Supplementary Information

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

Christian A. Bauer, Edward Y. Kim, Francesco Marangoni, Esteban Carrizosa, Natalie M. Claudio and Thorsten R. Mempel

1. Supplementary Video Guide
2. Supplementary Video Legends
2. Supplementary Figures
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)
Video S3: HA-Treg migrating in tumor stroma and parenchyma II
Video S4: HA-Treg interacting with TRITC-dextran-labeled phagocytes
Video S5: HA-CTL migrating in tumor stroma and parenchyma
Video S6: HA-Treg interacting with CD11c+ DC in tumor tissue
Video S7: HA-Treg and HA-CTL migrating in tumor stroma and parenchyma
Video S8: Co-localization of motile HA-Treg and arrested HA-CTL

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 50 µm). 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 55 µm). 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 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+ CD25hi 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 HA515-523 and HA107-119 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 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\textsubscript{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\textsuperscript{+}, mostly CD62L\textsuperscript{-} 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⁻ CD11bhi cells was analyzed. Black lines: without HA-Treg; red histograms: with HA-Treg; grey histograms: isotype control staining.