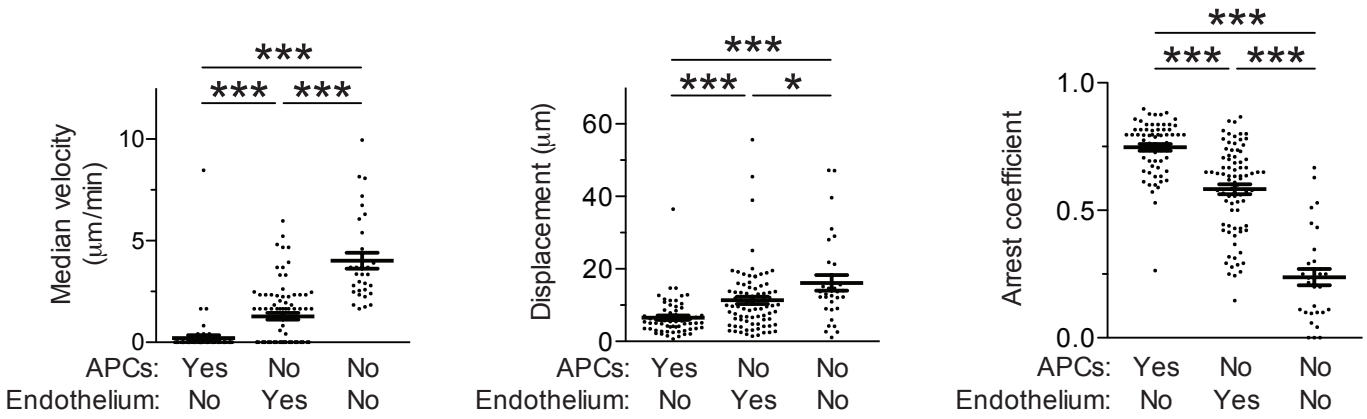

Supplemental Figure 1. Motility parameters of OT-I effector T cells arrested in the capillary lumen of B6-OVA kidney grafts that lack H-2Kb expression on the endothelium (n = 65), APC (n = 81), or both (n = 31). Same experimental setup as Figure 4A.

ONLINE METHODS

Mice

C57BL/6J (B6) (Thy1.2, CD45.2, H-2^b), B6.PL-Thy1a/CyJ (Thy1.1, CD45.2, H-2^b), BALB/cJ (BALB/c) (H-2^d), C57BL/6J-Tg(CAG-OVA)916Jen/J (CD45.2, H-2^b) (B6-OVA), C57BL/6-Tg(TcraTcrb)1100Mjb/J (CD45.2, H-2^b) (OT-I), and B6 CD11c-YFP mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Cancer Institute (NCI). B6.Cg-Tcra^{tm1Mom} Tg(TcrLCMV)327Sdz (P14) mice (CD45.2, H-2^b) and the B6 H2Kb$^{-/-}$ were purchased from Taconic. OVA+CD11cYFP+ mice and OVA+H2Kb$^{-/-}$ mice mice were generated by cross breeding B6-OVA mice with either B6-CD11cYFP or B6-H2Kb$^{-/-}$ mice respectively. H2kB6-Ly5.2/Cr (Thy1.2, CD45.1, H-2^b) were purchased from National OT-I and P14 mice bred onto the Rag-/-Thy1.1 and Rag-/-Thy1.2 backgrounds. Alymphoplasia mice (Map3k14$^{aly/aly}$, Thy1.2, H-2^b) (*aly/aly*) were purchased from CLEA (Osaka, Japan) and bred onto a B6 CD45.1 congenic background. All animals were maintained under SPF conditions.

Surgical procedures and bone marrow chimeras

Splenectomies and heterotopic transplantation of vascularized heart and kidney grafts were performed as previously described[1-3]. Heart graft rejection was defined as cessation of palpable heartbeat and was confirmed by histological analysis. Bone marrow chimeras were generated by irradiating recipient mice with 1000cGy from a Nordion Gamma Cell 40 cesium source. The same day mice were injected retro-orbitally with 1 x 10^7 donor bone marrow cells. After an 8-week reconstitution period, blood was phenotyped to verify appropriate reconstitution. Chimerism was consistently greater than 90 – 95% in the blood and was confirmed in selected

kidneys. All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) regulations of the University of Pittsburgh.

Generation, isolation and adoptive transfer of effector and memory T cells

Polyclonal Cells. Polyclonal CD4 and CD8 effector and memory T cells were generated as described[4]. Briefly, B6 (CD45.2) mice on the Thy1.1 and Thy1.2 backgrounds were immunized i.p. with 2 x 10^7 BALB/c splenocytes on days 0 and 21. One or 6-8 wks after the first immunization, spleen and lymph node cells were harvested to obtain effector and memory T cells, respectively, and enriched for T cells by negative selection, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen), treated or not with 200 ng/ml pertussis toxin (PTx) (Sigma) for 30 minutes at 37° C (5% CO_2), and $CD44^+CFSE^+$ $CD4^+$and $CD8^+$ effector and memory T cells were sorted on a BD Aria Plus high-speed sorter (purity ~95%). To study migration, equal numbers of PTx treated (Thy1.1, CD45.2) and untreated (Thy1.2, CD45.2) CD4 and CD8 effector or memory T cells (2-3 x 10^6 each) were co-transferred i.v. into B6 (Thy1.2, CD45.1) or splenectomized *aly/aly* (*aly/aly*-spleen, Thy1.2, CD45.1) recipients of BALB/c heart allografts. To study allograft rejection, the same number of PTx treated or untreated memory T cells were transferred separately into *aly/aly*-spleen recipients.

Monoclonal (TCR-tg) cells. Dendritic cells (DCs) were generated by culturing bone marrow cells with IL-4 and GM-CSF (Peprotech) for 8 days. DCs were stimulated with 100 ng/ml LPS overnight and pulsed with either 10 μg/ml OVA (SIINFEKL) or 5 μg/ml LCMV (KAVYNFATM) peptides (Genscript) for 2 hours at 37° C. 2-3x10^6 OVA or LCMV peptide-

pulsed DCs were injected i.v. with $5x10^5$ OT-I (Thy1.1, CD45.2) or P14 (Thy1.2, CD45.2) T cells, respectively, into B6 (Thy1.2, CD45.1) mice based on published method[5]. Five days later, spleen and lymph node cells were labeled with CFSE, treated with PTx or not, and OT-1 and P14 effectors recovered by high-speed sorting $CD45.2^+CD8^+CFSE^+$ cells. Flow analysis confirmed that > 95% of these cells were $CD44^+$ and were OVA or LCMV MHCI-tetramer positive (Beckman-Coulter). OT-I and/or P14 effectors, with or without PTx treatment, were then co-transferred i.v. (1-2 x 10^6 each) into B6 (Thy1.2, CD45.1) recipients of either B6-OVA or B6 heart grafts. To study the role of VLA-4 in migration, OT-I effector T cells were incubated prior to transfer with 100 μg/ml monoclonal rat anti-mouse VLA-4 antibody (PS/2, BioXCell) on ice for 20 minutes. In addition, recipients received 250 μg PS/2 i.p. on the day of cell transfer, and 1 and 2 days later. For the intravital imaging studies, cells were labeled prior to transfer with 2.0mM CFSE, cell tracker orange (CTO), or cell tracker violet (CTV) (Invitrogen) and high-speed sorted by gating on $CD45.2^+CFSE/CTO/CTV^+$ cells and using the following dump channel: $CD4^+CD45R/B220^+CD11c^+CD11b^+CD49b^+Ly\text{-}76^+CD16/32^+F4/80^+$. Flow analysis confirmed that sorted populations were > 95% $CD8^+CD44^+$ and were tetramer positive. OT-I and/or P14 effectors, with or without PTx treatment, were then co-transferred i.v. (7-10 x 10^6 each) into B6 (Thy1.2, CD45.1) recipients of either a B6-OVA or B6 kidney grafts.

Analysis of cell migration by flow cytometry

Heart grafts, kidney grafts, spleen, and lymph nodes were harvested at the indicated time points after T cell transfer. Lymphocytes were isolated from heart and kidney grafts as previously described[4]. Total number of recovered lymphocytes was determined and the transferred polyclonal and monoclonal T cells enumerated by flow cytometry by gating on the

$CD45.2^+Thy1.1^+$ and $CD45.2^+Thy1.2^+$ populations after live/dead cell discrimination and exclusion of non-T cells ($CD45R/B220^+CD11c^+CD11b^+CD49b^+Ly\text{-}76^+CD16/32^+$ $F4/80^+$ cells). All fluorochrome- or biotin-tagged antibodies were purchased from BD Pharmingen, eBioscience, Biolegend, or R&D Systems: BDCD90.1 (OX-7), CD90.2 (30-H12), CD45.2 (104), CD45.1 (A20), CD8a (53-6.7), CD4 (RM4-5), CD44 (IM7), CD62L (MEL-14), CD45R/B220 (RA3-6B2), CD11c (HL3), CD11b (M1/70), CD49b (DX5), Ly-76 (TER-119), CD16/32 (2.4G2), and F4/80 (BM8). Fixable live/dead Aqua cell stain (405nm excitation) was purchased from Invitrogen. Flow acquisition was performed on LSRII and LSRFortessa analyzers (BD Biosciences), and data analyzed using Flowjo software (Treestar Corp.).

Multi-photon intravital microscopy and image analysis

Multi-photon intravital microscopy was performed on transplanted kidneys using an established method[3]. An Olympus FluoView FV1000 microscope with a Mai Tai DeepSee femtosecond-pulsed laser (Spectra-Physics) tuned and mode-locked to 825nm was used for all experiments. The recipient mouse was anesthetized with isoflurane and oxygen and placed on a heat pad to maintain core body temperature at 37°C. An i.v. line was inserted in the external jugular vein to provide 5% dextrose lactated ringer's solution for hydration and 500kDa dextran conjugated with either FITC or Rhodamine for visualization of blood vessels in the transplanted kidney. The kidney graft was extraverted from the abdominal cavity with intact vascular connection and immobilized in a custom cup mount[3]. A coverslip was placed on top of kidney and z-stacks were visualized with a 25x water immersion objective (SP1 NA:1.05) 25mm to 55mm below the kidney capsule. 12 slices were acquired at a step size of 2.7mm. Brightness and laser power

were adjusted based on the imaging depth and kept below phototoxic levels. Dwell time was set to 8μs/pixel and resolution was a maximum of 512x512. The scanning area was cropped to adjust for a 30 second-long stack that was then repeatedly scanned up to 60 times for a maximum imaging time of 30 minutes per location. These settings were repeated up to five locations per transplanted kidney. T cells were enumerated at 7 independent time points per z-stack. All acquired movies were analyzed using Volocity software (Perkin-Elmer). Drift was corrected using the blood vessels as a reference point. Cells present in the field of view for at least five time points (> 2 min) were tracked in the *x*, *y*, and *z* direction for the duration of each video. Cells were determined to have firmly adhered if they were arrested for >30 sec in the capillary lumen and transmigrated if the majority or all of the cell body had moved outside the capillary lumen at any time during the course of their tracking. The motility parameters were plotted using Volocity.

In vitro migration assay

5μm pore size chemotaxis assay chamber (Millipore) was utilized with or without 0.5μg/ml IP-10 in medium in the bottom chamber. $5x10^4$ OT-I effector T cells, isolated as described above, with or without pre-treatment with pertussis toxin were placed in the top chamber of each well for a total of 7-12 wells per group. The chamber was incubated at 37° C for 3 hrs after which the cells in the bottom chamber were collected and counted using a hemocytometer and trypan blue exclusion. The assay was repeated using OT-I effector T cells re-stimulated SIINFEKL-pulsed DCs at 37°C for two hours prior to use in the chemotaxis assay. This restimulation protocol was sufficient to induce IFNγ production in 60% of the cells.

Statistical Methods

Statistical analysis of allograft survival was calculated using the log-rank test. All other experiments were analyzed using unpaired *t* test (2-tailed) for samples with normal distribution and the Mann-Whitney test (2-tailed) for samples with a non-Gaussian distribution. All statistical calculations were amde in *GraphPad Prism 5.0c*. Significance was set at $P<0.05$.

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