Supplemental Figures

Mapping immune processes in intact tissues at cellular resolution

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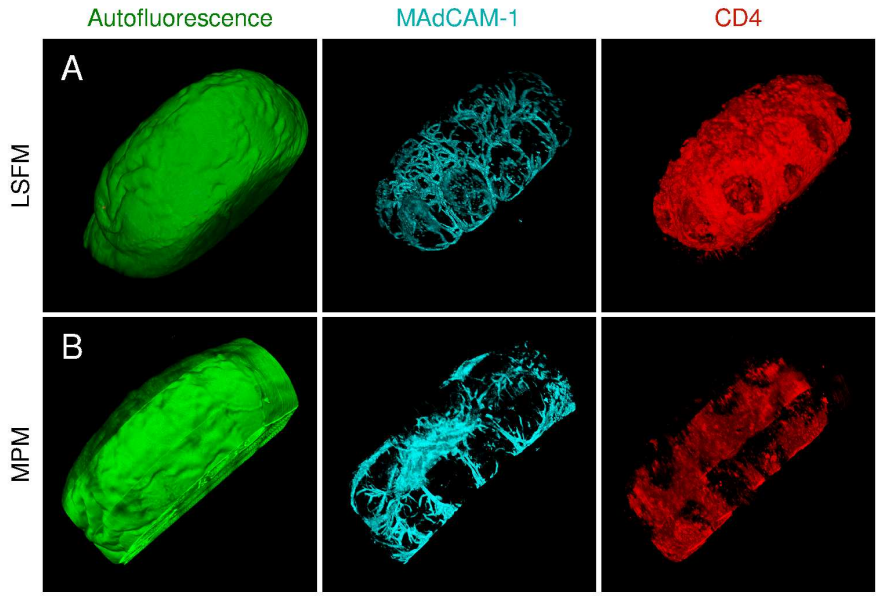
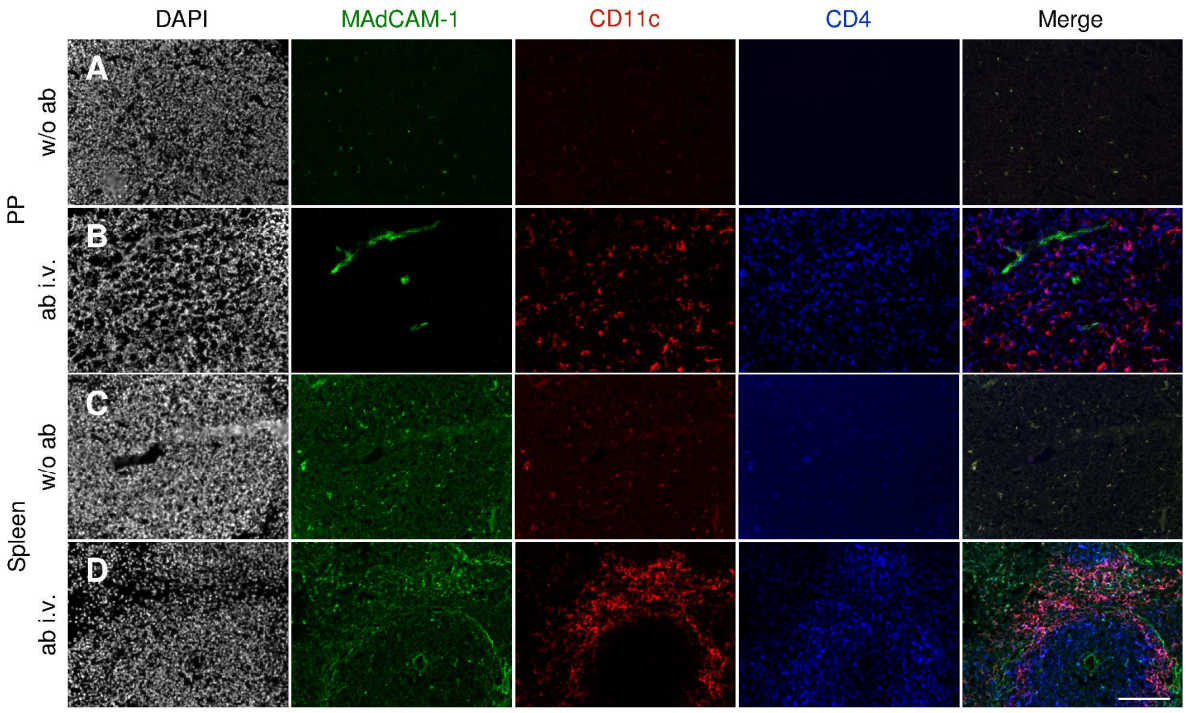
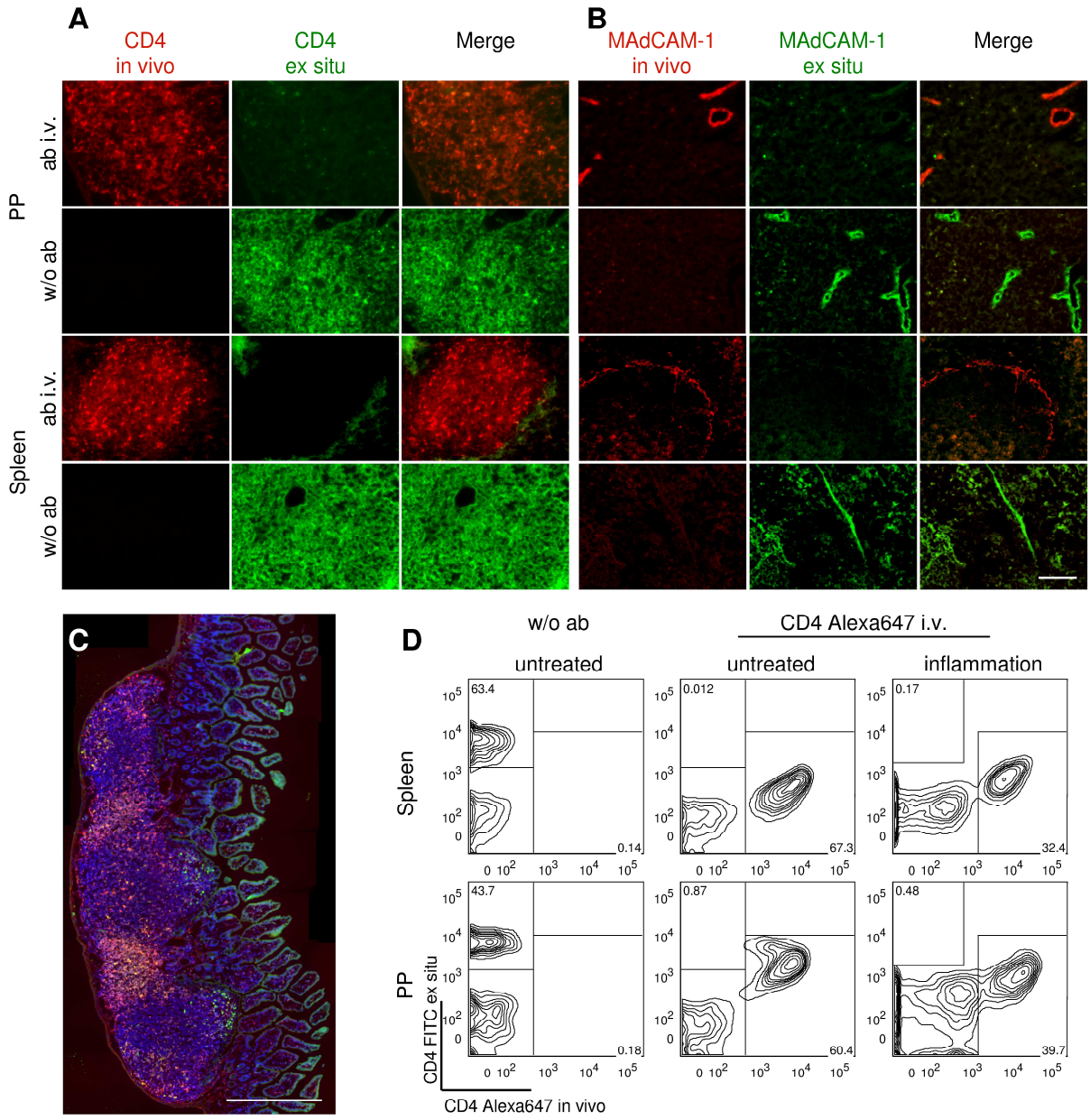


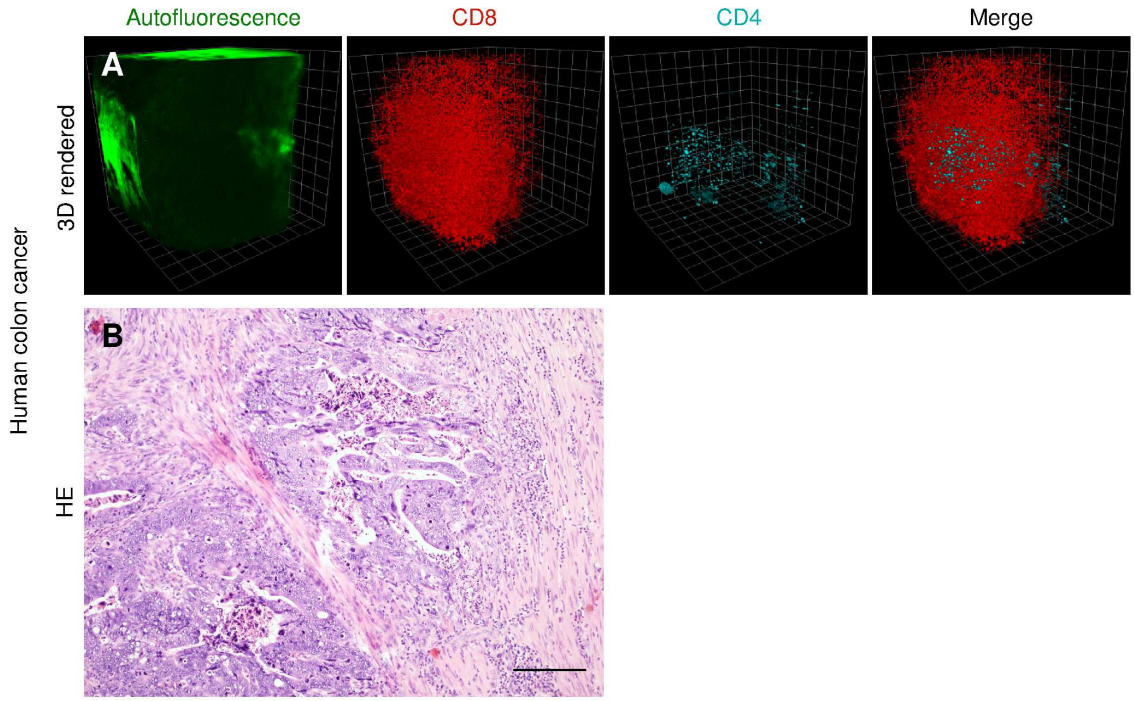
Supplemental Figure 1: Comparison of LSFM and MPM for whole PP imaging. (**A**) LSFM allowed imaging of whole PP using a x5 objective with 0.66 adapter (5µm increment, 300 optical sections / detection channel) within 12 minutes. (**B**) Due to low photon efficiency, MPM imaging of the same PP required a x20 objective (5µm increment, 243 optical sections / detection channel) and acquisition time took approximately 6 hours.



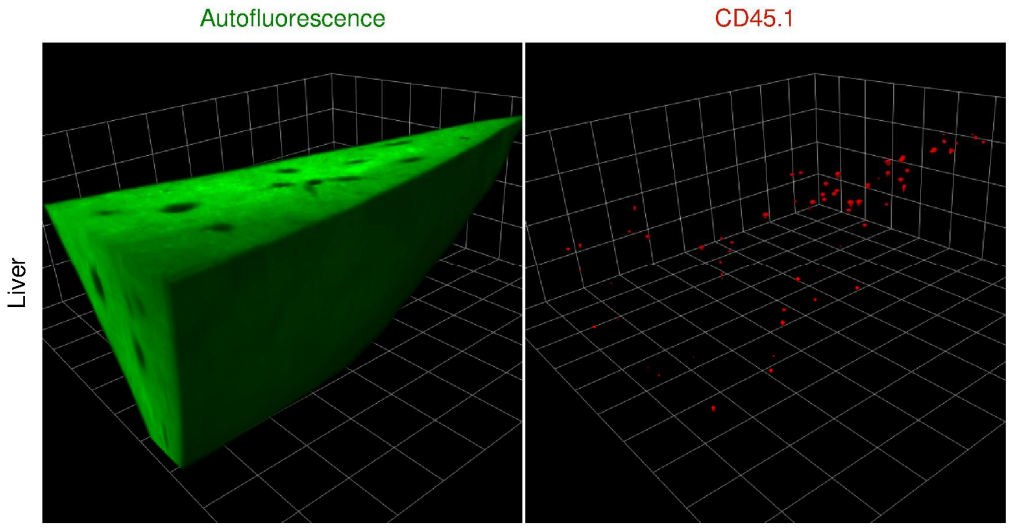
Supplemental Figure 2: Antibody in vivo staining. Antibodies were injected i.v. 2.5 h (CD4, CD11c) or 0.5 h (MAdCAM-1) before perfusion. Specific antibody distribution was confirmed by conventional immunofluorescence microscopy. PP of (**A**) an untreated mouse and (**B**) of a mouse intravenously injected with fluorescent labeled antibodies (MAdCAM-1 (green), CD11c (red) and CD4 (blue). Spleen of (**C**) an untreated mouse (w/o antibody) and (**D**) a mouse injected with antibodies. Sections were stained with DAPI ex situ (Scale bar: 100 µm). Abbreviations: ab = antibody, i.v. = intravenously, w/o = without.



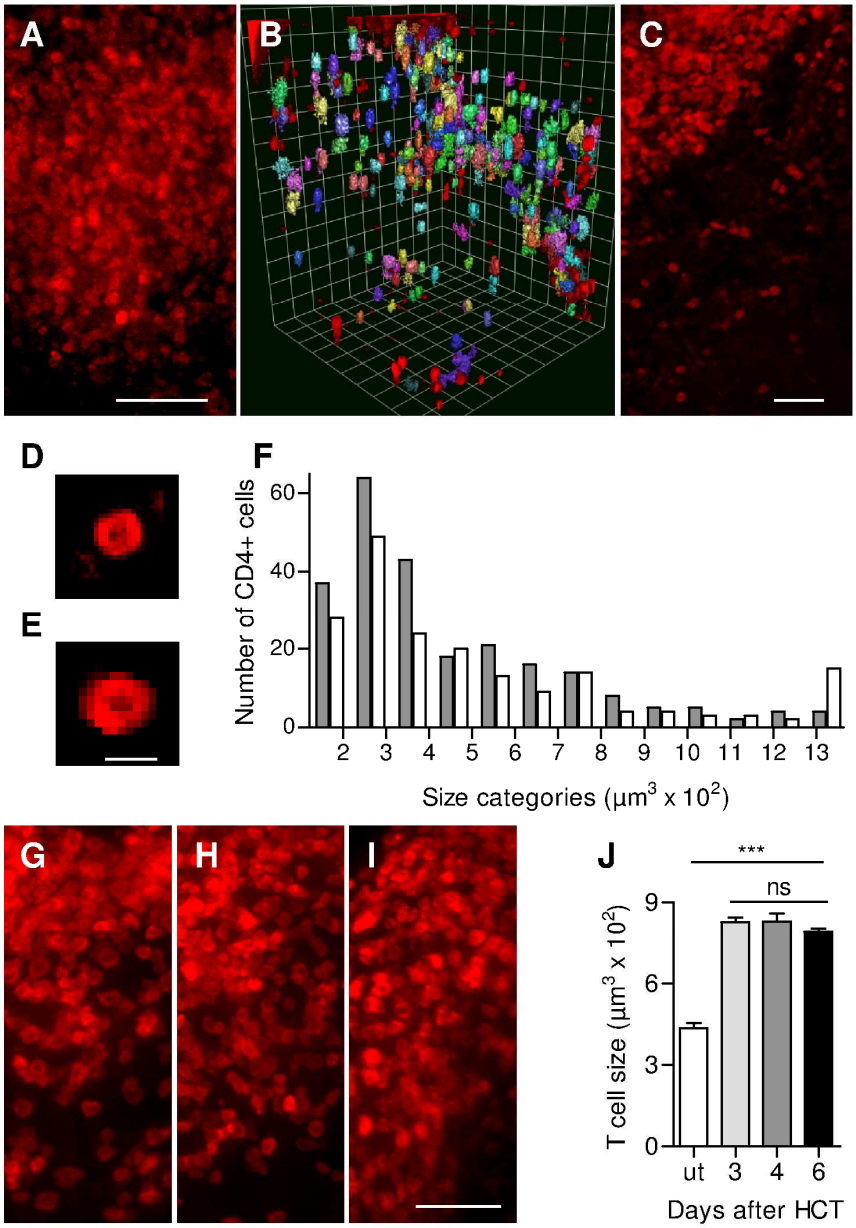
Supplemental Figure 3: Distribution and specificity of i.v. antibody staining. To confirm antibody i.v. staining in an untreated BALB/c mouse (**A**) CD4 (red) was injected 2.5 h and (**B**) MAdCAM-1 (red) was injected 0.5 h before perfusion and post-section staining was performed with DAPI and either with CD4 (green) or MAdCAM-1 (green), respectively (x20, scale bar: 100 µm). Sections from mice injected with antibody (ab i.v.) were compared to sections from mice that did not receive an antibody injection (w/o antibody). (**C**) Tiling of a whole PP cross-section with surrounding mucosal tissue indicates specific whole tissue distribution of i.v. injected CD4 (red) antibody (x20, stitched from 30 images, scale bar: 500 µm). (**D**) Confirmation by flow cytometry of i.v. antibody staining in inflamed and untreated tissues. CD4-Alexa647 antibody was injected in untreated mice or in mice at day+6 after allo-HCT (inflammation). 2.5 h after injection spleen and PPs were digested in presence of CD4-FITC antibody and subsequently analyzed with flow cytometry. Cell frequencies demonstrate efficiency of i.v. staining (> 98 %) of cellular events. Abbreviations: ab = antibody, i.v. = intravenously, w/o = without.



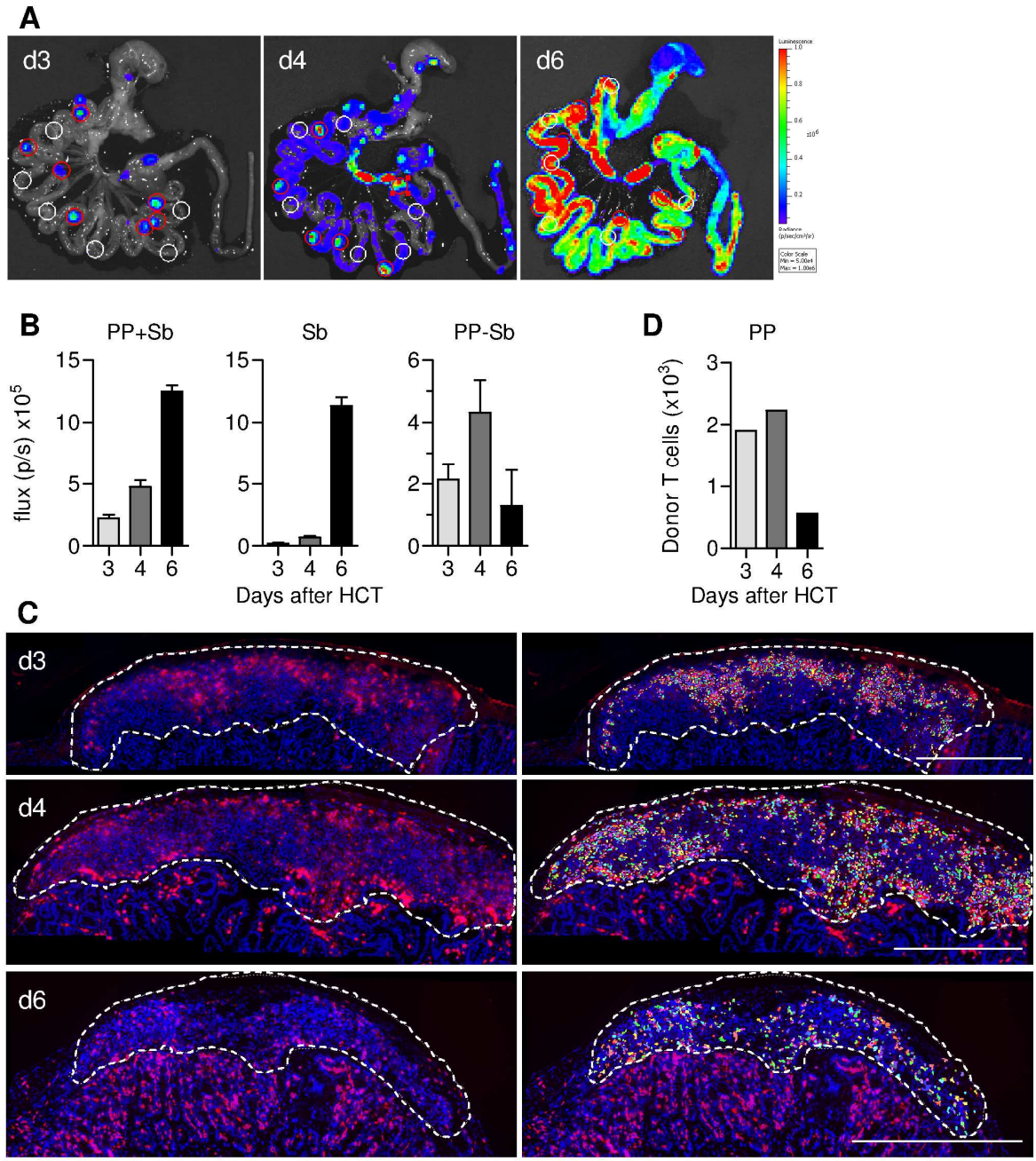
Supplemental Figure 4: LSFM imaging of human colon cancer tissue. (**A**) 3D reconstruction of a moderate differentiated human colon adenocarcinoma specimen with tumor infiltrating CD8+ T cells (red) and CD4+ T cells (cyan). (**B**) Histopathologic image of a sample from the same specimen stained with hematoxylin and eosin (HE) after formalin fixation and paraffin embedding confirmed the lymphocytic infiltration of the cancer tissue (scale bar: 500µm).



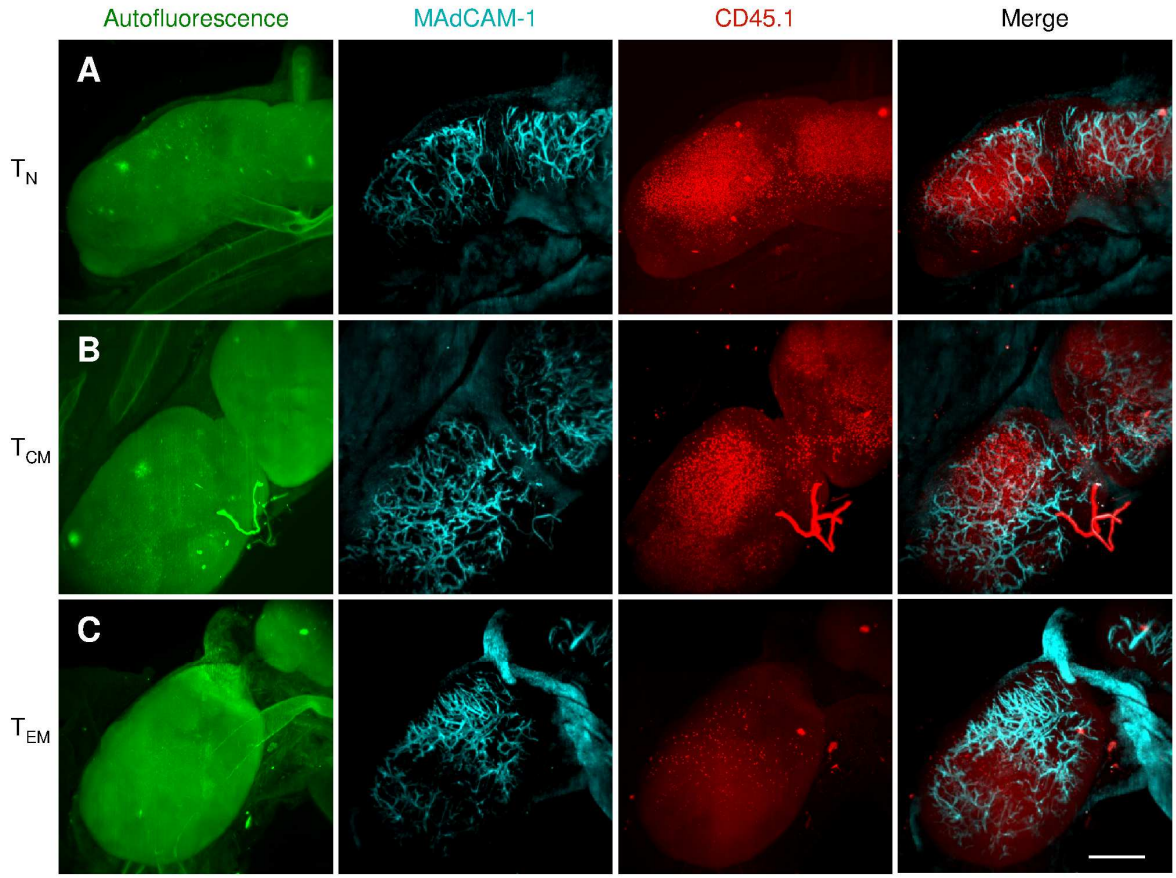
Supplemental Figure 5: Imaging of single donor T cells in the liver 60h after allo-HCT. Bone marrow together with luciferase^{+} CD45.1^{+} transgenic T cells were transplanted into myeloablatively irradiated allogeneic recipients to induce acute GVHD. Detection of single infiltrating T cells (red) in the liver (autofluorescence in green) by LSFM (unit: 246µm).



Supplemental Figure 6: Quantification of T cells in intact PPs. (A) Clustering of T cells within a PP (CD4, red) impede automated cell counting (z-projection scale bar: 50 µm). **(B)** To calculate cell volumes, single CD4+ cells are rendered (colored objects) and measured at less dense areas. **(C)** Comparison of cell imaging with confocal microscopy and LSFM measurements (z-projection; scale bar: 50 µm). Representative single cells imaged with **(D)** LSFM and **(E)** confocal microscopy using a x20 objective (z-projection scale bar: 10 µm). **(F)** Size distribution of measured CD4+ cells shown for LSFM (grey bars; mean volume 435.6 µm³ ± 19.7, N = 233) and confocal microscopy (white bars; mean volume 561.1 µm³ ± 33.4, N = 195). **(G)** Donor T cells on day +3, **(H)** day +4 **(I)** and day +6 after allo-HCT (z-projections scale bar: 50 µm). **(J)** Mean T cell volume was increased during activation (day +3 mean volume 826.9 µm3 ± 17.2 N = 816; day +4 mean volume 830.2 µm3 ± 27.9 N = 301, day +6 mean volume 788.8 µm3 ± 15.2 N = 976). Statistics: ANOVA, Bonferroni corrected (ns = P>0.05, *** = P<0.001).



Supplementary Figure 7: Visualizing and quantifying cellular changes within Peyer´s Patches after allo-HCT. Bone marrow together with CD90.1$^+$ luciferase$^+$ transgenic T cells were transplanted into irradiated allogeneic recipients to induce acute GVHD. (A) Ex vivo BLI was performed at the transition from GVHD initiation phase to effector phase (day +3 and day +4 after allo-HCT) and at the effector phase (day+6). (B) Emitted photons of donor T cells in PPs (red circles) or intestinal mucosa (Sb, white circles) were quantified respectively. (C) PPs from day +3, day +4 and day +6 were stained for donor T cells with CD90.1 (red) and DAPI (blue) and whole PP sections were imaged in high-resolution with IFM (x20) and stitched together. Donor T cells were automatically counted with the Software Volocity (colored objects) by determining nuclei with a high MFI in the red channel, indicating cellular staining. (D) Quantification of whole PP sections. (scale bar: 500 µm)



Supplemental Figure 8: Homing capacity of individual T cell subsets to mesenteric lymph nodes (mLN). LSFM imaging of whole mLNs allowed the detection of donor T cell subsets 20 h after transfer. Accordant to the homing capacity to PPs, T cell numbers in the mLNs revealed a better homing capacity of (**A**) TN and (**B**) TCM over (**C**) TEM (z–projection scale bar: 500µm).

Supplemental Videos

Mapping immune processes in intact tissues at cellular resolution

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Supplemental Video Legends

Supplemental Video 1: Virtual journey through an intact Peyer's patch by multicolor LSFM. Three-dimensional reconstruction of approximate 1500 optical sections created by LSFM. Tissue autofluorescence (green) revealed microanatomical details of the intestinal mucosa and submucosa such as villi, crypts and a PP with its subepithelial dome regions. Within the PP, $CD4^+$ T lymphocytes (red) colocalize to high endothelial venules (MAdCAM-1, cyan).

Supplemental Video 2: Optical sectioning of an intact Peyer's patch by multicolor LSFM. Whole PPs were imaged by creating approximate 1500 optical sections (x5 Objective, 5 µm increments) in multiple colors (Overlay: tissue autofluorescence, green; MAdCAM-1, cyan; $CD4^+$ T cells, red). The optical sections were used for 3D tissue reconstruction shown in the **Supplemental Video 1**.

Supplemental Video 3: Multicolor LSFM of diverse murine organs. A C57Bl/6 mouse was injected i.v. with fluorescent labeled antibodies CD31 (cyan) and LYVE-1 (red). 3D reconstruction of (**a**) heart, (**b**) testicle, (**c**) skin and (**d**) mLNs are shown.