Supplementary Materials for

Critical role of CD206+ macrophages in promoting a cDC1-NK-CD8 T cell anti-tumor immune axis

Arja Ray[1,2], Kenneth H. Hu[1,2,#], Kelly Kersten[1,2,##], Tristan Courau[1,2], Nicholas F. Kuhn[1,2], Itzia Zaleta-Linares[1,2], Bushra Samad[2,3], Alexis J. Combes[1,2,3,4], Matthew F. Krummel[1,2,3*]

Affiliations:
[1]Department of Pathology, [2]ImmunoX Initiative, [3]UCSF CoLabs, [4]Department of Medicine, University of California, San Francisco, CA 94143, USA. [#]Current Address: Department of Immunology, The University of Texas MD Anderson Cancer Center and James P Allison Institute. [##]Current Address: Cancer Metabolism and Microenvironment Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA.

***Corresponding Author:**
Matthew F. Krummel, Ph.D.
513 Parnassus Avenue, HSW 512
San Francisco, CA 94143-0511
matthew.krummel@ucsf.edu
Tel: (415) 514-3130
Fax: (415) 514-3165

Materials and Methods:

Mice: All mice were treated in accordance with the regulatory standards of the National Institutes of Health and American Association of Laboratory Animal Care and were approved by the UCSF Institution of Animal Care and Use Committee. *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$ mice in the C57BL6/J background were custom-generated from Biocytogen Inc. and then maintained heterozygous (bred to C57BL6/J wild type mice) at the UCSF Animal Barrier facility under specific pathogen-free conditions. C57BL6/J, C57BL6/J CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J), Csf1r^{Cre} (C57BL/6-Tg(Csf1r-cre)1Mnz/J) mice were purchased for use from Jackson Laboratories and maintained in the same facility in the C57BL6/J background. For adoptive transfer experiments, CD45.1^{het}; OT-I^{het} (denoted simply as CD45.1; OT1) mice were used. Mice of either sex ranging in age from 6 to 14 weeks were used for experimentation.

Depletion of select immune cell populations: For depletion of CD206-expressing macrophages, 500ng (20ng/g body weight, assuming an average 25g weight for each mouse) diphtheria toxin (DTx; List Biological Laboratories) in 100µL 1X PBS was injected intraperitoneally into each mouse – for both *Csf1r*^{Cre}; *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$ (DTR) and *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$ (WT) groups - at every time point. For the early depletion regime, injections were started 2 days after adoptive transfer of T cells and continued every 2-3 days till endpoint, while for the late depletion regime, injections began at d10 after T cell injection and continued till endpoint. For testing the effects of DTx in tumor-free tissue, similar dosing of DTx as the early depletion regime was implemented without tumor injection, and the skin (ectopic tumor site) and skin-draining lymph nodes were isolated for analysis. Mice were found to be healthy and without frank health issues with 6 doses of 500ng DTx (early depletion regime), but were monitored nevertheless throughout the experiment, as per IACUC guidelines.

For depletion of neutrophils, mice were treated with 200µg/dose of anti-Ly6G antibody (Clone 1A8, InvivoMAb) in PBS intraperitoneally every 2-3 days starting one dose after the beginning of

DTx treatment and coincident with DTx treatment thereafter. Control mice were similarly treated with the corresponding isotype control antibody (Clone 2A3, InvivoMAb).

Mouse tumor digestion and flow cytometry: Tumors from mice were processed to generate single cell suspensions as described previously(*18*). Briefly, tumors were isolated and mechanically minced on ice using razor blades, followed by enzymatic digestion with 200 µg/mL DNAse (Sigma-Aldrich), 100U/mL Collagenase I (Worthington Biochemical) and 500U/mL Collagenase IV (Worthington Biochemical) for 30 min at 37°C while shaking. Digestion was quenched by adding excess 1X PBS, filtered through a 100µm mesh, spun down and red blood cells were removed by incubating with RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) at room temperature for 10 mins. The lysis was quenched with excess 1X PBS, spun down and resuspended in FACS buffer (2mM EDTA + 1% FCS in 1X PBS) to obtain single cell suspensions. Similarly, tumor draining lymph nodes (dLN) were isolated and mashed over 100µm filters in PBS to generate single cell suspensions. For counting absolute numbers of cells, CountBright Absolute Counting Beads were added to the cell suspensions prior to staining, while noting the total weight of the tumor and the fraction of the total tumor cell digest used for staining.

For each sample, 2.5-3 million cells/sample were stained in a total of 50µL of antibody mixture for flow cytometry. Cells were washed with PBS prior to staining with Zombie NIR Fixable live/dead dye (1:500) (Biolegend) for 20 min at 4°C. Cells were washed in FACS buffer followed by surface staining for 30 min at 4°C with directly conjugated antibodies diluted in FACS buffer containing 1:100 anti-CD16/32 (Fc block; BioXCell) to block non-specific binding. Antibody dilutions ranged from 1:100-1:400, optimized separately. After surface staining, cells were washed again with FACS buffer. For intracellular staining, cells were fixed for 20 min at 4°C using the IC Fixation Buffer (BD Biosciences) and washed in permeabilization buffer from the FoxP3 Fix/Perm Kit (BD Biosciences). Antibodies against intracellular targets were diluted in permeabilization buffer

681 containing 1:100 Fc Block and cells were incubated for 30 min at 4°C followed by another wash
682 prior to readout on a BD LSRII or Fortessa Cytometer.
683
684 **Processing and flow cytometry analysis of other mouse organs:** To phenotype cells from
685 lymphoid organs, inguinal, axillary and brachial (tumor-draining) lymph nodes were isolated, pried
686 open with tweezers (lymph nodes) or cut into small pieces (spleen) and digested with the same
687 digestion cocktail as above, intermittently pipetting with cut P1000 pipette tips to enhance
688 mechanical digestion. The resulting suspensions were then filtered using 100µm filter, washed
689 with 1X PBS to generate single cell suspensions. For splenic digests, RBC lysis was performed
690 as described above before staining for flow cytometry.
691 For lung digests both lobes were isolated, cut into small pieces with scissors and minced by using
692 gentleMACS dissociator (Miltenyi Biotec) in RPMI. Next, the mixture was spun down and
693 resuspended in the digestion mixture described above and allowed to digest with shaking at 37°C
694 for 20 mins, following which, the remaining tissue was either minced again using the gentleMACS
695 dissociator and/or directly mashed over a 100µm filter in FACS buffer to generate a single cell
696 suspension, ready to be processed for staining and flow cytometry.
697 Skin digestion was done as previously described(*37*). Briefly, mice were shaved and depilated
698 prior to removal of dorsal skin. The skin was then rid of fat, minced with scissors and razor blade
699 in the presence of 1 ml of digest media (2 mg/ml collagenase IV (Roche), 1 mg/ml hyaluronidase
700 (Worthington), 0.1 mg/ml DNase I (Roche) in RPMI-1640 (GIBCO). The minced skin was then
701 moved to a 50 ml conical with 5 ml additional digest solution and incubated at 37°C for 45 min
702 with shaking and intermittent vortexing before being washed and passed through a 70µm strainer
703 prior to staining.
704
705 **Flow cytometry Data Analysis:** Analysis of flow cytometry data was done on FlowJo and later
706 plotted on GraphPad Prism or R. Relative MFI of the Venus reporter was calculated by subtracting

the background average MFI of the same channel in WT samples from those in each DTR sample. For analysis of a shift in relative abundance of a population x (Fig. 2), the $\log_2$ (% x of CD45 in WT/ % x of CD45 in DTR) was calculated and plotted as a heatmap, such that positive values indicate depletion and negative values indicate enrichment.

Tumor injections and adoptive transfer of CD8 T cells into tumors: The B78chOVA and MC38chOVA cancer cell lines, as previously described(*11, 18*), were generated by incorporating the same mcherry-OVA construct used to establish the PyMTchOVA spontaneous mouse line(*38*). For tumor injections, the corresponding cells were grown to near confluency (cultured in DMEM with 10% FCS (Benchmark) and 1% PSG (Gibco)) and harvested using 0.05% Trypsin-EDTA (Gibco) and washed 3x with PBS (Gibco). The number of cells to be injected per mouse was resuspended in PBS to a final volume of 50µL per injection. The suspension was injected subcutaneously into the flanks of anesthetized and shaved mice. Tumors were allowed to grow for 14–21 days unless otherwise noted, before tumors and tumor-draining lymph nodes were harvested for analysis. CD8 T cells were isolated from CD45.1;OT-1;Cd69-TFP mice using the EasySep Negative Selection Kit (Stem Cell Bio), resuspended in 1X PBS at 10X concentration 100µL was injected into each tumor-bearing mice. For B78chOVA 1 million and for MC38chOVA tumors, 200,000 CD8 T cells were injected retro-orbitally into each mouse either 5d (B78chOVA), 7d (MC38chOVA) post tumor injection. Tumor measurements were done by measuring the longest dimension (length) and approximately perpendicular dimension (width) using digital calipers, rounded to one decimal place each. For experiments using the transgenic PyMTchOVA strain, mammary tumor-bearing females in the age range of 15 to 24 weeks were used when mice developed at least 2 palpable tumors.

Spatial single cell RNA Sequencing and Analysis: Spatial scSeq of immune cell populations at the tumor edge, interface and interior zones was performed using ZipSeq, as previously

described(*11*), with the additional condition of DTx treatment integrated into the dataset. Briefly, B78chOVA tumors subcutaneously grown in Csf1rCre; CD206$^{LSL\text{-}Venus\text{-}DTR}$ mice d12 post adoptive transfer of 1 million CD2dsRed; OT-I CD8 T cells with (DTx) and without (Control) DTx treatment (early depletion regime) were harvested and sliced into 160µm slices using a Compressotome (Precisionary Instruments VFZ-310-0Z). Imaging, spatial barcoding, subsequent digestion, sorting, encapsulation (10X Genomics) and library construction, CellRanger processing and alignment were performed as described previously(*11, 19*). The two separate sequencing runs (Control and DTx) were assembled and integrated into a single data structure using Harmony(*39*). The final object underwent scaling and then scoring for cell cycle signatures (S and G2M scores as computed using Seurat's built-in CellCycleScoring function. The object then underwent regression for cell cycle effects (S and G2M score as described in the Seurat vignette) and percent mitochondrial reads before PCA.

Relative abundance from scSeq data was calculated by: log2 (% of each cluster (cell type) within a tumor region (Edge, Mid, Inner) in the Ctrl / (% of the same cluster in the same region in the DTx treated group), thereby yielding positive values for depletion and negative values for enrichment. While abundances were calculated with the broad clusters from the overall object, the lymphoid clusters were isolated to a separate object, re-clustered to further probe for individual gene expression (*Cxcr3, Flt3l, Xcl1*) in the resulting subsets.

Transwell Assay of CD8 T cell migration: For transwell assays, subcutaneously injected B78chOVA tumors grown for 14 days and then harvested, digested, and sorted for CD206+ vs. CD206- TAMs. 3 days before the sort, CD8 T cells from a B6 mouse were harvested and stimulated in vitro with anti-CD3/anti-CD28 Dynabeads (Thermo Fisher) for 24h, taken off the beads and rested in 10U/mL IL-2 for an additional 48h to produce effector-like CD8 T cells. Post-sort, 500,000 activated T cells were plated in 75µL T cell media (RPMI + 10% FCS + 50µM β -

759 marcaptoethanol) on top of a 5µm transwell insert (Corning), allowed to settle for 30mins and
760 subsequently, 10,000 sorted CD206-, CD206+ TAMs or no TAMs were added to the bottom well
761 to induce T cell migration. Cells at the bottom were collected at 3h, mixed with CountBright
762 absolute counting beads, stained and analyzed by flow cytometry to quantify the number of CD8
763 T cells migrated. Total number of CD8 T cells migrated in each condition was normalized to the
764 average number of cells migrated in the no TAM condition.
765
766 **Human tumor samples:** All tumor samples were collected with patient consent after surgical
767 resection under a UCSF IRB approved protocol (UCSF IRB# 20-31740), as described
768 previously(*23*). In brief, freshly resected samples transported in ice-cold DPBS or Leibovitz's L-
769 15 medium before digestion and processing to generate a single-cell suspension. The five most
770 well-represented cancer indications in this collection were included in the cohort: Colorectal
771 cancer (CRC), gynecological cancers (GYN), head and neck cancer (HNSC), kidney cancer
772 (KID), lung cancer (LUNG). Clinical data including survival of patients were obtained through
773 regular clinical follow-up at UCSF.
774
775 **Transcriptomic analysis of human tumors:** All tumor samples were collected under the UCSF
776 Immunoprofiler project as described(*23*). Briefly, tumor samples were thoroughly minced with
777 surgical scissors and transferred to GentleMACS Tubes containing 800 U/ml Collagenase IV and
778 0.1 mg/ml DNase I in L-15/2% FCS per 0.3 g tissue. GentleMACS Tubes were then installed onto
779 the GentleMACs Octo Dissociator (Miltenyi Biotec) and incubated for 20 min (lymph node) or
780 35 min (tumor) according to the manufacturer's instructions. Samples were then quenched with
781 15 mL of sort buffer (PBS/2% FCS/2mM EDTA), filtered through 100µm filters and spun down.
782 Red blood cell lysis was performed with 175 mM ammonium chloride, if needed. Freshly digested
783 tumor samples were sorted by FACS into conventional T cell, Treg, Myeloid, tumor and in some
784 cases, stromal compartments and bulk RNA-seq was performed on sorted cell fractions. mRNA

was isolated from sorted fractions and libraries were prepared using Illumina Nextera XT DNA Library Prep kit. The libraries were sequenced using 100bp paired end sequencing on HiSeq4000. The sequencing reads we aligned to the Ensembl GRCh38.85 transcriptome build using STAR(*40*) and gene expression was computed using RSEM(*41*). Sequencing quality was evaluated by in-house the EHK score, where each sample was assigned a score of 0 through 10 based on the number of EHK genes that were expressed above a precalculated minimum threshold. The threshold was learned from our data by examining the expression distributions of EHK genes and validated using the corresponding distributions in TCGA. A score of 10 represented the highest quality data where 10 out of 10 EHK genes are expressed above the minimum threshold. The samples used for survival analysis and other gene expression analyses had an EHK score of greater than 7 to ensure data quality. Ensemble gene signatures scores were calculated by converting the expression of each gene in the signature to a percentile rank among all genes and then determining the mean rank of all the genes in the signature (*17*). The corresponding gene list for obtaining the stimulatory dendritic cell score is as described before(*10*).

TCGA analyses. Survival analyses using the TCGA dataset was performed using the TCGA sub-cohort described in(*23*). Briefly, tumor RNAseq counts and TPM along with curated clinical data for 13 cancer types (BLCA, COAD, GBM, GYN (grouping OV, UCEC and UCS), HNSC, KIRC, LIHC, LUAD, PAAD, SARC and SKCM) was filtered down to include primary solid tumors and metastatic samples only, to parallel the IPI cohort samples. This reduced the TCGA sample set to 4341 tumor samples. $CD206^{Replete}$ gene scores were generated by first normalizing (using percentiles) the expression values of each gene composing the signature across all patients, followed by averaging these normalized values for each patient. The same method was used for deriving $CD206^{Depleted}$ gene scores and we then calculated the ratio of $CD206^{Replete}/CD206^{Depleted}$ gene scores by dividing each score value for each patient. For survival analysis, patients were

split into either $CD206^{Replete}$ gene score ^{HIGH} vs ^{LOW} (top/bottom 20% respectively, n=861) or ($CD206^{Replete}$:$CD206^{Depleted}$ gene signature ratio)^{HIGH} vs ($CD206^{Replete}$:$CD206^{Depleted}$ gene signature ratio)^{LOW} (top/bottom 20% respectively, n=861) and analyzed using a log-rank test.

Two-photon imaging of tumor slices: Tumor slices (adjacent to the ones used for spatial barcoding by ZipSeq) were fixed in 2% paraformaldehyde (PFA; Sigma), washed and left overnight in 1X PBS before imaging on a custom-made 2-photon microscope as previously described(*10*) to visualize the Venus reporter and CD2dsRed marked CD8 T cells and fibrous collagen by second harmonic generation (SHG). Dual laser excitations at 800nm and 950nm were used to excite the requisite fluorophores.

Statistical Analysis: Statistical analysis was done in GraphPad Prism or in R. For testing null hypothesis between two groups, either Student's t tests and or the non-parametric Mann-Whitney U tests were used, depending on the number and distribution of data points. Likewise, for testing null hypotheses among 3 or more groups, ANOVA or non-parametric tests were performed, followed by post-hoc test, correcting for false discovery rates (threshold = 0.05) in multiple comparisons. Unless otherwise mentioned, data are representative of at least 2 independent experiments.

Supplementary Figures and Figure Legends:

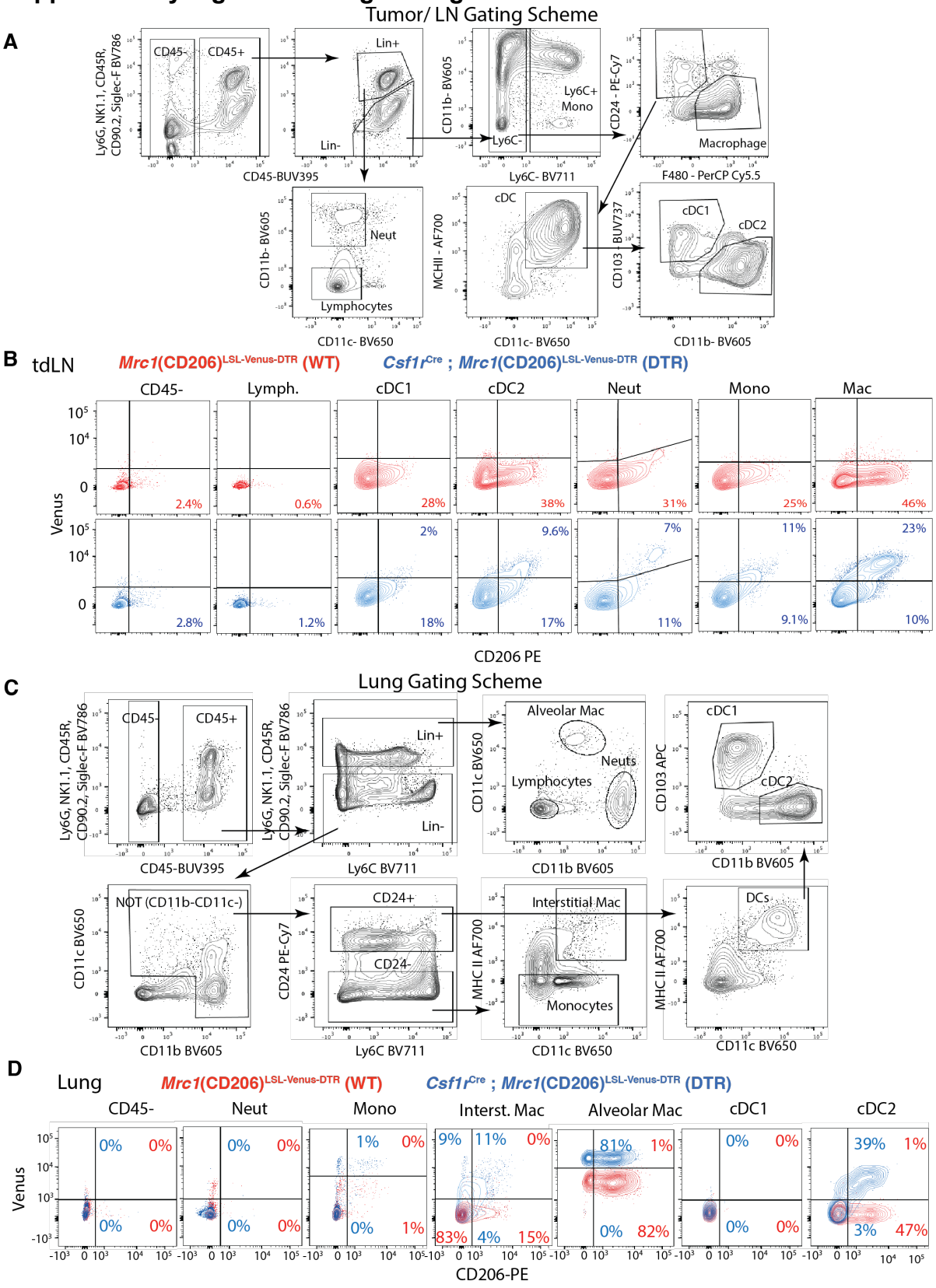
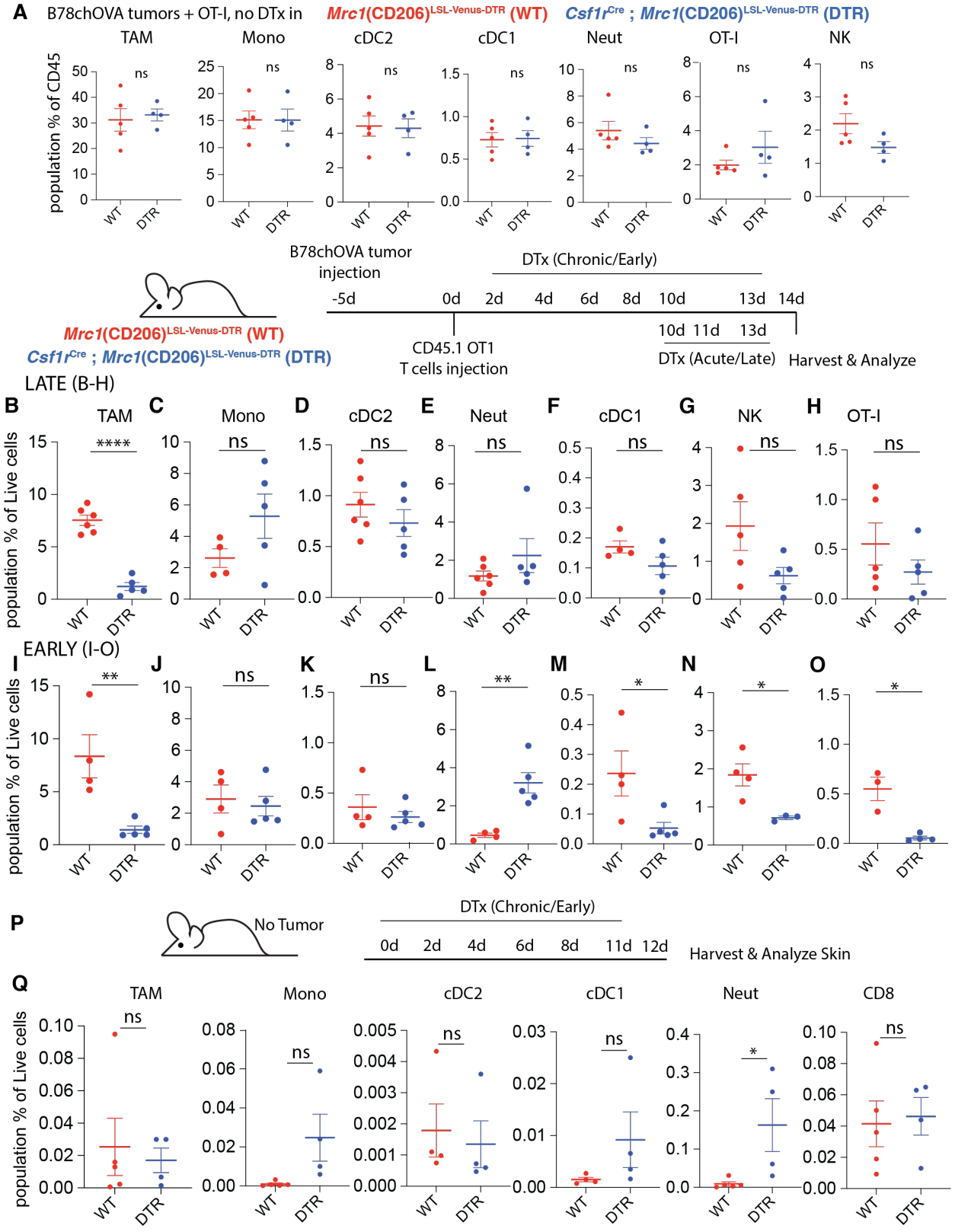


Fig. S1: Representative flow cytometry gating scheme to identify myeloid cells and lymphocytes from **(A)** tumor and tdLN and **(C)** lung;Flow cytometry plots showing reporter (Venus) and CD206 expression in different immune cells in (**B**) d18 B78chOVA tdLN and (**C**) lung in WT (red; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$) and DTR (blue; *Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$) mice.



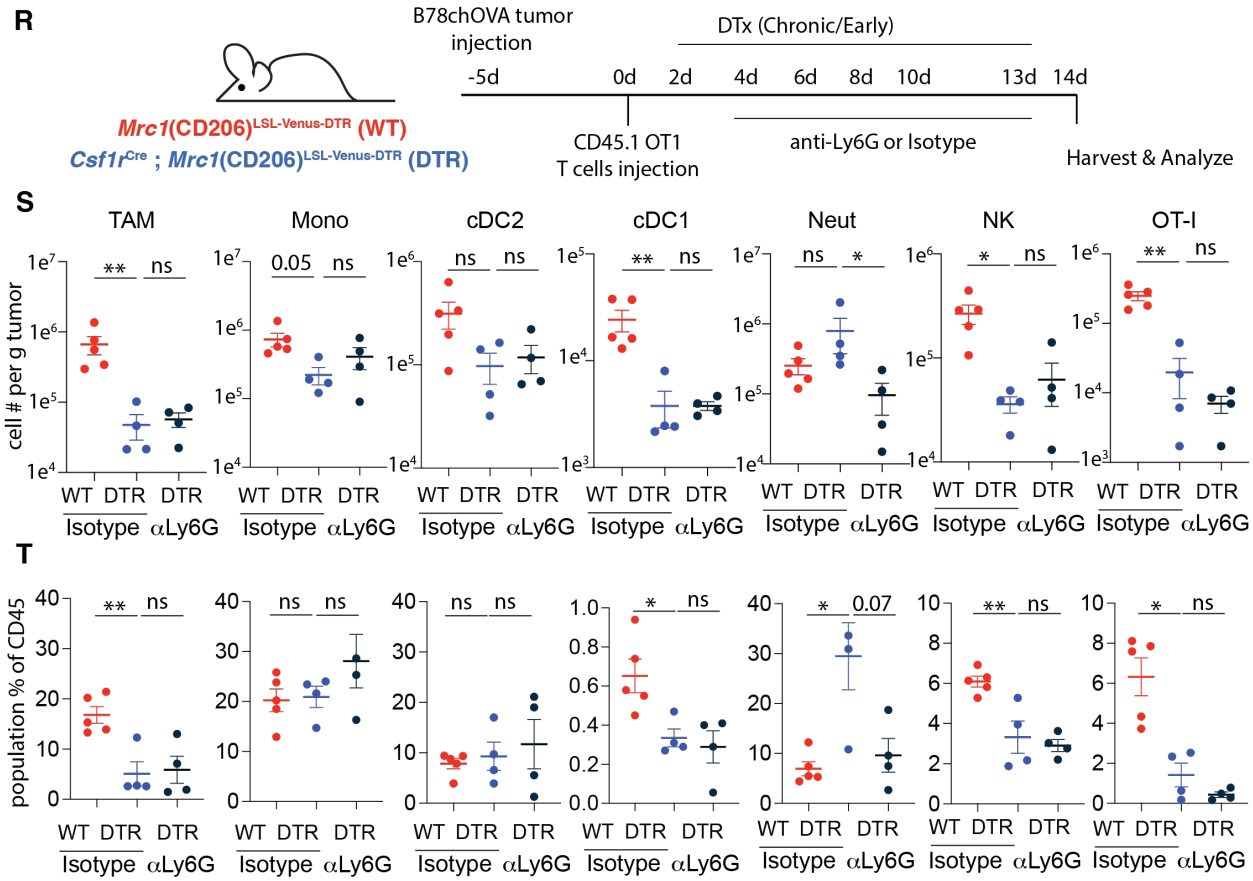
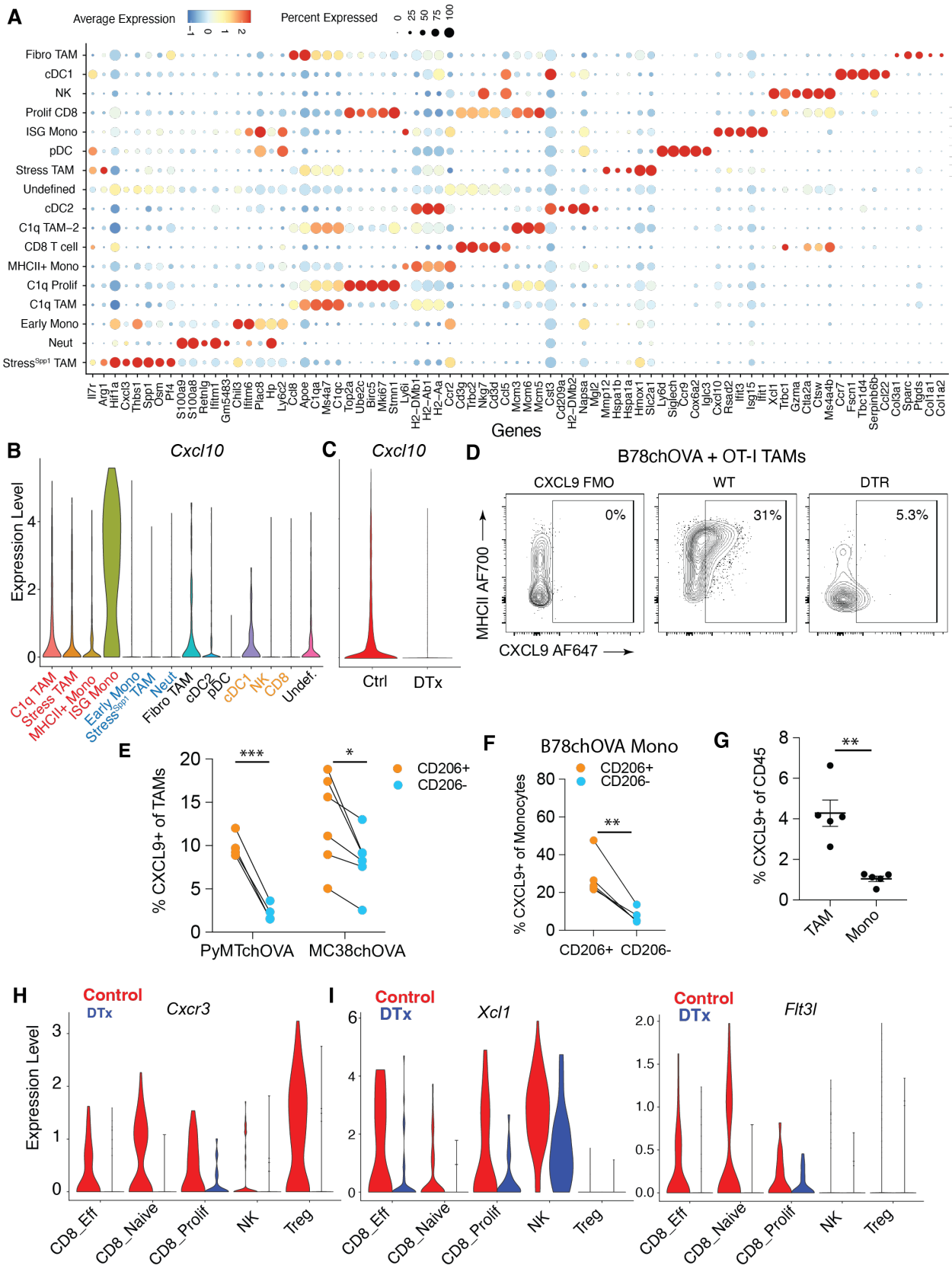
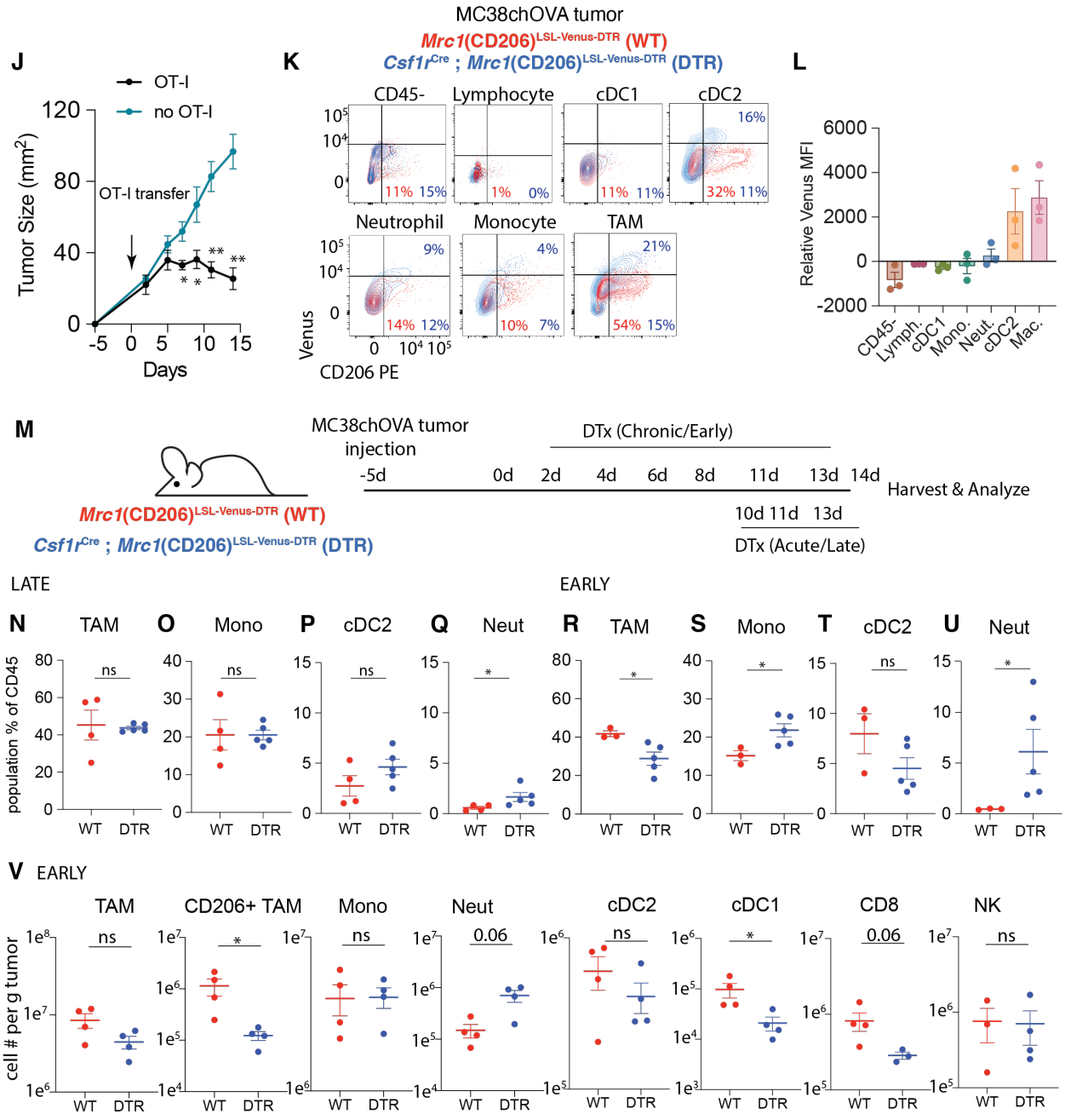


Fig. S2: (A) Relative abundance of different immune populations as a percentage of CD45+ cells in *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$ (WT) and *Csf1r*^{Cre}; CD206$^{LSL\text{-}Venus\text{-}DTR}$ (DTR) mice with B78chOVA tumors (day-5), OT-I adoptive transfer (day 0) and harvest at d14 without DTx administration; Schematic representation of the experimental setup for tumor injection, OT-I T cell adoptive transfer, early and late diphtheria toxin administration and analysis; Relative abundance of different immune populations as a percentage of live cells with **(B-H)** late and **(I-O)** early depletion regimens. (**P**) Schematic representation of the experimental setup for analysis of skin in *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$ (WT; red) and *Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$ (DTR; blue) mice with DTx administration; **(Q)** Relative abundance of different immune populations in the skin as a percentage of live cells; (**R**) Schematic representation of the experimental setup for B78chOVA tumor injection, OT-I T cell adoptive transfer, and early diphtheria toxin administration with either isotype control or anti-Ly6G antibody treatment and analysis; Abundance of different immune populations as (**S**) cells per g of tumor and **(T)** percentage of CD45+ cells in WT and DTR mice. ****p<0.0001, **p<0.01, *p <0.05, ns = no significance by Student's t-tests or Mann-Whitney test, or ANOVA with post-hoc test correcting for false discovery (*alpha < 0.05, ** alpha < 0.01). Bar graph data are shown as mean +/- SEM.





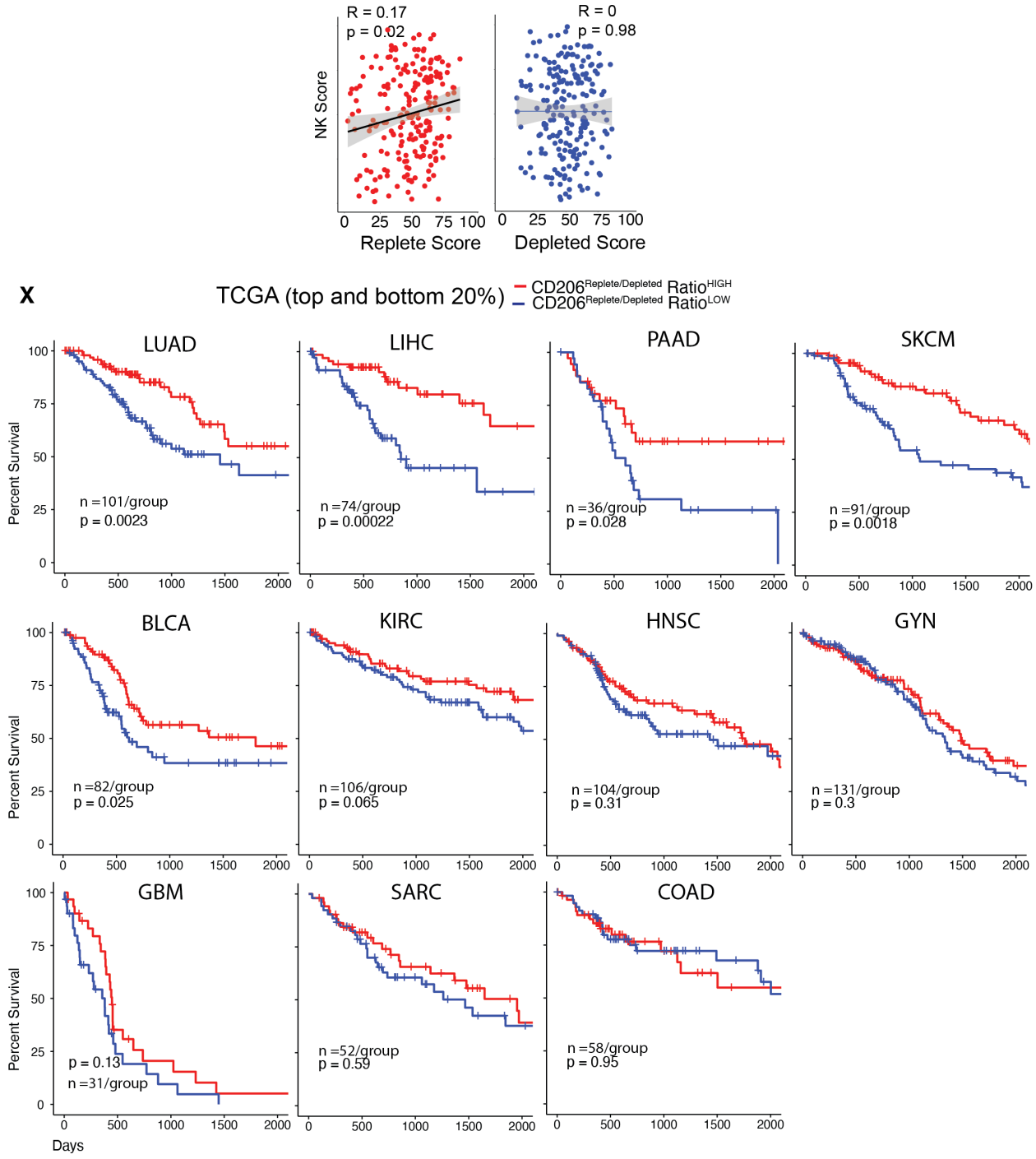


Fig. S3: (A) Dotplot representing top5 differentially expressed genes and select other genes in each immune cell cluster identified from a harmonized dataset of spatially barcoded Control and DTx treated B78chOVA tumors d12 post adoptive transfer of CD2dsRed; OT-I cells; *Cxcl10* expression **(B)** aggregated across treatment conditions by cluster and **(C)** aggregated across clusters by treatment; **(D)** Representative flow cytometry plots showing CXCL9 expression in

B78chOVA TAMs with or without DTx mediated depletion; **(E)** CXCL9 expression in PyMTchOVA and MC38chOVA (both without OT-I adoptive transfer) TAMs split by their CD206 expression; **(F)** CXCL9 expression in OT-I treated B78chOVA (d14 post adoptive transfer) monocytes and **(G)** relative abundance of CXCL9+ TAMs and Monocytes in the same context; Violin plot representing **(H)** *Cxcr3*, **(I)** *Xcl1* and *Flt3l* expression in the lymphoid compartment in Control and DTx treated conditions; **(J)** Representative time course of MC38chOVA tumor size with or without adoptive transfer of OT-I T cells; **(K)** Overlaid flow cytometry plots showing reporter (Venus) and CD206 expression in different immune cells in MC38chOVA tumors in WT (red; $Mrc1^{\text{LSL-Venus-DTR}}$) and DTR (blue; $Csf1r^{\text{Cre}}$; $Mrc1^{\text{LSL-Venus-DTR}}$) mice and **(L)** quantification of relative reporter expression (DTR – WT) in the different subsets. **(M)** Schematic representation of the experimental setup for early and late CD206+ TAM depletion in MC38chOVA tumors using $Mrc1^{\text{LSL-Venus-DTR}}$ (WT) and $Csf1r^{\text{Cre}}$; $Mrc1^{\text{LSL-Venus-DTR}}$ (DTR) mice; Relative abundance of different immune populations as a percentage of CD45+ cells with **(N-Q)** late and **(R-U)** early depletion regimens. **(V)** Abundance of different immune populations as total number of cells per g of MC38chOVA tumor in WT and DTR mice by the early DTx administration regimen; **(W)** Scatter plots of the $CD206^{\text{Replete}}$ and $CD206^{\text{Depleted}}$ Mono/Mac score per patient with the NK cell score (Pearson R and p value for the null hypothesis that there is not a correlation are noted); **(X)** Kaplan-Meier survival curves of patients grouped by the value of the $CD206^{\text{Replete}}$: $CD206^{\text{Depleted}}$ signature ratio (top and bottom 20%) from TCGA split by indications , p values for the log-rank test are noted for each curve in (X) bar graph data are mean +/- SEM, ****p<0.0001, **p<0.01, *p <0.05, ns = no significance by paired ratio t-tests (E, F) or unpaired t-tests or Mann-Whitney test.