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A Transendocytosis Model of CTLA-4 Function Predicts Its Suppressive Behavior on Regulatory T Cells

Tie Zheng Hou,* Omar S. Qureshi,[†] Chun Jing Wang,* Jennifer Baker,[†] Stephen P. Young,[†] Lucy S. K. Walker,* and David M. Sansom*

Manipulation of the CD28/CTLA-4 pathway is at the heart of a number of immunomodulatory approaches used in both autoimmunity and cancer. Although it is clear that CTLA-4 is a critical regulator of T cell responses, the immunological contexts in which CTLA-4 controls immune responses are not well defined. In this study, we show that whereas CD80/CD86-dependent activation of resting human T cells caused extensive T cell proliferation and robust CTLA-4 expression, in this context CTLA-4 blocking Abs had no impact on the response. In contrast, in settings where CTLA-4⁺ cells were present as “regulators,” inhibition of resting T cell responses was dependent on CTLA-4 expression and specifically related to the number of APC. At low numbers of APC or low levels of ligand, CTLA-4-dependent suppression was highly effective whereas at higher APC numbers or high levels of ligand, inhibition was lost. Accordingly, the degree of suppression correlated with the level of CD86 expression remaining on the APC. These data reveal clear rules for the inhibitory function of CTLA-4 on regulatory T cells, which are predicted by its ability to remove ligands from APC. *The Journal of Immunology*, 2015, 194: 2148–2159.

Activation of T cells in secondary lymphoid organs takes place at the interface between T cells and APC. Typically, APC at sites of infection upregulate CD80 and CD86 in response to signaling by TLR or other microbial pattern recognition receptors and migrate to lymph nodes (1–3). As a result, APC increase both in number and level of costimulatory molecule expression, resulting in the initiation of T cell responses in a CD28-dependent manner (4–6). CD28 signaling is important in the expansion, survival, and helper function of T cells (7–10). Against this background, the inhibitory receptor CTLA-4 shares the same ligands with CD28 but opposes T cell responses such that the absence of CTLA-4 results in autoimmune T cell activation with accompanying tissue infiltration and destruction (11, 12). The expression of CTLA-4 on both regulatory T cells (Treg) as well as activated T cells raises the issue of the mechanism by which CTLA-4 acts and the immunological context where inhibition takes place. A surprisingly large number of models of CTLA-4 function have been proposed, including both cell-intrinsic and cell-extrinsic mechanisms (13–15). However, the ability of these models to predict CTLA-4 functional behavior is variable. For example, despite popular perceptions of CTLA-4 as

an inhibitory signal for T cell activation, a consistent body of literature indicates that the major function of CTLA-4 *in vivo* is via a cell-extrinsic pathway (13), that is, that CTLA-4 influences the cells around it rather than the cells expressing it. Therefore, although the role of CTLA-4 as a negative regulator is well established, the context for its effective function is not. Ultimately, understanding how to predictably measure and understand CTLA-4 function in humans has considerable implications in autoimmune settings as well as other disorders involving immune dysregulation.

We recently proposed a model for CTLA-4 function whereby the central feature was the ability of CTLA-4 to capture ligands (CD80 and CD86) from APC and degrade them inside the CTLA-4-expressing cell (16). Such a mechanism is a form of cell-extrinsic ligand competition that makes several predictions for CTLA-4 function. Most obvious is that CTLA-4 function should be evident only when it depletes ligands to below a level sufficient for CD28 costimulation. A corollary of this concept is that the amount of ligand on the APC relative to the amount of CTLA-4 on T cells should dictate whether the threshold for CD28 costimulation is achieved. Accordingly, in situations where the supply of ligand is limited then consumption by CTLA-4 should be more functionally effective and vice versa.

We therefore set out to test how parameters such as the number of APC and their relative ratio to CTLA-4⁺ cells affected the ability of CTLA-4 to regulate T cell activation. Using a model system, we demonstrate that the efficacy of suppression by CTLA-4 is dictated by the total amount of costimulatory molecules in the system. Under conditions favoring CTLA-4 function there was effective depletion of costimulatory ligands sufficient to suppress T cell responses, and the degree of suppression was tightly correlated with the observed downregulation of ligands on APC. In contrast, under unfavorable conditions with high levels of ligands, CTLA-4 continued to function; however, its impact on T cell proliferation was minimal because sufficient ligand still remained. Based on this model system, we tested the ability of natural Treg to suppress T cell responses. We observed that in accordance with our model, CTLA-4-dependent suppression was profoundly influenced by the ratio of APC/Treg and corresponded

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Abbreviations used in this article: CHO, Chinese hamster ovary; DC, dendritic cell; Treg, regulatory T cell.

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with the level of CD86 downregulation. In contrast, no CTLA-4-dependent inhibition was observed during stimulation with CD3/CD28 Abs, showing that CTLA-4 suppressive function was strictly dependent on ligand-driven T cell activation. Taken together, these data establish immunological contexts that predict the functional capacity of CTLA-4 that are consistent with a model whereby an important function of CTLA-4 is to deplete its ligands from APC in a T cell-extrinsic manner.

Materials and Methods

Cell lines and culture

Chinese hamster ovary (CHO) cells transfected with human CD80, CD86, CTLA-4, and Jurkat cells transduced with human CTLA-4 as previously described (17) and CHO cells with CD86GFP fusion protein with GFP tagged to the C terminus of CD86 were previously described (16). CHO transfectants were maintained in DMEM (Invitrogen) complete culture media containing 10% FBS (Biosera), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin and streptomycin (Invitrogen). Jurkat cells were maintained in RPMI 1640 (Invitrogen) complete culture media containing 10% FBS (Biosera), 1% L-glutamine (Sigma-Aldrich), and 1% penicillin and streptomycin (Invitrogen). All cells were cultured at 37°C in 5% CO₂ as previously described (18).

Preparation of human T cells and dendritic cells

PBMC were isolated from fresh human blood leukocyte cones, provided by the National Blood Transfusion Services (Birmingham and Colindale, U.K.), by density centrifugation on Ficoll-Paque Plus (GE Healthcare). Human monocytes and T cells were isolated using an EasySep kit (StemCell Technologies) according to the manufacturer's instructions. Briefly, to deplete CD4⁺CD25⁺ Treg, CD4⁺CD25⁻ T cells were purified by negative selection using an EasySep human CD4⁺CD25⁻ T cell enrichment kit. Naive T cells were purified by negative selection using an EasySep Human CD4⁺CD25⁻ T cell and then naive CD4⁺ T cell enrichment kit. Treg were purified by negative selection using an EasySep human CD4⁺ T cell and then CD4⁺CD127^{low}CD49d⁻ Treg enrichment kit. Cells were collected, washed, and used in T cell stimulation experiments. CD3⁻CD11c⁺CD14⁺ monocytes were purified by negative selection using a human monocyte enrichment kit. To generate dendritic cells (DC), monocytes were differentiated by culturing in RPMI 1640 (Invitrogen) DC culture media containing GM-CSF at 20 ng/ml and IL-4 at 100 ng/ml (PeproTech) at 37°C for 7 d.

Flow cytometry

T cells were labeled with anti-human CD3 FITC; CD4 PE, allophycocyanin, or PB; CD11c allophycocyanin; CD14 PerCP; CD25 PE; CD45RA PE; CD45RO FITC; CD49d FITC; CD80 PE; CD86 FITC; CD127 PE-Cy7; and CTLA-4 PE (BD Pharmingen) and Foxp3 allophycocyanin (eBioscience) Abs at the recommended concentrations at 4°C in RPMI 1640 buffer with 2% FBS. CTLA-4 staining was carried out on fixed cells using 2% paraformaldehyde and permeabilized T cells with 0.05% saponin. FOXP3 intracellular staining was performed using a FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. For intracellular cytokine staining, after 5 d of stimulation samples were restimulated with PMA (50 ng/ml), ionomycin (1 μM), and brefeldin A (5 μg/ml) for 4 h to induce cytokine production, and the cells were then fixed with 3% paraformaldehyde and permeabilized in a 0.1% PBS-saponin solution before being stained for IL-2 production. For cell sorting, Far Red⁺ CHO-CD86GFP cells were purified to >98% purity on a MoFlo sorter (Dako) before being used for T cell stimulation. All stained cell after washes were acquired on CyAn flow cytometer (Dako) using Summit software. Data analysis was performed using FlowJo software (Tree Star).

BIAcore and Ab blocking assay

Anti-CTLA-4 binding affinity was determined using surface plasmon resonance using a BIAcore 3000 instrument. CTLA-4Ig was bound through amine coupling to a CM5 chip as a target for Ab binding. Increasing concentrations of tremelimumab, BNI3, 11G1, and 10A8 were then passed over the chip at 25°C for 30 min and the amount of binding was detected. The maximal response from each dilution was plotted as a graph and the affinity of the Ab calculated from the slope. In assays of blocking CTLA-4 binding to its ligands, various dilutions of the Abs were incubated with CTLA-4Ig that had been prelabeled with a PE fluorophore. To this, CHO cells expressing either CD80 or CD86 were added for 30 min on ice. After washing, the detection of bound CTLA-4Ig was carried out by flow cytometry.

Immunofluorescence staining and confocal microscopy

For colocalization of CD86 and CTLA-4, CHO cells were incubated together for 16 h on a poly-L-lysine coated coverslip in a 24-well plate. Cells were then fixed with methanol at -20°C for 20 min. Nonspecific binding sites were blocked by incubation in blocking solution, which consisted of 5% donkey serum (Sigma-Aldrich) in PBS at room temperature. Cells were incubated with anti-CD86 primary Ab (B7-2, C19 Ab; Santa Cruz Biotechnology) and anti-CTLA-4 (BNI3; BD Pharmingen), washed, and then labeled with donkey anti-goat Alexa Fluor 546 and anti-mouse Alexa Fluor 488 secondary Abs (Molecular Probes). Imaging was carried out using a Zeiss LSM 510 or a Zeiss LSM 780 inverted laser scanning confocal microscope using a ×63 oil immersion objective with excitation at 488 and 543 nm. Constant laser powers and acquisition parameters were maintained throughout individual experiments for analysis. Digital images were prepared using ImageJ (Wayne Rasband, National Institutes of Health). All confocal images shown are representative of at least 30 micrographs.

CD86 transendocytosis assays

CD86 transfer from donor CHO cells expressing CD86-GFP fusion protein into recipient CHO cells expressing CTLA-4 was measured by flow cytometry. Donor CD86-GFP cells were labeled with CellTrace Far Red DDAO-SE labeling kit (Molecular Probes) according to the manufacturer's instructions. In brief, CD86GFP⁺ CHO cells were incubated together with CTLA-4⁺ or control CHO cells in round-bottom 96-well plates at 37°C at various ratios for times stated. Cells were then disaggregated by pipetting, singlets were gated, and GFP was measured in both donors (Far Red⁺) and recipients by FACS. Anti-CD86 was also used to stain for residual CD86 expression on the donor cells in some cases.

T cell proliferation assays

Resting T cells were washed with PBS and incubated with a CellTrace Violet cell proliferation kit (Molecular Probes) according to the manufacturer's instructions. The reaction was quenched followed by PBS wash and cells were resuspended at 1.8 × 10⁶ cells/ml before use as responder T cells. All T cell proliferation assays were performed in 250 μl RPMI 1640 complete culture media. Responder T cells (0.9 × 10⁵) were stimulated with soluble anti-CD3 Ab (OKT3; hybridoma from American Type Culture Collection) at 0.5 μg/ml or Dynabeads human T-activator CD3/CD28 (Invitrogen) in 96-well flat-bottom plates. To provide costimulation signals, monocyte-derived DC or fixed CHO transfectants expressing human CD80 and CD86 were added at the indicated ratio as previously described (16). Cells were cultured for 5 d in presence or absence of 10 μg/ml CTLA-4Ig (abatacept [Orencia]; Bristol-Meyers Squibb) to block CD80 and CD86. Division of responder T cells was measured by the dilution of violet dye using flow cytometry. Live proliferating T cell counts were performed using counting beads (Dako) and analyzed using FlowJo software.

T cell suppression assays

In suppression assays, unlabeled CTLA-4⁺ Treg, Jurkat cells, or T cell blasts were used as suppressors and added into violet-labeled responder T cells in the presence or absence of anti-CTLA-4 blocking Abs (tremelimumab provided by Pfizer) at 40 μg/ml. When using purified Treg or T cell blasts, these were negatively selected (untouched). When using Jurkat cells, their proliferation was inhibited by incubating with mitomycin C (100 μg/ml; Sigma-Aldrich) at 37°C for 1 h prior to adding into responder T cell culture. Activated T cells (T blasts) were generated by simulating naive CD4 T with DC at a DC/T ratio of 1:10 and soluble anti-CD3 Ab (OKT3; hybridoma from American Type Culture Collection) at 0.5 μg/ml at 37°C for 7 d. To deplete DC, cells were incubated with anti-CD11c PE followed by anti-PE microbeads. CD11c⁺ DC were discarded by passing through an MS column (Milteni Biotec), and negative selected CTLA-4⁺ T blasts were then collected. T cell proliferation was measured as above.

Results

CTLA-4 expression does not intrinsically restrain primary T cell responses

In an attempt to understand the settings where CTLA-4 inhibits T cell responses that are specifically driven by the natural CD28 ligands, we established costimulation assays using cells transfected with either CD80 or CD86. In these experiments T cells were stimulated by either ligand in the presence of anti-CD3, and

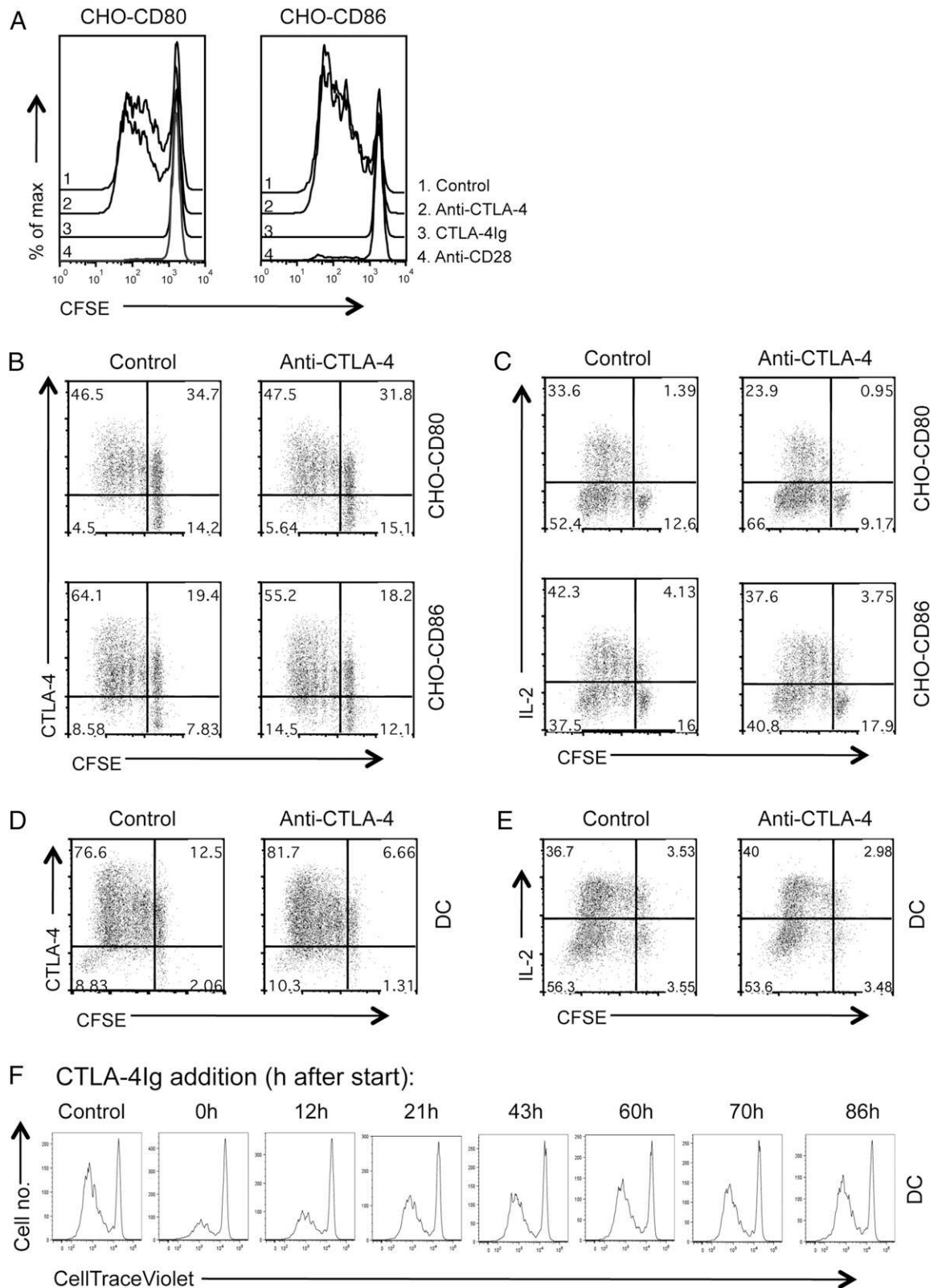


FIGURE 1. CTLA-4 blockade has little impact on resting T cell proliferation or IL-2 production. CFSE-labeled responder CD4 T cells were stimulated with 0.5 $\mu\text{g/ml}$ soluble anti-CD3 and CHO-CD80- or CHO-CD86-expressing cells (**A–C**) or monocyte-derived DC (**D** and **E**). (**A**) T cell stimulations were carried out in the presence or absence anti-CTLA-4 Ab (tremelimumab; 40 $\mu\text{g/ml}$), CTLA-4 Ig (abatacept; 10 $\mu\text{g/ml}$), or anti-CD28 Ab (10 $\mu\text{g/ml}$) for 5 d and analyzed by flow cytometry. (**B** and **C**) Cells stimulated as in (**A**) were analyzed for cell division and total CTLA-4 expression in the presence or absence of CTLA-4 blockade or for IL-2 production. In (**D**) and (**E**), T cell responses were stimulated with monocyte-derived DC in the presence of anti-CD3 and analyzed for CTLA-4 expression (**D**) and IL-2 production (**E**). For cytokine analysis, cultures were restimulated at day 5 with PMA/ionomycin in the presence of brefeldin A for 4 h. The data presented are representative of five independent experiments. (**F**) CD80/CD86 control commitment of CD4 T cells in the first 24–48 h. CellTrace Violet-labeled naive CD4 conventional T cells were stimulated by soluble anti-CD3 (0.5 $\mu\text{g/ml}$) and human monocyte-derived DC in the presence of CTLA-4Ig (abatacept at 10 $\mu\text{g/ml}$) at indicated times after culture setup. Data are representative of three separate experiments.

responses were modulated by blocking with CTLA-4Ig or anti-CD28 Ab. This revealed that T cell proliferative responses were completely ligand- and CD28-dependent (Fig. 1A). Strikingly, blockade with anti-CTLA-4 had no effect on ligand-stimulated T cell responses (Fig. 1A). Given the lack of efficacy of anti-CTLA-4 Ab, we questioned whether T cells activated in this manner expressed CTLA-4 (Fig. 1B). The data showed that despite robust CTLA-4 expression both prior to and during cell division, blocking anti-CTLA-4 had no impact on cell division or indeed IL-2 production (Fig. 1C), both of which are proposed targets for CTLA-4 signaling. Given the reductionist nature of this experiment, we further tested whether the same effect was seen using cultured DC to stimulate T cells. As shown (Fig. 1D, 1E), parallel data were obtained using DC, indicating little or no impact of CTLA-4 blockade in these settings. Thus, despite robust CTLA-4 expression prior to cell division and clear evidence that the natural ligands were effective in driving CD28 costimulation, CTLA-4 was not effective at inhibiting primary T cell responses. To study the kinetics of CD80/CD86 costimulation, we blocked CD80/CD86 by CTLA-4Ig during T cell stimulation driven by DC and anti-CD3 at different time points. Intriguingly, the inhibitory effect on T cell proliferation correlated with the time of CTLA-4Ig addition (Fig. 1F). These data indicate that CD80/CD86 costimulation is strictly required for T cell commitment to divide in the first 12 h time window.

To ensure that these results were not related to lack of effective blockade by anti-CTLA-4, we compared a number of different Abs for their binding to CTLA-4 using surface plasmon resonance and their capacity to block CTLA-4 interaction with its ligands. All Abs tested were effective at binding to CTLA-4 with affinities in the low nanomolar range (Fig. 2A, Table I). To confirm the capacity of anti-CTLA-4 Ab to block CTLA-4–ligand interactions, CTLA-4Ig was used to bind cells expressing CD80 or CD86 and detected by flow cytometry. The ability of the various CTLA-4 Abs to disrupt CTLA-4Ig binding to ligands was then tested. All Abs tested were capable of blocking CTLA-4 binding to both CD80 and CD86 and completely ablate ligand binding at a concentration of ~ 7 nM (Fig. 2B, 2C). Furthermore, at 10-fold lower concentrations nearly 80% binding to both CD80 and CD86 was blocked by tremelimumab and BNI3 (Fig. 2B, 2C). Given that we used a concentration of ~ 270 nM ($40 \mu\text{g/ml}$) in our functional assays, this suggests that CTLA-4 was fully blocked in our experiments. Because tremelimumab was the highest affinity and most effective blocking Ab (albeit broadly similar to other anti-CTLA-4 Abs), this was used in subsequent functional experiments to try and identify contexts where CTLA-4 function was evident.

Determining the parameters that affect transendocytosis of ligands into CTLA-4-expressing cells

Because well-characterized blocking Abs for CTLA-4 did not affect T cell responses in standard costimulation assays, we tested their ability to block ligand downregulation by CTLA-4, which occurs during the process of transendocytosis. In this instance, CTLA-4-expressing cells acquire CD80 and CD86 from the surface of donor cells resulting in ligand depletion (16). We initially tested the ability of anti-CTLA-4 to block ligand transfer as assessed by confocal microscopy. In these experiments anti-CTLA-4 potently inhibited transendocytosis as shown by the lack of CD86-GFP transfer to CTLA-4-expressing cells (red) as well as the retention of CD86-GFP on the donor cell surface (Fig. 3A). We also used flow cytometry to measure transendocytosis, allowing us to simultaneously monitor downregulation of GFP-ligand from labeled donor cells (Far Red) and track GFP uptake by the CTLA-4⁺ recipient CHO cells. This analysis showed that

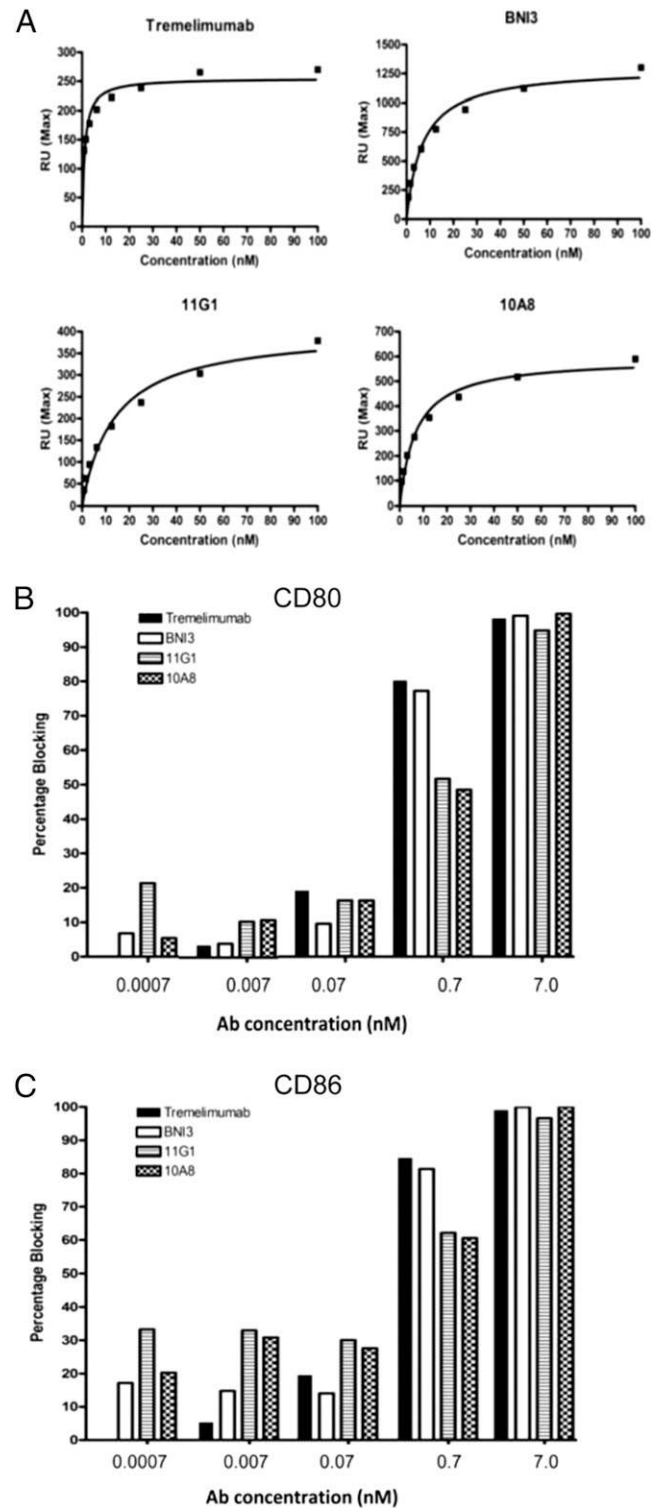


FIGURE 2. Characterization of blocking anti-CTLA-4 Abs. (A) Analysis of anti-CTLA-4 Abs binding to CTLA-4Ig using surface plasmon resonance. The maximum response unit for each concentration of anti-CTLA-4 was plotted for four different anti-CTLA-4 clones. (B and C) Anti-CTLA-4 Abs were tested for their ability to block CTLA-4Ig binding to CD80 or CD86. CHO cells expressing either CD80 (B) or CD86 (C) were incubated with $0.1 \mu\text{g/ml}$ CTLA-4Ig in the presence of increasing concentrations (nM) of tremelimumab, BNI3, 11G1, and 10A8 anti-CTLA-4 Abs. Percentage blocking is calculated based on the mean fluorescence intensity of unblocked CTLA-4Ig staining.

coincubation of CD86-expressing cells with CTLA-4-expressing cells resulted in the loss of GFP from the CD86 donors and uptake

Table I. Calculated Ab-binding affinity to CTLA-4

Ab	K_D (nM)
Tremelimumab	1.051
BN13	6.908
11G1	13.89
10A8	6.891

The K_D value is determined using the nonlinear regression, one-site-binding model in Prism.

of GFP by CTLA-4-expressing but not control recipients (Fig. 3B). To further explore the rate of depletion of CD86-GFP by CTLA-4 over time, we measured the expression of remaining GFP on the CD86 donors at various time points. At a 4:1 donor/recipient ratio, ~2–3% of GFP was depleted per hour. At a 4:16 ratio with increased amounts of CTLA-4 present, the GFP depletion rate was ~2 to 4-fold faster than that at a 4:1 ratio (Fig. 3C). This suggests that the more CTLA-4-expressing cells there were, the faster they depleted their ligand CD86.

A number of interesting characteristics were evident from these experiments when we varied parameters predicted to dictate CTLA-4 function, such as the level of ligand expression, the relative ratios of CTLA-4/CD86-expressing cells, and the contact time between cells. As shown in Fig. 3D and 3E, downregulation of ligand from the CD86⁺ donors (*left panels*) was compared with acquisition of GFP by the recipient CTLA-4⁺ cells (*right panels*) at 3 and 24 h. Additionally, donor CD86-GFP cells expressing either high and low levels of ligand were also compared (*top versus bottom rows*). In cocultures with higher numbers of CD86-GFP donors relative to the number of CTLA-4 “regulators” (4:0.25 ratio; *lane 4*), downregulation of CD86 was limited especially at shorter coculture times (Fig. 3D, *left panels*). However, as we increased the number of CTLA-4⁺ cells (4:16 ratio; *lane 7*) or increased the coculture time (Fig. 3E; 24 h), more substantial removal of CD86 was achieved. In control experiments with untransfected recipients (no CTLA-4) (*lane 8*), no GFP downregulation was observed. Interestingly, when we analyzed GFP uptake into the CTLA-4 recipients (Fig. 3D, 3E, *right panels*), we found a greater capture of CD86-GFP per CTLA-4⁺ cell at low CTLA-4 cell numbers (*lane 4*) compared with higher numbers (*lane 7*). Thus where CD86-bearing donor cells were abundant, each CTLA-4 recipient acquired more ligand per cell despite relatively ineffective downregulation of the donor. In contrast, at low numbers of ligand-bearing cells (and thereby relatively increased CTLA-4) each CTLA-4 recipient acquired less CD86-GFP per cell but achieved much more significant ligand downregulation overall.

From a perspective of immune regulation, the most relevant parameter is removal of ligand from the APC and therefore the total amount of CD86 costimulation available for T cell stimulation. We calculated this available costimulatory potential as the product of cell number times the number of ligand molecules per cell. When viewed in this way, decreases in total CD86 in the donor population were matched by proportional increases of GFP acquired within the recipient cells (Fig 3F–H). This reinforces the conclusion that ligand loss from the CD86⁺ donor is a direct consequence of transfer to the CTLA-4 recipient via transendocytosis. Moreover, ratios at which ligand depletion was relatively ineffective at early time points (Fig. 3F, *lane 6*, 3h) nevertheless led to substantial downregulation at later time points (Fig. 3G, *lane 6*, 3 versus 24 h), indicating that transendocytosis is an active time-dependent process. With low levels of ligand, at later times downregulation occurred to such a marked extent that saturation was reached; that is, increasing the number of CTLA-4⁺ recipients did not reduce ligand levels further (compare Fig. 3F and 3G, *lower left panels*,

lanes 4–7). Therefore, as shown in Fig. 3H, at early time points a robust correlation exists between the amount of ligand removed from the donors and the total amount detected in recipients. However, at later times and particularly at lower starting amounts this correlation breaks down as the ligand supply becomes exhausted. Overall, measuring total ligand downregulation revealed that control by CTLA-4 is most effective under conditions where removal is favored by low amounts of initial ligand expression, higher numbers of CTLA-4-expressing cells, or by longer contact times.

T cell responses are directly proportional to level of ligand downregulation

To determine the functional significance of different efficiencies of downregulation by CTLA-4, we recovered and purified the CD86-GFP-expressing cells after contact with CTLA-4⁺ cells. The ligand-expressing cells were then fixed to preserve their level of costimulatory molecule expression. These cells were then used to stimulate purified naive human CD4⁺CD25⁻ T cells in a typical CD28 costimulation assay. As shown in Fig. 4A, cells that were efficiently depleted of CD86 whether by increasing CTLA-4 ratios or by increasing contact time were much less able to costimulate T cell responses. In contrast, those exposed to insufficient CTLA-4 regulation, for example, having high level of CD86 initially or shorter contact times, retained the ability to stimulate responses in a CD86-dependent manner. For example, conditions 5 and 6 resulted in marked depletion of CD86 at 24 h and yielded cells that were extremely poor at costimulating T cell proliferation in contrast to the same ratio tested at 3 h (Fig. 4A). Moreover, as predicted, the control of T cell responses by CTLA-4 was also substantially more marked when starting with lower levels of ligand on the APC. As shown in Fig. 4B, very modest ratios of CTLA-4/CD86 could suppress T cell responses, especially at later time points where initial ligand levels were low. Furthermore, when the total CD86 level remaining was plotted against the number of proliferating T cells, a striking linear relationship was observed (Fig. 4C). Taken together, these data indicate that the overall reduction of costimulatory ligand determines the functional outcome of CTLA-4 transendocytosis. This, in turn, is controlled by the relative amounts of CD86 and CTLA-4 present and their contact time. Given that we observed a direct relationship between the amount of costimulation available and the magnitude of the T cell response, this suggests that reducing ligand expression is a sensitive and proportional way of controlling the commitment of T cells to a response.

Suppression by CTLA-4 does not require a specialized cell type

Given the above data, we wanted to test the extent to which different CTLA-4-expressing cells could affect T cell costimulation. We therefore tested the ability of different CTLA-4-expressing cell types to act as regulators of T cell responses stimulated by DC. As shown in Fig. 5A, CTLA-4⁺ Jurkat cells but not control Jurkat cells were able to suppress T cell responses in a manner that could be abrogated by anti-CTLA-4 Ab. Moreover, when T cell responses were stimulated by Ab-coated beads rather than by natural ligands, CTLA-4⁺ Jurkat cells were unable to inhibit the response, consistent with the ability of anti-CD28 Ab to bypass the requirement for ligand-driven costimulation (Fig. 5B). Similarly, we also investigated whether CTLA-4 on activated conventional CD4 T cells (T blasts) showed any suppressive function. CTLA-4⁺ blasts were also able to restrict T cell division in a CTLA-4-dependent manner, particularly at lower DC/T blast ratios (Fig. 5C). Taken together, these data suggest that, independent of T cell type, CTLA-4 can act extrinsically to reduce

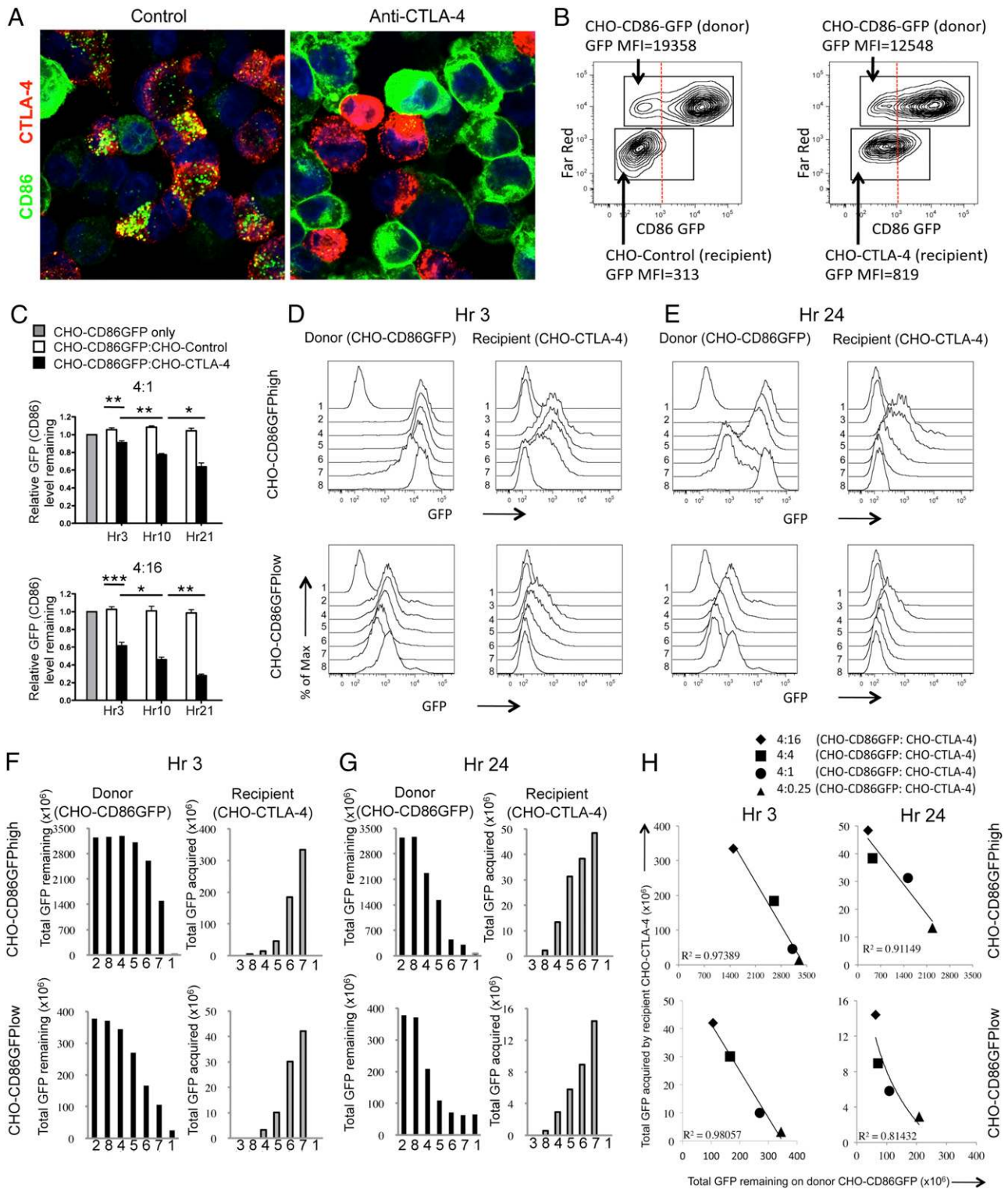


FIGURE 3. Characterization and blocking of CD86 transendocytosis. **(A)** CTLA-4-mediated acquisition of CD86 is blocked by anti-CTLA-4. Confocal micrographs (original magnification $\times 63$) show adherent CHO-CD86 cells and CHO-CTLA-4 cells after overnight incubation in presence or absence of anti-CTLA-4 blockade. CD86 (green) and CTLA-4 (red) were detected by Ab staining. Colocalization of CD86 and CTLA-4 is shown in yellow. **(B)** Depletion of CD86 from CD86⁺ cells is associated with concomitant CD86 acquisition by CTLA-4⁺ cells detected by flow cytometry. Far Red-labeled donor CHO-CD86-GFP cells were cocultured with recipient CHO-CTLA-4 (right panel) or CHO-control (left panel) at 37°C for 20 h. After coculture, singlet CTLA-4⁺ cells were analyzed for CD86-GFP acquisition based on Far Red⁻ cells, and loss of GFP from donors was determined by gating on Far Red⁺ cells. **(C)** Depletion of CD86 by CTLA-4 over time. CHO-CD86-GFP cells were cultured with CHO-CTLA-4 as in (B) at 4:1 (upper panel) and 4:16 (lower panel) donor/recipient ratio for 3, 10, and 21 h. The mean fluorescence intensity of GFP remaining on donor cells was used to calculate relative expression level. Bar graph shows mean \pm SEM of three experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by unpaired, two-tailed *t* test. **(D and E)** Mean fluorescence intensity of GFP on donors or recipients are shown for both high and low densities of ligand expression at 3 (D) and 24 h (E). **(F and G)** CD86 remaining on donors is inversely correlated with CD86 acquired by CTLA-4 on recipients. Conditions in (D)–(G) are numbered as: 1, CHO-control only; 2, CHO-CD86GFP only; 3, CHO-CTLA-4 only; 4, 4:0.25 (CHO-CD86GFP/CHO-CTLA-4); 5, 4:1 (CHO-CD86GFP/CHO-CTLA-4); (Figure legend continues)

CD86 levels from DC and this is sufficient to mediate suppression. Moreover, as predicted by our model, the efficiency of CTLA-4-dependent downregulation and its suppressive effect on T cell responses are highly sensitive to the number of DC (and consequently the overall amount of ligand) present.

Transendocytosis predicts CTLA-4-dependent Treg suppressive function

To determine whether CTLA-4-dependent suppression by natural Treg was consistent with data from our models, we tested the ability of purified human CD4⁺CD25⁺ T cells to regulate T cells in the presence of variable numbers of DC. As shown in Fig. 6A, high numbers of DC (8:1 ratio) resulted in most T cells entering division, although the number of cell divisions was rather low. At these ratios, CTLA-4Ig also had limited impact, indicating that when driven by abundant APC, T cell responses are less CD28-dependent. However, as DC numbers were reduced, responses became increasingly CD28-dependent as judged by the impact of CTLA-4Ig. Moreover, at these reduced numbers of DC, Treg suppression was clearly evident and CTLA-4 blockade substantially reversed the impact of Treg suppression. Again, it was notable that anti-CTLA-4 had no effect on control T cell responses in the absence of Treg or when T cells were stimulated using CD3/CD28 beads (Fig. 6B). To exclude the possibility that CTLA-4-dependent Treg suppression at low DC numbers was simply due to lower overall magnitude of T cell stimulation, we repeated these experiments keeping DC numbers fixed but this time increasing Treg. Again, increasing the ratio of Treg/DC resulted in more effective Treg suppression. This indicates that the relative ratio of Treg/DC is the critical factor influencing CTLA-4-based suppression rather than the DC number per se or indeed the Treg/responder ratio. As predicted, the degree of suppression was also correlated with the extent of downregulation of CD86 expression on the DC (Fig. 6C). Overall, these data show that CTLA-4 function on Treg is effective at controlling T cell responses in a manner that relates to the extent of ligand downregulation on DC. In turn, ligand downregulation is a function of Treg/DC ratio and level of ligand depletion as predicted by transendocytosis as a mechanism.

Discussion

CTLA-4 is an essential regulator of T cell function that in combination with the CD28 pathway represents a critical decision point in T cell activation. Manipulation of this checkpoint has led to the successful development of new therapeutics that are approved for use in autoimmunity (19) and cancer settings (20). Despite this success, the mechanistic basis for how the CTLA-4 checkpoint works has been uncertain, and this has hampered the design of assays that can predictably measure CTLA-4 function *in vitro*. Consequently, at present there are no widely adopted assays available for human use that measure CTLA-4 function. In the present study, we report a set of robust basic principles underpinning CTLA-4 function that can both aid assay design as well as interpretation of the many experiments where CTLA-4, CD28, and their ligands are manipulated. The data presented in this study suggest that control of T cell responses by CTLA-4 is highly compatible with a model based on the quantitative control of ligand expression.

A number of potential mechanisms of CTLA-4 action have been proposed (13–15), raising the issue of how much each mechanism of action contributes to CTLA-4 physiological function. This issue is not easily resolved by standard experimental approaches; for example, mutagenesis frequently affects more than one postulated mechanism. Accordingly, mutations in the CTLA-4 cytoplasmic domain that might affect signaling can also impact protein expression levels at the cell surface (21) as well as transendocytosis (16). Thus, most experiments to date are not decisive, and the issue of how CTLA-4 functions still remains unresolved. An alternative approach is to take each model at face value and determine the extent to which it provides useful predictions of CTLA-4 behavior. For example, intrinsic functions, such as the delivery of inhibitory signals by CTLA-4, would indicate that upon interaction with ligands, CTLA-4 will trigger inhibitory signals such that T cell activation or IL-2 production is impaired (22, 23). However, as shown in the present study, although CTLA-4 is robustly observed during T cell activation, it is difficult to demonstrate by CTLA-4 blockade that its expression intrinsically influences T cell responses. Accordingly, in our experiments despite T cell activation occurring in a ligand-driven and CD28-dependent manner, T cells proceed through cell division expressing high levels of CTLA-4, but show no evidence for inhibition as determined by blocking CTLA-4 Abs. These data suggest that, at the very least, there are significant contextual constraints to observe CTLA-4 ligands delivering T cell-intrinsic inhibition.

Considerable data also exist that a cell-extrinsic function of CTLA-4 is required to prevent autoimmune disease *in vivo* (24–29), suggesting that CTLA-4 expressed by T cells works by regulating other cells. Consistent with this, a number of studies have suggested that CTLA-4 can alter the level of CD80 and CD86 expression on APC (16, 29–32). We recently reported that such observations could be explained by transendocytosis, a process that results in the physical capture of ligands by CTLA-4-expressing cells (16). In the experiments described in the present study, we tested whether transendocytosis was a useful model on which to design assays capable of detecting CTLA-4 function. Data using cells transfected with CD86 or CTLA-4 provided initial insights into how such a mechanism might operate to regulate T cell responses. Several features were apparent from this model: specifically, the efficacy of CTLA-4 suppression correlates extremely well with its ability to deplete sufficient ligand from the ligand-bearing cells, thereby inhibiting CD28 costimulation. This ability is in turn influenced by the ratio of CTLA-4:ligand expression. In practical terms, this suggests that the number of ligand-bearing APC and their level of ligand expression relative to the number of CTLA-4-expressing regulatory cells can broadly dictate whether CTLA-4 is effective at controlling the T cell response. Using such model systems we could demonstrate that the amount of ligand removed was highly correlated with the amount of ligand acquired by the CTLA-4-expressing cells, suggesting that transendocytosis was the likely mechanism of downregulation. Recently, concepts allowed us to conduct quantitative analysis of ligand uptake and suppression to reveal functional defects in Treg from human patients with heterozygous CTLA-4 mutations. This reveals that clinically significant functional defects occur in the setting of ~50% loss of CTLA-4 expression, a concept entirely in keeping with the models proposed in this study

6, 4:4 (CHO-CD86GFP/CHO-CTLA-4); 7, 4:16 (CHO-CD86GFP/CHO-CTLA-4); 8, 4:16 (CHO-CD86GFP/CHO-control). Total fluorescence intensity of GFP was calculated as cell number times GFP mean fluorescence intensity on both donors and recipients following transendocytosis as shown in (D) and (E). (H) Plots show the relationship between total GFP acquired by CTLA-4-expressing cells versus GFP remaining on the donors. Data are presented at 3 and 24 h using both high- and low-density donors.

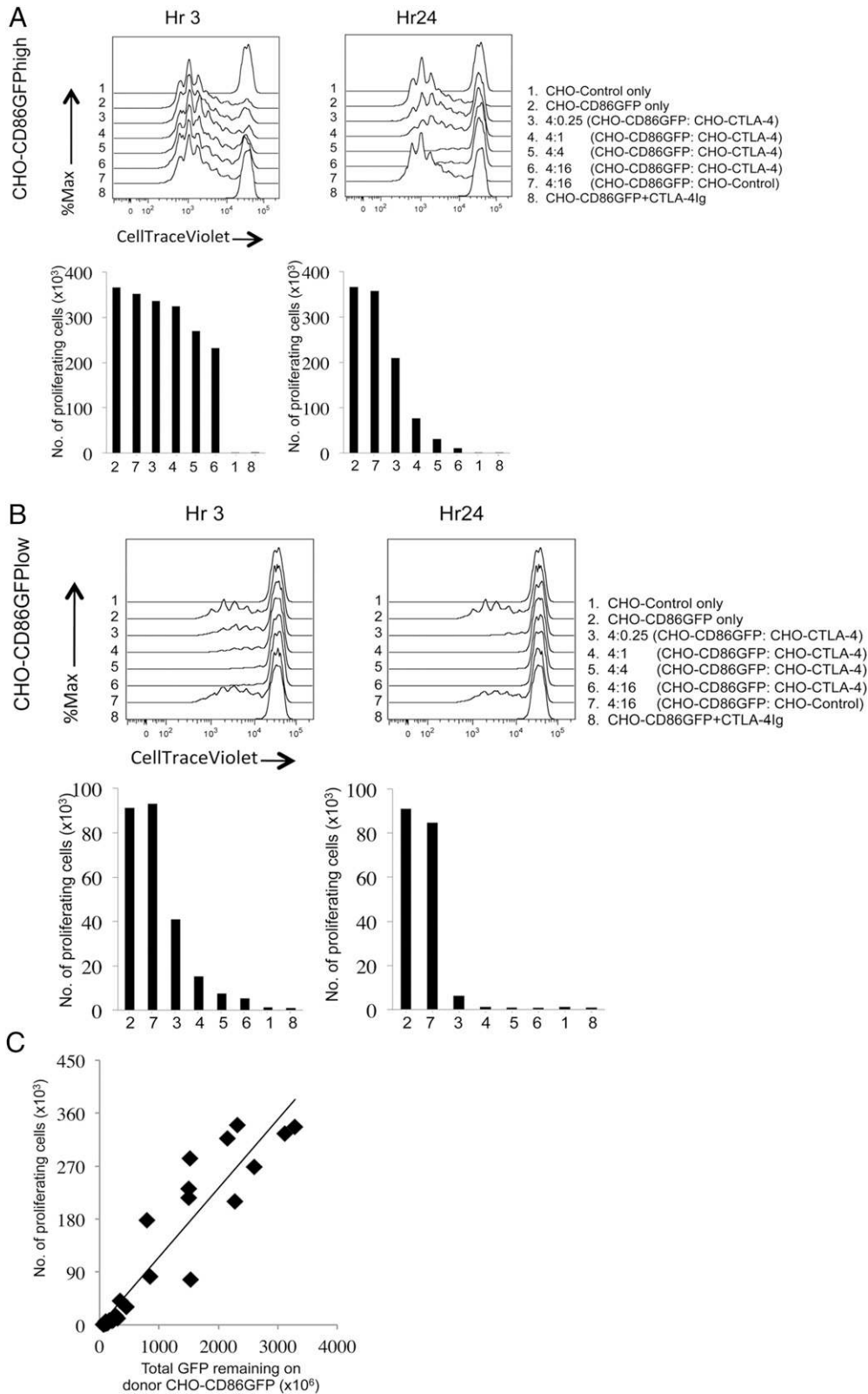


FIGURE 4. CTLA-4-mediated downregulation of CD86 is functionally relevant for T cell activation. (**A** and **B**) CellTrace Violet-labeled human naive CD4⁺ T cells were stimulated with CHO-CD86GFP cells, which were FACS sorted following transendocytosis as performed in Fig. 3 in the presence of 0.5 μ g/ml soluble anti-CD3. Cell division was monitored using CellTrace Violet dilution at day 5 using flow cytometry. Live proliferating T cell counts were determined using counting beads and the total cell numbers are shown in bar charts. Correlation between total CD86 remaining on the APC and total proliferating T cell number is shown in (**C**). The data presented are representative of three independent experiments.

(33). Importantly, the data from model transendocytosis experiments were highly consistent with the data obtained using DC- and Treg-expressing ligand and receptor at natural levels.

Downregulation of CD86 by CTLA-4 was observed in the presence of Treg and reversed by CTLA-4 blockade, and again the level of downregulation of CD86 was dependent on the relative

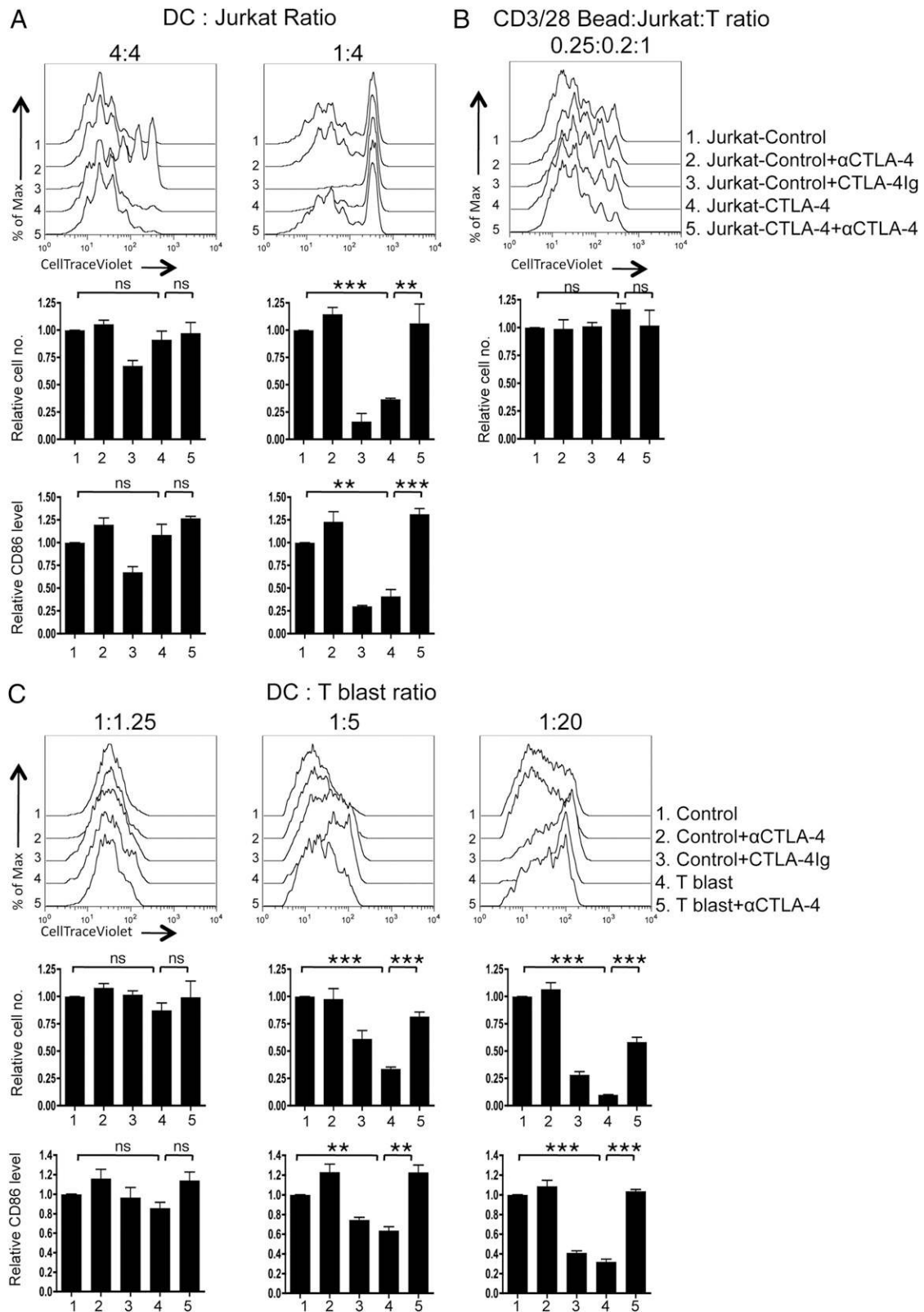


FIGURE 5. CTLA-4 suppression via DC does not require specialized cell type. **(A)** Violet-labeled human naive T cells were stimulated using DC in the presence of anti-CD3 and suppression was measured in response to CTLA-4⁻ (control) or CTLA-4-transduced Jurkat cells. Assays were carried out in the presence or absence of anti-CTLA-4 or in the presence of CTLA-4Ig (10 μ g/ml) to establish costimulation dependence. Monocyte-derived DC were used for costimulation at the indicated ratio. **(B)** Violet-labeled naive T cells were stimulated with anti-CD3/CD28-coated beads to provide a ligand-independent stimulus and suppression of CTLA-4⁺ Jurkat cells was monitored as in (A). Cell division was monitored by dye dilution at day 5 using flow cytometry and the relative numbers of dividing T cells are shown in bar chart. Expression levels of CD86 on CD11c⁺ DC are shown in mean fluorescence intensity by flow cytometric analysis using anti-CD86 Abs. **(C)** Activated T cells expressing CTLA-4 were used as suppressor cells in the same manner as in (A). Bar chart depicts mean \pm SEM; $n = 3$. Data are representative of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ by unpaired, two-tailed t test.

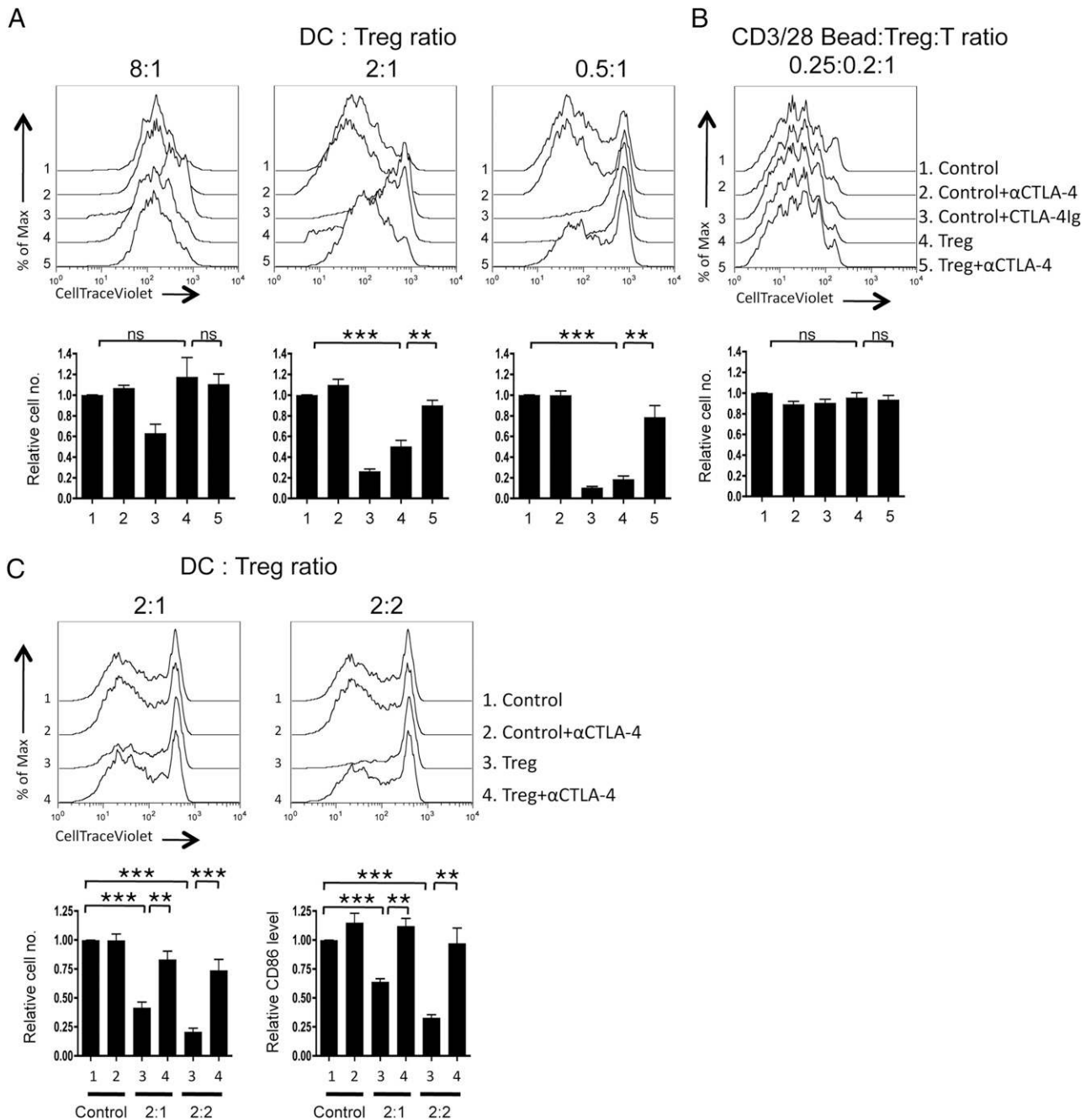


FIGURE 6. Transendocytosis predicts the behavior of CTLA-4-dependent suppression by Treg. **(A)** CellTrace Violet–labeled human naive T cells were stimulated with soluble anti-CD3 (0.5 μg/ml) and human monocyte-derived DC for costimulation at the indicated ratios in the absence or presence of Treg. Anti-CTLA-4 (40 μg/ml) or CTLA-4Ig (10 μg/ml) was used to block CTLA-4 or ligands, respectively. Cell division was monitored by CellTrace Violet dilution at day 5 using flow cytometry. Relative numbers of dividing T cells were calculated and are shown in bar charts. **(B)** Naive T cells were stimulated with CD3/CD28 beads and responses were measured as in **(A)**. **(C)** Suppression assays were carried out as in **(A)** with the exception that the DC numbers remained fixed and the Treg numbers increased, thereby maintaining equivalent stimulation of responder T cells. Relative numbers of dividing T cells were calculated and are shown in bar charts along with expression levels of CD86 on CD11c⁺ DC and are shown as mean fluorescence intensity. Bar graph shows mean ± SEM; *n* = 3–5. FACS data are representative of five experiments performed. ***p* < 0.01, ****p* < 0.001 by unpaired, two-tailed *t* test.

ratios of DC:Treg correlating with the level of suppression. Therefore, we propose that CTLA-4 effectively sets an activation threshold by controlling the amount of CD28 ligation available via cell-extrinsic ligand depletion.

The data presented in this study clearly emphasize the importance of ligand binding to CTLA-4. Indeed, our studies highlight that in order for CTLA-4 to regulate a T cell response, the response needs to be both ligand- and CD28-dependent. One simple test of such a property is whether a T cell response is sensitive to blockade

by CTLA-4Ig. Accordingly, when the response is not suppressed by CTLA-4Ig, then, in our experience, CTLA-4-dependent Treg function is inevitably ineffective. Such a situation is seen in our control experiments where T cells are stimulated using CD3/CD28 beads and are not suppressed by abatacept or by Treg. Accordingly, in our view, suppression assays that use anti-CD28 to stimulate T cell responses are unsuitable for measuring CTLA-4 function. These concepts are consistent with the fact that disease caused by CTLA-4 deficiency is dependent on both the presence

of ligands and on CD28 (34–36), suggesting that CTLA-4 controls ligand-driven stimulation *in vivo*.

One attractive feature of transendocytosis as a model of CTLA-4 function is that it presents a cogent argument for why CD28 and CTLA-4 share ligands. Indeed, if this were not the case, then this mechanism could not control CD28 function. This point is highlighted by the fact that CTLA-4 on Treg cannot control Ab-stimulated proliferation of T cells where ligands are not used to stimulate responses. Moreover, this also suggests that CTLA-4-dependent mechanisms are not able to control CD28-independent responses, which may be relevant when considering the loss of CD28 that occurs during extensive or chronic T cell stimulation observed in autoimmune diseases (37, 38). Additionally, our observation of transendocytosis by non-Treg fits well with recent data showing that CTLA-4 does not have to be expressed by Foxp3-expressing Treg in order for it to function in a suppressive manner (25, 39). Indeed, recent data from the Sakaguchi and colleagues (32) provide further support for this concept by showing that CTLA-4 is a key component in generating Treg behavior.

Our results also suggest that the functional impact of CTLA-4 on controlling T cell responses is likely to be highly variable depending on the degree of ligand downregulation achieved. Accordingly, in the steady-state, ligand levels may be relatively low and sufficiently controlled by CTLA-4 expressed by Treg that activation of self-reactive T cells is precluded. Removal of CTLA-4 entirely or selectively on Treg is sufficient to reveal underlying self-reactivity (29, 40). Conversely, our data would suggest that during robust priming of the immune system it is possible for CTLA-4 to be present on Treg but ineffective in restraining a T cell response owing to overwhelming levels of ligand expression both due to increased APC and increased levels of ligand expression. Under such situations loss of CTLA-4 function is predicted to be of limited impact, as has been observed experimentally (41). Additionally, control of ligand levels by CTLA-4 may also shape the magnitude and differentiation of the T cell response by potentially restricting cytokine production (42). Thus, one interpretation of CTLA-4 function is that it can act as a rheostat, sensitive to both the number and activation state of DC and therefore the “context” for immune regulation.

Despite our emphasis of ligand binding in order for CTLA-4 to function, a number of reports suggest that CTLA-4 may work in a ligand-independent manner (43, 44). It is reported ectopic overexpression of a splice variant CTLA-4 that lacks a ligand-binding region appears to protect mice from autoimmune disease models (45). Also, transduction of the cytoplasmic domain of CTLA-4 was shown to prevent animals from developing inflammatory conditions (46, 47). Although there may be distinct ligand-independent effects of CTLA-4, as pointed out above, the disease phenotype caused by CTLA-4 deficiency is largely ligand-dependent. Additionally, it is notable that the cytoplasmic domain of CTLA-4 is efficiently recruited to clathrin coated pits via AP-2 (48–50). One possibility, therefore, is that expression of the cytoplasmic domain may have effects on the trafficking of a number of receptors, for example, the transferrin receptor, which is highly upregulated during T cell activation and is also internalized via AP-2. Thus, ligand-independent CTLA-4 may mediate effects either via impact on non-CTLA-4-related pathways such as iron transport or alternatively by influencing expression of full-length CTLA-4 itself. Indeed, recent studies (51) have indicated that the function of the ligand-independent form requires co-expression of the full-length molecule in some settings. Moreover, it is clear that expression of the ligand-independent form is generally insufficient to prevent disease caused by loss of full-length CTLA-4, suggesting that the function of the ligand-dependent

molecule is dominant (44, 51). Taken together, although ligand-independent forms of CTLA-4 can alter disease outcomes in some settings, their contribution to the overall function of CTLA-4 *in vivo* may be limited.

Ultimately, a precise understanding of the key mechanisms of CTLA-4 function may reveal new opportunities for further targeting of this key immune regulator. The success of anti-CTLA-4 in clinical trials has again focused attention on how such effects may be mediated. Recent evidence that Treg provide a key mechanism of anti-CTLA-4 efficacy is supported by the demonstration in tumor therapy that effectiveness required Treg depletion dependent on the Fc region of the anti-CTLA-4 Ab (52). An improved understanding of CTLA-4 function also impacts our use of related strategies such as ligand targeting therapies, for example, abatacept and its high-affinity variant belatacept. Whether CTLA-4 provides distinct inhibitory signals based on its ligation or whether its effects are achieved predominately via limiting CD28 function clearly affects such approaches. The data presented in the present study provide support for the view that a clear function of CTLA-4 is the cell-extrinsic control of CD28 ligands on APC. Moreover, this function of CTLA-4 appears to conform to precise and predictable rules that govern its function on Treg.

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Disclosures

The authors have no financial conflicts of interest.

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