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Trans-endocytosis of CD80 and CD86: a molecular basis for the cell extrinsic function of CTLA-4 **

Omar S. Qureshi¹, Yong Zheng¹, Kyoko Nakamura¹, Kesley Attridge¹, Claire Manzotti¹, Emily M. Schmidt¹, Jennifer Baker¹, Louisa E. Jeffery¹, Satdip Kaur¹, Zoe Briggs¹, Tie Z. Hou¹, Clare E. Futter², Graham Anderson¹, Lucy S.K. Walker¹, and David M. Sansom^{1,*}

¹MRC Centre for Immune Regulation, School of Immunity and Infection, Institute of Biomedical Research, University of Birmingham Medical School, Birmingham, B15 2TT, UK.

²Department of Cell Biology, University College London Institute of Ophthalmology, University College London, London, EC1V 9EL, UK.

Abstract

CTLA-4 is an essential negative regulator of T cell immune responses whose mechanism of action is the subject of debate. CTLA-4 also shares two ligands (CD80 and CD86) with a stimulatory receptor, CD28. Here we show that CTLA-4 can capture its ligands from opposing cells by a process of trans-endocytosis. Following removal, these costimulatory ligands are degraded inside CTLA-4-expressing cells resulting in impaired costimulation via CD28. Acquisition of CD86 from antigen presenting cells is stimulated by TCR engagement and observed *in vitro* and *in vivo*. These data reveal a mechanism of immune regulation whereby CTLA-4 acts as an effector molecule to inhibit CD28 costimulation by the cell-extrinsic depletion of ligands, accounting for many of the known features of the CD28-CTLA-4 system.

Keywords

CTLA-4; CD86; T cell; dendritic cell; suppression

The T cell protein CTLA-4 is essential to the prevention of autoimmune disease (1-3). Although the molecular basis for CTLA-4 action has been suggested to be a cell-intrinsic inhibitory signal(4) possibly mediated by the cytoplasmic domain (5), a cell-extrinsic function for CTLA-4 is clearly evident from *in vivo* models (6-13). Therefore a molecular explanation of CTLA-4 function compatible with such a cell-extrinsic mechanism is needed. The intercellular transfer of a ligand from one cell to its receptor on a different cell is observed in both immune settings and elsewhere (14-19). Because of its highly endocytic behaviour, we tested whether CTLA-4 could potentially act in such a manner in order to deplete its ligands and thereby extrinsically inhibit T cell activation via CD28. We cultured CTLA-4-expressing (CTLA-4⁺) CHO cells with donor CHO cells expressing a C-terminally tagged CD86 protein (CD86-GFP). Using a flow cytometric assay we observed substantial transfer of CD86 into CTLA-4⁺ cells (Fig. 1A and fig. S1). This finding was confirmed by confocal microscopy where acquisition of ligand by CTLA-4 caused the appearance of CD86 containing vesicles within the CTLA-4⁺ cell (Fig. 1B). Treatment with the lysosomal inhibitor bafilomycin A caused an increase in detectable CD86-GFP after transfer (Fig 1A),

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*To whom correspondence should be addressed. D.M.Sansom@bham.ac.uk.

which indicated degradation of the transferred ligand inside the CTLA-4⁺ cell. Accordingly, CD86 did not appear on the cell surface of the recipient CTLA-4⁺ cell (fig S2). Interestingly, bafilomycin A treatment did not result in an increase in CTLA-4 expression, which suggested that although CTLA-4 could capture and deliver ligand for degradation, CTLA-4 itself was not degraded (fig. S3). Overall, this process appeared different to the generalized intercellular exchange or 'trogocytosis' reported for other receptors (20-22), where transferred proteins are detected at the cell surface.

To investigate the time course of CD86 acquisition we performed live-cell imaging of CTLA-4⁺ CHO cells interacting with CHO cells expressing CD86-GFP (fig. S4A, Movies S1 and S2). Within minutes of cell contact we observed a marked concentration of CD86-GFP at the site of cell-cell contact, from which CD86 positive vesicles emanated into the CTLA-4 expressing cell (Movies S1 – S4). Quantitation of this process revealed a significant depletion of GFP fluorescence from the plasma membrane of the CD86 donor cell and a corresponding increase in GFP inside the CTLA4⁺ recipient cell (fig. S4B). Kinetic analysis by flow cytometry also revealed that over 50% of CTLA-4⁺ cells acquired ligand within 3h (fig. S5). Furthermore, we estimated the number of CTLA-4 molecules and CD86 molecules expressed by our cell lines to determine the stoichiometry of CD86 depletion (fig. S6). This showed that a ratio of approximately 1:8 (CTLA-4:CD86) molecules was sufficient for functionally relevant depletion.

To confirm that trans-endocytosis of ligand was not an artifact of using GFP-fusion proteins we also performed experiments with wild type CD86 expressed in CHO cells. CHO-CD86 cells were cultured alone or with CHO-CTLA-4 cells then stained for CD86 and CTLA-4 using antibodies (Fig. 1C). In the absence of CTLA-4, CD86-expressing cells display a characteristic plasma membrane staining pattern (fig. S7A), however in the presence of CTLA-4, CD86 containing vesicles were observed inside CTLA-4 recipient cells (Fig. 1C, fig. S7A). Moreover, because CD86 was stained using an antibody against the cytoplasmic domain this indicated that the whole ligand had been transferred into the CTLA-4⁺ cell. Analysis of immunofluorescence images showed no significant acquisition of CTLA-4 by the CD86-expressing cells (fig. S7B). In addition, studies using the membrane dye PKH26 revealed that trans-endocytosis was associated with transfer of small amounts of membrane lipids (fig. S7C). Taken together these data indicated that protein transfer was unidirectional and appeared to involve transfer of membrane lipid. Because the C-terminus of CTLA-4 is required for endocytosis (fig. S8) (23, 24), and shows a remarkable degree of evolutionary conservation, we tested the contribution of this region to CD86 trans-endocytosis, by deleting the C-terminus of CTLA-4 (CTLA-4 del36). Although CD86 still localized to regions of cell-cell contact (Fig. 1D), wild-type-CTLA-4 was much more effective at depleting CD86 than CTLA-4 del36 (Fig. 1E). As expected, when the CD86-depleted cells from these experiments were used to stimulate T cells, those exposed to wild-type CTLA-4 had impaired stimulatory capacity compared to those exposed to non-endocytic CTLA-4 (Fig. 1F). Together, these results indicate that by a process of trans-endocytosis CTLA-4 removes CD86 from neighboring cells resulting in impaired T cell responses.

To test these observations in a more physiological setting we activated human CD4⁺CD25⁻T cells in the presence of monocyte-derived dendritic cells to allow the induction of CTLA-4 and looked for evidence of trans-endocytosis. In the absence of T cells, CD86 was robustly expressed on the surface of dendritic cells (Fig. 2A). In the presence of activated T cells, however, CD86 on the plasma membrane of DCs was reduced and instead found in a punctate pattern that co-localized with CTLA-4 (Fig. 2B). Importantly, incubation with a blocking anti-CTLA-4 antibody prevented the down-regulation of CD86 on the DC as well as the appearance of CD86 in CTLA-4⁺ vesicles in the T cell (Fig. 2, B and C and fig. S9). Although CD86 was downregulated in a CTLA-4-

dependent manner, expression of other molecules on the DC such as CD40 was unaffected (fig. S9). These results indicated that CTLA-4-mediated trans-endocytosis was specific to CD80 and CD86. To establish whether CTLA-4 was sufficient to confer this function to T cells we also generated a Jurkat cell line expressing CTLA-4 (fig. S10A). Jurkat cells transduced with CTLA-4 acquired the ability to capture CD86-GFP (fig. S10B). Analysis by electron microscopy also showed acquired CD86 in distinct intracellular vesicles within CTLA-4⁺ Jurkat cells (fig. S10C). The use of a number of blocking reagents, including CTLA-4-Ig and anti-CD28, confirmed the specificity of CD86-GFP transfer to Jurkat cells (fig. S11 and fig. S12) and demonstrated that CD28 was not capable of trans-endocytosis. We have previously shown that transfection of resting human T cells with CTLA-4 confers suppressive activity (8). We therefore tested whether this approach also conferred the ability to capture CD86 from the APC. CTLA-4-transfected (but not mock transfected) resting T cells exhibited specific sequestration and internalisation of CD86, from the DC (Fig. 2, D and E), but had no effect on HLA-DR expression. Taken together, these data demonstrated CTLA-4 expression by T cells was sufficient to confer the ability to remove CD86 from DCs.

Because TCR-engagement leads to enhanced trafficking of CTLA-4 to and from the plasma membrane (fig. S13, A and B) (25-27) we predicted that TCR stimulation should enhance CD86 acquisition. To test this, human CTLA-4⁺ T cell blasts were incubated in the presence of CD86-GFP-expressing CHO cells with or without anti-CD3. TCR stimulation increased the acquisition of CD86-GFP in a manner that was blocked by anti-CTLA-4 and enhanced by bafilomycin (Fig. 3, A). Similarly, staphylococcal enterotoxin B (SEB)-reactive T cell blasts incubated with dendritic cells also showed enhanced acquisition of CD86 (Fig. 3, B and C).

We next determined whether CD4⁺CD25⁺ regulatory T cells (Treg) could acquire CD86 from APC because CTLA-4 is constitutively expressed on Treg cells and is re-cycled upon stimulation (fig. S13, C and D). DC were incubated overnight with purified human CD4⁺CD25⁻ T cells or CD4⁺CD25⁺ Treg cells and anti-CD3. In the presence of CTLA-4⁺ Treg, CD86 was downregulated from the APC surface and observed in intracellular puncta inside the Treg. In contrast, CD86 remained on the plasma membrane of DC in the presence of CD4⁺CD25⁻ T cells that lacked CTLA-4 (Fig. 3, D and E). To test whether downregulation of CD86 by Treg cells affected T cell stimulation, we stimulated Treg with DCs in the presence or absence of a blocking anti-CTLA-4 antibody. We then re-purified these DCs and used them to stimulate CFSE-labelled responder T cells. This revealed that blocking CTLA-4 on regulatory T cells maintained the stimulatory capacity of the DC compared to where CTLA-4 was available to deplete ligands (fig. S14 A). Moreover, suppression could be overcome by restoring co-stimulation using CD86-expressing transfectants (fig. S14 B). We also observed that T cell blasts could act as suppressor cells in a CTLA-4-dependent manner (fig. S14 C) again indicating that depletion of costimulatory molecules by CTLA-4 has functional consequences.

Our data suggest a model where antigen stimulation of either T cells or Treg promotes the removal and degradation of CD80 and CD86 from antigen presenting cells by CTLA-4. To test whether this process could also take place *in vivo* we generated a system to study trans-endocytosis in mice. We first established that mouse CD4⁺ T cells stimulated *in vitro* could acquire CD86-GFP from CHO cell targets (fig. S15A and B). We next developed an *in vivo* protocol (fig. S16) in which DO11.10 TCR transgenic T cells (specific for a peptide fragment of chicken ovalbumin (OVA) presented by I-A^d) were transferred into Balb/c *Rag2*^{-/-} mice, which 3 weeks prior, had been reconstituted with CD86-GFP-transduced *Rag2*^{-/-} bone marrow. Recipient mice therefore lacked lymphocytes, except the adoptively transferred DO11.10 T cells, and expressed CD86-GFP on their antigen presenting cells.

Seven days after OVA immunization, mice were re-challenged with OVA peptide in the presence of the lysosomal inhibitor, chloroquine. T cells were then harvested and immediately analyzed by confocal imaging. This revealed CD86-GFP in endosomal compartments of antigen-stimulated, but not unstimulated, T cells (Fig. 4A). Moreover, internalized CD86-GFP was restricted to CD4⁺CD25⁺ T cells (Fig. 4B), consistent with the expression of CTLA-4 by these cells. These cells did not express Foxp3 (fig. S17A, left panel). The amount of CD86-GFP transfer was extensive because the mean GFP fluorescence inside T cells approached the amounts on donor cells themselves (fig. S17B). To establish the requirement for CTLA-4, we tested CD4⁺ cells from *Ctla4*^{+/+} or *Ctla4*^{-/-}, *Rag2*^{-/-}DO11.10 mice bred to mice that express OVA under the control of the rat insulin promoter (Rip-mOVA). These mice were useful because they develop OVA-specific Treg cells and we have shown that those deficient in CTLA-4 fail to regulate diabetes (13). After *in vivo* re-challenge with OVA peptide, CD86-GFP acquisition was only observed in CD4⁺CD25⁺ T cells capable of CTLA-4 expression but not those from *Ctla4*^{-/-} mice (Fig. 4C). Moreover, CD4⁺CD25⁺ T cells from these mice were almost entirely Foxp3⁺ (fig. S17A right panel). Overall, analysis indicated that approximately 25-40% of wildtype CD4⁺CD25⁺ T cells acquired ligand (fig. S17C). No transfer of control (unfused) GFP molecules was observed (fig. S17D). Together these data indicate that both Foxp3⁺ and Foxp3⁻ T cells are capable of CD86 acquisition *in vivo*.

The CTLA-4 molecule plays a critical role in suppressing autoimmunity and maintaining immune homeostasis; however, its precise mechanism of action has been a subject of debate. Recent data have provided evidence that CTLA-4 can perform a non-redundant effector function for Treg, requiring a cell extrinsic mechanism of action (9, 13). Here we demonstrate a cell-extrinsic model of CTLA-4 function which operates by the removal of co-stimulatory ligands from APCs via trans-endocytosis. Importantly, using both human and mouse T cells we establish that trans-endocytosis of ligand occurs in precisely the same settings where we have demonstrated CTLA-4-dependent regulation (8, 13). Moreover, this mechanism is specific for CTLA-4/CD28 ligands and operates in an antigen-dependent manner *in vivo*. Our data are also compatible with several studies demonstrating reduced levels of costimulatory ligand expression in the presence of CTLA-4-expressing Treg cells (9, 28, 29) and consistent with a role for CTLA-4 on both activated and regulatory T cells (9, 30, 31). Taken together, these results not only provide a widely applicable basis for CTLA-4 function but they also offer cogent explanations for long-standing paradoxes in the field, namely, how CTLA-4 can function in a cell-extrinsic manner, why CTLA-4 shares ligands with the stimulatory receptor CD28 and why CTLA-4 exhibits endocytic behaviour. Whilst not excluding other mechanisms of CTLA-4 action, we suggest that CTLA-4 carries out the same molecular functions whether expressed by T cells or by Treg; a concept which has significant implications for our understanding of immune homeostasis. Together these data provide a new framework for studies of CTLA-4 and should facilitate our understanding of its immunoregulatory role in the key settings of cancer, HIV infection and autoimmune disease.

Supplementary Material

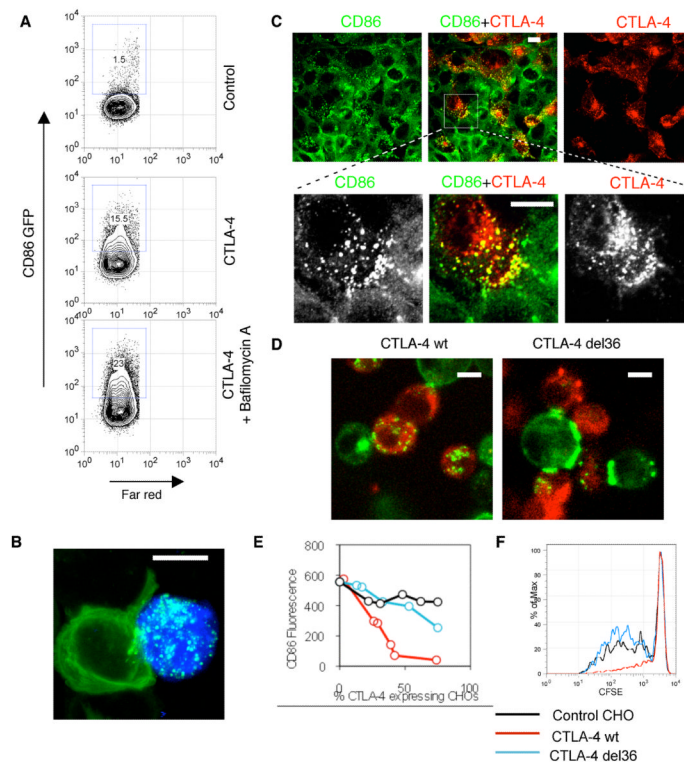
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

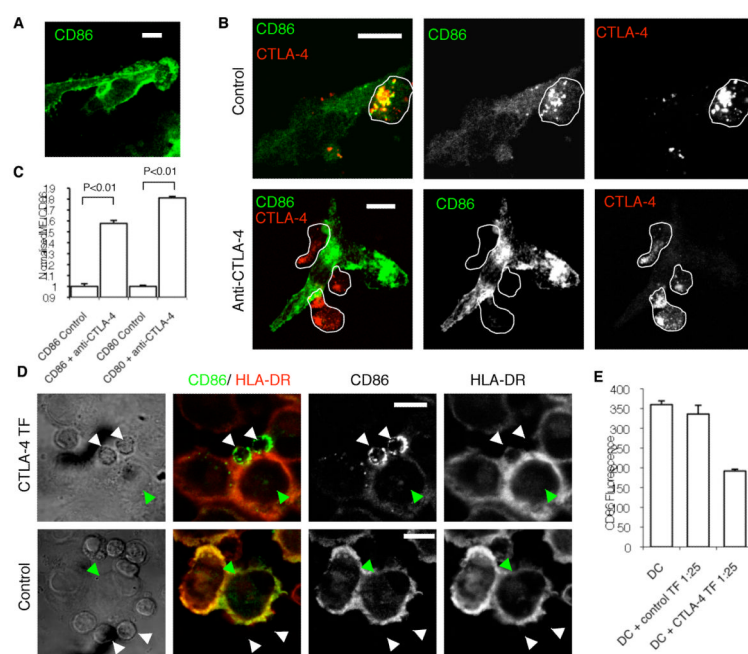
34. We would like to thank Arlene Sharpe for the generous gift of *Ctla4*^{-/-} mice, Bill Heath for the rip-mOVA mice and Gordon Freeman for the I-A^d expressing CHO cells. This work was supported by the BBSRC (OQ), MRC (LSKW, YZ, KA,ES and KN), Wellcome Trust (CM, TH, ZB) Arthritis Research UK (LJ). LSKW is an MRC Senior Fellow.

References and Notes

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**Fig. 1.**

CTLA-4 mediated acquisition of co-stimulatory molecules. **(A)** Flow cytometric analysis of CD86-GFP transfer into CTLA-4 expressing cells. CHO cells expressing CD86-GFP (Far Red labeled) were co-cultured with CHO controls or with CTLA-4⁺ CHO cells in the presence or absence of 10 nM Bafilomycin A. Singlet CTLA-4 expressing cells were analyzed for GFP acquisition by excluding Far Red⁺ donor cells from analysis (see fig. S1) **(B)** Projection of a confocal z-stack showing a CTLA-4⁺ CHO cell (blue) in contact with a CD86 GFP-expressing CHO cell (green) in the presence of Bafilomycin A (BafA). GFP inside the CTLA-4 cell appears as cyan puncta. **(C)** Confocal micrographs of adherent CD86-expressing CHO cells and CTLA-4⁺ CHO cells after overnight incubation. CD86 (green) and CTLA-4 (red) were detected by antibody staining. Co-localization of CD86 and CTLA-4 is shown in yellow. Lower panels show an enlargement of the boxed area, with single color images shown in white for equal contrast. CHO-CD86 cultured alone are shown in fig. S7A. **(D)** Confocal images of cells expressing wild type (wt) CTLA-4 or CTLA-4 lacking the cytoplasmic domain (del36) (red) incubated for 2 hours with CD86-GFP (green) expressing cells. **(E)** Flow cytometric analysis of CD86 surface expression on CHO-CD86 cells co-incubated with increasing numbers of untransfected (control), wild-type CTLA-4 or CTLA-4 del36 cells (expressed as % CTLA-4⁺ cells in the co-culture). Surface CD86 was detected by antibody staining. **(F)** Response of CFSE-labelled CD4⁺CD25⁺ T cells stimulated in the presence of anti-CD3 antibody with CD86-expressing cells fixed after co-culture with CTLA-4 WT or CTLA-4 del36. All data are single representatives of 3 or more independent experiments, Scale bars are 10 μ m.

**Fig. 2.**

Human T cells use CTLA-4 to remove CD86 from dendritic cells. **(A)** Typical CD86 expression on a human monocyte-derived dendritic cell (DC) cultured in the absence of T cells. **(B)** DC cultured for 72h with anti-CD3-activated CD4⁺CD25⁻T cells (outlined in white) stained with anti-CD86 (green) and anti-CTLA-4 (red). Single staining is shown as white for equal contrast. Cells were co-cultured in the absence or presence of blocking anti-CTLA-4. **(C)** Quantitation of surface CD80 and CD86 expression on DCs after co-culture with T cells in the presence or absence of anti-CTLA-4 determined by flow cytometry. Data show MFI change pooled from >5 experiments with SEM. **(D)** Confocal micrographs of allogeneic DCs co-cultured overnight with CTLA-4-transfected (CTLA-4 TF) or control resting CD4⁺CD25⁻human T cells. Cultures were fixed and stained with anti-CD86 (green) or anti-HLA-DR (red). White arrowheads highlight position of T cells, green arrowheads highlight DCs. White images show single color staining for contrast. **(E)** Mean fluorescence intensity of surface CD86 on DCs after incubation with either CTLA-4-transfected or control T cells as determined by flow cytometry. All data are representative of at least 3 independent experiments. Error bars represent the SEM. Scale bars = 10 μ M.

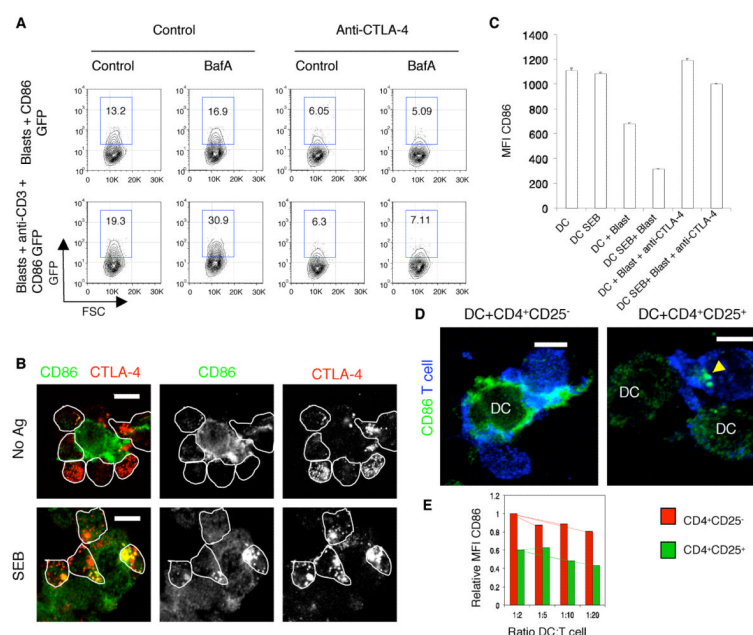
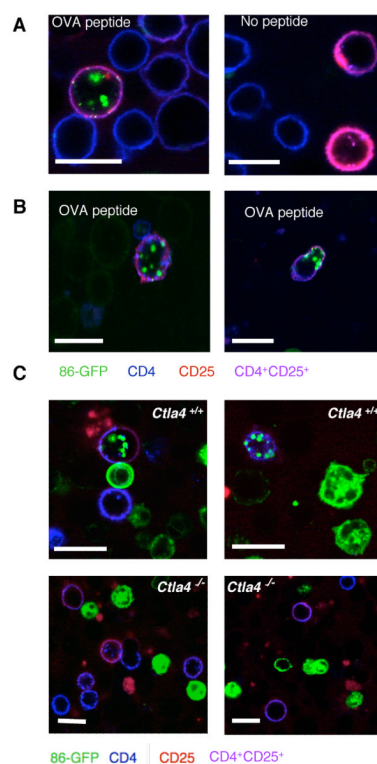


Fig. 3. TCR stimulation promotes CTLA-4 trafficking and trans-endocytosis of CD86. **(A)** Acquisition of CD86-GFP from CHO cells by human CD4⁺ T cell blasts in the presence or absence of anti-CD3 stimulation. Right hand panels show the effect of anti-CTLA-4 antibody on GFP uptake. **(B)** SEB-specific CD4⁺ T cell blasts were incubated with either unpulsed or SEB-pulsed DCs. Cells were fixed and stained with anti-CD86 (green) and anti-CTLA-4 (red). Yellow indicates colocalization. Single colors are shown in white for equal contrast. **(C)** Surface levels of CD86 on DCs incubated with SEB-specific T cell blasts for 16h as determined by flow cytometry. **(D)** CD4⁺CD25⁺ (Treg) or CD4⁺CD25⁻ T cells were incubated with DCs and anti-CD3 overnight, fixed, stained using anti-CD86 (green), anti-CD3 for T cells (blue), and visualized by confocal microscopy. Yellow arrow indicates CD86 puncta within T cells. **(E)** Surface levels of CD86 on DCs incubated with CD4⁺CD25⁺ (Treg) and CD4⁺CD25⁻ T cells overnight determined by flow cytometry. All data are representative of at least 3 independent experiments. Error bars represent the SEM. Scale bars = 10 μ M.

**Fig. 4.**

In vivo capture of CD86 by CTLA-4. Balbc *Rag2*^{-/-} mice were reconstituted with CD86-GFP transduced Balbc *Rag2*^{-/-} bone marrow to permit the development of APC expressing CD86-GFP. 3wk later mice were injected with DO11.10 CD4⁺ T cells and immunized as described in figure S16. **(A)** 6h after i.v. OVA peptide re-challenge, splenocytes were harvested, labeled at 4°C for CD4 (blue) and CD25 (red) and immediately imaged by confocal microscopy. Representative images of T cells from OVA peptide challenged or unchallenged mice are shown. **(B)** Representative images of CD4⁺ T cells purified from spleen after treatment *in vivo* with peptide showing CD4 and CD25 staining. **(C)** CD4⁺ T cells from either *Ctla4*^{+/+} or *Ctla4*^{-/-} (*Rag2*^{-/-} DO11.10 Rip-mOVA) mice were injected into mice that previously received CD86-GFP *Rag2*^{-/-} bone marrow cells. Cells were re-challenged with OVA *in vivo* as above. Splenocytes were isolated and immediately analyzed for CD86-GFP (green), CD25 (red) CD4 (blue) by confocal microscopy. Two representative panels are shown for both *Ctla4*^{+/+} and *Ctla4*^{-/-} conditions. Data are representative of 3 independent experiments. Scale bars = 10 μM.