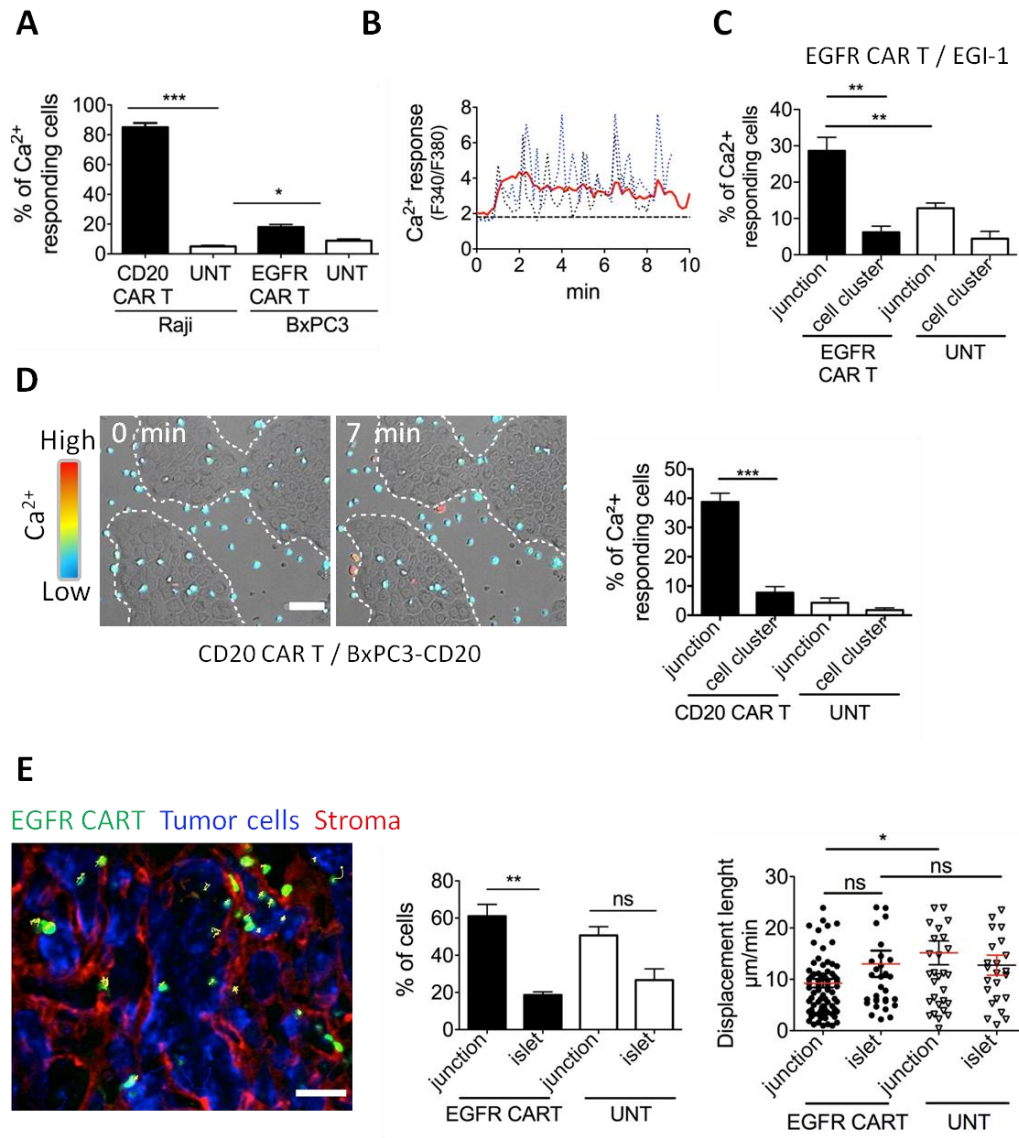


Figure S1



Supplemental Figure 1. CAR T cell responsiveness is dependent on the spatial orientation of carcinoma cells

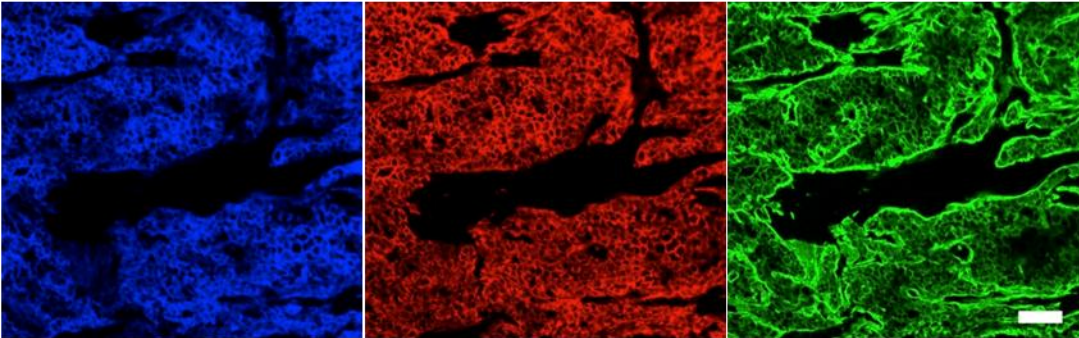
(A) Percentage of Ca²⁺ responding untransduced, CD20 and EGFR CAR T cells in contact with, respectively, Raji B cells or BxPC3 carcinoma cells. Results are shown as mean ± SEM; n = 100-150 cells/condition from 4-5 independent experiments; Student test: **P < 0.05, ***P < 0.001. **(B)** Ca²⁺ levels of two single low affinity EGFR CAR T cells (dotted blue lines) plotted against time. Red thick lines show average Ca²⁺ responses (15-20 cells.) Basal Ca²⁺ levels are indicated by black dotted lines. Note the presence of Ca²⁺ oscillations of large amplitude. **(C)** Proportion of Ca²⁺ responding EGFR CAR T and untransduced cells at the periphery or within the tumor cell cluster of the cholangiocarcinoma cell line EGI-1. Mean ± SEM; n = 30-50 cells/condition from 3 independent experiments; Student test: **P < 0.01. **(D)** Ca²⁺ response of CD20 CAR T cells contacting CD20-transduced BxPC3 tumor cells. CD20-BxPC3 cells were placed on glass coverslips the day before the experiment. CD20 CAR T cells loaded with fura 2-AM were added before image recording. **(D, left)** Snapshots of a time lapse showing Ca²⁺ increases in CD20 CAR T cells after their contacts with CD20-BxPC3 tumor cells. Scale bar: 50 μm. See also **Movie S4**. **(D, right)** Proportion of Ca²⁺ responding CD20 CAR T and untransduced cells at the periphery or within the tumor cell cluster of CD20-BxPC3 cells. **(E)** Concentration and migration of EGFR CAR T cells in human renal cell carcinoma tumor slices. **(E, left)** Representative images of EGFR CAR T cell distribution in a human renal cell carcinoma slice stained for EpCAM (tumor cells) and fibronectin (stroma). White dotted lines delineate tumor islets. See also **Movie S8**. **(E, middle)** Proportion of EGFR CAR and untransduced T cells in the stroma, tumor-stroma junctions and tumor islets of human renal cell carcinomas, represented as mean ± SEM; n = 2 independent experiments; Student test: **P < 0.01. **(E, right)** Displacement of EGFR CAR and untransduced T cells in the stroma, tumor-stroma junctions and tumor islets of human renal cell carcinomas represented as mean ± SEM; n = 2 independent experiments; Student test: *P < 0.05. Scale bar: 50 μm

Figure S2

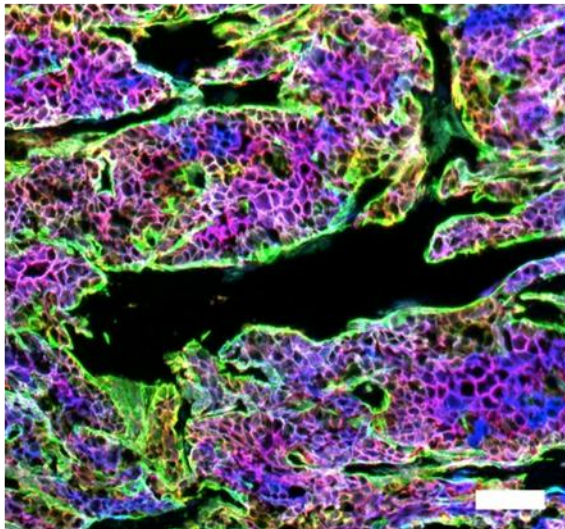
EpCAM

EGFR

CD104



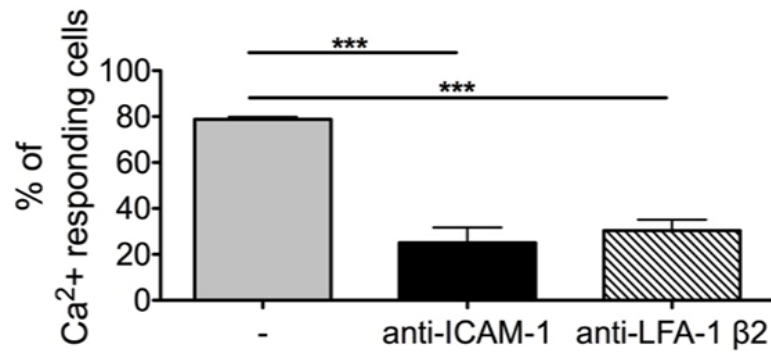
overlay



Supplemental Figure 2. Distribution of EpCAM, EGFR and CD104 in BxPC3 tumors.

Fixed cryosection (8- μ m thick) from a BxPC3 tumor (derived from a subcutaneous tumor cell implantation into NSG mice) immunostained with the indicated antibodies. Scale bar: 100 μ m.

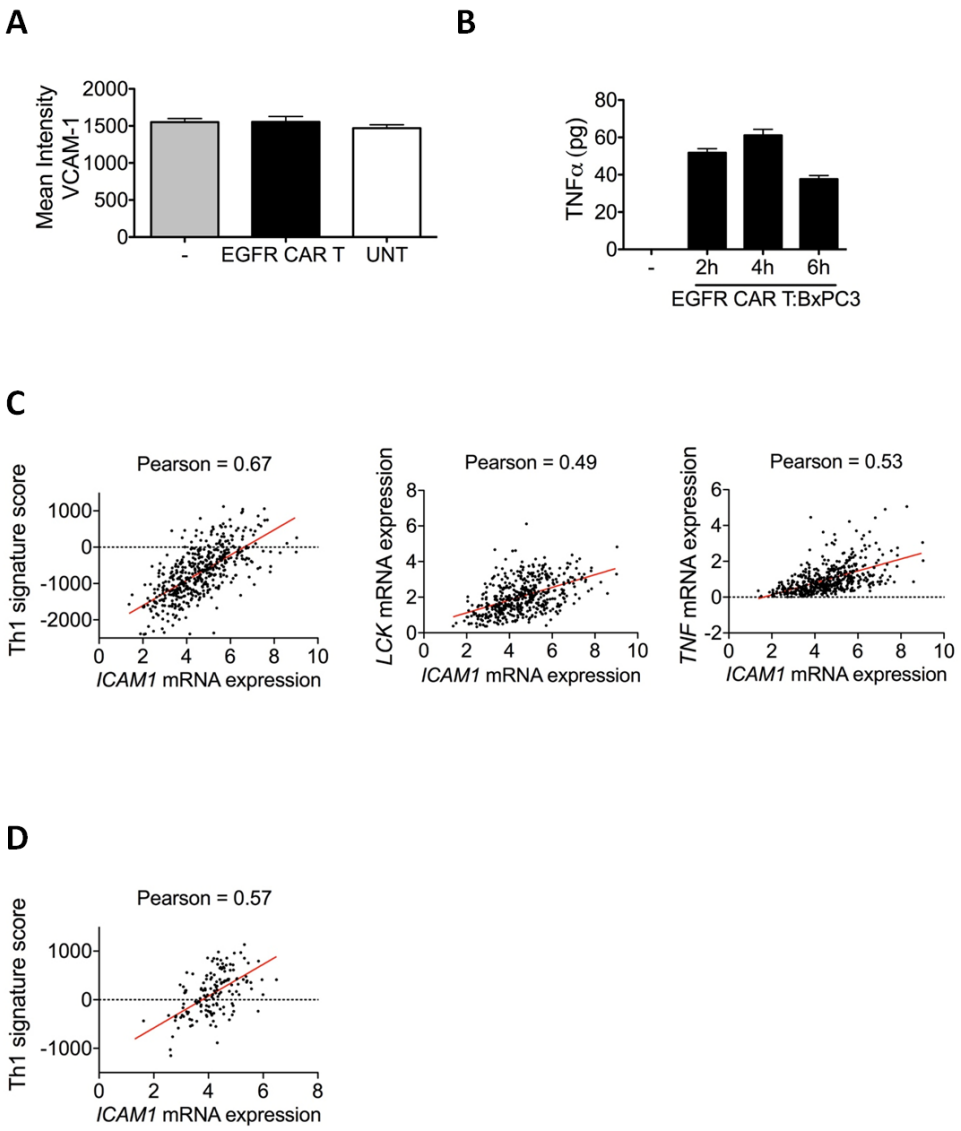
Figure S3



Supplemental Figure 3. Raji B cell-induced CD20 CAR T cell Ca²⁺ response is dependent on ICAM-1.

Proportion of Fura-2-loaded CD20 CAR T cells that increases their Ca²⁺ during their contacts with Raji B cells. Where indicated, ICAM-1 and LFA-1 were blocked with monoclonal antibodies (clones HA58 and TS1/18).

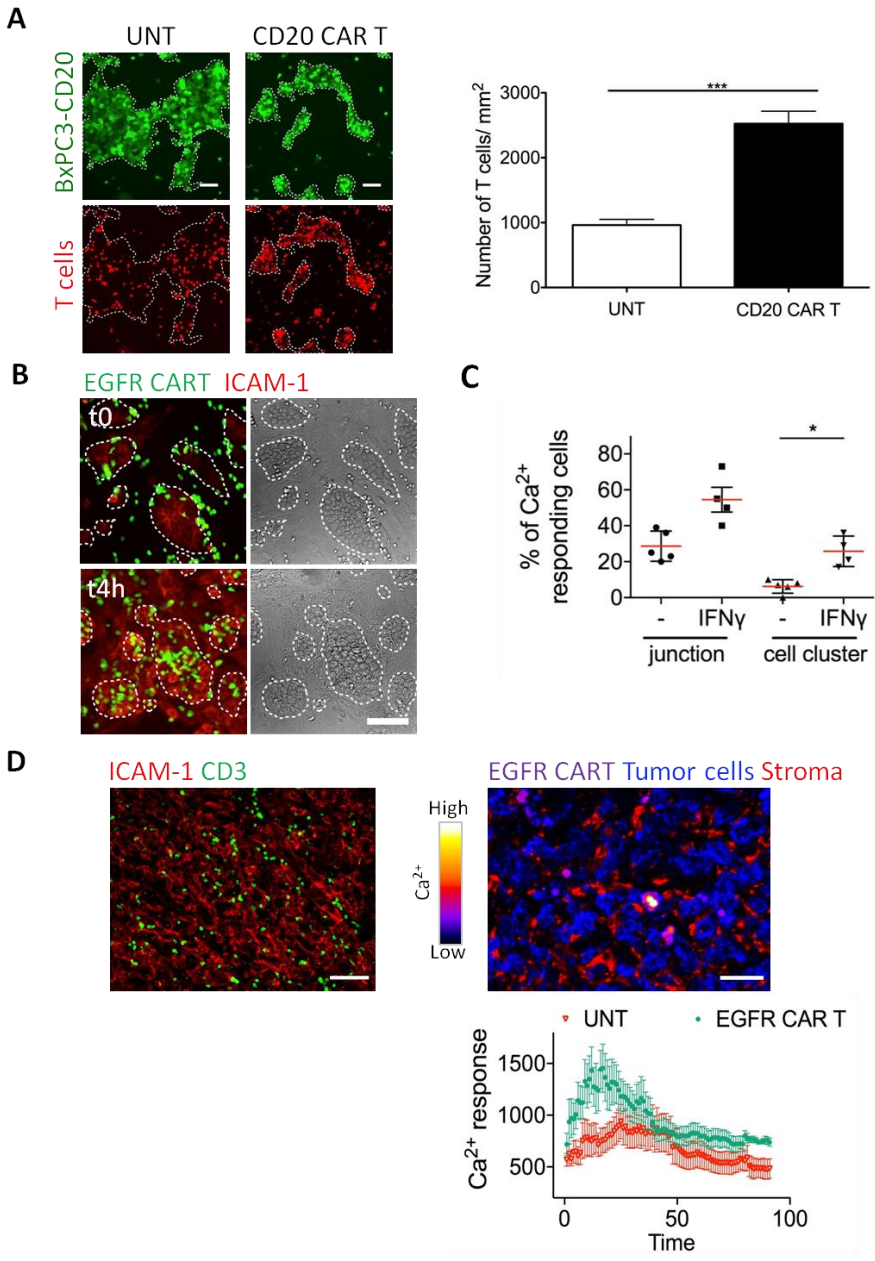
Figure S4



Supplemental Figure 4. ICAM-1 expression is associated with elevated T cell infiltration in human tumors

(A) EGFR CAR T cells did not mediate VCAM-1 expression by carcinoma cells. BxPC3 cells were left untreated, cultured with untransduced or EGFR CAR T cells for 4 h at the Effector: Target ratio of 5:1 and stained for VCAM-1. The histogram shows BxPC3 level of VCAM-1 fluorescent intensity. Mean \pm SEM; n = 3 independent experiments. (B) Amount of TNF α in the supernatant of EGFR CAR T - BxPC3 co-cultures determined by ELISA. Mean \pm SEM; n = 3. (C) Pearson correlation coefficients of ICAM-1 mRNA expression from the TCGA lung squamous cell carcinoma dataset with a Th1 signature score (left), Lck (middle) and TNF (right). (D) Pearson correlation coefficients of ICAM-1 mRNA expression from the TCGA pancreatic adenocarcinoma dataset with a Th1 signature score. R values are shown in the plots. P*** < 0.001 for all plots.

Figure S5



Supplemental Figure 5. CAR T cell-induced ICAM-1 expression by tumor cells facilitates lymphocyte activation and entry into tumor islets

(A, left) Snapshots showing untransduced and CD20 CAR T cells in relation to BxPC3-CD20 tumor cells at 4 h. Scale bar: 100 μ m. (A, right) Concentration of untransduced and CD20 CAR T cells in BxPC3-CD20 tumor cell regions. Mean \pm SEM; n = 3; Student test: ***P < 0.001. (B) Snapshots showing EGFR CAR T cells 4 h after adding them to BxPC3 - fibroblast coculture. Cells were stained for ICAM-1 before taking pictures. (C) Proportion of Ca²⁺ responding Fura-2-loaded EGFR CAR T cells at the junction and in clusters of EGI-1 cholangiocarcinoma cells pretreated or not with 10 ng/ml of IFN γ for 4 h. (D) Ca²⁺ responses of EGFR CAR T cells in human renal tumor slices positive for ICAM-1. (Left) A confocal picture of a human renal tumor slice stained with the indicated antibodies. (Right) A confocal picture of a slice from the same tumor showing the Ca²⁺ level of EGFR CAR T cells. Anti-EpCAM-1 and fibronectin Abs were used to visualize tumor cells and the stroma. See also **Movie S9**. (Lower) Average Ca²⁺ responses of untransduced and EGFR CAR T cells (15-20 cells) in human renal tumor slices plotted against time, represented as mean \pm SEM.

Supplementary Videos

Movie S1. Ca²⁺ responses of Fura-2-loaded CD20 CAR T cells during their interaction with Raji B cells. Cells were imaged with a wide-field microscope. By comparison, the behaviour of untransduced T cells is shown on the right. [Ca²⁺]_i of fura-2-loaded CD20 CAR T cells is displayed in color ranging from blue (low level) to red (high level). Frame interval is 10 s. A still image is shown in Fig. 1A.

Movie S2. Ca²⁺ responses of Fluo-4-loaded CD20 CAR T cells introduced into a slice made from a Raji tumor. Cells were imaged with a confocal spinning disk microscope. By comparison, the behaviour of untransduced T cells is shown on the right. [Ca²⁺]_i of CD20 CAR T cells is displayed in color ranging from violet (low level) to high (high level). T cell trajectories are shown white dotted lines. Frame interval is 15 s. A still image is shown in Fig. 1C.

Movie S3. Ca²⁺ responses of Fura-2-loaded EGFR CAR T cells during their interaction with BxPC3 tumor cells. Note that CAR T cells are activated at the periphery of tumor cell regions. Cells were imaged with a wide-field microscope. [Ca²⁺]_i of fura-2-loaded CD20 CAR T cells is displayed in color ranging from blue (low level) to red (high level). Frame interval is 10 s. A still image is shown in Fig. 2A.

Movie S4. Ca²⁺ responses of Fura-2-loaded CD20 CAR T cells during their interaction with CD20-expressing BxPC3 tumor cells. Same as in Movie S3. A still image is shown in Fig. S2D.

Movie S5. EGFR CAR T cells accumulate and stop at the periphery of BxPC3 tumor islets. CAR T cells were introduced into a slice made from a BxPC3 tumor that was subsequently stained for EpCAM (tumor cells) and fibronectin (stroma). Cells were imaged with a confocal spinning disk microscope. The animation represents a three-dimensional (3D) reconstruction of a sequential z series. Frame interval is 20 s. A still image is shown in Fig. 2B.

Movie S6. Ca²⁺ responses of Fluo-4-loaded EGFR CAR T cells in a vibratome section of a BxPC3 tumor. The slice was stained for EpCAM (tumor cells) and fibronectin (stroma) before imaging with a confocal spinning disk microscope. [Ca²⁺]_i of CD20 CAR T cells is displayed in color ranging from violet (low level) to high (high level). White arrows indicate two CAR T cells that increase their Ca²⁺ at the periphery of tumor islets. Frame interval is 15 s. A still image is shown in Fig. 1C.

Movie S7. Distribution and migration and EGFR CAR T cells in a vibratome section of a human lung tumor. The slice was stained for EpCAM (tumor cells) and fibronectin (stroma) before imaging with a confocal spinning disk microscope. The animation represents a three-dimensional (3D) reconstruction of a sequential z series. Frame interval is 20 s. A still image is shown in Fig. 2D.

Movie S8. Distribution and migration and EGFR CAR T cells in a vibratome section of a human renal tumor. Same as in Movie S7. A still image is shown in Fig. S1E.

Movie S9. Ca²⁺ responses of Fluo-4-loaded EGFR CAR T cells in a vibratome section of a human lung tumor. Same as in Movie S6. A still image is shown in Fig. S5D.