Supplementary Methods

Mice

C57BL/6, BALB/c, IL-10-/-, CD11c-DTR-EGFP mice (on BALB/c background), and CD11c-YFP mice (on B6 background) were purchased from The Jackson Laboratory. Foxp3-mRFP (FIR) mice were kindly provided by Dr. Yisong Wan. All mice were maintained at the animal facility at The University of North Carolina in Chapel Hill. All experiments were performed in accordance with protocols approved by The University of North Carolina Institutional Animal Care and Use Committee.

Transplantation systems

Donor T cell-depleted (TCD) bone marrow (BM) cells were prepared as previously described[1]. CD25-deplected splenic T cells were prepared using negative selection of CD25-, CD19-, MHCII-, γδTCR-, CD11c-, CD11b-, CD49b-, and Ly6G-expressing cells by antibodies coupled to biotin and streptavidin-coupled ferromagnetic beads. Then the cells were purified through a magnetic activated cells sorter (MACS) column (Miltenyi Biotec). T cell purity was measured by flow cytometry before transplantation and had 90∼95% purity. Recipient mice on BALB/c background were irradiated at 800 rads and B6 background mice at 950 rads one day prior to transplantation with 3∼5 million of donor T cells (or 3 million total T cells with 2 million of T_{reg}) and 4∼5 million TCD BM cells. Donor WT or IL-10 $^{-/-}$ T_{reg} cells were prepared as previously described using a column purification method[2]. Briefly, splenic T cells were purified through negative selection onto CD4 columns by Cedarlane. These purified $CD4^+$ T cells were stained with biotin-coupled anti-B220,

anti-CD49b, anti-CD8, anti-γδTCR, and PE-coupled CD25 and then negatively-selected using streptavidin Dynal beads and Dynal magnets (Invitrogen). Finally the cells were positively-selected using anti-PE microbeads through MACS column (Miltenyi). The term eT_{regs} is used to indicate the source of T_{reg} cells that is used clinically realizing that this is composed of both natural and inducible T_{regs} with approximately 70% of the eT_{regs} expressing Helios suggestive but not conclusive for natural T_{regs}.

Generation of inducible T_{regs}

CD4 T cells were purified from C57BL/6 lymph nodes and spleens using negative selection of $CD25^-$, $CD8^-$, $CD19^-$, $\gamma\delta TCR^-$, and CD11b-expressing cells by MACS. $CD25^-$ $CD4^+$ T cells were then cultured in a 24-well plate in DMEM containing 10% FBS, 1% L-glutamine, 1% Pen-strep, 1% non essential amino acids, 10mM Hepes with 100 U/ml of IL-2 and 10 ng/ml of TGF-β for 4 days. Purity of iT_{regs} was greater than 85% (Suppl. Fig. 2d).

Intravital imaging

CD11c-DTR-EGFP mice (BALB/c background) were lethally irradiated (800 cGy). On the next day, they were transplanted with TCD BM cells, CMTPX (Invitrogen)-labeled naïve T cells and unlabeled naïve T cells from C57BL/6 mice. In different experiment settings, the mice were transplanted with different combination of labeled T cells, labeled T_{regs}, or labeled iT_{regs} with different unlabeled control cells. The cell combinations are indicated in the figure legends. For some experiments,

two hours after transfer, the recipient mice were injected with anti-CD62L Ab (MEL-14) to synchronize T cell movement in the LN[3]. At 2-8 and 18-24 hours after transplantation, recipient mice were anesthetized and the popliteal LN imaged using FV1000MPE microscope (Olympus). The body temperature was maintained at 37 °C by an electric warm plate. A plaster caste was put on a hind leg to minimize the respiratory movements and microscopic surgery was done to expose the popliteal LN [4]. T cell-DC interactions in this LN were observed and XYZ/time-lapse movies were recorded. The LN was excited at 860 nm. 5 z-planes separated by 3-5 μm were collected at 60 time points so that each movie was approximately 30-40 minutes. Images were analyzed using Imaris and Matlab for velocity, cell interactions, cell confinement, and cell arrest.

Flow cytometry of dendritic cells

Lymph nodes or spleen were minced in magnesium- and calcium-free Hank's balanced-salt solution containing 5% FCS and 10 mM HEPES, pH 7.4 (Gibco), then were digested for 35 min at 37 °C with collagenase A (1 mg/ml; Roche) and DNase I (0.2 mg/ml; Sigma-Aldrich). EDTA (final concentration, 20 mM) was added to the cells for 5 min at 25 °C. Single-cell suspensions were prepared with 70 μm Cell Strainer nylon mesh (Falcon) and red blood cells were lyzed by ACK lysis buffer. Cells were stained for 20 min at 4 °C with florescence-labeled antibodies in Dulbecco's PBS (Gibco) containing 3% FCS, 5 μg/ml of Fc block and 10 mM EDTA. Flow cytometric acquisition was performed on a Cyan flow cytometer using Summit software (Dako) or LSRII using Diva software (BD). αCD11c-APC.Cy7 (HL3),

αCD54-APC (YN1/1.7.4), αCD86-FITC (GL1), αCD40-PE (1C10), αCD80-FITC(16-10A1), αMHCII-ef450 (AF6-120.1), and αCD70-PE (FR70) were either from eBioscience or BD pharmingen.

Regulatory T cell suppression assay

BALB/c DCs used as stimulator cells for T_{con} cell proliferation were prepared as previously described [5]. LN or spleen single cell suspensions were layered over RPMI-1640 medium containing 10% (vol/vol) FCS and 17% (wt/vol) Nycodenz (Accurate Chemical and Scientific) and centrifuged at 450*g* for 20 min at 25 °C. Low-density cells at the interface were collected and washed. DCs were then purified from these low-density cells by negative selection of CD3-, CD19-, Ly6G-, TER119-, γδTCR-, CD49b-expressing cells using MACS columns (Miltenyi). C57BL/6 T_{cons} and T_{regs} were purified as described above. T_{cons} were labeled with CFSE according to manufacturer's instructions (Invitrogen). DCs and T_{cons} were cultured at a 1:5 ratio in 96-well round bottom plates with or without T_{regs} (1:1 ratio to T_{cons}) for 2-3 days. CFSE dilutions of T_{cons} were analyzed on MACSQuant (Miltenyi). In some experiments, T_{regs} were labeled with CMTPX (Invitrogen) according to manufacturer's instructions to evaluate the effect of CMTPX on T_{reg} suppressor function.

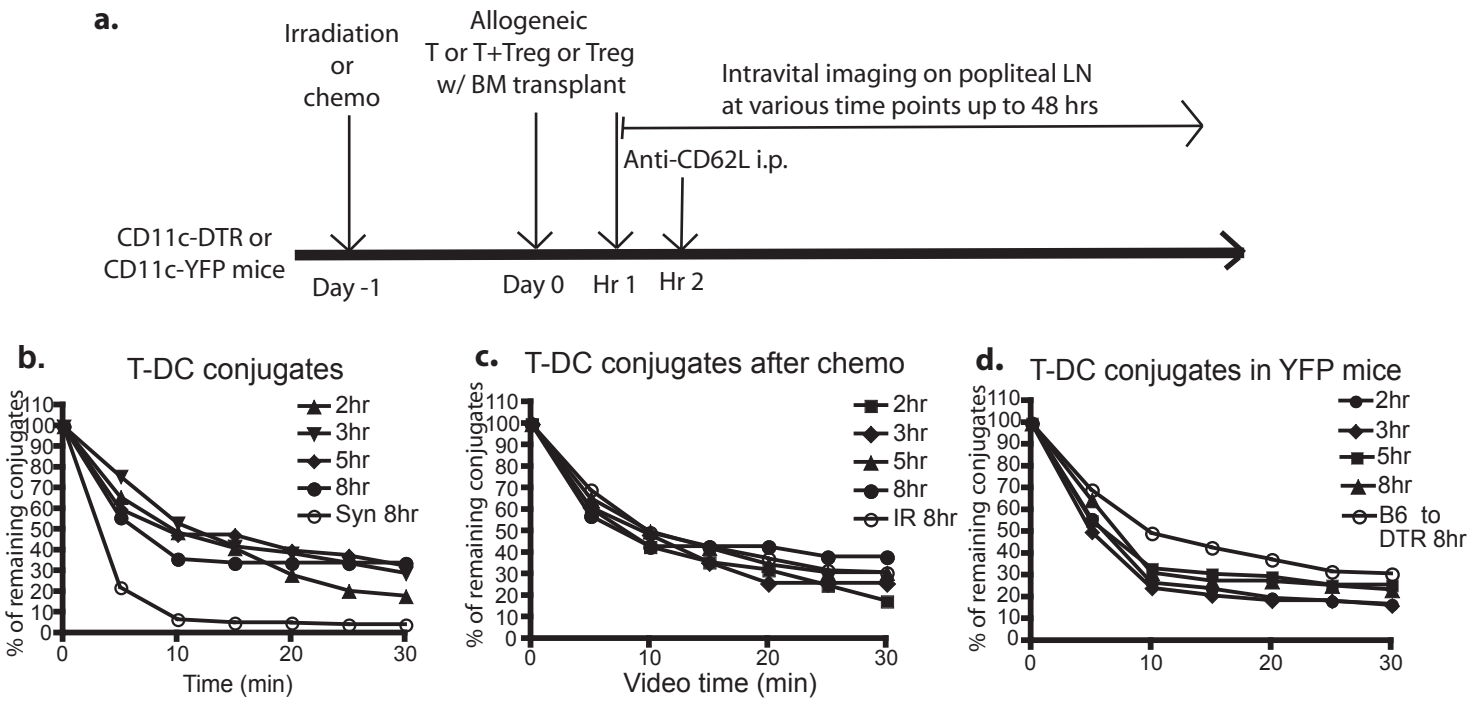
Statistical Analysis

All the comparisons between velocity, displacement ratio and contact time were tested by Mann-Whitney test using Prism software. The differences between mean

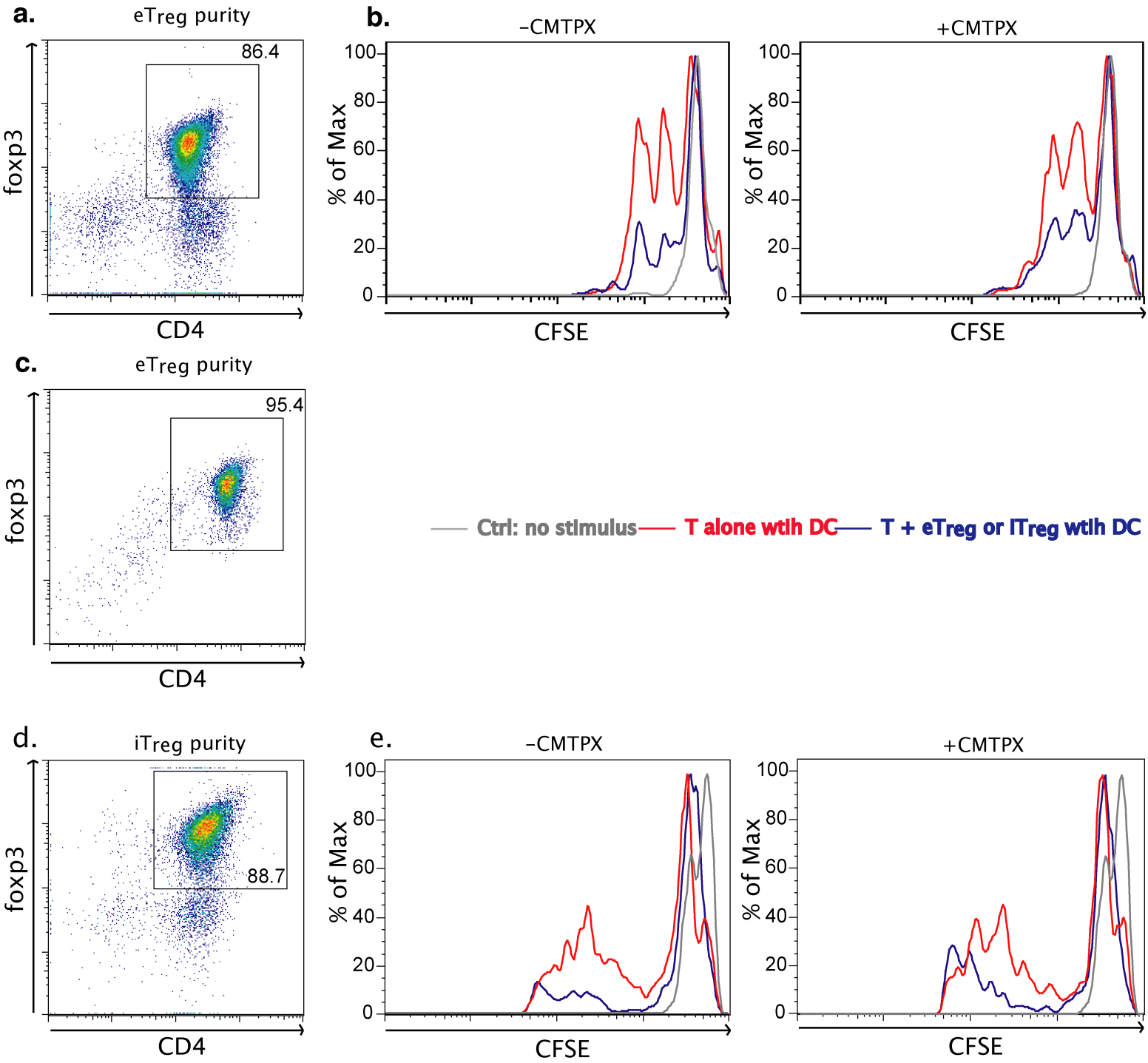
fluorescence intensity of DC molecules, numbers of DCs, and percentages of $7AAD^+$ DCs were compared by student's t test. $P < 0.05$ was considered significant.

References for Methods

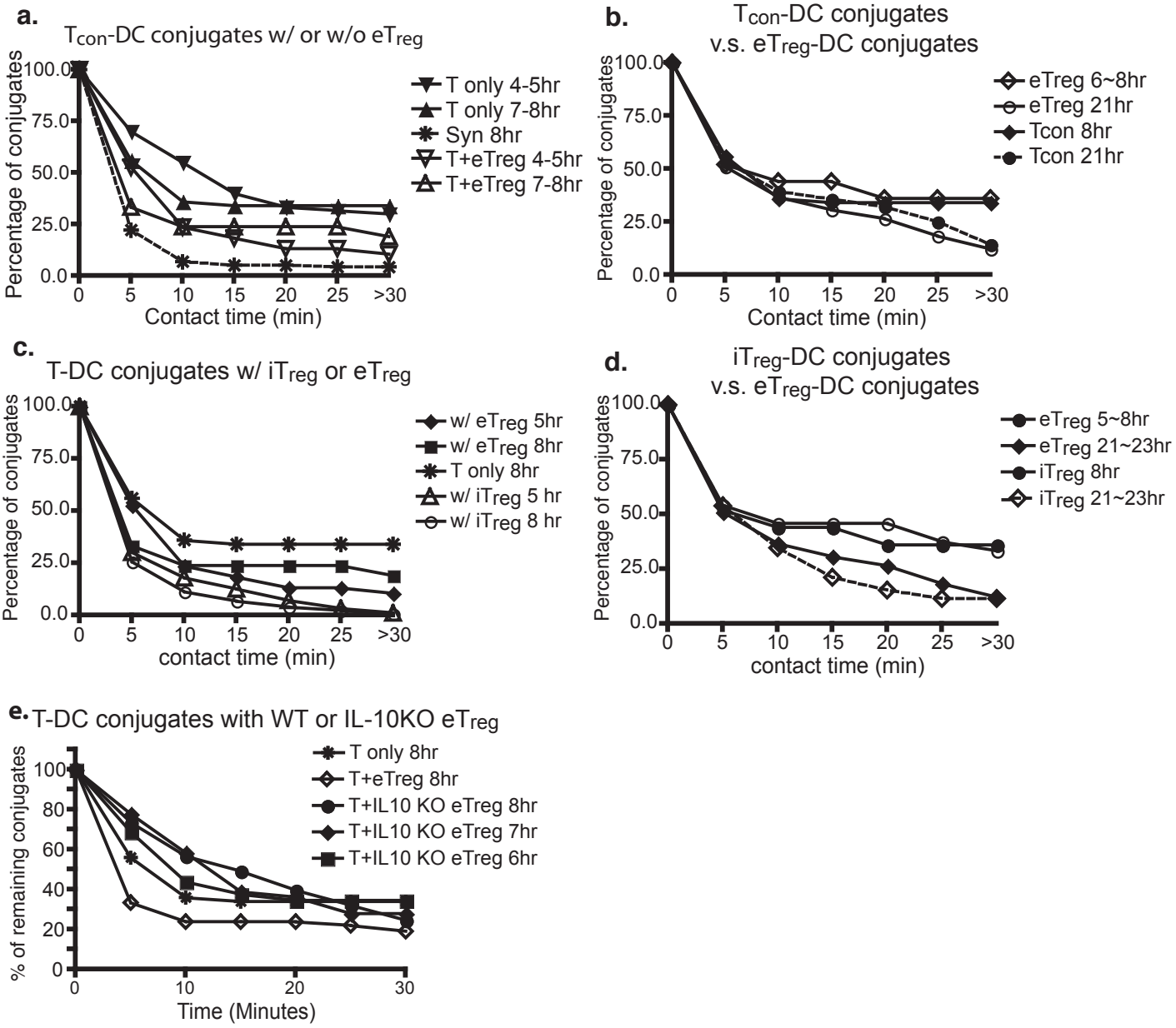
1. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, et al. Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. J Immunol 2004;173:845-54.
2. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, et al. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 2004;104:3804-12.
3. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature 2004;427:154-9.
4. Celli S, Bousso P. Intravital two-photon imaging of T-cell priming and tolerance in the lymph node. Methods Mol Biol 2007;380:355-63.
5. Nakano H, Lin KL, Yanagita M, et al. Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. Nat Immunol 2009;10:394-402.



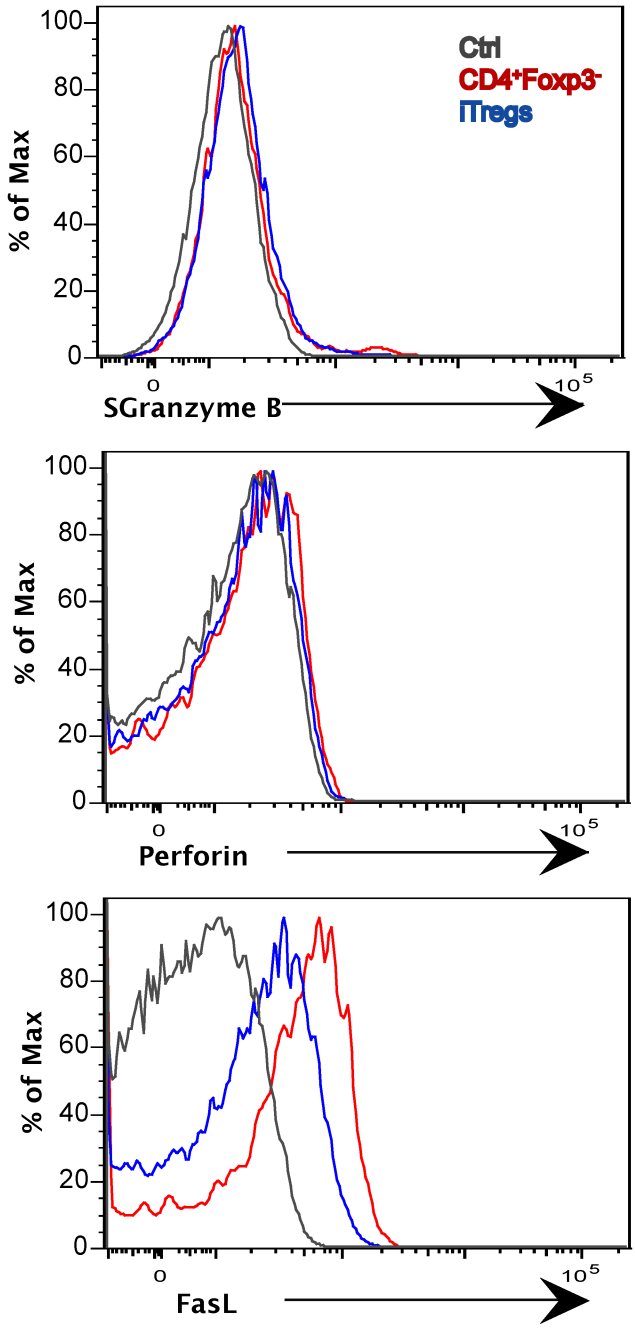
Supplementary figure 1. Donor T cells display stable contacts with DCs from 2 hours post-transplant. Intravital imaging of donor T cells (labeled with CMTPX) and host DCs in the popliteal LNs of irradiated CD11c-DTR-EGFP mice (BALB/c background) or CD11c-YFP mice (C57BL/6 background). (a) Schematic plan for our experimental process. (b) CD11c-DTR-EGFP mice were irradiated before receiving red B6 donor T cells. This panel shows the percentage of remaining conjugates of T_{con}-DC that last for 5, 10, 15, 20, 25, or 30 minutes. Each line represents a different time point post-transplant. (c) Percentage of remaining conjugates of T_{con}-DC that last for 5, 10, 15, 20, 25, and 30 minutes in CD11c-DTR-EGFP treated with cytoxan and transplated with red T cells one day later. (d) Percentage of remaining conjugates of T_{con}-DC that last for certain time in irradiated CD11c-YFP mice. This is a Balb/c to C57Bl/6 model. Note: The percentage of remaining T-DC conjugates does not represent the frequency of allogeneic reactive T cells due to several reasons: (1) T cells in the imaging field (100~150 μm below LN surface) may not be representative of all T cells infused. (2) There are a low level of non-specific long interaction between T-DC when DC are activated[3]. (3) CD11c promoter may also be activated in host T cells and macrophages.



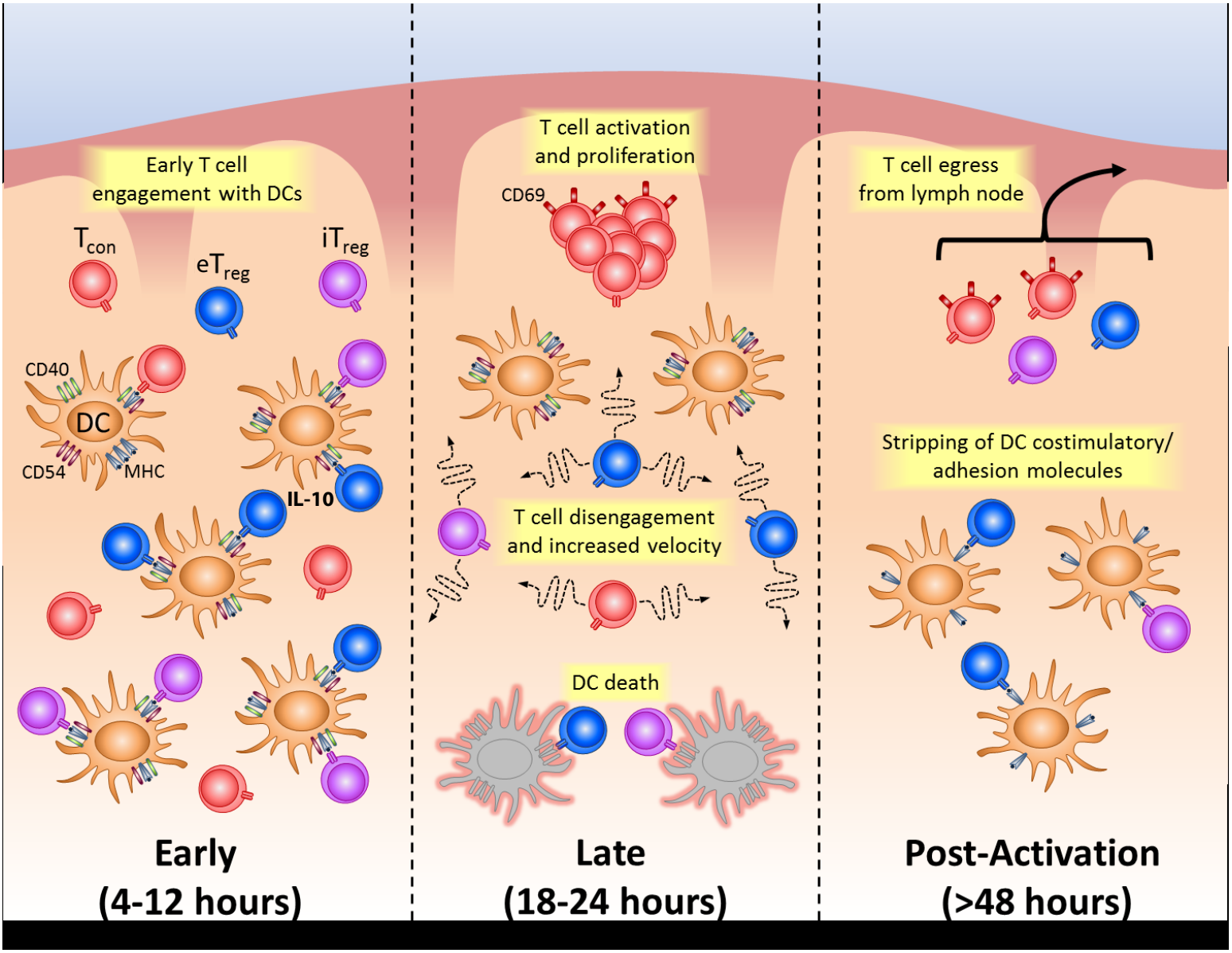
Supplementary figure 2. Suppression of T cell proliferation by endogenous Tregs and inducible Tregs. (a, c& d) eTregs or iTregs were purified or generated according to the methods section and stained for CD4 first. Then intracellular Foxp3 was stained by foxp3 staining kit according to the manufacturer's manual (eBioscience). Single cell suspensions were analyzed by MACSQuant (Miltenyi). (a&c) The purity of eTreg and (d) The purity of iTreg shown by CD4 and Foxp3 expression. (b&e) T cells were cultured with allogeneic DCs (250,000 T to 50,000 DC) for 2 or 3 days in the presence or absence of eTregs or iTregs (250,000)(labeled with CMTPX or unlabeled). Whole culture was stained for CD4 and CD8 before analyzed on MACQuant. CFSE dilutions of T_{cons} in the presence or absence of eTregs (b) or iTregs (e) are shown.



Supplementary Figure 3. eTreg and iTreg disrupt Tcon-DC interaction while their own interactions with DC are not affected by Tcon. Intravital imaging of donor T cells or eTreg, or iTreg (labeled with CMTPX) and host DCs in the popliteal LNs of irradiated CD11c-DTR-EGFP mice. (a) Percentage of remaining conjugates of Tcon-DC that last for 5, 10, 15, 20, 25, and 30 minutes with or without Tregs (unlabeled). Each line present different time points or conditions. (b) Percentage of remaining conjugates of eTreg-DC that last for certain time in comparison to conjugates of Tcon-DC. (eTreg imaging was acompanied by unlabeled Tcon in order to keep consistent cell number). (c) Percentage of remaining conjugates of Tcon-DC that last for 5, 10, 15, 20, 25, and 30 minutes in the presence of iTregs or eTregs (unlabeled). (d) Percentage of remaining conjugates of iTreg-DC that last for certain time in comparison to conjugates of eTreg-DC. (e) Percentage of remaining conjugates of Tcon-DC that last for 5, 10, 15, 20, 25, and 30 minutes in the presence of IL-10 -/- or WT eTregs (unlabeled).



Supplementary Figure 4. iTregs also express FasL. Flow analysis of iTregs that were generated fror $CD4^+CD25^-$ spleen cellls in culture for four days. iTregs are gated on $CD4^+foxp3^+$ while $CD4^+foxp3^-$ cells were used for comparison. The expressions of granzyme B, perforin, and fasL are shown. Ctrl: Ab isotype control on total $CD4^+$ cells.



Supplementary figure 4. DC:T cell interactions within the lymph node following allogeneic transplantation. During the early phase (4-12 hours) following T cell transfer (far left panel), donor conventional T cells (T_{con}), endogenous regulatory T cells (eT_{reg}), and induced T_{reg} (iT_{reg}) enter the lymph node from the circulation and engage recipient dendritic cells (DC) expressing major histocompatibility-peptide complexes (MHC), costimulatory molecules such as CD40, and adhesion molecules such as CD54. Both eT_{reg} and iT_{reg} exhibit more superior DC engagement in comparison to T_{con}. By 18-24 hours after transfer (middle panel), donor T cells start disengaging from DCs and increase their migratory velocity. Stimulated T_{con} undergo proliferation and upregulation of activation markers, while eT_{reg} and iT_{reg} induce DC death. After 48 hours (far right panel), eT_{reg} and iT_{reg} induce loss of costimulatory and adhesion molecules from the DC plasma membrane.