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Acquisition of Suppressive Function by Activated Human CD4+CD25− T Cells Is Associated with the Expression of CTLA-4 Not FoxP3

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The role of CTLA-4 in regulatory T cell (Treg) function is not well understood. We have examined the role of CTLA-4 and its relationship with the transcription factor FoxP3 using a model of Treg induction in human peripheral blood. Activation of human CD4+CD25− T cells resulted in the appearance of a de novo population of FoxP3-expressing cells within 48 h. These cells expressed high levels of CTLA-4 and cell sorting on expression of CTLA-4 strongly enriched for FoxP3+-expressing cells with suppressive function. Culture in IL-2 alone also generated cells with suppressive capacity that also correlated with the appearance of CTLA-4. To directly test the role of CTLA-4, we transfected resting human T cells with CTLA-4 and found that this method conferred suppression, similar to that of natural Tregs, even though these cells did not express FoxP3. Furthermore, transfection of FoxP3 did not induce CTLA-4 and these cells were not suppressive. By separating the expression of CTLA-4 and FoxP3, our data show that FoxP3 expression alone is insufficient to up-regulate CTLA-4; however, activation of CD4+CD25− T cells can induce both FoxP3 and CTLA-4 in a subpopulation of T cells that are capable of suppression. These data suggest that the acquisition of suppressive behavior by activated CD4+CD25− T cells requires the expression of CTLA-4, a feature that appears to be facilitated by, but is not dependent on, expression of FoxP3.


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3Abbreviations used in this paper: Treg, regulatory T cell; DC, dendritic cell; PPD, purified protein derivative.

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occurred before T cell division. These cells also expressed CTLA-4, and there was a strong correlation between the level of CTLA-4 and FoxP3 expression. Interestingly, we also observed that treatment of CD25+/H11002 T cells with IL-2 alone could up-regulate FoxP3 in the absence of CTLA-4, but that these cells were not suppressive. In contrast, sorting of activated CTLA-4+/H11001 T cells strongly enriched for FoxP3 expression and these cells were suppressive in functional assays. To determine whether CTLA-4 was capable of suppression we transfected CTLA-4 into CD25+/H11002 FoxP3+/H11002 T cells. This method did not induce FoxP3 but could confer suppressive activity. Conversely, expression of FoxP3 alone did not up-regulate CTLA-4 and these cells were not suppressive. Taken together these data provide evidence that a bona fide population of CTLA-4+/FoxP3+ Tregs is induced upon human T cell activation, which is regulatory, and that CTLA-4 but not FoxP3 expression is critical for suppressive function.

Materials and Methods

Purification of T cells

Human CD4+CD25+ and CD4+CD25- T cells were purified by either cell sorting or using specific anti-CD25 microbeads. PBMC were isolated from fresh buffy coats, provided by The National Blood Transfusion Services (Birmingham, U.K.), using Ficoll-Paque density centrifugation. CD4+ T cells were isolated by incubating PBMC with human CD4+ T cell enrichment mix according to the manufacturer’s instructions (StemSep; StemCell Technologies). To purify CD25+ T cells, CD4+ cells were incubated with anti-CD25 microbeads (Miltenyi Biotec) at 4°C for 30 min. CD4+CD25+ T cells that did not bind to the column were collected from the flow-through and stained 36 h later for Foxp3 with the Abs shown. CD4+CD25+ T cells stimulated with CD3/CD28 coated beads were stained 36 h later for Foxp3 with the Abs shown. CD4+CD25+ T cells sorted on a MoFlo cytometer (DakoCytomation). Where cells were sorted for CTLA-4 expression, stimulated T cells were labeled for 1 h at 37°C with anti-CTLA-4 PE, placed on ice, and stained for 30 min with CD25 allophycocyanin.
Cells were sorted on a MoFlo cytometer (DakoCytomation) based on the expression of CTLA-4 and CD25.

Flow cytometry

CD25-PE Cy5, CD69-FITC, CTLA-4-PE, and CTLA-4-allophycocyanin were purchased from BD Pharmingen. Anti-Foxp3-PE (clone PCH101) was from eBioscience. The 206D-Alexa Fluor 488 FoxP3 Ab was purchased from Biolegend. Cells were stained for total CD25 and using anti-CDTA-4 on fixed and permeabilized cells and recycling CTLA-4 in live cells at 37°C. Cells were acquired on a BD FACSCalibur and analyzed using FlowJo software.

T cell stimulation

Purified CD25+ T cells were stimulated with anti-CD3/anti-CD28 coupled Dynabeads (Dynal) or mature dendritic cells (DCs) (10:1 or 100:1 ratio of T cells to DCs). DCs were grown from monocytes using GM-CSF and IL-4 as previously described (15). For purified protein derivative (PPD) stimulation, autologous DC were generated as described and 5 × 10^5 DCs were added to 10^5 purified CD25+ T cells. Cells were cultured for 5 days and pulsed with 1 μCi/well of [3H]thymidine during the last 16 h of culture, and incorporation was measured by scintillation counting. In some experiments CD25+ T cells were stimulated using CD80 or CD86 transfectants plus anti-CD3 (1 μg/ml) as previously described (24) and analyzed by FACS at the time points indicated. For IL-2 treatment, CD4+CD25+ T cells were cultured in RPMI 1640 medium containing IL-2 (10 ng/ml) for the times shown. Fresh IL-2 was added to the culture every 2 days.

Suppression assays

Functional suppression assays were performed using an MLR in which induced Tregs (1 × 10^6) were cocultured with responder T cells at a ratio of 1:1 in the presence of monocyte-derived DCs. Blocking anti-CTLA-4 Ab (BNI3-Pharmingen) was used at 10 μg/ml where indicated. For time course experiments, IL-2-treated CD4+CD25+ T cells were harvested at each time point, frozen in 40% FCS, 10% DMSO, and 50% RPMI 1640, and stored in liquid nitrogen. At the end of the IL-2 culture, all samples were thawed on the same day and added to the 96-well containing responder T cells and DCs to measure inhibition. Assays were incubated for a further 5 days, and during the last 16 h [3H]thymidine was added at 1 μCi/well. [3H]Thymidine incorporation was measured by scintillation counting, and proliferative responses were expressed as the mean of triplicate wells ± SD. Results shown are representative of a minimum of three experiments performed.

PCR analysis of gene expression

Foxp3 expression was measured by RT-PCR and real-time RT-PCR. Total RNA extracted from purified CD4+CD25+ T cells treated with IL-2 with TRIZol reagent (Invitrogen) and first-strand cDNAs were synthesized by reverse transcription (Superscript First-strand Synthesis System for RT-PCR kit; Invitrogen). Quantitative mRNA expression was measured by real-time PCR with PRISM 7700 sequence detection system (PE Applied Biosystem), and the TaqMan Mastermix kit with β-actin mRNA was used as internal control. The primers and the internal fluorescence TaqMan probes were designed as follows: Foxp3 (forward) 5'-GAGAAGCTGAGTGCCATGCA-3', (reverse) 5'-AAGAGCCCTTGTGGATGAT-3', and probe 5'-FAM-ATGGCACTGACCAAGGCTTCATTG-eclipse dark-3', and β-actin 5'-CCTGCACCCAGGACAATCC-3', 5'-GCGGATCCACACGAGTACT-3', and probe 5'-VIC-ATCGAGATCATGTGCTCTGGACGGCCA-Yahima yellow-3'. The program used for amplification was 45 cycles of 15 s at 95°C and 1 min at 60°C.

CFSE labeling

To determine cell division, T cells were washed twice with PBS and incubated with 2.5 μM CFSE for 10 min at 37°C, agitating gently. The labeling reaction was quenched by the addition of RPMI 1640 containing 10% FCS. The cells were washed three times with medium and resuspended in medium at a concentration of 2 × 10^6 cell/ml.

T cell transfection

CD4+CD25+ T cells freshly isolated from buffy coat were transfected with plasmids containing full-length CTLA-4 and Foxp3 cDNA using Amaxa human T cell nucleofector kit according to the manufacturer’s protocol. Briefly, 4 × 10^6 CD4+CD25+ T cells were resuspended in the 100 μl of nucleofector solution. The 3 μg of plasmid DNA was added and transfected using program U014 or V024. At 24 h postnucleofection, cells were analyzed for expression by flow cytometry and used for functional assays by cocultured with responder T cells and DCs as described.

Results

CTLA4 expression induced after activation of CD4+CD25+ T cells is strongly correlated with Foxp3 expression

During T cell activation experiments, we observed that human CD25+ T cells showed a significant increase in Foxp3 mRNA, as measured by RT-PCR (Fig. 1a). To confirm this observation and to analyze the expression of Foxp3 protein, we carried flow cytometric analysis to determine Foxp3 expression under different activation conditions. Purified CD4+CD25+ T cells depleted of endogenous CD25+ Tregs were stimulated with anti-CD3/CD28 beads or anti-CD3 plus CD80 or CD86 transfectants and examined for Foxp3 induction. This revealed that activation of CD4+CD25+ cells resulted in the appearance of a distinct population (in ~10–30%) of Foxp3½-high-expressing cells (Fig. 1b). This increase was seen with both anti-CD3/CD28 Ab stimulation and importantly with costimulation using transfectants expressing the natural
CD28/CTLA-4 ligands. It was also clear that some cells expressed lower levels of FoxP3 staining. However, whether this reflects genuine differences in FoxP3 protein levels is the subject of debate (21, 25). To further validate our FoxP3 staining we therefore activated T cells and compared FoxP3 expression using a second FoxP3 Ab 206D (Fig. 1c). Staining with both 206D and PCH101 revealed induction of FoxP3 in a similar fraction of the cells. Notably, the presence of a significant population of nonactivated cells provided an internal negative population. Dual staining with both Abs clearly showed that these Abs recognized the same cell populations expressing FoxP3, providing no evidence that PCH101 erroneously identifies FoxP3+ cells. Finally, we also analyzed how the level of FoxP3 affected the expression of CTLA-4 because CTLA-4 expression is a notable feature of FoxP3+ Tregs. Activated CD25− T cells were therefore gated on low medium or high expression of FoxP3 as shown in Fig. 1b and analyzed for CTLA-4 expression. This process revealed a strong correlation between FoxP3 and CTLA-4 expression (Fig. 1d), with cells gated for high levels of FoxP3 expressing much higher levels of CTLA-4. Taken together these data were consistent with the concept that FoxP3 enhances CTLA-4 expression and that activation of human CD4+CD25− T cells induced a population of cells expressing FoxP3 and high levels of CTLA-4.

Because purified CD25− T cells contain a small population of FoxP3-expressing cells that cannot be depleted by removing...
CD25+ cells we sought to establish whether the increase in CTLA-4high FoxP3+ cells resulted from the expansion of pre-existing Tregs or whether it was due to de novo expression. We therefore performed kinetic experiments to determine the expression of CTLA-4 and FoxP3. Purified CD4+CD25- T cells were stimulated using CD3/CD28 beads and then stained for FoxP3 and CTLA-4 at various time points. This process revealed that significant FoxP3 expression was observed by 24 h (Fig. 2a), which is before T cell division takes place in this system. Interestingly, we also observed that although there is initially a strong coexpression of CTLA-4 and FoxP3 by 120 h, the majority of CTLA-4+ cells clearly lacked FoxP3. This observation suggested that before cell division there is a strong correlation between CTLA-4 and FoxP3 expression, but that over time FoxP3 expression diminishes, whereas CTLA-4 is maintained. To support these kinetic experiments we also performed CFSE labeling studies. Again this experiment confirmed that an increase in FoxP3 could be observed by 24 h in the absence of cell division (Fig. 2b), demonstrating that these cells are indeed induced FoxP3-expressing cells. Overall these data demonstrated that activation of human CD4+CD25- T cells induced a distinct population of FoxP3-expressing cells that, initially, strongly coexpress CTLA-4 but eventually expression of FoxP3 gradually diminishes.

**Effect of IL-2 on FoxP3 and CTLA-4 expression**

Because IL-2 is established as an important factor in Treg homeostasis, and has been proposed to influence CTLA-4 expression, we investigated the effect of IL-2 on the expression of FoxP3 and CTLA-4 in our in vitro system. The addition of blocking Abs to IL-2 resulted in a significant decrease in the percentage of cells expressing FoxP3 (Fig. 3a) as well as a decrease in the level of CD25, indicating that IL-2 is an important factor for maintaining inducible FoxP3+ cells. Interestingly, the inhibitory effect of blocking IL-2 on CD25 expression was more apparent in the FoxP3+ cells, suggesting that FoxP3 may be able to sustain CD25 expression in the absence of IL-2. In addition, we observed that, FoxP3+ cells maintained CTLA-4 expression in the absence of IL-2. To complement these studies, we examined the effect on FoxP3 and CTLA-4 expression of supplementing CD4+CD25- T cells cultures with IL-2 (Fig. 3b). This revealed, that CD4+CD25- cells cultured in IL-2 could acquire expression of FoxP3 by 48 h. However, in this system in which there was no overt T cell stimulation, FoxP3 expression could be induced without CTLA-4. Accordingly FoxP3 was evident by 48 h, whereas CTLA-4 expression was not observed until later time points. To examine the functional effects of treatment with IL-2, we tested whether these cells could influence T cell alloreponses stimulated by cultured DCs. CD25- T cells were harvested each day from IL-2 culture and frozen. At the end of the experiment, T cells were thawed and tested simultaneously in a suppression assay. The results of this analysis revealed that cells cultured in IL-2 for 24–48 h, which lacked CTLA-4 expression, did not possess suppressive capacity (Fig. 3c); however, from day 3 onward there was evidence of substantial suppression of T cell responses, which correlated with up-regulation of FoxP3.

**CTLA-4 expression identifies functional Tregs**

The described experiment suggested that CTLA-4 expression was associated with T cell suppression. We therefore conducted cell sorting experiments based on CTLA-4 expression. By staining cells for CTLA-4 at 37°C, we could detect live cells that traffic CTLA-4 to the plasma membrane. This effectively labeled CTLA-4+ cells but did not require cell permeabilization, which precludes subsequent functional analysis. Because we had observed that up to 48 h after stimulation with CD3/CD28 beads, blocking Abs to IL-2, we were able to acquire expression of FoxP3 by 48 h, whereas CTLA-4 expression was not observed until later time points. To examine the functional effects of treatment with IL-2, we tested whether these cells could influence T cell alloreponses stimulated by cultured DCs. CD25- T cells were harvested each day from IL-2 culture and frozen. At the end of the experiment, T cells were thawed and tested simultaneously in a suppression assay. The results of this analysis revealed that cells cultured in IL-2 for 24–48 h, which lacked CTLA-4 expression, did not possess suppressive capacity (Fig. 3c); however, from day 3 onward there was evidence of substantial suppression of T cell responses, which correlated with up-regulation of FoxP3.

**FIGURE 4.** Sorting for CTLA-4 expression at 48 h identifies functional Tregs. a, CD4+CD25- T cells stimulated with CD3/CD28 beads were stained for CTLA-4 (37°C) or CD25 and sorted into the regions (R1-R3) shown by the populations CD25-/CTLA-4¬ (R1), CD25+/CTLA-4- (R2), and CD25+/CTLA-4+ (R3). b, Cells were sorted into the regions shown in a and recovered for mRNA analysis of FoxP3 expression by PCR. c, CD4+CD25- T cells stimulated and sorted as shown in a were compared with natural Tregs for their ability to suppress alloreponses. Fresh CD4+CD25- T cells were stimulated by DC at different ratios (1:10 or 1:100 ratio for DC to T cells) in the presence of sorted populations shown. Proliferative responses were determined by titrated thymidine incorporation as measured by scintillation counting at day 5 of culture. Data are representative of three experiments.
CTLA-4 expression is largely confined to FoxP3+ cells, we stimulated CD25− T cells for 48 h and sorted on the basis of CTLA-4 expression. Cells were sorted into three populations CD25+/CTLA-4− (R1), CD25+/CTLA-4− (R2), and CD25+/CTLA-4− (R3) populations as shown in Fig. 4a. Populations were then analyzed for expression of FoxP3 by RT-PCR and for functional suppression. This analysis demonstrated that the CTLA-4+ cells labeled at 37°C contained the high levels of FoxP3 mRNA in comparison to CTLA-4− cells (Fig. 4b). Furthermore, CTLA-4+ cells were very effective at suppressing alloresponses, whereas CTLA-4− cells were not (Fig. 4c). These data clearly demonstrate that selecting CTLA-4+ cells at 48 h after stimulation, i.e., before cell division, enriches for a fraction of functionally competent Tregs that have been induced following stimulation. We therefore concluded that induced CTLA-4+ FoxP3+ cells generated during in vitro stimulation were functional and possessed suppressive capacity similar to natural Tregs.

To determine whether CTLA-4 expression was sufficient to confer suppression to CD4+CD25− T cells, we transfected resting T cells with CTLA-4 cDNA using the Amaxa Nucleofector and used them in a suppression assay. This revealed that cells transfected with CTLA-4 efficiently expressed CTLA-4 protein (Fig. 5a) compared with mock transfected cells. Furthermore, these cells themselves were unresponsive to stimulation (data not shown). Strikingly, CTLA-4-transfected T cells potently suppressed the activation of normal resting CD4+CD25− T cells (Fig. 5b). Furthermore, CTLA-4 transfected cells did not up-regulate FoxP3, indicating that FoxP3 expression was not required to for this regulatory function. Conversely, we also transfected FoxP3 into resting T cells and observed that FoxP3 expression alone did not induce CTLA-4 and nor did these cells have suppressive capacity. Taken together these data suggested that the acquisition of suppressive function by Tregs requires CTLA-4 but not FoxP3 expression. Finally to confirm the role of CTLA-4 in suppression we conducted experiments in the presence of anti-CTLA-4 Ab. This analysis showed that anti-CTLA-4 was able to reverse the suppressive capacity of CTLA-4-transfected T cells (Fig. 5c).

To confirm that suppression by CTLA-4-transfected cells reflected inhibition of responder T cells rather than differences in proliferation by the transfected cells, we labeled responder T cells with CFSE and monitored cell division. This result clearly showed that the suppressive effect of CTLA-4-transfected cells seen in thymidine incorporation assays reflects a decrease in responder T cell division as measured by CFSE (Fig. 6a). Finally, we also tested whether the CTLA-4-mediated suppression could affect Ag-specific responses other than alloantigen. We therefore stimulated
T cells with autologous DCs plus the recall Ag PPD. Once again the addition of CTLA-4-transfected T cells compared with mock transfected T cells resulted in clear inhibition of PPD responses and similar data were also seen when stimulating with the superantigen staphylococcal enterotoxin B (data not shown). Overall we concluded that expression of CTLA-4 conferred suppressive activity in several different T cell stimulation settings.

Discussion
The role of CTLA-4 in Treg function has been the subject of considerable debate. In this study, we investigated the relationship between CTLA-4 and FoxP3 induced during human T cell activation. We provide evidence of an initial strong correlation between FoxP3 expression and that of CTLA-4 in the early stages of activation and that isolating these cells demonstrates suppressive function, consistent with induced Tregs. Furthermore we provide evidence that suppressive function relates to CTLA-4 expression rather than to FoxP3 expression.

It is now clear that human CD4+CD25− T cells can be induced to express FoxP3 by T cell stimulation without additional cytokines (7, 8, 23). Nonetheless, the issue of whether these induced FoxP3+ cells reflect the induction of functional Tregs remains controversial. We examined the regulation of both CTLA-4 and FoxP3 on activated human CD4+CD25− T cells and observed a strong correlation between expression of CTLA-4 and FoxP3 at early time points (24–48 h). This tight relationship is consistent with the view that CTLA-4 is a possible target of FoxP3 transcriptional regulation that may occur in cooperation with other transcription factors such as NF-AT (16). However, we also observed that normally activated T cell blasts can express CTLA-4 in the absence of FoxP3 at later time points. Thus, although FoxP3 is not absolutely required for CTLA-4 expression, our data suggest that FoxP3 may facilitate the expression of CTLA-4 before cell division and that following cell division FoxP3 is not required. This concept is in keeping with the fact that natural FoxP3+ Tregs maintain CTLA-4 expression in the absence of overt stimulation. In contrast, T cell blasts appear to express higher levels of CTLA-4 with increasing cell divisions (24, 26). Therefore the regulation of CTLA-4 expression in Treg and nonregulatory T cell blasts appears to differ, in both kinetics and the dependence on FoxP3. We suggest that FoxP3 expression may facilitate the early expression of high levels of CTLA-4, which could then contribute to the “anergic” status typically ascribed to Tregs in vitro (27). In keeping with this suggestion, we observe that CTLA-4–transfected T cells are relatively unresponsive to normal stimulation.

By exploiting the relationship between CTLA-4 and FoxP3 early after T cell activation, we have been able to purify induced FoxP3+ cells and assess their suppressive function. Strikingly, we observed that cell sorting on CTLA-4 expression selected for functionally suppressive T cells, expressing FoxP3. Thus our observations appear similar to those observed originally by Walker et al. (7). One possible reason for the differences between our conclusions and those who find that CD25− cells induced to express FoxP3 are not suppressive (8, 22, 28) is that it has not been possible previously to isolate these FoxP3+ cells effectively. Consequently, proliferation or cytokine production from contaminating nonregulatory T cells may mask any regulatory effects. In some studies, T cells with induced FoxP3 have been analyzed for their intrinsic ability to produce cytokines such as IL-2 and IFN-γ as a measure of FoxP3 function. The results have led to the conclusion that because these cytokines are still transcribed in FoxP3+ cells, FoxP3 is therefore not functionally active (8, 22). However, interpretation of this analysis is complicated by the relative timing of cytokine gene expression when compared with FoxP3. Thus, if cytokine genes are transcribed before FoxP3 is induced, it might appear that FoxP3 expression does not inhibit cytokine gene expression and is therefore nonfunctional. In contrast, we find clear evidence that sorting of CTLA-4+ FoxP3+ T cells identifies induced FoxP3+ cells that are capable of suppressing alloresponses.

Experiments directly expressing FoxP3 using retroviral vectors to infect CD25− T cells have also yielded conflicting results, regarding the role of FoxP3 in suppression. It was concluded that FoxP3 expression confers suppression (29), whereas another study found it insufficient (28). In our experiments, transfection of FoxP3 into resting T cells did not confer Treg activity. We suggest that whether FoxP3 confers suppression may depend on its ability to induce CTLA-4. Our ability to transfect CTLA-4 and confer suppression strongly supports a role for CTLA-4. Importantly, we found clear evidence that FoxP3 expression alone is not sufficient for the induction of CTLA-4 in resting T cells. Thus, the level of CTLA-4 expression achieved during T cell stimulation may underlie the differences in suppression observed in several reports. It is noteworthy that in studies using retroviral FoxP3 transduction, the conditions used involve “preactivation” of T cells prior transduction. However, these are quite variable and make use of soluble anti-CD28 Abs or anti-CD3 Abs and APC (28, 29). Nonetheless, these conditions are designed not to induce FoxP3 themselves, and
it is therefore likely that CTLA-4 is not also effectively induced. Consequently, variable levels of CTLA-4 expression caused by preconditioning of T cells before transduction may affect the conclusions from these studies.

An additional possibility is that CTLA-4 expression and suppressive capacity may be influenced by the level of FoxP3 expression during activation. Transgenic mice generated by Wan and Flavell (30), expressed low levels of FoxP3 and demonstrated impaired suppression. Notably, these cells also expressed much lower levels of CTLA-4 compared with normal FoxP3+ T cells. Indeed, in other studies, lack of suppressive function also correlates with low levels of CTLA-4 expression in Tregs. For example in FoxP3-null T cells, it is clear that despite undergoing aspects of Treg differentiation, these cells were nonfunctional and again CTLA-4 expression was highly impaired (31). Likewise in a FoxP3 retroviral transduction study (28), very modest CTLA-4 expression was achieved; however, in experiments in which some suppression was observed, this correlated with higher expression of CTLA-4 (28).

We directly addressed the role of CTLA-4 expression in human Treg function by transfecting CTLA-4 directly into resting human T cells. This experiment allowed CTLA-4 expression before T cell activation, thereby mimicking the expression pattern of CTLA-4 seen in Tregs. Strikingly, we observed that T cells acquired potent suppressive capabilities demonstrating that CTLA-4 may be a direct mediator of Treg function. This finding is consistent with a number of murine studies that also indicate a functional role for CTLA-4 on Treg on Treg. Anti-CTLA-4 Abs can clearly abrogate Treg function in settings in which CTLA-4 expression was restricted only to Tregs (12). Furthermore, bone marrow chimera experiments demonstrated a suppressive role for CTLA-4-expressing cells consistent with that of Tregs (32), a finding further supported by more recent chimera experiments (33). Although our data are supportive of a role for CTLA-4 in Treg function, the level of expression obtained by transfection is higher than the level found in natural Tregs. Therefore, experiments using variable levels of CTLA-4 expression would be useful to further validate our findings.

The question of how CTLA-4 may affect the function of Tregs is at present unresolved. However, several distinct mechanisms are possible (see review in Ref. 34). In the context of the present experiments, perhaps the most plausible mechanisms include the activation of IDO pathway or direct competition for CD28 ligands on APC. However it is also possible that CTLA-4 ligation signals the generation of additional downstream effectors in the Tregs. To date, we have been unable to demonstrate a role for IDO in our system and therefore prefer other models in which CTLA-4 affects the APC because we observe clear dose effects of APC numbers on the efficacy of CTLA-4-mediated suppression. Whether CTLA-4 expressed on activated T cells acts in a regulatory capacity is unknown. However, although it is not impossible that activated CTLA-4+ T cells could act in a regulatory manner, it is notable that Tregs have significantly higher levels of CTLA-4. Furthermore the ability of T cell blasts to make cytokines such as IL-2 could serve to override regulatory potential conferred by CTLA-4 expression.

Despite good evidence for CTLA-4 in Treg function, some data from CTLA-4 knockout mice have suggested that CTLA-4 expression is not absolutely required for suppression, indicating that alternative suppressive mechanisms can operate in the absence of CTLA-4 (10, 12). Overall, however, a significant body of data indicates that CTLA-4 is an important component of Treg suppression and is likely to represent a significant therapeutic target for modulating Treg behavior.

Disclosures
The authors have no financial conflict of interest.

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