation (5). Some of the best studied are the naturally arising CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> that have been shown to prevent and even cure colitis in the T cell transfer model (6–8). In addition to their role in intestinal homeostasis, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> play a key role in dominant tolerance to self-Ags and can also impede host protective immune responses to tumors and pathogens (9–11). Recently, expression of the transcription factor FoxP3 has been shown to be a useful marker for naturally arising T<sub>R</sub>. FoxP3 plays a key functional role in CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> development, as mice with natural or induced mutations in this gene lack T<sub>R</sub> and develop a fatal multorgan inflammatory disease (12–14). Similarly, loss of function mutations in FOXP3 have been shown to be responsible for the human autoimmune and inflammatory disease, immune polyendocrine X-linked enteropathy, Diabetes and chronic intestinal inflammation with several features resembling IBD are found in nearly all patients, and gastrointestinal symptoms are typically the reason for the initial clinical presentation, providing evidence that T<sub>R</sub> also contribute to intestinal homeostasis in humans (15, 16).

Recently, it has been proposed that the inhibitory receptor CTLA-4 plays a functional role in T<sub>R</sub> activity (6, 17, 18). This receptor belongs to the same family as CD28 and binds to the same ligands, B7-1 and B7-2. CTLA-4 is up-regulated upon T cell activation, and its activity as a negative regulator of T cell responses is now well-described (19). In vitro, ligation of CTLA-4 on activated CD4<sup>+</sup> T cells suppresses IL-2 production and limits cell cycle progression (20, 21). In vivo, blockade of CTLA-4 leads to increased T cell-mediated immunity in a number of model systems including Ag-specific responses (22), parasitic infection (23), and autoimmune disease (24–26). Manipulation of the B7:CTLA-4 pathway is also an attractive target for stimulating antitumor immunity (27–29). In a recent clinical trial, metastatic melanoma patients were treated with a humanized anti-CTLA-4 mAb in conjunction with two modified gp100 melanoma-associated Ags; this led to cancer regression in a proportion of patients (3 of 14). However, anti-CTLA-4 treatment also resulted in autoimmune disease (6 of 14) including the development of enterocolitis (30). These findings are consistent with work in animal models, demonstrate a critical role for CTLA-4 in the regulation of peripheral tolerance in humans, and give further impetus to understanding how CTLA-4 may be important for regulating tolerance to colonic Ags.

Among resting CD4<sup>+</sup> T cells, CTLA-4 is expressed primarily by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub>, being detectable on ~50% of these cells as compared with <1% of naïve CD4<sup>+</sup>CD45RB<sup>hi</sup> cells (6, 17). The expression of CTLA-4 on T<sub>R</sub> has been linked to regulation of organ-specific autoimmune disease in vivo, and there is some evidence to suggest that CTLA-4 is required for the suppressive

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3 Abbreviations used in this paper: IBD, inflammatory bowel disease; T<sub>R</sub>, regulatory T cell; WT, wild type; KO, knockout; MLN, mesenteric lymph node.
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+CD45RBhigh and CD4+CD25+ populations led to development of colitis, suggesting a key role for CTLA-4 in Treg-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+CD25+ Treg 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 Treg cells in the intestine.

Materials and Methods

Mice

BALB/c wild-type (WT), B7-1/-B7-2-deficient (B7-1/-B7-2 knockout [KO]), and B7-1/-B7-2/CTLA-4-deficient (B7-1/-B7-2/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.C57Bl10D2.Ly9.2 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 anti-rat coated Dynabeads (Dynal). CTLA-4 KO bone marrow was then mixed in a 1:1 ratio with bone marrow taken from BALB/c.C57Bl10D2.Ly9.2 mice and injected i.v. into gamma-irradiated (5.5 Gy, 550 rad) BALB/c.C57Bl10D2.Ly9.2 mice. Eight weeks later, T cell reconstitution was assessed by analysis of expression of the Ly9 allele in peripheral blood. For additional experiments, CTLA-4+/− Treg were sorted based on expression of CD4, CD25, and Ly9.1.

Purification of CD4+ T cells

CD4+ 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+ cells were depleted by using anti-CD8, anti-B220, anti-H-2, and anti-Mac-1 Abs, together with anti-rat coated Dynabeads (Dynal). Purified CD4+ T cells were stained with anti-mouse CD4-PE-CyChrome (clone RM4.5; BD Biosciences), anti-mouse CD45RB-FITC (clone 16A; BD Biosciences), and anti-mouse CD25-PE (clone PC61; BD Biosciences). Subpopulations of CD4+ cells were sorted by using three-color sorting on a FACSVantage (BD Biosciences) or MoFlo (DakoCytomation) cell sorter. T cells were sorted into CD4+CD45RBhighCD25− and CD4+CD45RBhighCD25+ subpopulations. Sorted populations were >98.5% pure on reanalysis. FACs analysis of the sorted CD4+CD45RBhigh population showed <1% FoxP3− 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-10R 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-lg (24 ng, 1399RU) was captured onto a sensor chip (CM5) using a cobalt-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 using anti-CTLA-4 Fab binding to a control cell lacking CTLA-4-lg.

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 × 105 CD4+CD45RB−/− cells alone or in combination with 1 × 105 CD4+CD25+ T cells. Control mice received 1 × 105 CD4+CD25− alone. In experiments where the pathogenic population came from B7-deficient mice, only 1 × 105 CD4+CD45RB−/− 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

Colonos 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, moderate 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) and 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− 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+CD25+ 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+CD25+ Treg 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+ T cell populations were isolated from CTLA-4−/− 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−/− mice, it was not possible to use these mice as a source of T cells for transfer experiments (35, 36). Therefore, the CTLA-4−/− mice used in this study were maintained on a B7-1/B7-2 KO background. The absence of B7-1/B7-2 expression prevents ligandation 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 B7-1/B7-2/CTLA-4−/− KO mouse strain provides a
a more definitive marker of TR is expressed by CD45RBhigh T cells alone (2.5–3.0% of CD4) deficient mice retain functional activity. This would not directly express around 10% of the CD4 cells, with the majority of CD25, and FoxP3. Mice were analyzed at 6–8 wk of age. Representative plots show log10 fluorescence and are gated on CD4+ lymphocytes.

FIGURE 1. CD4+CD25+FoxP3+ 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, CD25, and FoxP3. Mice were analyzed at 6–8 wk of age. Representative plots show log10 fluorescence and are gated on CD4+ lymphocytes.

unique tool to analyze the function of CTLA-4 on both regulatory and colitogenic T cells during the development of colitis.

To confirm that CD25+T R can be generated in the absence of CTLA-4, splenocytes were isolated from WT, B7-1/B7-2 KO, and B7-1/B7-2/CTLA-4 KO mice and stained for expression of CD4, CD25, and FoxP3. In WT BALB/c mice, CD4+CD25+ cells comprise around 10% of the CD4+ T cell population (Fig. 1). FoxP3, a more definitive marker of T R is expressed by ~15% of CD4+ cells, with the majority of CD25+ cells also expressing FoxP3 (Fig. 1). In contrast, in both B7-1/B7-2 KO and B7-1/B7-2/CTLA-4 KO mice, only 1–2% of CD4+ cells express CD25 (Fig. 1). In the same way the frequency of FoxP3+ cells is significantly reduced (2.5–3.0% of CD4+ cells) but again the majority of CD4+CD25+ cells still express FoxP3, confirming the suitability of CD25 as a T R marker in these mice. The reduction in CD4+CD25+ T R frequency in B7-1/B7-2 KO mice is most likely due to the lack of CD28 ligation, as previous studies have shown that blockade of B7-1/B7-2 or loss of CD28 resulted in a reduction in both thymic and peripheral CD4+CD25+T R (38–40).

CTLA-4-deficient T R prevent colitis

We next determined whether CD4+CD25+ T R from CTLA-4-deficient mice retain functional activity. This would not directly exclude a role of CTLA-4 on WT T R, as genetically modified mice often develop alternative mechanisms to compensate for the loss of a key molecule. To check for CD4+CD25+ T R function, this population was isolated from WT, B7-1/B7-2 KO, and B7-1/B7-2/CTLA-4 KO mice and transferred alone or in combination with WT CD4+CD45RBhigh cells to SCID mice. None of the isolated CD25+ populations were pathogenic, since transfer of WT, or B7-1/B7-2 KO, or B7-1/B7-2/CTLA-4 KO CD4+CD25+ cells alone to SCID recipients did not elicit any colonic inflammation (Table I). As previously described, WT CD25+ T R inhibited the development of colitis when cotransferred with WT CD4+CD45RBhigh cells (Table I, Fig. 2) (6). Similarly, CD4+CD25+ T R from both B7-1/B7-2 KO and B7-1/B7-2/CTLA-4 KO mice were able to protect mice from the induction of colitis by WT CD4+CD45RBhigh. Reducing the number of CD4+CD25+ T cells transferred failed to reveal any difference in the potency of these populations (data not shown). Thus, CTLA-4-deficient CD4+CD25+ cells retain the ability to prevent disease.

The role of CTLA-4 expression on the potentially pathogenic CD4+CD45RBhigh cells was also investigated. In early experiments we noted a rapid but transient wasting disease following transfer of B7-deficient CD45RBhigh cells that did not occur in recipients of WT CD45RBhigh cells (data not shown). There was no correlation between this early wasting and the presence or absence of CD25+ T R, or the later development of colitis, and in later experiments it was avoided by reducing the number of CD45RBhigh cells transferred. This modification did not affect the incidence or severity of colitis.

Both WT and B7-1/B7-2 KO CD4+CD25+T R were able to inhibit colitis when cotransferred with B7-1/B7-2/CTLA-4 KO CD4+CD45RBhigh cells (Table I, Fig. 2). Similarly, B7-1/B7-2/CTLA-4 KO CD4+CD25+ cells could mediate protection from colitis induced by B7-1/B7-2 KO CD45RBhigh cells. However, when CTLA-4 was absent on both colitogenic and regulatory cells the majority of recipient mice went on to develop disease (Table I, Fig. 2). This would suggest that CTLA-4 expressed by both the colitogenic T cells and by the T R has an impact on the protection from colitis. This is consistent with multiple roles for CTLA-4 in both the activation of effector T cells and in mediating T R activity.

<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+CD5RBhigh</td>
<td>CD4+CD25+</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>None</td>
<td>16 (16)</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>WT</td>
<td>0 (8)</td>
</tr>
<tr>
<td>None</td>
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<td>0 (5)</td>
</tr>
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<td>B7-1/B7-2/CTLA-4 KO</td>
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</tr>
<tr>
<td>None</td>
<td>B7-1/B7-2/CTLA-4 KO</td>
<td>5 (6)</td>
</tr>
</tbody>
</table>

*B. C. C. 17. acid mice received CD4+ T cell subsets as described. Mice were sacrificed 6–8 wk after T cell transfer and colons were taken for histological analysis. Data show incidence of colitis (colitis score ≥2) and mean colitis score of those mice with colitis. Data are pooled from four independent experiments.
chimeras were generated using CTLA-4 KO (Ly9.1^+^) and BALB/c.C57Bl10D2.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^+^ T_R could be recovered from these mice using expression of the congenic marker Ly9.1. The sorted CTLA-4 KO CD4^+^CD25^+^ T_R contained a similar frequency of FoxP3^+^ cells as the counterpart WT CD4^+^CD25^+^ T_R and were also able to prevent colitis induced by transfer of WT CD4^+^CD45RB**high** cells to BALB/c.RAG2 KO mice (Fig. 3). This confirms our observation that T_R 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^+^ T_R is largely independent of IL-10 (43), as IL-10-deficient T_R 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^+^ T_R (Fig. 3 and Ref. 43). By contrast, administration of an anti-IL-10R mAb completely abrogated suppression mediated by CTLA-4-deficient CD4^+^CD25^+^ T_R (Fig. 3).

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

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

We have previously reported that administration of anti-CTLA-4 mAb abrogates CD4^+^CD25^+^ T_R-mediated suppression of colitis (6, 18). However, whether the Ab functions via effects on effector T cells, T_R, or both is not known. To investigate this issue, the outcome of anti-CTL4-a mAb administration was examined in transfer experiments where expression of CTLA-4 was restricted to the colitogenic or T_R cells. Suppression of colitis mediated by B7-1/B7-2/CTLA-4 KO CD4^+^CD25^+^ T_R was not affected by anti-CTLA4 mAb treatment (Fig. 4A). In addition, while WT CD4^+^CD25^+^ T_R were able to prevent colitis induced by CD4^+^CD45RB**high** cells from B7-1/B7-2/CTLA-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 T_R-mediated control of colitis via its effects on T_R and not colitogenic effector cells.

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

Next, we investigated the possibility that the anti-CTLA-4 treatment was somehow eliminating the T_R population. Anti-CTLA-4
mAb bound to the surface of T<sub>R</sub> might lead to deletion of this population, or it might cross-link CTLA-4 providing an agonistic signal, inhibiting T<sub>R</sub> 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<sup>+</sup>CD45RB<sup>high</sup> cells and WT CD4<sup>+</sup>CD25<sup>+</sup> 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 transfused mice that received a control Fab. These results indicate that the functional effects of anti-CTLA-4 administration are independent of the Fc portion of the Ab, ruling out Ab-induced cross-linking of CTLA-4 and generation of an agonistic signal, as well as Fc-mediated cellular depletion, as mechanisms of action.

**Accumulation of CD4<sup>+</sup>CD25<sup>+</sup>T<sub>R</sub> is not inhibited by the presence of anti-CTLA-4**

It was possible that blockade of CTLA-4 on T<sub>R</sub> inhibited a positive signal required for T<sub>R</sub> proliferation and accumulation. To assess the effect of the mAb on T<sub>R</sub> accumulation, expression of an allotypic marker was used to distinguish the progeny of the CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD45RB<sup>high</sup> populations following transfer. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> were purified from WT BALB/c (Ly9.1) mice and transferred together with CD4<sup>+</sup>CD45RB<sup>high</sup> cells from BALB/c.C57Bl10D2 Ly9.2 congenic mice to immunodeficient recipients. After 2 wk, CD4<sup>+</sup> T cells from the spleen were analyzed for expression of allotypic markers. Mice that received anti-CTLA-4 mAb had an increased number of total CD4<sup>+</sup> T cells, with both Ly9.1<sup>+</sup> and Ly9.2<sup>+</sup> cells being increased ~2-fold (Fig. 6A, data not shown). However, the proportion of CD4<sup>+</sup>CD25<sup>+</sup> progeny (Ly9.1<sup>+</sup> CD4<sup>+</sup> cells) vs CD4<sup>+</sup>CD45RB<sup>high</sup> progeny was similar in mice that had received anti-CTLA-4 to that seen in control mice, a pattern that was maintained at later time points (Fig. 6B). These data show that the ability of anti-CTLA-4 to suppress T<sub>R</sub> activity is not the result of impaired accumulation of the CD4<sup>+</sup>CD25<sup>+</sup> population.

**CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> are present in GALT of colitic mice**

It remained possible that the presence of anti-CTLA-4 was in some way able to disrupt the homing of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub>. To determine whether this was the case, the GALT from T cell-transferred SCID mice was analyzed for the presence of T<sub>R</sub>. Frozen mesenteric lymph node sections from mice that had received CD4<sup>+</sup>CD45RB<sup>high</sup> cells in combination with CD4<sup>+</sup>CD25<sup>+</sup> cells and anti-CTLA-4 mAb were stained for the presence of CD3<sup>+</sup> cells in anti-CTLA-4-treated mice compared to WT CD4<sup>+</sup>CD25<sup>+</sup> cells. Again, some mice also received anti-CTLA-4 mAb. Mice were sacrificed 6–8 wk after transfer and colons taken for histological analysis. Data show colitis scores for individual mice taken from two to three independent experiments.

**Discussion**

CTLA-4 has long been known to play an important role in controlling immune responses (19). Although many mechanisms have...
been reported for CTLA-4 function in vitro, evidence for their presence in vivo is still 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+ Treg populations, and administration of anti-CTLA-4 mAb has been linked to a loss of Treg-mediated suppression, both in vitro and in vivo (6, 17, 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 Treg. Despite the problems associated with isolating CD4+ T cells from CTLA-4-deficient mice (35, 36), previous studies have demonstrated that protection is dependent upon CTLA-4 expression (46), 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 Treg, but that its presence on CD4+ CD25+ Treg 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+ Treg 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 demonstrated that administration of anti-CTLA-4 mAb overcame the activity of Treg, 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 Treg, but that its presence on CD4+ CD25+ Treg plays an important role in the functional activity of this population.

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 Tregs; nor does it cross-link CTLA-4 providing an agonist 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 Treg progeny following transfer in vivo. Instead, absolute numbers of CD25+ Tregs were increased along with splenomegaly, although the frequency of Treg progeny as a percentage of the transferred CD4+ T 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 from anti-CTLA-4-treated mice. Single-cell suspensions from spleen and mesenteric lymph nodes were stained for flow cytometric analysis. Plots show log10 fluorescence and are gated on CD3+ CD4+ lymphocytes. Proportion of CD4+ T cells expressing Foxp3 in the spleen and mesenteric lymph nodes of anti-CTLA-4 or control mice. Each point represents a single mouse analyzed by flow cytometry as in B.
were found to be refractory to TR-mediated suppression in vitro. Importantly, administration of the anti-CTLA-4 mAb did not overcome the regulatory activity of CTLA-4 deficient CD4+CD25+ TR, 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 TR function by preventing a CTLA-4-mediated signal through B7-1/CD28 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+ Treg has similar effects, suggesting that this interaction may be important for the suppressive activity of these cells (52). Our findings that CD4+CD25+ Treg are able to suppress colitis induced by CTLA-4-deficient CD4+CD45RBhigh 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 Treg that is crucial for TR-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+CD25+ cell-mediated suppression. In a recent report, B7-deficient CD4+CD25+ cells were found to be refractory to TR-mediated suppression in vitro and in vivo (53). Whether CTLA-4 expressed by Treg was important in these interactions is not clear. Indeed, while this may represent an other mechanism by which CD4+CD25+ Treg influence T cell responses, it does not appear to be essential, since in our model B7-1/B7-2/CTLA-4 KO CD4+CD45RBhigh T cells remained susceptible to suppression by WT TR, through a CTLA-4-dependent mechanism.

PD-1, another member of the CD28/CTLA-4 family, has also been linked to TR-mediated prevention of colitis. CD4+CD25+PD-1+ T cells expressing high levels of FoxP3 and CTLA-4 have been shown to prevent colitis in the CD4+CD45RBhigh transfer model (54). This protection, like that mediated by CD4+CD25+ T cells, was overcome by anti-CTLA-4 but not by anti-PD-1 Abs. Although the role of PD-1 in TR 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+ Treg to exert their suppressive phenotype. However, Treg 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+ Treg 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 Treg cells rely on IL-10 more heavily than the WT Treg population, as administration of an anti-IL-10 mAb abrogated the protection mediated by these cells. Although CTLA-4-deficient Treg can compensate for the absence of CTLA-4, this compensation is not complete. Thus, CD4+CD25+ T cells from B7-1/B7-2/CTLA-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+CD45RBhigh cells. By contrast, B7-1/B7-2/CTLA-4 KO CD4+CD45RBhigh cells were susceptible to regulation by WT and B7-1/B7-2 KO CD4+CD25+ cells. One explanation could be that the CD4+CD45RBhigh population from B7.1/B7.2/CTLA-4 KO mice is less susceptible to regulation than that from WT or B7.1/B7.2 KO mice. Thus, the combination of CTLA-4 deficiency in both effector and Treg 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 led 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 perturbations (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+CD25+ TR function without changes in FoxP3 expression.

The clinical studies illustrate that the anti-CTLA-4 mAb treatment may also have an impact on TR-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 by 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 TR-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 TR-mediated self-tolerance.

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**Disclosures**

The authors have no financial conflict of interest.

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