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Supplemental information

CX3CR1 deficiency-induced TIL tumor restriction as a novel addition for CAR-T design in solid malignancies

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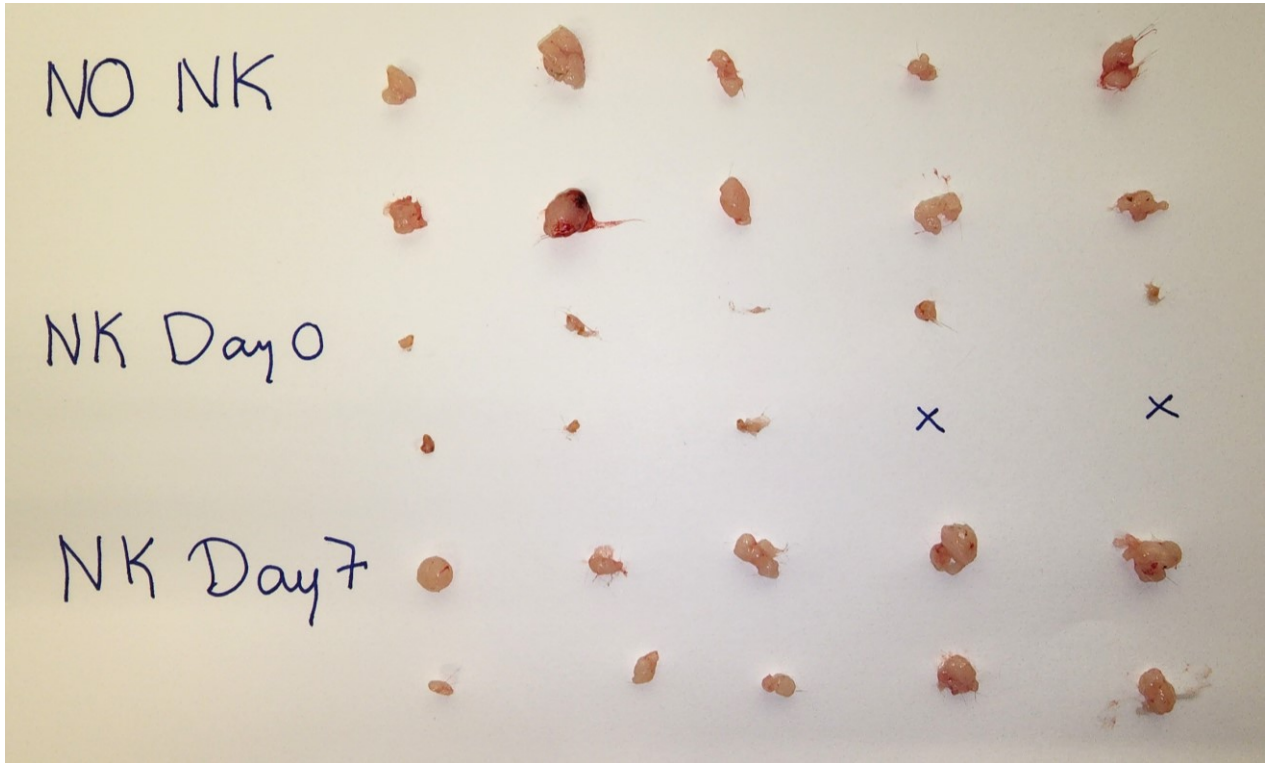
Supplemental Figure 1

A



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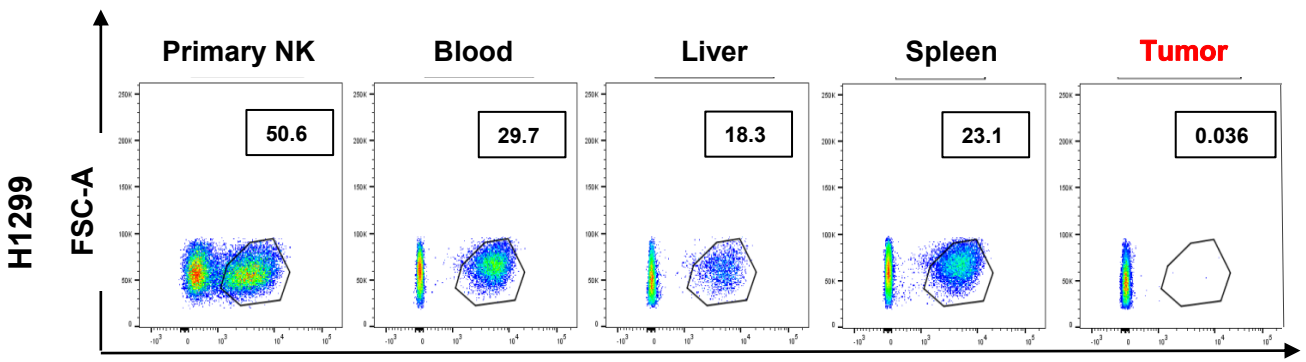
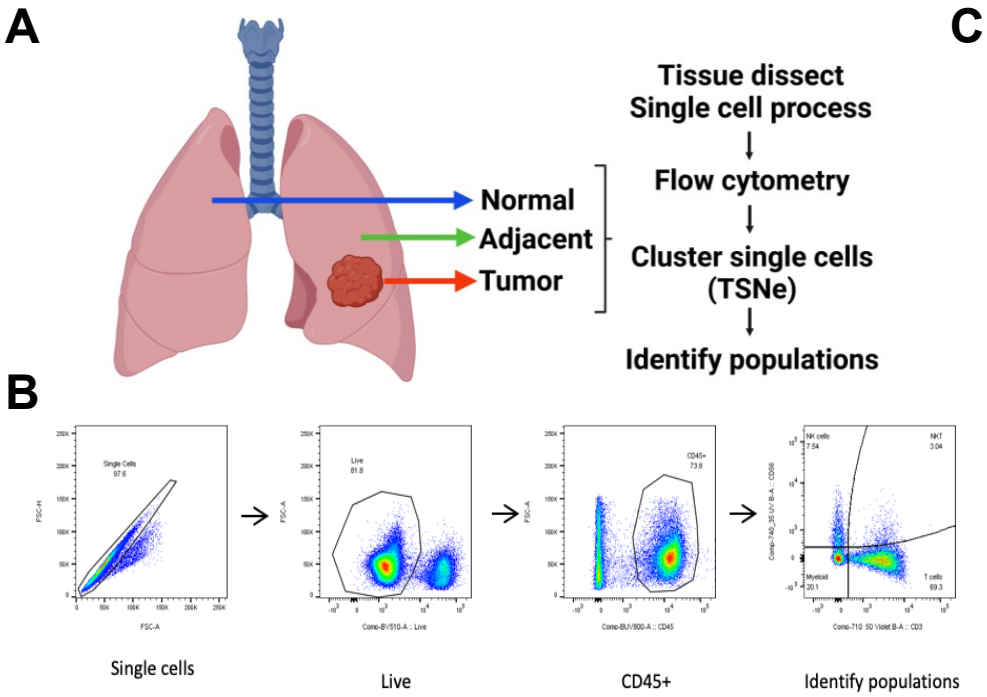
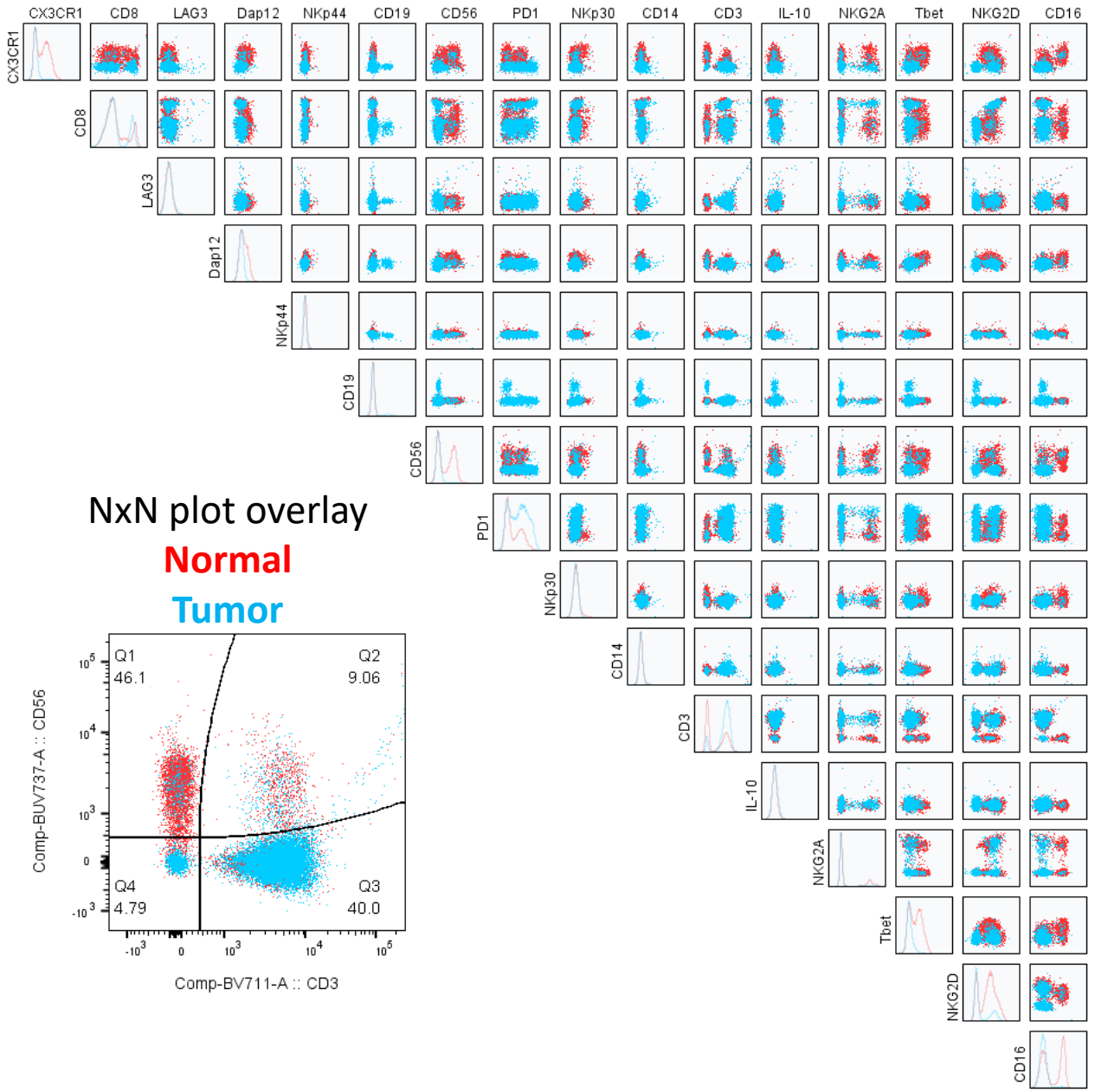


Restricted infiltration of NK cells in A549 and H1299 growing tumor. A) Tumors isolated from the mice shown in ***figure 1A*** and quantified via luciferase in ***figure 1B***. X in NK Day 0 denotes the absence of any tumor cells. **B)** Repeat of experiment as in ***figure 1C-D*** using luciferase-expressing H1299 lung cancer cell line inoculated into NSG mice. When tumor was palpable, isolated primary human NK cells are added and after 5 days tissues were collected and mononuclear cells were analyzed by flow cytometry for the presence of $hCD45^{+}$ cells to assess for infiltration into different tissues (representative figure of n=3).

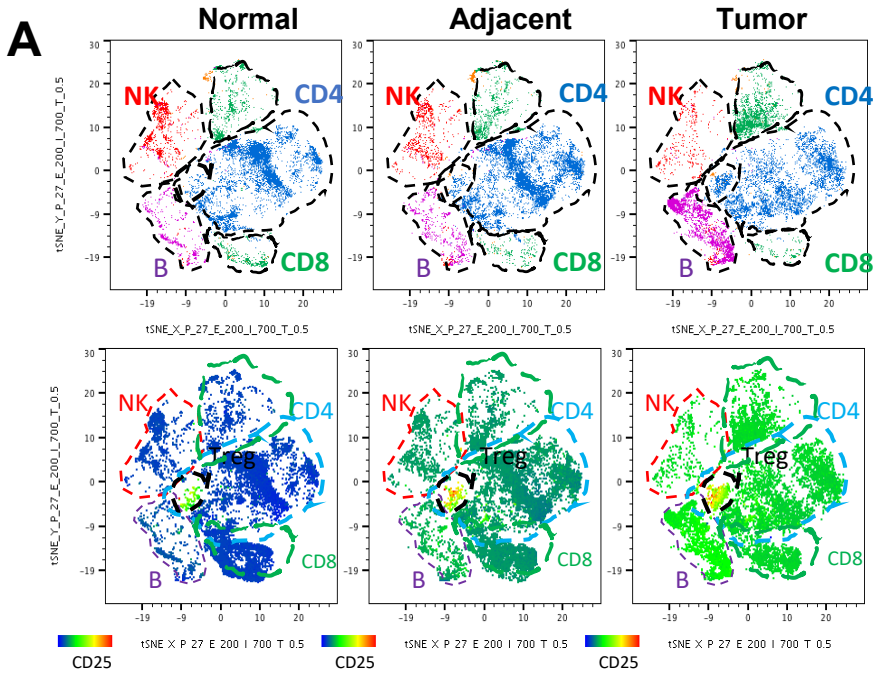
Supplemental Figure 2

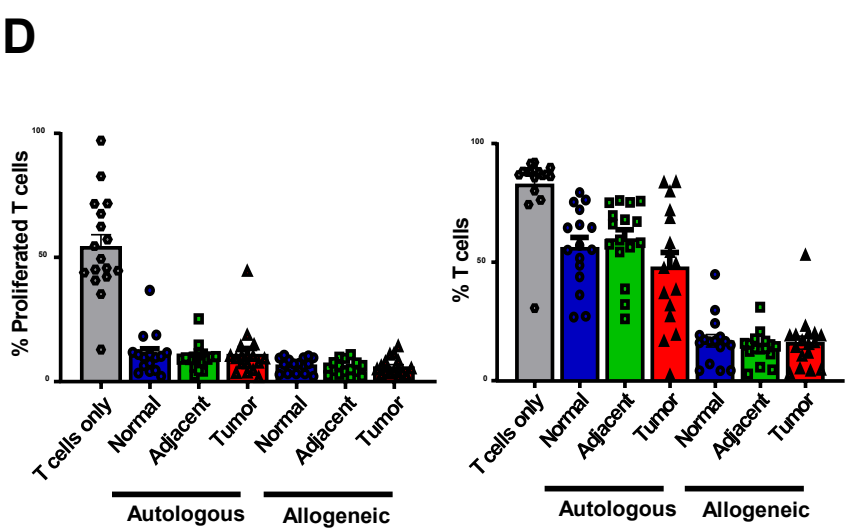


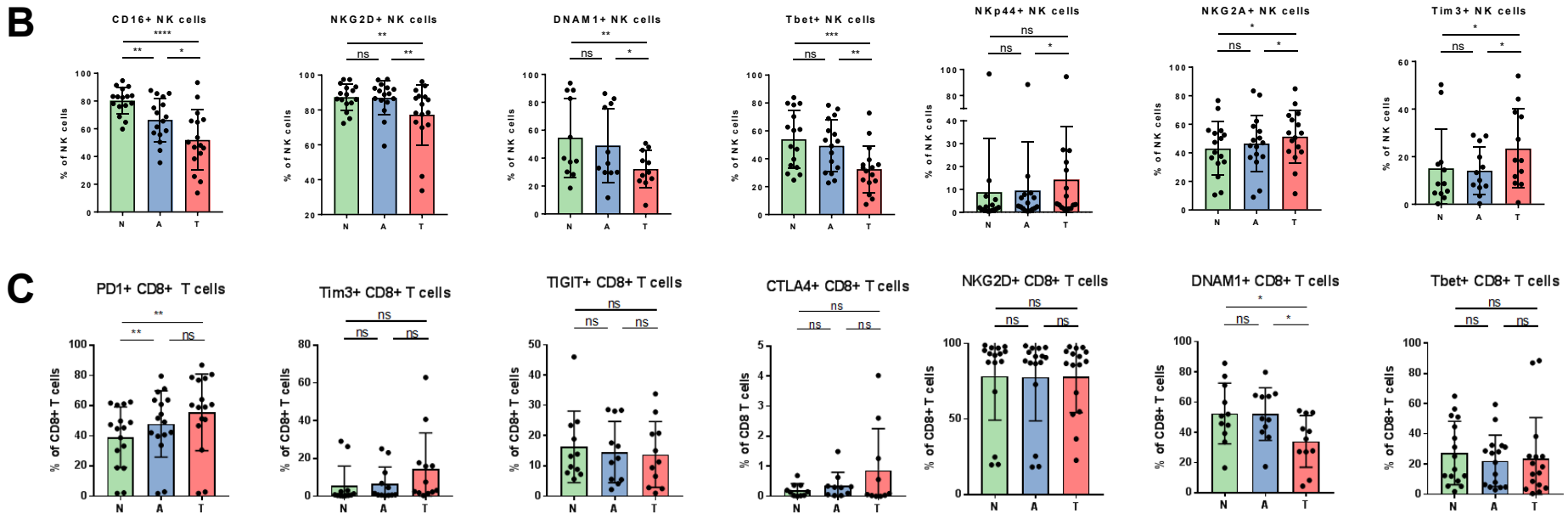


Schematics of lymphocyte populations collection and flow analysis (STAR METHODS: Patient specimens and flow cytometry). A) Schematic representation of primary lung tissue isolation locations. Developed with Biorender. **B)** Gating strategy of leukocytes isolated from the different primary tissues as in A. **C)** NxN plot overlay of the panel used for tSNE analysis comparing tumor versus normal cells from primary lung cancer specimens, as shown (n=16). Main populations included NK cells: $CD3^{-}CD56^{+}$, T cells: $CD3^{+}CD56^{-}$, NKT cells: $CD3^{+}CD56^{+}$, B cells: CD19 & immature myeloid sub-populations defined in the main figure.

Supplemental Figure 3

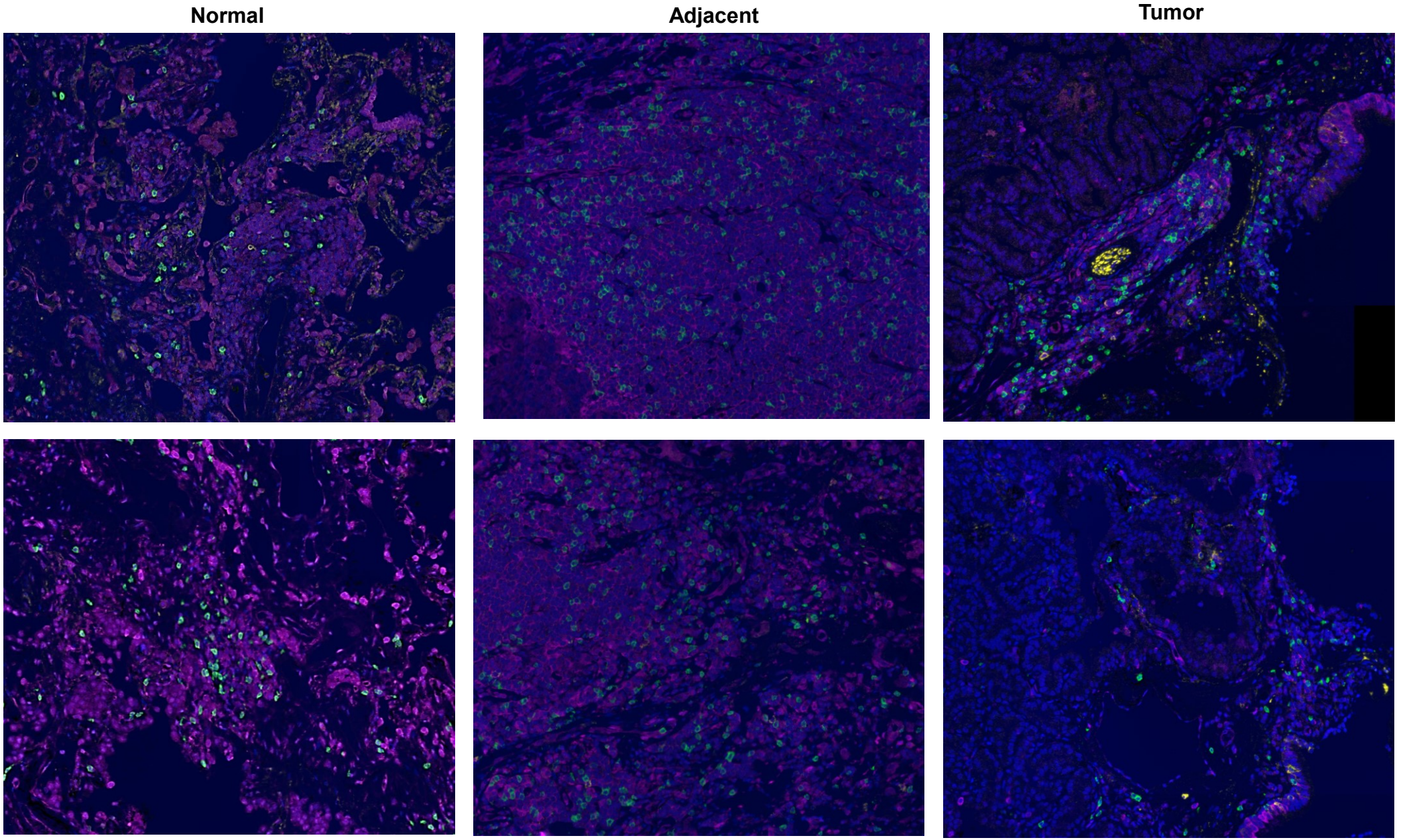




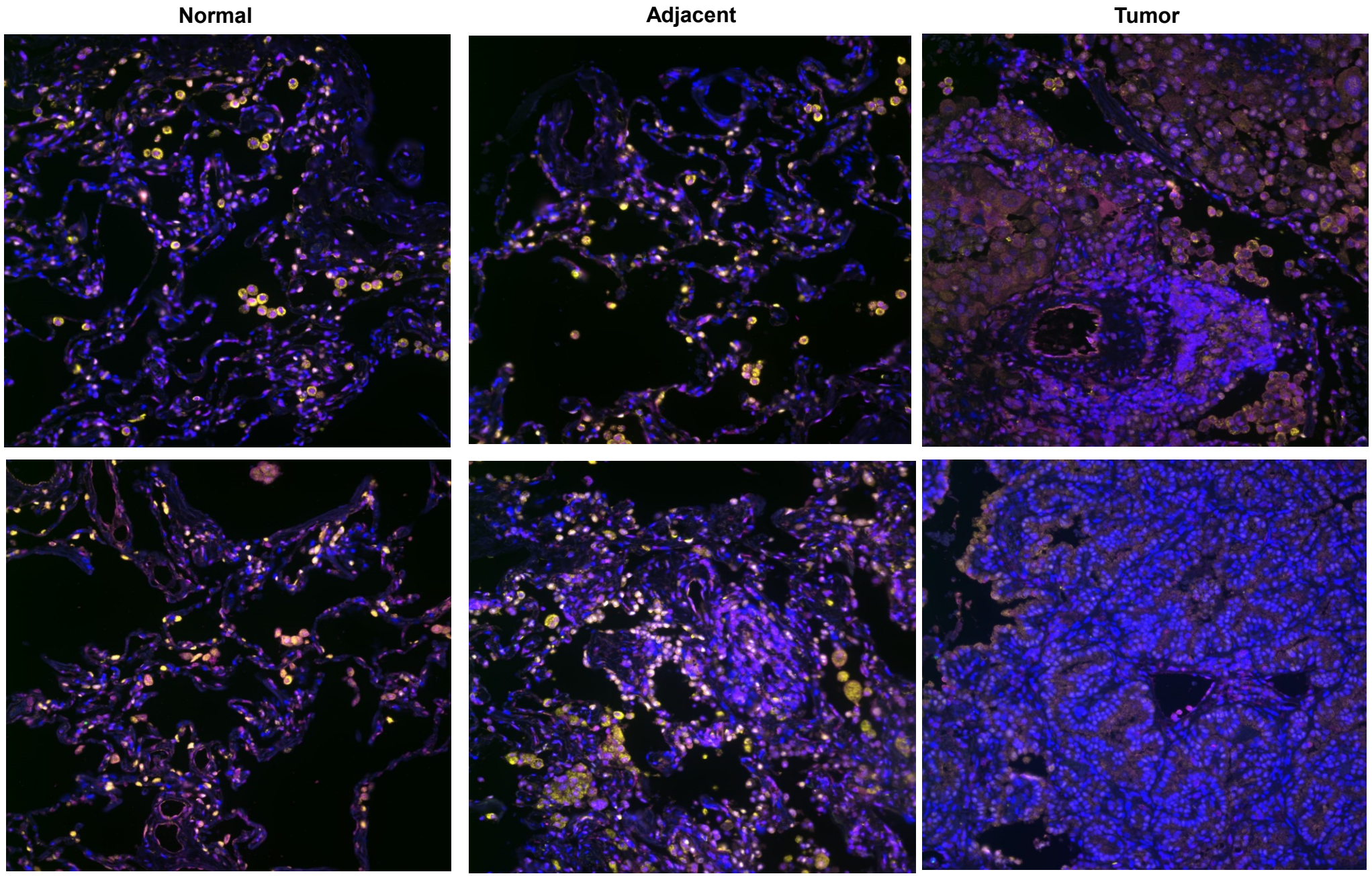


Analysis of lymphocyte populations in primary tumors (STAR METHODS: Patient specimens and flow cytometry). A) Representative TSNe analysis of lymphocyte populations in different locations in the same primary specimen and T reg ($CD25^+$, bottom panel). **B)** Changes in different receptors in NK cells from primary lung cancer specimens, as shown (n=16). **C)** Changes in different receptors in $CD8^+$ cytotoxic T cells cells from primary lung cancer specimens, as shown (n=16) from the same panel as ***figure 2*** **D)** Enriched $CD11b^+$ cells were isolated from mononuclear cells from lung tissues from 16 specimens away normal tissue, adjacent normal tissue or tumor tissue. Afterwards they were co-cultured for 72 hours with either autologous T cells, or an allogeneic T cells, stained with CFSE to measure proliferation by flow cytometry. T cell to MDSC ratio was 4:1. We also measured the percentage of T cells after the assay. Both panels compared with Kruskal-Wallis ANOVA test.

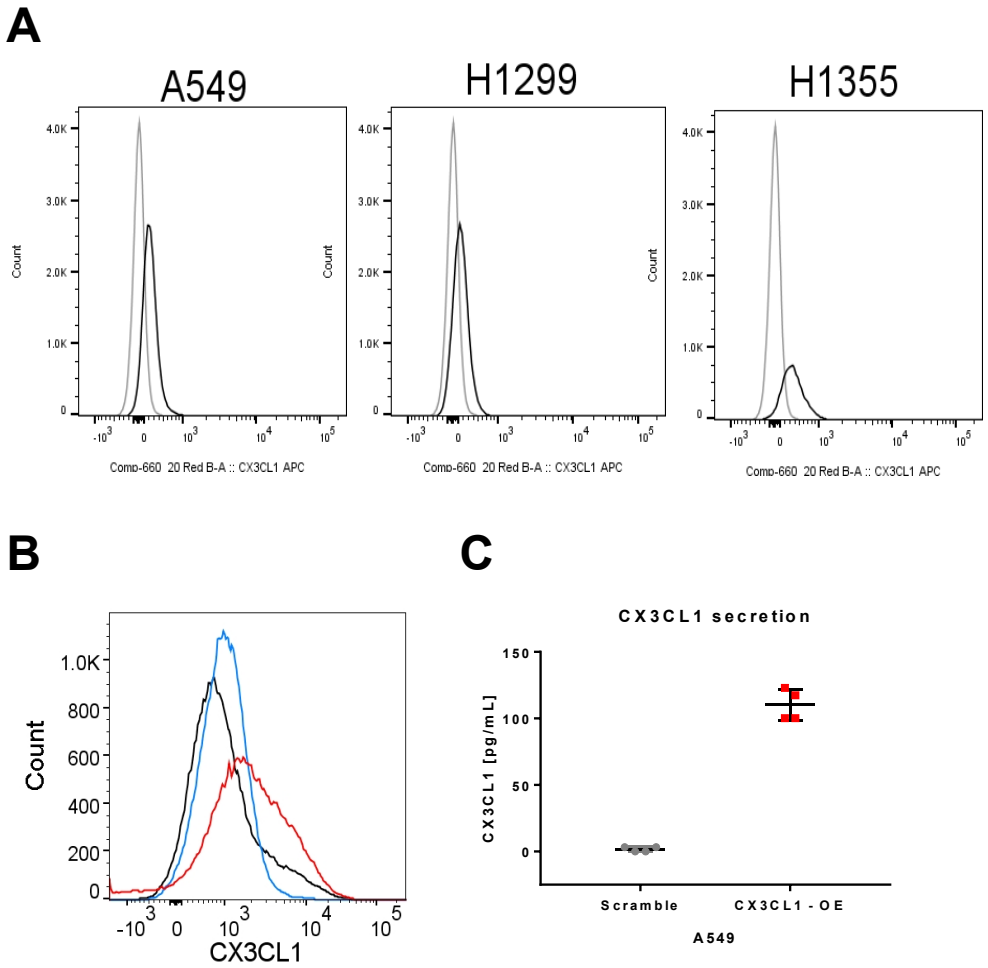
Supplemental Figure 4: Complete (lower magnification) image as in main ***figure 2D***



Supplemental Figure 5: Complete (lower magnification) image as in main ***figure 2E***

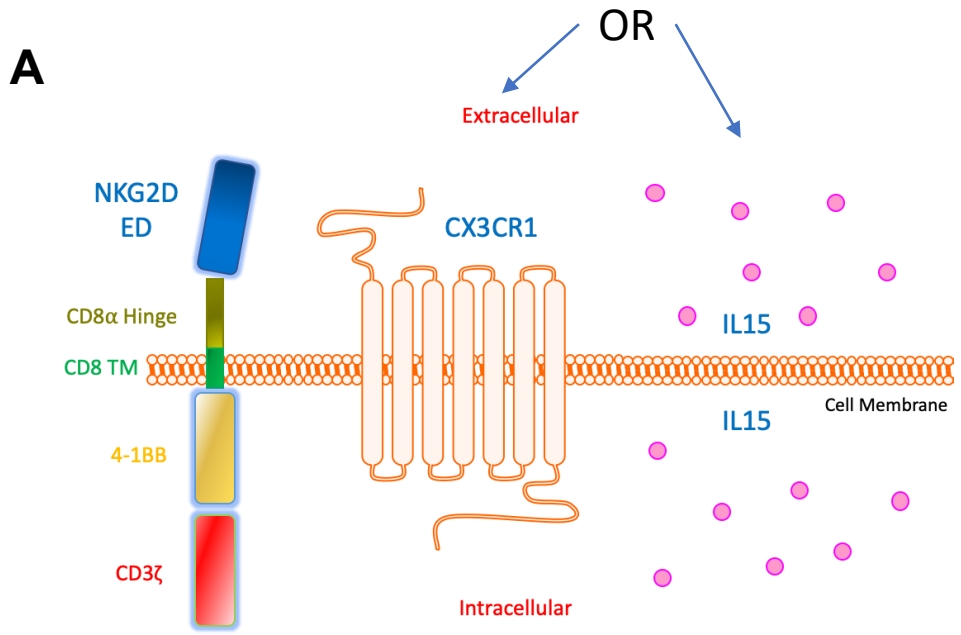


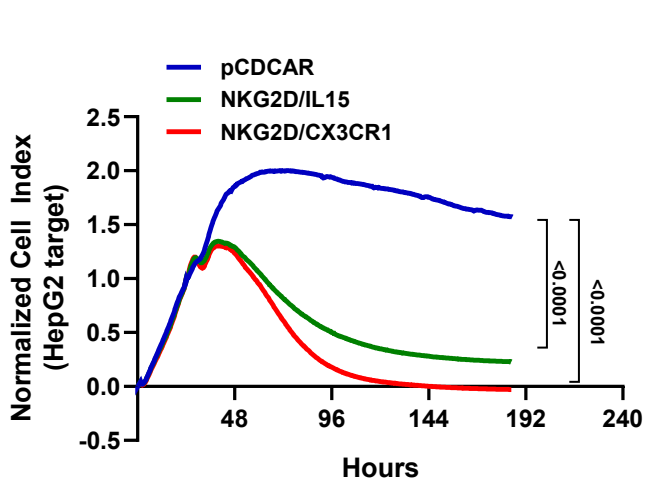
Supplemental Figure 6

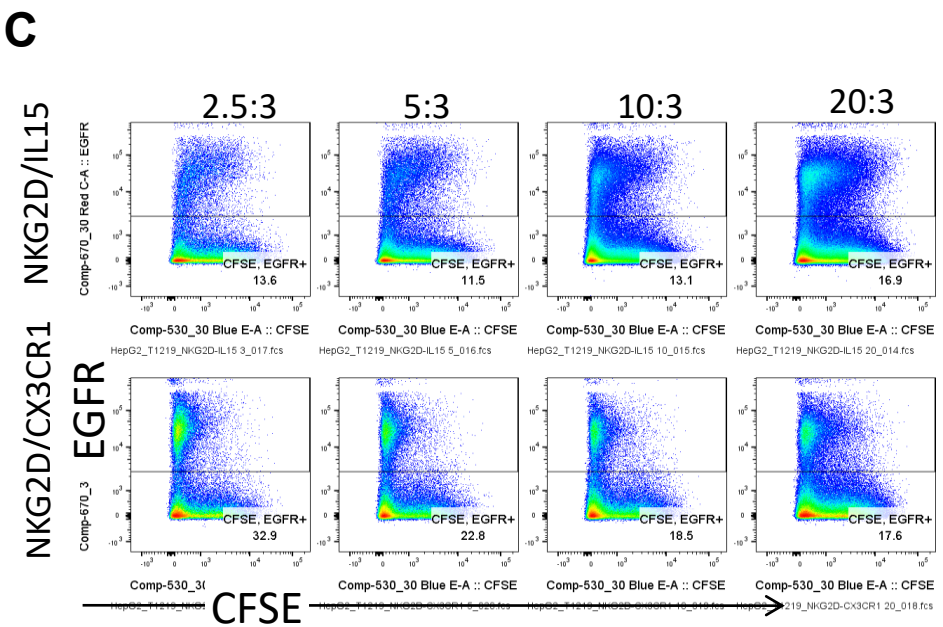


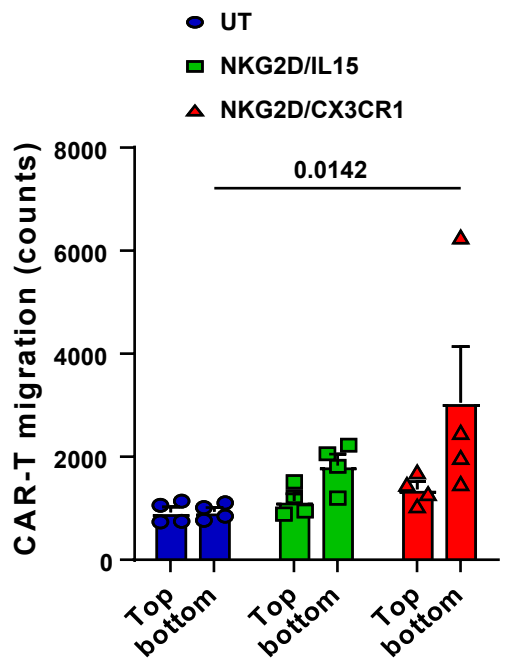
Expression of CX3CL1 in cell lines (STAR METHODS: Cell lines and flow cytometry). A) Flow cytometric quantification of CX3CL1 expression in cell lines A549, H1299 and H1355. **B)** Flow cytometric analysis of CX3CL1 expression in A549 (blue) or A549 overexpressing CX3CL1 (red) against isotype control (grey). **C)** CX3CL1 secretion in A549 cells with or without CX3CL1 overexpression.

Supplemental Figure 7



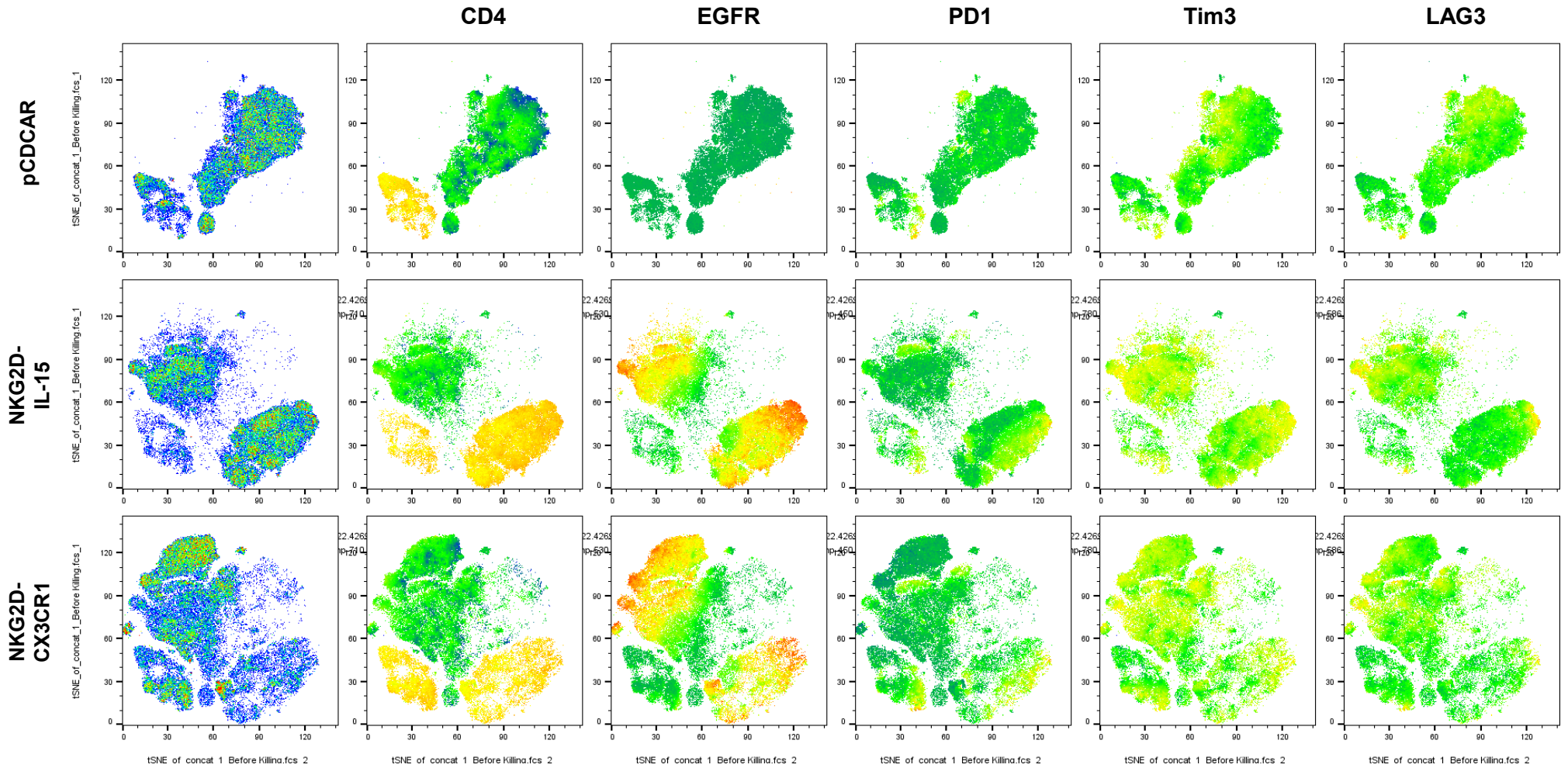






CX3CR1 co-expression in NKG2D CAR-T design (STAR METHODS: CAR-T sections). A) Schematic representation of NKG2D CAR-T co-expression with either CX3CR1 or IL-15 after transfection with our constructs. **B)** CAR-T cytotoxic assay of HepG2 liver cancer cell line as a target to complement the experiment shown in ***figure 4B*** analyzed with one way ANOVA. **C)** CFSE-label proliferation of CAR-T cells ($EGFR^{+}$) cells after co-culture with A549 cells at the ratio shown isolated from the experiment in ***figure 4C***. Representative figure of n= 3. **D)** Mobilization of CAR-T cells (as shown) towards A549 cells. A549 cells were seeded confluently at the bottom of a 24 plate with trans-well inserts (5um pore size). Five hundred thousand CAR-T cells were seeded at the top and the media was changed for non-supplemented media and 200uL of FBS added at the bottom of the wells. Cells were allowed to migrate for 48 hours and measured by flow cytometry (n=4).Significance was defined by 2way ANOVA with multiple comparison analysis.

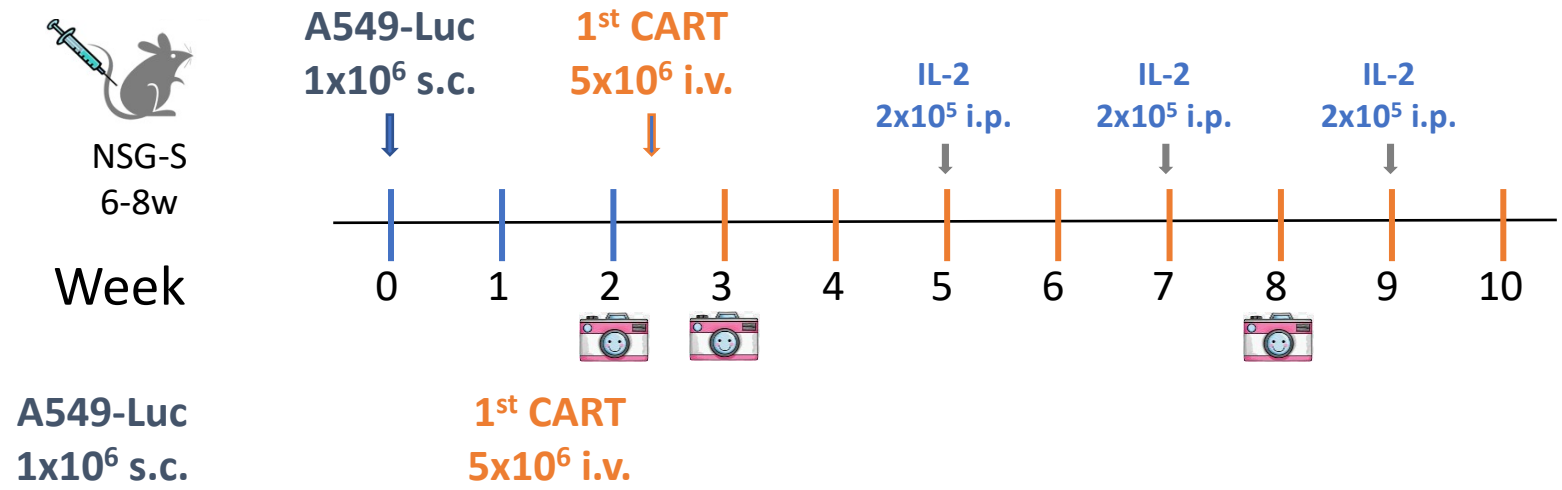
Supplemental Figure 8



Phenotypic tSNE analysis of checkpoint receptors in CAR-T prior to tumor. tSNE analysis of expression of CD4, EGFR, PD1, Tim3, LAG3, in CAR-T cells prior to co-incubation with target cells (post-co-incubation tSNE is in ***figure 4D***. First column shows the event distribution pattern in pseudocolor to highlight cellular density, the second column shows the expression (yellow) of CD4 which we can infer that the green T cells are CD8 expressing cells. EGFR is only positive in CAR-T containing T cells. Expression of PD1, Tim3 and LAG-3 is minimal in these cells.

Supplemental Figure 9

A



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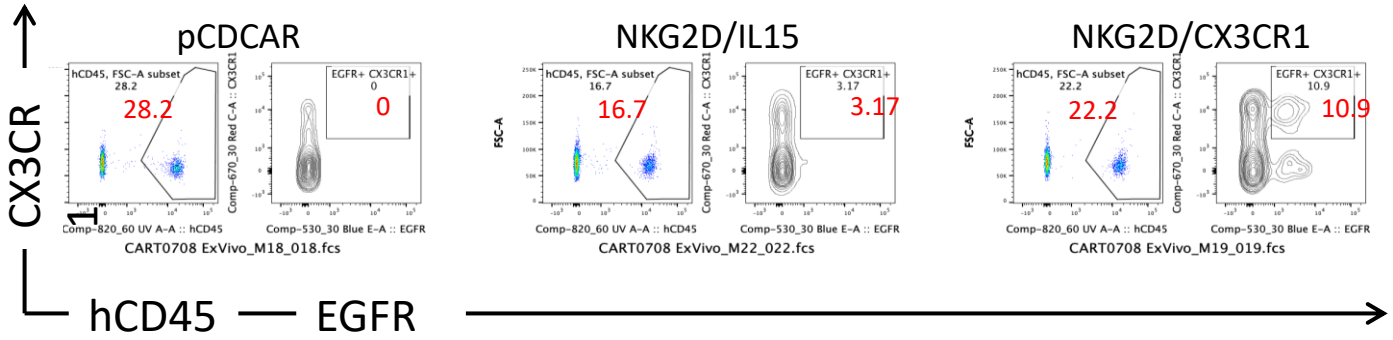
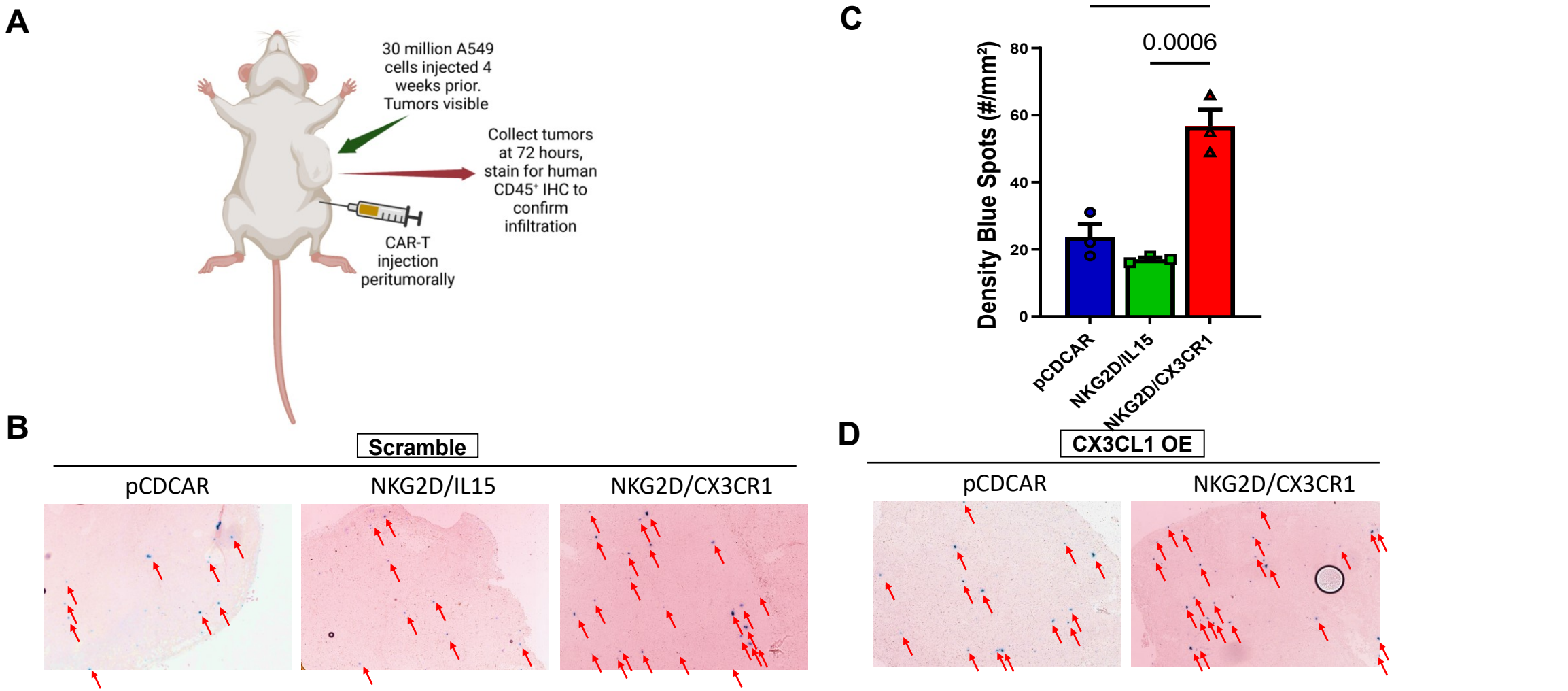


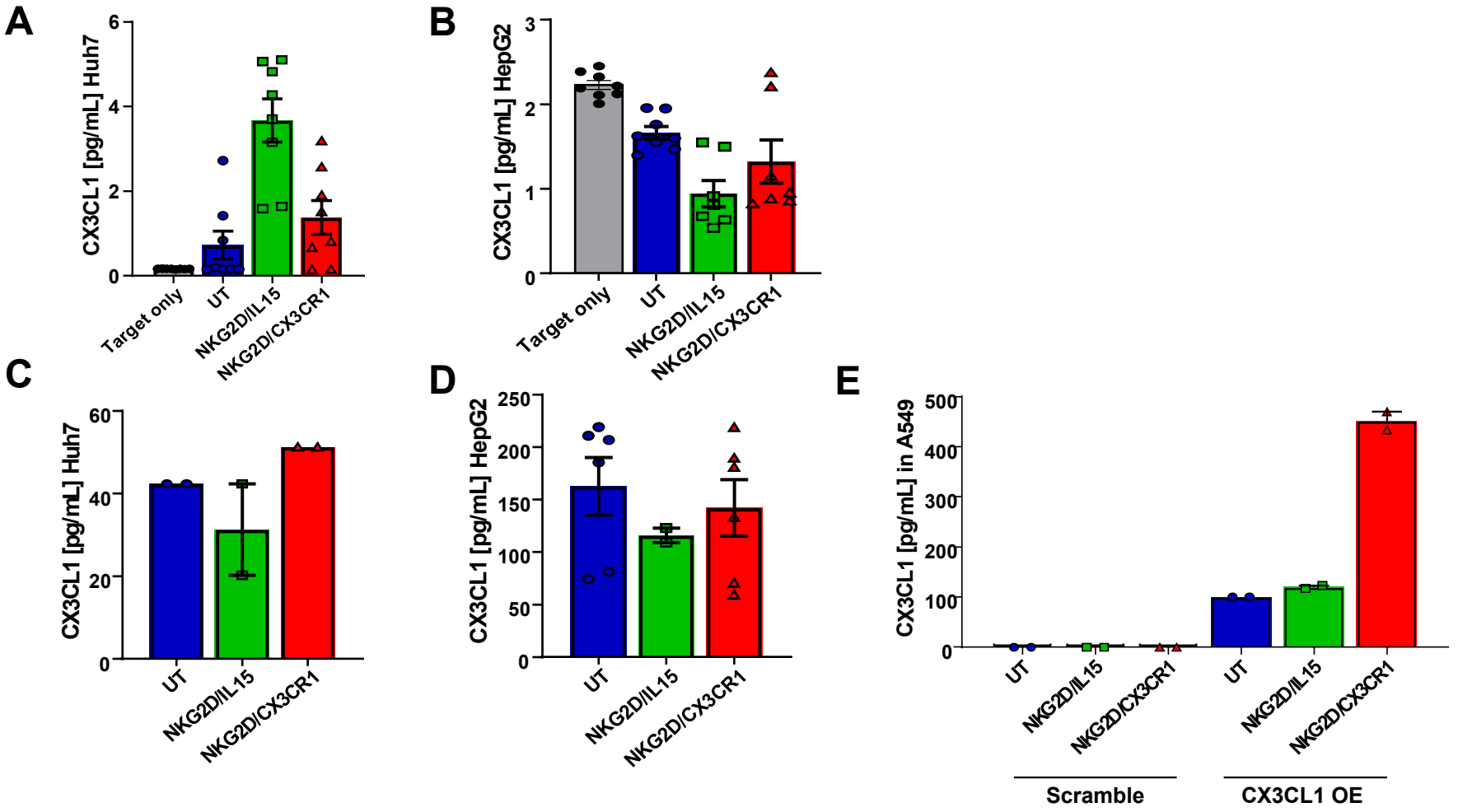
Testing CX3CR1-expressing CAR-T in solid tumor models in vivo. A) Schematic representation of in vivo CAR-T testing using A549-luciferase subcutaneous tumors in NSG-S mice shown in ***figure 5***. (a standard procedure to maintain the viability of human T cells in NSG mice, and akin to the protocol used in patients undergoing adoptive T-cell therapy) **B)** flow cytometric analysis of circulating CAR-T in the peripheral blood of mice a week after injection.

Supplemental Figure 10



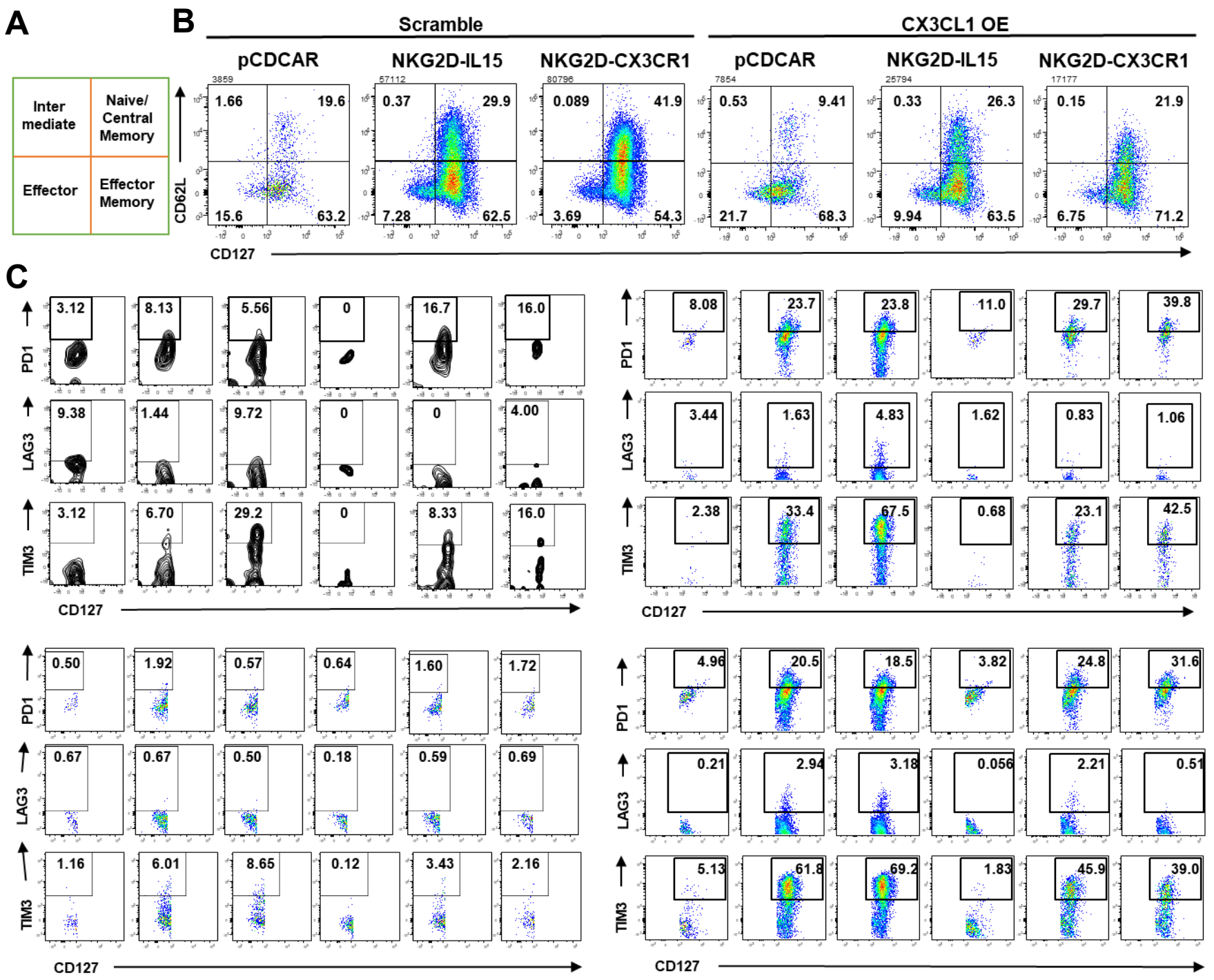
Short term infiltration assay of CAR-T into stablished A549 tumors. A) Schematic representation of CAR-T peritumoral injection prior to short term (72 hour) collection for assessment of infiltration. Developed with Biorender. **B)** Infiltration of primary human CD45⁺ CAR-T cells into A549 (Scramble) tumors grafted onto NSG mice for one month prior. The image represents paraffin tissue slides immune-stained with human CD45 (CAR-T cells strained in blue and pointed with red arrows to aid in visualization) and counterstained pink (representative figure). **C)** Quantification of hCD45⁺ CAR-T infiltration of experiment as in B. Significance was estimated by ANOVA and multiple comparison analysis (n=3). **D)** For confirmation NKG2D-CX3CR1 CAR-T was also tested on CX3CL1 overexpressing A549 cells (representative figure).The data in these figures compliments the information shown in ***figure 5B-D.***

Supplemental Figure 11



Expression of CX3CL1 in target cell lines after CAR-T challenge (STAR Methods): ELISA). A) CX3CL1 secretion in Huh7 cells alone of co-cultured with human CAR-T cells expressing vector (UT), NKG2D-IL15 CAR or NKG2D-CX3CR1 CAR. Measurements of 4 trials in duplicates. Please note the axis is set at 6 while the axis in C is set at 60. **B)** CX3CL1 secretion in HepG2 cells alone or co-cultured with human CAR-T cells expressing vector (UT), NKG2D-IL15 CAR or NKG2D-CX3CR1 CAR. Measurements of 4 trials in duplicates. Please note the axis is set at 3 while the axis in D is set at 250. CX3CL1 secretion after rechallenge with T cells isolated from mice treated with the CAR-T (when available) indicated using the same target cells as in the tumor: **C)** Huh7 cells, **D)** HepG2 cells and **E)** A549 cells with or without CX3CL1 overexpression. Error bars are the SEM of the number of experimental animal circulating human T cells tested.

Supplemental Figure 12



CX3CR1 co-expression in NKG2D CAR-T phenotypic characterization and exhaustion profile. A) Schematic representation of T cell phenotypic populations analyzed with CD62L/CD127. **B)** Percentages of CAR-T phenotypic populations after encounter in vivo with A549 tumors with or without CX3CL1 overexpression. Numbers show percentages of each population from the total number of live T cells. **C)** Exhaustion characterization (PD1, LAG3 and TIM3 expression) in the four subpopulations shown in B. Each quadrant represents the populations described in A and the plots are in the same order shown in B. The number of the percentage of cells gated from each quadrant shown. This is a representative analysis of mice shown in experiment in ***figure 5A and B***.