Supporting online materials and methods

Cell purification. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were purified from the peripheral blood of healthy human donors between ages of 16 and 75 years (*1*) (New York Blood Center, NY, NY) or from 25 patients with Rheumatoid arthritis in different stages (accordingly to disease activity score (DAS); fig. S12) as previously described(*2*). The New York University Institutional Review Board has reviewed the use of human specimens for this study. Briefly, the whole blood was incubated (20 min, 22°C) with RosetteSep™ human $CD4^+$ T cell enrichment cocktail (StemCell Technologies, Vancouver, BC, Canada). The remaining un-sedimented cells were loaded onto Ficoll-Paque Plus (Amersham Bioscience, Piscataway, NJ), isolated by density centrifugation, and washed with PBS. In second round of purification, $CD4^+$ T cells were separated into $CD25^-$ and $CD25^+$ populations with magnetically coupled mAb against human CD25 (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified cells were cultured in RPMI containing antibiotics and 10% heat-inactivated FBS. Human APCs were obtained by depleting T cells from PBMCs with CD3+ depletion kit (StemCell Technologies, Vancouver, BC, Canada) and treated with Mitomycin C 0.2 mg/ml for 30 min, washed and resuspended in complete medium. $CD4^+CD25^{hi}$ T cells were separated from total $CD4^+$ T cells according to their CD25 expression by means of high-speed FACS sorting. $CD4^+$ T cells were incubated for 30 minutes with anti-CD25 PE-labeled antibodies (Miltenyi Biotec). Sorting was performed by using a MoFlo cytometer (BeckmanCoulter, Brea, CA). Umbilical cord blood (UCB) $CD25^+$ and $CD25^-$ $CD4^+$ T cells were isolated from frozen UCB units

(National Placental Blood Program, New York Blood Center) by positive selection using directly conjugated anti-CD25 magnetic microbeads. Cells were cultured with anti-CD3/CD28 mAb-coated Dynabeads for 18 to 21 days and split every 2 to 3 days. Recombinant IL-2 (300 IU/ml; Chiron, Emeryville, CA) was added on day 3 and maintained for culture duration.

Planar lipid bilayers. Planar lipid bilayers were prepared in parallel-plate flow cells as described previously (*3-5*). Liposomes that contained biotin-CAP-phosphatidylethanolamine (Avanti Polars Lipids, Alabaster, AL) were mixed with liposomes containing Cy5-ICAM-1-GPI and placed on an acid Piranha solution cleaned 40 mm round glass coverslip to form the planar bilayers with final density 0.01 mol% biotin and ICAM-1 250 molecules/μm^2. After blocking streptavidin (4μg/ml) and Alexa Fluor 546-labeled monobiotinylated anti-CD3ε monoclonal antibody (5 μg/ml, OKT3, eBioscience, San Diego, CA) were reacted sequentially with the biotinylated lipid bilayers. The flow cell containing the bilayers was warmed up to 37^{o}C, cells were injected in 500 μl of HEPES-buffered saline containing 1% human serum albumin, and images were collected for 30 min on a custom automated Olympus IX-70 inverted fluorescence microscope.

Microscopy. All TIRF imaging was performed on the custom automated Olympus IX-70 inverted fluorescence microscope using the 60X/1.45 N.A. TIRF objective from Olympus. TIRF illumination was set up and aligned according to the manufacturer's instructions as previously described (*6*). Briefly, cells interacted with the bilayers for 6 min at 37^{o}C, fixed with 2% PFA, permeabilized with 0.05% Triton-X 100, and blocked. Cells were incubated with rabbit

polyclonal antibodies to phospho-Src tyr 416 (Cell Signaling Technologies, Beverly, MA), phospho-ZAP-70 tyr 319 (sc-12946-R, Santa Cruz Biotech, Santa Cruz, CA), PKC-θ (sc-212; Santa Cruz Biotech) or Carma-1 (Card 10, C-12, Santa Cruz Biotech) for 20 min, and then incubated with fluorescently tagged goat anti-rabbit Fab_2 (Invitrogen, Carlsbad, CA). Controls included the use of nonimmune species-matched IgG. Measurement of signaling was done by determining fluorescence intensity on the entire cell-bilayer contact area as detected in the IRM channel. Average fluorescence intensity was measured using IP-Lab software (Biovision, Exton, PA) and background fluorescence was subtracted from average intensity. Confocal microscopy was carried out on a Zeiss LSM 510 Meta imaging system (63x 1.4 NA; Zeiss, Jena, Germany) using appropriate factory-set filters and dichroics for different fluorophores. Fluorophore saturation was cautiously avoided during acquisition. Acquisition settings were maintained constant throughout the experiment. Images were acquired using LSM (Zeiss) software and were analyzed and reconstructed in 3D using Imaris software (Bitplane, Saint Paul, MN). The images were further processed using Photoshop under identical contrast settings.

In vitro suppression assays. $CD4^+CD25^+$ T cells were treated or not, washed, and added at different ratios (1:9,1:3,1:1 or $5X10^4$:$5X10^5$, $1.25X10^5$:$5X10^5$, $5X10^5$:$5X10^5$, respectively) to $CD4^+CD25^-$ T cells at final concentration $2X10^6$/ml (cytokine secretion) or $2X10^5$/ml (proliferation). The cells were co-cultured on anti-CD3 mAb (5 μg/ml) pre-coated 24-well plates for 24-48 hr (cytokine secretion), or 96 hr (proliferation). Human TNF-α (210-TA) and

neutralizing antibodies against TGF-β RII (AF-241-NA) were purchased from R&D Systems Inc (Minneapolis, MN). and added to co-cultures were indicated. Cytokine secretion was determined by ELISA as previously described (*1*), using Human IFN-γ Cytoset^{tm} and Human IL-10 Cytoset^{tm} (Biosource; Camarillo, CA) and IL-17 and IL-4 (Invitrogen). Proliferation was assessed by Alamar Blue^{tm} assay (Invitrogen) (*7*) or by CFSE dilution as previously described (*8*).

CFSE labeling. We added CFSE to the cell suspensions (1X10^{7}cells/ml) at a final concentration of 5 μM, 37 ^{o}C, for 30 min; we stopped the reaction with FCS at a final concentration of 10%. We washed cells twice with PBS and resuspended them with complete RPMI media.

Flow cytometry. Indicated populations of T cells were stained (30 min, 4^{o}C) with PE-labeled anti-CD25 (Miltenyi Biotec) and FITC-labeled anti-CD127 (eBioscience) antibodies and washed with PBS (containing 0.05% BSA and 0.05% sodium azide). For intracellular staining, cells were fixed and permeabilizied with Fixation/Permeabilization buffer set (00-5523; eBioscience), washed, and stained (30 min, 4^{o}C) with primary antibodies (PE-labeled Foxp3 (PCH101) or PKC-θ (C-18)). Then, the cells were incubated (30 min, 4^{o}C) with a FITC-conjugated secondary antibodies (Jackson ImmunoResearch Lab. Inc., West Grove, PA). We analyzed samples in a FACSCalibur machine (BD, Franklin Lakes, NJ).

Inhibitors. The PKC-θ inhibitors: compound 20, BIX02508, BIX02509, BIX02510, and BIX02511 were provided by Boehringer-Ingelheim Pharmaceuticals, Inc (Rigdefield, CT) and dissolved in DMSO(*9, 10*). T cells

were pretreated with indicated concentrations of the inhibitors or DMSO control for 1, 5, 15, 30 or 60 min at 37^{o}C and washed three times. The NF-κB (#481408) and IKK (#401474) inhibitors were purchased from Calbiochem (San Diego, CA).

RNA interference. SiRNA duplexes (siRNAs) were synthesized and purified by Qiagen Inc (Valencia, CA) as described(*11*). The PKC-θ target sequences were: siRNA1 (5'-AAACCACCGTGGAGCTCTACT-3') and siRNA2 (5'-AAGAGCCCGACCTTCTGTGAA-3'); control siRNA was purchased from Qiagen (1027281). Transfections of freshly purified T cells were performed using the human T cell Nucleofector kit (Amaxa Biosystems, Lonza, Basel, Switzerland). Transfected cells were cultured in RPMI 1640 containing 10% FCS on immobilized anti-CD3 antibodies for 48-72 hours. Tranfection efficiency was controlled by evaluating PKC-θ levels using Western Blot analysis.

Western Blot. We lysed cells in RIPA buffer supplemented with protease and phosphatase inhibitors. We loaded equal amounts of protein on an SDS-PAGE gel and transferred to nitrocellulose membrane. The membranes were blocked, probed with the specific antibodies overnight, washed, and stained with secondary antibodies from Li-Cor, Inc. (Lincoln, NE) Immunoreactive protein bands were visualized using an Odyssey Infrared Imaging system. Anti-alpha actin antibodies were used as loading controls.

Mice and mouse cells. We housed all mice under specific pathogen-free conditions at the Skirball Institute Central Animal Facility, New York University School of Medicine (NYUSM). We used the C57BL/10.PL (Thy1.2), Thy1.1 congenic C57BL/10.PL Thy1.1 and C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice (Thy1.2) at 6-12 weeks of

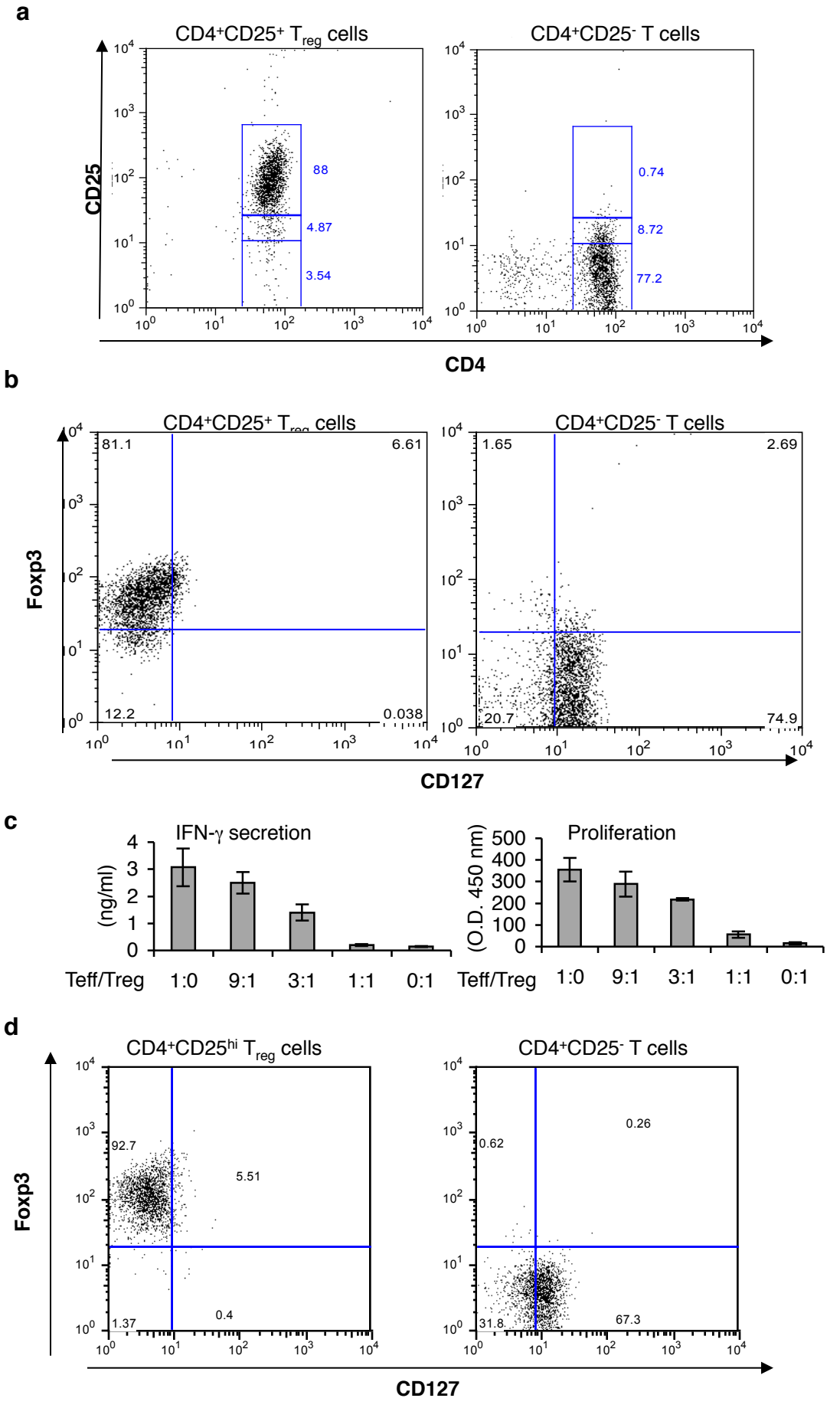
age from Jackson Laboratory. We purified $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes from spleen of wild-type C57BL/10.PL mice by magnetic cell separation using Miltenyi reagents. The purity of the $CD25^+$ population was about 90%. We purified $CD4^+CD25^-CD45RB^{high}$ fractions by cell sorting in a MoFlo cytometer (BeckmanCoulter) at NYUSM. The purity of MoFlo-sorted fraction was >95%.

Treg expansion in vivo. Foxp3+ Treg were purified from Foxp3-GFP knockin mice (*12*) by cell sorting in a MoFlo cytometer (BeckmanCoulter) at NYUSM. Sorted cells were treated with C20 for 30 min at 1μM and washed. We intravenously injected C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice with 1.5 X 10^6 sorted $CD4^+CD25^-$ $CD45RB^{high}$ T cells in combination with 5 X 10^5 of Foxp3-GFP Treg. Numbers of Foxp3-GFP Treg were evaluated in spleen and mesenteric lymph nodes after 7 days by Flow Cytometry.

T cell transfer model of colitis. For T cell transfer model of colitis, we intravenously injected C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice with 5 X 10^5 sorted $CD4^+CD25^-CD45RB^{high}$ T cells alone or in combination with 0.125 X 10^5 of $CD4^+CD25^+$ T cells that were pretreated or not as indicated. We weighed mice twice a week and inspected them for clinical sighs of colitis such as diarrhea, rectal prolapse and ruffled fur. We carried out scoring by histological examination, as previously described (*13, 14*). We histochemically stained paraffin-embedded sections with H&E. Images were acquired with an Axioplan 2 fluorescent microscope (Zeiss).

We determined P values by Mann-Whitney test or two-tailed t-test by using the GraphPad Prism software (San Diego, CA)

Supplementary figure 1



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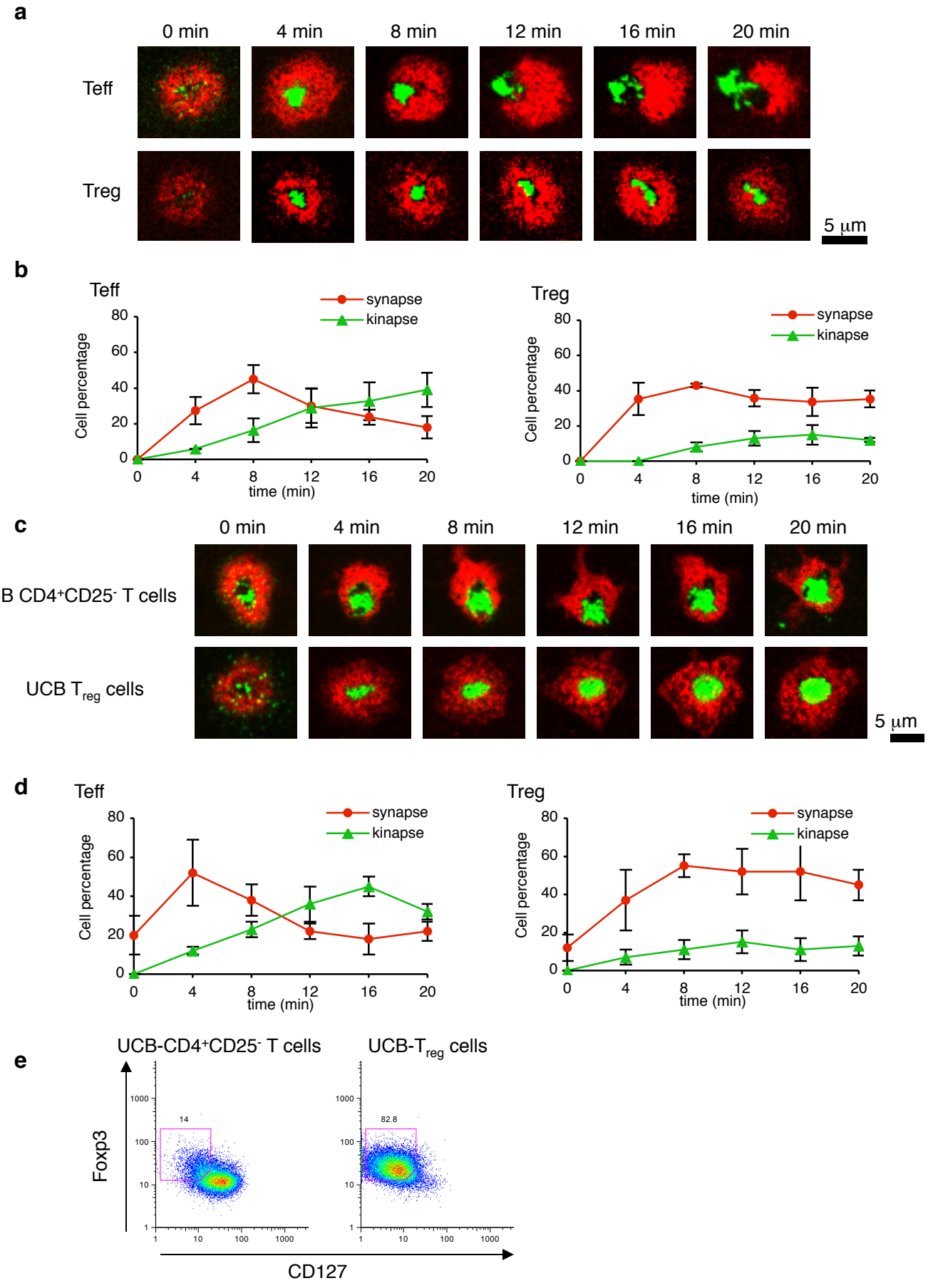

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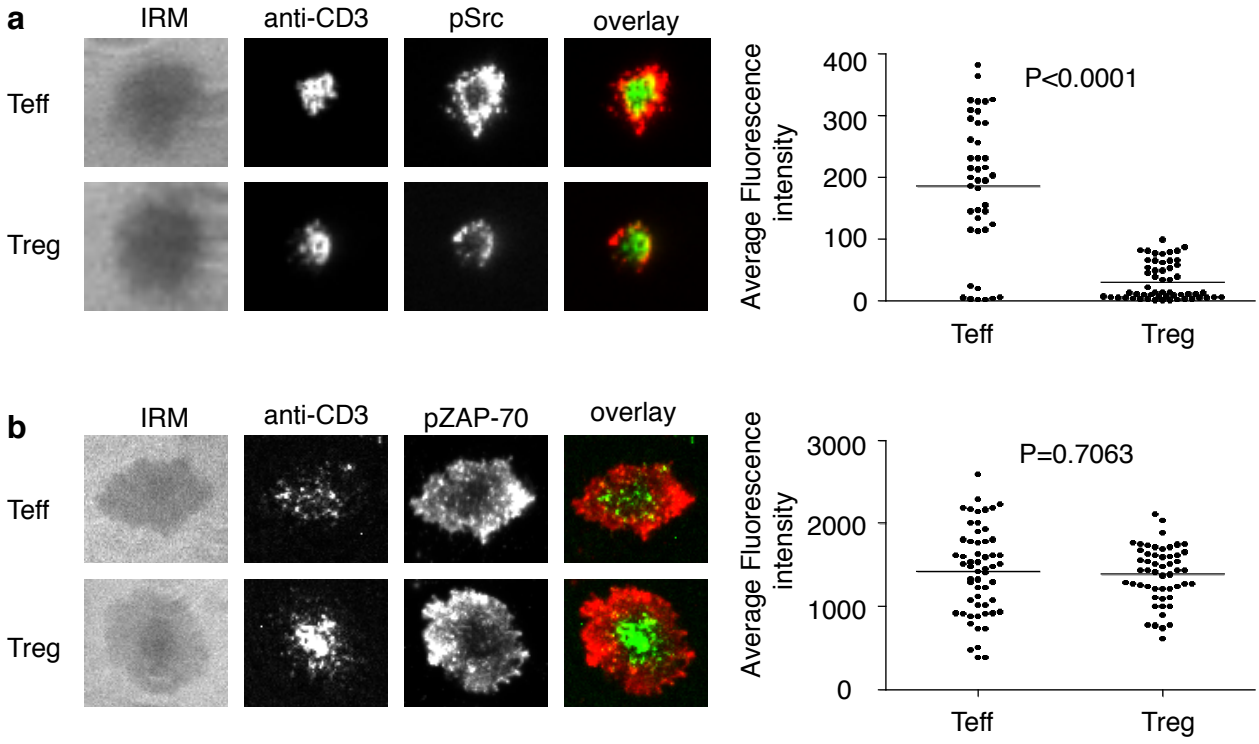

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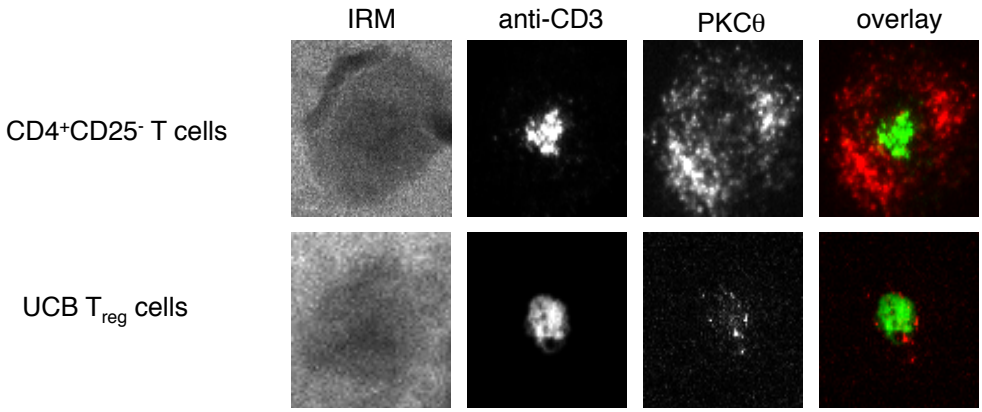

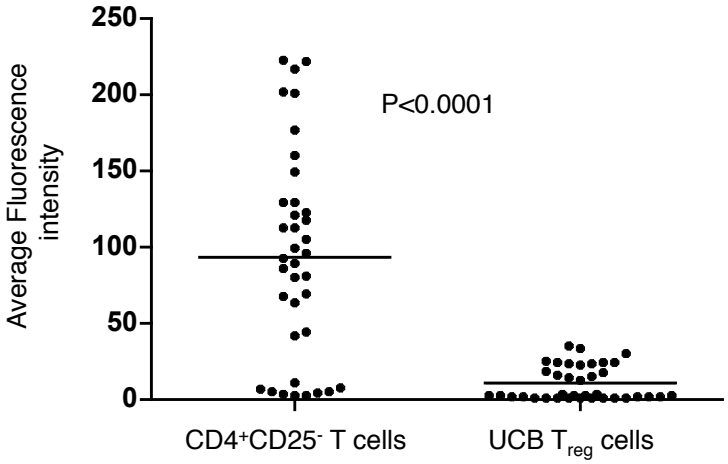

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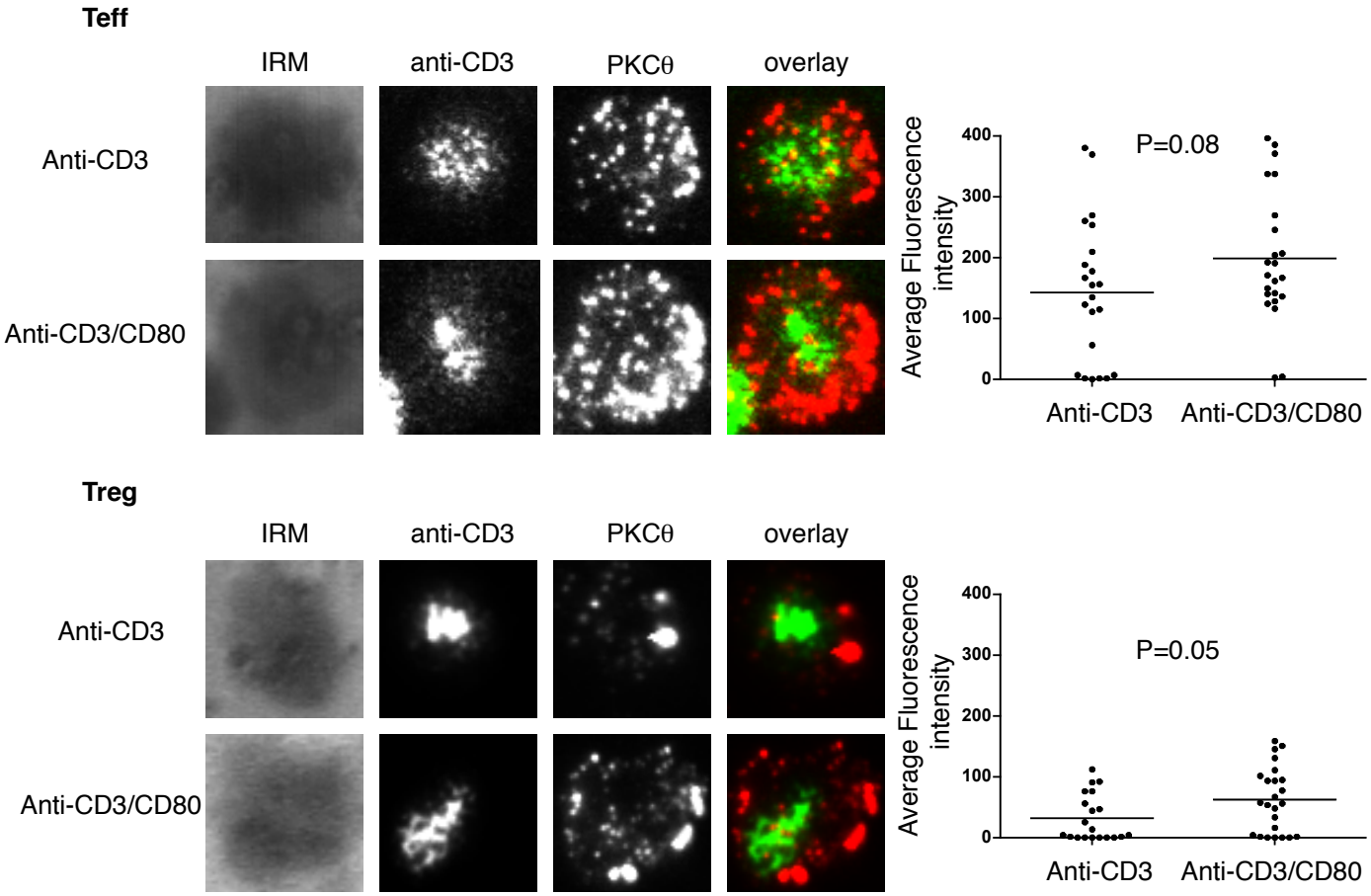

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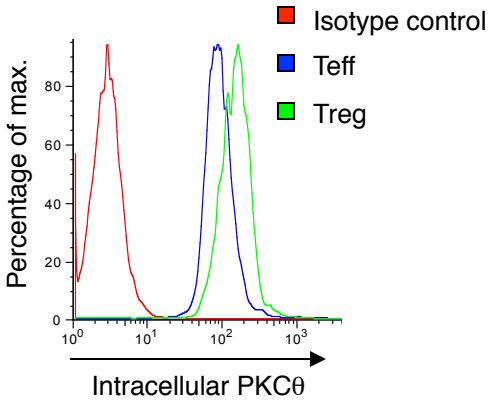

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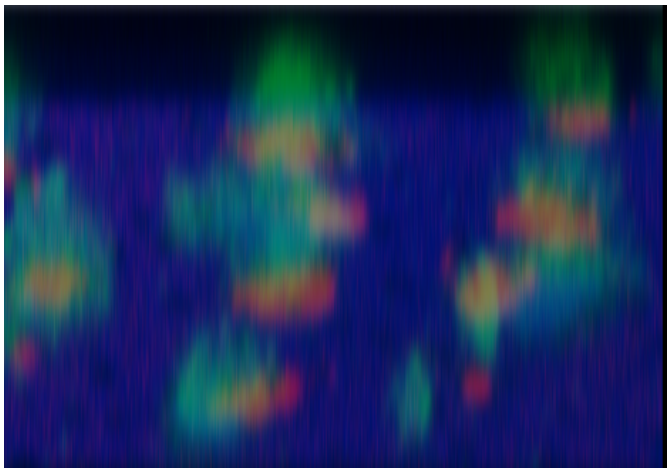
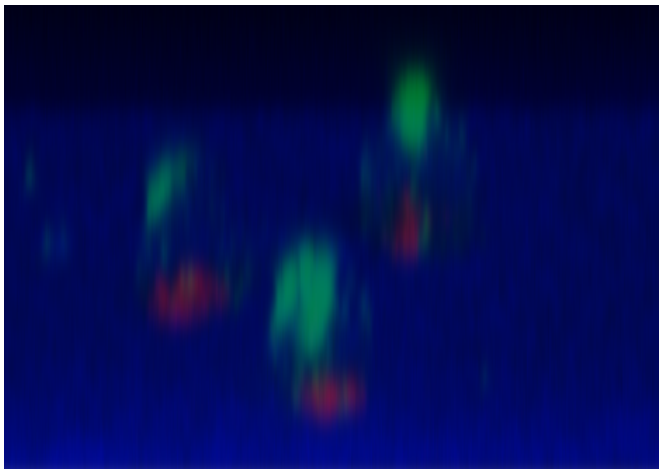


Teff

Green: PKC-θ

Red: anti-CD3

Blue: ICAM-1

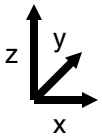


Treg

Green: PKC-θ

Red: anti-CD3

Blue: ICAM-1



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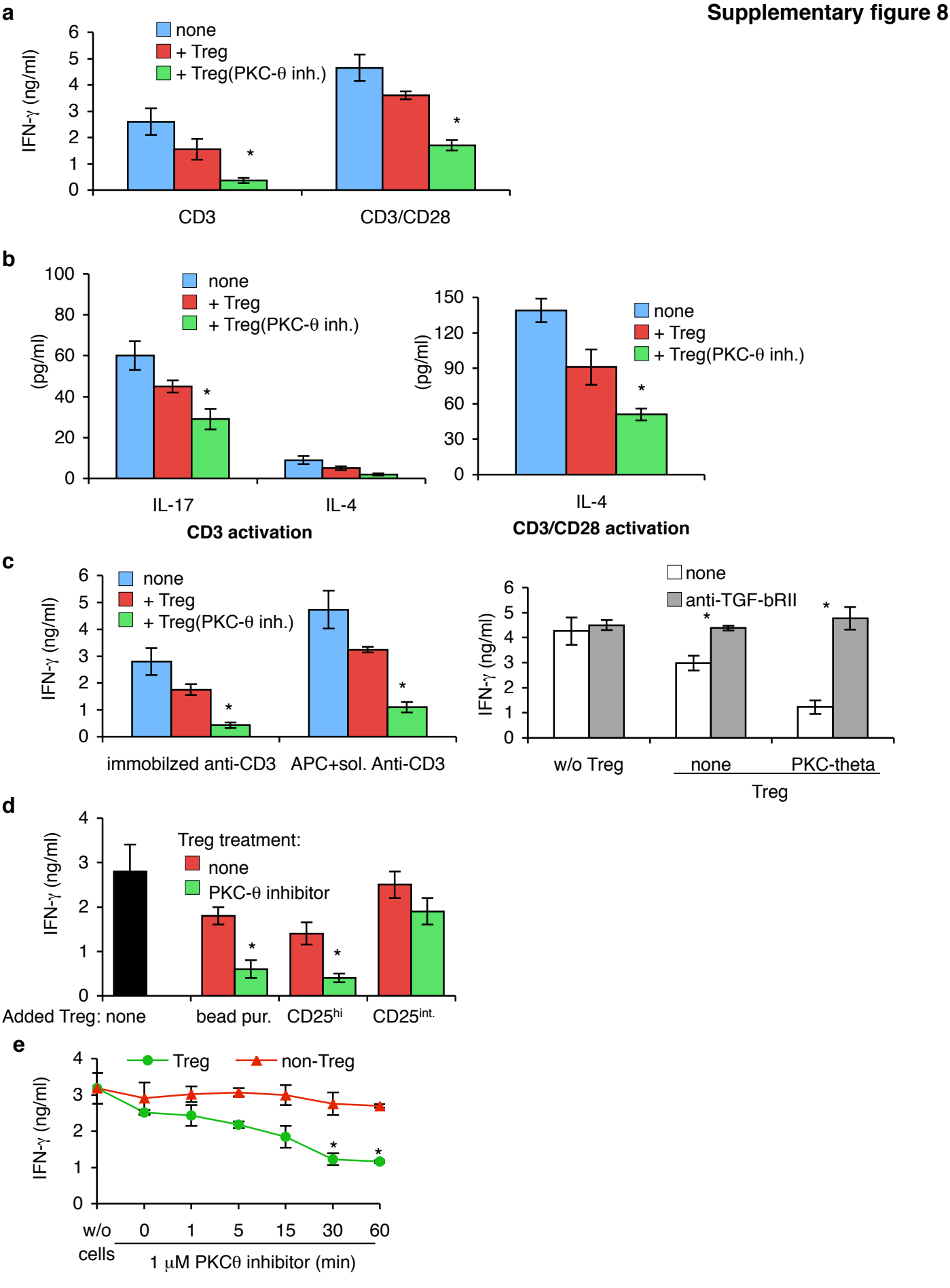

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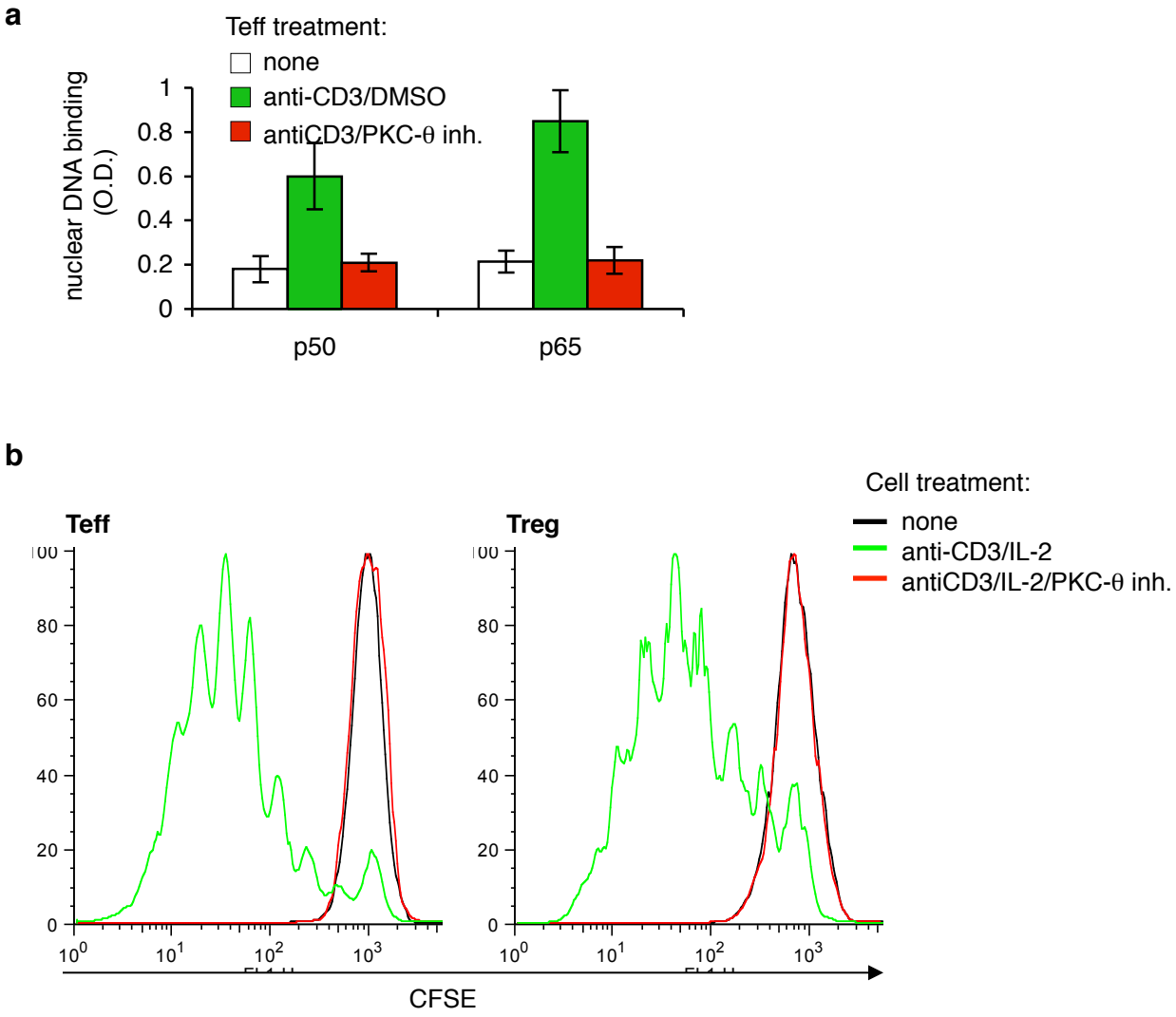
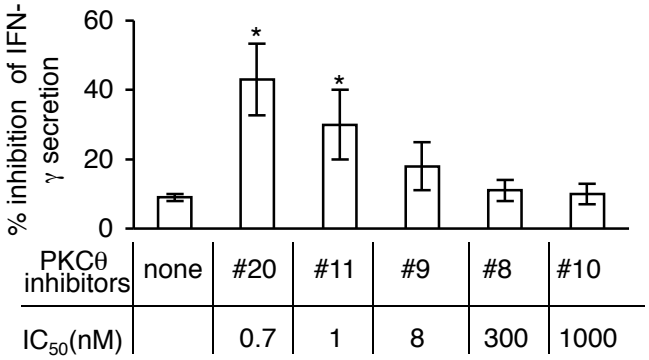


Supplementary figure 10

a



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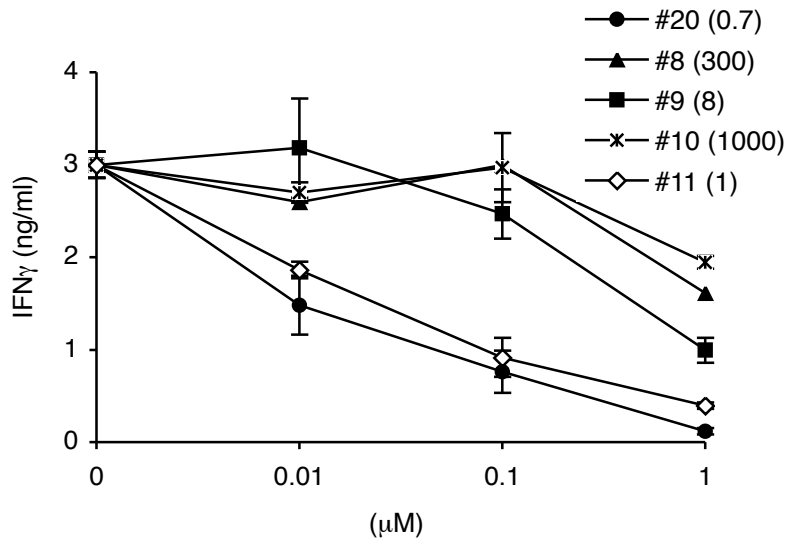

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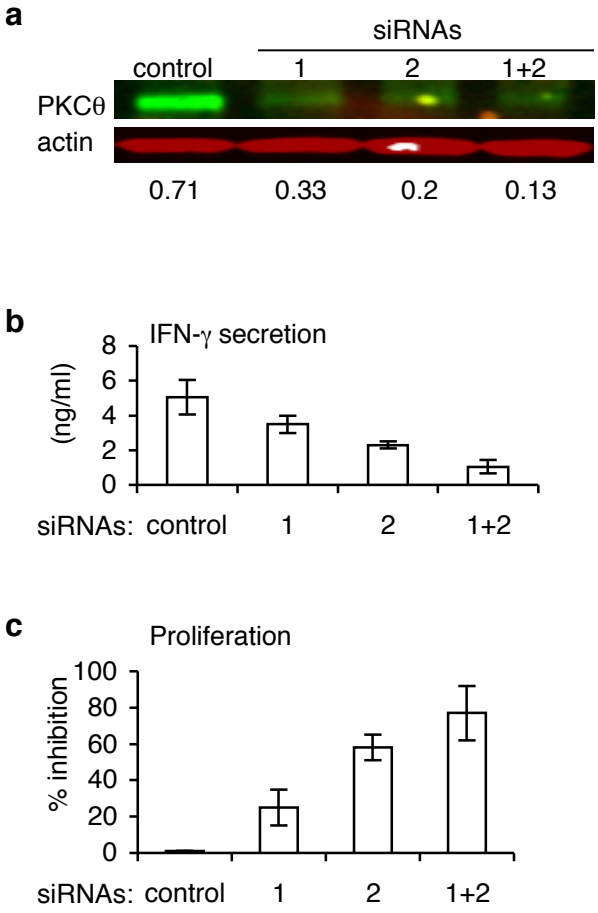
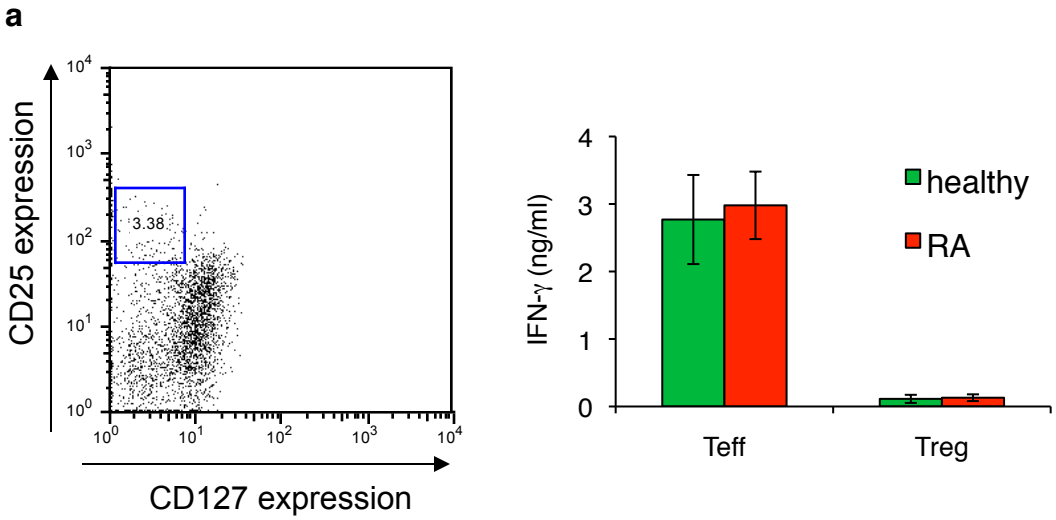
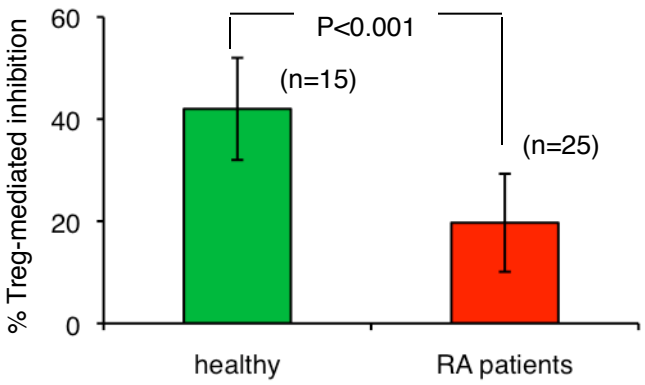


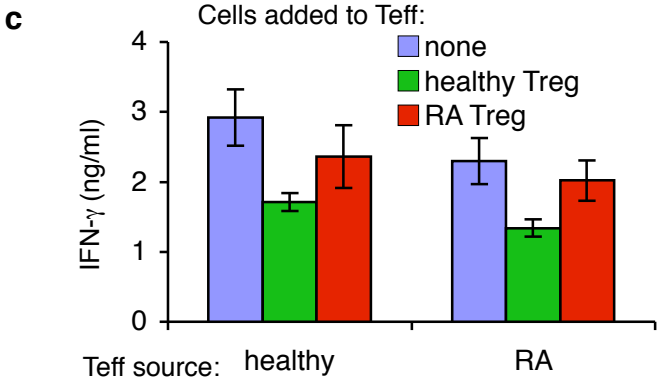
Supplementary figure 12

sample	age	gender	ethnicity	DMARD	DAS score	ESR
100486	38	M	Asian	MTX	0.84	3
100489	42	F	Hispanic	MTX	1.75	11
100480	59	M	NHW	MTX		15
100485	51	M	Hispanic	none	3.61	12
100479	76	F	NHW	MTX	3.7	20
100469	59	F	Hispanic	none	3.77	73
100470	46	F	Hispanic	MTX	3.81	12
100471	38	M	Asian	none	3.9	4
100487	24	F	NHW	none	4.26	44
100464	44	F	Hispanic	none	4.36	19
100488	61	F	Hispanic	none	4.48	52
100442	62	F	Hispanic	MTX	4.49	27
100481	59	F	Hispanic	MTX	4.6	38
100441	61	M	Hispanic	MTX	4.88	14
100477	46	F	Asian	MTX	4.89	18
100463	52	F	AA	MTX	5.59	93
100476	48	F	NHW	MTX	5.98	8
100483	48	F	NHW	MTX	5.99	12
100484	52	F	AA	MTX	6.08	30
100473	50	F	AA	MTX	6.53	14
100491	50	F	Hispanic	MTX	6.64	12
100492	76	F	AA	MTX	6.64	32
100482	69	F	NHW	none	7.35	
100493	58	F	Hispanic	MTX	7.45	62
100494	70	F	Hispanic	MTX	8.17	60

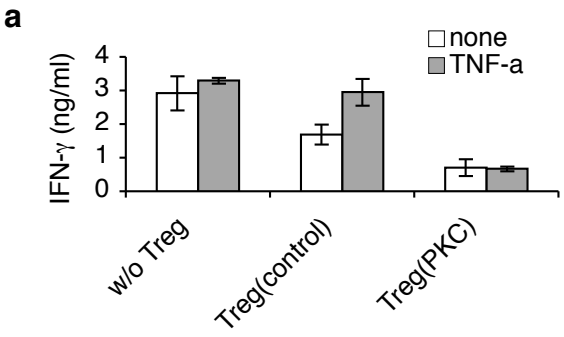
Supplementary figure 13

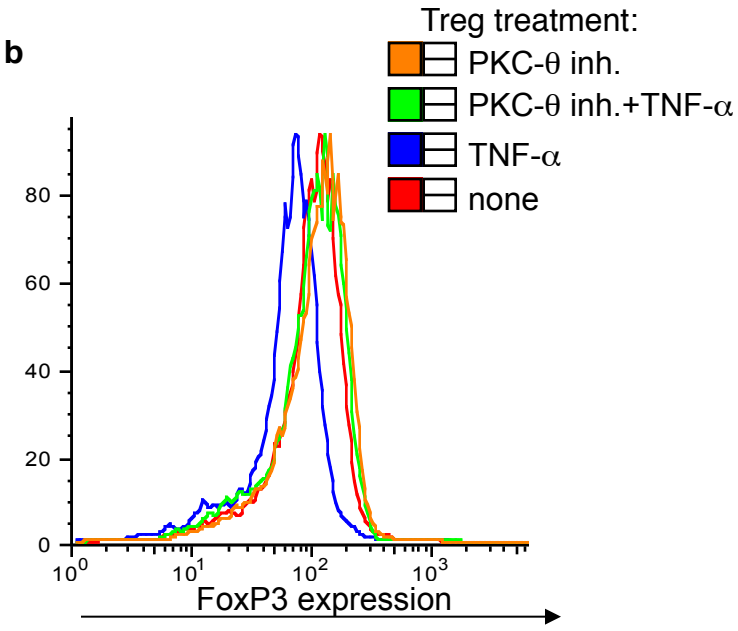






Supplementary figure 14





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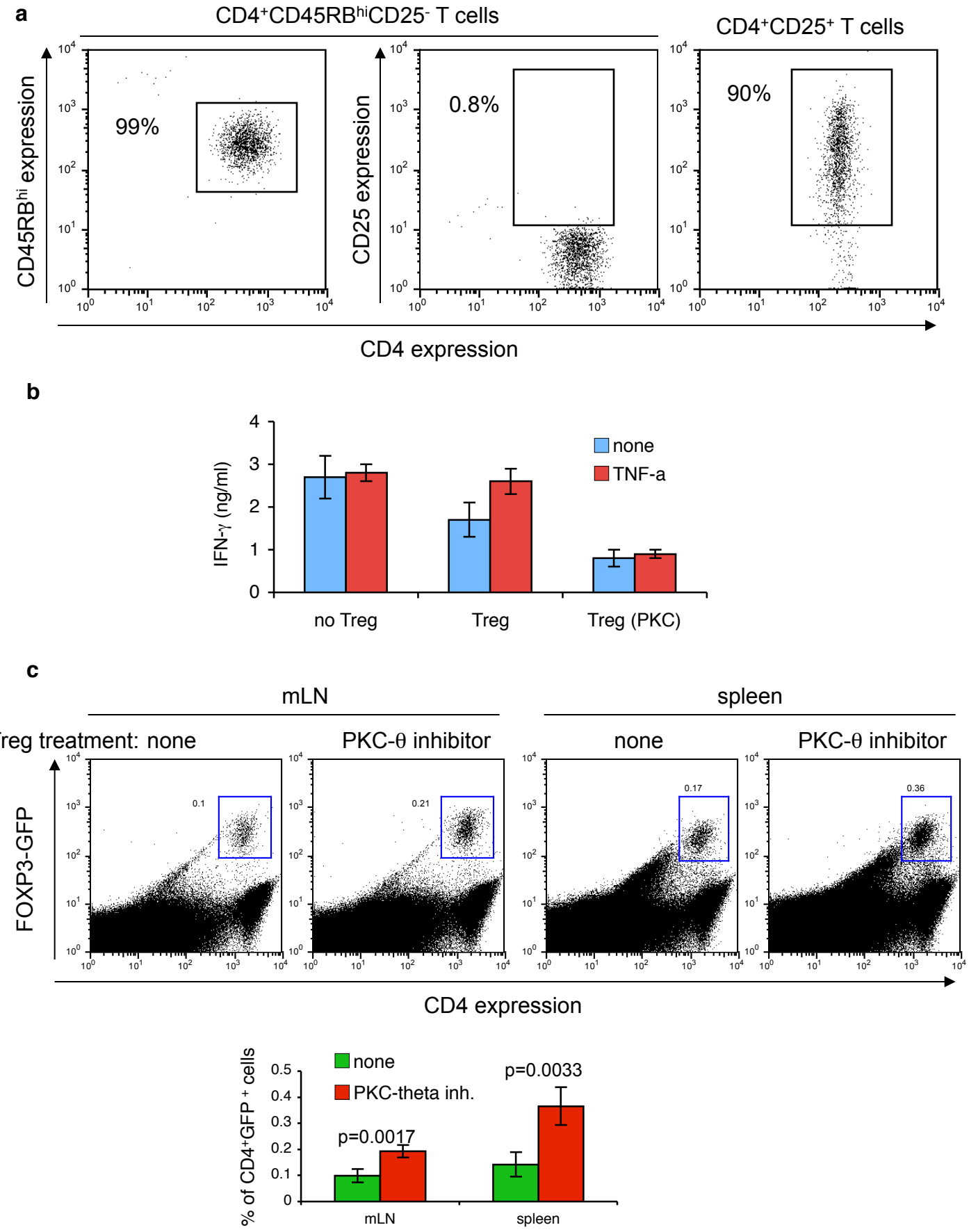

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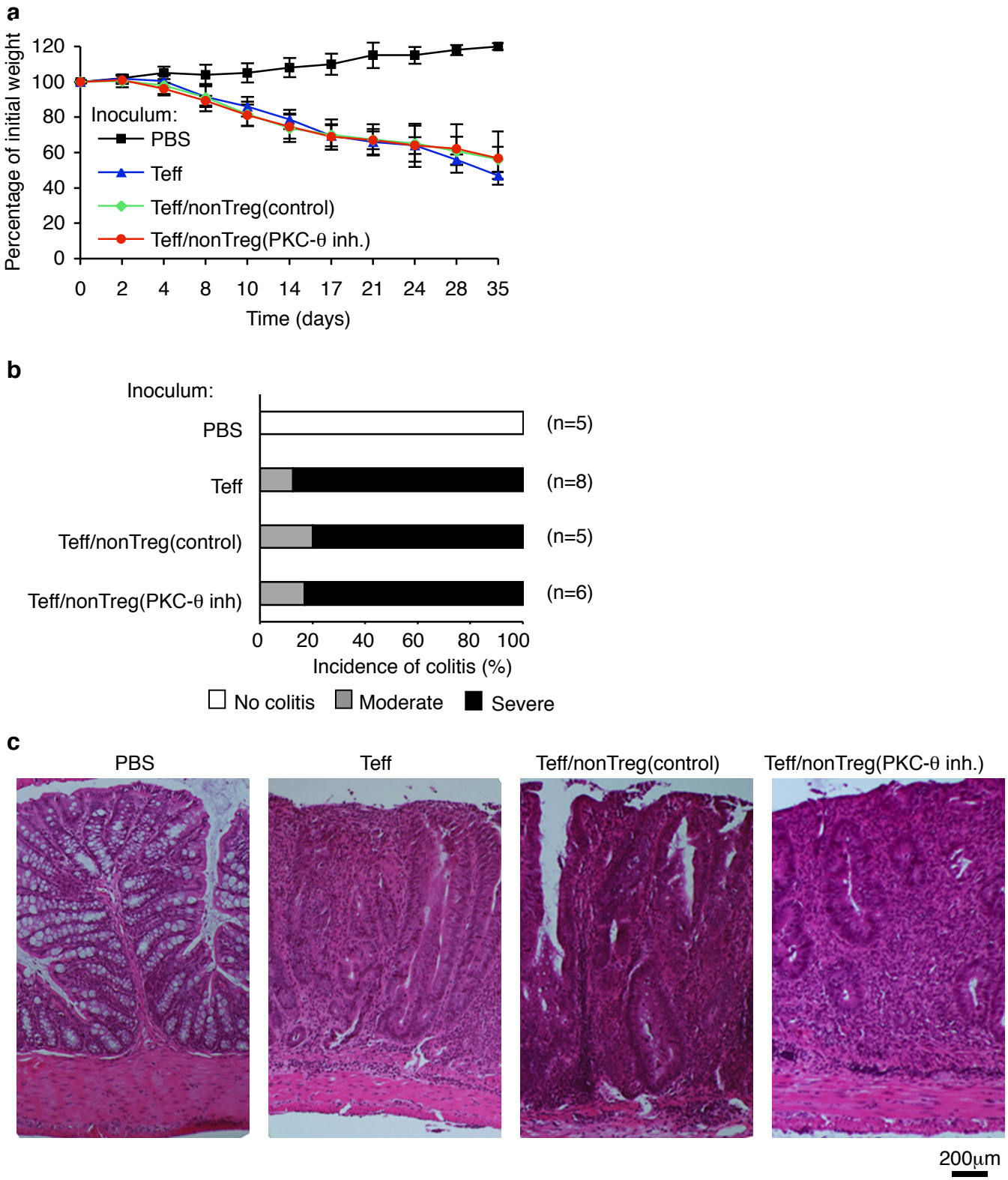

Supplementary figure legends

Figure 1. Purification and characterization of human Treg. (a) Treg were purified from healthy donors by positive selection (about 90% of $CD4^+CD25^{hi}$). Greater than 80% of Treg represents $Foxp3^+CD127^-$ (b). $CD4^+CD25^+$ T_{reg} cells suppress IFN-γ secretion and proliferation of Teff in concentration-dependent manner (c). (d) $CD4^+CD25^{hi}$ Treg were purified by sorting. Greater than 90% of cells represents $Foxo3^+CD127^-$.

Figure 2. Human Treg form more stable IS compared to Teff. Freshly purified human blood Treg and Teff (a, b) or umbilical cord blood (UCB)-derived Treg and Teff and expanded by using anti-CD3/28 mAb-coated beads (c,d) were introduced into bilayers containing anti-CD3 mAb (green) and ICAM-1 (red). Images of the same fields were acquired over 20 min. Representative images of Teff and Treg are shown (a, c). Synapse morphology was defined as accumulation of anti-CD3 antibodies in center of symmetrical ICAM-1 ring (see panel a, Treg, all times). Kinapse morphology was defined as migratory synapse with broken and asymmetrical ICAM-1 focal zone (see panel a, Teff 12-20 minutes). The percentages of cells forming the different morphologies were calculated, and summary of three independent experiments is shown (b, d). (e) Greater than 80% of expanded UCB-derived Treg represents $Foxo3^+CD127^-$.

Figure 3. Src kinase recruitment to IS is reduced in Treg. Teff and Treg were introduced into bilayers containing anti-CD3 mAb (5 μg/ml) and ICAM-1 at 250 molecules/μm^2 for 6 min, fixed, permeabilized, stained for phosphorylated Src kinase activation loop Y394 (a) or phosphorylated Zap70 interdomain A Y319

(b) and imaged by TIRFM. Images of randomly selected fields are shown. The average intensity of staining was measured. Data are representative of four different experiments. P values were calculated by Mann-Whitney test.

Figure 4. PKC-θ recruitment to IS is reduced in expanded Treg. Human umbilical cord blood (UCB)-derived Treg expanded by using anti-CD3/28 mAb-coated beads were introduced into bilayers containing anti-CD3 mAb and ICAM-1 for 6 min, fixed, permeabilized, stained for PKC-θ and imaged by TIRFM. Images of randomly selected fields are shown. The average intensity of staining was measured. Data are representative of three different experiments. P values were calculated by Mann-Whitney test

Figure 5. Co-stimulation through CD28 up-regulates the recruitment of PKC-θ to IS. Teff and Treg were introduced into bilayers containing anti-CD3 mAb (5 μg/ml) and ICAM-1 at 250 molecules/mm^2 with or without CD80 at 200 molecules/mm^2 for 6 min, fixed, permeabilized, stained for PKC-θ and imaged by TIRFM. Images of randomly selected fields are shown. The average intensity of staining was measured. Data are representative of three different experiments. P values were calculated by Mann-Whitney test.

Figure 6. Treg express higher levels of intracellular PKC-θ compared to Teff. Expression levels of PKC-θ were determined by FACS analysis in permeabilized cells. One representative experiment of four is presented.

Figure 7. PKC-θ is sequestered in the distal pole of Treg IS. Teff and Treg were introduced into bilayers containing anti-CD3 mAb (5 μg/ml) and ICAM-1 at 250 molecules/μm^2 for 6 min, fixed, permeabilized, stained for PKC-θ and

imaged by Confocal Microscopy. PKC- θ (green); anti-CD3 (red); ICAM-1 (blue). Images of randomly selected fields are shown. One representative experiment of three is presented.

Figure 8. Treatment with PKC-θ inhibitor up-regulates Treg function in vitro. Treg were treated with PKC-θ inhibitor at 1 μM for 30 min **(a-d)**, or at 1 μM for 0-60 min **(e)**, washed three times and mixed with $CD4^+CD25^-$ T (Teff) cells at 1:3 **(a-d)** or 1:9 **(e)** ratio and plated on immobilized anti-CD3 mAb **(a-e)** alone, anti-CD3/anti-CD28 mAb **(a, b)** or on APCs with soluble anti-CD3 mAb (5μg/ml) **(c)** in presence (right panel) or absence (left panel) of neutralizing antibodies against TGF-β RII (20 μg/ml). The supernatants were analyzed for IFN-γ **(a, c, d, e)** IL-4 or IL-17 **(b)** secretion after 24-48 hours. The means±SD of three different experiments are shown. P values were calculated by t-test.

Figure 9. Treatment with PKC-θ inhibitor reduces NF-kB activation and TCR and IL-2 stimulated proliferation in Treg and Teff. Treg and Teff were treated with 1 μM of PKC-θ inhibitor (C20) for 30 min, washed, stimulated by anti-CD3 antibodies and p65/p50 specific binding to NF-κB consensus sequence was determined by ELISA (a). Proliferation was tested by CFSE dilution after 72 hr on anti-CD3 antibodies and IL-2 (100 U/ml) (b). One representative experiment of three is presented.

Figure 10. Analogs of C20 up-regulate Treg function accordingly to their IC_{50} values. **(a)** Purified Treg were treated with 1 μM of analogs of PKC-θ inhibitor with different IC_{50} values as indicated on graph, washed three times, mixed with Teff cells at 1:9 ratio and plated on immobilized anti-CD3 mAb. **(b)**

$CD4^+$ T cells were incubated with indicated doses of inhibitors on immobilized anti-CD3 antibodies for 48 hours. IFN-γ levels in supernatants were determined by ELISA. Summary of three independent experiments is presented.

Figure 11. Reduction of PKC-θ protein by specific siRNA down-regulates IFN-γ secretion in $CD4^+$ T cells. $CD4^+$ T cells were transfected with siRNA targeting PKC-θ, or with control siRNA and plated on anti-CD3 mAb. After 48 hours the PKC-θ expression was measured by Western blot (a) analysis and IFN-γ levels in supernatants were determined by ELISA (b). Summary of three independent experiments is presented.

Figure 12. The clinical and demographic details of studied RA patients. M - Male; F - Female; NHW – Non-Hispanic White; AA – African American; DMARD - Disease Modifying anti-Rheumatic Drug; MTX - methodexate; DAS - Disease Activity Score; ESR - Erythrocyte Sedimentation Rate. The increased levels of the ESR is strongly associated with serious underlying disease, such as RA. However, the relative utility of this test to measure disease activity has decreased as new methods of evaluating disease have been developed. ESR is not always correlated with DAS score and decreased Treg function in RA patients. Some patients have low ESR level with high DAS score and almost absence in Treg function and the opposite occurred too.

Figure 13. Treg purified from RA patients failed to suppress IFN-γ secretion in Teff. **(a)** $CD4^+CD25^{hi}CD127^{lo}$ Treg represent about 3%±0.95 of total $CD4^+$ T cells in RA patients and do not secrete IFN-γ in response to anti-CD3 activation. One representative experiment of five is shown. **(b)** Freshly purified

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Treg from healthy donors and RA patients were mixed with $CD4^+CD25^-$ T cells at ratio 1:3, and plated on anti-CD3 mAb. The supernatants were analyzed for IFN-γ after 24-48 hours. % Treg-mediated inhibition was calculated as: 1- (level of IFN-γ in presence of Treg / level of IFN- γ in absence of Treg) X 100%. n - number of patients. **(c)** Treg from healthy donors or RA patients were mixed with $CD4^+CD25^-$ T cells from healthy donors or RA patients at ratio 1:3, and plated on anti-CD3 mAb. The supernatants were analyzed for IFN-γ after 48 hours. Summary of three independent experiments is shown.

Figure 14. Inhibition of PKC-θ protects Treg from inactivation by TNF-α. **(a)** Treg from healthy donors were treated with PKC-θ inhibitor as described and co-culture with $CD4^+CD25^-$ T cells with or without TNF-α (50 ng/ml). IFN-γ secretion was determined after 48 hours. **(b)** Untreated or PKC-θ inhibitor-treated Treg were incubated for 24 hours with TNF-α (50 ng/ml) and Foxp3 expression was determined by FACS. One representative experiment of three is shown.

Figure 15. Inhibition of PKC-θ up-regulates and protects the function of murine Treg in vitro and induces expansion in vivo. **(a)** Purity of mouse Treg and $CD4^+CD45RB^{hi}CD25^-$ T cells. **(b)** Freshly purified murine Treg were treated or not with 1 μM PKC-θ inhibitor C20 for 30 min, washed three times, mixed with $CD4^+CD45RB^{hi}CD25^-$ T cells at ratio 1:3, and plated on immobilized anti-CD3 mAb with or without 50 ng/ml of mTNF-α. The supernatants were analyzed for IFN-γ after 48 hours. Average of three independent experiments is shown. **(c)** Freshly purified murine Foxp3-GFP Treg were treated or not with 1 μM PKC-θ inhibitor C20 for 30 min, washed three times, mixed with $CD4^+CD45RB^{hi}CD25^-$ T

cells at ratio 1:3, and injected in C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice. Foxp3-GFP Treg were evaluated in spleen and mesenteric lymph nodes 7 days later by FACS. Summary of two independent experiments is shown.

Figure 16. Inhibition of PKC-θ in $CD4^{+}CD25^{-}$ T cells (non-Treg) did not protect mice in colitis model. Colitis was induced in C57BL/10.PL TCR $\alpha^{-/-}$ $\beta^{-/-}$ mice as described in extended on-line Materials and Methods. Weight (A), colitis score (B) and representative colon histology (C) are shown. Numbers in parentheses indicate number of mice. Combined data of three independent experiments are presented.

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