Supplemental Data

CD44 Mediates Successful Interstitial Navigation by Killer T Cells and Enables Efficient Antitumor Immunity

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Supplemental Experimental Procedures

Generation and transduction of CTL

Single cell suspensions from spleens of TCR transgenic OT-I animals were stimulated with SIINFEKL peptide (1μg ml^{-1}) for 2 hours, washed and placed into culture. After 2 days, rmIL-2 (20 ng ml^{-1}) was added, and cells were cultured for an additional 6 days.

In studies involving retroviral transduction, a similar protocol was used with the exception that that the cells were exposed to SIINFEKL peptide together with IL-2 for 24h. Production of retrovirus and retroviral transduction was carried out as described previously (Pearce et al., 2003). Transduction efficiency of T cells in all experiments was ~50%.

Generation of retroviral plasmids

In experiments where the migration of OT-I and OT-Ix*Cd44*$^{-/-}$ CTL in identical tumor regions was compared, T cells were transduced with MigR1-based retroviral plasmids containing ECFP or YFP coding sequences (Mrass et al., 2006). In rescue experiments, *Cd44*$^{-/-}$-T cells were transduced with retroviral plasmids containing full-length CD44 or CD44 mutants (a schematic overview of the CD44 constructs is provided in **Figure S9)**. The constructs were generated as follows: CD44 coding sequences were subcloned into the BglII/EcoRI site of retroviral MigR1 vector (Pear et al., 1998) or fused in-frame at their C-terminus to the GFP coding sequence of retroviral vector FGM, a MigR1 variant,

lacking the internal ribosome entry site sequences. The coding sequence of full length murine CD44 was amplified by PCR from mouse splenocyte cDNA with high fidelity *Pfx* polymerase (Invitrogen) using the primers, FLfwd: 5'-ATGAAGATCTCCACCATGGACA AGTTTTGGTGGCACACAGCTTG with FLrev: 5'-ATGCTGAATTCCTACACCC CAATCTTCATGTCCACACTC for MigR1 or FLfusionrev: 5'-ATGCTGAATTC TCACCCCAATCTTCATGTCCACACTCTG for FGM. The PCR product corresponds to murine CD44 cDNA (BC005676). For the generation of CD44 mutants the MigR1 plasmid containing full length CD44 was used as a template. To generate CD44ΔECD the primer ΔECDfwd: 5'-ATGAAGATCTCCACC*ATGGACAAGTTTTGGTGGCACACAGCTTGGGGACTTTGCC TCTTGCAGTTGAGCCTGGCA*CAGGACAGTGGAGTGACCACAAC-3' (signal peptide is shown in Italic) was used with FLrev or FLfusionrev primers. To generate CD44ΔICD, ΔICDrev: 5'-ATGCTGAAT TCCTACTGCCCACACCTTCTCCTACTA TTGAC-3' or ΔICDfusionrev: 5'-ATGC TGAATTCCCTGCCCACACCTTCTCCTACT ATTGAC-3' for C-terminal fusion were used with FLfwd primer. All constructs were verified by sequencing.

Supplemental Figures

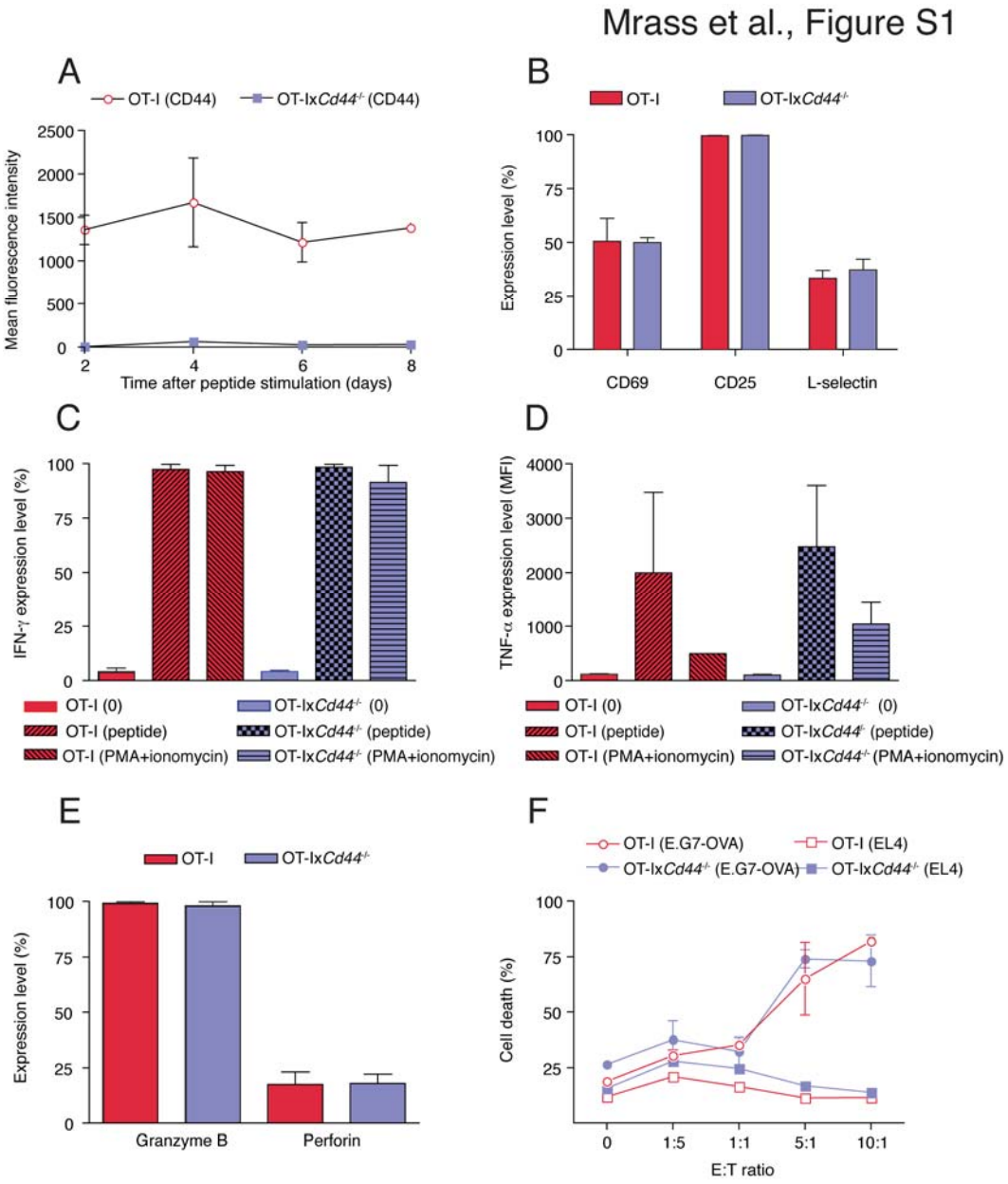

Figure S1. Phenotypic and functional characterization of in vitro-activated OT-I and OT-Ix*Cd44*$^{-/-}$ effector T cells

T lymphocytes from OT-I and OT-Ix*Cd44*$^{-/-}$ mice were stimulated with cognate peptide antigen and cultured in the presence of IL-2.

(A, B) Flow cytometry was used to determine the expression level of CD44 on days 2, 4, 6 and 8 after activation, as well as the activation markers CD25, CD69 and L- selectin on

day 8 of culture (n=5 experiments).

(C, D) On day 8 after peptide stimulation, T lymphocytes were restimulated with cognate peptide antigen or PMA and ionomycin. Production of IFN-γ and TNF-α was determined by intracellular staining using flow cytometry (n=3 experiments).

(E) The expression level of granzyme B and perforin of T lymphocytes was determined by flow cytometry on day 8 of culture.

(F) Graded numbers of T lymphocytes on day 8 of culture were incubated with EL4 or E.G7-OVA tumor cells to determine antigen specific cytotoxicity. Death of the tumor cells was determined using a cytolysis assay.

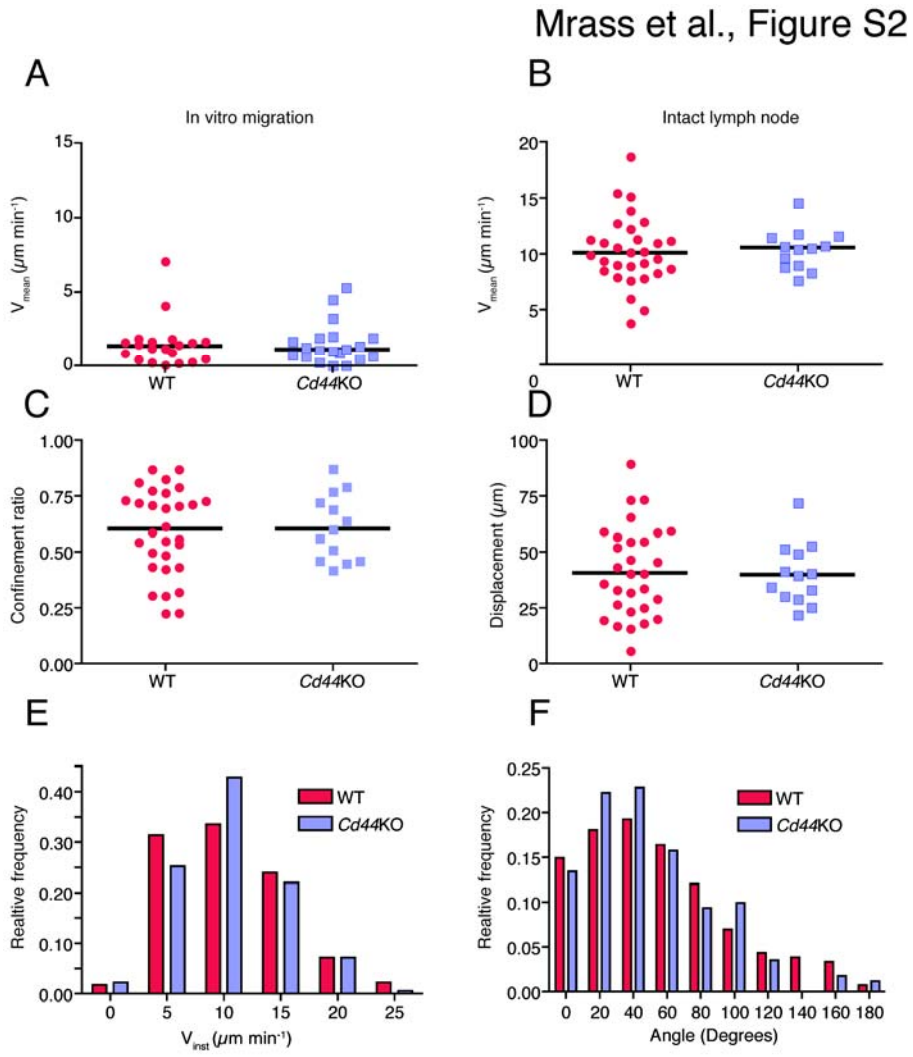

Figure S2. Migration of naïve T cells.

(A) Single cell suspensions from spleens of OT-IxDPE^{GFP} or OT-Ix*Cd44*$^{-/-}$xDPE^{GFP} mice were plated on collagen gel, and time-lapse sequences generated with fluorescence microscopy. GFP^{+} T cells were tracked and the velocity was determined.

(B-F) Lymph nodes from OT-Ix*Cd44*$^{-/-}$xDPE^{GFP} mice were explanted and imaged with 2-photon microscopy. GFP^{+} T cells were tracked and motility parameters determined. The obtained values were compared to previously published data from CD44-wildtype T cells (Mrass et al, 2006).

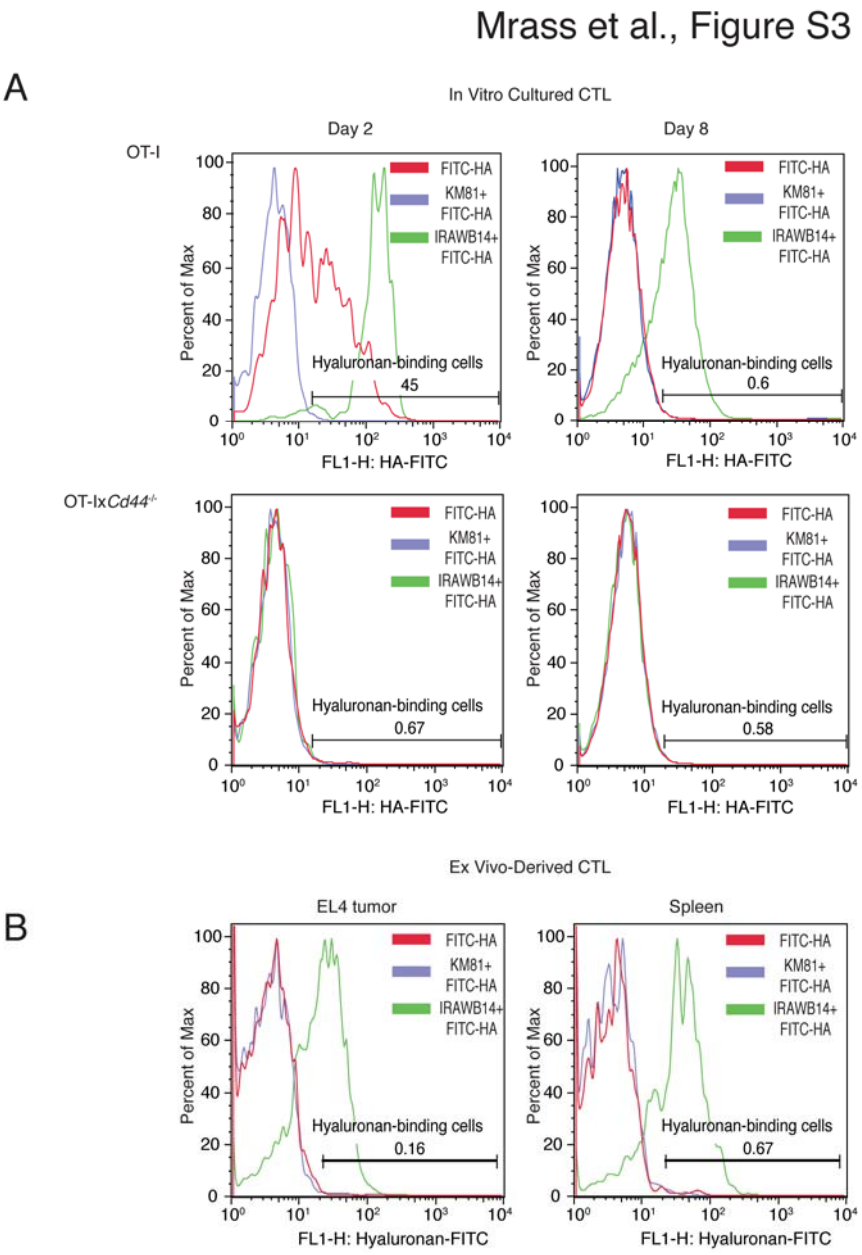

Figure S3. Hyaluronic acid-binding activity of effector CTL from OT-I and OT-Ix*Cd44*$^{-/-}$ mice

(A) T lymphocytes from OT-I or OT-Ix*Cd44*$^{-/-}$ mice 2 or 8 days after stimulation with cognate peptide antigen were incubated with FITC-HA. Some samples were preincubated with antibodies inducing (IRAWB14) or blocking (KM81) the HA binding capacity of CD44 prior to adding HA-FITC. HA binding activity was measured using flow cytometry.

(B) Effector T lymphoytes generated from OT-I or OT-Ix*Cd44*$^{-/-}$ mice were adoptively

transferred into EL4 tumor-bearing mice. After 3 days, single cell suspensions from spleen and tumor tissues were generated followed by incubation of the samples with FITC-HA, IRAWB14 and KM81, as in (A). Antigen-specific T cells were identified using anti-CD8 antibody and an MHC class I-SIINFEKL tretramer. HA binding activity of $CD8^{+}tetramer^{+}$ T cells was quantified by flow cytometry.

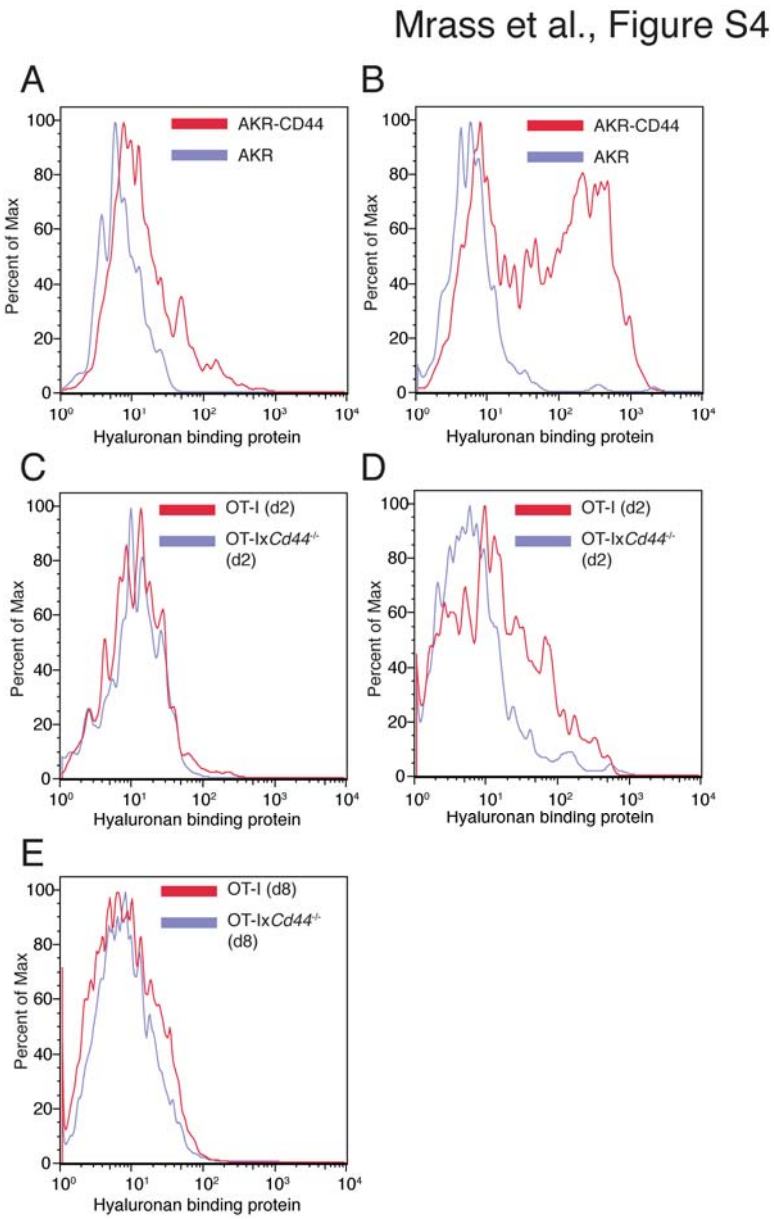

Figure S4. Measurement of endogenous HA binding to T lymphocytes

(A, B) As a positive control of the assay, AKR and AKR-CD44 cells were incubated with biotinylated HA-binding protein (HABP) followed by exposure to PE-streptavidin, either in the absence (A) or presence (B) of preincubation with hyaluronic acid.

(C-E) In vitro stimulated T lymphocytes from OT-I or OT-Ix*Cd44*$^{-/-}$ mice at day 2 or 8 of culture were incubated with biotinylated HA-BP followed by PE-streptavidin. In (D) this was preceded by an incubation of the cells with HA.

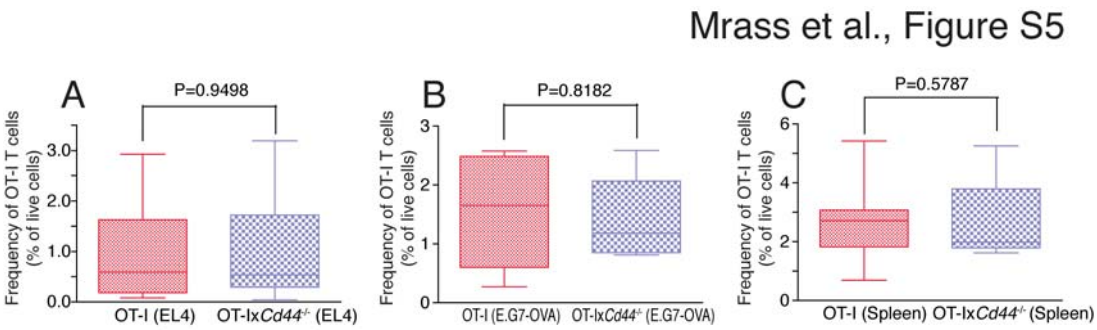

Figure S5. In vivo trafficking of OT-I and OT-Ix*Cd44*$^{-/-}$ effector T lymphocytes

C57BL/6 mice were injected subcutaneously with either E.G7-OVA or EL4 tumor cells (10^6) into both flanks. Nine days later, CTL ($2x10^7$) generated from OT-IxDPE^{GFP} or from OT-Ix*Cd44*$^{-/-}$xDPE^{GFP} mice were adoptively transferred. On day 3 (A, C) or day 4 (B) after adoptive transfer, single cell suspensions from EL4 (A; n=20) and E.G7-OVA tumors (B; n=6) or spleen (C; n=10) were generated. The percentage of GFP$^+$ T cells in gated live cells was determined by flow cytometry.

Mrass et al., Figure S6

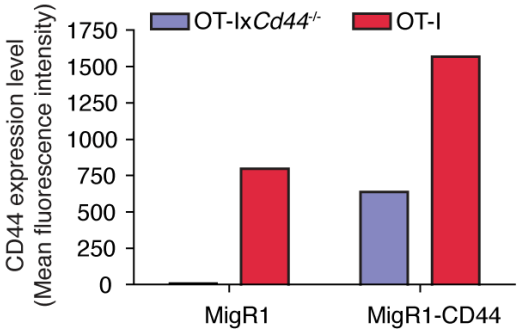

Figure S6. CD44-expression levels on T lymphocytes after retroviral introduction

Peptide-activated T cells derived from OT-I or OT-Ix*Cd44*$^{-/-}$ mice were transduced with MigR1 or MigR1-CD44 retroviruses. Seven days after transduction, CD44 cell surface expression levels were determined with flow cytometry.

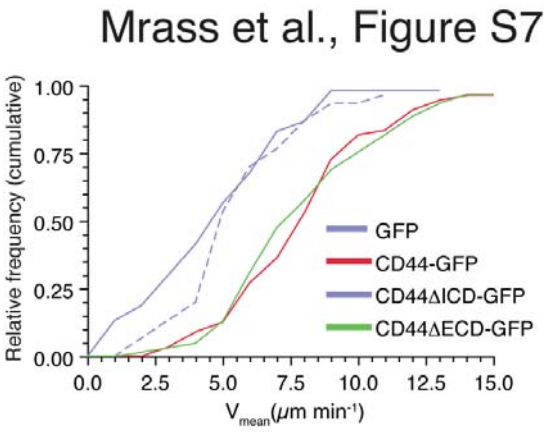

Figure S7. Migratory properties of T lymphoytes after introduction of CD44-GFP fusion molecules

Peptide-activated T cells derived from OT-Ix*Cd44*$^{-/-}$ mice were transduced with retroviruses carrying GFP or the indicated CD44-GFP fusion proteins. Motility assays on collagen gels were carried out as in Figure 3A-3C.

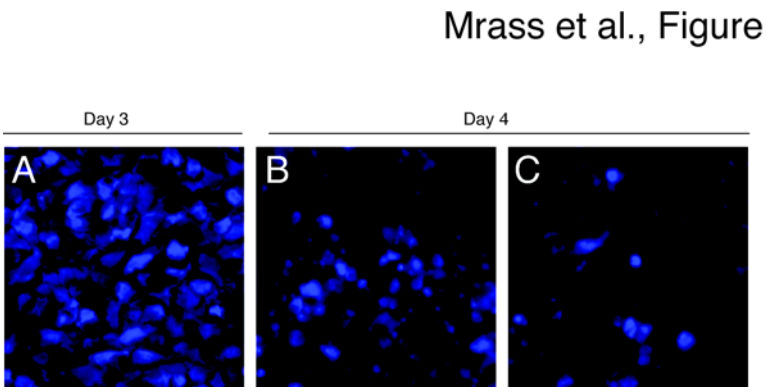

Figure S8. Tumor cell density after adoptive transfer

C57BL/6 mice were injected subcutaneously into the flank with E.G7-OVA-ECFP tumor cells (10^6). After 9 days $2x10^7$ CTL generated from OT-I splenocytes were adoptively transferred. 3 (A) and 4 (B, C) days later, the tumors were explanted and subjected to immuno-fluorescence microscopy. Note that the selected region on day 3 after adoptive transfer contains a high density of tumor cells. In contrast, on day 4 the density has decreased and a high frequency of cells shows signs of apoptosis such as rounding.

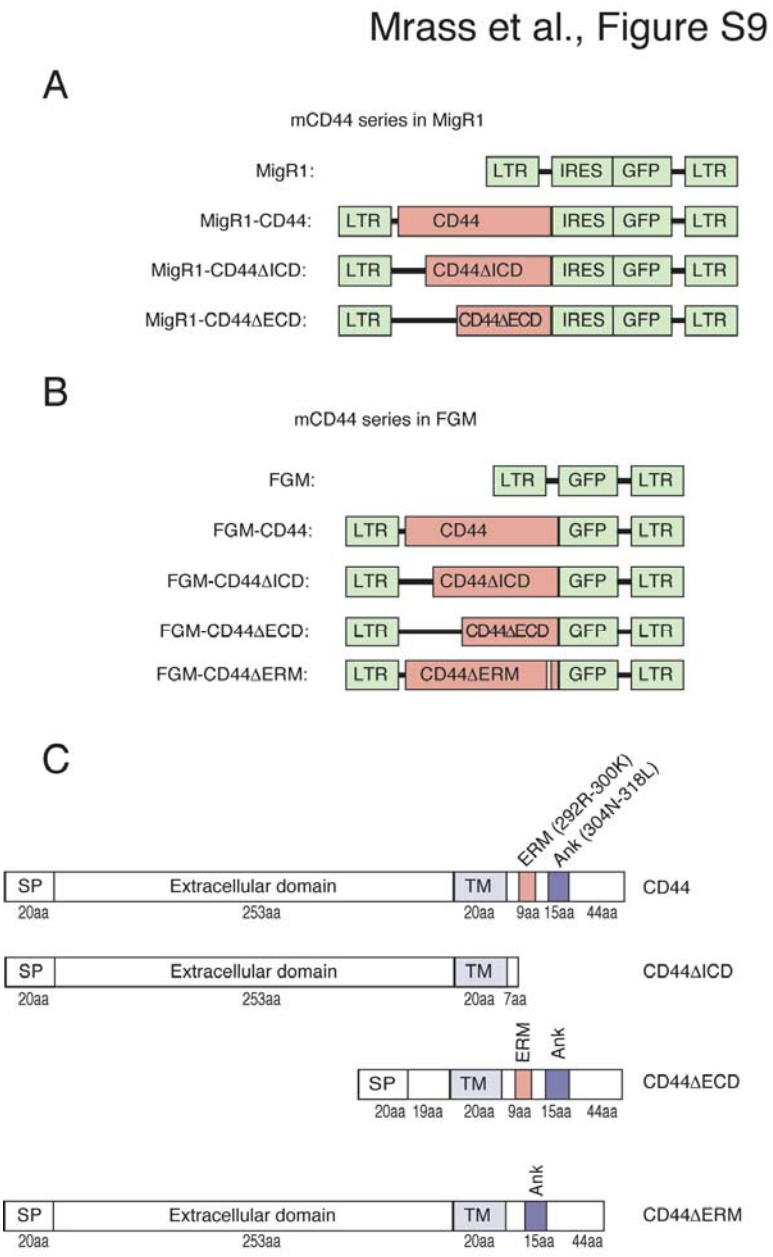

Figure S9. Schematic representation of retroviral constructs

(A, B) Structure of MigR1 and FGM derivatives. The derivatives contain insertions encoding for the various CD44 variants (red). Sequence elements of the vector backbone are shaded in green.

(C) Structure of the sequences encoding for the various CD44 variants. SP: signal peptide, TM: transmembrane region, Ank: ankyrin-binding domain

Supplemental References

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