

Anti-HVEM mAb therapy improves antitumoral immunity both *in vitro* and *in vivo*, in a novel transgenic mouse model expressing human HVEM and BTLA molecules challenged with HVEM expressing tumors

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Supplementary methods :

Tumor Cell Lines

NCIH2291 and NICH2405 are lung adenocarcinoma cell lines purchased from ATCC and grown in RPMI supplemented with 10% FCS. HT29 is a colorectal cancer cell line grown in DMEM supplemented with 10% FCS. All cell lines were confirmed to be free of mycoplasmas using the MycoAlert detection kit (Lonza, Basel, Switzerland). Cells were detached in PBS EDTA 5mM without enzymatic solution to avoid HVEM cleavage.

Peripheral Blood Mononuclear Cells (PBMCs)

Human peripheral blood was obtained from Etablissement Francais du Sang (EFS) after obtaining informed consent from the donor. Human peripheral blood mononuclear cells (PBMC) were isolated using a Ficoll media (Eurobio) centrifuged at 800 × g for 30 min at room temperature with no break or acceleration. Cells were recovered from the interface with plasma, washed twice in PBS, counted and frozen in RPMI supplemented with 20% FCS and 10% DMSO until experimentation.

HVEM and PD-L1 expression

Phenotypic expression was assessed by flow cytometry. HVEM expression was measured on CD4⁺ and CD8⁺ T cells in resting or activated conditions. 100 000 PBMC per well were distributed in 96 wells flat bottom plate, with donor specific OKT3 concentration (Ultra-LEAF Purified anti-human CD3 Antibody, Biolegend) and the following mABs when indicated: IgG1, anti-HVEM18-10, and/or anti-PD-L1 3.1 at 10 µg/ml in a final volume of 200µL RPMI supplemented with 10% FCS and 30 UI/ml IL-2 (Proleukine, Novartis). Noteworthy, OKT3 concentration was determined in advance for every PBMC donor, ranging from 5 to 50 pg/ml to obtain a sub-optimal T cells activation. Negative controls were PBMC without OKT3 and mAbs. T cell differentiation subsets were determined as: naive (CD27⁺CD45RA⁺), effector memory (CD27⁺CD45RA⁻), central memory (CD27⁻CD45RA⁻) and TEMRA (CD27⁻CD45RA⁺) (Table1). HVEM and PD-L1 expression on tumor cell lines was established twice with appropriate isotype control and viability staining.

Co-culture and proliferation assays

After thawing and overnight resting in RPMI supplemented with 10% FCS, PBMC were stained with Cell Trace Violet (CTV, ThermoFisher) according to manufacturer instructions. Briefly PBMC were washed in PBS twice. CTV staining was performed at 37°C and 5% CO₂ for 15 minutes precisely with 1µL CTV for 10 to 15 million PBMC/ml of PBS. Then, PBMC were washed twice in RPMI supplemented with 10% FCS. PBMCs were stimulated as described above. Briefly, PBMC plated and the following mABs: IgG1, anti-HVEM18-10, and/or anti-PD-L1 3.1 and IL-2. When tumor cell lines were cultured with PBMC, they were seeded in wells 24 hours before experimentation with 50 000 cells per wells to obtain a confluence around 60%. NCIH2405 and HT29 were treated with mitomycin C at 10µg/ml (Sigma Aldrich) during 3 hours and washed three times before adding PBMC. After 72H incubation (37°C, 5% CO₂) PBMC were

recovered, washed in PBS and stained at 4°C during 20 minutes with viability staining and the following antibodies: CD45, CD3, CD4, CD8, TCR- $\gamma\delta$, CD25, (Table 2). Acquisition was performed on FACS LSR II (Becton Dickinson). Application settings and sphero-beads (BD Biosciences) were used to ensure reproducible results between experiments. Data acquisition on LSRII was performed with BD DIVA software and data analysis was conducted with FlowJo (Treestar, Becton-Dickinson) software v.10.

Knock-in mouse model

To produce B6-Tnfrsf14^{tm1Ciphe} KI mice in which the mouse *tnfrsf14* exon 1 was replaced with the human *tnfrsf14* exon 1, ES cells were electroporated with the vector 1477_hTNFRSF14_03_v1 (containing the human cDNA sequence TNFRSF14-001 ENST00000355716 corresponding to human TNFRSF14 exon 1). ES cells were then cultured 8 days in 96 well plate under G418 selecting conditions (250 μ g/mL) and screened for proper vector integration by PCRs using primers 1477_ScES_RH5_Fwd: CTCCACTGCTGCTGCTGCTCTT and 1477_ScES_RH5_Rev: GTCCCCAAACTCACCTGAA. Southern blot analysis was used to confirm the unique integration to the correct locus. ES validated clones were then injected into Balb/cN blastocysts. Germ-line transmission, proper deletion of the Neo cassette and the presence of the sequence coding for human Tnfrsf14 was assessed by PCR (see genotyping). To produce Btla^{Tm1Ciphe} mice, exon 2 from WT mouse was substituted by human exon 2.(17) The same method as described above was used to replace murine exon 2 with Btla-001 (ENSMUST00000102802) sequence. B6-Tnfrsf14^{tm1Ciphe} and Btla^{Tm1Ciphe} mice were then crossed to obtain double KI mice.

Genotyping

HuBTLA^{+/+}HuBTLA^{+/+} genotyping was performed using the following PCR primers: Fwd, 5'-TGCAATGATACCTATGGTCC -3'; Rev, 5'-TGACTGTTCTGATCTGGGG -3'; with expected band sizes at 537 bp for WT alleles and 616 bp for KI. HVEM^{hu KI} genotyping was performed using the following PCR primers: Fwd KI, 5'-CCTTACATGTTTTACTAGCCAG -3'; Fwd WT, 5'-CTGCCTCTAACAGACTTCAGT -3'; Rev, 5'-TGAAGGTGTTGTCTGTAGGG -3'; with expected band sizes at 198 bp for WT alleles and 259 for KI.

Tumor preparation for Mass cytometry and immunoprofiling

Tumors were resected from huBTLA^{+/+} and DKI mice which received anti-HVEM18-10, anti-CTLA-4 or isotype treatment. Tumors were collected and digested using the Tumor Dissociation Kit (Miltenyi Biotech). Digested tumors were mechanically disrupted using the GentleMACS Octo Dissociator (Miltenyi Biotech) to obtain a single-cell suspension, followed by isolation of CD45⁺ TILs using the CD45 (TIL) MicroBeads kit (Miltenyi Biotech). The cells were counted before proceeding for cell surface staining. Then, tumor cells were stained for viability using Cisplatin for 10 min at 37°C, washed and stained using the panel described in supplemental table 3. Finally, cells were incubated in the presence of Iridium, a DNA intercalator allowing the identification of cells. Stained cells were then acquired on a Helios mass cytometer (Cy-TOF, Fluidigm) and analyzed using the OMIQ software platform (OMIQ).

Luminescent Label Assay :

The *CellTiter-Glo*[®] Luminescent Cell Viability Assay (Promega) was used to determine the number of viable cells in culture based on quantitation of the ATP present.

The intracellular amount of ATP was determined according to the manufacturer's procedures. The 96-well plate and the CellTiter-Glo[®] reagent was first equilibrated at room temperature for 30 min. Then an equal volume of CellTiter-Glo[®] Reagent is added to the volume of cell culture medium present in each well (100 μ L) for every tested condition. We mix contents for 2 minutes on an orbital shaker at 400 rpm to induce cell lysis. The plate is incubated at room temperature for 10 minutes to stabilize luminescent signal. Luminescence is recorded using a GloMax[®]-Multi+ Detection System (Program parameters: orbital shaking: 30sec, integration time: 0.1 sec, 3 reads every 5min). Data is analyzed on Prism Software.

Unsupervised CyTOF data clustering and phenotypic analysis

CyTOF data files were exported (Helios program, Fluidigm), debarcoded and live cells were gated in FlowJo (Treestar, BD). Live cell .fcs files were exported and analyzed using OMIQ online platform (OMIQ) (18). T cells were manually identified (TCRb⁺CD3⁺). T cell-gated data were subsampled to maximum equal available cell number (6000 T cells) and were subjected to an arcsinh transformation (co-factor 5). Clustering of tumor infiltrating T cells was performed using PhenoGraph (19), with the following parameters: Euclidean distance metric with K (nearest neighbor factor) =30 for cluster identification at the per mouse level. PhenoGraph clusters (n=19) were gated and displayed on a UMAP (Euclidian distance, neighbor factor 15, minimum distance 0.4) for phenotypic analysis. T cell marker expression was represented in a heatmap in function of the 19 clusters, where marker expression (columns) and clusters (rows) were subjected to hierarchical clustering using Euclidian distance (OMIQ).

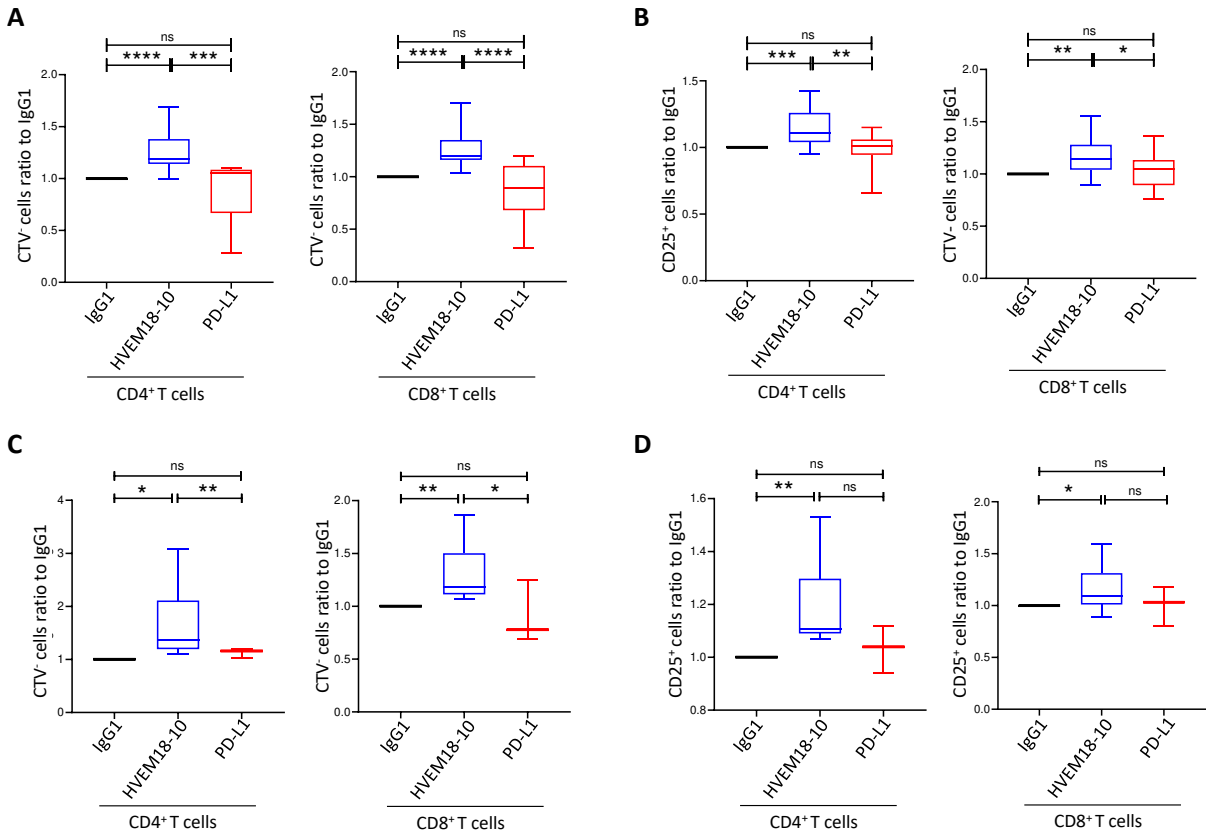
Flow cytometry for draining lymph node phenotyping.

Mice which completely rejected tumors after re-challenge had their draining lymph node (LN) resected. LNs were dissociated and stained using the panel in supplemental table 4. Stained cells were then acquired on a LSR II cytometer (Becton-Dickinson) and analyzed using the OMIQ software platform (OMIQ).

Statistical analysis

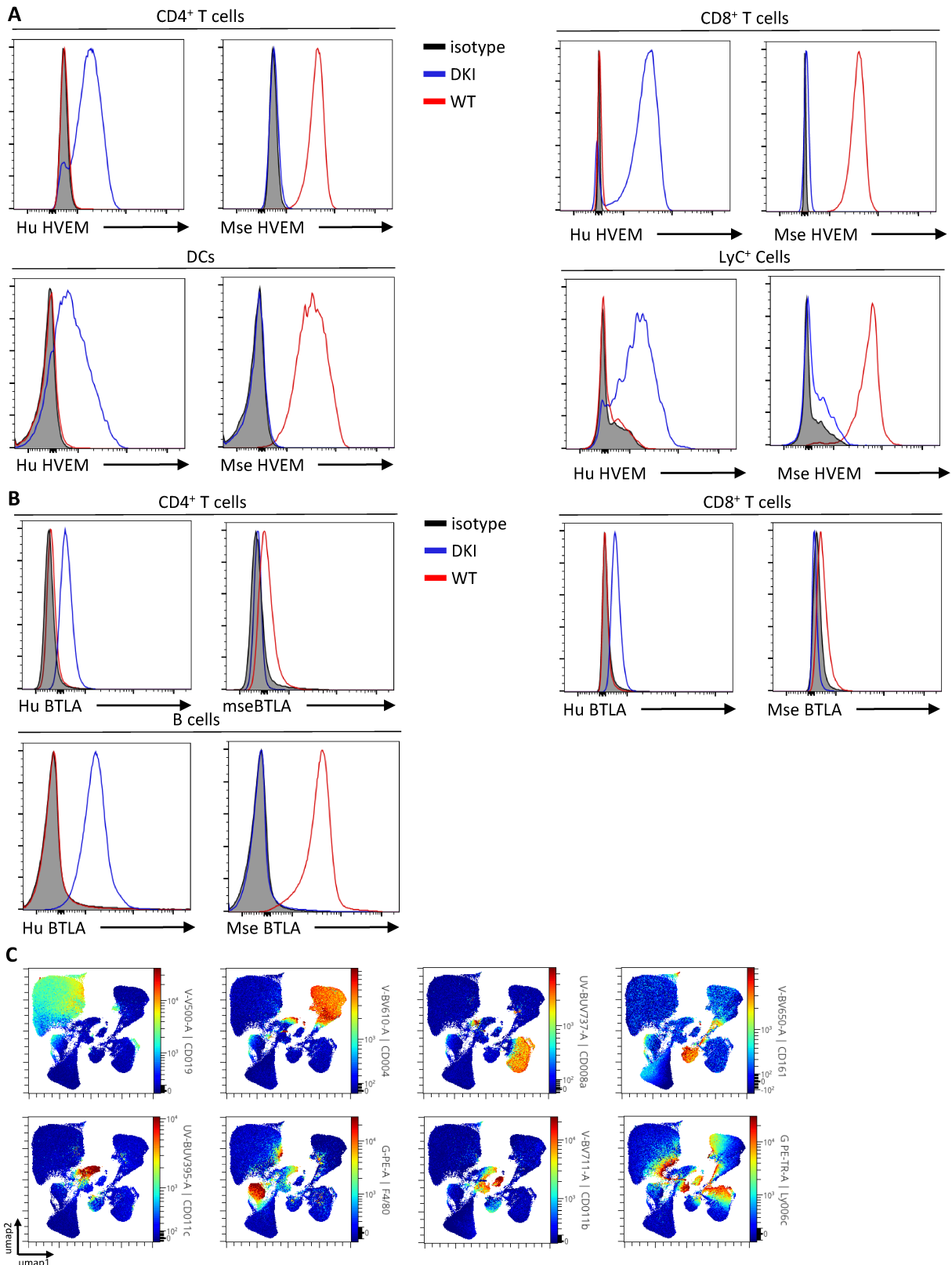
GraphPad Prism software was used to analyze and graph samples. For multiple comparison, two-way ANOVAs were performed and for comparison of condition pairs mann-whitney test was performed. *: p -val<0.05; **: p -val<0.01; ***: p -val<0.005; ****: p -val<0.001.

Supplementary figures and legends :



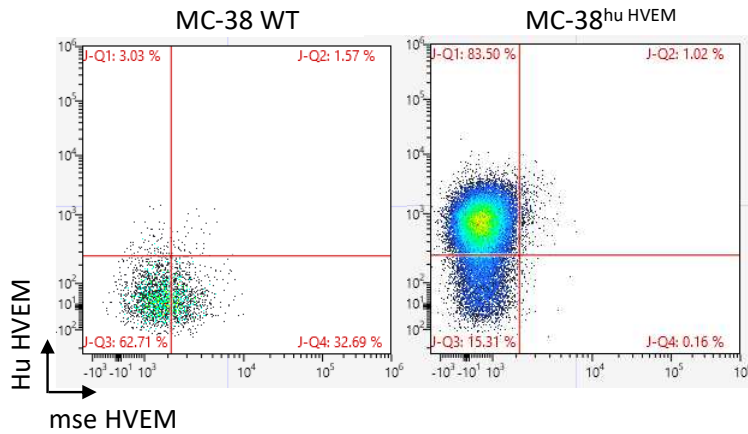
Supp.Figure.1 anti-HVEM18-10 enhances T cells response against PD-L1 negative lung and colorectal cancer cell line

PD-L1 negative lung cancer NCIH2405 (A-B) or colorectal cancer HT29 (C-D) cell lines were seeded 24 hours before the experiment. Then, cultured PBMC from healthy donors for 72h with OKT3 stimulation and treated or not (IgG1) with anti-HVEM 18-10 antibody (blue bars/lines) or anti-PD-L1 (red bars). (A,C) proliferation profile of T cells by Cell TraceViolet staining (A for NCIH2405 and C HT29) and CD25 expression (B for NCIH2405 and D for HT29). Bar plots are the Mean \pm SEM of different healthy donors samples, (A-B n=15; C-D : HVEM18-10 condition n=8 ; PD-L1 condition n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$ (Student's t-test)

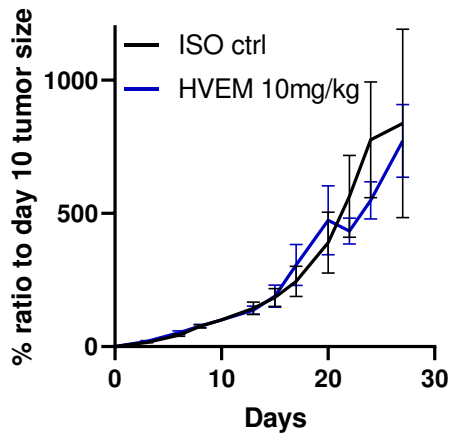


Supp. Figure 2 (associated to Fig5): hBTLA^{+/+} and hBTLA^{+/+}hHVEM^{+/+} Mice show similar immune cell phenotypes. A. Human or mouse HVEM expression was assessed on CD4⁺, CD8⁺ T cells, DCs (CD11c+MHC class II+) and LyC⁺ cells from DKI (Blue), WT mice (red) or isotypic Ig control (black) B. Human or mouse HVEM expression on CD4⁺, CD8⁺ T and B cells cells from DKI (Blue), WT mice (red) or isotypic Ig control (black) C. Individual marker expression for the gating of each individual immune cell population.

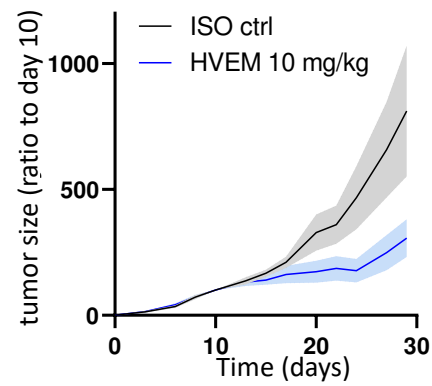
A.



B.

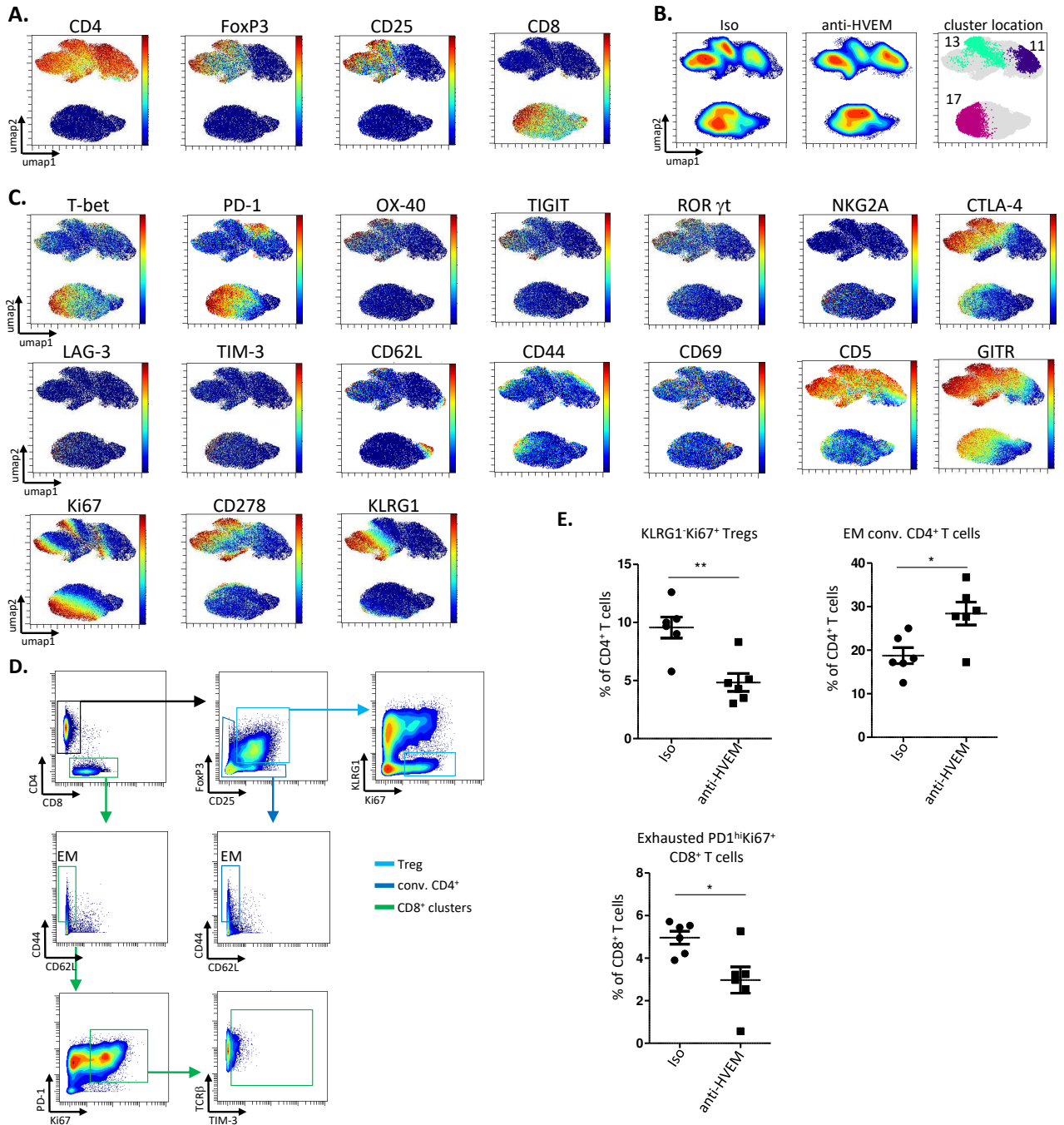


C.



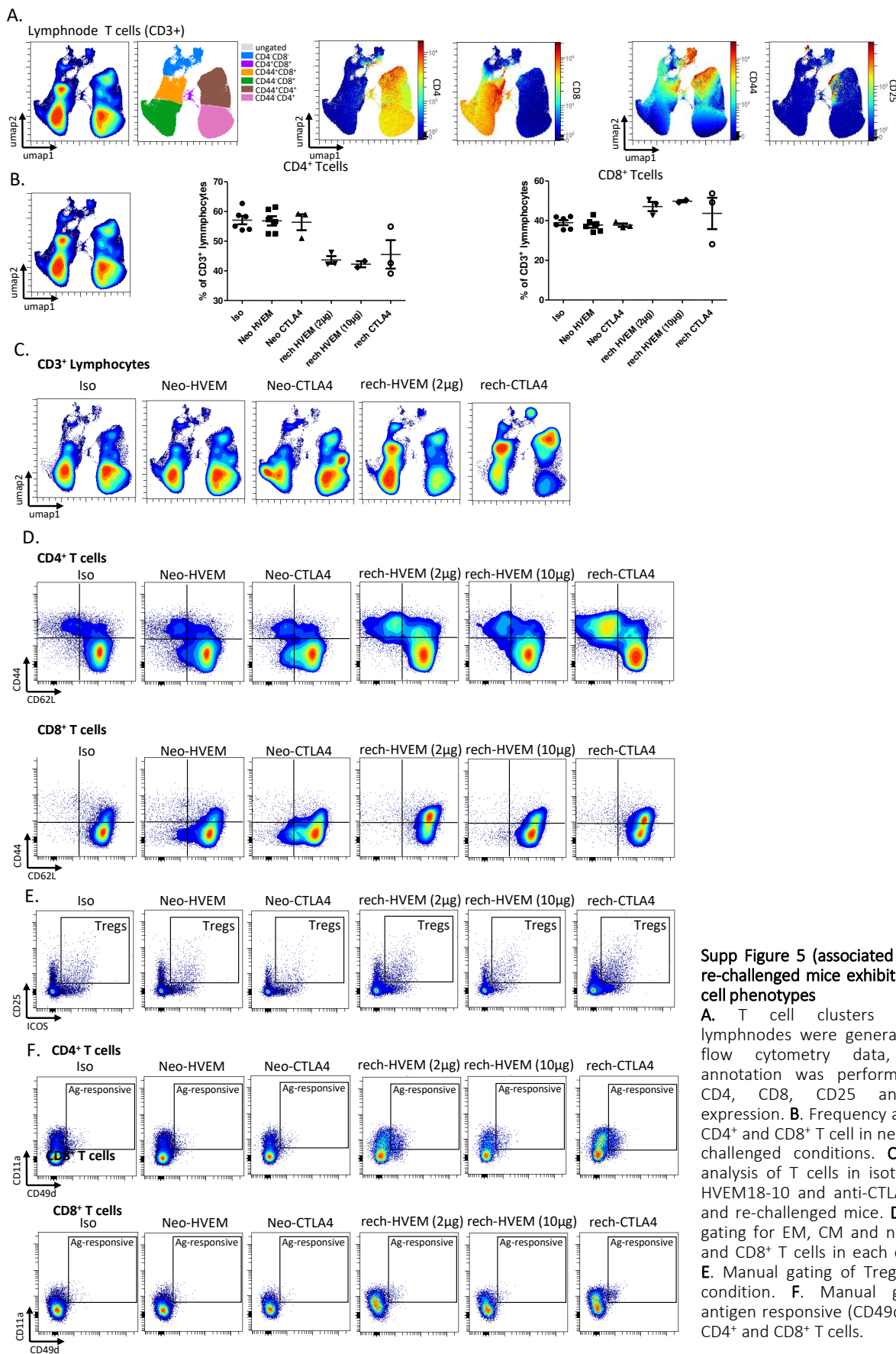
Supp. Figure 3 blocking Trans-BTLA-HVEM binding *in vivo* is sufficient to decrease solid tumor growth

A. MC38-WT or MC-38 1E10 clone expression of Human or mouse HVEM in cytometry B. Measure of tumor growth showed a ratio to size of tumor at randomization day. Colorectal cancer cells MC-38^{hu} HVEM were injected (0.5×10^6) at day 0, and Iso ct (black) or anti-HVEM 18-10 antibody 10 mg/kg (blue).



Supp Figure 4 (associated to Fig7) : Individual marker expression, significantly modulated cluster location and manual gating validation of clusters 11, 13 and 17.

A. Main gating marker UMAP for the identification of Tregs, conventional CD4⁺ T cells and CD8⁺ T cells. **B.** Density UMAP and significantly altered cluster location. **C.** Phenotype and function marker UMAPs used to generate the heat map in Figure 8. **D.** Strategy allowing the gating of PhenoGraph clusters 11 (EM conv. CD4⁺ T cells), 13 (KLRG1⁺Ki67⁺ Tregs) and 17 (exhausted PD-1^{hi}Ki67⁺CD8⁺ T cells) for manual validation. **E.** Statistical validation of manually gated clusters (from D). **p*.val<0.05; ***p*.val<0.01.



Supp Figure 5 (associated to Fig8) : re-challenged mice exhibit specific T cell phenotypes

A. T cell clusters in mice lymphnodes were generated using flow cytometry data, T cell annotation was performed using CD4, CD8, CD25 and CD44 expression. **B.** Frequency analysis of CD4⁺ and CD8⁺ T cell in neo- and re-challenged conditions. **C.** Density analysis of T cells in isotype, anti-HVEM18-10 and anti-CTLA-4 neo- and re-challenged mice. **D.** Manual gating for EM, CM and naive CD4⁺ and CD8⁺ T cells in each condition. **E.** Manual gating of Tregs in each condition. **F.** Manual gating of antigen responsive (CD49d⁺CD11a⁺) CD4⁺ and CD8⁺ T cells.