Supplementary Information

Dynamic regulatory T cell-APC interactions locally promote intratumoral CTL dysfunction

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Supplementary Video Guide

Video S1: HA-Treg migrating in tumor stroma and parenchyma I
Video S2: HA-Treg migrating in tumor stroma and parenchyma I (slices)
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Video S6: HA-Treg interacting with $CD11c^+$ DC in tumor tissue
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Supplementary Video legends

Supplementary Video 1: HA-Treg migrating in tumor stroma and parenchyma I. A recording from a CT26HA tumor implanted into a dorsal skinfold chamber and infiltrated by GFP-expressing HA-Treg (green) and HA-CTL (not visualized). CT26HA tumor cells express histone H2B-Cerulean, which labels their nuclei (blue). The animal was injected with 150 kDa TRITC-dextran 10 min before the recording to monitor perfusion of the tumor blood vessels (red). Each individual frame is a maximum intensity projection of 11 z-stacks spaced 5 μm apart (total thickness of 50μm). Scale bar = 50 μm. Time is shown in minutes and seconds.

Supplementary Video 2: HA-Treg migrating in tumor stroma and parenchyma I (slices). Individual optical sections from same recording as shown in video 1A highlight that HA-Treg (green) deeply infiltrate the tumor parenchyma. Numbers at top indicate depth below tissue surface. Scale bar = 100 μm. Time is shown in minutes and seconds.

Supplementary Video 3: HA-Treg migrating in tumor stroma and parenchyma II. A recording from a CT26HA tumor implanted into a dorsal skinfold chamber and infiltrated by GFP-expressing HA-Treg (green) and HA-CTL (not visualized). CT26HA tumor cells express histone H2B-Cerulean, which labels their nuclei (blue). The animal was injected with 150 kDa TRITC-dextran 2 hours before the recording to monitor perfusion of the tumor blood vessels and to subsequently label intratumoral phagocytes (red). Each individual frame is a maximum intensity projection of 12 z-stacks spaced 5 μm apart (total thickness of 55μm). Scale bar = 50 μm. Time is shown in minutes and seconds.

Supplementary Video 4: HA-Treg interacting with TRITC-dextran-labeled phagocytes. Magnified views from Videos 1 and 2. Top panels show HA-Treg transiently arresting in the vicinity of phagocytes, while bottom panels show HA-Treg migrating freely in areas largely devoid of dextran-uptake. Scale bar = 20 μm. Time is shown in minutes and seconds.

Supplementary Video 5: HA-CTL migrating in tumor stroma and parenchyma. A recording from a CT26HA tumor implanted into a dorsal skinfold chamber, infiltrated by histone H2B-mRFP-labeled HA-CTL (shown in green). CT26HA cell express H2B-Cerulean, which labels their nuclei (blue). The animal was injected with Qtracker 655 non-targeted quantum dots to monitor perfusion of tumor blood vessels (red). Each

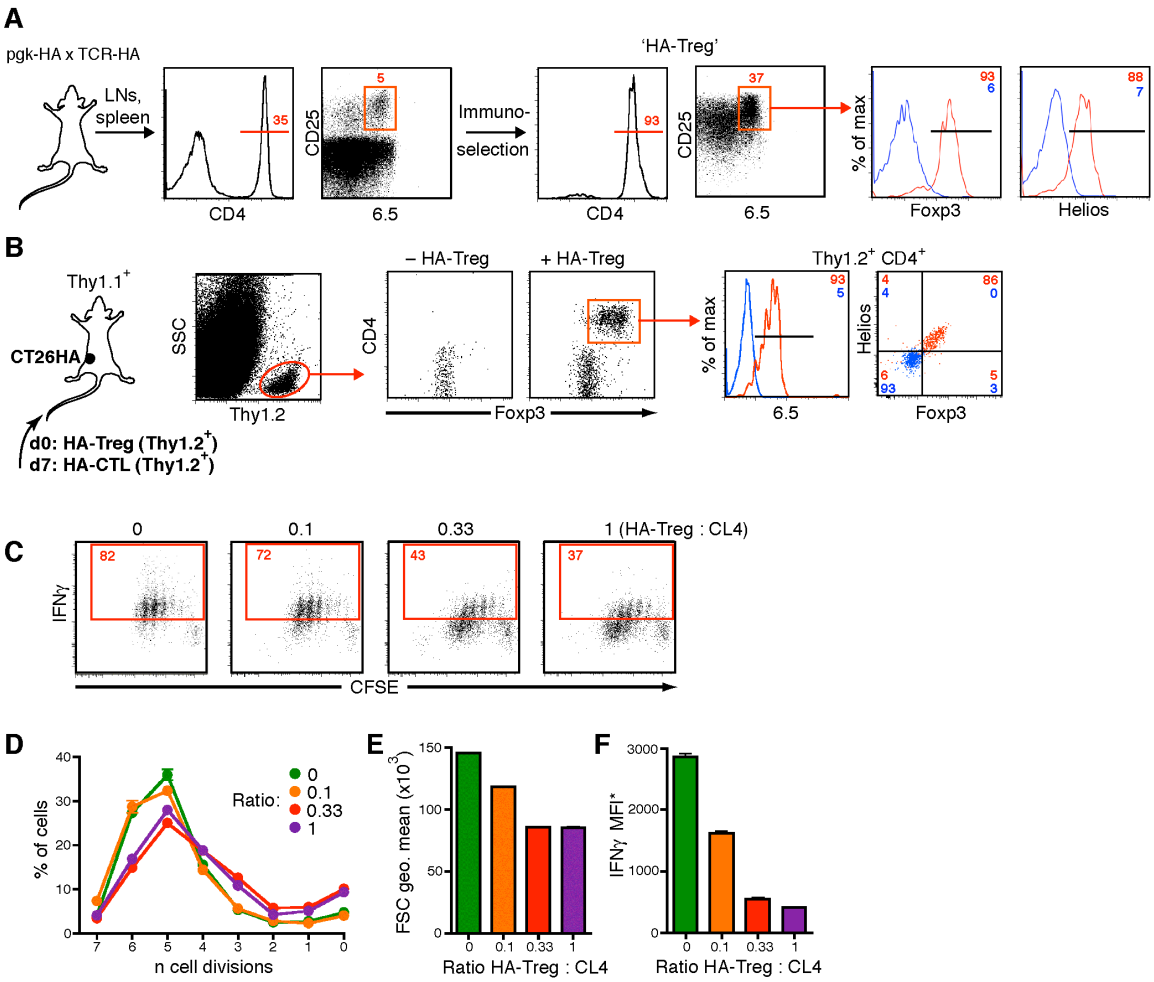
individual frame is a maximum intensity projection of 12 z-stacks spaced 4 μm apart (total thickness of 44μm). Scale bar = 50 μm. Time is shown in minutes and seconds.

Supplementary Video 6: HA-Treg interacting with CD11c$^+$ DC in tumor tissue. Recordings from a CT26HA tumor implanted into a dorsal skinfold chamber on a F1 (CD11c-mCherry x Balb/C) animal. Infiltrating HA-Treg (green) transiently interrupt their migratory activity to undergo unstable interactions with mCherry$^+$ DC (red). Tumor cells express H2B-Cerulean, which labels their nuclei (blue). Note the characteristic tethering of HA-Treg to mCherry$^+$ cells during disengagement (2nd panel from left). Scale bar = 20 μm. Time is shown in minutes and seconds.

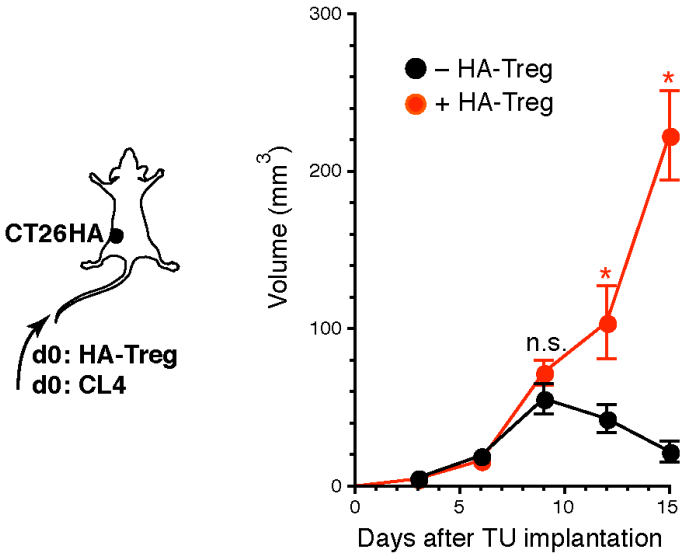
Supplementary Video 7: HA-Treg and HA-CTL migrating in tumor stroma and parenchyma. A recording from a CT26HA tumor implanted into a dorsal skinfold chamber and infiltrated by GFP-expressing HA-Treg (green) and tdTomato-expressing HA-CTL (red). CT26HA tumor cells express histone H2B-Cerulean, which labels their nuclei (blue). The animal was injected with Qtracker 655 non-targeted quantum dots to monitor perfusion of the tumor blood vessels (white). Each individual frame is a maximum intensity projection of 11 z-stacks spaced 5 μm apart (total thickness of 50μm). Scale bar = 50 μm. Time is shown in minutes and seconds. Time is shown in minutes and seconds.

Supplementary Video 8: HA-Treg migrating in close vicinity to HA-CTL. Magnified views of from Video 5 (Blue and white channel omitted for clarity). Each panel shows an arrested HA-CTL (red) and a HA-Treg (green), who continuously localize at close distance to each other, but do not form a stable cell-cell interface suggestive of a direct interaction. Scale bar = 10 μm. Time is shown in minutes and seconds.

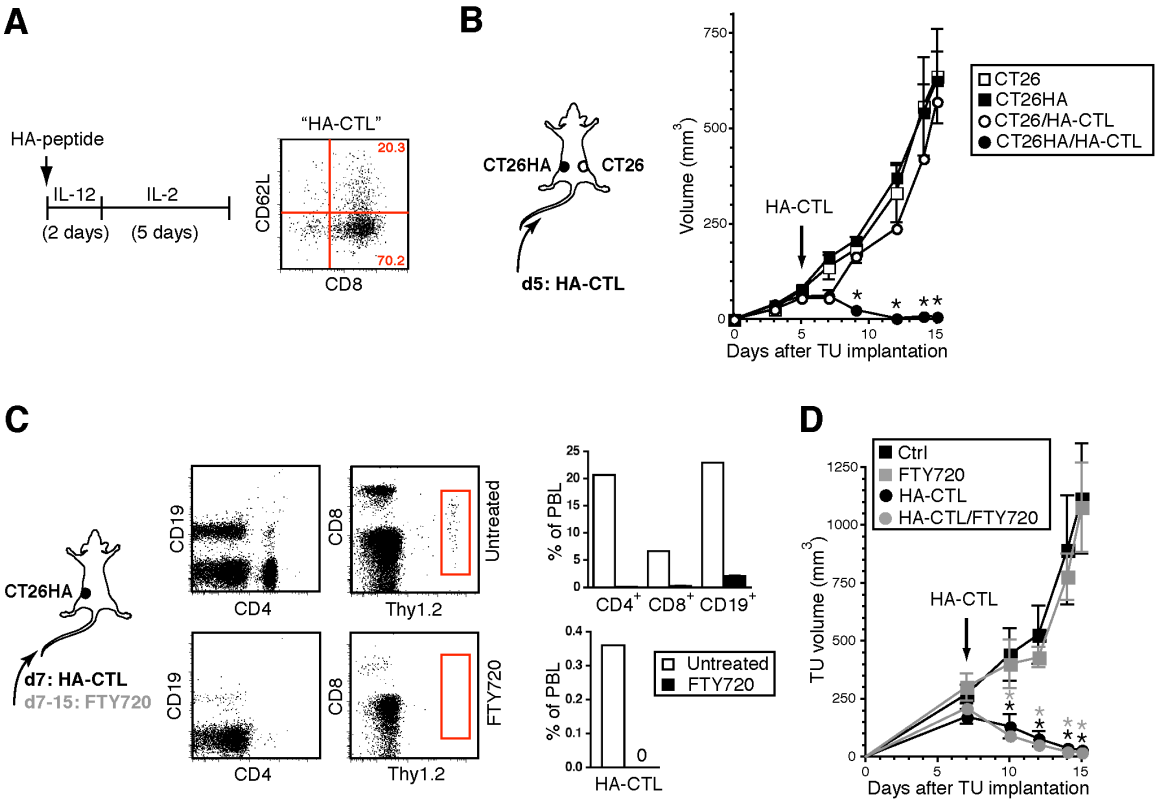
Supplementary Figures



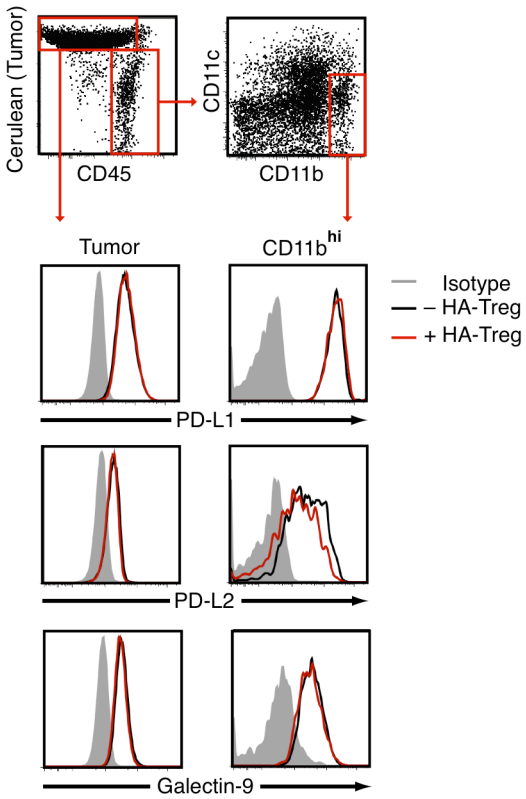
Supplementary Figure 1. Foxp3$^+$ HA-specific Treg cells ("HA-Treg") that suppress CD8 T cells activation *in vitro*. (**A**) LN and spleen cells were harvested from Thy1.2$^+$ pgk-HA x TCR-HA mice and enriched by immuno-magnetic selection for CD4$^+$ CD25^{hi} cells. Typically >1/3 of these stained with the clonotypic antibody 6.5 identifying T cells expressing the TCR-HA receptor at high density. (**B**) HA-Treg continue to express Foxp3, the TCR-HA, and Helios in CT26HA tumors 9 days after transfer. (**C-F**) CFSE-labeled, naïve CL4 T cells were co-cultured with varying numbers of HA-Treg and a fixed number of APC in presence of $HA_{515-523}$ and $HA_{107-119}$ peptides. HA-Treg have a modest effect on proliferation (**D**), but a pronounced effect on cell size (**E**) and IFN-γ-expression (**F**) of CL4 T cells. Graphs in E, F indicate means and SD.



Supplementary Figure 2. Growth of CT26HA tumors in the flank after injection at day 0 with naïve HA-specific CL4 $CD8^+$ T cells with out or without HA-Treg. The experiment shown is representative of two with similar results; data represent n=5 animals/group; Means ±SEM are shown; * indicates $p<0.05$.



Supplementary Figure 3. Adoptively transferred HA-CTL reject CT26HA tumors without need for reactivation in tumor-draining LNs. (**A**) Splenocytes from CL4 TCR transgenic mice were loaded with $HA_{515-523}$ peptide for 1 hour and cultured with mouse IL-12 for 2, and with mouse IL-2 for the subsequent 5 days. This produced a population of $CD8^+$, mostly $CD62L^{neg}$ effector T cells ("HA-CTL"). (**B**) HA-CTL transferred into mice implanted 5 days earlier with both CT26 and CT26HA tumors reject HA-expressing, but not control CT26 tumors. HA-expression did not alter growth kinetics of CT26HA compared to CT26 tumors in absence of HA-CTL transfer. (**C**) Treatment of tumor-bearing mice with the functional sphingosine-1-phosphate receptor antagonist FTY720 starting at the time of HA-CTL transfer prevents egress of lymphocytes from LNs and thus caused profound lymphopenia. It also prevents the egress of HA-CTL that had migrated to tumor-draining LNs and thereby their appearance in peripheral blood 3 days after transfer. Thus FTY720 treatment prevented HA-CTL that migrated to tumor-draining LNs from subsequently entering tumors and contributing to their rejection. (**D**) FTY720 treatment did not affect the ability of HA-CTL to reject CT26HA tumors, indicating that HA-CTL that directly migrated to tumors sufficed to reject these. FTY720 did not affect tumor growth in animals that did not receive HA-CTL. Data in (B) and (D) represent for 3 mice per group. All graphs indicate means, B and H show SEM; * indicates $p<0.05$ against all other groups in (B) and $p<0.05$ in comparison between Ctrl and HA-CTL (black symbols) or FTY720 and HA-CTF/FTY720 (grey symbols) in (D).



Supplementary Figure 4. HA-Treg do not alter expression of PD-L1, PD-L2, and Galectin-9 on APC in tumor tissue. (**A**) BALB/c mice were injected with HA-Treg or not and subsequently implanted with CT26HA tumors in the flanks. On day 7 HA-CTL were transferred and 3 days later expression of PD-L1, PD-L2, and galectin-9 on Cerulean^{+} tumor cells and CD11c^{-} CD11b^{hi} cells was analyzed. Black lines: without HA-Treg; red histograms: with HA-Treg; grey histograms: isotype control staining.