

Supplemental information

**Batf3⁺ DCs and the 4-1BB/4-1BBL axis
are required at the effector phase in the tumor
microenvironment for PD-1/PD-L1 blockade efficacy**

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Supplementary Figure 1 - Ziblat A. et al

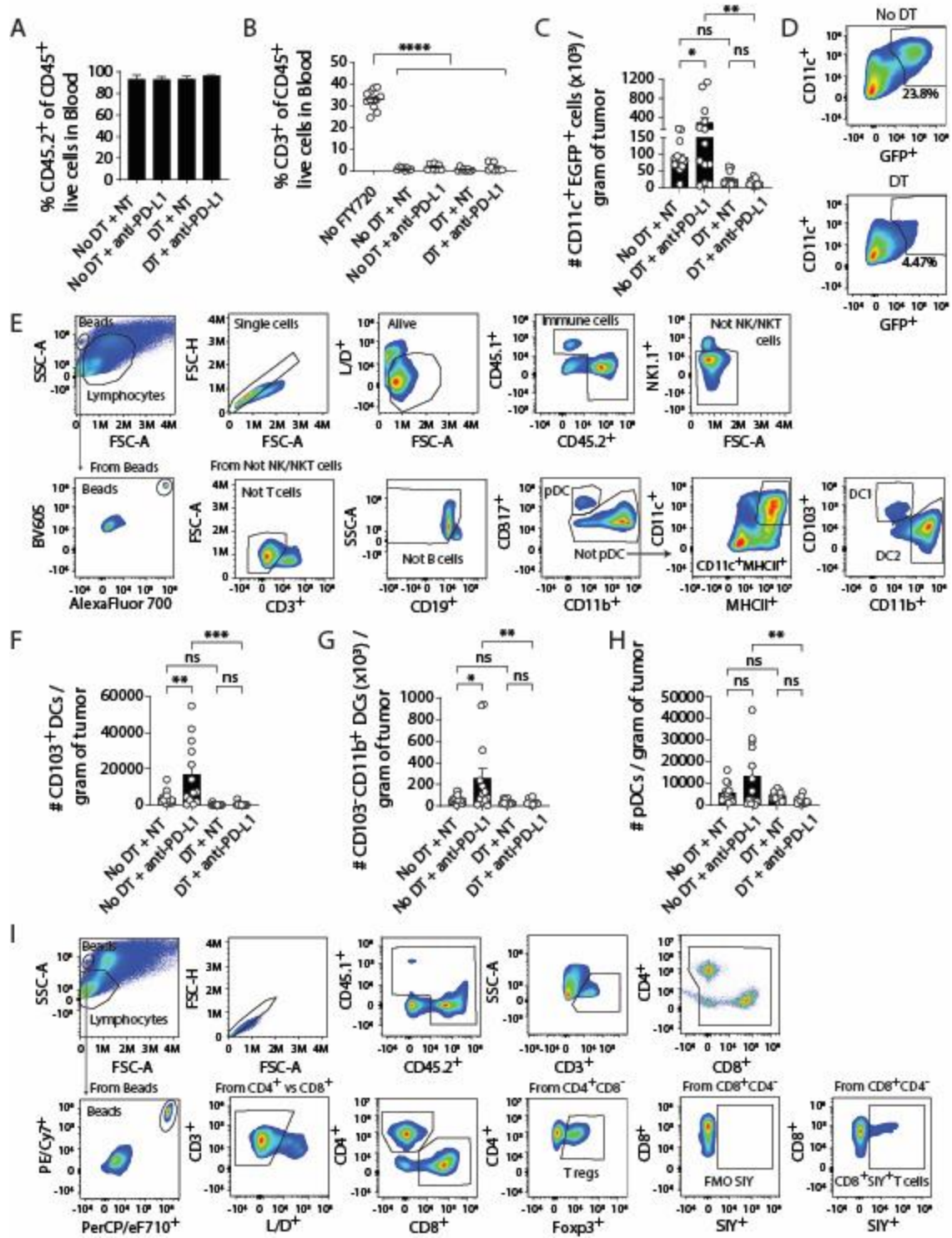


Fig. S1. Experimental design, experimental controls and flow cytometry gating strategies used for the chimeric experiments, related to Figure 1. (A) Percentage of chimerism analyzed in the blood of all mice before tumor injection. (B) FTY720 efficacy evaluated by analyzing the percentage of CD3⁺ T cells in blood in each group at endpoint, one of two independent experiments is shown, n≥8 per group. (C) DT efficacy analysis counting the number of CD11c⁺EGFP⁺ cells per gram of tumor in each group. (D) Representative flow cytometry dot plots showing CD11c⁺EGFP⁺ cells without (top panel, NT) and with DT treatment (bottom panel, DT). (E) Representative flow cytometry gating strategy used to analyze the number of pDC, DC2s and DC1s in the TME. (F-H) Number of DC1s (F), DC2s (G) and pDC (H) per gram of tumor in the TME at the end of the experiment. Two independent experiments, n=13 per group (A, C, F-H). (I) Representative flow cytometry gating strategy used to analyze the number of T cells in the TME. The gate to count the number of beads was done with different fluorophores in the axes in different experiments, selecting in each case the fluorophores that gave the best visualization/separation of the beads. Bar graphs represent the mean values of the indicated data points, and the error bars represent SEM. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. One-way ANOVA with Dunnett's post-test using "No FTY720" as control group (B) and One-way ANOVA with Bonferroni's post-test (A, C, F-H) were used for statistical analysis.

Figure S2 - Ziblat A. et al

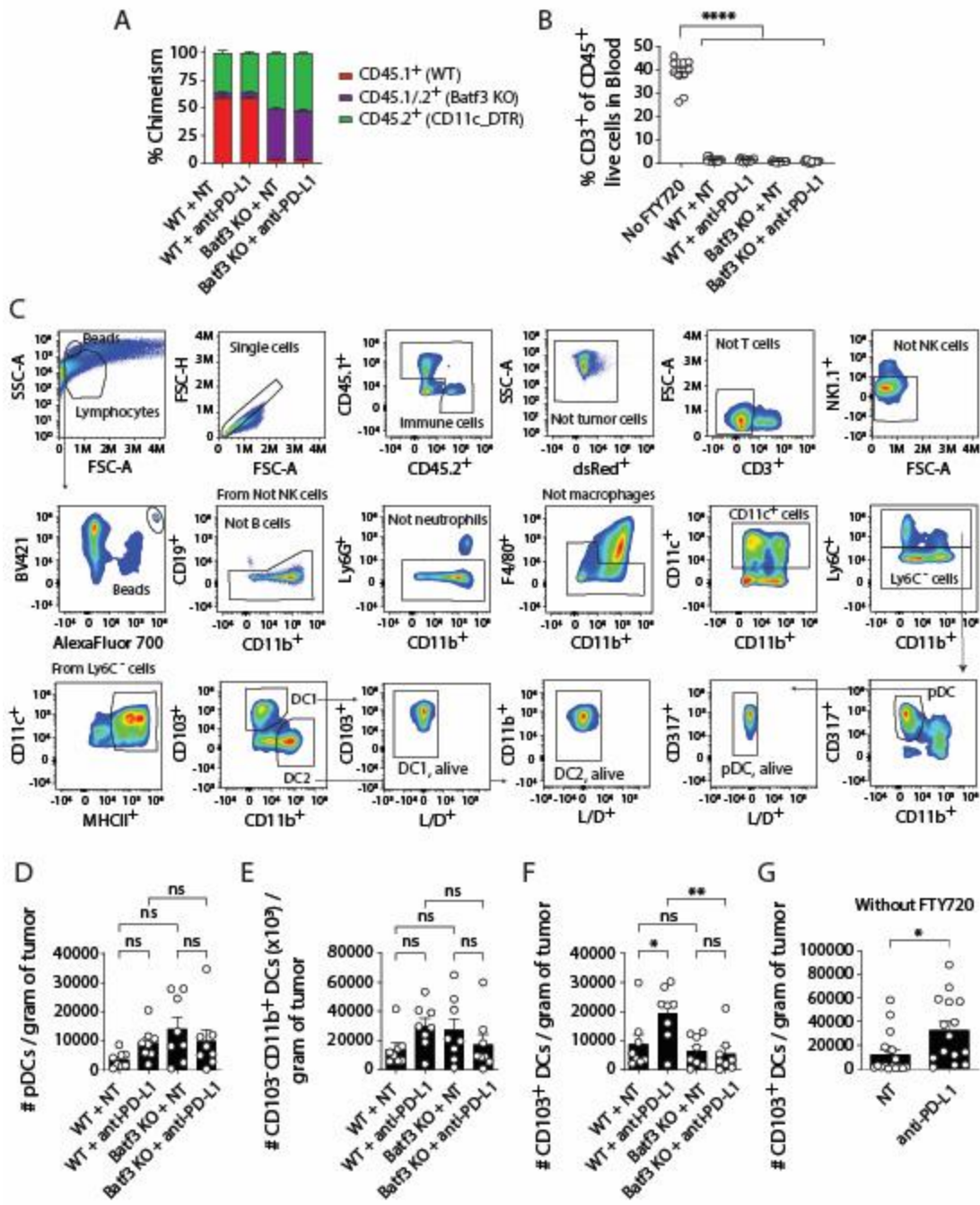


Fig. S2. Experimental controls and flow cytometry gating strategy used for the mixed BM chimera experiments, related to Figure 2. (A) Percentage of chimerism analyzed in the blood of all mice before tumor injection. Two independent experiments, $n \geq 15$ per group. (B) FTY720 efficacy evaluated by analyzing the percentage of CD3⁺ T cells in blood in each chimeric group at endpoint. Two independent experiments, $n \geq 15$ per group. (C) Representative flow cytometry gating strategy used to analyze the number of pDC, DC1s and DC2s in the tumors. (D-F) Number of pDC (D), DC2s (E), DC1s (F) per gram of tumor in each group. One of three independent experiments is shown, $n=8$ per group. (G) Number of DC1s per gram of tumor at day 24, from B6 mice injected with B16.SIY with or without anti-PD-L1 treatment, and without FTY720 treatment. Bar graphs represent the mean values of the indicated data points, and the error bars represent SEM. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. One-way ANOVA with Dunnett's post-test using "No FTY720" as control group (B), One-way ANOVA with Bonferroni's post-test (D-F) and t -test (G) were used for statistical analysis.

Supplementary Figure 3 - Ziblat A. et al

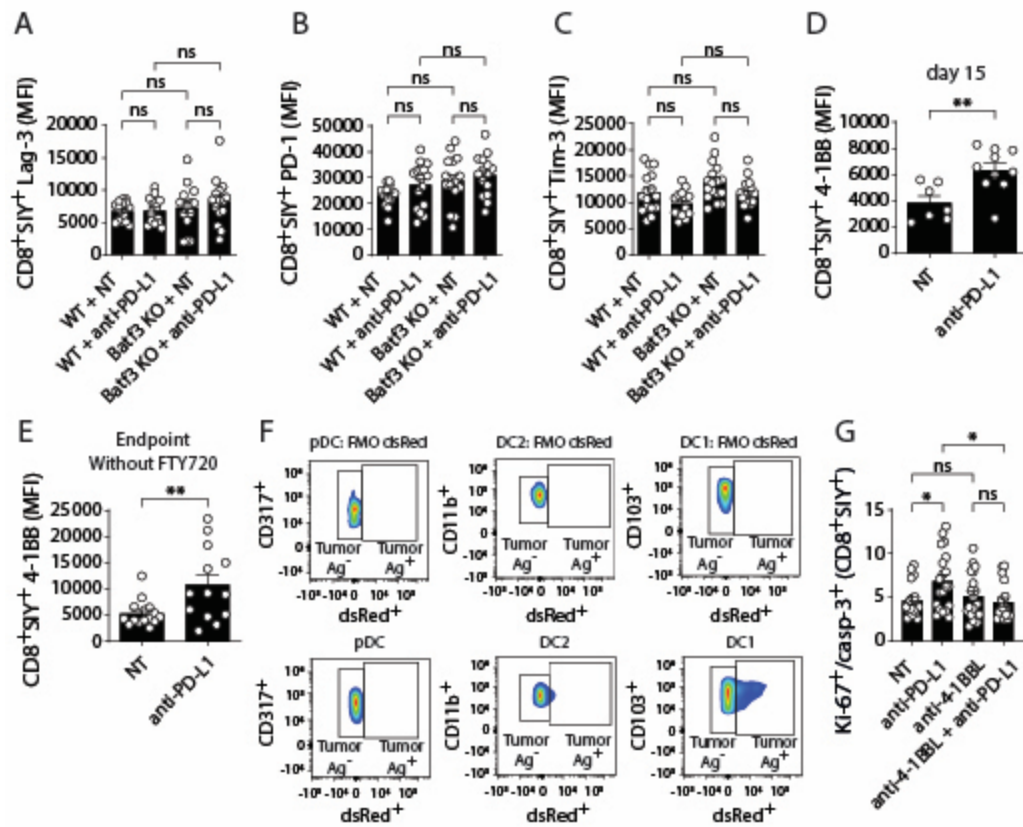


Fig. S3. Expression levels of LAG-3, PD-1 and Tim-3 on Ag-specific CD8⁺ T cells in the presence or absence of Batf3⁺ DCs, 4-1BB expression upon anti-PD-L1 treatment at different time points, gating strategy used for detection of tumor-derived material acquisition by DCs and Ag-specific CD8⁺ T cell proliferation to apoptotic ratio in different conditions, related to Figure 3. (A-C) Lag-3 (A), PD-1 (B) and Tim-3 (C) expression on Ag-specific CD8⁺ T cells in the presence or absence of Batf3⁺ DCs, with or without anti-PD-L1 treatment. Experimental design is shown in Figure 2A and gating strategy on S1I. Chimeric mice were injected with B16.SIY (A and B) or with MC38.SIY (C). Two independent experiments, n=16 per group (A and B) and n≥14 (C). (D-E) 4-1BB expression on Ag-specific CD8⁺ T cells at day 15 of tumor injection on B6 mice treated with FTY720 from day 7 of tumor injection (D) or in B6 mice at day 21 of tumor injection without FTY720 treatment (E). (F) Fluorescence minus one (FMO) for dsRed, performed by injecting mice with B16F10 (the parental tumor cell line which does not express dsRed) and representative plots of dsRed expression on pDC, DC1s and DC2s at day 9 of tumor injection, one day after one dose of anti-PD-L1 treatment. (G) Ag-specific CD8⁺ T cell proliferation (Ki-67⁺) to apoptotic (active-casp-3⁺) ratio at day 15, in mice treated with FTY720 from day 7 of tumor injection and treated with 3 doses of anti-PD-L1 in the absence or presence of anti-4-1BBL blocking mAb. Three independent experiments, n≥18. Bar graphs represent the mean values of the indicated data points, and the error bars represent SEM. ns, not significant; *, p<0.05; **, p<0.01. One-way ANOVA with Bonferroni's post-test (A-C and G), and *t*-test (D and E) were used for statistical analysis.

Supplementary Figure 4 - Ziblat A. et al

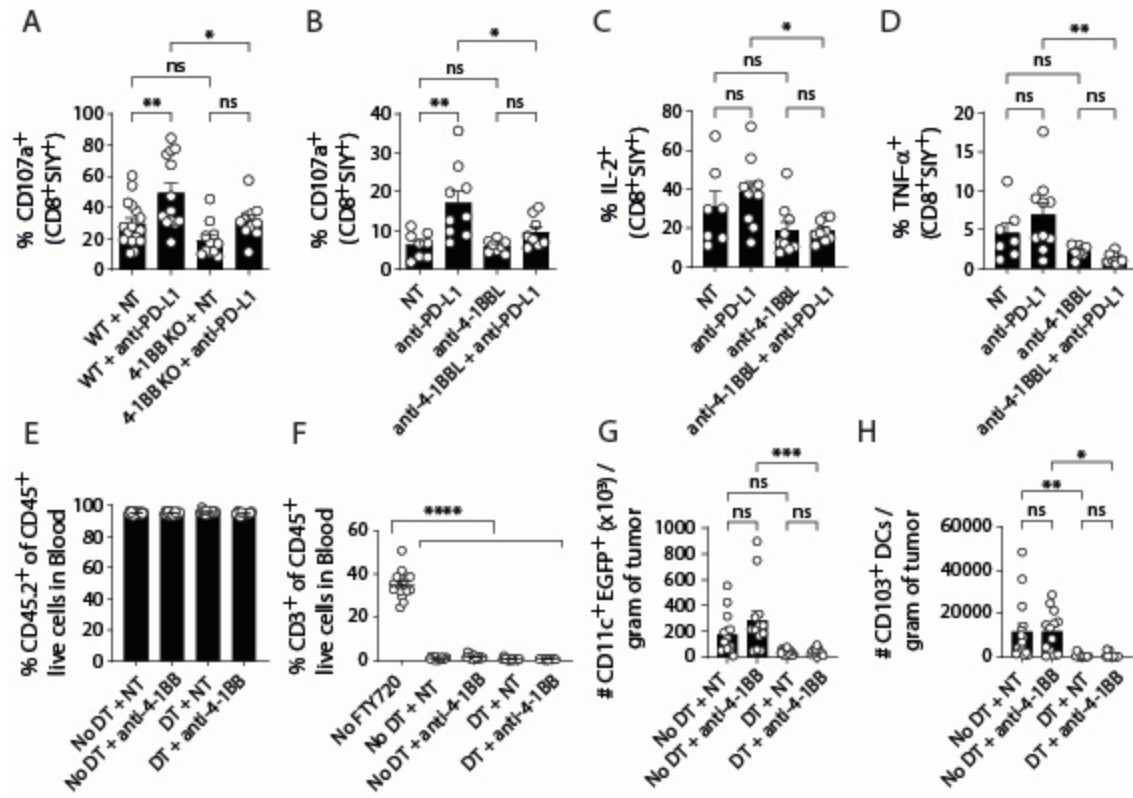


Fig. S4. 4-1BB/4-1BBL axis is needed for Ag-specific CD8⁺ T cell functional reinvigoration and controls related to Figure 4. (A) Percentage of Ag-specific CD8⁺ T cells that degranulated at day 21 of B16.SIY tumor injection in WT or 4-1BB KO mice, with or without anti-PD-L1 treatment. Two independent experiments, n≥10 per group. (B) Percentage of Ag-specific CD8⁺ T cells that degranulated at day 15 of MC38.SIY tumor injection in B6 mice, with or without anti-PD-L1 treatment in the absence or presence of anti-4-1BBL blocking mAb and treated with FTY720 from day 7 of tumor injection. One independent experiment, n≥8 per group. (C-D) Percentage of Ag-specific CD8⁺ T cells (CD8⁺SIY⁺) that expressed IL-2 (C) or TNF-α (D) in the TME at day 15 after B16.SIY tumor injection, in each group of mice. Experimental design depicted on Figure 4A. One independent experiment, n≥7. (E) Percentage of chimerism analyzed in the blood of all chimeric mice before tumor injection. Two independent experiments, n≥12 per group. (F) FTY720 efficacy in the chimeric mice evaluated by analyzing the percentage of CD3⁺ T cells in blood in each group at endpoint. Two independent experiments, n≥12 per group. (G) DT efficacy analysis counting the number of CD11c⁺EGFP⁺ cells per gram of tumor in each group. Two independent experiments, n≥12 per group. (H) Number of CD103⁺ DCs per gram of tumor in the chimeric mice treated or not with diphtheria toxin (DT) and treated or not with anti-4-1BB agonistic mAb. Bar graphs represent the mean values of the indicated data points, and the error bars represent SEM. ns, not significant; *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. One-way ANOVA with Bonferroni's post-test (A-E, G and H) and with Dunnett's post-test using "No FTY720" as the control group (F) were used for statistical analysis.

Supplementary Figure 5 - Ziblat A. et al

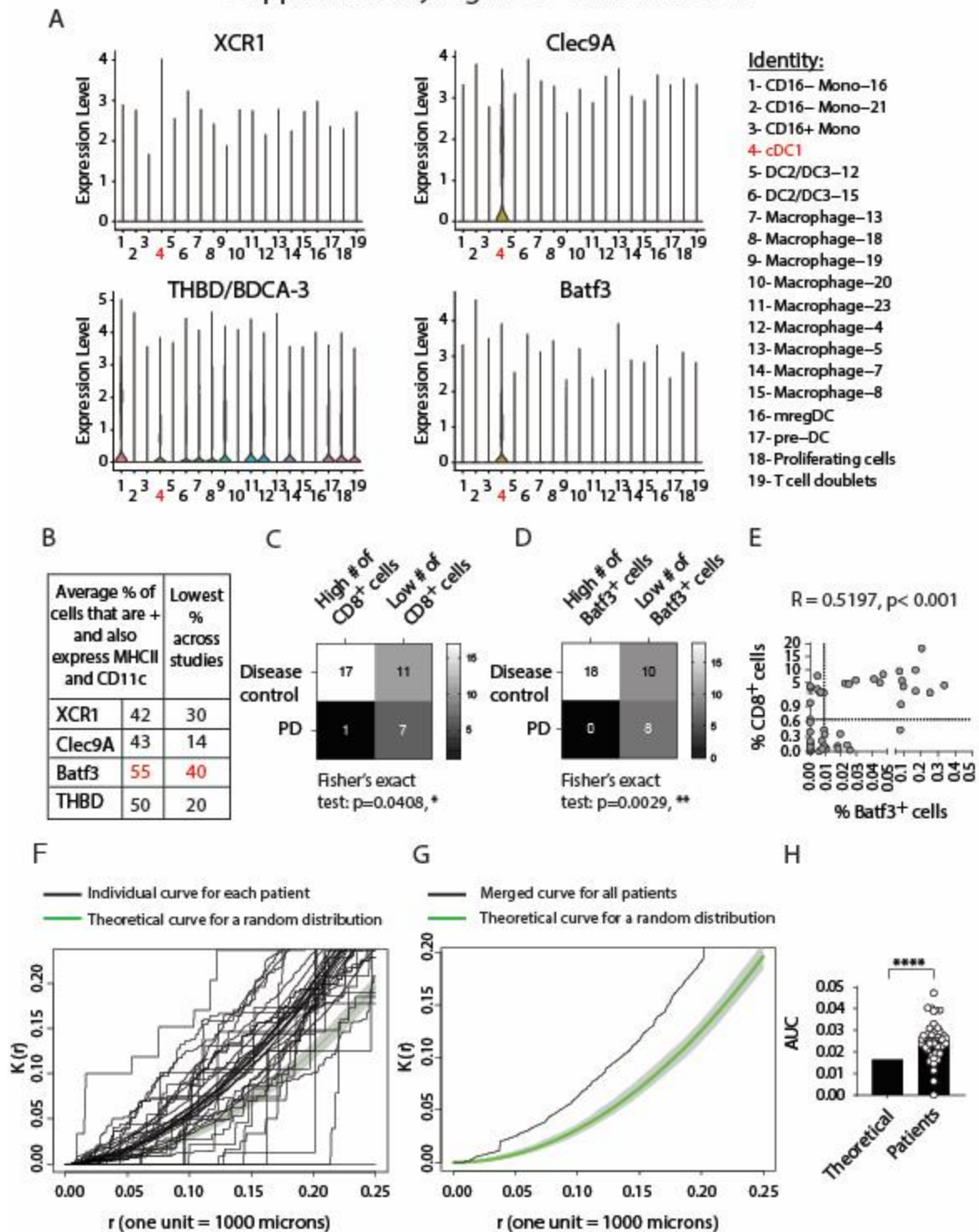


Fig. S5. Selection of the best gene to identify DC1s in human tumor samples, melanoma patients with higher number of CD8⁺ and Batf3⁺ cells have a higher disease control rate, and there is a correlation between the number of CD8⁺ and Batf3⁺ cells and these cells are clustering in primary bladder cancer samples, related to Figure 5. (A) Violin plots showing the expression of DC1 markers (XCR1, Clec9a, BDCA-3/THBD and Batf3) on different myeloid populations. Analysis performed on scRNAseq data from Mulder *et al*²⁸. (B) Average percentage of cells that are positive for the DC1 marker and simultaneously express MHCII and CD11c genes (left column) and lowest percentage of cells that express the DC1 gene and MHCII and CD11c, across all the data sets analyzed (right column). Analysis performed on scRNAseq data from several public data^{28,31–33,102}. (C, D) Heat map representation of the number of patients with high or low number of CD8⁺ cells (C) or Batf3⁺ cells (D) in melanoma and their clinical response to checkpoint blockade. (E) Correlation between the number of CD8⁺ cells and Batf3⁺ cells in 47 samples of human bladder cancer. Each dot on the graph represents one patient and shows the number of each cell type. Dotted lines indicate the median for each cell type. (F) Spatial analysis of the mIF images with the Kcross function that measures the number of CD8⁺ cells within certain distance from Batf3⁺ cells, normalizing by CD8⁺ cells density. Each black curve represents an individual patient, and the green curve indicates the theoretical curve based on a random distribution of cells. Gray area indicates the confidence interval of the theoretical curve. Curves above the upper border of the confidence interval of the theoretical curve for a random distribution indicate CD8⁺ cells and Batf3⁺ cells aggregation/clustering. A total of 41 patients were included in the analysis as they had at least one region of interest with at least one Batf3⁺ cell and two CD8⁺ cells. (G) Spatial analysis where the black line represents the merged curve for all patients and the green line is the theoretical curve for a random distribution of the cells, with the confidence interval in gray. (H) Comparison of the patient's area under the curve (AUC) and the AUC from the theoretical curve. A Fisher's exact test was used for the comparison of the disease control rate in high and low CD8⁺ cell and Batf3⁺ cell numbers groups (C and D), a Spearman's correlation was used to analyze the correlation between CD8⁺ cells and Batf3⁺ cells percentages (E) and a one sample *t*-test was used for the comparison of AUC (H). *, *p*<0.05; **, *p*<0.01; ***, *p*<0.001; ****, *p*<0.0001.

Supplementary Figure 6 - Ziblat A. et al

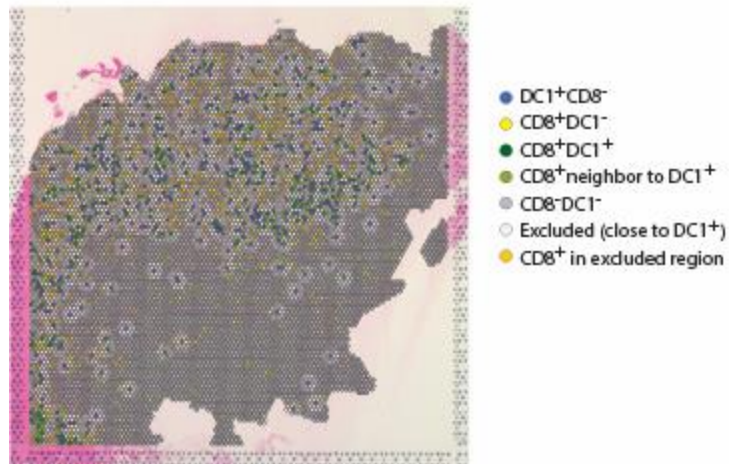


Fig. S6. Strategy used for spatial transcriptomics analysis of human bladder tumor samples, related to Figure 6. Representative image showing the spatial distribution of the Visium spots colored differentially according to the presence/absence of CD8⁺ T cell and DC1 gene markers. In grey, spots that do not express either of the markers. In dark green, spots which express both, CD8⁺ T cell and DC1 markers. In light green, CD8⁺ spots that are next to a DC1⁺ spot. In blue, DC1⁺CD8⁻ spots. In white, spots within 2 spots from a DC1s⁺ spot. In yellow, CD8⁺DC1s⁻ spots, considered distant from DC1s (at least 3 spots from any DC1⁺ spot). In orange CD8⁺ spots that were removed from the analysis for being not distant enough nor close enough to DC1s.