Supporting Information

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SI Materials and Methods

Animals. Twelve-week-old female BALB/c mice and female C57BL/6 mice were purchased from The Jackson Laboratory. The mice were housed under pathogen-free conditions. All mouse experiments were approved by the University Committee on Animal Resources at the University of Rochester.

DNA Constructs and Cloning. Cytoplasmic domains of the *bovine rhodopsin* (NM_001014890) (gift from Daniel Oprian, Brandeis University) were replaced by those of *human CXCR4* (NM_003467), and *mCherry* sequence was incorporated into the 3′-terminal region through GGG-linker. Two-step PCR has been used to produce the chimeric DNA construct. First, the final six DNA fragments were amplified, either cloning site (*NheI*/*BamHI*) or overhangging sequences between 27–42 nt which include 24-nt overlapping sequences were added at 5′- and 3′-termini. Four DNA fragments were amplified from the *bovine rhodopsin* as a template with following primers: primer set #1 (For 5′-TTTGCTAGCGCCACCATGAACGGTACCGAAGGCCC AAAC and Rev 5′-GCTTCTCAGTTTCTTCTGGTAACCCATGACGTACAGCGTGAG GAAGTTGAT) to produce 225-nt fragment, primer set #2 (For 5′-GGTTACCAGAAG AAACTGAGAAGCATGCTCAACTACATCCTGCTCAACCTG and Rev 5′-CTTCCTT GGCCTCTGACTGTTGGTGGCGTGGACCACCACCACGTACCGCTCGATCGC) to produce 264-nt fragment, primer set #3 (For 5′-ACCAACAGTCAGAGGCCAAGGAAG CTGTTGGCCATCATGGGCGTCGCCTTCACC and Rev 5′-CTGGTGGCCCTTGGA GTGTGACAGCTTGGAGATGATAATGCCATAGCAGAAGAAGATGACAAT) to produce 285-nt fragment, and primer set #4 (For 5′-CTGTCACACTCCAAGGG CCACCAGAAGCGCAAGGCCCTCCGTATGGTTATCATCATGGTCATC and Rev 5′-CTGGGCAGAGGTCCGGAACTGCTTGTTCATCATGAT) to produce 240-nt fragment. C-terminal cytoplasmic domain of the *human CXCR4* was amplified with primer set #5 (For 5′-AAGCAGTTCCGGACCTCTGCCCAGCACGCACTCACC and Rev 5′-AAAGGATCCTTAGGCAGGCGCCACTTGGCTGGTCTCTGTGCTGGAGTG AAAACTTGAAGACTC) to produce 177-nt fragment. The DNA product of primer set #5 was gel-purified and reamplified with primer set #6 (For 5′-AAGCAGTTCCGGACCTCTGCCCAGCACGCACTCACC and Rev 5′-CTTGCTCA CGCCGCCCCCGGCAGGCGCCACTTGGCTGGTCTC) to produce 183-nt fragment including GGG-linker sequence at 3′-terminus. The mCherry gene was amplified with primer set #7 (For 5′-CCTGCCGGGGGCGGCGTGAGCAAGGGCGAGGAGGATAAC and Rev 5′-AAAGGATCCTTACTTGTACAGCTCGTCCATGCC) to produce 732-nt fragment with GCC-linker sequence at 5′. PCR products of primer sets #1–4, #6, and #7 were gel-purified and sequenced. Each product was pooled and used as templates (2 ng/μL) for an additional assembly-PCR with primers (For 5′-TTTGCTAGCGCCACCATGAACGGTACCGAAGGCCCAAAC and Rev 5′-AAA GGATCCTTACTTGTACAGCTCGTCCATGCC). The final PCR product of 1,809-nt assembled DNA fragment was gel-purified and cloned into pcDNA3.1/Hygro(+) with *NheI*/*BamHI*. The blue opsin (bOpsin) mCherry construct was kindly provided by N. Gautam (Washington University School of Medicine).

PA-CXCR4 Expression in T Cells. OT-I T cells were prepared from lymph nodes using a negative selection method. To express WT CXCR4-HA or PA-CXCR4, the respective constructs were transfected into 5×10^6 cells using an Amaxa Mouse T-cell Nucleofector Kit (Lonza) following the manufacturer's protocol. The transfected mouse T cells were incubated overnight. To label WT CXCR4-HA, the transfected cells were fixed, permeabilized, and labeled with a rabbit anti-HA Ab (Sigma) or a donkey Alexa488 anti-HA Ab (Invitrogen). The transfected cells expressing WT CXCR4-HA or PA-CXCR4 were then imaged.

The *PA-CXCR4-mCherry* sequence was also cloned into the pMIGR-GFP vector. PA-CXCR4-mCherry and GFP control retroviruses were generated using the Phoenix-ecotropic packaging cell line (from Indiana University National Gene Vector Biorepository). Infected mouse T cells were cultured for 5 d in 10 U/mL rhIL-2 (National Cancer Institute)-containing RPMI with 10% (vol/vol) FBS, 1% glutamine, 1% penicillin-streptomycin, 1% MEM Nonessential Amino Acids (Gibco), 2% (vol/vol) Hepes, and 0.1% 50 mM 2-mercaptoethanol (Sigma-Aldrich). The cells were sorted based on their GFP signal and cocultured with irradiated splenocytes for an additional 5 d.

Signal Transduction Assays. HEK293T cells (ATCC) were transfected with the pcDNA3.1-PA-CXCR4-mCherry plasmid using Lipofectamine 2000 (Invitrogen) on a Delta T culture dish (Bioptech). The CXCR4-HEK293T stable cell line was a gift from Martine Biard-Piechaczyk (Centre National de la Recherche Scientifique). To measure intracellular calcium release, the cells were stained with 2 μg/mL Fluo-4 AM (Molecular Probes) at 37 °C for 30 min and then incubated at room temperature for an additional 10 min. The cells were resuspended in Leibovitz's L15 medium (Gibco) containing 2 mg/mL glucose and protected from light. To excite Fluo-4 AM and activate PA-CXCR4, the cells were imaged under a GFP HC HiSN Zero Shift Filter set [with excitation wavelength (450–490 nm), dichroic mirror (495 nm), and emission filter (500–550 nm)] for 15 min. Later, 2 μM ionomycin (Invitrogen) was added to induce maximum intracellular calcium release.

To measure the cAMP level, HEK293T cells stably expressing PA-CXCR4 or WT CXCR4 were first incubated with 10 μM forskolin (Invitrogen) at 37 °C for 30 min and then stimulated with light or incubated with 1 μM CXCL12 for 5 min. The cells were washed with PBS and immediately lysed in cell lysis buffer. cAMP level in the lysates was determined using a cAMP Elisa Kit (Parameter cAMP Assay, R&D). For mouse T cells, OT-I T cells were transfected with PA-CXCR-mCherry or mCherry as described above.

Western Blot. Retroviral tranduced OT-1 T cells or HEK293T cells stably expressing PA-CXCR4, WT CXCR4, or bOpsin were serum starved (0.5% serum) for 24 h and then incubated with 30 nM CXCL12 or exposed to light (505 nm) for the indicated time. Total cell lysates were prepared, and Western blot analysis was performed using rabbit polyclonal antibodies specific for phospho-PAK1 (Thr423)/phospho-PAK2 (Thr402), PAK1/PAK2, phosphor-Myosin Light Chain 2 (Ser-19), Myosin Light Chain 2, phospho-Akt (Ser473), and pan-Akt (Cell Signaling).

Rap1 FRET Assay. Both Rap1-Raichu FRET sensor and PA-CXCR4-mCherry were transfected into 5×10^6 GD25 cells using Amaxa Nucleofector Solution V (Lonza) following the manufacturer's protocol. The transfected cells were grown on Delta T dishes overnight. Cells that were positive for CFP, YFP, and mCherry expression were used to measure FRET during light stimulation. Images of DIC, CFP, YFP, FRET, and mCherry in the cells were acquired every 10 s with 2 × 2 binning through a 60× oil

immersion objective lens at 37 °C through a dual-view image splitter (Photometrics) and a CFP/YFP dual-band filter set (Chroma). Following imaging of DIC, CFP, YFP, FRET, and mCherry for several minutes, the Mosaic system was applied to stimulate a part of the cell body. Immediately after light stimulation for 1–3 min, images of DIC, CFP, and YFP were captured to measure FRET in the same cell. As a negative control, the transfected cells were treated with 100 nM PTX for 4 h before imaging. Data analysis was performed with the Autoquant Imaging algorithm of AutoDeblur.

In Vivo Light Stimulation. PA-CXCR4–expressing CD4 T cells were prepared from the lymph nodes of DO11.10 T-cell receptor (TCR)-transgenic mice by negative selection. To label with CFSE, cells were incubated with 0.2 mM CFSE in prewarmed PBS buffer at 1×10^6 cells per mL for 5 min at 37 °C and washed two times. Cells were then resuspended at 1×10^6 cells per mL in PBS buffer. An insulin syringe (29G1/2, 0.3 mL) was filled with CFSE-labeled PA-CXCR4-mCherry–expressing T cells and connected to a syringe pump. For the femoral vein imaging, TNFα (0.5 μg in 10 μL) was intramuscularly injected into the left thigh of BALB/c mouse 24 h before imaging. The mice were initially anesthetized using an i.p. injection of pentobarbital sodium at a dose of 65 mg/kg, and the hair on the skin of the thigh was removed. The mice were subsequently placed on a custom-designed platform. The skin of the thigh of the TNFα-injected mice was cut, and the femoral vein was exposed. The syringe needle was then carefully inserted into the left femoral vein of the mice. The exposed, needle-inserted femoral vein was covered with PBS buffer. After placing the mice on the microscope stage for imaging, anesthesia was maintained with isoflurane for restraint and to avoid psychological stress and pain in the animal during imaging. The core body temperature of the mice was maintained using a warming pad set to 37 °C. The syringe pump ran at a very slow rate (20 μL/h) to permit i.v. injection of CFSE-labeled PA-CXCR4–expressing T cells into the femoral vein with a minimal effect on blood flow in the femoral vein. To visualize firm adhesion of PA-CXCR4–expressing T cells in the light-stimulated blood vessels, we used an FV1000-AOM two-photon system (Olympus) (for imaging) equipped with single-photon lasers (for light stimulation). For two-photon imaging, a MaiTai HP Ti:Sa Deep See laser system (Spectra-Physics) was tuned to 900 nm for GFP and second harmonic generation (SHG). Images were acquired at a resolution of 256 × 256 pixels with a pixel dwell time of 2 μs and a step size of 2 μm to a depth of 80 μm every 30 s. CFSE and SHG were visualized using band-pass filters with 519/25 nm and 390/40 nm bandwidths, respectively. For light stimulation, the argon laser was tuned to 488 nm and powered at 0.48 mW/mm^2 for 10 min. Light stimulation was continuously performed at a ROI of a 300-μm circle using a step size of 2 μm to a depth of 80 μm without intervals during the time period. Volocity software (PerkinElmer) was used to track the movements of the leukocytes.

For long-term in vivo light exposure in freely moving animals, an optical fiber (core = 200 μm; Doric Lens) was inserted into a protector and glued to the mouse ear skin. The fiber was coupled to an LED system (Doric Lens) through a joint rotator (Doric Lens). The peak light output during 505-nm light stimulation was estimated to be 3.76 mW/mm^2 at the tip of the attached fiber. Experiments were conducted in long-term drug administration chambers (Coulbourn Instruments) with continuous food and water supply.

In Vivo Homing Assay. CD4$^+$ T cells were purified by negative selection from DO11.10 TCR transgenic mice and cocultured with irradiated splenocytes coated with 1 μg/mL $OVA_{323–339}$ peptide (Mimotopes) in and 10 unit/mL IL-2 containing media. T cells were infected with PA-CXCR4 retrovirus 48 h after isolation as described above. After 5 d of culturing, T cells were then sorted based on GFP intensity and cocultured with irradiated splenocytes for additional 5 d. Before transfer, cell culture were layered over Ficoll and centrifuged to remove splenocytes and dead cells. Five million PA-CXCR4–expressing CD4 T cells were CFSE labeled and adoptive transferred to recipient BALb/c mice via retroorbital injection. Mice ear were immunized by 10 μg $OVA_{323–339}$ peptide emulsified with 10 μL complete Freund's adjuvant (Sigma-Aldrich) and subjected to light activation for 24 h, 48 h, or 72 h in long-term drug administration chambers as described above. Mouse ear pinnas with the light exposure site was cut into 0.5-mm^2 pieces and digested with collagenase (Roche) at 37 °C for 30 min. Spleen and cervical lymph nodes were harvested and filtered through a 40-μm cell strainer. The spleno-erythrocytes were lysed by ACK lysis buffer (Gibco), and samples were incubated with a combination of APC-conjugated anti-mouse CD3ε (Clone 145–2C11; eBioscience) and PE-conjugated anti-CD4 (Clone RM4-5; eBioscience). Flow cytometry was performed using FACSCalibur (BD Biosciences).

B16/OVA Tumor. B16-OVA cells were resuspended in 50 μL HBSS$^{-/-}$ and 1×10^5 cells were intradermally injected into one ear pinna of a recipient C57BL/6 mouse. Tumor growth was monitored every other day from day 5 after tumor injection. At day 7, 5×10^6 PA-CXCR4$^+$CD8 T cells were transferred via retroorbital injection into tumor-bearing recipients, and an optic fiber was attached to the melanoma site at the ear to provide light stimulation for another 7 d. The mice were housed in the long-term drug administration chambers described above and killed at day 14 or 21 posttumor injection.

Flow Cytometry. Mouse ear tumors with a light exposure site were cut into 0.5-mm^2 pieces and digested with collagenase/dispase (Roche) at 37°C for 30 min. The spleen and LNs were harvested, and spleno-erythrocytes were lysed in ACK lysis buffer (Gibco). The samples were incubated with a combination of PerCP-Cy5.5 anti-CD11b (Clone M1/70; eBioscience), FITC anti-Ly6G (Clone 1A8; Biolegend), BV650 anti-NK1.1 (Clone PK136; Biolegend), BV570 anti-Ly6C (Clone HK1.4; Biolegend), eFluor450 anti CD45.1 (Clone A20; eBioscience), PE-Cy7 anti-CD3e (Clone 145-2C11; Biolegend), APC anti-CD4 (Clone RM4-5; BD Bioscience), and PE anti-CD8a (Clone Ly2; BD Bioscience), and flow cytometry analysis or cell sorting was performed.

Immunostaining. At day 14 after tumor injection, mice were anesthetized using an i.p. injection of pentobarbital sodium and i.v. injected with 10 μg Alexa647 anti-CD31 (m390) Ab (Biolegend). The mice were euthanized 30 min after the Ab injection. The earflap containing the melanoma site was excised as a square and placed in filtered PBA buffer (1 g BSA and 100 mg sodium azide in 100 mL PBS buffer). The ear tissue was blocked by adding Fc-block (Pharmingen) in 1 mL PBA buffer at 4 °C for 10 min. After washing, the ear tissue was labeled with 5 μg Alexa488 anti-CD45.1 Ab (Biolegend) and 5 μg Cy3 anti-CD8 polyclonal Ab (Bioss) at 4 °C for 24 h and fixed. Ear tissue was pinned on a silicone piece and covered with PBS buffer. The ear tissue was then imaged using two-photon microscopy to visualize SHG, Alexa647 anti-CD31 at 800 nm, and Alexa488 anti-CD45.1 and Cy3 anti-CD at 900 nm at a resolution of 512 × 512 pixels, with a pixel dwell time of 2 μs and a step size of 1 μm to a depth of up to 200 μm. Volocity software (PerkinElmer) was used to analyze the locations of fluorescent cells and vessels.

RT-PCR. Digested tumor tissues were sorted based on CD45.1 and CD8 expression. mRNA was isolated from tumor-infiltrating T cells, and cDNA was synthesized using a Gene Expression Cells-to-Ct Kit (TaqMan). Real-time PCR analysis was performed as described in the manufacturer's instructions, with primers and probe sets from TaqMan Gene Expression Assays (TaqMan).

Statistics. All statistical tests were done with GraphPad Prism. Many analyses used the nonparametric Mann–Whitney *u* test. For multiple analyses of variance, the Kruskal–Wallis test was used with Dunn's posttest.

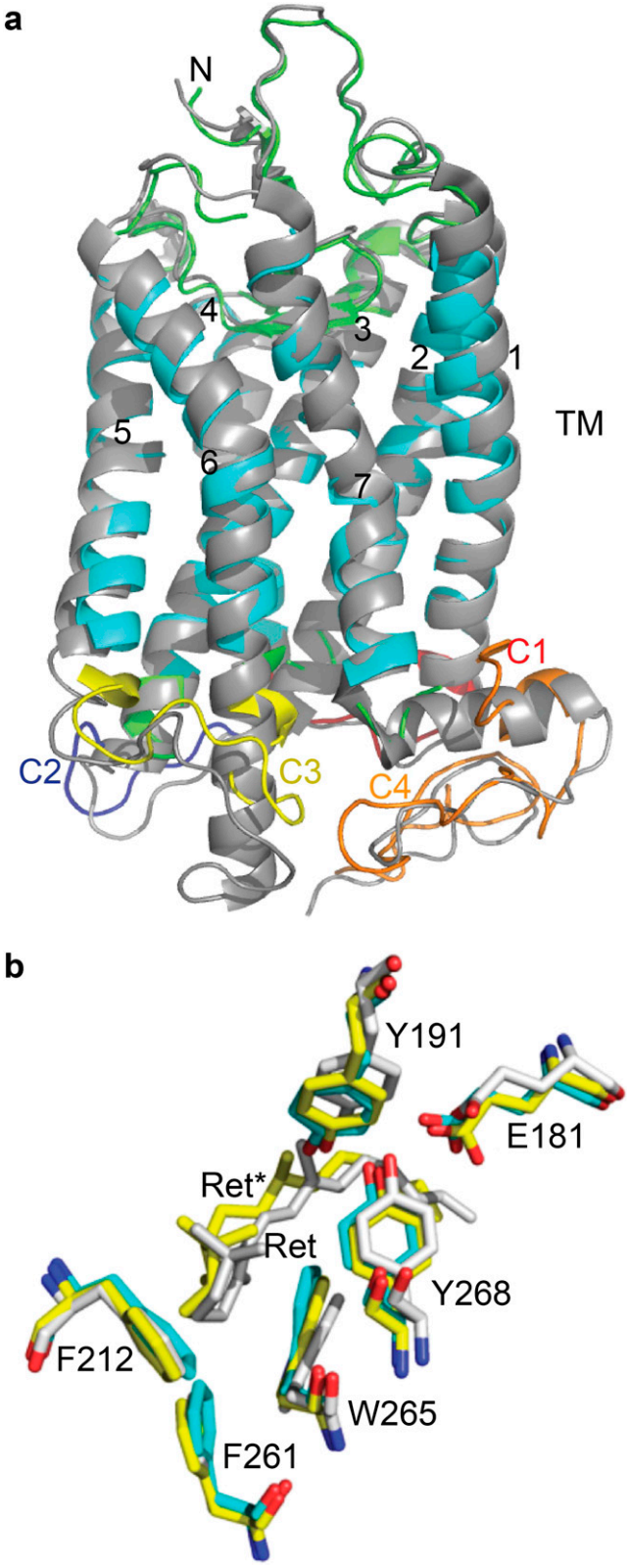


Fig. S1. Structural homology modeling of PA-CXCR4. (*A*) Superposition of bovine rhodopsin (gray, PDB ID: 1U19) and PA-CXCR4 (green, extracellular domain; cyan, transmembrane domain; red, blue, yellow, and orange, cytoplasmic loops). TM helices are numbered from 1 to 7. (*B*) Superposition of retinals (Ret, bound to rhodopsin; Ret*, bound to PA-CXCR4) and their binding site residues (gray, rhodopsin; yellow, retinal-free PA-CXCR4; cyan, retinal-bound PA-CXCR4).

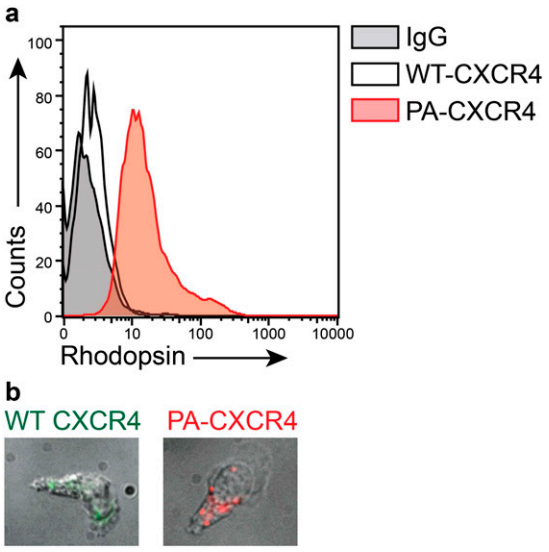


Fig. S2. Expression of PA-CXCR4. OT-I T cells were transfected with either WT CXCR4-HA or PA-CXCR4-mCherry. (*A*) For flow cytometry, cells were surface stained with rhodopsin antibody (clone 1D4) targeting N-terminal epitope followed by a PE-conjugated secondary antibody. (*B*) For microscope imaging, cells expressing WT CXCR4-HA were fixed and permeabilized and then stained with Alexa488 conjugated anti-HA Ab. Expression of PA-CXCR4 was visualized by mCherry signal.

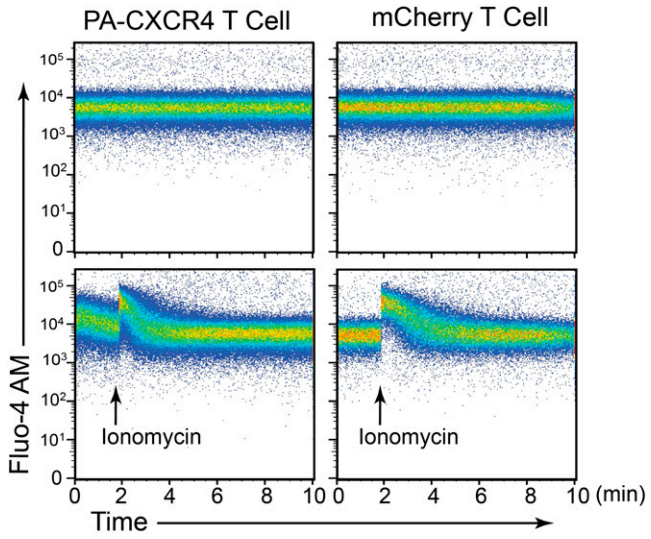


Fig. S3. Baseline calcium in PA-CXCR4–expressing T cells. Mouse OT-I CD8 T cells expressing PA-CXCR4 or mcherry control vector were loaded with Fluo-4 AM for 30 min at 37 °C. Baseline calcium was measured by flow cytometry.

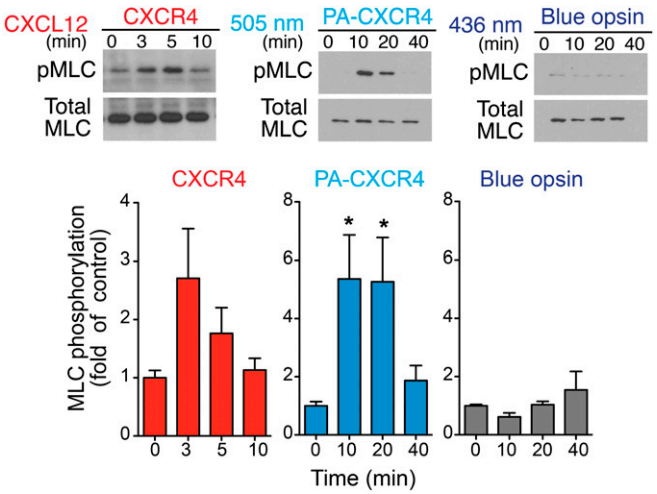


Fig. S4. Phosphorylation of myosin light chain in HEK293T cells. The 293T cells stably expressing WT CXCR4 were stimulated by 30 nM CXCL12 (CXCR4). PA-CXCR4-mCherry expressing cells were stimulated by 505-nm light (PA-CXCR4). Blue opsin-mCherry–expressing cells were stimulated by 436-nm light (Blue opsin). Densitometric analysis of phospho-MLC was normalized to total protein level and expressed as mean fold increase compared with control (time = 0 min). Data are expressed as mean ± SEM from three independent experiments. *$P < 0.05$.

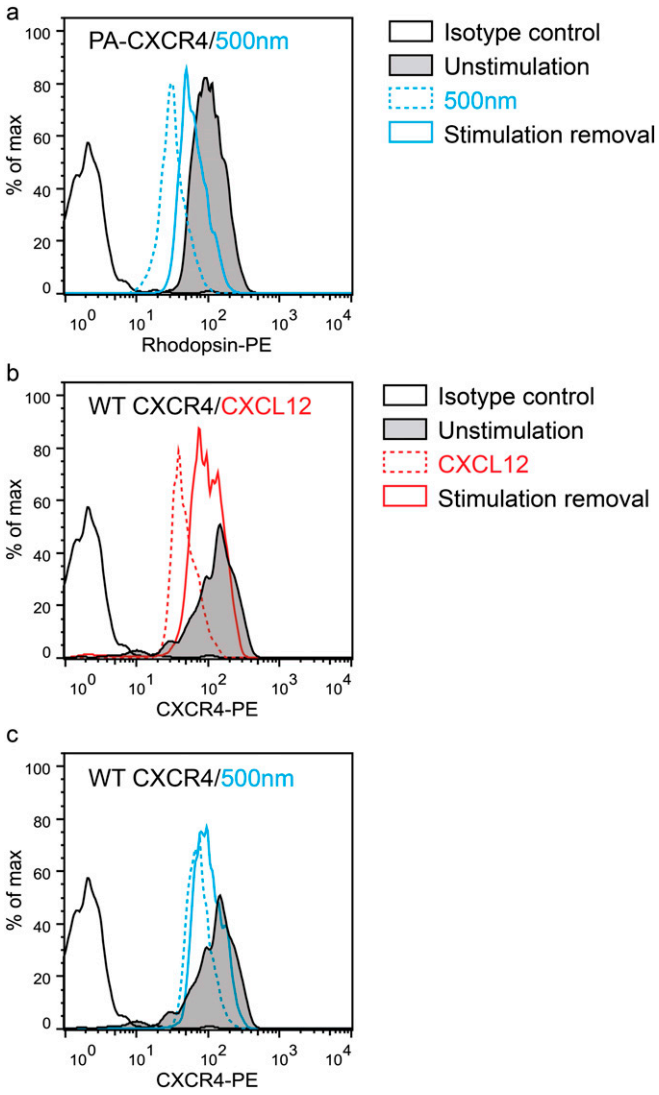


Fig. S5. Recycle of PA-CXCR4 in T cells. Mouse T cells expressing PA-CXCR4 (*A*) or CXCR4 (*B* and *C*) were illuminated with 505-nm light (blue) or incubated with 500 nM CXCL12 (red) for 30 min. To restore receptor surface expression, cells were washed and incubated in dark for 2 h. Cells were stained with anti-rhodopsin antibody (clone 1D4) followed by a PE-conjugated secondary antibody or PE-conjugated anti-CXCR4 antibody (clone 184).

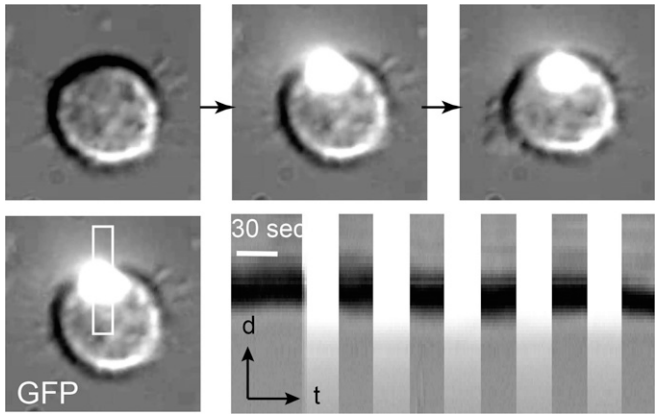


Fig. S6. Effect of light activation on T-cell polarization. GFP-expressing mouse T cells were plated on ICAM-1 coated dish. Every 30 s, 505-nm light was applied to cells. Kymograph pictures at the light activation site were taken in the same manner as in Fig. 2*A*. Experiment was repeated on 20 individual cells; representative pictures are shown.

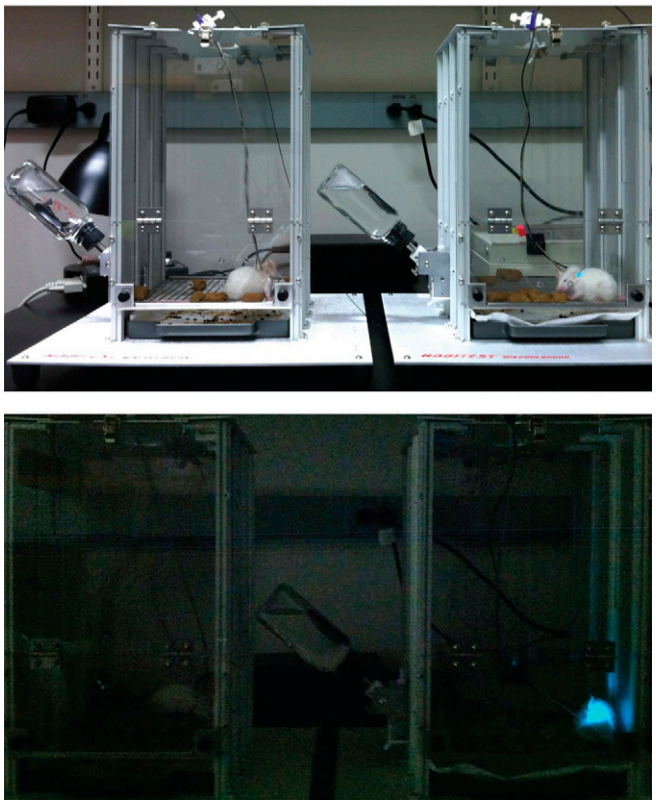


Fig. S7. In vivo light activation setup.

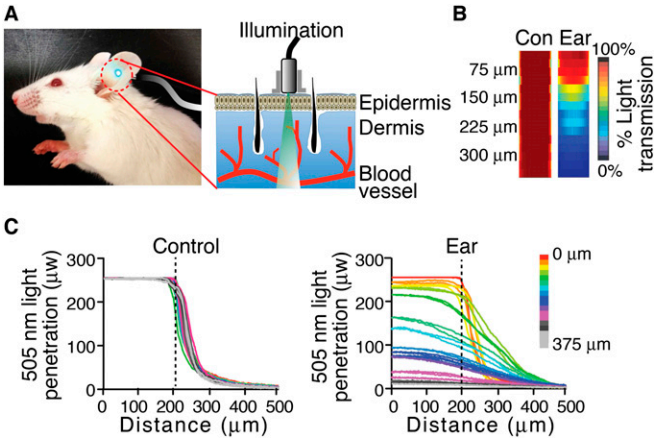


Fig. S8. Light propagation in the mouse ear. (*A*) A freely moving mouse with an optic fiber implanted on the ear skin (*Left*). Schematic of the in vivo optical stimulation setup (*Right*). (*B*) Measured relative transmission of vertical light power from an optic fiber (core = 200 μm, NA = 0.37), shown as a function of the % of light transmission from the fiber tip to the ear skin (rainbow colors). Fixed, whole mouse ear tissue was cut into fifteen 25-μm-thick horizontal sections. An optical fiber emitting 3.67 mW/mm^2 light at 505 nm was lowered into the first block of the ear tissue sections (outer skin), and light power was measured on the underside of the block, giving a transmission fraction for the tissue sample. This measurement was repeated for a range of whole-ear thicknesses by stepping the fiber through stocks of the block. Con; control sections with OCT fixative only. (*C*) Measured absolute transmission of vertical light power and horizontal spreading of diffracted light from an optic fiber (r = 200 μm, NA = 0.37) attached skin area (dotted line), shown as a function of the distance from the fiber tip to the ear skin (rainbow colors). Control, control sections with OCT fixative only.

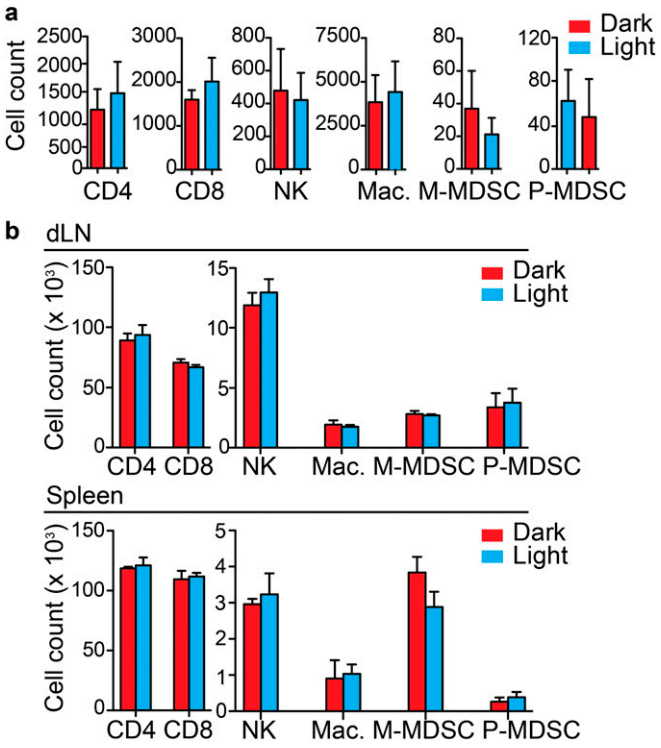


Fig. S9. Changes in leukocyte populations at the tumor. PA-CXCR4–expressing OT-1 CD8 T cells were adoptively transferred to the tumor bearing ears of B16/OVA recipient mice. Seven days after light activation, tumors and lymphoid organs were harvested from control (dark) and test mouse (light). FACS staining was performed to show immune cell populations per total 1×10^6 cell count in tumor (*A*) or in draining lymph node and spleen (*B*).

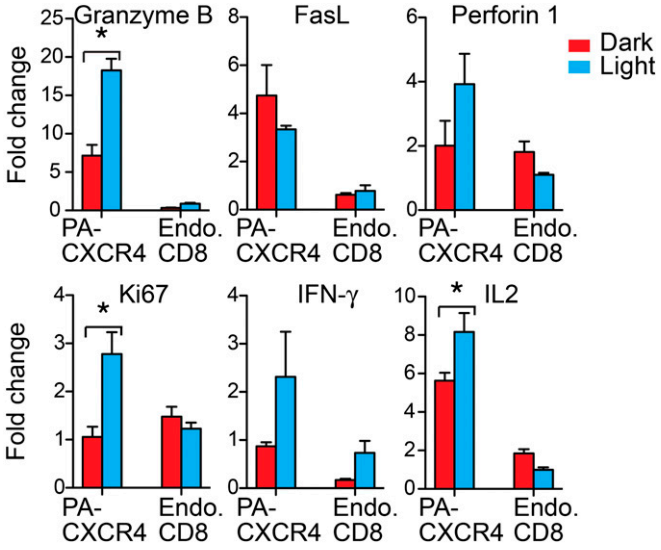
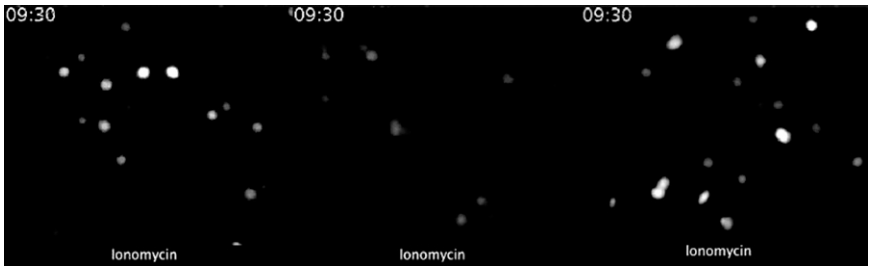
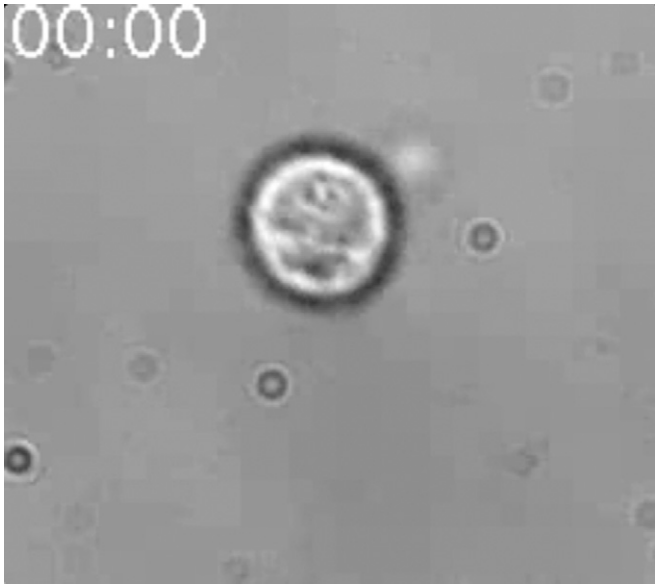


Fig. S10. Light stimulation and functions of cytotoxic T cells (CTLs). PA-CXCR4–expressing OT-1 CD8 T cells were adoptively transferred to the tumor-bearing ears of B16/OVA recipient mice. Seven days after light activation, tumors were harvested from control (dark) and test mouse (light). mRNA was purified from OT-1 T cells sorted from the tumor. mRNA from three mice was pooled together and assayed for real-time PCR. Data are expressed as fold increases relative to the mRNA levels in one selected sample within the endogenous light group. $*P < 0.05$.



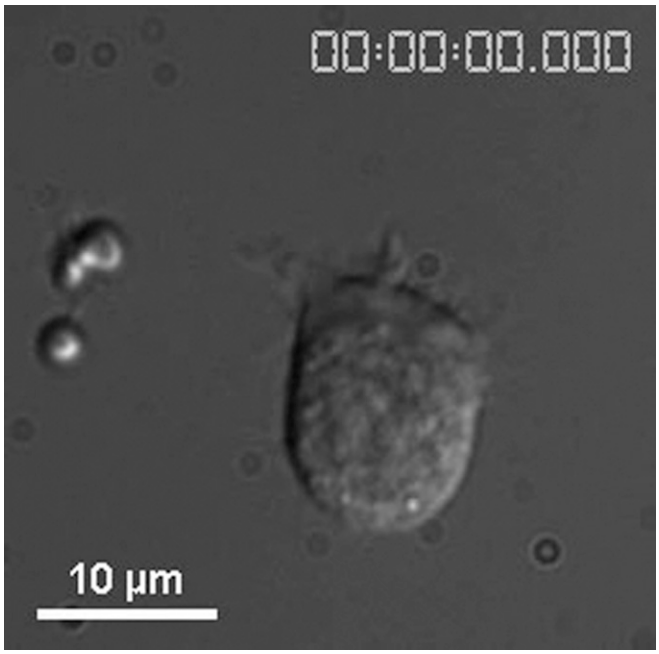
Movie S1. Ca^{2+} release by light stimulation. Mouse T cells were transiently transfected with mCherry or PA-CXCR4-mCherry and then stained by Fluo-4 AM. PA-CXCR4–expressing cells (white circles) were stimulated and imaged with 488-nm light.

Movie S1



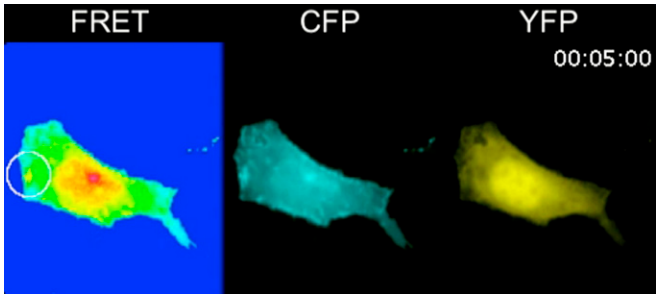
Movie S2. T-cell polarization. Mouse T cells were transduced with PA-CXCR4 and stimulated with 505-nm light.

Movie S2



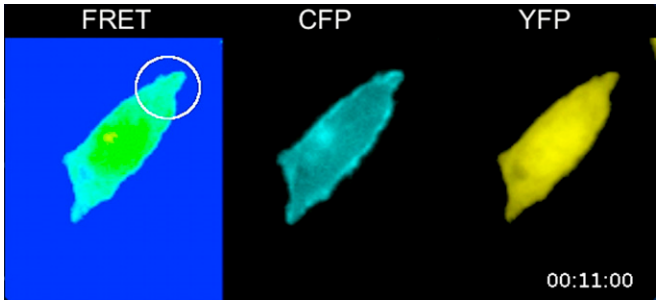
Movie S3. T-cell polarization. Mouse T cells were transduced with GFP and stimulated with 505-nm light.

Movie S3



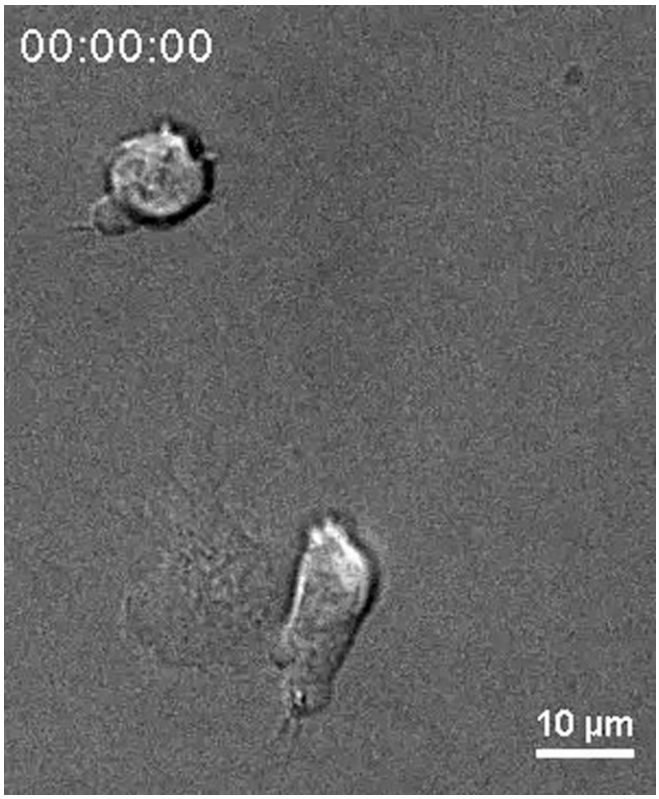
Movie S4. Rap1 activation. GD25 cells were transfected with both Rap1-Raichu FRET sensor and PA-CXCR4. Rap1 activation was measured by FRET signal after local stimulation with 505-nm light.

Movie S4



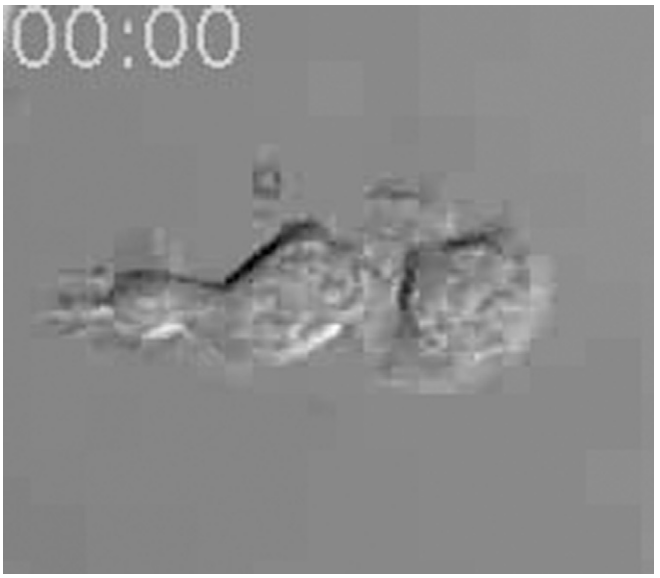
Movie S5. Rap1 activation. GD25 cells were transfected with both Rap1-Raichu FRET sensor and PA-CXCR4. Cells were pretreated with PTX and Rap1 activation was measured by FRET signal.

Movie S5



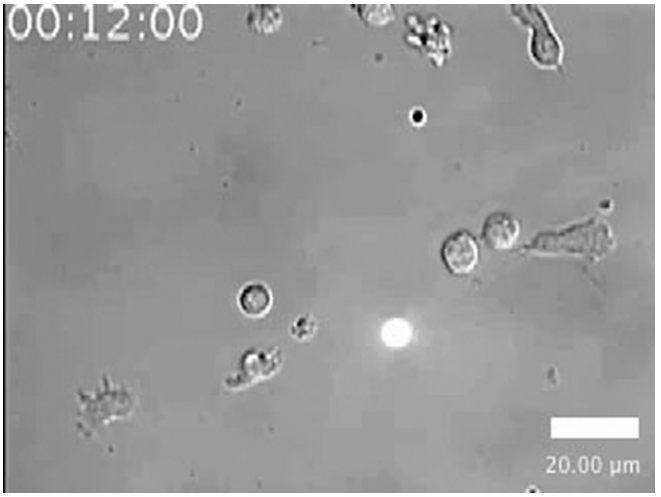
Movie S6. Directed T-cell migration and controlled stop by light. PA-CXCR4–expressing T-cell migrated following light stimulation and then stopped when the light placed on the uropod.

Movie S6



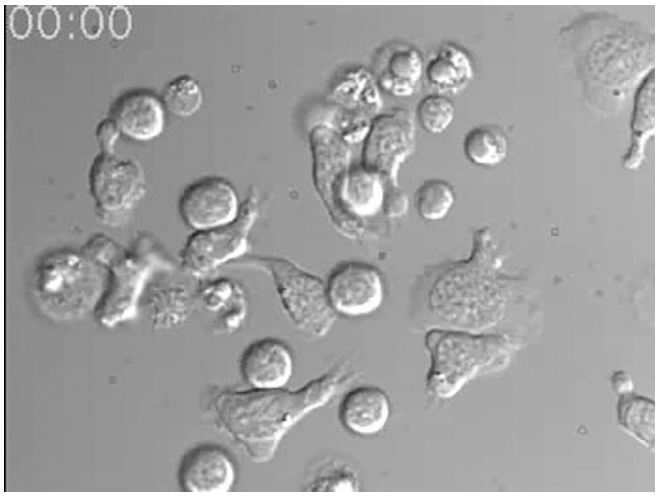
Movie S7. Reverse polarization of T-cell migration. Direction of T-cell migration was reversed by light stimulation on the uropod.

Movie S7



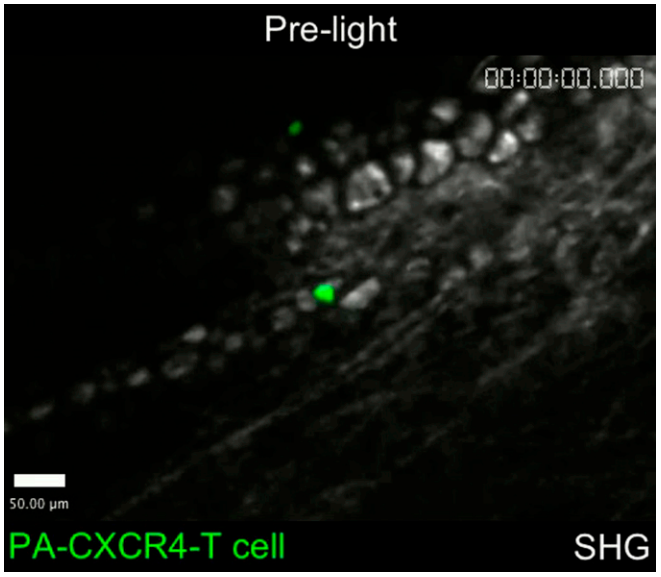
Movie S8. Phototaxis of T cells. Directional migration of PA-CXCR4–expressing T cells toward a small light spot on ICAM-1–coated plate.

Movie S8



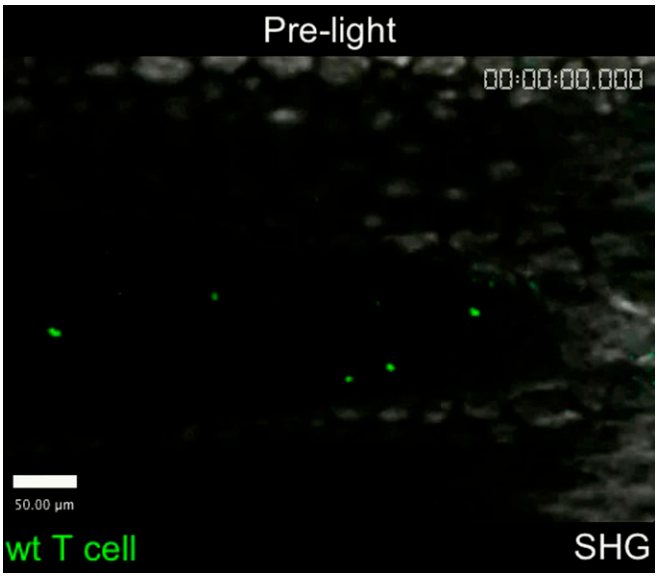
Movie S9. Phototaxis of T cells. Directional migration of PA-CXCR4–expressing T cells toward a large light spot on ICAM-1–coated plate.

Movie S9



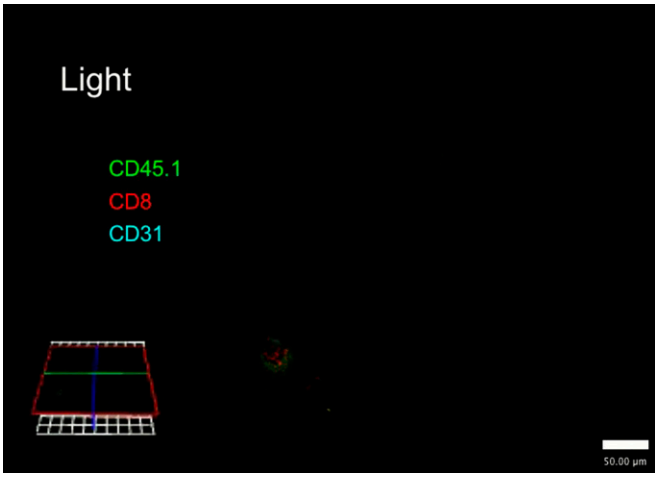
Movie S10. Firm adhesion of T cells in the mouse femoral vein. CFSE-labeled PA-CXCR4–expressing CD4 T cells were transferred and TNFα-treated femoral vein was imaged before (pre) and after (post) stimulation with 488-nm light for 10 min.

Movie S10



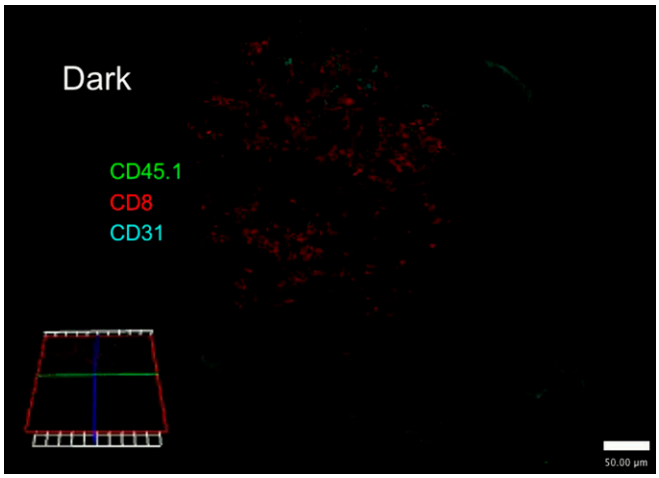
Movie S11. Firm adhesion of T cells in the mouse femoral vein. CFSE-labeled CD4 T cells were transferred and TNFα-treated femoral vein was imaged before (pre) and after (post) stimulation with 488-nm light for 10 min.

Movie S11



Movie S12. Recruitment of PA-CXCR4^{+}OT-I CTLs to the tumor. A 3D visualization of PA-CXCR4^{+}CD45.1^{+}CD8^{+}OT-I CTLs at the B16/OVA tumor site after 7 d of light stimulation. PA-CXCR4^{+}OT-I CTLs (CD45.1^{+}, green), endogenous CD8 T cells (CD8^{+}, red), and blood vessels (CD31^{+}, cyan) are shown.

Movie S12



Movie S13. Recruitment of PA-CXCR4^{+}OT-I CTLs to the tumor. A 3D visualization of PA-CXCR4^{+}CD45.1^{+}CD8^{+}OT-I CTLs at the B16/OVA tumor site without light stimulation. PA-CXCR4^{+}OT-I CTLs (CD45.1^{+}, green), endogenous CD8 T cells (CD8^{+}, red), and blood vessels (CD31^{+}, cyan) are shown.

Movie S13