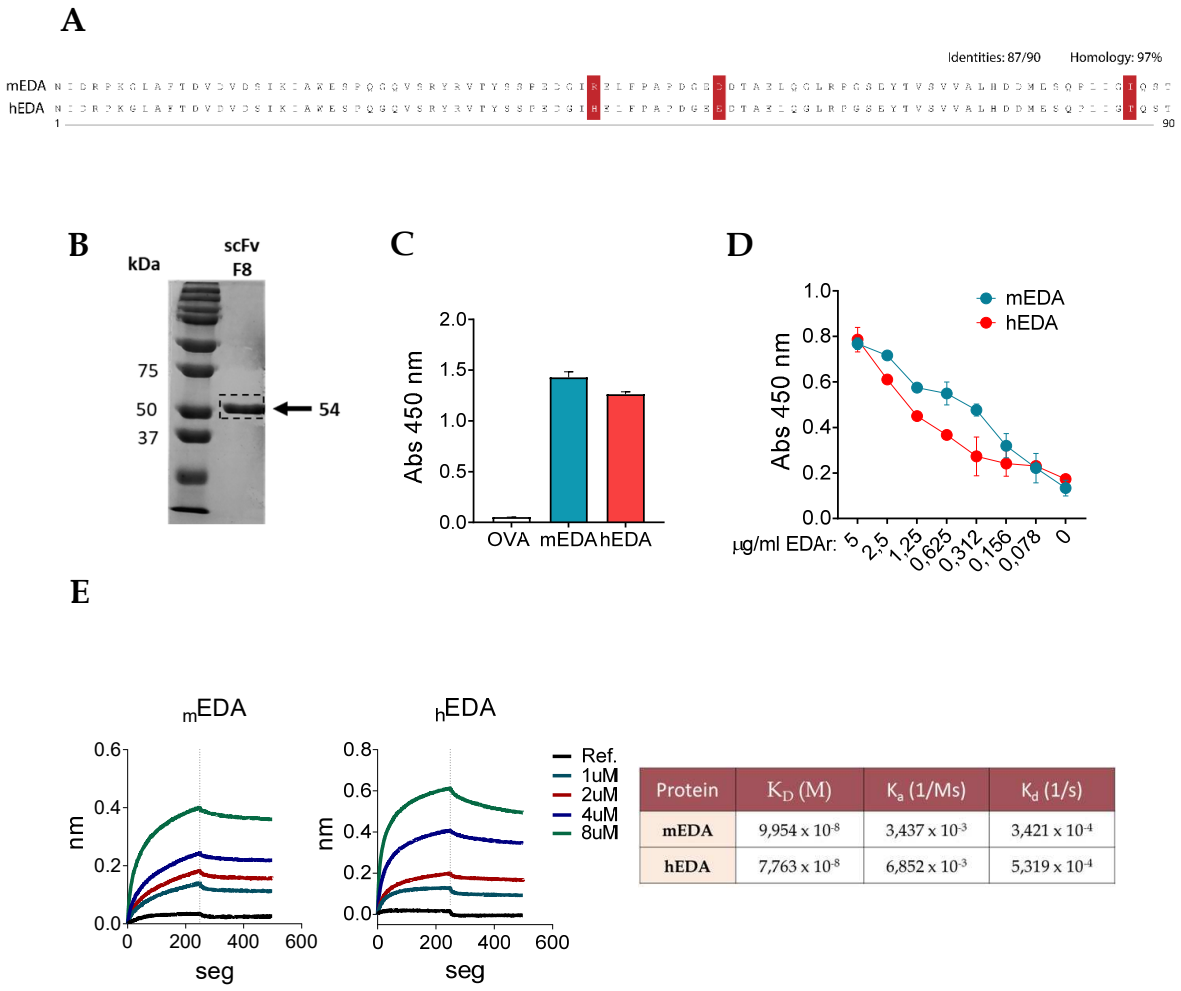
BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)

Supplementary Figures:
Targeting the Extra Domain A of Fibronectin for Cancer Therapy with CAR-T cells.

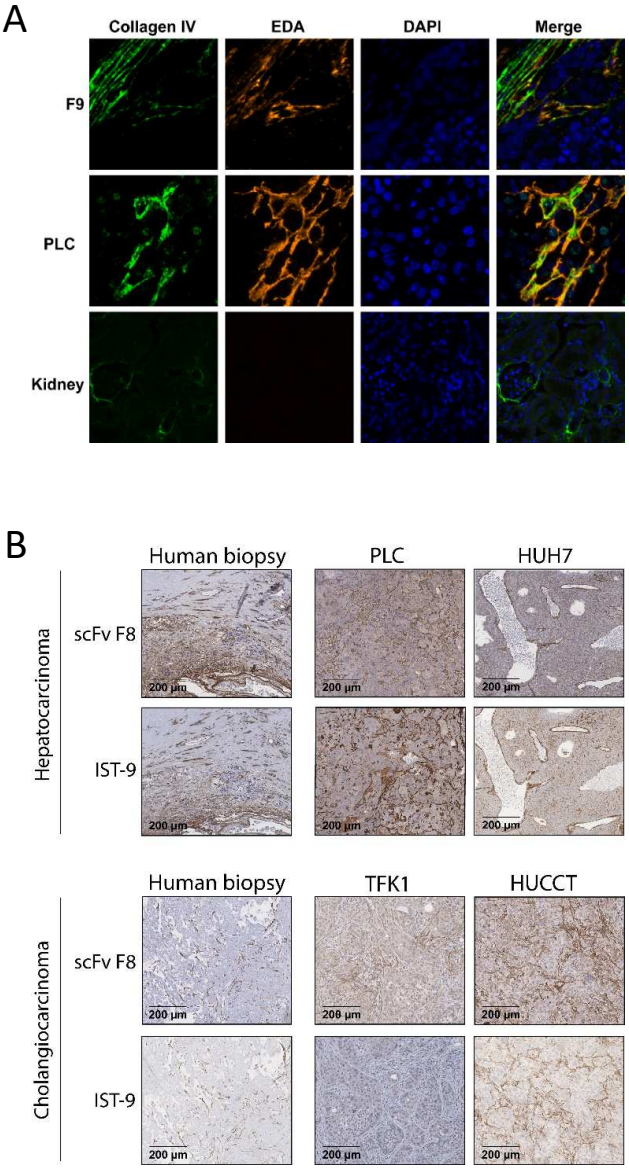
Martín-Otal Celia, Lasarte-Cia Aritz, Serrano Diego, Casares Noelia, Conde Enrique, Navarro Flor, Sanchez-Moreno Inés, Gorraiz Marta, Sarrión Patricia, Calvo Alfonso, De-Andrea Carlos, Echeveste José, Vilas Amaia, Rodriguez-Madoz Juan Roberto, San Miguel Jesús, Prosper Felipe, Hervás-Stubbs Sandra, Lasarte Juan José, Lozano Teresa



Protein	K_D (M)	K_a (1/Ms)	K_d (1/s)
mEDA	9,954 x 10^{-8}	3,437 x 10^{-3}	3,421 x 10^{-4}
hEDA	7,763 x 10^{-8}	6,852 x 10^{-3}	5,319 x 10^{-4}

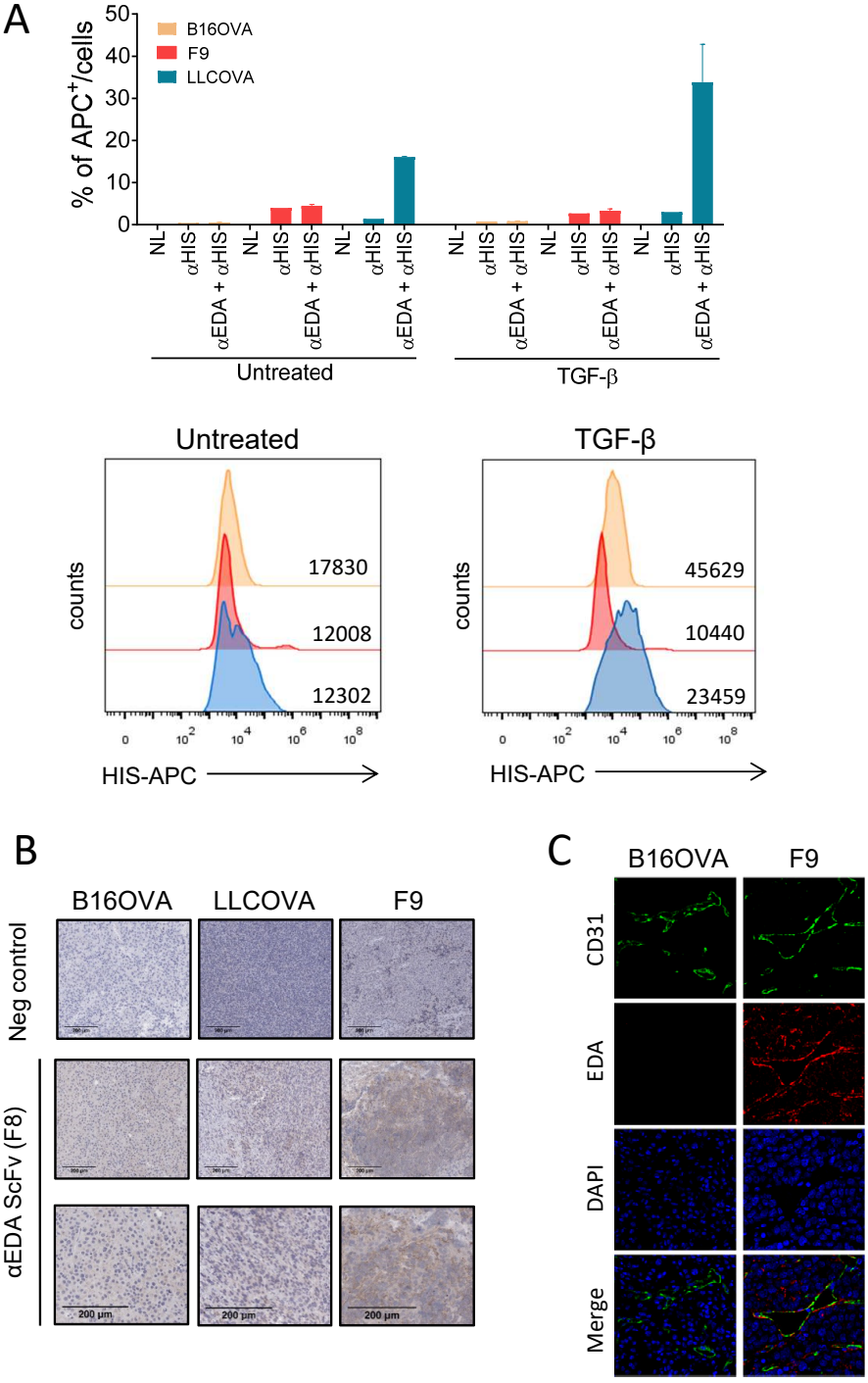
Supplementary Figure 1: Production and characterization of the anti EDA scFv F8. (A) Human and mouse EDA aminoacid sequence alignment. (B) Coomassie blue staining of the polyacrylamide gel with the purified scFv F8. (C, D) Recognition of human and mouse EDA proteins coated to ELISA plates by scFv F8 antibody fragment. (D) Titration experiment for EDA recognition using different human or mouse EDA concentrations coated to the ELISA plates. (E) Analysis of equilibrium dissociation constant (KD) of scFv F8 to soluble EDAs by using Bio-Layer Interferometry (BLI) technique. scFv F8 was coated to the AR2G sensor and different concentrations of the recombinant proteins are tested. Ref: Control buffer.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



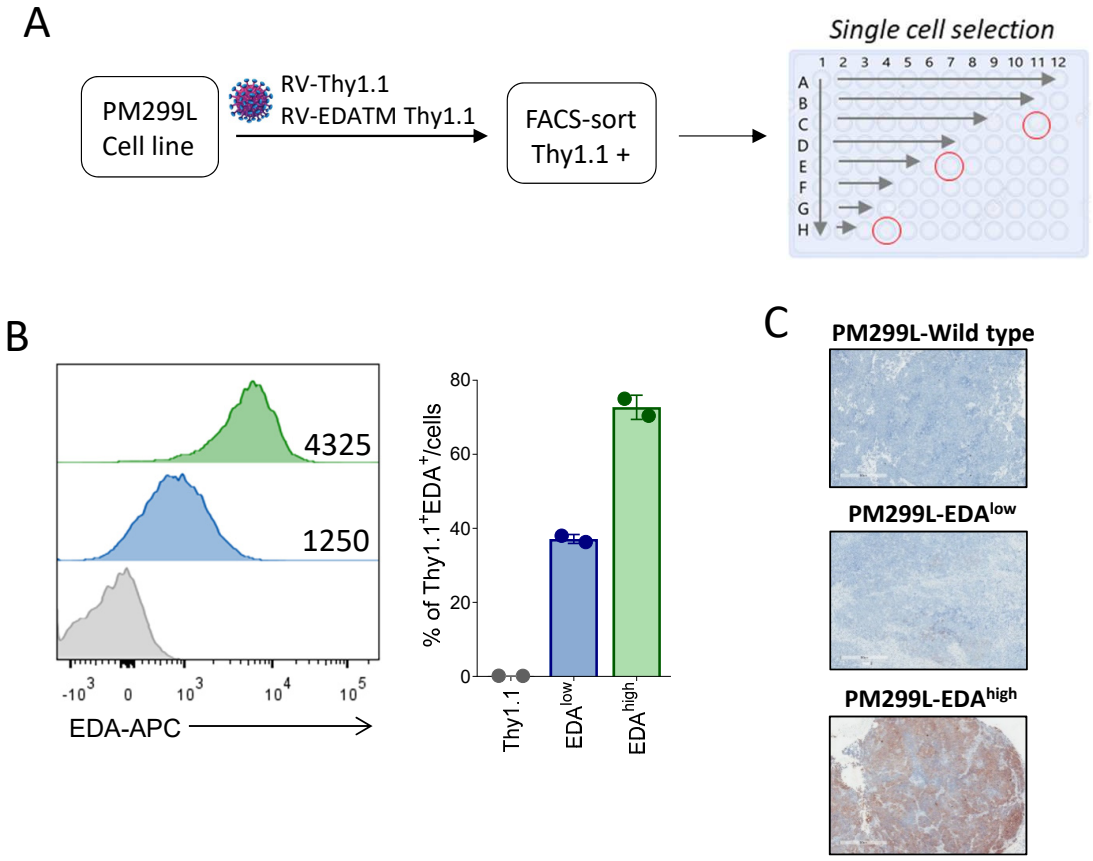
Supplementary Figure 2: Measurement of EDA expression in tissues: (A) Co-localization experiments of EDA and type IV collagen in F9 and PLC tumors as well as normal kidney. Paraffin-embedded tissue sections were labeled with anti-Collagen-IV, EDA antibodies and DAPI for nuclear counterstain and studied by confocal microscopy. **(B)** EDA expression in tumor samples from NSG mice challenged with human hepatocarcinoma and cholangiocarcinoma cell lines using the anti-human EDA IST-9 antibody and the scFv F8 that detects the murine and the human versions of EDA protein. Human biopsies from patients with hepatocarcinoma or cholangiocarcinoma were used as controls for EDA staining with each antibody.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



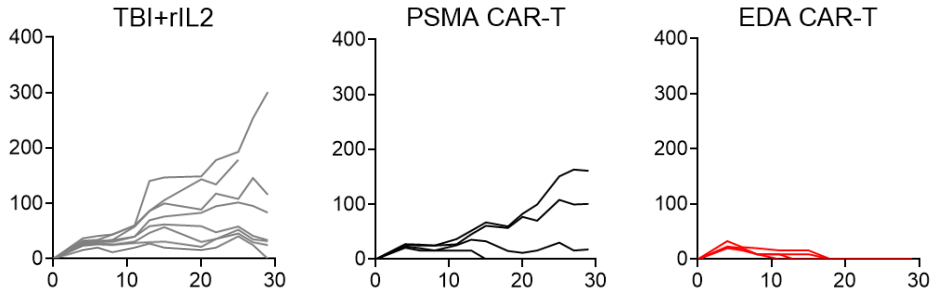
Supplementary Figure 3. Evaluation of EDA expression in different tumor murine cell lines and tumors. (A) B16-OVA melanoma, LLC-OVA lung carcinoma and F9 teratocarcinoma were cultured in vitro in the absence or presence of TGF-β for 16 h and stained for the analysis of EDA expression by flow cytometry (upper panel). Representative histograms of the flow cytometry analysis summarizing the results obtained (lower panel). **(B)** EDA expression in tumor tissues induced in mice after subcutaneous injection of the indicated cell lines at day 15 after tumor challenge. Representative examples of the immunohistochemical analysis. **(C)** EDA/CD31 co-localization experiments in B16-OVA and F9 derived tumor sections at day 15 after tumor challenge.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



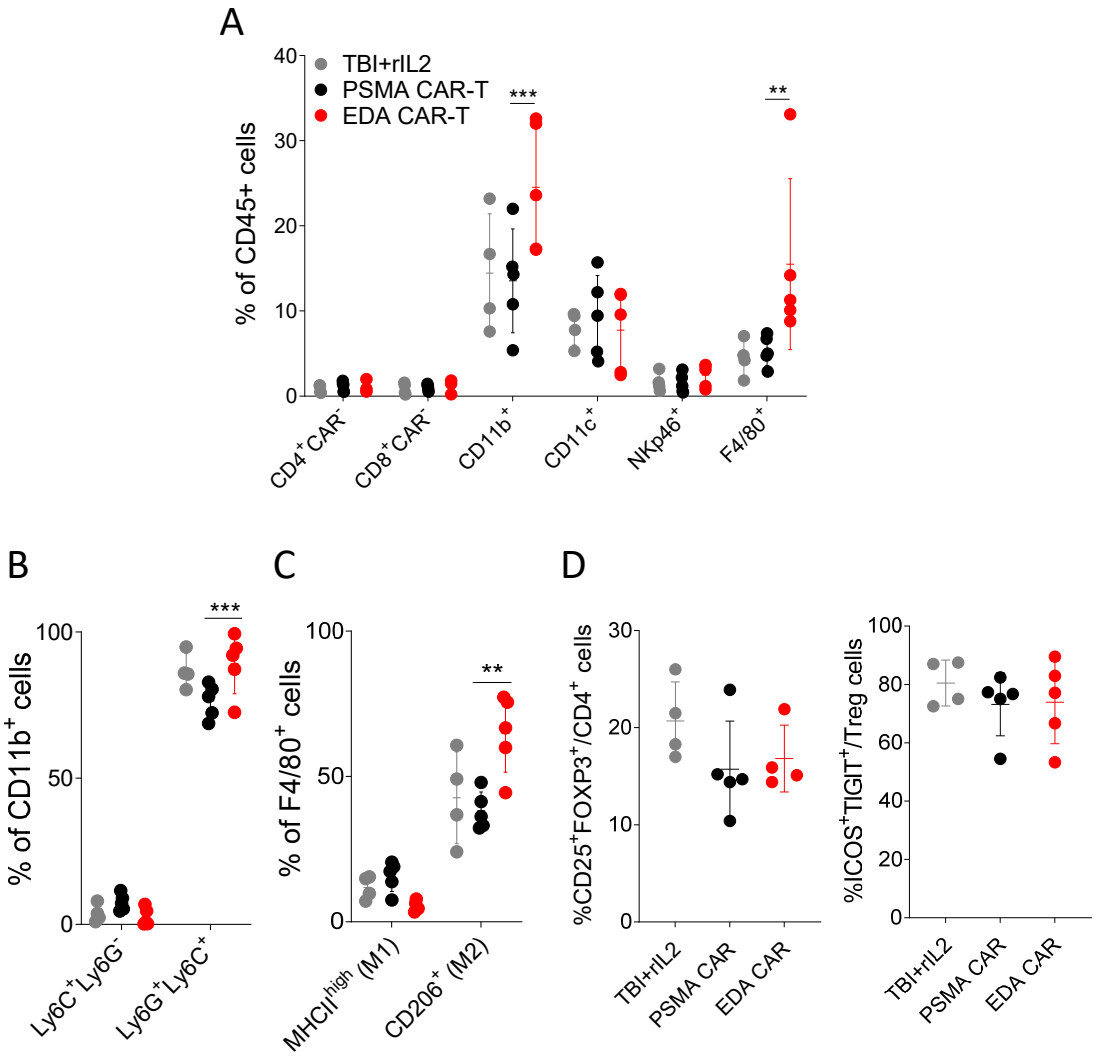
Supplementary Figure 4. Generation and characterization of EDA expressing PM299L tumor cell clones. **(A)** Schematic representation of the procedure for clone selection. **(B)** Flow cytometry analysis of EDA expression levels of different PM299L EDA expressing clones. Numbers in histograms indicate mean fluorescence intensity. **(C)** Immunohistochemical analysis of EDA expression of tumor tissues obtained from C57BL/6 mice challenged with the indicated PM299L EDA expressing clones at day 14 after injection . PM299L-EDA^{high}, PM299L-EDA^{low} and PM299L-wt (Thy 1.1) as EDA^{-} control cell line were used.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



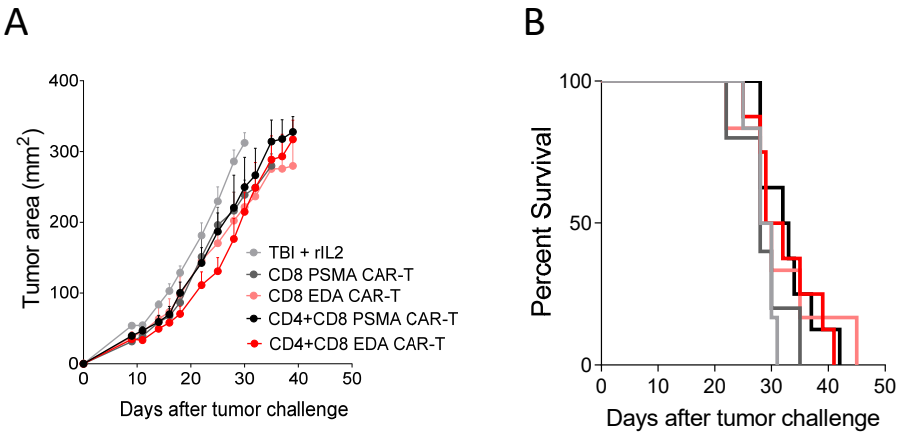
Supplementary Figure 5. Antitumor activity of EDA CAR-T therapy in EDA+ tumors. C57BL/6 J mice were injected subcutaneously with 2×106 cells PM299L-EDAhigh tumor cells. At day 7, mice bearing tumors were treated by adoptive transfer with 9×106 EDA CAR-T or with PSMA CAR-T cells as a control. Tumor growth for individual mice is plotted.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



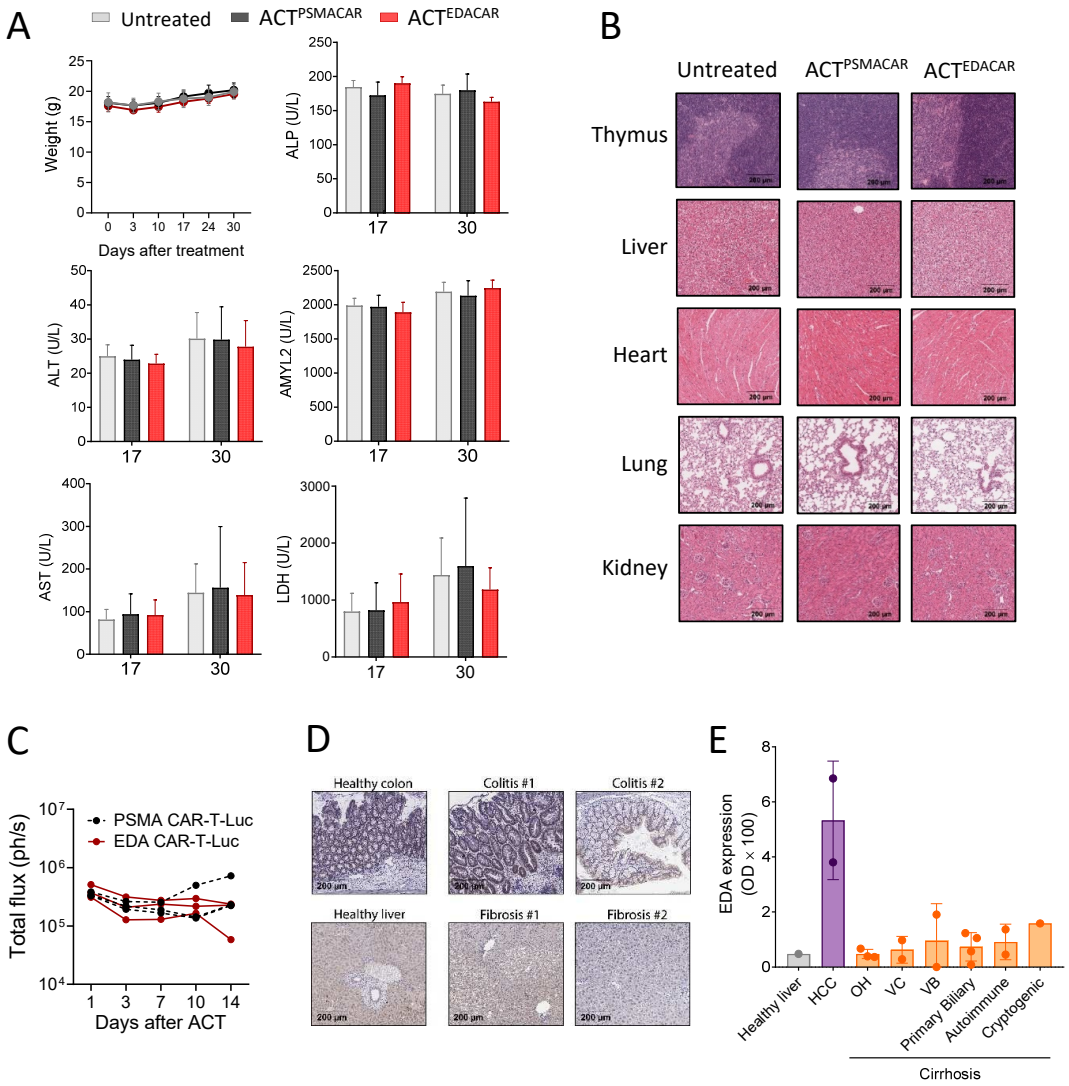
Supplementary Figure 6. Flow cytometry analysis of leukocyte infiltration into the F9 tumors treated with EDA CAR-T, PSMA CAR-T or treated only with TBI+rIL2. **(A)** Percentage of CD4$^+$ CAR$^-$, CD8$^+$CAR$^-$, CD11b, CD11c, NKp46 or F4/80$^+$ cells in total CD45$^+$ cells. **(B)** Percentage of Ly6G$^+$Ly6C$^-$ and Ly6G$^+$Ly6C$^+$ in total CD11b$^+$ cells. **(C)** Percentage of MHCII^{high} (M2) and CD206 (M2) cells in total F4/80$^+$ cells. **(D)** Percentage of CD25$^+$Foxp3$^+$ cells in total CD4$^+$ cells and percentage of ICOS$^+$TIGIT$^+$ cells in total Treg cells . (n = 5 mice per group). **p<0.01, ***p<0.001. One-way ANOVA with Bonferroni multiple comparison. The mean and SD for each condition are plotted

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



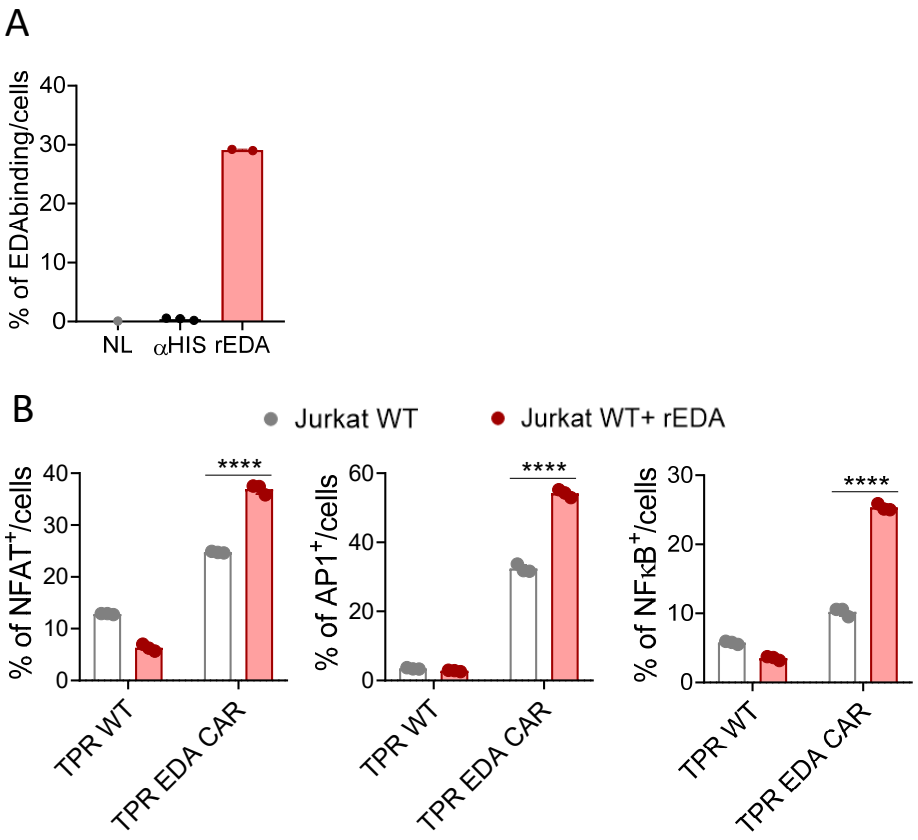
Supplementary Figure 7. Lack of efficacy of EDA CAR-T cells in mice bearing B16OVA tumor cells. C57BL/6 mice bearing tumors were treated 7 days after tumor challenge with $CD8^+$ or a mixture of $CD4^+$ and $CD8^+$ EDA CAR-T or PSMA CAR-T lymphocytes. **(A)** Mean tumor growth at different time points and **(B)** Kaplan–Meier plot of survival are plotted. (n = 6-8 mice per group).

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



Supplementary Figure 8. Lack of toxicity after EDA CAR-T cell administration in mice. *Naïve* C57BL6 J mice were treated with 1×10^7 CAR-T cells (5×10^6 CD8 and 5×10^6 CD4). (**A**) Body weight was measured periodically during all the follow up and serum samples were analyzed at day 17 and day 30 to measure biochemical parameters; AST: aspartate aminotransferase, ALT: alanine aminotransferase, serum albumin, AMYL2: alpha Amylase, Urea, Creatinine, CRPLX: C-reactive protein, ALP: alkaline phosphatase, LDH: lactate dehydrogenase (**B**) H&E staining of thymus, liver, hearth, lung, and kidney tissue sections obtained at day 30 of the follow up (scale bar 200 µm; right, scale bar 50 µm). **(C)** Naive C57BL6 were injected with EDA CAR -T luciferase (EDA CAR-T Luc) and PSMA CAR-T luciferase (PSMA CAR-T luc) and luciferase activity was analyze over time. total flux (photons/s) is ploted at day 1,3, 7, 10, 14 after treatment. **(D)** EDA expression in an animal model of liver fibrosis induced by CCL4 treatment and a model of inflammatory colitis induced by DSS administration. The corresponding tissue sections from untreated healthy mice were used as controls. **(E)** EDA expression detected in human samples from a healthy liver, from 3 patients with alcoholic cirrhosis, 2 patients with HCV and 2 HBV related cirrhosis, 4 primary biliary cirrhosis, 2 autoimmune and 1 cryptogenic cirrhosis as compared to that observed in patients with hepatocarcinoma (HCC).

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance placed on this supplemental material which has been supplied by the author(s)



Supplementary Figure 9. Flow cytometry analysis of the activation of NFAT, AP1 and NF-kB in the triple parameter reporter Jurkat TPR cell line expressing the EDA-CAR in response to stimulation with wild type Jurkat cells previously incubated with EDA protein. (A) Measurement of EDA binding to wild type Jurkat cells. Cells were incubated with 25 µg/ml of EDA protein for 30 min at 37 ºC, washed and labeled with anti-His-APC labelled antibodies. EDA binding to Jurkat cells was then measured by flow cytometry. **(B)** Activation of NFAT, AP1 or NF-kB in Jurkat TPR reporter cell line in response to their co-culture with wild type Jurkat cells previously incubated with EDA. ****p<0.0001. Two-way ANOVA with Bonferroni multiple comparisons test. NL: non labelled