CTLA-4 mediated transendocytosis of co-stimulatory molecules primarily targets migratory dendritic cells

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One Sentence Summary: CTLA-4 transendocytosis is elicited by self-antigens and downregulates costimulatory ligands on migratory dendritic cells.
Abstract: CTLA-4 is a critical negative regulator of the immune system and a major target for immunotherapy. Yet precisely how it functions *in vivo* to maintain immune homeostasis is not clear. As a highly endocytic molecule, CTLA-4 can capture costimulatory ligands from opposing cells by a process of transendocytosis (TE). By restricting costimulatory ligand expression in this manner, CTLA-4 controls the CD28-dependent activation of T cells. Regulatory T cells (Tregs) constitutively express CTLA-4 at high levels and in its absence show defects in TE and suppressive function. Activated conventional T cells (Tconv) are also capable of CTLA-4-dependent TE, however the relative use of this mechanism by Tregs and Tconv *in vivo* remains unclear. Here we set out to characterize both the perpetrators and cellular targets of CTLA-4 TE *in vivo*. We found that Tregs showed constitutive cell surface recruitment of CTLA-4 *ex vivo* and performed TE rapidly following TCR stimulation. Tregs outperformed activated Tconv at TE *in vivo*, and expression of ICOS marked Tregs with this capability. Using TCR-transgenic Tregs that recognise a protein expressed in the pancreas, we showed that presentation of tissue-derived self-antigen could trigger Tregs to capture costimulatory ligands *in vivo*. Finally, we identified migratory dendritic cells (DCs) as the major target for Treg-based CTLA-4-dependent regulation in the steady state. These data support a model in which CTLA-4 expressed on Tregs dynamically regulates the phenotype of DCs trafficking to lymph nodes from peripheral tissues in an antigen-dependent manner.
Introduction

CTLA-4 is essential to prevent aberrant T cell responses against self-proteins. Mice genetically deficient in CTLA-4 develop a lymphoproliferative syndrome (1, 2) with T cell reactivity to tissue-specific self-antigens, such as PDIA2 (protein disulfide isomerase-associated 2), an enzyme expressed in pancreatic acinar cells (3). Individuals harboring CTLA-4 mutations exhibit multiple autoimmune features (4, 5) reflecting the importance of this pathway in controlling T cell responses to self-proteins in humans. Thus, data from mice and humans suggest that CTLA-4-dependent regulation can be elicited by self-antigens and is essential to temper self-reactivity. Nevertheless, the ability of self-antigens to mobilise CTLA-4 expression and initiate CTLA-4-dependent functions has not been directly tested.

Considerable CTLA-4 functional activity is known to map to the Foxp3+ regulatory T cell (Treg) population, and ablating CTLA-4 in this compartment results in lethal immune dysregulation (6). It has been proposed that the CTLA-4 pathway represents a core mechanism of Treg suppression (7) that is indispensable for normal immune homeostasis. Accordingly, loss of CTLA-4 impairs Treg function in multiple models (3, 6, 8-14) and conversely CTLA-4 over-expression can confer suppressive capacity (15, 16). Recently, single cell RNA-seq analysis confirmed Ctla4 as a component of the core gene signature shared by all Tregs, independent of additional phenotypic variation (17) such as that associated with tissue residency at particular anatomical sites (18). The capacity to utilise the CTLA-4 pathway therefore appears to be hardwired into the Treg lineage.
Treg-expressed CTLA-4 functions in a cell-extrinsic manner to control the CD28-dependent activation of naïve T cells by restricting their access to costimulatory ligands. This function can be mediated by ligand competition, given the higher affinity of CTLA-4 for the ligands (CD80 and CD86) it shares with CD28 (19). CTLA-4 is also able to capture its ligands from antigen presenting cells (APC) by transendocytosis (TE), targeting them for degradation within the recipient cell (20). Notably, multiscale spatiotemporal modelling of T cell-APC interactions suggests that simple ligand competition by CTLA-4 is not sufficient to interrupt CD28 engagement, with TE being required to effectively eliminate the costimulatory signal (21). Additional mathematical modelling indicates that ligands need to be of optimal affinity to maximise TE, and that the process is a quantitative one, dictated by expression levels of ligands and receptors (22, 23). Tregs from humans with heterozygous CTLA-4 deficiency show a quantitative defect in TE and suppressive function (5), while in model systems where APCs have been subjected to TE, ligand loss is proportional to the number of CTLA-4-expressing cells and the amount of remaining ligand directly correlates with the number of T cells that can be induced to proliferate (23). Thus, establishing the identity of the cellular partners involved in CTLA-4 regulation in vivo, their expression of ligands and receptors, and their response to stimulation remain important issues in understanding CTLA-4 biology.

Therefore, in this study we set out to analyse the kinetics of CTLA-4 trafficking in Tregs and activated conventional T cells (Tconv) and compare the capacity of these cell subsets to perform CTLA-4-dependent TE in vitro and in vivo. Tregs showed constitutive recruitment of CTLA-4 to the cell membrane and performed TE rapidly following TCR stimulation. Conversely, Tconv relied upon de novo synthesis of CTLA-4 to establish a similar functional capability. We found
that while both Tregs and activated Tconv were inherently capable of CTLA-4-dependent TE, in a competitive scenario *in vivo* TE was restricted to Tregs and was a property of the Treg fraction characterised by high ICOS expression. Further, by combining the expression of GFP-tagged ligands *in vivo* with a TCR transgenic approach, we showed that tissue-expressed self-antigens were sufficient to elicit CTLA-4-dependent TE. Finally, using gene-deficiency, antibody blockade and adoptive transfer approaches we identified lymph node (LN) migratory dendritic cells (DCs) as the major target of CTLA-4-dependent ligand down-regulation *in vivo*.
Results

Constitutive cycling of CTLA-4 in Tregs

According to the TE model, the biologically relevant fraction of CTLA-4 is the cycling pool of CTLA-4 molecules that traffic to the plasma membrane and are available for ligand-binding within a given time frame. However, the dynamics of CTLA-4 cycling in murine Tregs and Tconv, including its modulation by TCR engagement and its relation to the surface and total CTLA-4 pools, has not been ascertained. We therefore quantified cycling CTLA-4 in CD4 T cells by assessing uptake of labeled anti-CTLA-4 Ab at 37°C for 2 hours. Unlike Tconv, a sizeable fraction of Tregs exhibited constitutive CTLA-4 cycling ex vivo in the absence of stimulation, despite very limited cell surface expression (Fig. 1A and B). Simple cell surface stains for CTLA-4 therefore greatly underestimate the quantity of functionally relevant membrane-exposed protein. Activation of Tregs with anti-CD3/CD28 beads for 6 hours significantly increased cycling CTLA-4 MFI (Fig. 1A and B), despite a transient but reproducible decrease in the total CTLA-4 pool (Fig. 1B). Tconv needed prolonged TCR stimulation for CTLA-4 to be induced, consistent with a requirement for de novo production, and the cycling and surface levels were lower than for Tregs at all timepoints examined in line with the lower total expression of CTLA-4 (Fig. 1, fig. S1). TE was assessed by using Chinese Hamster Ovary (CHO) cells expressing GFP-tagged CD80 as a source of ligand (20) and measuring ligand capture by flow cytometry. Consistent with the CTLA-4 cycling data, only Tregs were capable of TE at 6 hours whereas both Tregs and Tconv exhibited TE following 24 hours of stimulation (Fig. 2A and B). Thus, Tconv acquired a similar capacity as Tregs to elicit TE in vitro once they had been stimulated to upregulate comparable levels of CTLA-4. Internalisation of captured ligands was confirmed by confocal microscopy (Fig. 2C and D) and
further corroborated by the accumulation of captured ligand in the presence of the lysosomal inhibitor Bafilomycin A1 (BafA) (fig. S2).

_Transendocytosis is CTLA-4-dependent and sensitive to low levels of TCR engagement_

To distinguish any non-specific ligand transfer from that driven by CTLA-4, we used Tregs isolated from mixed bone marrow (BM) chimeric mice harboring both wildtype and CTLA-4-deficient cells. These animals lack the T cell hyperactivation phenotype seen in germline CTLA-4-deficient mice and therefore allow CTLA-4-deficient cells to be studied in a comparable activation state to wildtype cells. CTLA-4-sufficient Tregs efficiently captured CD80-GFP from CHO cells whereas CTLA-4-deficient Tregs present in the same well, distinguished by congenic markers, failed to capture ligand (Fig. 3A and B). Addition of BafA further increased the GFP signal in CTLA-4-sufficient Tregs (Fig. 3A and B). The flow cytometry data were corroborated by confocal microscopy, confirming ligand internalisation by CTLA-4-sufficient Tregs (fig. S3).

TE could be elicited at low levels of TCR engagement, with marked ligand uptake at anti-CD3 Ab concentrations of approximately 30 ng/ml (Fig. 3C and fig. S4). Interestingly, a low level of ligand capture was evident even in the absence of anti-CD3 Ab. Subdividing Tregs into effector and resting populations, based on CD45RB and CD62L expression (fig. S5), revealed that this was entirely attributable to the effector Treg subset (Fig. 3C and fig. S4), consistent with their higher expression and cycling of CTLA-4 (fig. S5). Using BM-derived DCs, we were able to demonstrate peptide dose-dependent ligand downregulation by TCR-transgenic Tregs in vitro and this was inhibited by anti-CTL4 Ab (fig. S6).

_Preferential transendocytosis by Tregs in vivo_
We previously showed that Tregs are able to capture GFP-tagged CD86 in vivo (20). In these experiments, GFP-tagged ligands were introduced into Rag2-/- BM cells that could give rise to APC following adoptive transfer to Rag2-/- hosts. Here we adopted a similar approach to extend our analysis to CD80, and to compare the ability of Tregs and Tconv to acquire ligands in vivo. CD4 T cells from DO11 x RIP-mOVA mice, that contain a mixture of ovalbumin (OVA)-specific Tconv and Tregs (24), were adoptively transferred into mice injected with CD80-GFP transduced Rag2-/- BM cells 3 weeks earlier. Antigen was provided by OVA/alum immunisation. Although OVA-specific Tconv upregulated CTLA-4 expression in response to immunisation (Fig. 4A), there was little evidence of TE. In contrast, the Treg population robustly captured GFP-tagged CD80 (Fig. 4A) and its intracellular localisation was confirmed by confocal analysis ex vivo (Fig. 4B). Cells that had captured ligand in vivo mapped to the antigen-specific (DO11+) Foxp3+ CD25+ CTLA-4+ ICOS+ population (Fig. 4C) and GFP acquisition positively correlated with Treg expression of ICOS and CTLA-4 (fig. S7).

_Tregs capture ligands in response to tissue-expressed self-antigen_

Together with our previous study (20), the above experiments establish that Tregs can capture both CD80 and CD86 in response to immunised antigen in vivo. However, a key unresolved question is whether TE can be triggered in response to self-antigens. To explore this issue, we first asked whether expression of CTLA-4 by Tregs was enhanced at sites of self-antigen recognition. We took advantage of the DO11 x RIP-mOVA mice, in which a T cell response is targeted against the OVA-expressing β-cells in the pancreas ultimately leading to diabetes induction (25). OVA-specific Tregs in the pancreas expressed markedly higher levels of CTLA-4 than those at distant sites (Fig. 5A). In contrast, although CTLA-4 was upregulated in Tconv
responding to pancreatic antigen, both the proportion of positive cells and the MFI of CTLA-4 staining were much lower than that observed in Tregs (Fig. 5A and fig. S8A). We noted a tight correlation between CTLA-4 and ICOS expression in Tregs across all tissues examined (Fig. 5B, Fig. 5C and fig. S8B), suggesting that ICOS marks Tregs with the highest potential to elicit CTLA-4-mediated TE.

We have previously shown that OVA-specific Tregs use CTLA-4 to prevent OVA-specific Tconv from causing diabetes in mice expressing the RIP-mOVA transgene (9). To directly test whether pancreas-expressed antigen was capable of triggering TE, we introduced Rag2−/− BM transduced with CD80-GFP into mice expressing OVA in the pancreas (RIP-mOVA/Rag2−/−). This permitted the development of CD80-GFP-bearing APC in a setting where OVA is presented as a tissue self-antigen. Three weeks following reconstitution, mice were injected with CD4 T cells from DO11 x RIP-mOVA mice (containing OVA-specific Tregs and Tconv). Antigen-specific Tregs isolated from the pancreas of these mice 7 days later showed acquisition of CD80-GFP, and this was most evident in the fraction expressing the highest levels of CTLA-4 (Fig. 5D and E). In contrast, antigen-specific Tconv showed little evidence of ligand acquisition. Treg ligand capture was stimulated by presentation of pancreatic OVA as revealed by comparison with cell transfers into littermate mice lacking OVA expression (Fig. 5D and E).

**Polyclonal Tregs modulate DC phenotype in a CTLA-4 dependent manner**

If presentation of endogenous self-antigens drives TE, we reasoned that polyclonal Tregs should be capable of acquiring ligand in response to self-peptides constitutively presented by APC in the steady-state. We therefore adoptively transferred polyclonal CD4 T cells into mice expressing
CD80-GFP and looked for evidence of ligand acquisition in splenic Tregs in the absence of exogenous stimulation. Seven days post transfer, Tregs, but not Tconv, had acquired GFP-tagged ligands in a manner that was blocked by injection of anti-CTLA-4 Ab (Fig. 6A and B). Since conventional DCs (cDCs) are recognised as key APC for the maintenance of peripheral tolerance by Tregs (26-29), we assessed if CD80-GFP expression within this population was impacted by the presence of T cells. In parallel with the increased GFP signal in Tregs, we noted a decreased GFP signal in cDCs and a concomitant decrease in antibody staining for CD80 (Fig. 6C and D). Levels of CD80 and GFP on macrophages were not altered (Fig. S9A). These data suggest that polyclonal Tregs constitutively perform CTLA-4-dependent TE and modulate the phenotype of cDCs in the steady state.

*Tissue migratory DCs are the primary targets for CTLA-4-based regulation in vivo*

Since our data implicated self-antigens as a trigger for TE, and cDCs as target cells, we hypothesised that migratory DCs, which transport antigen from tissues, could be an important player in this process. Migratory DCs have been shown to be responsible for the traffic of self-antigens from a range of peripheral tissues including skin (30), stomach (31) and endocrine pancreas (30). To test the involvement of migratory DCs, we used CTLA-4 deficiency or blockade, reasoning that APC under CTLA-4 dependent control should be identifiable by an increase in costimulatory ligand expression upon disruption of this interaction. We subdivided DCs into migratory versus resident using MHC Class II and CD11c expression as shown by others (32-34) and took advantage of the gating strategy published by Guilliams et al. (35) to characterise cDC subsets (fig. S10, fig. S11). Mice deficient in CTLA-4 showed a dramatic increase in CD80 and CD86 on migratory DCs (MHCIIhiCD11cint) within LN while resident DCs
(MHCII$^{\text{int}}$CD11c$^{\text{hi}}$) remained unchanged (Fig. 7A and B). We observed that both cDC1 and cDC2 populations within the migratory DC population were affected. Since CTLA-4-deficient mice develop a lymphoproliferative syndrome with widespread immune activation (fig. S12), the altered phenotype of migratory DCs might have been influenced by ongoing inflammation since birth. To address this caveat, we performed short-term (1 day) blockade with anti-CTLA-4 Ab, which elicited the same pattern of changes, with migratory DCs showing increased expression of CD80 and CD86 and resident DCs remaining unchanged (Fig. 7C and D). Only at a later timepoint (day 4) did resident DCs show differences, with increased CD80 in the cDC2 subset. These data are consistent with steady state CTLA-4-dependent downregulation of CD80, and to a lesser extent CD86, on migratory DCs in LNs. In line with regulation being limited to tissue DC, splenic DCs showed only modest changes as a result of CTLA-4 deficiency or blockade, similar to LN resident DCs (fig. S13). Macrophages did not show altered CD80 and CD86 expression following CTLA-4 blockade (fig. S9C). To confirm that migratory DCs were targeted by CTLA-4, we used FITC skin painting to identify DCs migrating from skin to draining LNs. FITC-labelled DCs that migrated to the LNs were MHCII$^{\text{hi}}$CD11c$^{\text{int}}$ and showed increased expression of CD80 and CD86 following CTLA-4 blockade, while resident DCs in the same LN remained largely unchanged (fig. S14). As a complementary approach, we sought to restore CTLA-4-dependent regulation by reconstituting Rag2-deficient mice with CD4 T cells. This resulted in the downregulation of CD80 and CD86 on LN migratory DCs while resident DC remained unchanged (Fig. 8, fig. S15). Again, changes to splenic cDC populations were minimal (fig. S16). If the T cells were CD25-depleted prior to transfer, downregulation of costimulatory ligands was greatly reduced, consistent with Tregs being the major elicitors of CTLA-4 function (Fig. 8). Expanding Tregs by injecting IL-2-complex (36) reduced cDC ligand expression
further (fig. S17). Collectively these data reveal a striking preference for migratory DCs as the target of CTLA-4-dependent ligand regulation by Tregs *in vivo*. 
Discussion

This study builds on our previous work on CTLA-4-dependent TE (20) and reveals that Treg CTLA-4 is constitutively trafficked to the cell membrane, permitting continuous TE by this population in the steady state. Self-antigens are a likely stimulus for this behavior, and we directly demonstrate that Treg encounter with self-antigen expressed in a peripheral tissue can drive high CTLA-4 expression and trigger capture of costimulatory ligands.

Although activated Tconv are capable of performing TE, we show that in a competitive scenario in vivo, where Tregs and Tconv specific for the same antigen co-exist, TE is almost entirely restricted to the Treg compartment. This holds true for responses to both tissue-antigen and immunised antigen and may reflect a failure of Tconv in these settings to achieve the very high CTLA-4 levels required for ligand capture. The ability of Tregs but not Tconv to acquire ligand was recapitulated when polyclonal T cells were studied, although here distinct TCR repertoires may add an additional variable. The Treg repertoire is believed to be skewed towards recognition of self-proteins (37, 38) and peripheral Tregs express higher levels of Nur-77 than Tconv, consistent with ongoing receipt of TCR signals (39, 40). Tregs are thought to continuously respond to self-antigen derived TCR ligands (24, 41), which promote their suppressive capacity (42, 43) and guide their anatomical location (24, 41, 44-46). Thus, superior TE by Tregs may reflect the preferential ability of Tregs to interact with and be stimulated by self-antigen-bearing APC, their higher levels of CTLA-4 and longer dwell times. All of these features are consistent with models of how CTLA-4 TE is thought to operate (22, 23, 47).
Antigen-specific Tregs capable of capturing ligands in vivo in our experiments were characterised by high ICOS expression. Furthermore, we identified a tight relationship between ICOS and CTLA-4 expression in Tregs, with ICOS positivity being restricted to Tregs bearing the highest levels of CTLA-4. Since only the latter were capable of appreciable ligand acquisition, ICOS effectively serves as a marker of Tregs with transendocytic potential, perhaps explaining the prognostic value of ICOS+ Tregs in some cancer settings (48). Interestingly, expression of ICOS, along with CD103 and TIGIT, has been suggested to identify pancreas-infiltrating Tregs that have recently been activated by antigens (49). Since ICOS and CTLA-4 are known to be highly sensitive to CD28 engagement (50, 51), together this suggests that the ICOShiCTLA-4hi phenotype of Tregs with TE potential may reflect a recent history of TCR and CD28 engagement.

Ligand capture by polyclonal Tregs in vivo, seen in our experiments, was accompanied by a loss of GFP (and ligand) from cDCs. Since the expression of the GFP-tagged ligand is driven by a ubiquitous viral promoter, and costimulatory ligand expression is therefore decoupled from endogenous gene regulation mechanisms, this supports the idea that ligand removal rather than altered gene expression is responsible. Numerous studies have reported CTLA-4 dependent downregulation of CD80 and CD86 in vitro (6, 20, 52, 53) however relatively few have documented this process in vivo (54). In the context of graft versus host disease, one study found that Tregs were able to decrease costimulatory ligand expression on cDCs in vivo and thereby inhibit fast-phase lymphopenia-induced proliferation (55). The capacity to downregulate CD80 and CD86 was linked to the Treg:DC ratio and was lost if Tregs were rendered CTLA-4
deficient. These changes are consistent with the TE model and suggest that Treg CTLA-4 acts to continuously dampen costimulatory ligand expression on DCs in the steady state.

Treg-driven downregulation of CD80 and CD86 on APCs has been documented by numerous groups in a wide variety of experimental systems (reviewed in (56)). Therefore, the observation that DCs interacting with Tregs in vivo bear a CD86hi phenotype (57) has constituted a paradox. The notion that the high expression of CD86 reflects the subset of DC (migratory rather than resident) goes some way towards resolving this paradox. Presentation of tissue self-antigens has been shown to map to the migratory DC subset in several systems including in the context of the gastric H+K+ ATPase autoantigen (31) and pancreatic islet antigen (30). Migratory DCs carrying tissue antigen to the draining LNs could conceivably pose the highest risk for triggering a Tconv response towards low-affinity self-antigens due to their high expression of MHC class II and costimulatory ligands, even in germ free or TLR pathway deficient mice (58). Such risk might invoke a particular need for Treg-mediated control; indeed it has been reported that migratory DCs entering LNs are actively intercepted by Tregs on and around the collagen fibres just under the LN capsule (59). Furthermore, imaging studies have revealed Tregs clustering with both migratory cDC1 in the T cell zone and migratory cDC2 that occupy the interfollicular space by virtue of EBI2-dependent chemotaxis (32, 57). Consistent with this, our experiments show non-discriminatory CTLA-4-mediated control of both migratory cDC1 and cDC2.

Using multiple approaches, we have shown that CTLA-4 selectively targets migratory DCs over other CD80/86-expressing cells such as resident DCs and CD64+F480+ macrophages. One limitation of our study is that it focuses on TE in the steady state and does not address whether
the target populations for TE change in the setting of infection. Inflammatory stimuli not only alter DC phenotype but also greatly increase DC migration rates, potentially skewing the DC:Treg ratio. Dynamic changes in cellular phenotypes and ratios may result in different cell types interacting with Treg and undergoing CTLA-4-dependent modification in infectious settings. Furthermore, inflammation-driven increases in expression of CD80 and CD86 may elevate them beyond the reach of TE-mediated control.

Collectively, these findings provide new insights into the steady-state operation of one of the most crucial mediators of Treg function. The concept that migratory DCs are continuously modified by Treg-expressed CTLA-4 in the steady state may be of particular relevance to the field of checkpoint immunotherapy since migratory cDC1s transport tumor antigens to draining LNs (60, 61). Understanding the cellular targets of CTLA-4 based regulation will be important for unravelling the synergy between therapeutic targeting of CTLA-4 and the PD-1/PD-L1 axis.

**Materials and Methods**

**Study design.** The goal of this study was to enhance our understanding of the cells that elicit CTLA-4-based immunoregulation *in vivo* and to identify the APC populations targeted by this process. The experimental approaches include flow cytometry to enumerate population frequencies and/or staining intensity, confocal microscopy to assess captured GFP-tagged ligands and adoptive transfer studies using genetically modified mice. Sample sizes were based on previous experiments or the limiting factor in a given experiment (e.g. how many donor mice available, or cell yields following purification). No outliers were excluded. The number of
replicates is provided in each figure legend. There was no randomization and no blinding. Littermate controls were used where appropriate.

**Mice.** BALB/c and DO11.10 mice were from The Jackson Laboratory. *Rag2*-/- mice were purchased from Taconic Farms. RIP-mOVA mice on a BALB/c background that express a membrane-bound form of OVA under the control of the rat insulin promoter (from line 296-1B) were a gift from W. Heath (Walter and Eliza Hall Institute, Melbourne, Australia). BALB/c CTLA-4-/- mice were kindly provided by A. Sharpe (Harvard, Boston, MA). DO11.10 and RIP-mOVA mice were crossed (DO11 x RIP-mOVA) as previously described (24). Mixed BM chimeric mice were made by transferring equal parts of T cell depleted wildtype and CTLA-4-/- congenically marked BM i.v. into irradiated (2 Gy) *Rag2*-/- recipients. Animals were allowed to reconstitute for at least 9 weeks. Mice were housed in individually ventilated cages with environmental enrichment (e.g. cardboard tunnels, paper houses, chewing blocks) at University College London Biological Services Unit. Experiments were performed in accordance with the relevant Home Office project and personal licenses following institutional ethical approval (University College London).

For experimental procedures 6-10 week old mice (male and female) were used, unless otherwise stated. All injections were carried out in the absence of anesthesia and analgesia, typically between 2pm and 11pm, and mice were returned to the home cage immediately following the procedure. The first injection for *in vivo* TE experiments was typically at 10am. The welfare of experimental animals was monitored regularly (typically immediately postprocedure, then at least every 2–3 days). No adverse events were noted during these experiments.
Flow cytometry. Cells were stained with the following antibody clones: B220 (RA3-6B2), CD3 (145-2C11), CD4 (GK1.5), CD11b (M1/70), CD11c (N418 and HL3), CD19 (1D3), CD25 (PC61), CD26 (H194-112), CD45 (30-F11), CD45RB (C363.16A), CD62L (MEL-14), CD64 (X54-5/7.1), CD69 (H1.2F3), CD80 (16-10A1), CD86 (GL-1), CD103 (M290), CD172a (P84), CTLA-4 (UC10-4F10-11), DO11.10 TCR (KJ126), F4/80 (BM8), Foxp3 (FJK-16s), ICOS (C398.4A), MHCII (M5/114.15.2), NKp46 (29A1.4), TCRβ (H57-597), Thy1.1 (OX-7), Thy1.2 (53-2.1), XCR1 (ZET). Acquisition was performed on LSRFortessa flow cytometer using the FACSDiva acquisition software (all from Becton Dickinson). Analysis of flow cytometry data was performed using FlowJo v10 (FlowJo LLC). MFI denotes geometric mean. The CRAN package Rtsne was used to compute t-SNE in R.

In vitro CTLA-4 cycling. CD4 cells were isolated from BALB/c LNs using positive magnetic selection (L3T4 MicroBeads, Miltenyi Biotec). Where stated, purified cells were cultured in the presence of anti-CD3 anti-CD28 activation beads (Invitrogen, Thermo Fischer Scientific) at a 2:1 T cell-to-bead ratio for up to 24 hours. Staining was carried out at 4°C to label surface CTLA-4; at 37°C for 2 hours to identify cycling CTLA-4; or after cell fixation/permeabilisation to stain the total CTLA-4 pool.

In vitro TE. CD80-GFP CHO cells were generated by transduction with pMP71 retroviral vectors carrying a CMV promoter driven eGFP-fused mouse CD80. Resulting transfectants were sorted (FACS Aria II, Becton Dickinson) for uniform GFP expression. CD4+ T cells from BALB/c LNs or mixed BM chimeric mice were mixed at a 1:1 ratio with CD80-GFP donor CHO
cells for up to 24 hours in the presence of soluble anti-CD3 and 100μM TAPI-2 where indicated. Where stated, cells were cultured in the presence of anti-CTLA-4 (4F10; 2B Scientific) at 100μg/ml and 25nM Balifomycin A1 (Sigma-Aldrich) was used for up to 6 hours prior to culture termination. For confocal analysis, CD4+CD25+ Treg (Regulatory T Cell Isolation Kit, Miltenyi Biotec) were cultured with CD80-GFP donor CHO cells. Live cells were stained and imaged in 8-well Nunc Lab-Tek II Chamber Slides (Sigma-Aldrich) on a C2+ Nikon confocal microscope running NIS Elements acquisition software. Image analysis was performed in FIJI (ImageJ).

**Generation of CD80-GFP mice.** GFP-tagged CD80 was inserted into a modified pDual lentiviral vector (gift from H. Stauss, UCL, London, UK), and placed under control of an SFFV promoter. Viral particles were produced in HEK293T cell line by co-transfection with pMD.G and pCMV8.91 vectors using FUGENE6 transfection reagent (Hoffman-La Roche). Harvested supernatant was concentrated by ultracentrifugation and relative viral titer estimated on HEK293T cells. *Rag2*-/ BM from 4-6 week old donor mice was enriched for Lin- cells using magnetic sorting (Lineage Cell Depletion kit, Miltenyi Biotec) and transduced at MOI of 3.3 in StemSpan serum free medium (STEMCELL Technologies) supplemented with murine SCF at 100ng/ml, 10ng/ml TPO and 50ng/ml Flt3L (all from Peprotech). After overnight transduction cells were injected i.v. into irradiated (3.5 Gy) 4-6 week old *Rag2*-/ mice.

**In vivo TE.** For immunisation-driven TE, CD4 cells were isolated from DO11 x RIP-mOVA mice and injected i.v. into CD80-GFP expressing *Rag2*-/ recipients. After 24 hours, mice received 100μg OVA323-339/alum i.p. At day 7, mice were challenged with 100μg of OVA peptide i.v. for 6 hours, in the presence of chloroquine to inhibit lysosomal degradation (600μg
i.p.) for the last 3 hours. Splenocytes were then analysed. For self-antigen driven TE, CD4 cells were isolated from BALB/c or DO11 x RIP-mOVA mice and adoptively transferred into CD80-GFP expressing Rag2-/- recipients expressing RIP-mOVA where indicated. CTLA-4 blockade was performed by i.p. injection of anti-CTLA-4 (4F10) at 500µg/mouse every 2-3 days where indicated. Control Ab-treated mice received hamster IgG. After 6 days mice were injected with 600µg chloroquine i.p. and harvested at day 7. Cells were isolated from pancreases by liberase/DNAse digestion and density separation as previously described (25). Spleens were digested with collagenase/DNAse (62).

**Statistical analysis.** Statistical analysis was performed using Graphpad Prism version 6, and p-values were calculated by two-tailed t test for the means with a 95% confidence interval. Analysis of more than two groups was performed by one-way ANOVA or two-way ANOVA if confound by two factors with a 95% confidence interval and corrected for multiple comparisons. Spearman’s correlation coefficients with 95% confidence intervals (two-tailed test) were calculated in Fig. 5; z-scores for comparison of correlation coefficients were calculated using Fisher’s r to z transformation.
Supplementary Materials

Supplementary Materials and Methods

Fig. S1. Surface, cycling and total CTLA-4 expression by Treg and Tconv.

Fig. S2. Effects of CTLA-4 blockade and inhibition of lysosomal acidification on TE.

Fig. S3. Internalisation of CD80-GFP by CTLA-4 sufficient Treg.

Fig. S4. TE by resting and effector Treg in response to different concentrations of anti-CD3 Ab.

Fig. S5. CTLA-4 expression and cycling by resting and effector Treg.

Fig. S6. CTLA-4-mediated downregulation of CD80-GFP on bone marrow-derived DCs

Fig. S7. GFP acquisition correlates with ICOS and CTLA-4 expression.

Fig. S8. CTLA-4 expression is increased at sites of self-antigen recognition and ICOS marks Treg with highest CTLA-4.

Fig. S9. CTLA-4 targets expression of CD80 and CD86 on cDCs but not macrophages.

Fig. S10. Gating strategy for identification of splenic cDC subsets.

Fig. S11. Gating strategy for identification of LN resident and migratory cDC subsets.

Fig. S12. Phenotype of Tconv and Tregs in CTLA-4-deficient mice.

Fig. S13. CD80 and CD86 expression on splenic cDC subsets following CTLA-4 ablation.

Fig. S14. Functional identification of migratory DCs by FITC skin painting.

Fig. S15. Comparison of CD80 and CD86 expression in settings of CTLA-4 blockade and RAG deficiency.

Fig. S16. CD80 and CD86 expression on splenic cDC subsets in Rag2-deficient mice with or without T cell transfer.

Fig. S17. Impact of Treg expansion on CD80 and CD86 expression on cDC subsets.

Table S1. Raw Data File
References and Notes:


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Figure Legends

Fig. 1. Constitutive cycling of CTLA-4 in Treg. CD4 T cells from BALB/c LNs were cultured in the presence or absence of anti-CD3/anti-CD28 beads at a 2:1 (T:Bead) ratio for 6, 12 or 24 hours and analysed by flow cytometry. (A) Representative FACS plots showing CTLA-4 expression by Tregs (CD4+Foxp3+) and Tconv (CD4+Foxp3-). CTLA-4 was stained on intact cells at 4°C (Surface), at 37°C for 2 hours (Cycling), or on fixed and permeabilised cells (Total). (B) Collated data showing mean ± SD (n=3-4); **p≤0.01, ***p≤0.001, ****p≤0.0001, ns=not significant, two-way ANOVA. Data are representative of at least four independent experiments. Raw data for 12 and 24 hours is shown in Fig. S1.

Fig. 2. TE by Tregs and Tconv *in vitro*. (A-B) CD4 T cells from BALB/c LNs were co-cultured with CD80-GFP expressing CHO cells at a 1:1 ratio in the absence of stimulation or with 0.8µg/ml anti-CD3 Ab for 6 or 24 hours. (A) Representative FACS plots showing total CTLA-4 expression and GFP uptake by Tregs (CD4+Foxp3+) and Tconv (CD4+Foxp3-). (B) Collated data (n=4) showing fraction of GFP-positive cells. (C-D) MACS purified Tregs were cultured overnight with CD80-GFP expressing CHO cells at 1:1 ratio, with or without anti-CD3 Ab; 25nM BafA was added for the final 4 hours of culture. Donor CHO cells were removed by magnetic separation and T cells imaged by confocal microscopy at 20x magnification. (C) Confocal images representative of at least 3 independent experiments. (D) Scoring of confocal images. Each point in unstimulated (n=446) and stimulated (n=323) conditions represents an individual cell from 11-12 separate images. Plots show mean signal intensity of CD25 and GFP and number of GFP fluorescence maxima (representing distinct GFP-filled punctae) per cell.
Graphs show mean + SD (CD25 Fluorescence, GFP Fluorescence), mean ± SD (GFP+ punctae); ***p≤0.001, ****p≤0.0001, ns=not significant, two-tailed paired (B) or unpaired (C) Student’s t tests. Data are representative of three independent experiments.

Fig. 3. TE is CTLA-4-dependent and constitutively active in effector Tregs. (A-B) CD4 T cells isolated from LNs of mixed BM chimeric mice containing CTLA-4-sufficient (WT) and CTLA-4-deficient (KO) cells were co-cultured with CD80-GFP expressing CHO cells at a 1:1 ratio for 6 hours in the presence of 0.8µg/ml anti-CD3 Ab. Lysosomal degradation was inhibited with 25nM BafA where indicated. (A) Representative FACS plots showing acquisition of CD80-GFP by WT and CTLA-4/- Foxp3+ Tregs. (B) Collated data from at least three independent experiments (n=6-9). Mean ± SD; **p≤0.01, ****p≤0.0001, paired two-tailed Student’s t test. (C) CD4 T cells from BALB/c LNs (n=5) were co-cultured with CD80-GFP expressing CHO cells at a 1:1 ratio for 6 hours in the presence of different anti-CD3 Ab concentrations. 100µM TAPI-2 was added to inhibit shedding of CD62L. Graphs show the frequency of GFP positive cells within all Treg (Total Treg), CD45RB+CD62L+ Treg (resting Treg) or CD45RB-CD62L- (effector Treg) and are representative of two independent experiments.

Fig. 4. Preferential TE by Tregs in vivo. CD80-GFP expressing mice were injected i.v. with 5-10 x 10^6 CD4 T cells from DO11 x RIP-mOVA mice and immunised with OVA/alum 24 hours later. 7 days post T cell transfer, mice were challenged with OVA peptide for 6 hours, in the presence of chloroquine to inhibit lysosomal degradation (600µg i.p.) for the last 3 hours. (A) Acquisition of CD80-GFP by DO11 Tconv (CD4+Foxp3-) and DO11 Tregs (CD4+Foxp3+) from spleens of immunised or unimmunised mice. Plots are representative of at least three
independent experiments. (B) Splenocytes were enriched for CD4+ T cells, stained for CD4 and CD25 and imaged at 20x magnification. Images are representative of at least four independent experiments. (C) tSNE dimensionality reduction analysis of CD3+CD4+ T cells in the immunised setting. GFP +ve cells are highlighted by the black gate. Colour axes shows median expression of GFP, Foxp3, CD25, CTLA-4, DO11 and ICOS in each cell.

Fig. 5. Ligand capture by Tregs in response to tissue-expressed self-antigen. (A) DO11 Tconv (CD4+Foxp3-) and Tregs (CD4+Foxp3+) from spleens, peripheral LN (axillary, brachial, inguinal and cervical; pLN), pancreatic lymph nodes (PanLN) and the pancreas of 12 week old DO11 x RIP-mOVA mice (n=3) were stained for intracellular CTLA-4 expression and analysed by flow cytometry. (B) Representative FACS plots showing expression of ICOS and CTLA-4 in DO11 Tregs and Tconv in the pancreas. (C) Correlation of ICOS and CTLA-4 expression in Tregs and Tconv from lymphoid tissues and the pancreas of DO11 and DO11 x RIP-mOVA mice (n=60 datapoints from 6 mice). Lines have been added to map linear relationships for visualisation purposes. p value denotes comparison of the z-transformed r values. (D-E) CD80-GFP expressing mice, or mock-transduced mice (GFP-), with or without pancreatic expression of OVA were injected i.p. with 5-10 x 10^6 CD4 T cells from DO11 x RIP-mOVA mice. 6 days post T cell transfer mice were injected with chloroquine (600µg i.p.). (D) Representative FACS plots showing acquisition of CD80-GFP by DO11 Tregs and DO11 Tconv in the pancreas at day 7. (E) Collated data from three independent experiments showing mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, multiple t tests.

Fig. 6. CTLA-4-dependent modulation of cDC phenotype by polyclonal Tregs. CD80-GFP expressing mice were injected i.v. with 5-10 x 10^6 BALB/c CD4 T cells. CTLA-4 was blocked
by i.p. injection of 500µg anti-CTLA-4 Ab every 2-3 days. 6 days post T cell transfer, mice were injected with chloroquine (600µg i.p.) and 24 hours later splenocytes were analysed by flow cytometry. (A) Representative FACS plots and (B) collated data showing acquisition of CD80-GFP by Tconv (CD4+Foxp3-) and Tregs (CD4+Foxp3+) (n=4-8). Data are representative of four independent experiments. (C) Frequency of the CD80+GFP+ population or (D) overall expression of CD80 within Lin-MHCII+CD11c+CD26+ cDCs in mice that received BALB/c CD4 T cells (+CD4) and controls (-CD4). Data show one representative experiment (n=2) of two independent experiments. Mean ± SD; **p≤0.01, ****p≤0.0001, ns=not significant, unpaired two-tailed Student’s t test.

Fig. 7. Impact of CTLA-4 ablation on CD80 and CD86 expression in LN cDC subsets. (A, B) LNs (axillary, brachial, inguinal and cervical) from 17-18 day old CTLA-4-/- mice or CTLA-4+/+ littermate controls were digested and cells stained for analysis by flow cytometry. (A) Representative FACS plots showing CD80 and CD86 expression on migratory and resident cDC subsets. (B) Collated data showing CD80 and CD86 expression on migratory and resident cDCs (n=3). (C,D) BALB/c mice were treated with anti-CTLA-4 Ab or control IgG (Ctrl) and harvested after 1 or 4 days (1 or 2 doses of 500 µg anti-CTLA-4 Ab respectively). LNs (axillary, brachial, inguinal and cervical) were digested and cells stained for cDC markers and CD80 and CD86. (C) Representative FACS plots and (D) collated data (n=3-4) are shown. Mean ± SD; **p≤0.01, ****p≤0.0001, ns=not significant. Statistical significance was determined by two-way ANOVA. Data show one representative experiment of three independent experiments.

Fig. 8. Impact of T cell transfer on CD80 and CD86 expression in LN cDC subsets. Rag2-/- recipient mice were injected with 6 x 10⁶ bulk CD4 T cells or 5.5 x 10⁶ CD25-depleted CD4 T
cells. Six days later, LNs (axillary, brachial, inguinal and cervical) were digested and cells stained for analysis by flow cytometry. (A) Representative FACS plots showing CD80 and CD86 expression on migratory and resident cDC subsets. (B) Collated data of CD80, CD86 and MHCII expression (n=3-4). Mean ± SD; *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001, two-way ANOVA. Data are representative of at least four independent experiments.
A

Unstimulated
Tconv  Treg

Surface

Cycling

Total

Treg  Tconv

CD4

B

Surface

Cycling

Total

CTLA-4 MFI

Fig. 1
Fig. 2
Fig. 3

A

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B

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C

**A**

**B**

**C**

- **Total Treg**
- **Resting Treg**
- **Effector Treg**

- **- BafA**
- **+ BafA**
Fig. 5
Fig. 6
Fig. 7
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**Fig. 8**

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Legend:
- **No transfer**
- **CD4+**
- **CD4+ CD25−**
Supplementary Materials

Supplementary Materials and Methods

Fig. S1. Surface, cycling and total CTLA-4 expression by Treg and Tconv.

Fig. S2. Effects of CTLA-4 blockade and inhibition of lysosomal acidification on TE.

Fig. S3. Internalisation of CD80-GFP by CTLA-4 sufficient Treg.

Fig. S4. TE by resting and effector Treg in response to different concentrations of anti-CD3 Ab.

Fig. S5. CTLA-4 expression and cycling by resting and effector Treg.

Fig. S6. CTLA-4-mediated downregulation of CD80-GFP on bone marrow-derived DCs.

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Fig. S8. CTLA-4 expression is increased at sites of self-antigen recognition and ICOS marks Treg with highest CTLA-4.

Fig. S9. CTLA-4 targets expression of CD80 and CD86 on cDCs but not macrophages.

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Fig. S11. Gating strategy for identification of LN resident and migratory cDC subsets.

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Fig. S15. Comparison of CD80 and CD86 expression in settings of CTLA-4 blockade and RAG deficiency.

Fig. S16. CD80 and CD86 expression on splenic cDC subsets in Rag2-deficient mice with or without T cell transfer.

Fig. S17. Impact of Treg expansion on CD80 and CD86 expression on cDC subsets.

Table S1. Raw Data File
Supplementary Materials and Methods

**In vitro TE from CD80-GFP BMDCs.** Bones (tibiae, femora, humeri, os coxa) were harvested from CD80-GFP expressing RAG2/-/- mice and cleared of tissue before stepwise washing in 70% ethanol and PBS. Bone marrow extraction was performed by crushing the bones, followed by red blood cell lysis and magnetic sorting to isolate CD80-expressing cells. 2 x 10^6 of the isolated cells were differentiated into BMDCs and matured with LPS (Peprotech) in a 10 cm petri dish as previously described (ref 63) CD4+CD25+ T cells isolated from DO11 x RIP-mOVA donor mice were cultured together with CD80-GFP BMDCs at 10:1 = Treg:DC ratio and stimulated by a range of OVA_{323-339} peptide concentrations. Where stated, cells were cultured in the presence of anti-CTLA-4 (4F10; 2B Scientific) at 100µg/ml. Following overnight culture cells were harvested and BMDCs analysed for CD80 and GFP expression by flow cytometry.

**FITC skin painting.** Mouse ears were painted with 20 µL of a 1% FITC (Sigma-Aldrich) and dibutyl phthalate (Sigma-Aldrich) solution (1 part 2% FITC in acetone and 1 part dibutyl phthalate, for a final concentration of 1% FITC). Draining cervical LNs and non-draining popliteal LNs were harvested at day 3, digested and flow cytometric analysis was performed to identify FITC+ migratory cDCs.

**In vivo expansion of Treg.** Treg were expanded by i.p. administration of IL-2 complex (IL-2c), consisting of 1µg IL-2 (eBioscience, Thermo Fisher Scientific) complexed to 5µg JES6-1A12 (eBioscience, Thermo Fisher Scientific). BALB/c mice were treated with IL-2c or PBS at day 0, 1 and 2, and harvested at day 5 for flow cytometric analysis of Treg and cDCs.
Fig. S1.
Fig. S1. Surface, cycling and total CTLA-4 expression by Treg and Tconv. CD4 T cells from BALB/c LNs were cultured in the presence or absence of anti-CD3/anti-CD28 beads at a 2:1 (T:Bead) ratio for 6, 12 or 24 hours and analysed by flow cytometry. Representative FACS plots showing CTLA-4 expression by Treg and Tconv. CTLA-4 was stained on intact cells at 4°C (Surface), at 37°C for 2 hours (Cycling), or on fixed and permeabilised cells (Total). Data are representative of at least four independent experiments.
Fig. S2.
Fig. S2. Effects of CTLA-4 blockade and inhibition of lysosomal acidification on TE. CD4 T cells purified from LNs of WT BALB/c mice were co-cultured with CD80-GFP expressing CHO cells at 1:1 ratio for 6 hours in the presence of 0.8µg/ml anti-CD3. CTLA-4 was blocked with 100µg/ml anti-CTLA-4 and 25nM BafA was added where indicated. (A) Representative FACS plots showing Treg acquisition of CD80-GFP. (B) Collated data from two independent experiments (n=5-6) showing percentage of GFP-positive Treg; Mean ± SD; ***p≤0.001, ****p≤0.0001, unpaired two-tailed Student’s t test.
Fig. S3.
Fig. S3. Internalisation of CD80-GFP by CTLA-4 sufficient Treg. CD4+CD25+ cells from WT/CTLA-4/- mixed (1:1 ratio) BM chimeras were co-cultured with CD80-GFP expressing CHO cells at 1:1 ratio overnight in the presence of 0.8µg/ml anti-CD3 to provide stimulation. 25nM BafA was added for the final 4 hours of culture. (A) Confocal images showing CD80-GFP acquisition by WT and CTLA-4/- Treg. (B) Scatter plot of GFP mean signal intensity of individual WT or CTLA-4/- cells (n=155-393) identified across 11-12 images. Mean + SD; **p≤0.01, ****p≤0.0001, ns=not significant, unpaired two-tailed Student’s t test. Data are representative of two independent experiments.
Fig. S4.
Fig. S4. TE by resting and effector Treg in response to different concentrations of anti-CD3 Ab. CD4 T cells purified from BALB/c LNs were co-cultured with CD80-GFP expressing CHO cells at a 1:1 ratio for 6 hours in the presence of a range of anti-CD3 concentrations. 100μM TAPI-2 was added to inhibit shedding of CD62L. 25nM BafA was added where indicated. Representative FACS plots showing total CTLA-4 expression and GFP uptake by CD45RB+CD62L+ resting Treg or CD45RB-CD62L- effector Treg.
Fig. S5.
Fig. S5. CTLA-4 expression and cycling by resting and effector Treg. CD4 T cells purified from BALB/c LNs were cultured in the presence or absence of anti-CD3/anti-CD28 beads at 2:1(T:Bead) ratio for 6 hours and analysed by flow cytometry. 100µM TAPI-2 was added to prevent shedding of CD62L. (A) Effector (CD62L-CD45RB-) and resting (CD62L+CD45RB+) populations within Treg after 6 hours stimulation in presence of TAPI-2. (B) Representative FACS plots showing CTLA-4 expression by CD62L- and CD62L+ Treg. CTLA-4 was stained on intact cells at 4°C (Surface), at 37°C for 2 hours (Cycling), or on permeabilised cells (Total). (C) Collated data of CTLA-4 expression (n=4). Mean ± SD; **p≤0.01, ****p≤0.0001, ns=not significant, multiple t tests. Data are representative of three independent experiments.
Fig. S6.
Fig. S6. CTLA-4-mediated downregulation of CD80-GFP on bone marrow-derived DCs. 2.5 x 10^5 purified CD4+CD25+ T cells from DO11 x RIPmOVA mice were cultured with LPS-matured CD80-GFP expressing BMDCs at 10:1 = T:DC ratio and in the presence of a range of OVA323-339 peptide concentrations. Anti-CTLA-4 at 100 µg/mL was added to inhibit CTLA-4 function. Following an overnight culture, BMDCS were assayed for GFP and CD80 expression by flow cytometry. (A) Representative FACS plots showing CD80 and GFP expression on BMDCs. (B) Charts demonstrate change in ligand levels over a range of antigen stimulation in control (open symbol) and anti-CTLA-4 treated (closed symbol) wells (n=2-4). Data are representative of more than three similar independent experiments.
Fig. S7.
Fig. S7. GFP acquisition correlates with ICOS and CTLA-4 expression. CD80-GFP expressing mice were injected i.v. with CD4 T cells from DO11xRIPmOVA mice and immunised with OVA/alum 24 h later. 7 d post T cell transfer, mice were challenged with OVA peptide for 6 h, in the presence of chloroquine to inhibit lysosomal degradation (600µg i.p.) for the last 3 h. (A) Principal component analysis of CD3+ CD4+ Treg cells in the immunised setting. Colour axes in top two plots show fluorescence intensity of DO11 TCR and GFP, respectively. Bottom eight plots show loess regression curves of fluorescence intensity of indicated markers along PC2. (B) Spearman correlation of GFP fluorescence intensity versus fluorescence intensity of indicated markers in CD3+ CD4+ Treg cells in the immunised setting. Colour axes and values shown represent correlation coefficients. Principal component analysis was performed in R on scaled and centered data.
Fig. S8.
Fig. S8. CTLA-4 expression is increased at sites of self-antigen recognition and ICOS marks Treg with highest CTLA-4. CD4 T cells from spleens, peripheral LN (axillary, brachial, inguinal and cervical; pLN), pancreatic lymph nodes (panLN) and pancreas of DO11 x RIP-mOVA mice were stained for total CTLA-4 expression and analysed by flow cytometry. (A) Collated data showing percentage of DO11+ Treg (CD4+Foxp3+) or DO11+ Tconv (CD4+Foxp3-) expressing CTLA-4 at different tissue sites (n=3); Mean ± SD; **p≤0.01, ***p≤0.001, ****p≤0.0001, multiple t tests. (B) Representative FACS plots showing co-expression of CTLA-4 and ICOS in DO11 Treg or DO11 Tconv populations. Data are representative of three independent experiments.
Fig. S9.
Fig. S9. CTLA-4 targets expression of CD80 and CD86 on cDCs but not macrophages. (A-B) CD80-GFP expressing mice were injected i.v. with 5-10 x 10⁶ BALB/c CD4 T cells. 6 days post T cell transfer, mice were injected with chloroquine (600 µg i.p.) and 24 hours later splenocytes were analysed for by flow cytometry. (A) Representative FACS plots showing expression of CD80-GFP on splenic MHCII+CD11c+CD26+ cDCs and CD64+F4/80+ macrophages. (B) Frequency of the CD80+GFP+ population in mice that received BALB/c CD4 T cells (+CD4) and controls (-CD4) in cDC (from Fig. 6C) and macrophages. (C) BALB/c mice were treated with anti-CTLA-4 Ab or control IgG (Ctrl). Spleens were harvested after 1 or 4 days (1 or 2 doses of 500 µg anti-CTLA-4 Ab respectively), digested and analysed for by flow cytometry. Plots show collated data of CD80 and CD86 expression on CD64+F4/80+ macrophages (n=3-4). Mean ± SD; ns=not significant, one-way ANOVA. Data are representative of at least two independent experiments.
Fig. S10.
Fig. S10. Gating strategy for identification of splenic cDC subsets. Splenocytes from BALB/c mice were stained with a panel for identification of APC populations. (A) Plots show the gating strategy implemented. Lineage exclusion stain contains CD3, CD19, B220 and NKp46; macrophage exclusion is performed on the basis of CD64 and F4/80 expression. cDC1 are identified as CD11c+CD26+ XCR1+; cDC2 are identified as CD11c+CD26+ CD172a+CD11b+. (B) Collated data of CD80 and CD86 expression on cDC1 and cDC2 (n=4), representative of at least six independent experiments. Plots show mean ± SD. **p≤0.01, ns=not significant, paired two-tailed Student’s t test.
Fig. S11.
Fig. S11. Gating strategy for identification of LN resident and migratory cDC subsets. LNs (axillary, brachial, inguinal and cervical) from BALB/c mice were digested and cells stained with a panel for identification of APC populations. (A,B) Plots show the gating strategy implemented. Lineage exclusion stain contains CD3, CD19, B220 and NKp46; macrophage exclusion is performed on the basis of CD64 and F4/80 expression. Migratory cDCs are identified as MHCII$^{hi}$CD11c$^{int}$ cells and LN resident cDCs as MHCII$^{int}$CD11c$^{hi}$. For both migratory and resident cDCs, cDC1 subset is identified as XCR1$^+$; cDC2 subset is CD172a$^+$CD11b$^+$. (C) Collated data of CD80 and CD86 expression on resident and migratory cDC1 and cDC2 (n=3), representative of at least six independent experiments. Plots show mean ± SD. *p≤0.05, **p≤0.01, ns=not significant, paired two-tailed Student’s t test.
Fig. S12.
Fig. S12. Phenotype of Tconv and Tregs in CTLA-4-deficient mice. Expression of CD69, CD25, CD62L and ICOS in CD4+Foxp3- Tconv or CD4+Foxp3+ Tregs from LNs (axillary, brachial, inguinal and cervical) and spleen of 17-18 day old CTLA-4-/- mice or CTLA-4+/- littermate controls. Collated data showing mean ± SD (n=5-17); **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns=not significant, unpaired two-tailed Student’s t test. Data are representative of at least four independent experiments.
Fig. S13.
Fig. S13. CD80 and CD86 expression on splenic cDC subsets following CTLA-4 ablation. (A,B) Spleens from 17-18 day old CTLA-4/- mice or CTLA-4+/- littermate controls were digested and cells stained for analysis by flow cytometry. (A) Representative FACS plots and (B) collated data showing CD80 and CD86 expression of splenic cDC subsets (n=3). (C,D) BALB/c mice were treated with a single dose of 500 µg anti-CTLA-4 (4F10) and harvested 24 hours later; or 2 doses at 48 hour intervals for a day 4 harvest. Spleens were digested and cells stained for analysis by flow cytometry. (C) Representative FACS plots and (D) collated data showing CD80 and CD86 expression of splenic cDC subsets (n=3-4). Plots show mean ± SD. *p≤0.05, **p≤0.01, ***p≤0.001, ns=not significant. Statistical significance was determined by two-way ANOVA.
Fig. S14.
Fig. S14. Functional identification of migratory DCs by FITC skin painting. BALB/c mouse skin was treated with 1% FITC and dibutyl phthalate on day 0, and at day 2, mice were injected i.p. with 500 µg anti-CTLA-4 Ab or control IgG (Ctrl). At day 3, cervical LNs (draining the site of FITC application) and popliteal LNs were digested and cells stained for cDC markers and CD80 and CD86. (A) Representative FACS plot shows gating for migratory and resident cDCs on the basis of CD11c and MHCII expression (lineage exclusion stain contains CD3, CD19, B220 and NKp46). (B) Representative FACS plots show FITC+ CD45+Lin-MHCII+ cells reaching the draining (cervical) LN fall within the migratory cDC gate. CD11b and CD103 expression by the FITC+ cells is also shown. Bottom panel shows absence of FITC+ cells within the non-draining (popliteal) LN. (C) Collated data showing CD80 and CD86 expression on FITC+ migratory cDCs, total migratory cDCs and total resident cDCs in the cervical LNs (n=3). Plots show mean ± SD. *p≤0.05, ****p≤0.0001, ns=not significant, two-way ANOVA. Data show one representative experiment of two independent experiments.
Fig. S15.
Fig. S15. Comparison of CD80 and CD86 expression in settings of CTLA-4 blockade and RAG deficiency. CD80 and CD86 expression on migratory DCs from WT, anti-CTLA-4 Ab treated WT, RAG-/- mice and CD4 T cell injected RAG-/- mice (Supplementary to Fig. 7C,D and Fig. 8). BALB/c mice were treated with anti-CTLA-4 Ab or control IgG (WT) and harvested after 1 (aCTLA-4 d1) or 4 days (aCTLA-4 d4) (1 or 2 doses of 500 µg anti-CTLA-4 Ab respectively). LNs (axillary, brachial, inguinal and cervical) were digested and cells stained for cDC markers and CD80 and CD86. Alternatively, Rag2-/- recipient mice were left untreated (RAG-/-) or received 6 x 10^6 CD4 T cells (RAG+CD4). Six days later, LNs (axillary, brachial, inguinal and cervical) were digested and cells stained for cDC markers and CD80 and CD86. CTLA-4 blockade and RAG deficiency experiments were performed on different days and cells were stained with the same antibody panels and acquired with the same FACS settings. CD86 MFI data at day 4 (lower right panel) suggests staining is comparable between experiments.
Fig. S16.
Fig. S16. CD80 and CD86 expression on splenic cDC subsets in Rag2-deficient mice with or without T cell transfer. Rag2/- recipient mice were injected with 6 x 10^6 CD4 T cells or 5.5 x 10^6 CD25-depleted CD4 T cells. Six days post T cell transfer, spleens were digested and cells stained for analysis by flow cytometry. (A) Representative FACS plots showing CD80 and CD86 expression on splenic cDC subsets. (B) Collated data of CD80 and CD86 expression (n=3-4). Plots show mean ± SD. *p≤0.05, **p≤0.01, ns=not significant, two-way ANOVA. Data are representative of at least four independent experiments.
Fig. S17.
Fig. S17. Impact of Treg expansion on CD80 and CD86 expression on cDC subsets. BALB/c mice were treated with IL-2 complex (IL-2c) or PBS (Control) on days 0, 1 and 2. At day 5, LNs (axillary, brachial, inguinal and cervical) were harvested, digested and cells stained for analysis by flow cytometry. (A) Representative FACS plots show Foxp3 and CD25 expression on CD4+ T cells. (B) Collated data showing percent and number of Treg (CD4+Foxp3+) (n=4). Plots show mean ± SD. ***p≤0.001, ****p≤0.0001, unpaired t test (C) Collated data showing CD80 and CD86 expression on MHCII\textsuperscript{hi}CD11c\textsuperscript{int} migratory and MHCII\textsuperscript{int}CD11c\textsuperscript{hi} resident LN cDCs (n=4). Plots show mean ± SD. *p≤0.05, ****p≤0.0001, ns=not significant, two-way ANOVA. Data show one representative experiment of two independent experiments.