Supplemental Methods

Cranial window surgery and brain multiphoton intravital microscopy. Unless otherwise stated, 8-9 days p.i., mice were anesthetized with Ketamine/Xylazine injected i.p. and placed onto a stereotactic surgical stage to immobilize the skull. Fur was removed from the top of the head using a chemical depilatory agent (Nair), 2% solution of 20 mg/mL Xylocaine (APP Pharmaceuticals, LLC) was injected subcutaneously above the skull, and then the skin above the skull removed. Craniotomies of ~4 mm in diameter were performed in the parietal bone of the skull using a 0.8 mm burr drill bit (Fine Science Tools), leaving the dura mater intact. A 5 mm diameter glass coverslip (Warner Instruments) was attached to the skull with 2-part tissue glue composed of powdered dental repair powder (Lang Dental Manufacturing Co. Inc.) and ethyl cyanoacrylate glue (Elmer's Products, LLC) allowing visualization of the underlying brain cortex. A small aluminum bar was attached by tissue glue to the frontal bones of the skull. After the glue was dry, the mouse was immobilized by attaching the metal bar to an imaging stage. Immersol W 2010 immersion medium was used between the cranial window and the 20x lens of the multiphoton microscopes. The medium and cranial window warmth were maintained using an objective warmer (Warner). Mice were imaged for ~30 min over several hours by time-lapse MP-IVM. Stacks of 11 square optical sections with 4 μm z step size were acquired on a multiphoton microscope with a 20x lens and 1x optical zoom every ~15 secs to provide image volumes of 40 μm in depth and 512 μm in width. The images were acquired on either a Bruker Ultima IV microscope equipped with Spectra-Physics MaiTai and MaiTai HP lasers using PrairieView software (Prairie Technologies) or on an Olympus MPE-RS equipped with Spectra-Physics Insight DS and MaiTai BB lasers using Olympus FV305-SW image acquisition software.

In order to stain for CD4 and CD8 cells, briefly, cranial windows were installed in WT mice, as described above. Immediately post-surgery, 10 μg each of PE-conjugated anti-CD4 (clone RM4-4, BioLegend) and BV421-conjugated anti-CD8 (clone 53-6.7, BioLegend) in 100 μL PBS r.o. At least 2 h later, the mice were anesthetized and QTracker655 injected r.o. and the brain imaged, as described above.

T cell transfer. Spleens and peripheral LNs of naïve Actin-TdTomato CXCR3$^{+/+}$ OT-I mice or Actin-CFP CXCR3$^{-/-}$ OT-I mice were harvested and homogenized using a 70 μm cell strainer. Untouched CD8^{+} T cells were isolated using EasySep Mouse CD8^{+} T cell negative selection kit according to the manufacture's instruction (STEMCELL Technologies). Purity of isolated CD8^{+} T cells was generally above 90%.

Flow Cytometric Analysis and intracellular cytokine staining. For T cell transfer experiments, on day 8 p.i, mice were injected iv. with 3 μg of anti-CD8a-BUV395 mAb (clone 53-6.7, BD Bioscience) and sacrificed 3 min later. Spleen, blood, and brain were isolated and processed for FACS staining. Spleens were processed using a 70 μm cell strainer and RBCs lysed using RBC lysing agent (Sigma). Whole blood was collected by cardiac puncture into tubes containing 0.5 M EDTA and RBCs lysed as above. Brains were removed, minced and placed in GentleMACs C tubes (Miltenyi) with Liberase TM (Roche) and DNase (Sigma-Aldrich) and digested in a 37°C water bath, and run on the GentleMACs machine three times. The cells were passed through a 70 μm cell strainer and resuspended in 30% Percoll. The brain cells were then centrifuged on 70% Percoll for 40 min at 800xg at RT. Myelin was removed from top layer and discarded, then the interface containing the leukocytes was removed and washed with PBS. Cells were blocked with

TruStain FcX anti-CD16/CD32 mAb (Biolegend) and Zombie Green in FACS buffer (PBS with 1% FCS and 0.05% NaN_3), washed with FACS buffer and incubated with fluorochrome-conjugated mAbs against CD8b (clone YTS156.7.7, Biolegend), CD44 (clone IM7, Biolegend), CD62L (clone MEL-14, BD Bioscience), CD25 (clone 3C7, BD Bioscience), VLA-4 (clone RI-2, Biolegend), LFA-1 (clone H155078, Biolegend), CD69 (clone HI.2F3, Biolegend), CD45.2 (clone 104, Biolegend), CD45 (clone 30-F11, Biolegend), CXCR3 (clone CXCR3-173, Biolegend) or appropriate isotype control for 30 min at RT.

For REX3 brain flow cytometry, brains were removed after intracardial perfusion with 20 mL of PBS containing 1 mM EDTA and cerebral cortices were dissected away from the underlying hippocampus and caudate putamen at the corpus callosum, processed into a single cell suspension as above. Cells were blocked as above and stained with eFluor 780 Fixable Viability Dye (eBioscience), followed by the fluorochrome-conjugated antibodies against F4/80 (clone BM8, BioLegend), Ly6C (clone HK1.4, BioLegend), CD11b (clone M1/70, BioLegend), CD11c (clone N418, BioLegend), CCR2 (clone 475301, R&D), CD45 (clone 30-F11, BD Biosciences) IA/IE (clone M5/114.15.2, BioLegend), DC-SIGN (clone MMD3, eBiosciences), CD3 (clone 17A2, BioLegend), CD19 (clone 6D5, BioLegend) CD4 (clone GK1.5, BioLegend), NK1.1 (clone PK136, BioLegend), CD8a (clone 53-6.7, ThermoFisher) or appropriate isotype control for 20 mins at 4°C. The cortices from 2-3 mice were pooled in each experiment in order to have enough leukocytes to analyze. All samples were acquired using either a Fortessa X-20 or a LSR-II flow cytometer (BD Biosciences) and analyzed by FlowJo Software (Tree Star).

To compare T cell populations from WT and $CXCR3^{-/-}$ mice, whole blood was obtained from mice

by cardiac puncture and immune cells isolate by spinning through Ficoll (GE Healthcare). Spleens were mechanically dissociated and passed through a 70μm strainer, the pellets washed with PAB (PBS and 1% BSA, 0.1% sodium azide), and stained in 200μl PAB. Cells were incubated for 15 min with TruStain fcX (anti-mouse CD16/32) (BioLegend) and stained with fluorochrome-conjugated mAbs against CD45 (clone 30-F11, BD Biosciences), CD3 (clone 17A2, BioLegend), CD4 (GK1.5, BioLegend), CD8a (clone 53-6.7, BioLegend), CD44 (clone IM7, BD Biosciences) and CD69 (clone H1.2F3, BD Biosciences) for 20 min at 4°C. For intracellular cytokine staining, PBMC or splenocytes plated in complete RPMI with PMA (Sigma-Aldrich) at 50ng/mL and Ionomycin (Sigma-Aldrich) at 2.5μg/mL for 6 h. After the first 2 h, 1μg/mL GolgiPlug (BD Cytofix/Cytoperm Kit; BD Biosciences) was added to the culture. The cells were then harvested from the plates, incubated for 15 min with TruStain fcX (anti-mouse CD16/32) (BioLegend), and stained with fluorochrome-conjugated mAbs against CD45 (clone 30-F11, BD Biosciences), CD3 (clone 17A2, BioLegend), CD4 (clone GK1.5, BioLegend), and CD8a (clone 53-6.7, BioLegend) for 30 min at 4°C. Cells were then permeablized using BD Cytofix/Cytoperm Kit (BD Biosciences) and stained with either fluorochrome-conjugated anti-IFNγ (clone XMG1.2, BD Biosciences) or Rat IgG1 isotype control (BD Biosciences) in Perm/Wash Buffer for 30 min at 4°C. In all cases, after staining, cells were resuspended in PAB and analyzed using an LSRII (BD Biosciences).

Whole mount histology. Cerebral cortices were dissected away from the underlying hippocampus and caudate putamen at the corpus callosum and small pieces of brain cortex were excised from the indicated mouse strains from uninfected or day 8 or 9 p.i. and placed in 200μL PAB staining buffer (PBS, 1% BSA, and 0.1% sodium azide). Samples were blocked using TruStain fcX (anti-mouse CD16/32; BioLegend) and the antibodies, AF488-conjugated anti-CD31 (clone 390,

BioLegend) and APC-conjugated anti-CD45 (clone 30-F11, BioLegend), were added directly to the tube at predetermined concentrations. The samples were then stained for 2 h at 4°C. The samples were then washed once in staining buffer. Stained cortex samples were then viewed using a fluorescence microscope connected to a monochrome CCD digital camera. Filters designed to detect AF488, RFP, APC, and BFP wavelengths were used to acquire alternate images of the same fields of view. Monochrome images were acquired and pseudo-colored using Zen Blue software (Zeiss, Oberkochen, Germany).

Whole mount staining for intravascular and extravascular cells. On day 8 post-infection, REX3 mice sacrificed and lung, pancreas and liver removed. Alternatively, anesthetized mice were injected with 3μg of APC-conjugated anti-CD45 mAb (clone 30-F11, BD Biosciences) r.o. and sacrificed 3 minutes later. Brain cerebral cortices were dissected away from the underlying hippocampus and caudate putamen at the corpus callosum and small 3-5mm pieces of tissue cortex were excised from the indicated tissues and placed in 200μL PAB staining buffer. Samples were blocked using TruStain fcX (anti-mouse CD16/32, BioLegend) and the non-brain organs stained with AF488-conjugated anti-CD31 (clone MEC13.3, BioLegend) and APC-conjugated anti-CD45 mAb (clone 30-F11, BD Biosciences) and the brain stained either with AF488-conjugated anti-CD31 or AF488-conjugated anti-CD45.2 (clone 104, BioLegend), were added directly to the tube at predetermined concentrations. The samples were then stained for 2 hrs at 4°C. The samples were then washed once in staining buffer. Stained cortex samples were then viewed using a fluorescence microscope connected to a monochrome CCD digital camera. Filters designed to detect AF488, RFP, APC, and BFP wavelengths were used to acquire alternate images of the same fields of view.

Monochrome images were acquired and pseudo-colored using Zen Blue software (Zeiss, Oberkochen, Germany).

Generation of bone marrow chimeric mice. Bone marrow was isolated from femurs of WT mice using a mortar and pestle, passed through a 70 μm strainer, and washed in PBS. Bone marrow cells (3×10^6) in PBS were injected i.v. via tail vein into lethally total body irradiated (1000 Rads) REX3 recipient mice on the same day. After injection, chimeric mice were allowed to reconstitute for a minimum of 8 weeks before experimental use.

Supplemental Data

Supplemental Table 1. Total T cells analyzed in MP-IVM experiments.

Fig 1A-E & Fig 2	Infected DPE-GFP (n=9)		Infected DPE-GFP CXCR3$^{-/-}$ (n=9)		Uninfected DPE-GFP (n=7)		Uninfected DPE-GFP CXCR3$^{-/-}$ (n=7)	
Location	Intravascular	Parenchymal	Intravascular	Parenchymal	Intravascular	Parenchymal	Intravascular	Parenchymal
Total analyzed	1,036	81	309	44	102	15	99	9

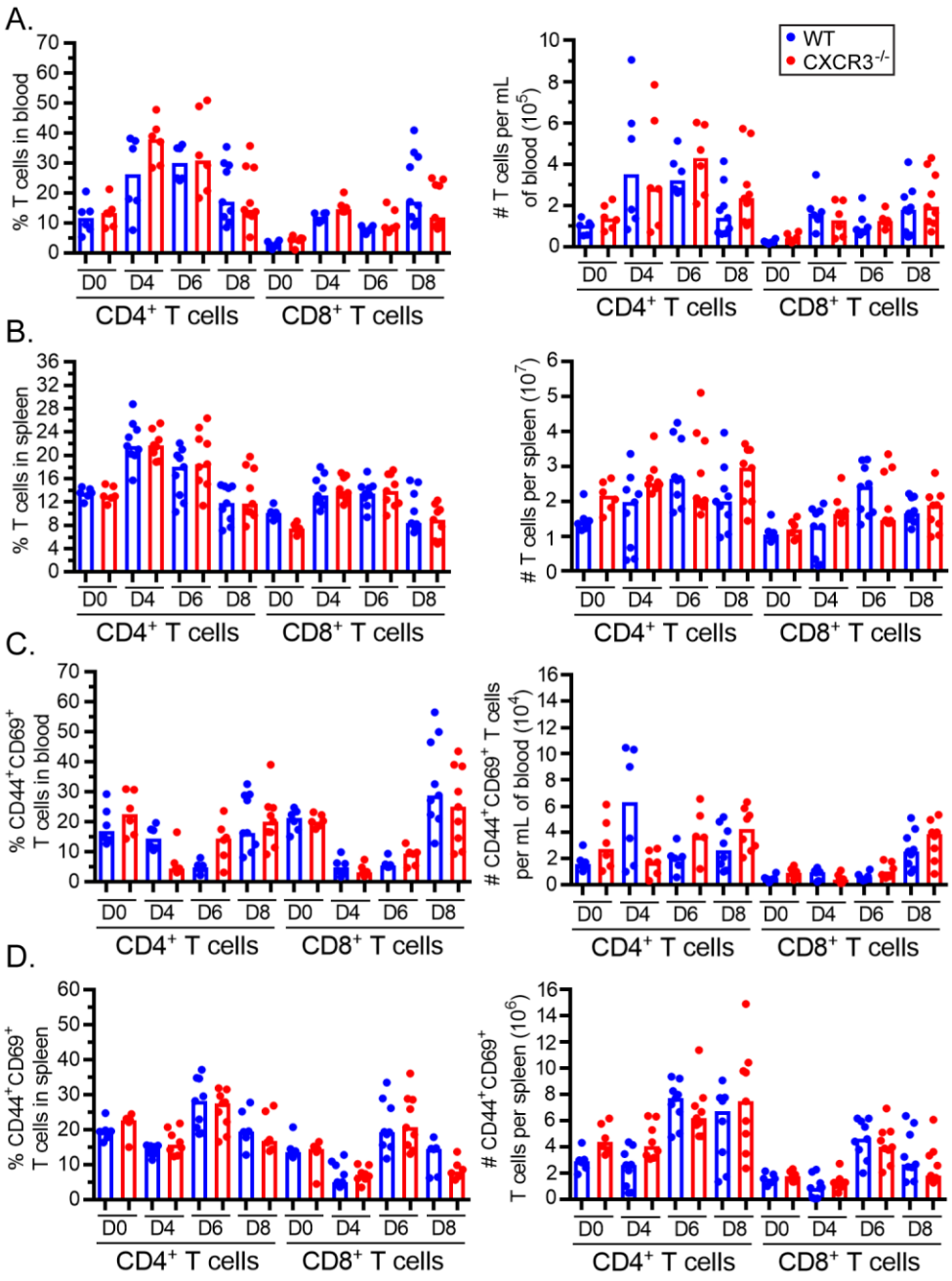
Fig 1C&D (n=16)	CD4 Intravascular	CD8 Intravascular
Total analyzed	229	1,147

Fig 3I-L (n=12)	WT OT-I		CXCR3$^{-/-}$ OT-I	
Location	Intravascular	Parenchymal	Intravascular	Parenchymal
Total analyzed	247	191	116	37

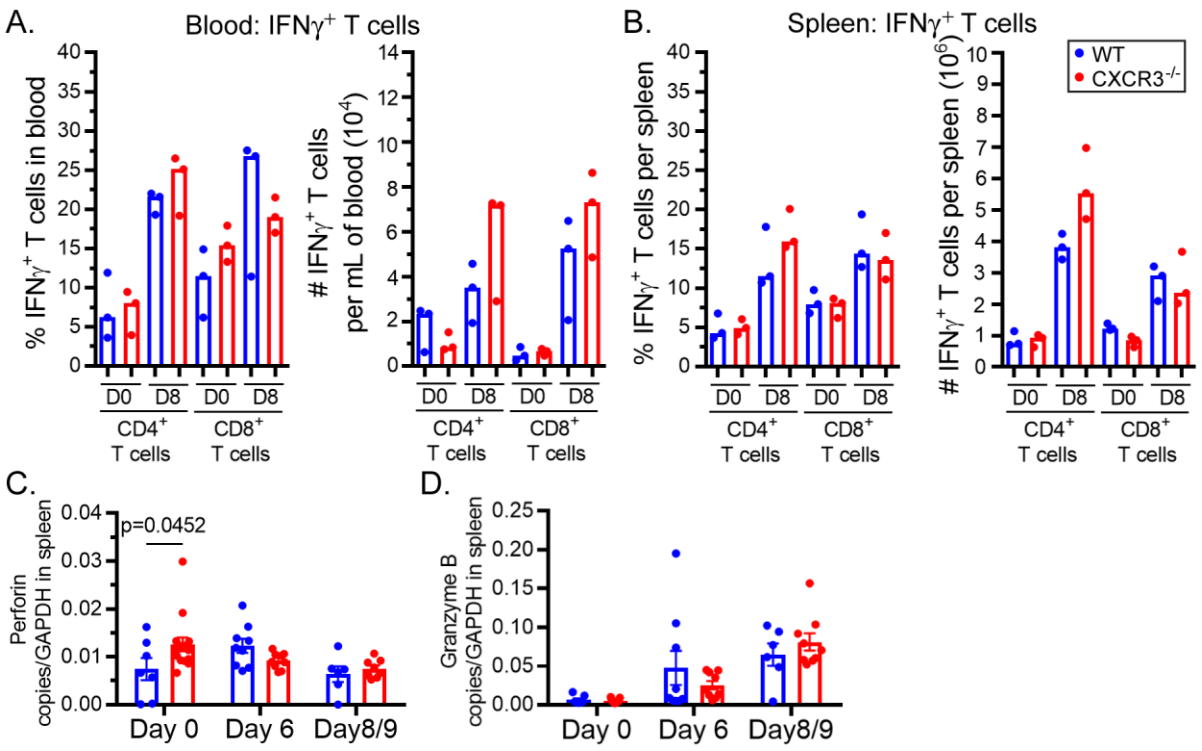
Fig 4	Pre-Ham IgG Intravascular (n=8)	Post-Ham IgG Intravascular (n=8)	Pre-αCXCL9&10 Intravascular (n=6)	Post-αCXCL9&10 Intravascular (n=6)	Pre-αCXCL9 Intravascular (n=6)	Post-αCXCL9 Intravascular (n=6)	Pre-αCXCL10 Intravascular (n=6)	Post-αCXCL10 Intravascular (n=6)
Total Analyzed	1,302	1,402	576	429	656	596	520	494

Fig 8B-D	DPE-GFP→C57BL/6 (n=5)	CXCR3$^{-/-}$→CXCR3$^{-/-}$ (n=10)	DPE-GFP→CXCL9$^{-/-}$ (n=5)	DPE-GFP→CXCL10$^{-/-}$ (n=5)
Location	Intravascular	Intravascular	Intravascular	Intravascular
Total Analyzed	343	203	555	197

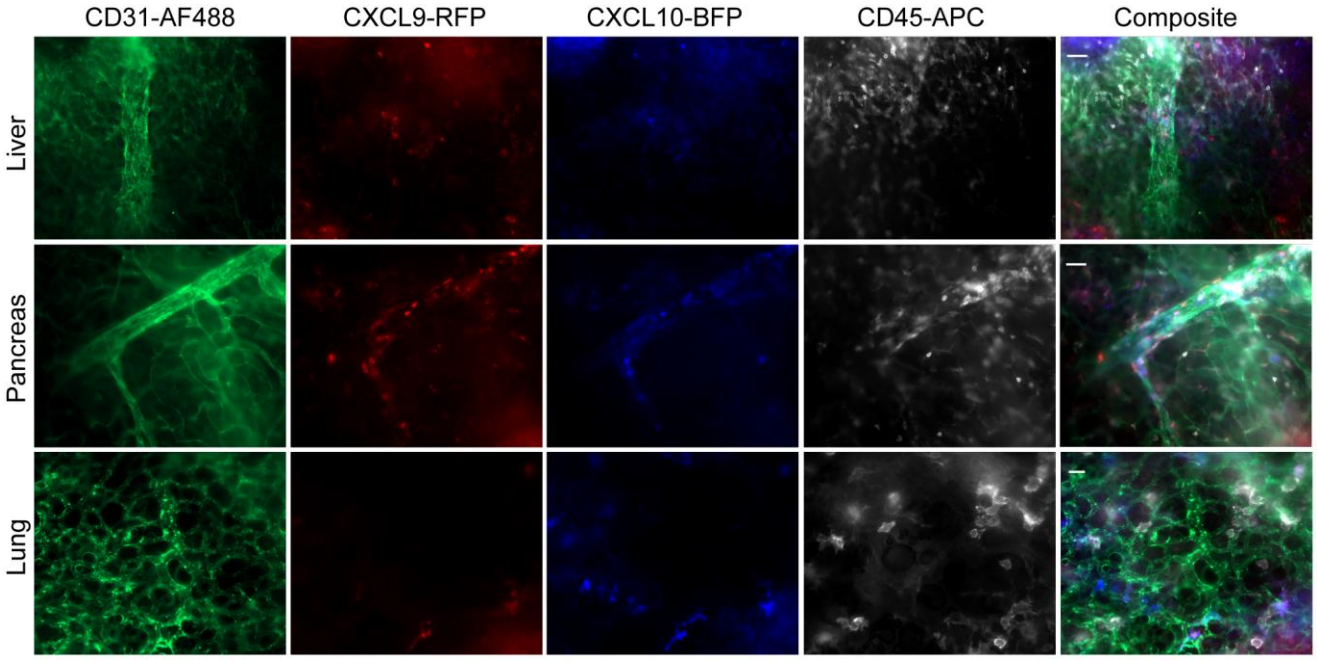
Supplemental Table 1. Total T cells analyzed in MP-IVM experiments. Table shows the total number of T cells analyzed per group in figures indicated in the top left cell. In addition, the number of mice studied in each experiments is indicated in parentheses where appropriate.



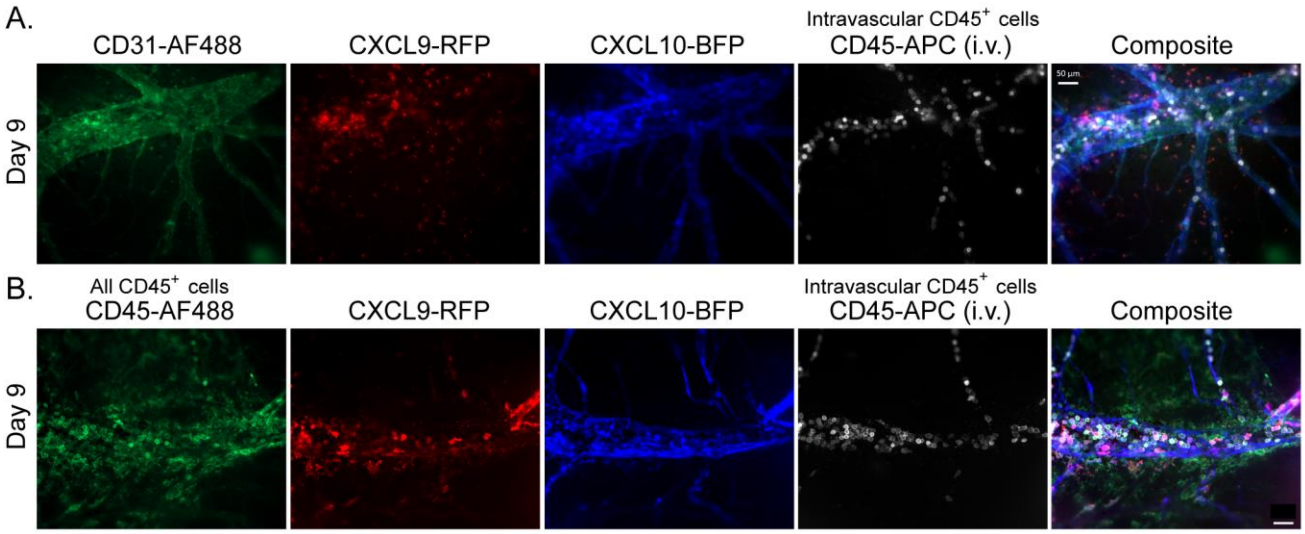
Supplemental Figure 1. Number and phenotype of T cells in the blood and spleen of WT and CXCR3$^{-/-}$ mice following PbA infection. WT (blue symbols) and CXCR3$^{-/-}$ (red symbols) mice were infected with PbA and on the indicated days p.i. CD4$^+$ and CD8$^+$ T cells in the blood and spleen were analyzed by flow cytometry. Quantitation of total CD4$^+$ and CD8$^+$ T cells from the (**A**) blood and (**B**) spleen, and activated (CD44$^+$CD69$^+$) CD4$^+$ and CD8$^+$ T cells from the (**C**) blood and (**D**) spleen. In each panel, left graph shows % cells per organ, and right graphs depicts # cells per organ. The number of mice/group total from 3 independent experiments were: Day 0= 6, Day 4= 6, Day 6= 6, Day 8= 9. Groups were compared using Kruskal-Wallis with Dunn's multiple comparison tests. Bars represent the median in all plots.



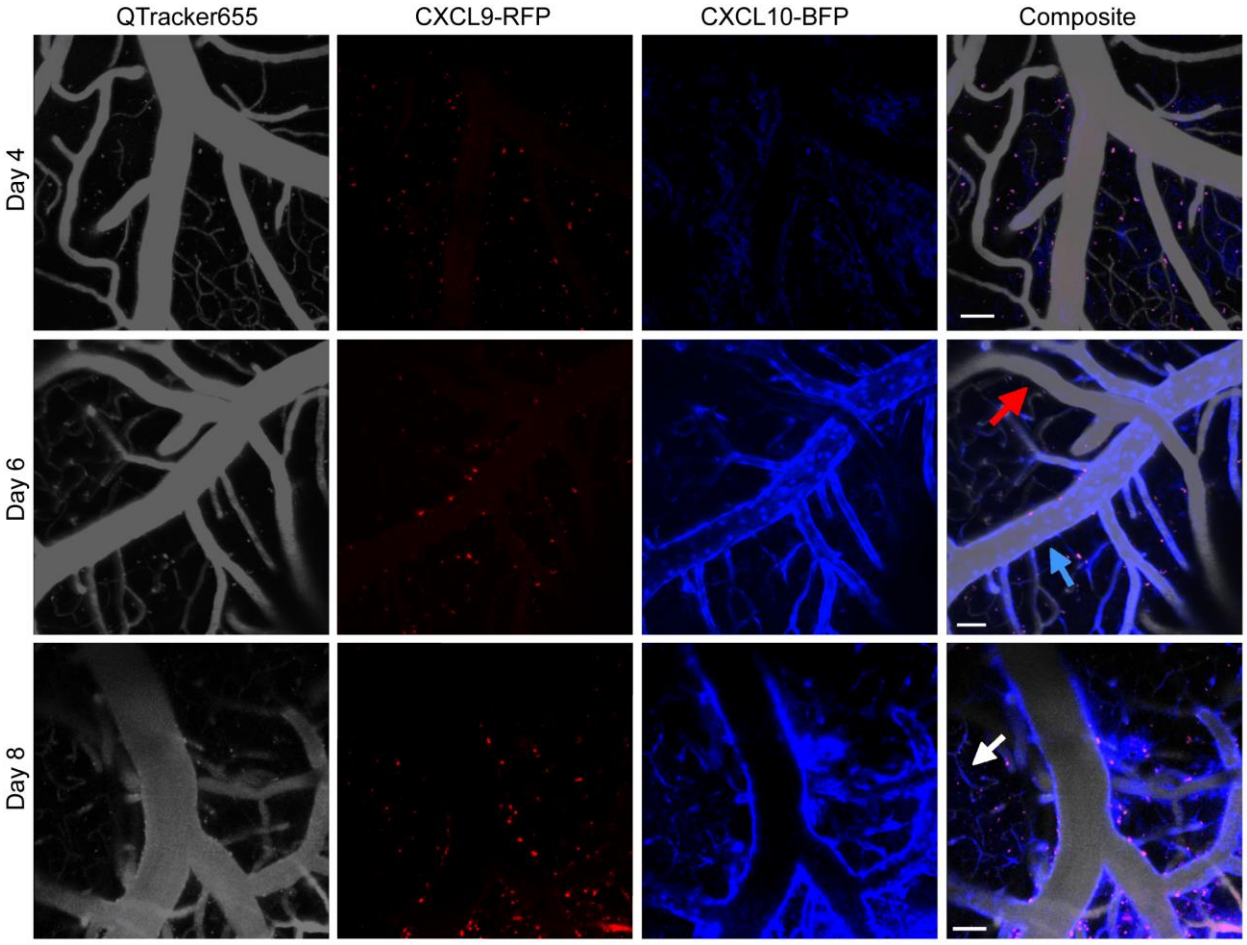
Supplemental Figure 2. Functional comparison of T cells from blood and spleen of WT and CXCR3$^{-/-}$ mice following PbA infection. WT (blue symbols) and CXCR3$^{-/-}$ (red symbols) mice were infected with PbA and sacrificed on the indicated days p.i. (**A**) Blood and (**B**) spleen were made into single-cell suspensions, stimulated for 6 h with PMA and Ionomycin and intracellular cytokine staining performed for IFNγ. The percent and number of IFNγ$^+$ CD4$^+$ and CD8$^+$ T cells were analyzed by flow cytometry. The number of mice/group total from 2 independent experiments= 6 for all groups. Groups were compared using Kruskal-Wallis with Dunn's multiple comparison tests. Bars represent the median in all plots. Spleens were analyzed by qPCR for the levels of (**C**) perforin and (**D**) granzyme B mRNA relative to GAPDH. The number of mice/group total from 3 independent experiments were: WT Day 0, 6, 8/9= 7, 8, 6 and CXCR3$^{-/-}$ Day 0, 6, 8/9= 15, 9, 9. The groups were compared using (**C** & **D**) 2way ANOVA with Bonferroni's multiple comparison tests. Bars represent the mean with SEM in all plots.



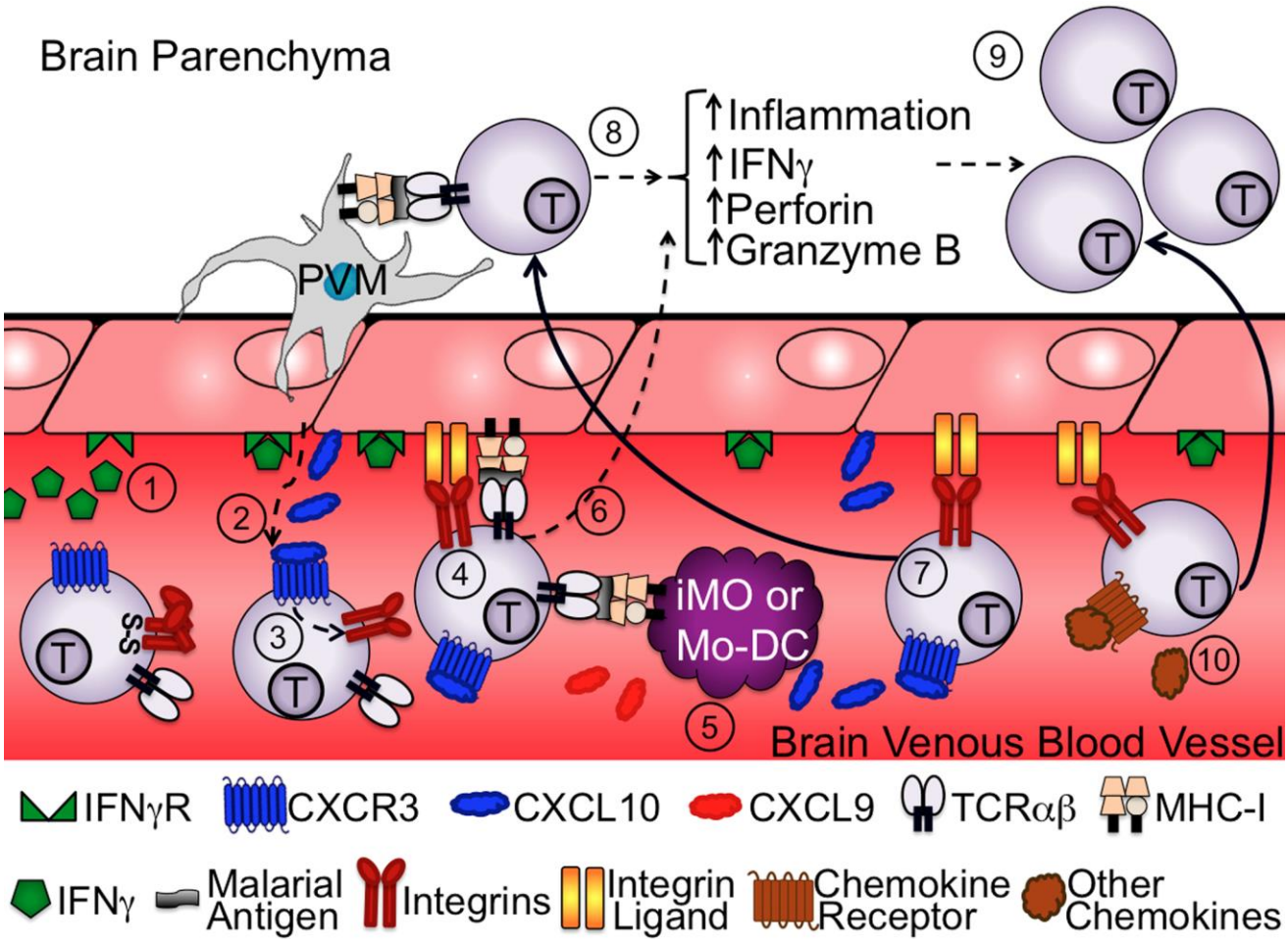
Supplemental Figure 3. Whole mount microscopy of organs in PbA-infected REX3 mice. Whole mount immunofluorescence was performed on the liver (top row), pancreas (middle row), and lung (bottom row) of PbA-infected REX3 mice on day 9 post-infection. Representative images are shown stained with anti-CD31-AF488 (green) and anti-CD45-APC (white). CXCL9-RFP is red and CXCL10-BFP is blue. Scale bar= 50 μm in liver and pancreas images and 20 μm in lung images. N= 6 mice from 3 independent experiments.



Supplemental Figure 4. Whole mount staining of intravascular immune cells in the brain of PbA-infected REX3 mice. Whole mount immunofluorescence was performed on the brain cortex of PbA-infected REX3 mice on d 9 p.i. 3 μg of anti-CD45-APC was injected r.o. into mice 3 min before sacrifice to label intravascular leukocytes. Representative images of whole mount preparations are shown stained with either (**A**) anti-CD31-AF488 (green) or (**B**) anti-CD45.2-AF488 (green). In both sets of images, intravascular CD45 staining is in white, CXCL9-RFP is red, and CXCL10-BFP is blue. Scale bar=50 μm. N= 6 mice from 3 independent experiments.



Supplemental Figure 5. CXCL10 expression in brain endothelial cells during the development of CM. Lethally irradiated REX3 mice were reconstituted with DPE-GFP bone marrow and infected with PbA. On d 4, 6, and 8 p.i., cranial window surgery was performed, QTracker655 (grey) injected r.o. and the cortices imaged by MP-IVM. In all sets of images, CXCL9-RFP is red and CXCL10-BFP is blue. Autofluorescence seen in the composite image is light pink or white. Red arrow indicates an arteriole, blue arrow indicates a venule, and white arrow indicates capillaries. Scale bar= 50 μm. N≥4 mice from 3 independent experiments.



Supplemental Figure 6. CXCR3-mediated T cell arrest leads to increase brain inflammation and the development of CM. 1) IFNγ-induced 2) CXCL10 production by brain endothelial cells activates CXCR3 on circulating $CD8^+$ T effector cells 3) inducing an upregulation of integrin affinity and 4) binding of effector T cells the brain endothelium. Cross presentation of malaria antigens by endothelial cells and 5) monocyte derived dendritic cells (Mo-DC) and inflammatory monocytes (iMO), which are also a source of CXCL9 and CXCL10, to these arrested $CD8^+$ T cells 6) induces their activation and release of perforin, granzyme and IFNγ. 7) Some arrested $CD8^+$ T cells transmigrate across the brain endothelium where 8) they can encounter malarial antigens presented by perivascular macrophages. The local activation of $CD8^+$ T cells and release of perforin, granzyme injures the brain endothelium, and the release IFNγ and other cytokines and chemokines 9) amplifies immune cell recruitment through CXCR3 as well as 10) non-CXCR3 chemokine pathways leading to increased inflammation, further endothelial damage and the development CM.

Supplemental Movie Legends

Supplemental Movie 1. T cells tracked in the brains of PbA-infected DPE-GFP and DPE-GFP CXCR3$^{-/-}$ mice. Brains of mice were imaged by MP-IVM and Qtracker655 (grey) injected r.o. to reveal patent blood vessels. GFP^{+} T cells were tracked in the brains of uninfected and d 8 PbA–infected DPE-GFP and DPE-GFP CXCR3$^{-/-}$ mice. The tracks in the example fields of view (FOVs) were color-coded according to the length of time visualized (0 to ~30 min). During the second play through, GFP^{+} perivascular macrophages were masked to more clearly visualize the tracked T cells. Scale bar=70 μm.

Supplemental Movie 2. Tracking CD4^{+} and CD8^{+} cells on the vessel lumen in the brains of PbA-infected WT mice. PE-conjugate anti-CD4 and BV421-conugated anti-CD8 mAbs were injected r.o. into a day 8 p.i. WT mouse 2 h prior to imaging. Qtracker655 (grey) injected r.o. to reveal patent blood vessels. Videos display the tracks of CD4 (red) and CD8 (cyan) T cells interacting with the brain vasculature. Scale bar=70 μm.

Supplemental Movie 3. Visualization of T cell attachment and detachment on brain endothelium in PbA-infected DPE-GFP and DPE-GFP CXCR3$^{-/-}$ mice. Zoomed in section of the movies in Video 1 to more clearly show T cell attachment and morphology. Left video shows T cells in the brain of a PbA-infected DPE-GFP mouse attaching to the brain vasculature for long periods of time and not detaching (blue arrows). Right video shows T cells in the brain of a PbA-infected DPE-GFP CXCR3$^{-/-}$ mouse, which attach (red arrows) for a short period and then detach from the endothelium (white arrows). Scale bar=20 μm