Supporting Online Material

Materials and Methods

Cell purification and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats (provided by the National Blood Transfusion Service, Birmingham, U.K) using Ficoll-paque density centrifugation. $CD4^+$ T cells were isolated by incubating PBMC with human $CD4^+$T cell-enrichment cocktail and magnetic colloid according to the manufacture's instruction (Stem Cell Technologies, France). To purify $CD25^+$ T cells $CD4^+$ cells were incubated with anti-CD25-microbeads (Miltenyi Biotech, Auburn, CA) at 4^oC for 30 minutes. $CD4^+CD25^-$ T cells which did not bind to the column, were collected from the flow-through and washed before use. $CD4^+CD25^+$ T cells were subsequently retrieved from the column. Human monocytes were purified from PBMC by negative selection using human monocyte enrichment mixture and magnetic colloid according to the instructions of the manufacturer (Stem Cell Technologies, France). To generate dendritic cells (DCs), monocytes (2×10^6 cells/ml) were cultured in RPMI 1640 medium containing 10% FCS and antibiotics with GM-CSF (PeproTech, Rocky Hill, NJ; 800 U/ml) and IL-4 (PeproTech; 500 U/ml). Additional medium containing GM-CSF and IL-4 was added every 2 days. To generate SEB-specific T cell blasts PBMCs were incubated with 1μg/ml SEB for 6 days. Live $CD4^+$ cells were then negatively purified as above. Where stated DCs were re-isolated from T cell cultures using anti-CD2 microbeads (Miltenyi) to deplete T cells.

DNA constructs and Transfectants

Full-length CTLA-4 cDNA was cloned into a CMV expression vector pCDNA3.1 as previously described (*25*). A CTLA-4 construct with the C-terminal 36 amino acids deleted was generated by PCR and cloned into the same vector to generate CTLA-4 del36. GFP-tagged CD80 and CD86 were generated by PCR to remove the stop codon and cloned into the EGFP-N1 vector (Clontech). Mouse CD86-GFP fusion was cloned

into modified (IRES-GFP deleted) MIGR1 vector (a gift from Dr. W.S. Pear, University of Pennsylvania, Philadelphia, PA).

CHO Transfectants

CHO cell lines expressing CD80, CD86 or CTLA-4 were generated by electroporation of human cDNAs cloned into a CMV expression vector. Cells were grown in DMEM containing 10% FBS as previously described (*25*). Cells expressing the plasmid were selected using G418 (500μg/ml) treatment and FACS. Cultures were maintained at 37°C in a humidified incubator containing 5% CO_2 and were passaged by trypsinization. CHO cells expressing IAd were obtained from Prof. Gordon Freeman (Dana Faber Cancer Institute) and transfected with mouse CD80-GFP using Amaxa nucleofection.

CTLA-4 expressing Jurkat Cells

CTLA4 was cloned into the MP71 retrovirus vector. Retroviral supernatants were obtained by transfecton of Phoenix-A packaging cells with retroviral vectors containing CTLA4 in combination with pCLampho, using the FUGENE6 transfection reagent (Roche Molecular Biochemical). Retroviral supernatants were harvested 48h after transfection and used for transduction of Jurkat cells. For transduction of Jurkat cells, non-tissue culture treated 6-well plates were coated with RetroNectin (TaKaRa) at 30 mg/ml and blocked by 2%BSA, 1 ml of retroviral supernatants were added to RetroNectin pre-coated wells and centrifuged at 1500 rpm at room temperature for 15 minutes. $2x10^6$ Jurkat cell were added and centrifuged at 2000 rpm at 30°C for 60 minutes. 24 hours post-infection, media was changed to fresh RPMI 1640 with 10% FCS. Jurkat cells were maintained in RPMI containing 10% FBS. 72 hours post-infection, cells were sorted by staining with CTLA4-PE (BD) using MoFlow.

Human primary T cell Transfectants

Purified human $CD4^+CD25^-$ T cells were transfected with wild-type CTLA-4 using the AMAXA nucleofector kit according to the manufacturers instructions.

Generation of retroviral supernatants and bone marrow transduction

CD86-GFP fusion sequence was cloned into modified (IRES-GFP deleted) MIGR1 vector (a gift from Dr. W.S. Pear, University of Pennsylvania, Philadelphia, PA). Retroviral superntants were prepared by transfecting retroviral vector into the packaging cell line Plat-E (*32*) using Lipofectamine 2000 (Invitrogen). To prepare bone marrow (BM) cells, adult $Rag2^{-/-}$ mice were treated with 150mg/kg 5-fluorouracil by intraperitoneal injection and total BM cells were flushed from femurs and tibias and used for retovirus infection.

Retrovirus infection of cells was performed as described previously (*33*). Fresh BM cells were cultured in IMDM supplemented with 20% FCS, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 50ng/ml stem cell factor, 50ng/ml IL-6, and 10ng/ml IL-3. After 48, 72, and 96 hr, cells were spin-infected with retrovirus by centrifuging the culture plate in the presence of 10ug/ml polybrene for 90 min.

Generation of CD86GFP $Rag2^{-/-}$ mice and CD4+ T cell transfer

Retrovirally transduced bone marrow was intravenously injected into irradiated (450 Rad) $Rag2^{-/-}$ mice. After 3 weeks, $CD4^{+}$ cells were isolated from DO11 mice or from CTLA-$4^{+/+}$ or CTLA-$4^{-/-}$ DO11xRip-mOVA/$Rag2^{-/-}$ mice and adoptively transferred I.V into Balb/c $Rag2^{-/-}$ mice at 1-5x10^{6} cells per recipient. After 24 hours, mice received 100μg of OVA/alum i.p. At day 8, mice were given 100μg of OVA peptide I.V followed by 600μg of chloroquine I.P, 3 hours later. After a further 3 hours spleens were harvested and digested with 5mg/ml collagenase dispase (Roche) prior to staining for confocal.

Flow cytometry assays

Flow cytometry was carried out using a Dako Cyan flow cytometer and acquired using Summit software. Compensation and analysis was performed using FlowJo software (Treestar inc.)

CD86-transfer

CD86 transfer into CTLA-4 expressing cells was carried out by labeling CD86 GFP expressing CHO cells with CellTrace Far Red DDAO-SE (Invitrogen). Cells were then incubated together with CTLA-4 expressing CHO cells for 3 hours in the presence or absence of 10 nM Bafilomycin A or 10 μM NH_4Cl. To identify GFP transfer to single, CTLA-4 expressing cells and exclude clusters containing CD86-GFP, cells were gated for low pulse width and negative for Far Red staining as shown in Supplementary figure S1. CD80 GFP transfer was assayed in a similar way. Where used, PKH 26 (Sigma) labeling was carried out by incubating CHO-CD86 cells according to manufacturer's instructions.

CFSE dilution

For assessing T cell proliferation, responder $CD4^+CD25^-$ T cells were labeled using 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes). CD4+ CD25- T cells were washed twice in PBS and resuspended at 2 x 10^7 cells/ml and an equal volume of CFSE in PBS added such that the final concentration was 2.5 μM. Cells were then incubated at RT for 10 minutes with gentle agitation and washed twice in normal growth medium. When stimulating with CD86 transfectants, CHO cells were fixed using 0.025% glutaraldehyde in PBS for 2-3 min and washed extensively. Fixed CHO cells and CFSE labeled responder cells with then incubated (1 CHO cell : 5 T cells unless otherwise specified) with anti-CD3 (0.5μg/ml) for 5 days before analysis by flow cytometry. When using Jurkat cells as suppressors, cells were incubated overnight with CHO cells and Jurkat cells removed using anti-CD2 microbeads (Miltenyi) prior to fixation. When using dendritic cells as stimulators, cells were cultured with $CD4^+CD25^+$ purified Tregs for 5 days with 0.5 μg/ml anti-CD3 (+/- anti-CTLA-4 20 μg/ml). DC were re-isolated by depleting CD2 positive cells with anti-CD2 microbeads (Miltenyi Biotech). Where indicated T cell blasts were used as CTLA4+ suppressor cells. Blasts were generated by CD3/CD28 bead stimulation for 5 days and added to along with CFSE-labelled responder T cells. Cells were stimulated with DC and anti-CD3(0.5μg/ml) for 5 days in the presence or absence of anti-CTLA-4 (20 μg/ml) before analysis by flow cytometry.

CD86 surface downregulation

For surface analysis of DC phenotypes, cells were collected into cold PBS and surface labeled at 4°C with antibodies against CD86, CD80, CD40 or CD11c directly conjugated to FITC, PE or APC (BD PharMingen, San Diego, CA). DCs were gated based on forward and side scatter, and CD11c labeling. DCs were incubated with T cells at the ratios indicated.

CTLA-4 Antibodies

Surface staining of CTLA-4 was carried out on ice for 30 min. Staining of cycling CTLA-4 was carried out by incubation at 37°C for 30min. Total CTLA-4 staining was carried out by fixing cells with 3.0% formaldehyde solution in PBS and permeabilizing with 0.1% saponin and then staining with anti-CTLA-4 PE (BNI3-Pharmingen) or anti-CTLA-4 C-19 antibody (Santa Cruz) followed by an appropriate secondary antibody. Where stated T cells were stimulated using anti-CD3 anti-CD28 beads (Invitrogen) for 2h prior to staining. To study CTLA-4 cycling in Treg, cells were incubated overnight with anti-CD3 anti-CD28 beads and CTLA-4 staining carried out at 37^{o}C. Where blocking anti-CTLA-4 was used unconjugated anti-CTLA-4 was used at 20μg/ml.

FOXP3 staining was performed using a FOXP3 staining kit (Ebioscience) according to the manufacturer's instructions.

Estimation of molecules of CD86 and CTLA-4

We used Quantum Simply Cellular anti-mouse IgG beads (Bangs Laboratories Inc, IN) according to the manufacturers instructions. Briefly, calibration curves were produced by titrating antibodies with beads of known antigen binding capacity. Flow cytometric staining of cells was then carried out using saturating titrations of antibody and used to estimate number of molecules from the calibration curves.

Confocal microscopy

Imaging was carried out using a Zeiss LSM 510 or a Zeiss LSM 780 inverted laser scanning confocal microscope using a 100x oil immersion objective with excitation at

488nm, 543nm and 633nm. Constant laser powers and acquisition parameters were maintained throughout individual experiments for analysis. Digital images were prepared using ImageJ (Wayne Rasband, NIH). For quantitation, cells were outlined and mean fluorescence intensity measured using ImageJ. Movies were prepared using ImageJ. All confocal images shown are representative of at least thirty micrographs taken from at least three independent experiments.

Live Cell Imaging

Cells were imaged using glass bottom culture dishes (MatTek). In single time point experiments CTLA-4 expressing cells were labelled with DDAO-SE and incubated with CD86-GFP CHO cells for 3h. For time course experiments CTLA-4 expressing CHO cells were identified by brief labeling with anti-CTLA-4 APC (BD) and incubated with CHO cells stably expressing CD86-GFP. Z-stacks were acquired as a time-series aproximately every 3 minutes for 90 minutes (Movie S1) or every 1 minute (Movie S2-4). Projections were then generated using ImageJ and converted into movies or single images. Movie S2 contains a single confocal plane.

Immunofluorescence staining

For co-localisation of CD86 and CTLA-4, CHO cells, were incubated together for 16 hours on a poly-L-lysine coated coverslip in a 24 well plate. Cells were then fixed with methanol at -20°C for 20 minutes. Non-specific binding sites were blocked by incubation in blocking solution (BS) which consisted of 5% donkey serum (Sigma) in PBS at room temperature (RT). Cells were incubated with anti-CD86 primary antibody (B7-2, C19 antibody, Santa Cruz, CA) and anti-CTLA-4 (BNI3 Pharmingen), washed and then labeled with donkey anti-goat Alexa 546 and anti-mouse Alexa 488 secondary antibodies (Molecular Probes).

Dendritic cells and T cells were added at a 1:3 ratio to poly-L-lysine coated coverslips in a 24 well plate. Cells were activated by the addition of anti-CD3 (Clone OKT3) at a concentration of 1 μg/ml and were incubated for 72h ($CD4^+CD25^-$) or overnight (CD4+CD25+) at 37 °C and 5 % CO_2. Where SEB stimulation was used , DC were

pulsed with 1μg/ml SEB for 2h and washed prior to use. Cells were centrifuged in a swing out rotor at 200 g for 10 minutes and fixed with methanol at -20°C for 20 minutes. Non-specific binding sites were blocked by incubation in blocking solution (BS). Cells were incubated with anti-CD86 primary antibody (B7-2, C19 antibody, Santa Cruz, CA), washed and then labeled with donkey anti-goat IgG Alexa 546. CTLA-4 was detected with anti-CTLA-4 APC. HLA-DR was labeled using anti-HLA-DR-FITC. For Treg experiments anti-CD3 was used to identify T cells and detected using anti-mouse IgG Alexa 633 secondary antibody.

Confocal staining of cycling CTLA-4 was carried out by incubating cells at 37°C with unconjugated anti-CTLA-4 for 30 minutes. Cells were then fixed, permeabilized and labeled with an anti-mouse Alexa 546. Total CTLA-4 was detected using a C-terminal anti-CTLA-4 antibody (Santa Cruz) detected with anti goat Alexa 488.

Following staining, coverslips were dried and mounted with Vectashield (Vector Laboratories, UK) prior to visualization by confocal microscopy.

Animals

DO11.10 TCR transgenic mice and BALB/C mice were purchased from The Jackson Laboratory. RAG-2-/- 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). DO11.10 mice, RIP-mOVA mice and RAG-/- mice were crossed as previously described. CTLA-4-/- mice on a BALB/c background were a gift from A. Sharpe (Brigham and Women's Hospital, Boston, USA). All mice were housed in the University of Birmingham Biomedical Services Unit and used according to Home Office and institutional regulations.

For *in vivo* transfer experiments, splenocytes were labelled in glass-bottom dishes (MatTek) at 4°C with anti-CD25 PE and CD4 APC for 15 minutes before imaging by

confocal microscopy. Quantitation of GFP fluorescence was carried out by outlining cells and measuring mean fluorescence in ImageJ.

Electron Microscopy

Cryo-immuno-EM was performed on cells fixed with 4% paraformaldehyde in 0.1M phosphate buffer pH 7.4, supported in 10% gelatin, and infused with 2.3M sucrose. Sections (70 nm) were cut at -120°C and picked up in 1:1 2.3M sucrose:2% methylcellulose. Mouse anti-HA (HA.11, Covance) was followed by rabbit anti-mouse intermediate antibody (Dako) and 10nm protein A gold and then contrast stained/dried in 1:9 4% uranyl acetate:2% methyl cellulose. Samples were viewed on a JEOL 1010 TEM, and images gathered with a Gatan OriusSC100B CCD camera.

Supplementary Figure Legends

Figure S1

(A) Gating strategy for analysis of CD86-GFP transfer to CTLA-4 expressing cells**.** CTLA-4-expressing cells and Far Red labelled CD86-GFP-expressing CHO cells (donor cells) were co-cultured for 3h. Following FACS acquisition of the total mixed population (i) cells were gated on low pulse width (ii) to exclude cell clusters. Far Red negative cells were then gated to exclude CD86-GFP donor cells from the analysis (iii). The resultant singlet CTLA-4 expressing cells were then analysed for GFP content (iv). **(B)** Depletion of CD86-GFP from donor cells is associated with concomitant GFP acquisition by CTLA-4 expressing recipient cells. CD86-GFP expressing CHO cells (donor cells) were co-cultured for 3h with control CHO or CTLA-4-expressing CHO cells. Compared with control CHO cells (upper panel), cells expressing CTLA-4 (lower panel, Far Red negative) acquire GFP. This is associated with a concomitant decrease in GFP signal on the CD86 donor cells (Far Red positive cells).

Figure S2

Staining for cell surface CD86 on CTLA-4 recipients post-transfer

Control CHO cells or CTLA-4 expressing CHO cells were incubated with CD86-GFP expressing CHO cells (Far Red labelled) for 3 hours. CD86 was then surface labelled by anti-CD86 PE at 4°C and gated using the strategy in Fig. S1. Whilst 36% of CTLA-4 positive cells capture CD86-GFP only 0.5% label for surface CD86. The CD86 staining of the donor cells following incubation with CTLA-4^{+} cells is shown in the lower panel, revealing a marked reduction in CD86 surface expression. Representative of 2 independent experiments.

Figure S3

Degradation of transferred CD86 in the absence of CTLA-4 degradation

(A). CTLA-4 expressing CHO cells were incubated alone (left panels) or with Far Red labelled CD86-GFP expressing CHO cells for 3 hours (right panels) in the presence or absence of the lysosomal inhibitor Bafilomycin A. Cells were then fixed and stained with anti-CTLA-4. Plots are gated on CHO-CTLA-4 (Far Red negative) cells. CD86 transfer

does not reduce CTLA-4 expression in GFP+ cells. Additionally, in the presence of Bafilomycin A the CD86-GFP signal increases (due to blockade of lysosomal degradation) however the expression of CTLA-4 is not affected (lower right panel). This suggests CTLA-4 is not degraded along with ligand. **(B)** Histogram overlays of the data in A showing CTLA-4 expression in the presence of absence of bafilomycin. Data are representative of 2 experiments.

Figure S4. Live imaging of CD86-GFP transfer

(A) Time-lapse (0-80 minutes) confocal micrographs of GFP-tagged CD86 (green) transfer into CTLA-4 expressing cells (stained red by pulsing anti-CTLA-4 APC) taken from Movie S1. White images show CD86 alone. (B) Quantitation of CD86 GFP mean fluorescence intensity of the cells shown in A. Fluorescence changes were analysed in the cells shown using Image J with each time point indicated by a circle.

Figure S5

Both CD80 and CD86 are acquired by CTLA-4 expressing cells.

CTLA-4 expressing CHO cells were incubated with Far Red-labelled donor CHO cells expressing either CD80-GFP or CD86-GFP. Cells were co-cultured for the times indicated in the presence or absence of ammonium chloride and analysed by FACS as shown in figure S1. Data are representative of at least 3 independent experiments.

Figure S6

Estimation of the number of CTLA-4 and CD86 molecules expressed in transfer experiments.

To quantify the number of CD86 molecules and CTLA-4 molecules expressed by cells, CD86 APC or CTLA-4 APC antibodies (BD Biosciences) were titrated on to beads that have known antibody binding capacity (ABC) (Quantum Simply Cellular anti-mouse IgG). The geometric mean fluorescence was determined for both CD86 and CTLA-4 antibodies generating a standard curve of MFI vs number of antibody molecules bound. This allows an estimation of the number of molecules expressed per cell for CD86 **(A)** or CTLA-4 **(B)**. Arrows intersecting standard curve show fluorescence values of CD86 on

CHO-CD86 transfectants or mature DC (A) and the values for CTLA-4 expressing Jurkat or CHO-CTLA-4 cells (B). CTLA-4 expressing Jurkat cells were incubated with CD86-expressing CHO cells overnight at various ratios and reduction in CD86 expression measured **(C)**. At a ratio of 1 Jurkat cell to 1 CHO cell which corresponds to a ratio of approximately 8 CD86 molecules to 1 CTLA-4 molecule we observe a 35% reduction in CD86 expression. This reduction was sufficient to observe a functional inhibition in T cell proliferation when these cells were used to stimulate CFSE-labelled responder T cells (**D**). Data are representative of 2 experiments.

Figure S7 Transfer between cells is unidirectional.

(A) CD86 expression pattern is punctate in cells cultured with CTLA-4. Confocal micrograph of adherent CHO-CD86 cells cultured overnight either alone or with CHO-CTLA-4 cells. Cells were fixed, permeabilised and stained with goat anti-human CD86 (C19) green. Dtata are representative of more than 20 independent experiments**(B)** Quantification of the confocal images. The cells shown in Fig. 1C were quantified by outlining cells (n=35) using Image J software and measuring fluorescence intensity of CTLA-4 and CD86. This shows a decrease in CD86 expression in the donor cell and an increase of CD86 in the CTLA-4 recipient. The CD86 donor cell does not acquire CTLA-4. **(C)** CHO-CD86 expressing cells were labelled with the lipophilic dye PKH26 to label the plasma membrane and then incubated with CHO-CTLA-4 cells. Transfer of membrane dye was monitored by flow cytometry to assess the level of plasma membrane exchange between CTLA-4 and CD86 cells. CTLA-4^{+} recipents were gated on those that acquired CD86-GFP(red box) and those which did not (black box). The PKH transfer associated with these populations was then analysed.Data are representative of 2 experiments.

Figure S8
Removal of the CTLA-4 C-terminus prevents endocytosis.

(**A**) CHO cells stably expressing either wild type CTLA-4 or CTLA-4 del36 (lacking the cytoplasmic domain) were initially incubated with an unlabelled anti-CTLA-4 antibody at 37°C for 30 minutes to allow labelling of internalising CTLA-4. Surface receptors were then stained red (anti-mouse Ig Alexa 594) at 4°C. Cells were then fixed and

permeabilized and stained with alexa 488 conjugated anti-mouse Ig. Cells that internalise the unlabelled CTLA-4 antibody at 37^{o}C antibody stain green. Bar chart shows quantification of plasma membrane (PM) to internalized (I) protein ratios of WT and mutant CTLA-4 plus SEM. PM/I ratios were determined by outlining cells using Image J(more 32 cells were analysed for each celltype). (**B**) Levels of surface expression of wild type and del36 CTLA-4 receptors found on cells used in Figure 1 E and F analysed by flow cytometry. Data are representative of 3 experiments.

Figure S9

CTLA-4 specifically downregulates CD80 and CD86 but not CD40 on dendritic cells

(**A**) Dendritic cells were incubated with human $CD4^{+}CD25^{-}$ T cells and anti-CD3 (0.5mg/ml) for 4 days to allow T cell expression of CTLA-4. Cultures were carried out in the presence or absence of anti-CTLA-4 (20 μg/ml). Dendritic cells were then gated using anti-CD11c and the levels of CD80, CD86 and CD40 analysed by flow cytometry. CD80 and CD86 levels were specifically increased by anti-CTLA-4 treatment whereas there was no change in CD40 levels.

Figure S10. Jurkat cell line expressing CTLA-4.

A). WT control or CTLA-4-transduced Jurkat cells were stained for total CTLA-4 expression and analysed by flow cytometry. Isotype control staining is shown in the filled histogram and compared with wild-type untransduced (dotted line) and transduced CTLA-4 staining (red line) demonstating that WT Jurkat cells do not express CTLA-4. **(B)** Acquisition of CD86-GFP by CTLA-4 transduced Jurkat cells as measured by FACS compared to control Jurkat. Lower panels show the effect of Bafilomycin (BafA) **(C)** Electron micrograph showing CD86-HA transfer to a CTLA-4 expressing Jurkat cell. 70nm thick cryosections were stained using anti-HA antibody and detected using rabbbit anti-mouse antibody followed by protein A gold**.** Data are representative of 3 experiments.

Figure S11. Blocking the interaction of CTLA-4 with CD86 prevents transfer.

(**A**) CTLA-4 expressing Jurkat cells were incubated for 3 hours with CD86-GFP expressing CHO cells in the presence of NH_4Cl. Cells were analysed as outlined in figure S1. Anti-CTLA-4 (10μg/ml), CTLA-4 Ig (10μg/ml) and CD80-Ig (10μg/ml) inhibited the transfer of CD86. (**B**) Pooled analysis (n=4) of GFP acquisition by CTLA-4 transduced Jurkat T cells with and without anti-CTLA-4 blockade. Error bars show SEM.

Figure S12. Anti-CD28 does not block CD86 transfer.

CTLA-4 expressing Jurkat cells were incubated for 3 hours with CD86-GFP expressing CHO cells in the presence of NH_4Cl. Cells were analysed as outlined in figure S1. The effect of anti-CTLA-4 (10μg/ml), anti-CD28 (10μg/ml) or anti-CD45RO (10μg/ml) on transfer of CD86-GFP was assessed. Data are representative of at least 3 experiments.

Figure S13. TCR stimulation drives CTLA-4 trafficking in T cells and Treg.

(A) Human CD4+ T cells were activated using anti-CD3 / anti-CD28 beads for 3 days. Cycling CTLA-4 was stained with anti-CTLA-4 labelling at 37^{o}C for 30 minutes using a mouse anti-CTLA-4 antibody. Cells were then fixed and stained for total CTLA-4 using goat anti-CTLA-4 C-terminal antibody. Cells were then stained with anti-mouse Alexa 555 and anti-goat Alexa 488. These data demonstrate that CTLA-4 remains in a recycling pool in activated T cells. (**B**) T cell blasts were re-stimulated with anti-CD3/CD28 beads for 1h and cycling and total CTLA-4 was detected by flow cytometry using anti-CTLA-4-PE. Whilst the total CTLA-4 level does not change, anti-CD3/CD28 stimulation increases the CTLA-4 cycling pool. **(C)** Total expression of CTLA-4 in resting human peripheral blood T cells gated on CD25 expression. Levels of CTLA-4 were determined by intracellular staining of $CD4^+$ T cells along with FoxP3. **(D)** Resting or activated (anti-CD3 and anti-CD28 beads) Tregs were stained for surface or cycling CTLA-4. Surface staining was carried out at 4 ^{o}C and cycling of CTLA-4 was detected by anti-CTLA-4-PE labelling at 37^{o}C. Data are representative of at least 3 experiments.

Figure S14. CTLA-4 downregulation of costimulatory molecules is functionally relevant. (A) Proliferation of CFSE-labelled $CD4^+CD25^-$ T cells 4d after stimulation by

DC re-isolated from culture with Tregs in the presence or absence of anti-CTLA-4 antibody. (B) CFSE-labelled $CD25^-$ T cells were stimulated with DC previously incubated with Treg. Cultures were supplemented with either control CHO cells or those transfected with CD86. (C) T cell blasts expressing CTLA-4 were used as suppressor cells. Dendritic cells were incubated with T cell blasts with or without anti-CTLA-4 and their capacity to stimulate proliferation of $CD25^-$ CFSE labelled responder CD4 cells was assessed. T cell responses to DC in the absence of blasts (left panel) and presence of blasts (right panel) are shown. Relative CD86 expression is shown. Data are representative of 3 experiments.

Figure S15. Mouse CD4+ CD25+ T cells can effectively capture CD86 in vitro.
(A) T cells from either WT or CTLA-4 –deficient mice ($Ctla4^{-/-}$) were incubated overnight with anti-CD3 (0.5μg/ml) and murine CD86-GFP expressing CHO cells. Transfer of CD86 was observed into wildtype T cells but not CTLA-4 knockout T cells. Wildtype cells did not acquire control GFP protein **(B)** DO11 T cells were incubated with CHO cells expressing $I\text{-}A^d$ and CD86-GFP either with or without OVA peptide and analysed by confocal microscopy. Cells were analysed for CD4 (blue) CD25(red) and CD86 (green) expression. Data are representative of 2 experiments.

Figure S16. Workflow of *in vivo* trans-endocytosis experiment
Figure illustrates the workflow for *in vivo* analysis of ligand trans-endocytosis.

Figure S17. Analysis of T cells from *in vivo* experiments
(A) FACS analysis showing Foxp3 staining of T cell populations shown in figure 4B and 4C. Plots are gated on CD4+CD25+ cells. (B) Quantification of confocal images from Fig. 4 shows that the mean GFP fluorescence of the CD86-GFP transferred to T cells approaches that of cells that were originally transduced with CD86-GFP indicating a high level of transfer. Fluorescence values were generated by measuring CD86-GFP content of recipient T cells and donor CD86-GFP+ cells using Image J software. Each circle represents an individual cell. (C) Quantification of *in vivo* experiments shown in Figure

4. Pooled data (n=2) from multiple confocal fields were scored for CD4+CD25+GFP+, CD4+CD25+GFP- and CD4+CD25- T cells. Approximately 100 cells were scored in each condition. Data show the requirement for CTLA-4 expression and peptide stimulation for CD86-GFP acquisition by T cells in vivo. (D) Representative images from *in vivo* experiments using control GFP transduced bone marrow. No endosomal GFP was observed in T cells in these experiments(n=2).

Supplementary Movie Legends

Movie S1. Acquisition of CD86 by CTLA-4 (1)
Time-lapse (0-80 minutes) confocal micrograph of CHO-CD86-GFP (green) transfer into CTLA-4 expressing CHO cells (red). Cells shown are also shown in figure S4.

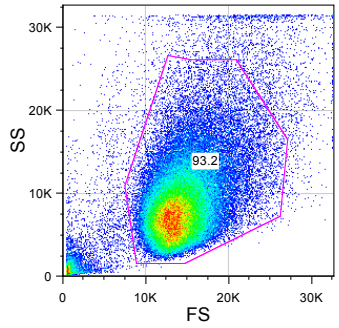
Movie S2. CD86–GFP acquisition by CTLA-4 at low power.
Time lapse (0-20 min) confocal images of multiple GFP-tagged CD86 (green) cells incubated with CHO-CTLA-4 –RFP expressing cells (red).

Movie S3. Close up of cells highlighted at beginning of movie S2. Note accumulation of CD86 at interface between cells and depletion of plasma membrane CD86 in cell to right of the image.

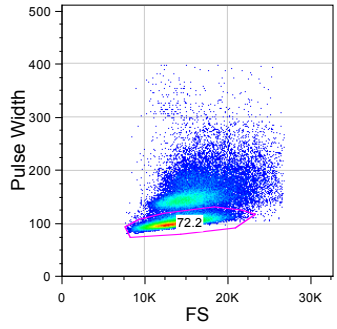
Movie S4. Maximum intensity projection showing acquisition of CD86 (green) by CTLA-4 expressing cells (red).

Figure S1A

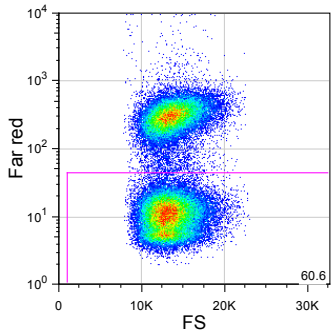
i Total acquisition gate



ii Singlet pulse width gate
- excludes clusters



iii Far red low - excludes CD86-GFP donor cells



iv GFP signal associated with CTLA-4 positive cells

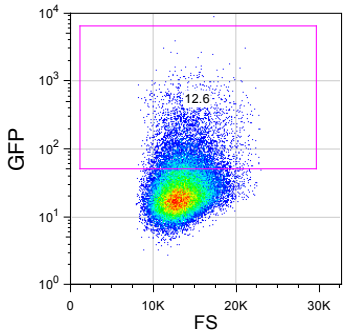
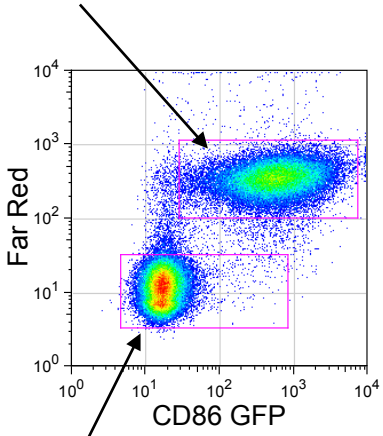


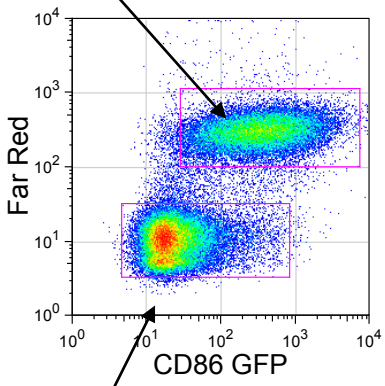
Figure S1B

CHO-CD86-GFP(donor)
GFP MFI = 827



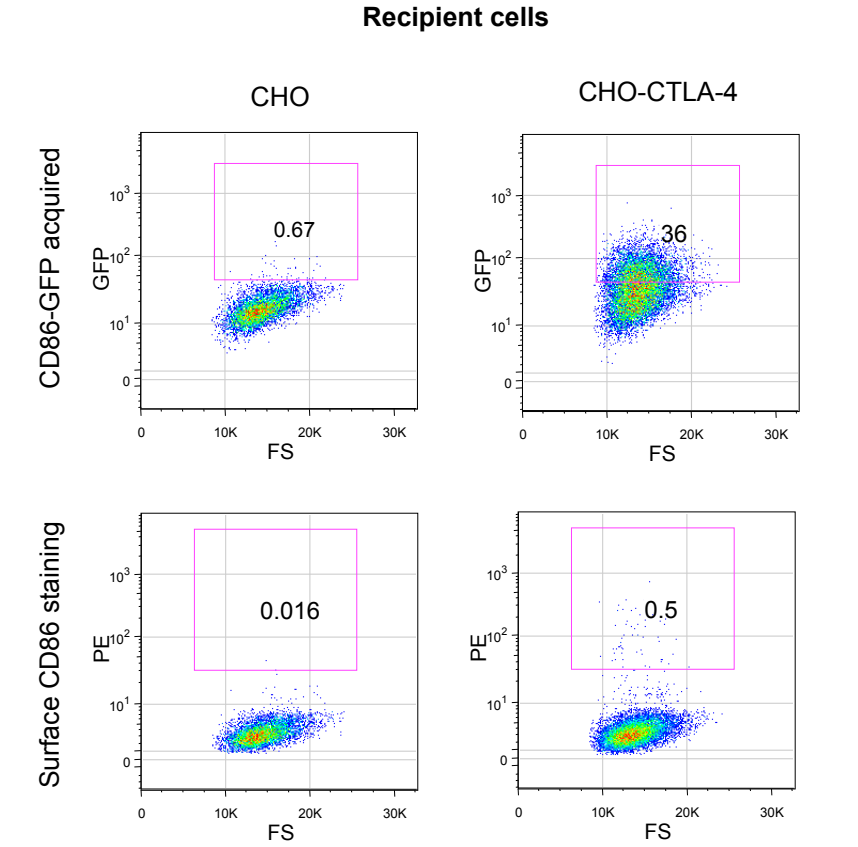
CHO (recipient)
GFP MFI = 19.2

CHO-CD86-GFP (donor)
GFP MFI = 594



CHO-CTLA-4 (recipient)
GFP MFI = 35.8

Figure S2


Recipient cells
CHO
CHO-CTLA-4
CD86-GFP acquired
GFP
0.67
36
FS
Surface CD86 staining
PE
0.016
0.5
0
10K
20K
30K

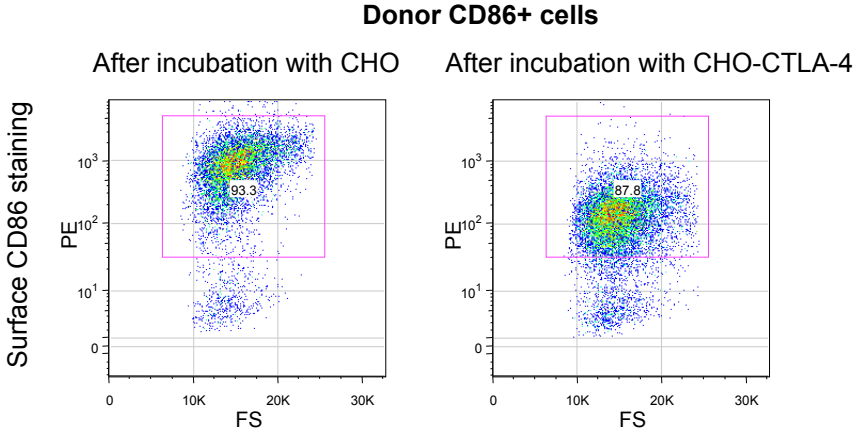
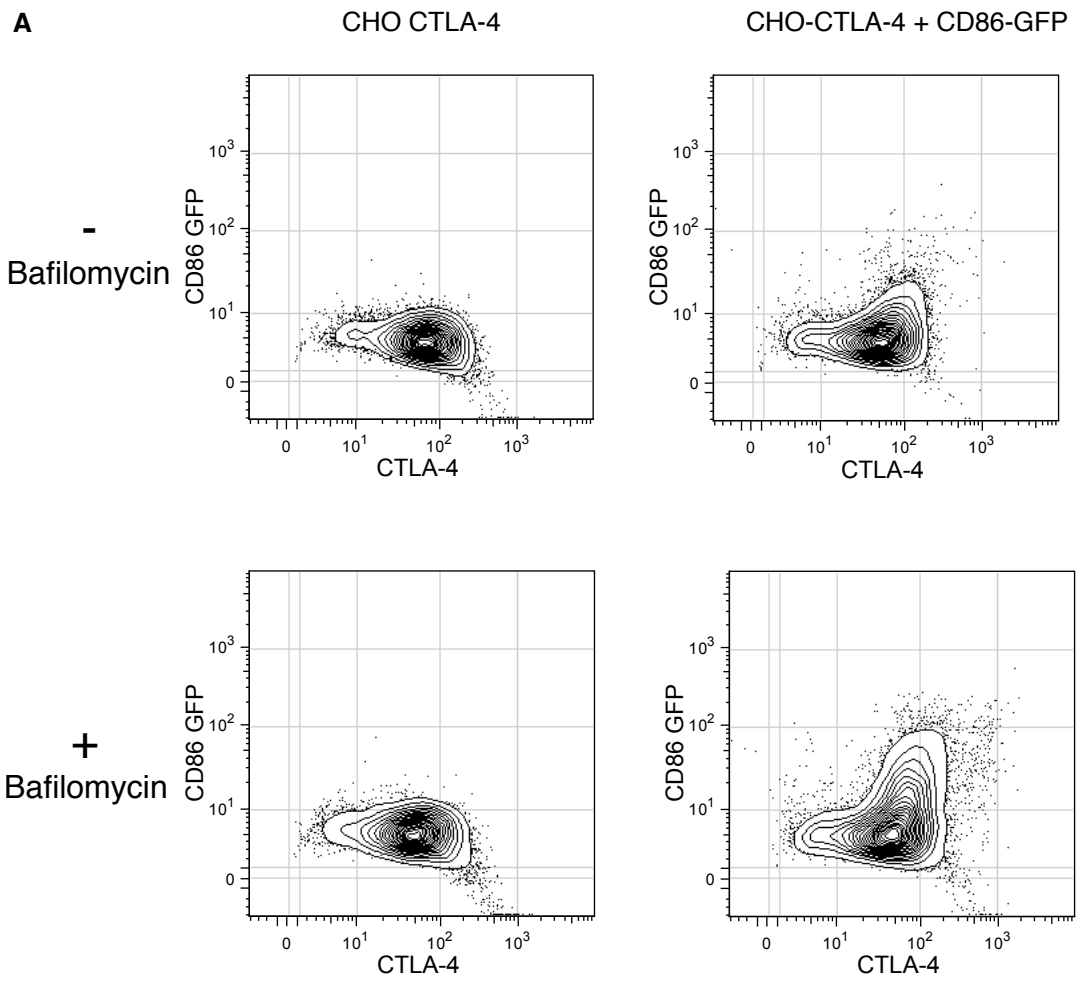

Donor CD86+ cells
After incubation with CHO
After incubation with CHO-CTLA-4
Surface CD86 staining
PE
93.3
87.8
FS
0
10K
20K
30K

Figure S3


A
CHO CTLA-4
CHO-CTLA-4 + CD86-GFP
-
Bafilomycin
+
Bafilomycin
CD86 GFP
CTLA-4
0
10^1
10^2
10^3

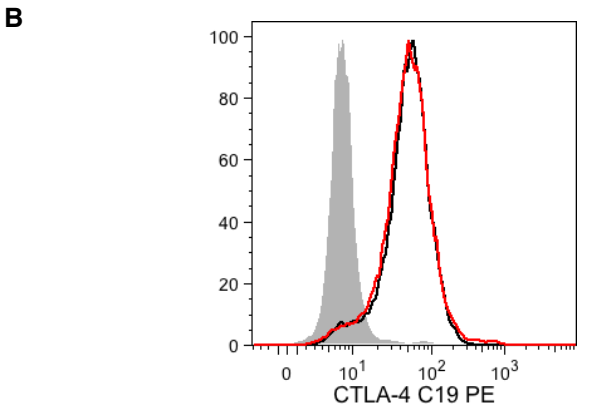
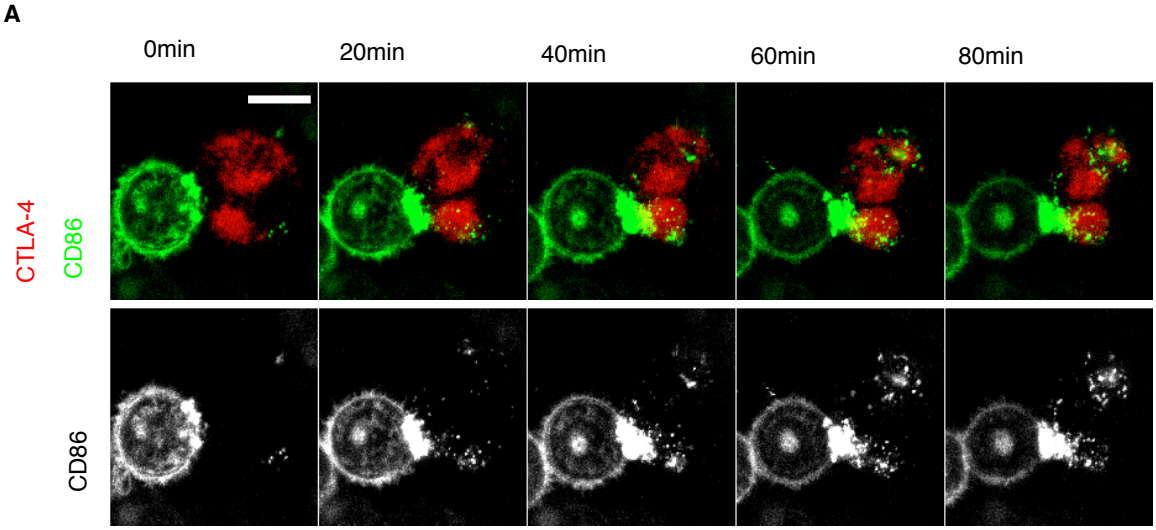

B
100
80
60
40
20
0
10^1
10^2
10^3
CTLA-4 C19 PE
CTLA-4 CHO Cells (unstained)
CTLA-4 CHO Cells
CTLA-4 CHO Cells + Bafilomycin A

Figure S4


A
0min
20min
40min
60min
80min
CTLA-4
CD86
CD86

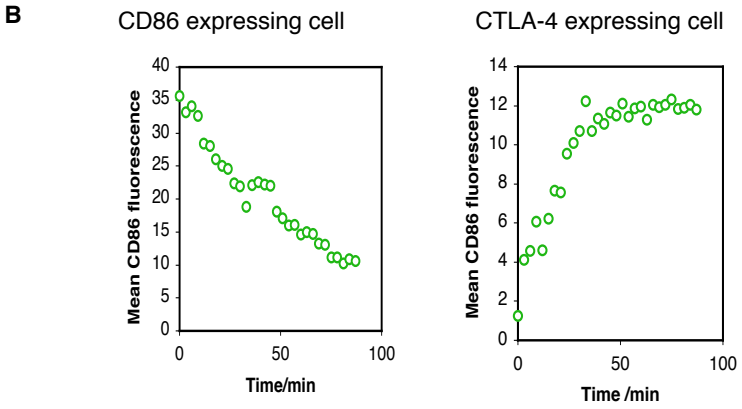

B
CD86 expressing cell
CTLA-4 expressing cell
Mean CD86 fluorescence
Time/min
Mean CD86 fluorescence
Time /min

Figure S5

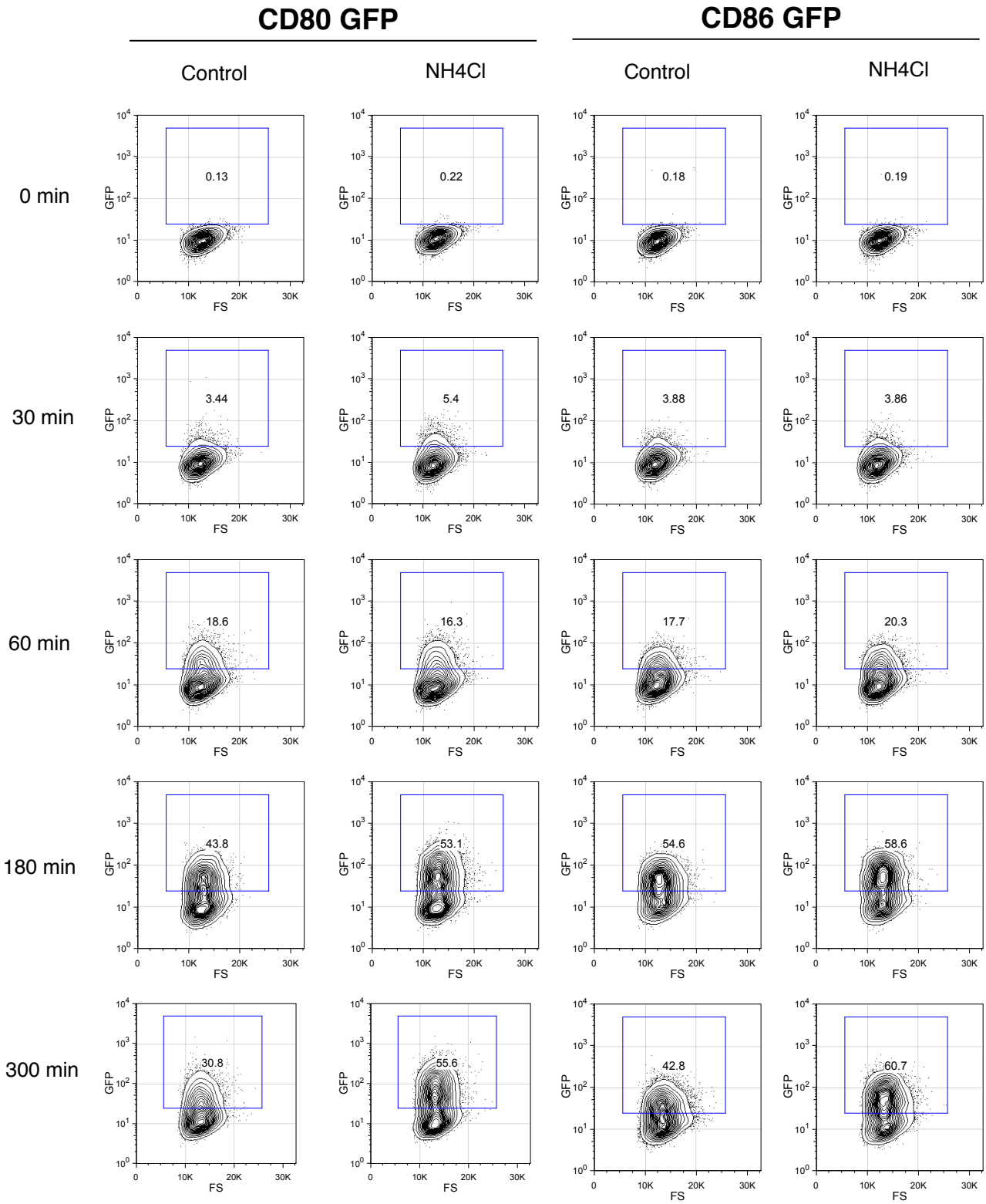
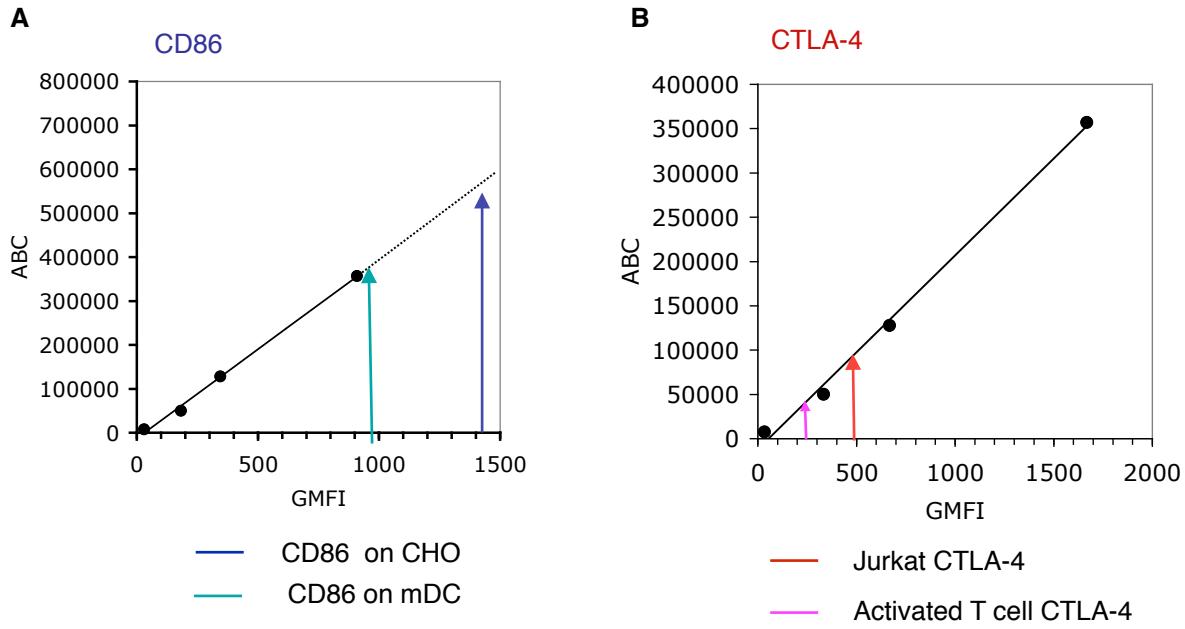

CD80 GFP
CD86 GFP
Control
NH4Cl
Control
NH4Cl
0 min
30 min
60 min
180 min
300 min
0.13
0.22
0.18
0.19
3.44
5.4
3.88
3.86
18.6
16.3
17.7
20.3
43.8
53.1
54.6
58.6
30.8
55.6
42.8
60.7
GFP
FS
0
10K
20K
30K

Figure S6


A
CD86
800000
700000
600000
500000
400000
300000
200000
100000
0
ABC
0
500
1000
1500
GMFI
CD86 on CHO
CD86 on mDC
B
CTLA-4
400000
350000
300000
250000
200000
150000
100000
50000
0
ABC
0
500
1000
1500
2000
GMFI
Jurkat CTLA-4
Activated T cell CTLA-4

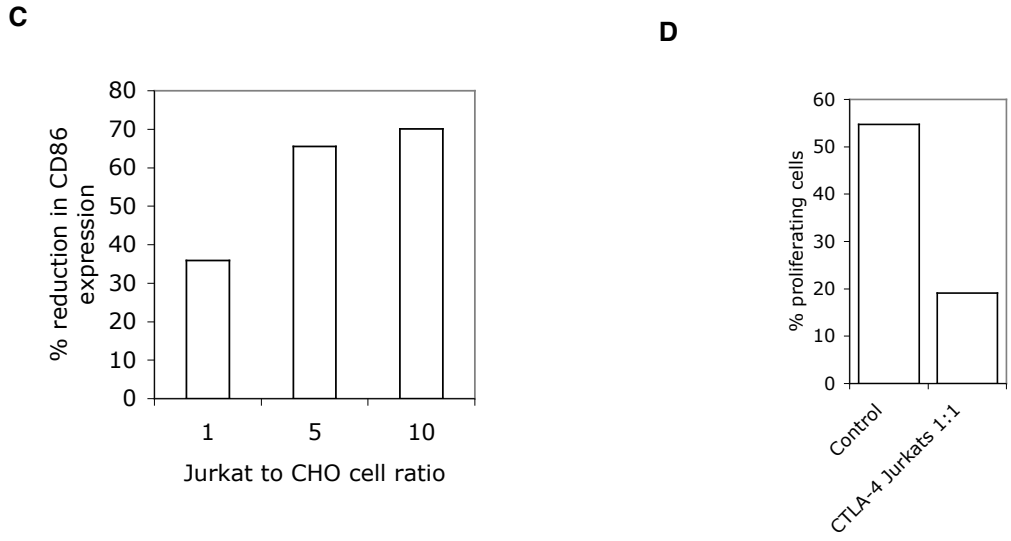

C
80
70
60
50
40
30
20
10
0
% reduction in CD86 expression
1
5
10
Jurkat to CHO cell ratio
D
60
50
40
30
20
10
0
% proliferating cells
Control
CTLA-4 Jurkats 1:1

Figure S7

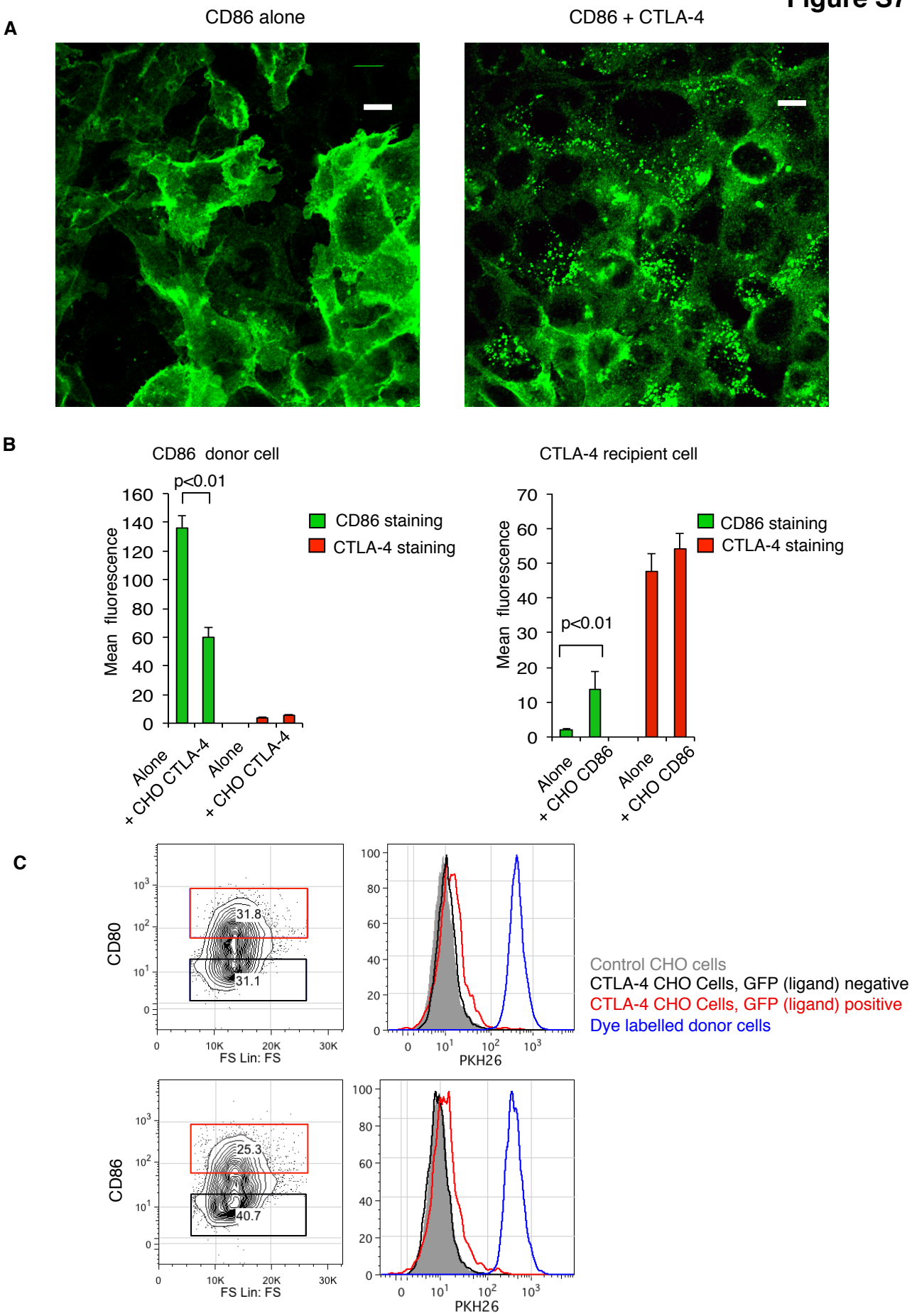
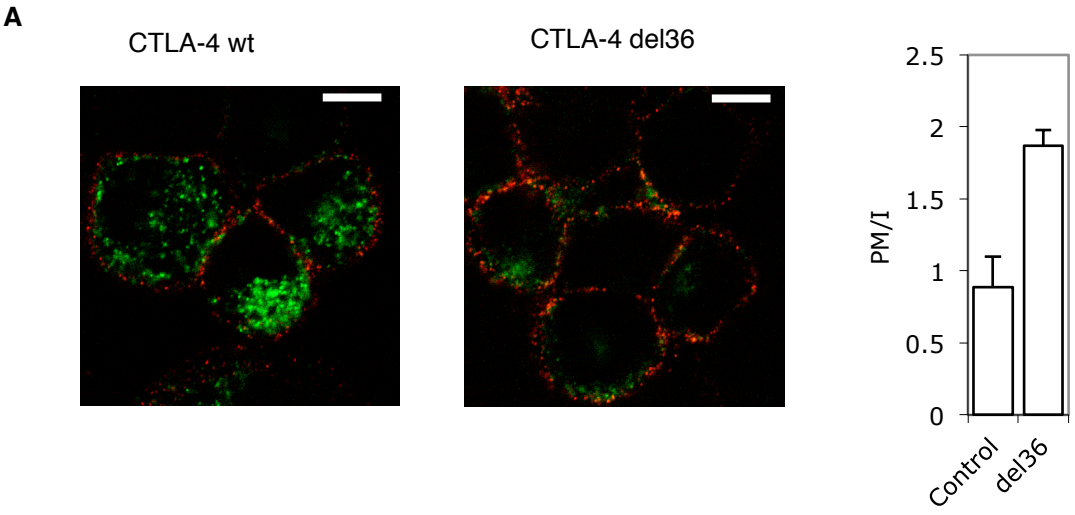

A
CD86 alone
CD86 + CTLA-4
B
CD86 donor cell
p<0.01
Mean fluorescence
CD86 staining
CTLA-4 staining
Alone
+ CHO CTLA-4
CTLA-4 recipient cell
p<0.01
+ CHO CD86
C
CD80
CD86
31.8
31.1
25.3
40.7
FS Lin: FS
PKH26
Control CHO cells
CTLA-4 CHO Cells, GFP (ligand) negative
CTLA-4 CHO Cells, GFP (ligand) positive
Dye labelled donor cells

Figure S8


A
CTLA-4 wt
CTLA-4 del36
2.5
2
1.5
1
0.5
0
PM/I
Control
del36

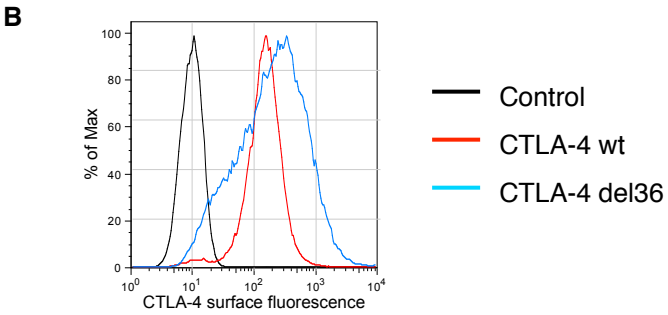

B
100
80
60
40
20
0
% of Max
10^0
10^1
10^2
10^3
10^4
CTLA-4 surface fluorescence
Control
CTLA-4 wt
CTLA-4 del36

Figure S9

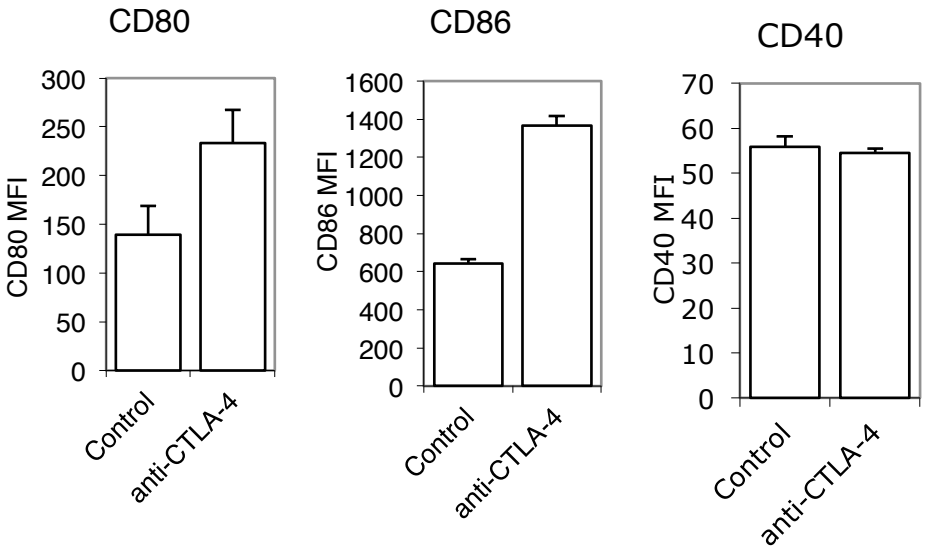
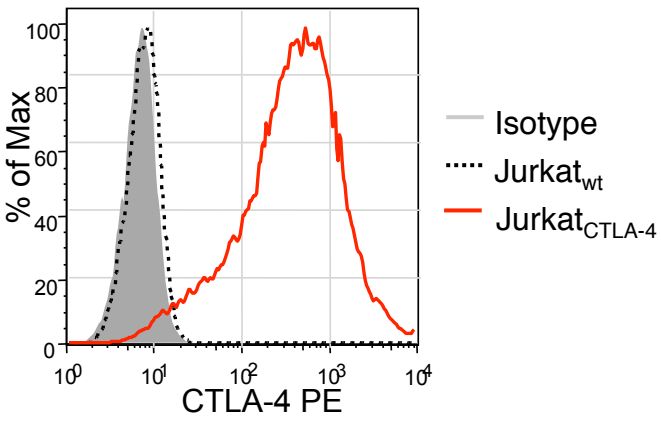
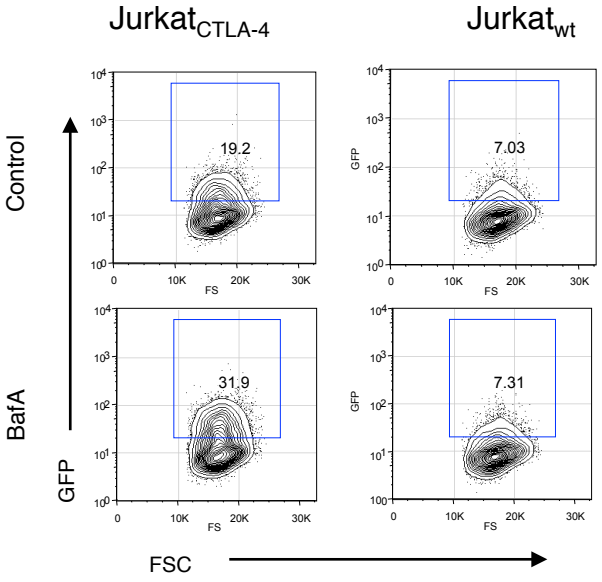

CD80
CD80 MFI
300
250
200
150
100
50
0
Control
anti-CTLA-4
CD86
CD86 MFI
1600
1400
1200
1000
800
600
400
200
0
Control
anti-CTLA-4
CD40
CD40 MFI
70
60
50
40
30
20
10
0
Control
anti-CTLA-4

Figure S10

A



B



C

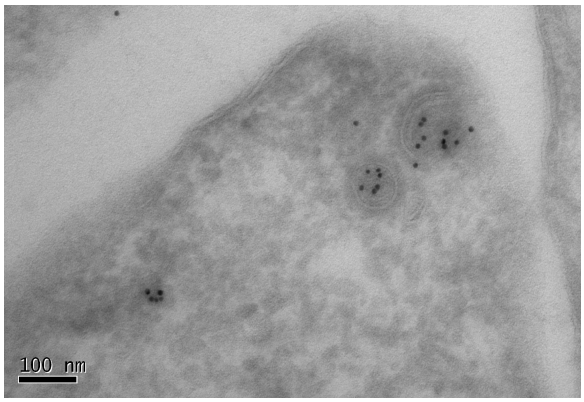


Figure S11

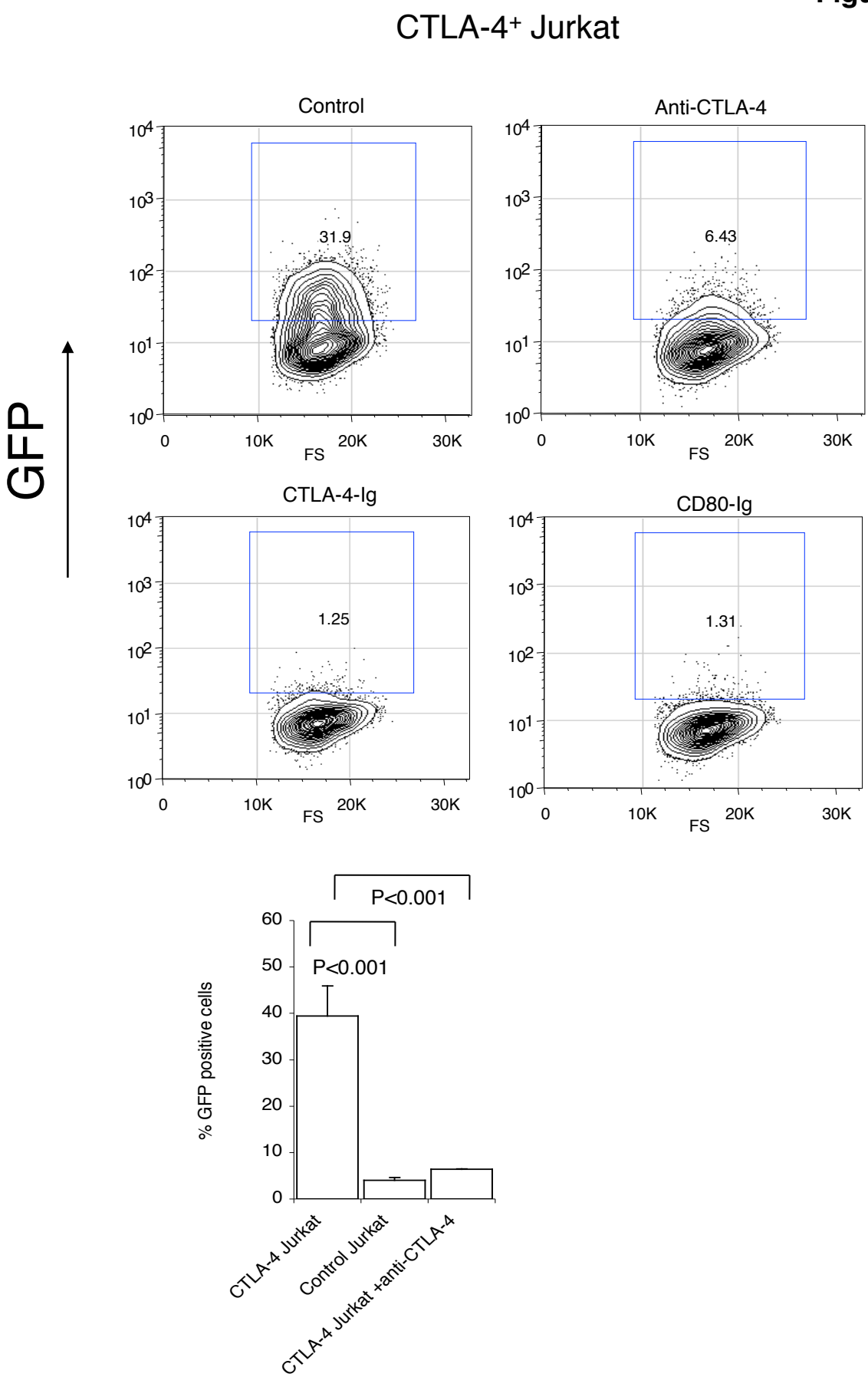

CTLA-4+ Jurkat
Control
Anti-CTLA-4
CTLA-4-Ig
CD80-Ig
GFP
FS
31.9
6.43
1.25
1.31
P<0.001
P<0.001
% GFP positive cells
CTLA-4 Jurkat
Control Jurkat
CTLA-4 Jurkat +anti-CTLA-4

Figure S12

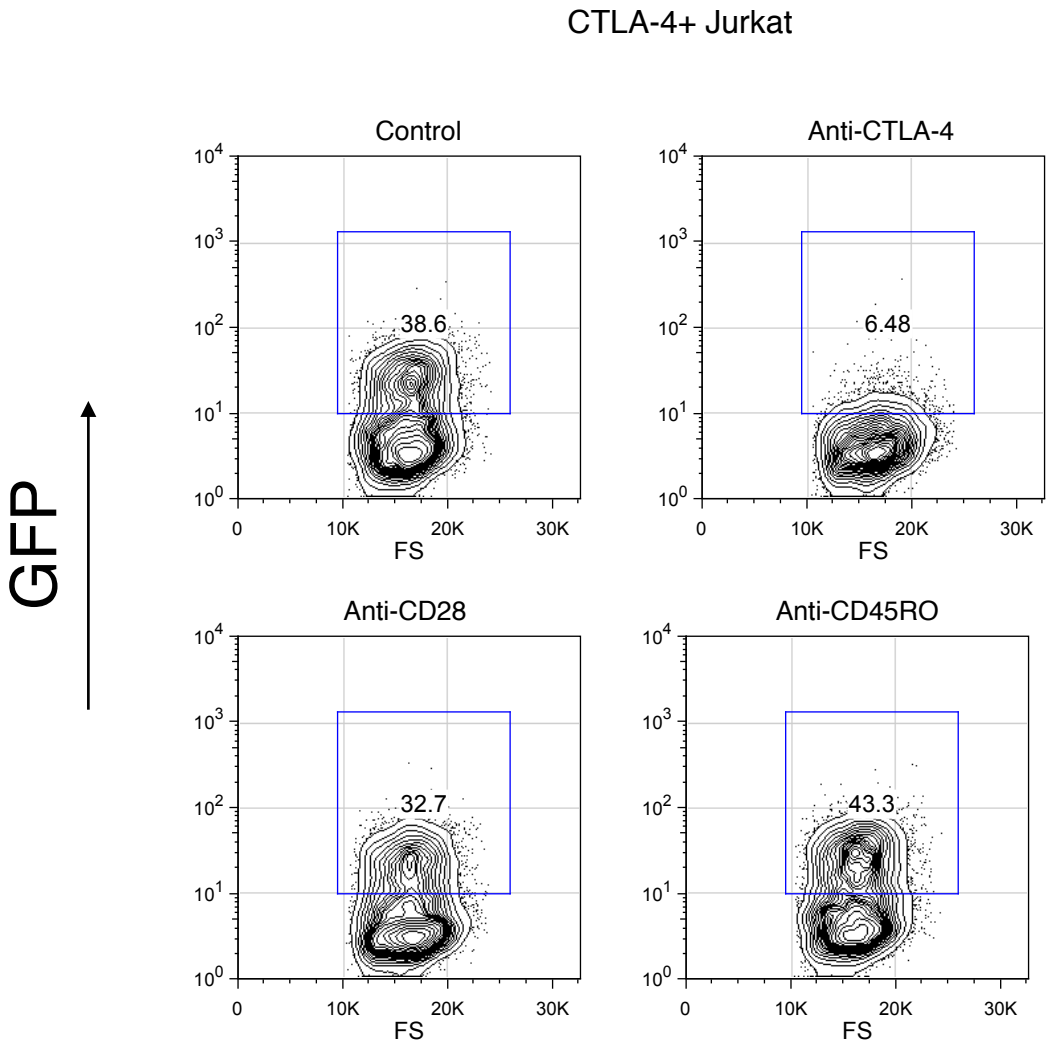

CTLA-4+ Jurkat
Control
Anti-CTLA-4
Anti-CD28
Anti-CD45RO
38.6
6.48
32.7
43.3
GFP
FS
0
10K
20K
30K
10^0
10^1
10^2
10^3
10^4

Figure S13

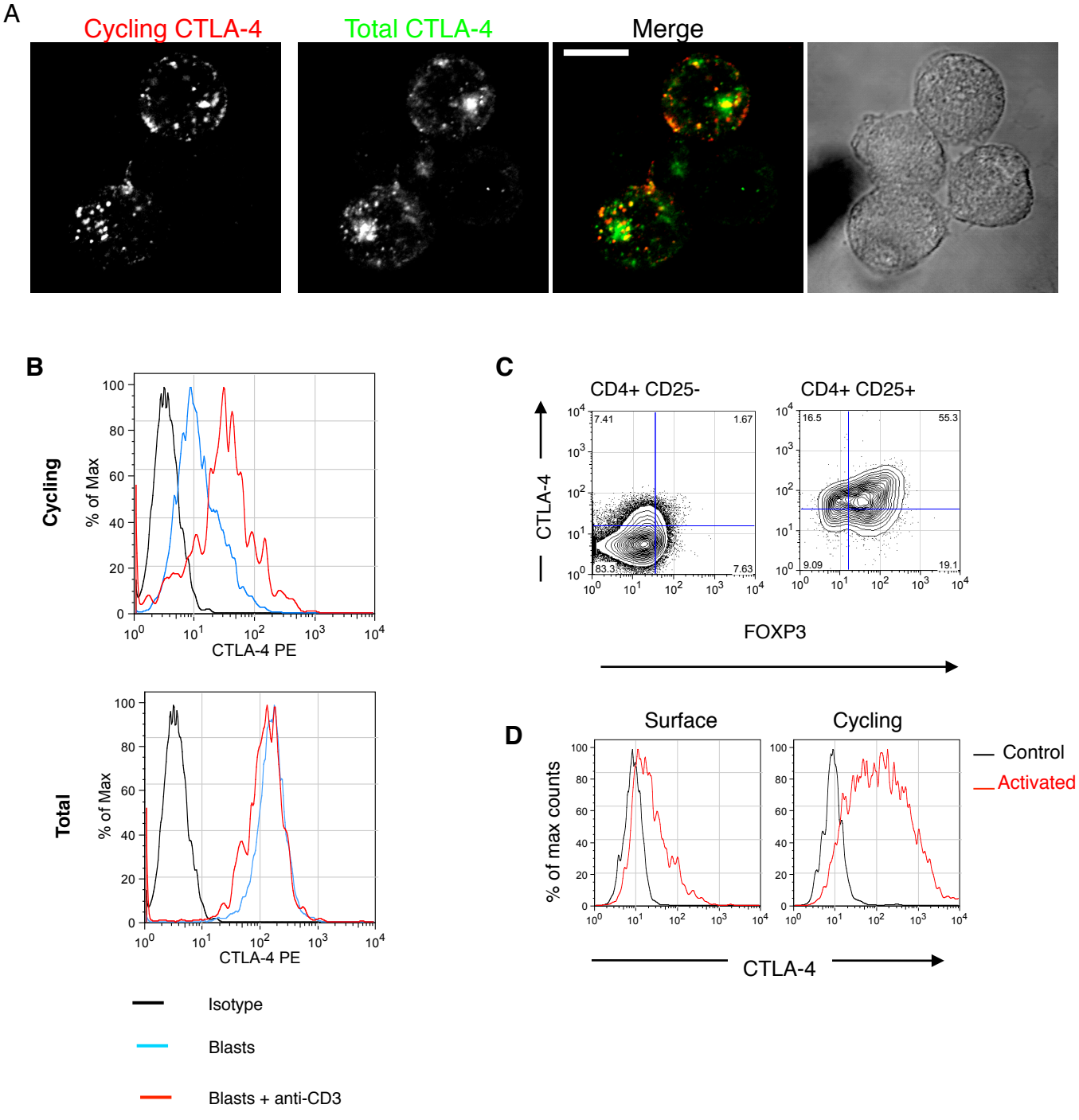

A
Cycling CTLA-4
Total CTLA-4
Merge
B
Cycling
% of Max
CTLA-4 PE
Total
% of Max
CTLA-4 PE
Isotype
Blasts
Blasts + anti-CD3
C
CD4+ CD25-
CD4+ CD25+
CTLA-4
FOXP3
7.41
1.67
83.3
7.63
16.5
55.3
9.09
19.1
D
Surface
Cycling
% of max counts
CTLA-4
Control
Activated

Figure S14

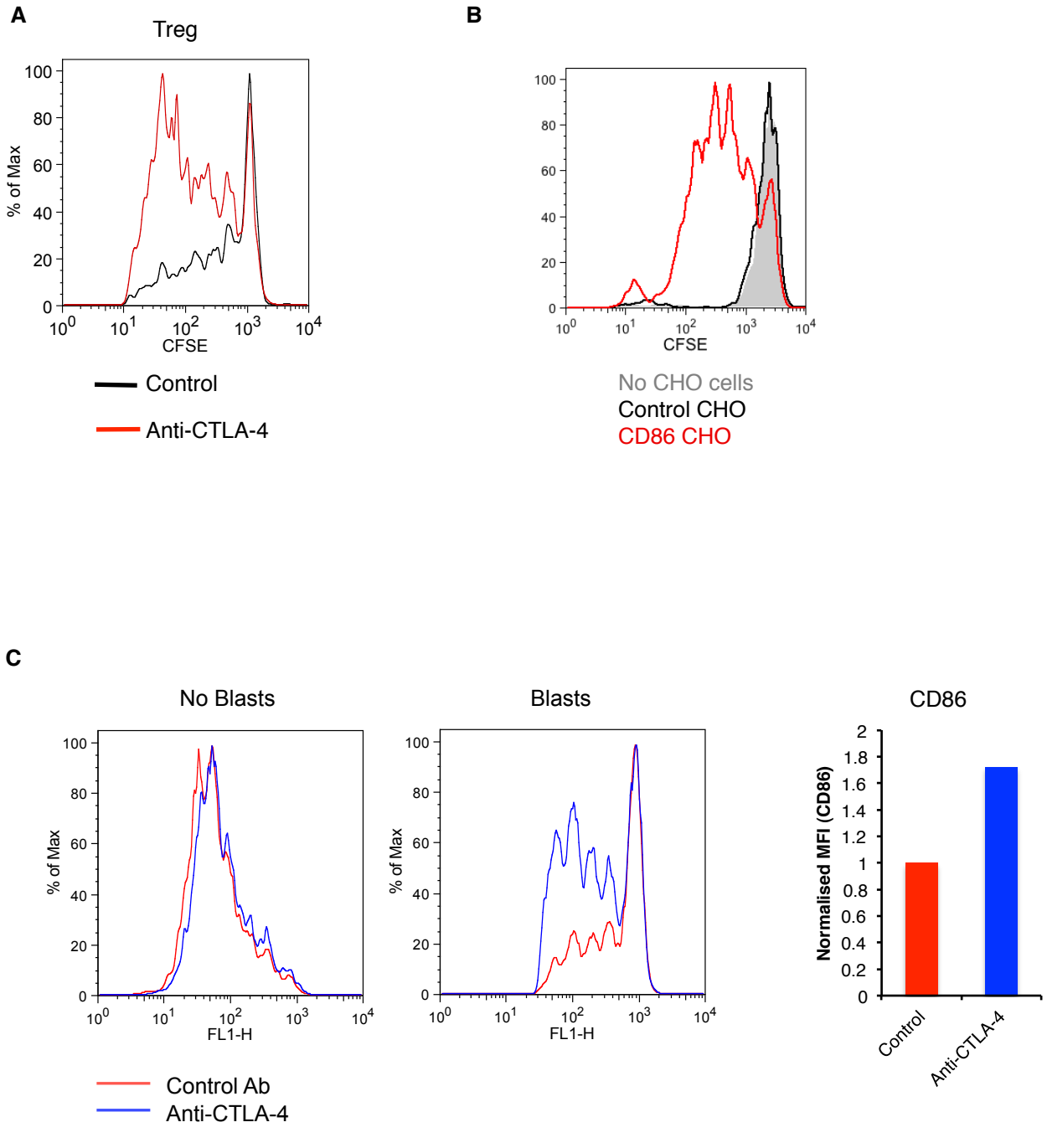

A
Treg
% of Max
CFSE
Control
Anti-CTLA-4
B
No CHO cells
Control CHO
CD86 CHO
C
No Blasts
Blasts
CD86
FL1-H
Control Ab
Anti-CTLA-4
Normalised MFI (CD86)
Control
Anti-CTLA-4

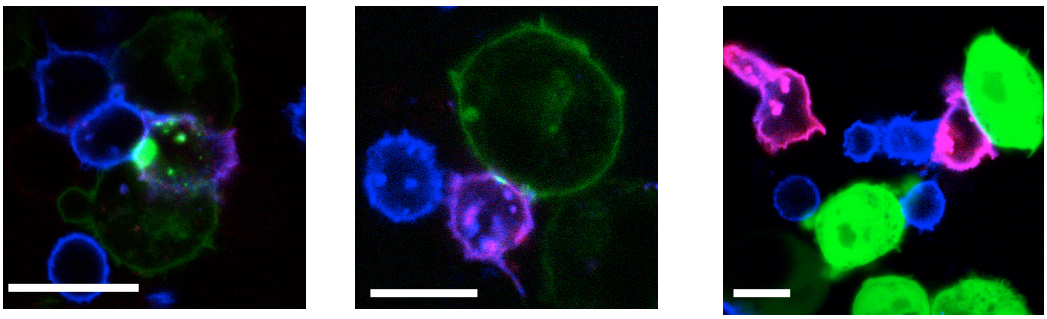
Figure S15

A

Ctla4 $^{+/+}$ CD86-GFP

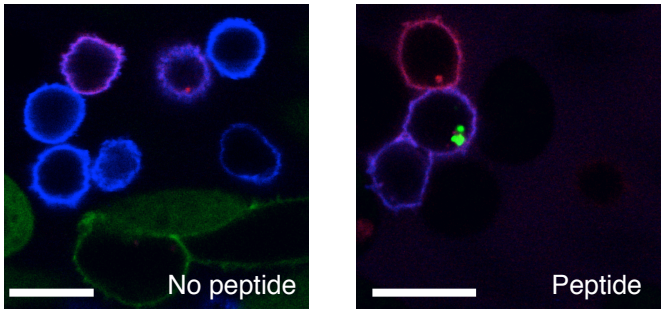
Ctla4 $^{-/-}$ CD86-GFP

Ctla4 $^{+/+}$ Control GFP



GFP CD4 CD25

B



CHO-IA^{d} -CD86-GFP

Figure S16

In vivo trans-endocytosis work flow

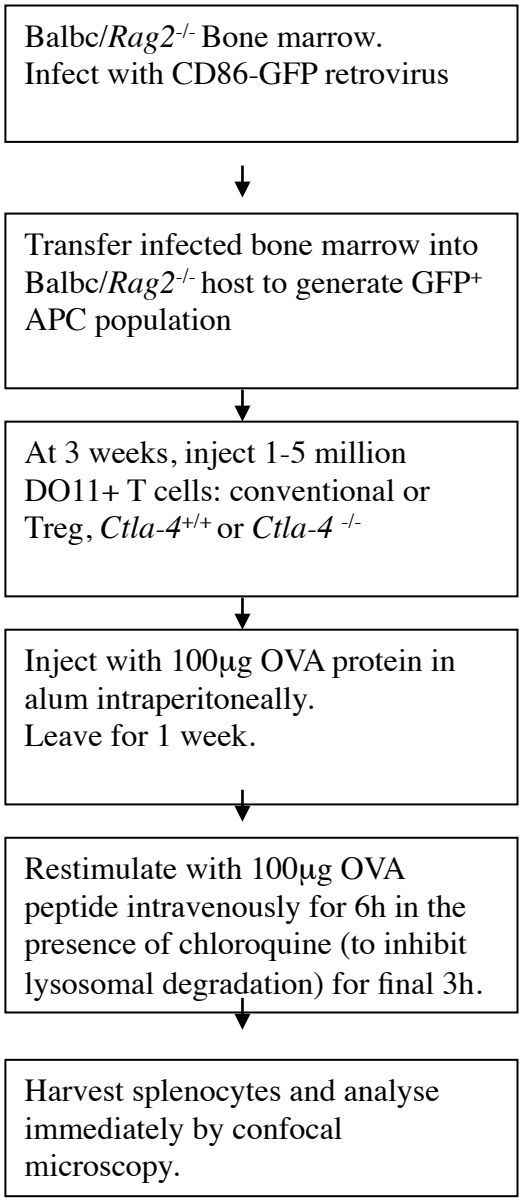
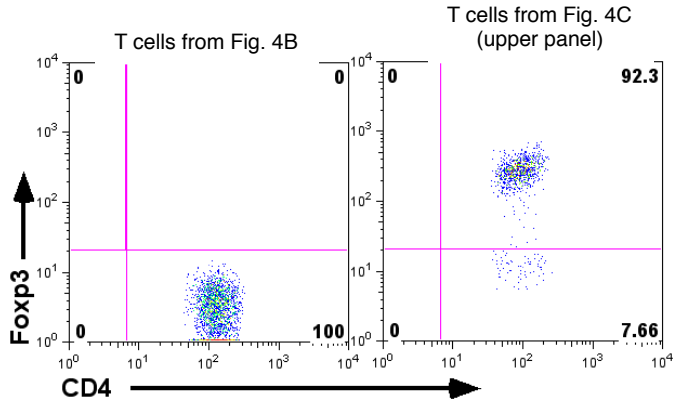
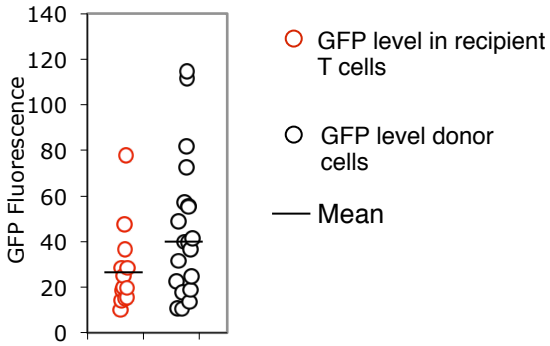


Fig. S17

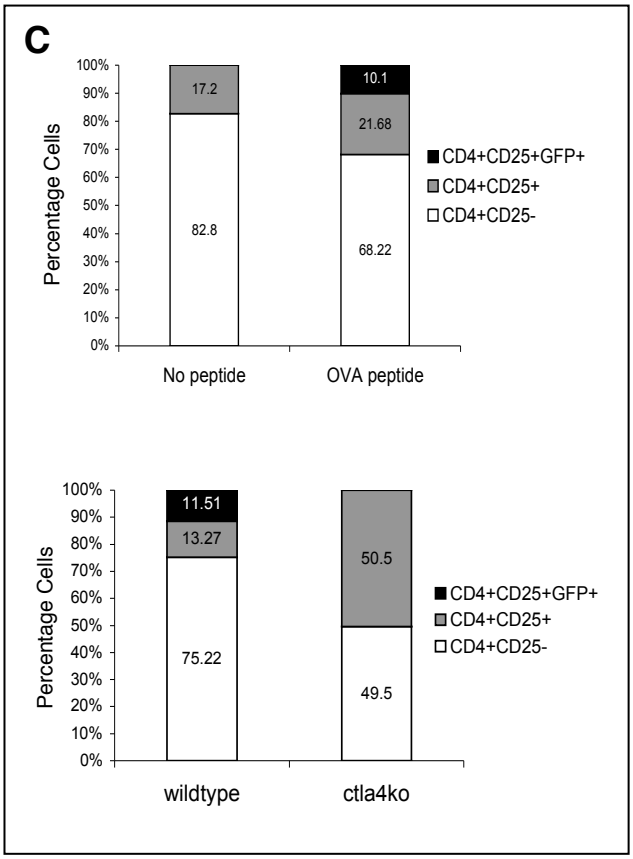
A


T cells from Fig. 4B
T cells from Fig. 4C (upper panel)
Foxp3
CD4
0
100
92.3
7.66

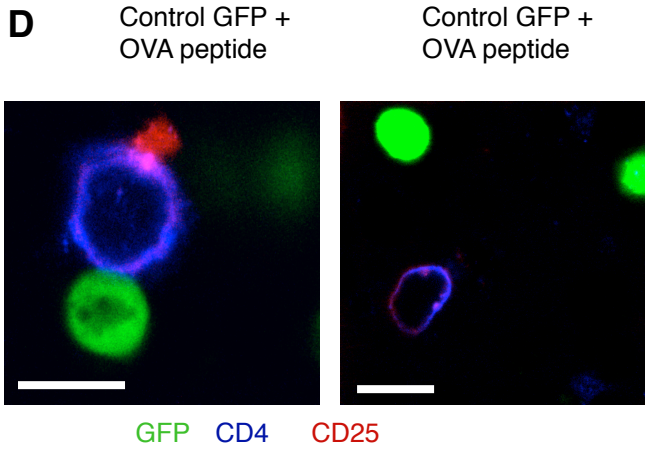
B


GFP Fluorescence
GFP level in recipient T cells
GFP level donor cells
Mean

C


Percentage Cells
17.2
82.8
10.1
21.68
68.22
No peptide
OVA peptide
CD4+CD25+GFP+
CD4+CD25+
CD4+CD25-
11.51
13.27
75.22
50.5
49.5
wildtype
ctla4ko

D


Control GFP + OVA peptide
Control GFP + OVA peptide
GFP CD4 CD25