

CCR10⁺ Epithelial Cells from Idiopathic Pulmonary Fibrosis Lungs Drive Lung Remodeling

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Supplemental Materials

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Short Running Title: CCR10⁺ epithelial cells promote fibrosis in IPF.

Supplemental Methods:

Immunohistochemistry:

Slides containing 4 μm sections were deparaffinized and hydrated by incubating them in two changes of xylene for five min each, followed by 2 changes of 100% ethanol for 3 min each, 70% ethanol for 2 min, 50% ethanol for 2 min, and distilled water for 5 min. Antigen retrieval was performed by incubating the slides in 10 mM Citric acid solution (pH 6.0) in an 80 °C oven overnight. The slides were subsequently washed in PBS and permeabilized in 10% methanol containing 0.4% H_2O_2 for 30 min. After permeabilization, IHC analysis on human lung biopsies or explants were performed using a rabbit anti-human CCR10 (Abcam Cat# ab30718), rabbit anti-EphA3 (Santa Cruz Biotech Cat# sc-919), rabbit anti-KRT5 mAb (clone EPR1600Y, Abcam, Cat# 75869) or mouse anti-human-KRT81 (Abcam, Cat# ab55407) antibodies. Dual color IHC was performed using a dual color IHC kit (Enzo Lifesciences), rabbit anti-human CCR10 (Abcam Cat# ab30718) and mouse anti-human EphA3 (Clone, SL2, Humanigen, Inc., Brisbane, CA) antibodies as recommended by the manufacturer. Images were acquired using an AxioCam MRc camera Zeiss AX10 microscope using a 5x (0.16 aperture) and 20x (0.8 aperture) lenses (Carl Zeiss Microscopy GmbH) at room temperature and Zen 2012 (Blue edition) v 1.1.2.0 software.

In vitro live cell imaging and proliferation analysis:

Normal and IPF CRC-expanded epithelial cells were cultured in a 96 well plate at a concentration of 2×10^4 cells/well in the presence or absence of 10 μM Y-27632 (STEMCELL technologies) as described in the "Culturing normal and IPF lung explant

derived airway epithelial cells” methods section. The next morning, cells were washed and stimulated with 1 μ M Hypomethylated CpG-ODN 2006 (CpG; Hycult Biotechnologies), 10 ng/ml IL-13 (R&D systems), 20 ng/ml IL-4 (R&D systems), 20 ng/ml IL-1 β (R&D systems), 20 ng/ml IL-18 (R&D systems) in complete medium (DMEM (Lonza) + 15% FBS (Atlanta Biological), 100 IU penicillin and 100 μ g/ml streptomycin (Mediatech, Manassas, VA), 292 μ g/ml L-Glutamine (Mediatech) and 100 μ g/ml of Primocin (Invivogen, San Diego, CA)). As a control, cells were cultured in senescent fibroblast conditioned supernatants. All stimulations were performed in the presence or absence of 10 μ M Y-27632. Cells were then imaged using an IncuCyte ZOOM live cell imaging system (Essen Biosciences) and cell confluency over time was quantified using IncuCyte ZOOM 2016B software (Essen Biosciences).

Soluble Collagen 1 ELISA:

Lung fibroblasts ($5-7 \times 10^3$ cells per well) were plated into a 96 well plate, treated with pre-clustered epithelial cell conditioned supernatants or cell-free conditioned supernatant controls for 72 hours. After 72 hours, lung fibroblast conditioned supernatants were diluted 1:5 in DPBS. Purified Collagen 1 (STEMCELL Technologies Inc), diluted in 1:5 DBPS diluted PEB medium, was utilized to generate an 8-point collagen 1 standard ranging from 200-0 ng/ml. Fifty microliters of the supernatants and standards were coated onto multisorb 96 well plates (Thermo Fisher Scientific) overnight at 4 °C. After coating, the plates were washed and blocked with 200 μ l of 3% BSA solution in DPBS (Lonza) for 1 h at room temperature on an orbital shaker. After blocking, plates were

washed and 50 μ l of 200 ng/ml biotin-conjugated anti collagen-1 antibody (Abcam Ab24821) was added to each well and the plates were incubated for 2 h at room temperature on an orbital shaker. After incubation, plates were washed and 50 μ l of HRP-conjugated streptavidin (R&D systems, diluted as recommended by the manufacturer) was added to each well and the plates were incubated for 30 min at room temperature on an orbital shaker. Plates were then washed, and the wells were developed by adding 100 μ l of TMB peroxidase substrate solution (Fitzgerald Industries International) until sufficient color is observed, after which the reaction was stopped by adding 50 μ l of 2N Sulfuric acid. Data were acquired by reading the absorbance at 405 nm using a Synergy H1 microplate reader (BioTek Instruments Inc.). To normalize to the number of cells in each well, 100 μ l of DAPI (300 μ M) was added to each well, cells were incubated with the DAPI solution for 15 minutes then washed twice with DPBS. DAPI fluorescence was acquired using a Synergy H1 microplate reader and was utilized to normalize the collagen 1 concentrations to the number of cells. The cell-free CS treated fibroblast replicate wells were then averaged, and the results were graphed as fold change relative to the average cell-free CS using GraphPad Prism version 7 (GraphPad Inc).

Single cell RNA sequencing and bioinformatic analysis:

IPF explant suspensions were cultured in CRC medium until epithelial colonies were apparent, cells were then transferred into PEB medium overnight and dissociated using Accutase dissociation solution (STEMCELL Technologies) as described above ("Culturing normal and IPF lung explant derived airway epithelial cells" section). Cells were then stained with BV421 anti-EpCAM and PE anti-CCR10 antibodies (Biolegend).

EpCAM⁺ CCR10⁺ and EpCAM⁺ CCR10⁻ epithelial cells were then FACS sorted using a BD ARIA III cell sorter (BD Biosciences). Sorted epithelial cells were spun down, counted and resuspended as recommended by the manufacturer (10x Genomics). All sequencing files have been uploaded onto Geo with an accession number of GSE115982. 3,575 CCR10⁺ and 3,863 CCR10⁻ epithelial cells were captured and a total of 317,604,987 reads were sequenced from EpCAM⁺ CCR10⁺ and 290,772,979 reads were sequenced from EpCAM⁺ CCR10⁻ cells. Sequenced reads were demultiplexed and aligned to the human transcriptome version GRCh38 using Cell Ranger Version 2.0.1 (10x Genomics). Aligned reads were normalized and analyzed via dimensionality reduction and clustering via Cell Ranger Version 2.0.1. Graph-based and K-mean clustering analysis was performed using Loupe Cell Browser software version 2.0.0 (10x Genomics). For population analysis, normalized .csv files were exported using Cell Ranger Version 2.0.1 and were then imported into SeqGeq software Version 1.3.0 (FlowJo LLC.). The resulting files from CCR10⁺ and CCR10⁻ cells were concentrated and then statistically analyzed using PCA and tSNE dimensionality reduction analysis. tSNE cell populations were then depicted based on the sample of origin (i.e. CCR10⁺ or CCR10⁻) and cell populations were gated based on the expression of CCR10, KRT5 and/or SCGB1A1. Significantly upregulated transcripts in CCR10⁺ KRT5⁺ versus CCR10⁻ KRT5⁺ or CCR10⁺ SCGB1A1⁺ versus CCR10⁻ KRT5⁺ or CCR10⁺ SCGB1A1⁺ versus CCR10⁺ KRT5⁺ cells were exported and uploaded into Ingenuity IPA (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>). Ingenuity canonical pathways and upstream regulators were then exported and are depicted in the supplemental tables.

Supplemental Figures:

Figure S1: Positive correlation between the number of EpCAM⁺ CCR10⁺ cells and hydroxyproline concentration in IPF CD3⁻ CCR10⁺ humanized NSG mice.

(A) CD3⁻ CCR10⁺ IPF lung cells were FACS sorted and intravenously administered into NSG mice. (B) Depicted are correlation analyses of hydroxyproline concentration and number of human CD45⁻ EpCAM⁺ CCR10⁺ cells in NSG lungs 35-days after cellular administration. n=5/group. p-values indicated. IPF: Idiopathic Pulmonary Fibrosis; FACS: Fluorescence-activated cell sorting; NSG: NOD Cg-Prkdc^{SCID} IL2rg^{Tm1wjl}Szi.

Figure S2: Expression of airway epithelial cell markers by cultured normal and IPF epithelial cells.

RNA was extracted from normal and IPF epithelial cells cultured in CRC medium. Depicted is the mean $\pm$ s.e.m. of epithelial lineage transcript expression from 3 normal and 3 IPF epithelial cell cultures. IPF: Idiopathic Pulmonary Fibrosis

Figure S3: Characterization of primary airway epithelial cell survival and proliferation in the presence or absence of Y-27632 and various stimuli.

CRC-expanded epithelial cells were cultured in a 96 well plate (2×10^4 /well) in the presence or absence of 10 μ M Y-27632 and stimulated with 1 μ M CpG, 10 ng/ml IL-13, 20 ng/ml IL-4, 20 ng/ml IL-1 β , 20 ng/ml IL-18 or senescent fibroblast conditioned supernatants. Cells were then imaged over a duration of 110 hours using an IncuCyte Zoom live cell imager and the confluence of the epithelial cells was quantified using

IncuCyte Zoom software version 2016B. Shown is the percent confluence of untreated (A-C, G-I & M-O) or Y27632 treated (D-F, J-L & P-R) cells stimulated with Sen C/S and CpG (A-F), IL-4/IL-13 (G-L) or IL-1 β /IL-18 (M-R). This experiment was performed with cells from three explanted lung tissues in triplicate. Sen C/S: senescent lung fibroblasts conditioned supernatants; CpG: Hypomethylated CpG-ODN 2006.

Figure S4: Conditioned supernatants from cultured IPF epithelial cells more consistently promote collagen 1 secretion by IPF lung fibroblasts.

Normal and IPF explant suspensions were cultured in PEB medium until epithelial cell colonies were apparent. After 2-3 passages, epithelial cells were cultured in 6 well plates for 24 hours, after which CS were collected and frozen at -20 °C. As a control, medium was added into an empty 6-well plate (cell free) and treated in a similar manner. Lung fibroblasts were then plated into 96 well plates for 24 hours, then washed and stimulated with 100 μ l of normal or IPF epithelial cell CS or cell-free CS. Fibroblasts were then incubated for 72 hours, after which the conditioned supernatants were collected and analyzed for collagen 1 secretion via ELISA. **(A-C)** Depicted is the mean fold change of secreted collagen 1 protein detected in normal (n=1; A) or IPF (n=2; B-C) lung fibroblast conditioned supernatants. Data shown are mean $\pm$ s.e.m. of PEC CS stimulated fibroblasts normalized to fibroblasts stimulated with cell-free medium controls (cell-free). *p $\leq$ 0.05 **p $\leq$ 0.01 relative to cell-free control via One-Way ANOVA corrected using Dunnett. PEB: PneumaCult Ex Plus medium + 10 μ M Y-27632; CS: conditioned supernatants; PEC CS: Pulmonary epithelial cell conditioned supernatant.

Figure S5: Population transcriptomic analysis of KRT5⁺ CCR10⁺ epithelial cells.

IPF epithelial cells were generated in CRC medium and then incubated in PEB medium overnight to promote CCR10 expression. Cells were then dissociated using Accutase and stained with fluorescent conjugated anti-EpCAM and CCR10 antibodies. EpCAM⁺ CCR10⁺ and EpCAM⁺ CCR10⁻ epithelial cells were FACS sorted and single cell RNA sequencing analysis was performed using the 10x genomics platform. **(A)** Resulting sequencing CSV files from CCR10⁺ and CCR10⁻ epithelial cells were imported into SeqGeq and were concentrated into one file. PCA followed by t-SNE analysis were performed on the concentrated file. Depicted is a t-SNE population plot for CCR10⁺ (red) versus CCR10⁻ (blue) cells. **(B-C)** Depicted are cells showing high expression of SCGB1A1 (B) or KRT5 (C). **(D)** Gating strategy to select CCR10⁺ KRT5⁺, CCR10⁻ KRT5⁺ & CCR10⁺ SCGB1A1⁺ epithelial cells. **(E-G)** Depicted is the fold change and q-values for differentially expressed transcripts in CCR10⁺ KRT5⁺ versus CCR10⁻ KRT5⁺ (E) CCR10⁺ SCGB1A1⁺ versus CCR10⁻ KRT5⁺ (F) and CCR10⁺ SCGB1A1⁺ versus CCR10⁺ KRT5⁺ epithelial cells (G). IPF: Idiopathic Pulmonary Fibrosis; CRC: Conditioned reprogrammed cell medium; PEB: PneumaCult Ex Plus medium + 10 μ M Y-27632; FACS: Fluorescence-activated cell sorting; PCA: Principle component analysis; t-SNE: t-Distributed Stochastic Neighbor Embedding; CSV: comma-separated values.

Figure S6: EphA3 is highly expressed by CCR10⁺ epithelial cells from IPF lung explants.

Normal and IPF lung explants cellular suspensions were stained with fluorescently conjugated anti-CD45, anti-EpCAM, anti-CCR10, biotin-conjugated anti-EphA3 antibodies followed by AF488 conjugated streptavidin and analyzed by flow cytometry. **(A-B)** Shown are representative dot plots from normal (A; n=10) and IPF (B; n=10) lung explants depicting CCR10⁺ and CCR10⁻ Epithelial cells (CD45⁻ EpCAM⁺; top), and the expression of cell-surface EphA3 protein in these populations (bottom). **(C-D)** Shown is the mean percentage of EphA3⁺ cells (C) and EphA3 GMFI (D) within CD45⁻ EpCAM⁺ CCR10⁺ and CD45⁻ EpCAM⁺ CCR10⁻ cells in normal and IPF lung explants. Data shown are mean $\pm$ s.e.m. n=10/group *p $\leq$ 0.05 **p $\leq$ 0.01 ***p $\leq$ 0.001 ****p $\leq$ 0.0001 via one-way ANOVA corrected with Tukey. IPF: Idiopathic Pulmonary Fibrosis; AF488: Alexa flour 488; GMFI: Geometric mean fluorescence intensity.

Figure S7: EphA3⁺ KRT5⁺ KRT81⁺ epithelial cells localize in honeycombing cysts and adjacent to fibroblastic foci in IPF lungs.

(A-F) IHC analysis of normal (A-B) or IPF (C-F) explanted lung tissues stained for EphA3 protein. Shown are representative images taken at 50x (top) and 200x (bottom) magnification. IgG control stained serial sections are shown in inlayed images. n=4 normal & n=18 IPF lung tissues. (G-J) IHC analysis in serial sections from IPF lung explants for KRT5 (A), EphA3 (B), KRT81 (C) protein expression. As a control, tissues were stained with non-immune IgG antibodies (D). Images were acquired at 200x magnification. n=3 IPF lung tissues. IPF: Idiopathic Pulmonary Fibrosis; IHC: Immunohistochemistry.

Figure S8: Xenografted human IPF cell flow cytometric gating strategy and controls.

Cellular suspensions were generated from human-cell xenografted NSG lung tissues and cells were blocked with human and mouse FC blocking antibodies. Cells were then stained with fluorescently conjugated anti-CD45, -EpCAM and -CCR10 or matching Isotype control antibodies. (A) Shown is the gating strategy for CD45⁻ EpCAM⁺ CCR10⁺ cells in humanized NSG lungs. (B-D) Representative dot plots showing Isotype antibody staining. (E-G) Representative dot plots showing CD45 staining in a CD45 FMO control (E), EpCAM staining in an EpCAM FMO control (F) and CCR10 staining in a CCR10 FMO control (G). This gating strategy was utilized to generate the results shown in Figures S1,

S2 and 5. IPF: Idiopathic Pulmonary Fibrosis; NSG: NOD Cg-Prkdc^{SCID} IL2rg^{Tm1wil}Szi; FC: FC receptor; Iso: Isotype control antibody.

Figure S9: Flow cytometric Isotype staining for xenografted human cells in NSG mice.

(A-D) Representative dot plots depicting isotype antibody staining of IPF cell-xenografted NSG lungs. These isotype controls were utilized to gate for cells depicted in Figure 8.

NSG: NOD Cg-Prkdc^{SCID} IL2rg^{Tm1wil}Szi; Iso: Isotype control antibody.

Figure S10: Human explant lung cell flow cytometric controls and gating strategy.

Relating to Figures 1, 6 & S7

Normal and IPF lung explant cell suspensions were stained with the indicated fluorescent probe-conjugated anti-human antibodies and analyzed by flow cytometry. **(A-C)** Representative dot plots of isotype stained human explant cells. **(D-E)** FMO controls for CCR10 gated CD45⁻ EpCAM⁺ (D) and CD45⁻ EpCAM⁻ cells (E). **(F-K)** To gate for various populations of cells fluorescent minus one (FMO) controls samples were stained with isotype control instead of an antigen specific antibody. Shown are representative dot plots of FMO samples stained with isotype control antibodies instead of anti-EpCAM (F), CD45 (G), CCR10 (H), PDGFRA (I) or EphA3 (J) antibodies. IPF: Idiopathic Pulmonary Fibrosis; Iso: isotype control antibody.

Supplemental Tables

Table S1: Differentially expressed transcripts in CCR10⁺ K-mean epithelial cell populations.

Table S2: Ingenuity Canonical pathways enriched in CCR10⁺ KRT5⁺ cells.

Table S3: Ingenuity Upstream regulators enriched in CCR10⁺ KRT5⁺ cells.

Table S4: Ingenuity Canonical Pathways enriched in SCGB1A1⁺ CCR10⁺ vs KRT⁺ CCR10⁻ cells.

Table S5: Ingenuity Upstream Regulators enriched in SCGB1A1⁺ CCR10⁺ vs KRT⁺ CCR10⁻ cells.

Table S6: Ingenuity Canonical Pathways enriched in SCGB1A1⁺ CCR10⁺ vs KRT⁺ CCR10⁺ cells.

Table S7: Ingenuity Upstream Regulators enriched in SCGB1A1⁺ CCR10⁺ vs KRT⁺ CCR10⁺ cells.

Supplemental Movies:

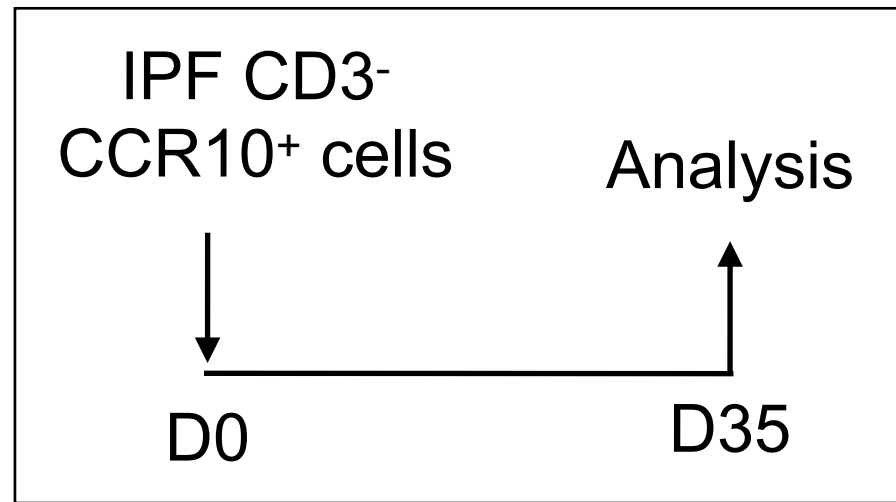
Movie S1: CRC medium cultured pulmonary epithelial cells in the presence of Y-27632.

Movie S2: PEB medium cultured pulmonary epithelial cells in the presence of Y-27632.

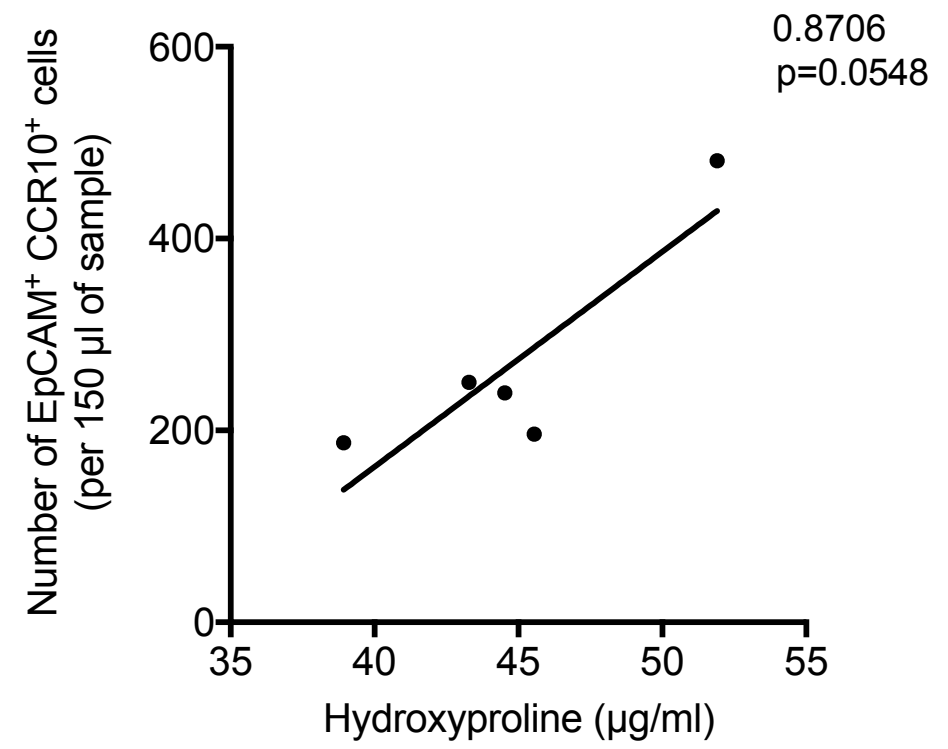
Movie S3: CRC medium cultured cells partial reversion into cuboidal cells after culture in PEB medium in the presence of Y-27632.

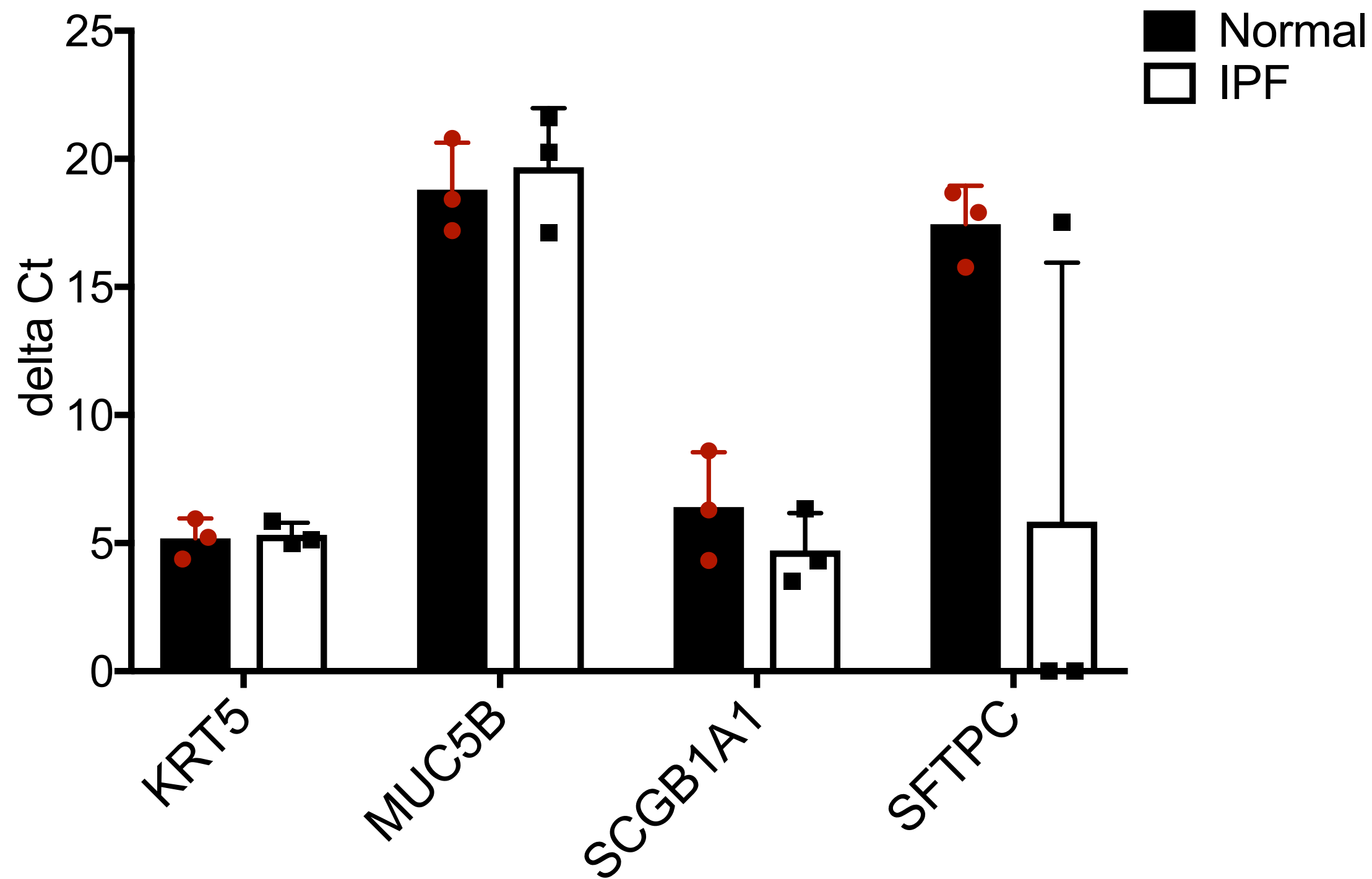
Movie S4: PEB medium cultured pulmonary epithelial cells in the absence of Y-27632.

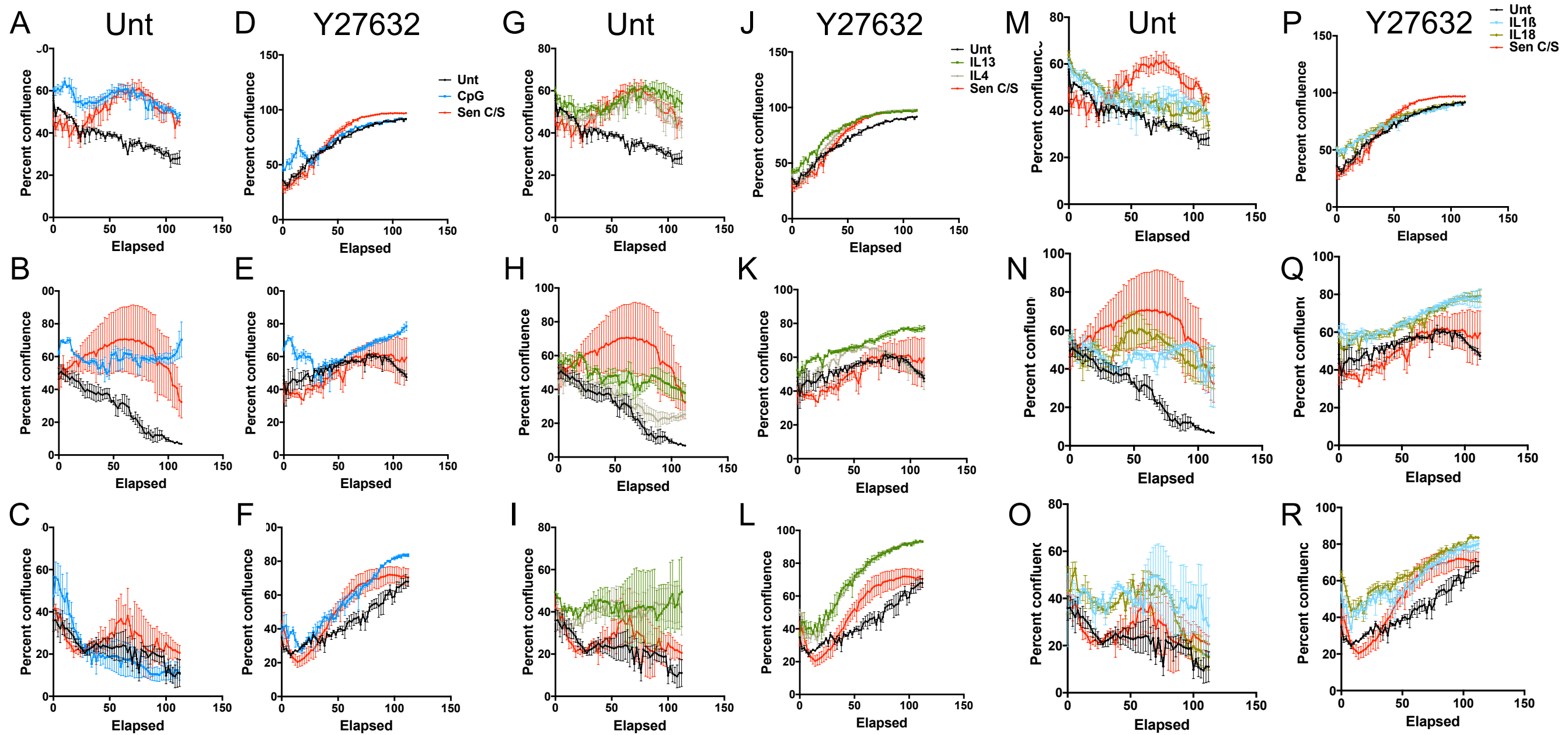
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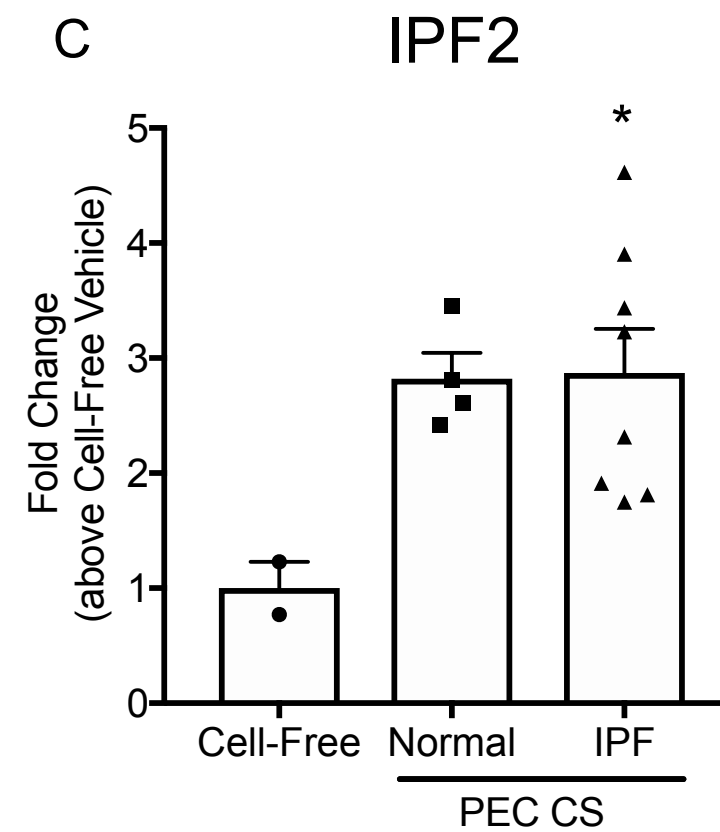
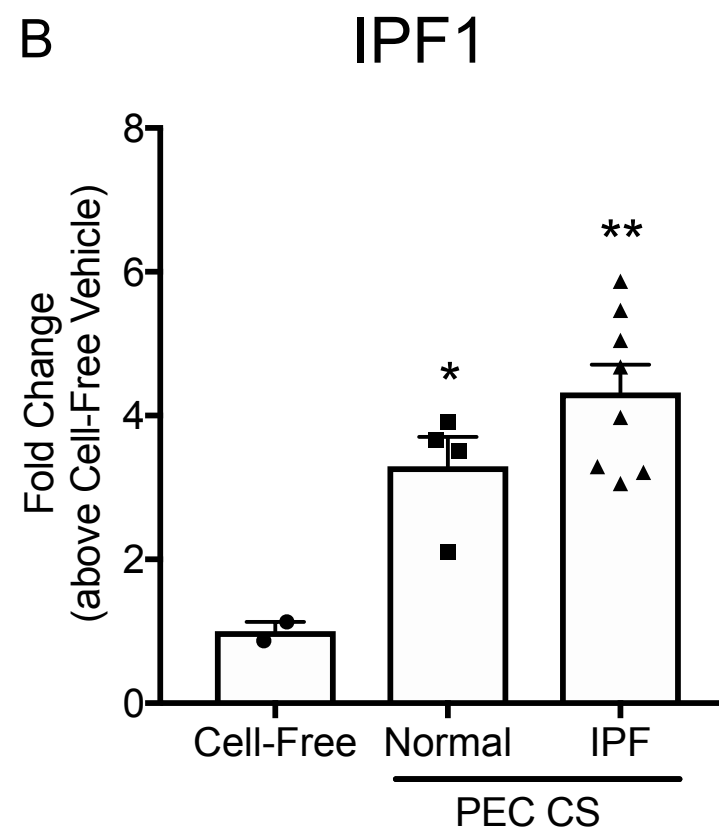
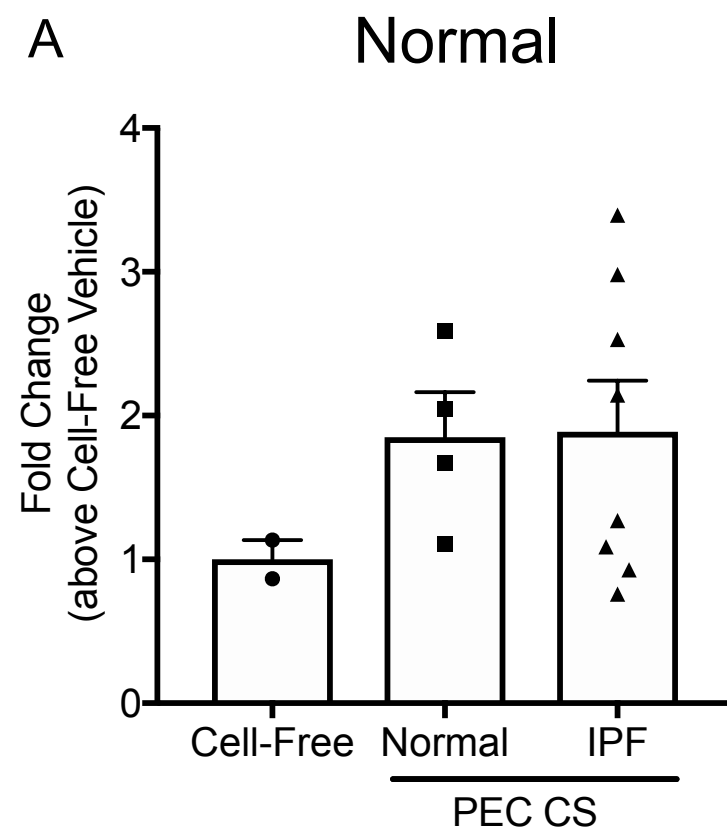


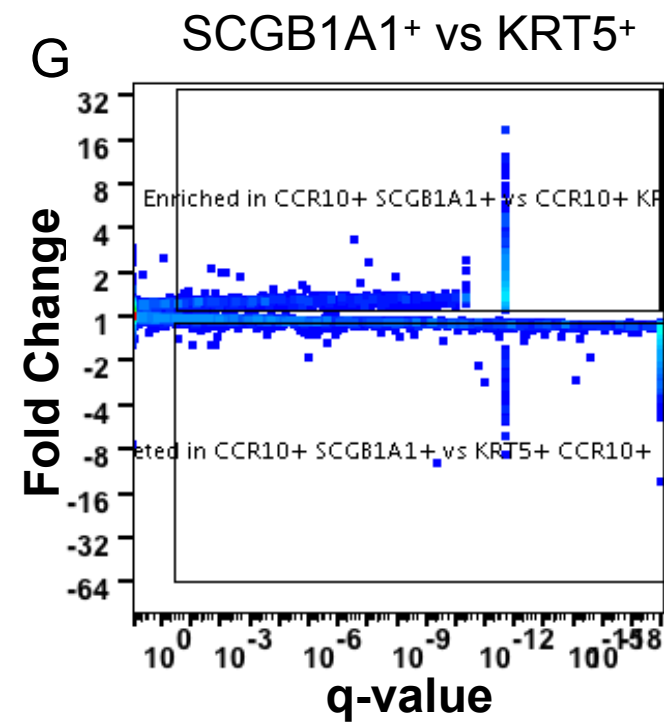
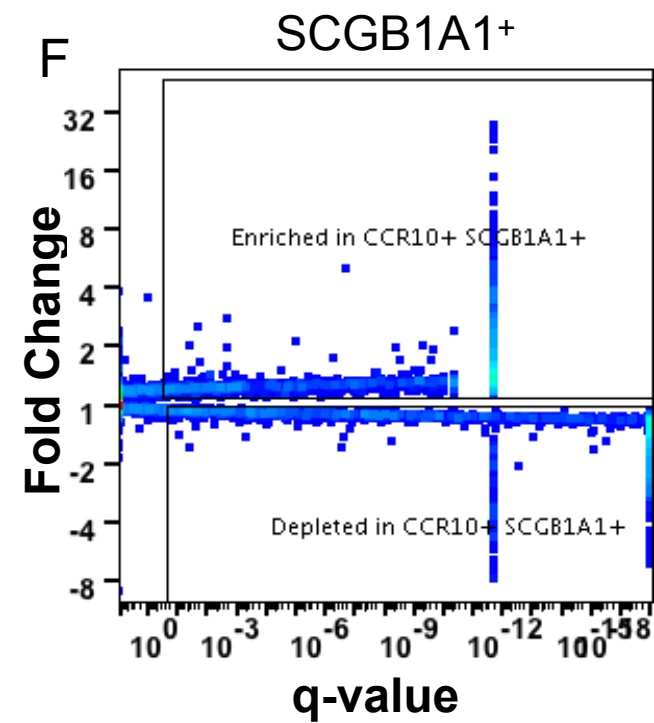
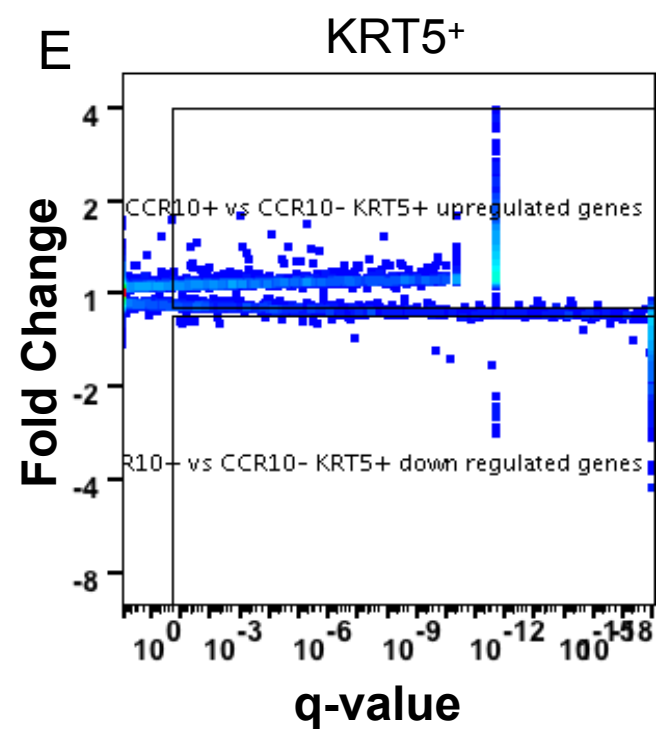
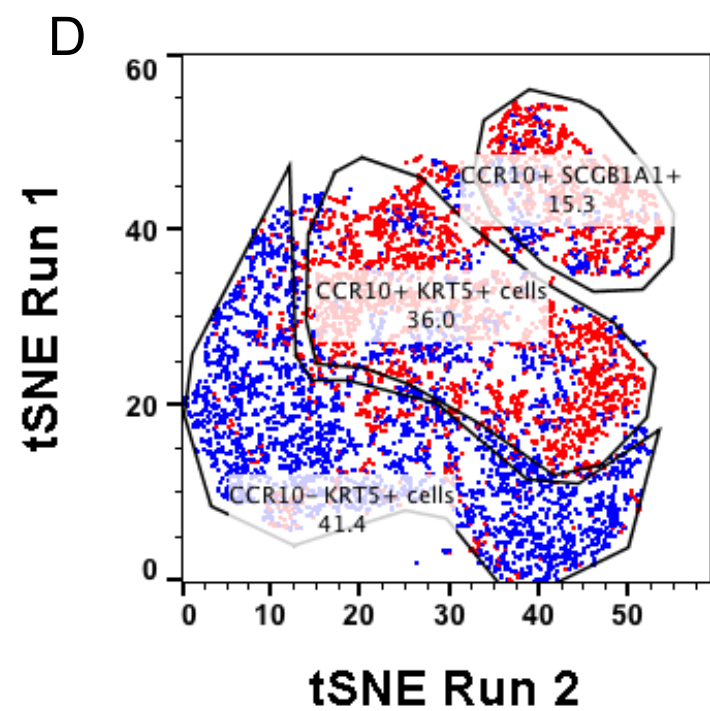
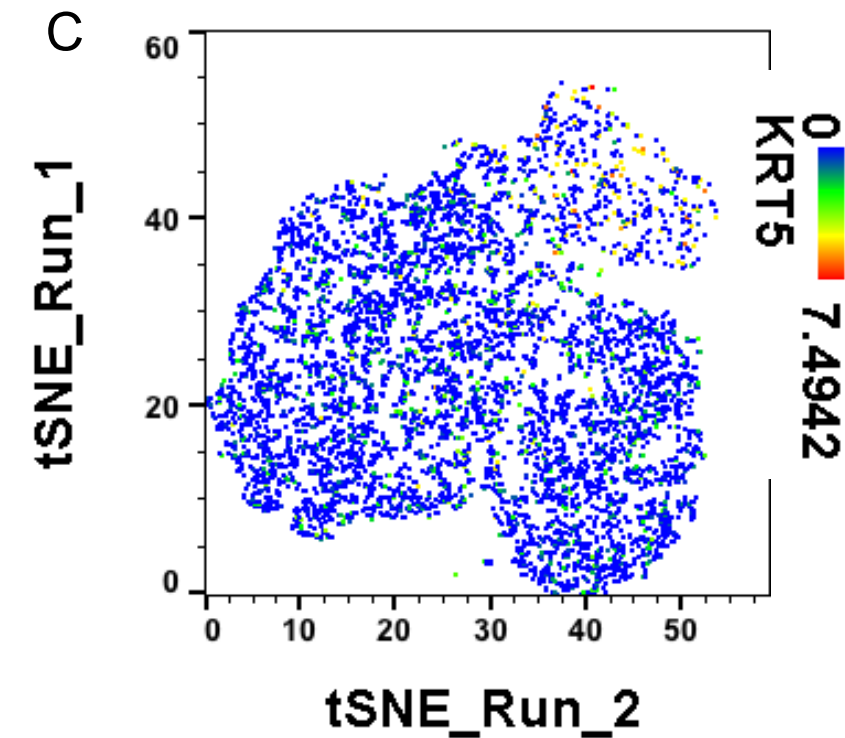
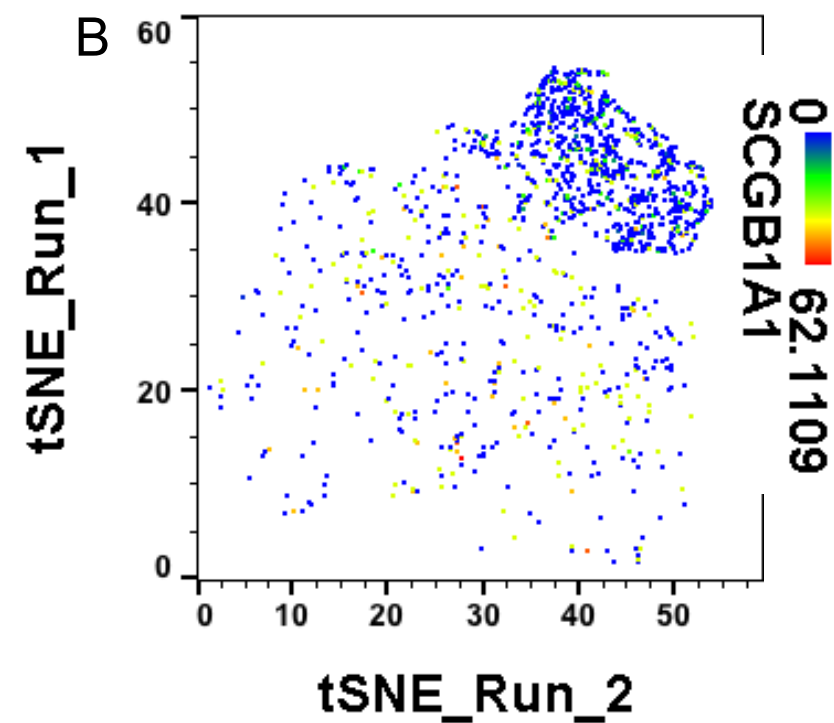
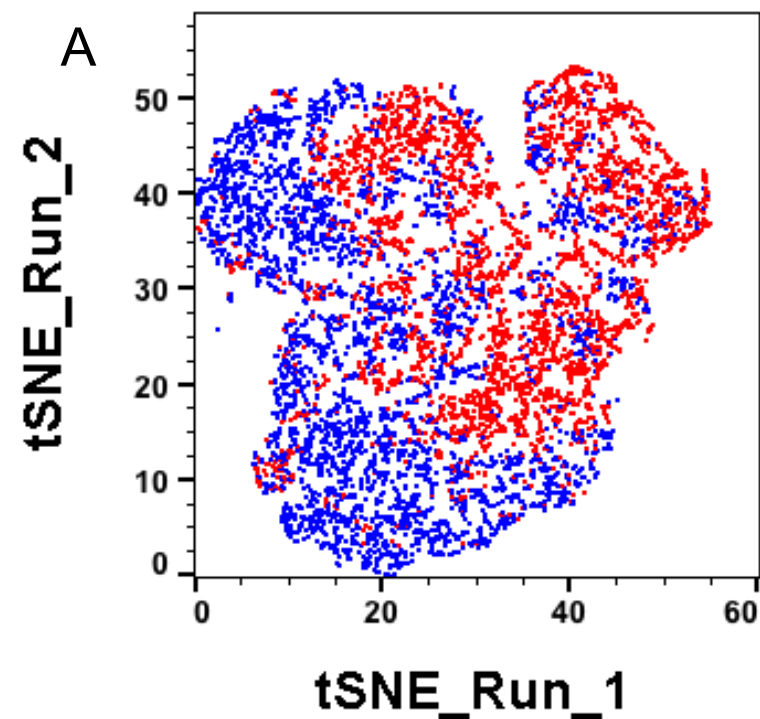
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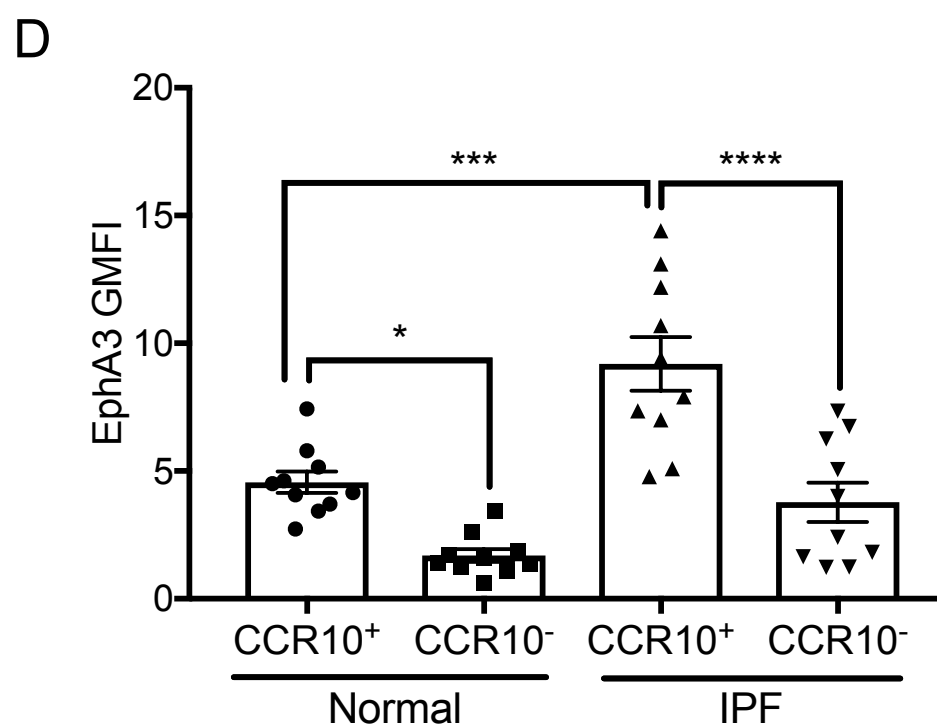
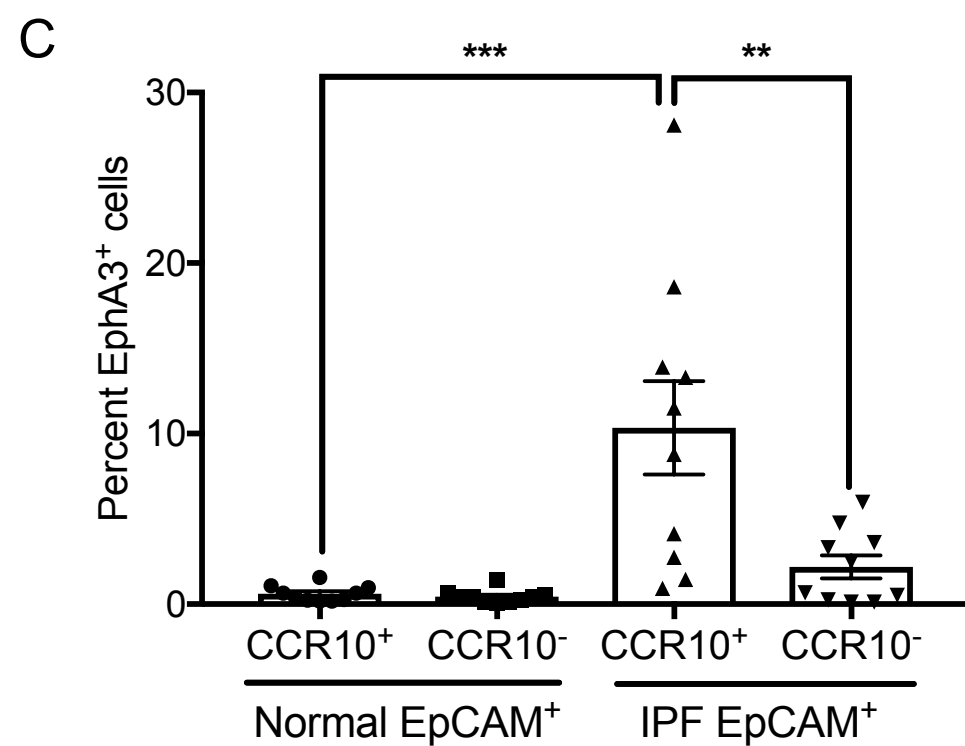
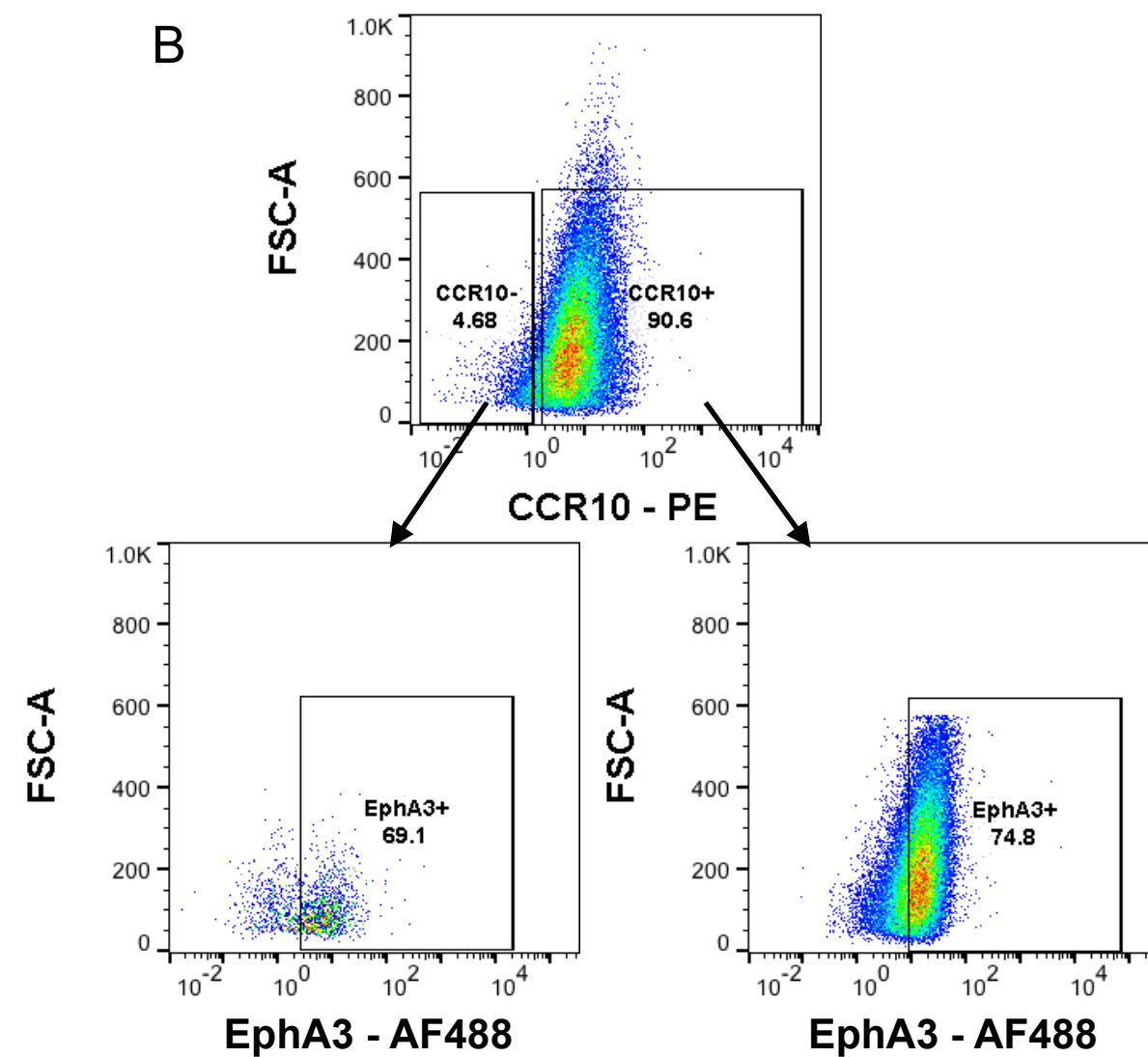
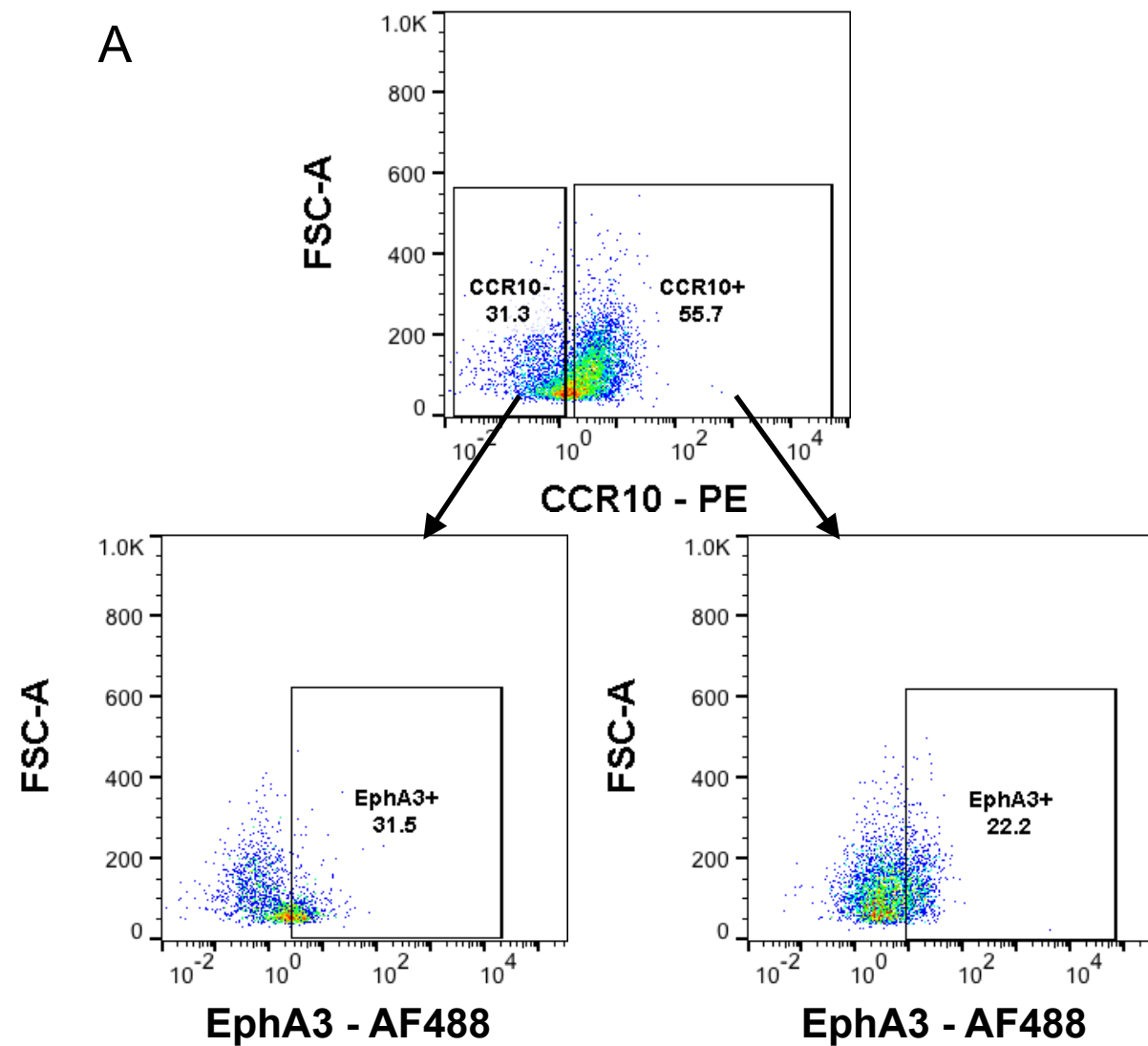






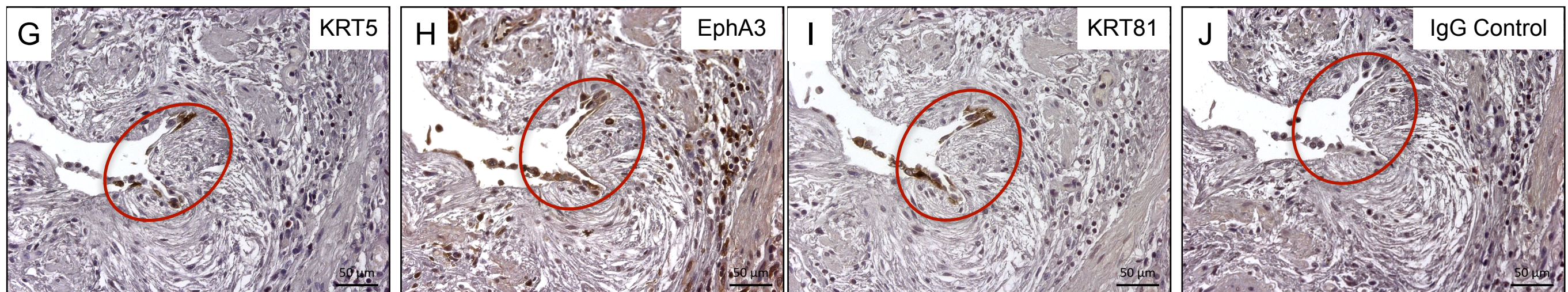
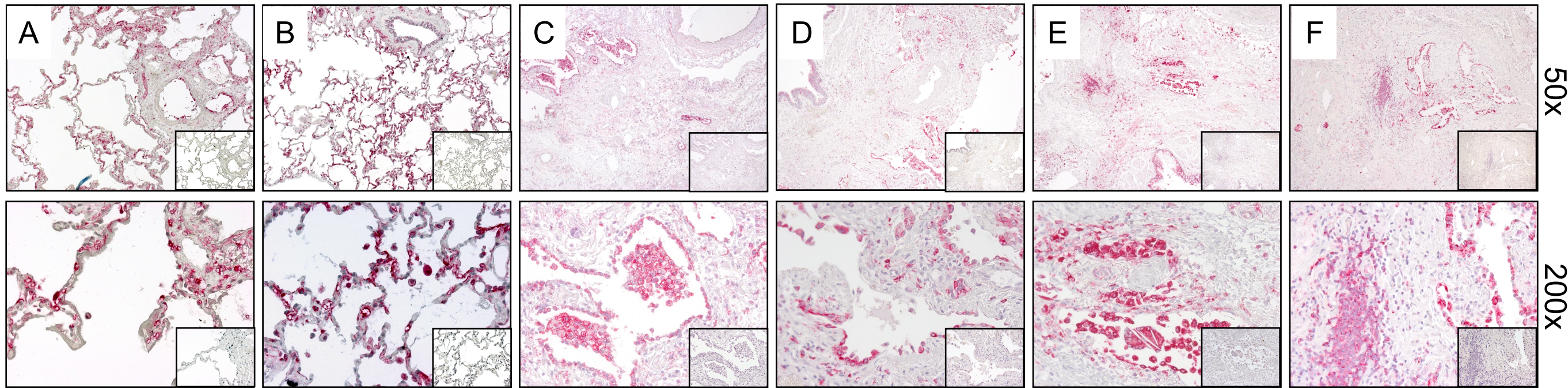




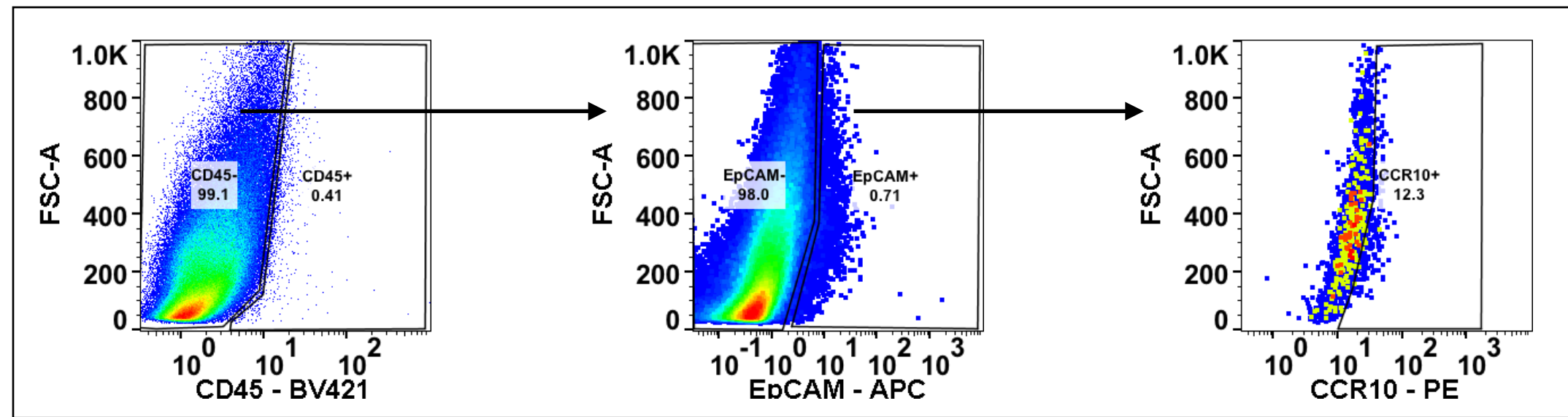


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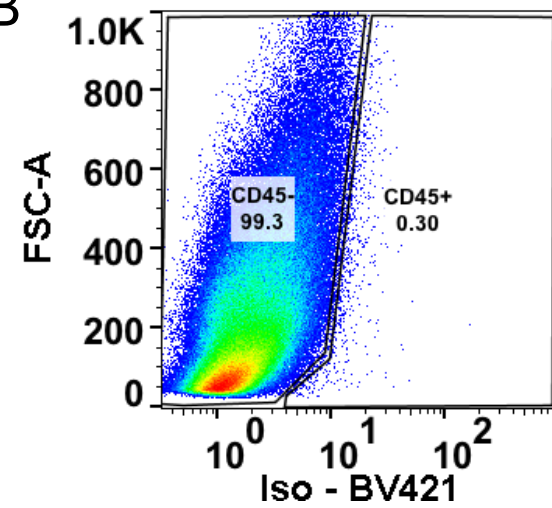
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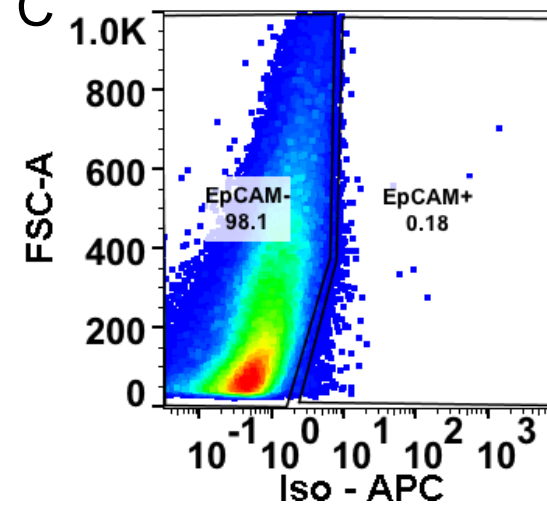
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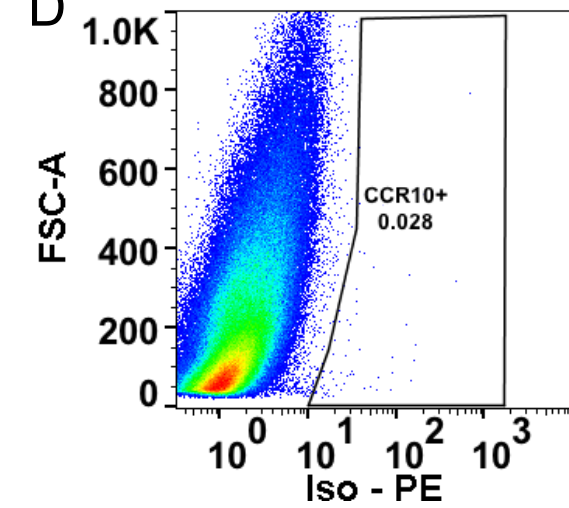
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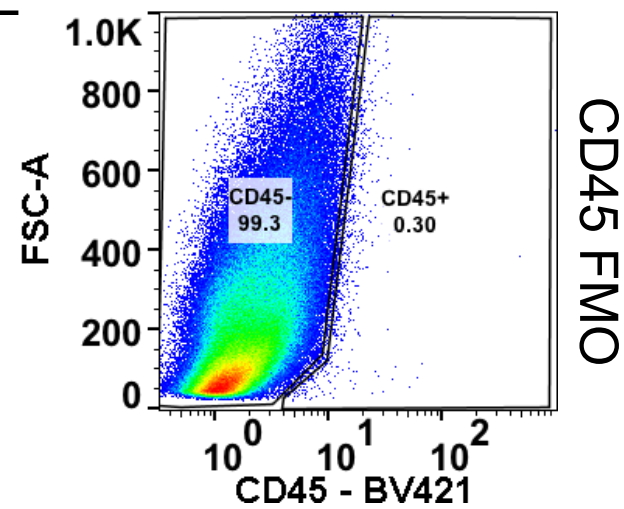
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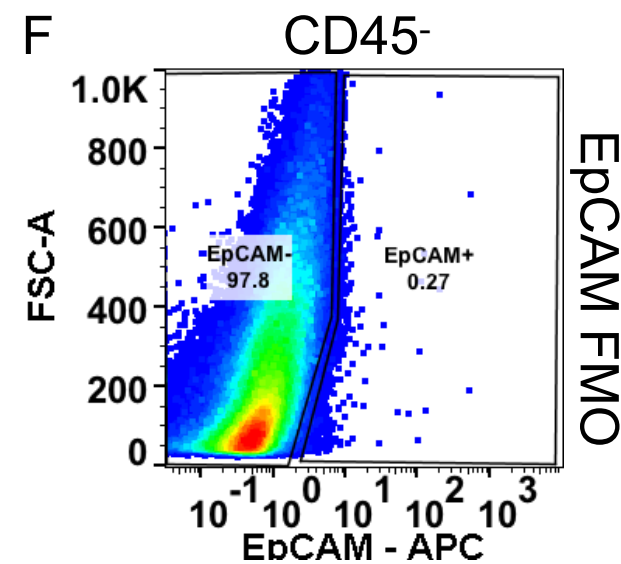


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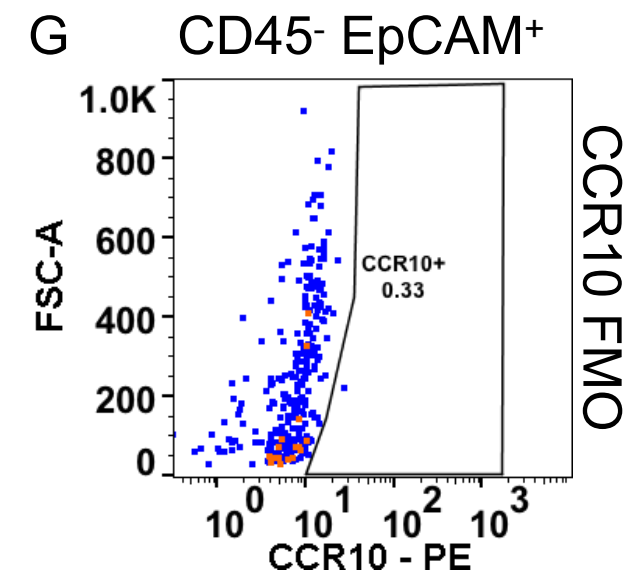
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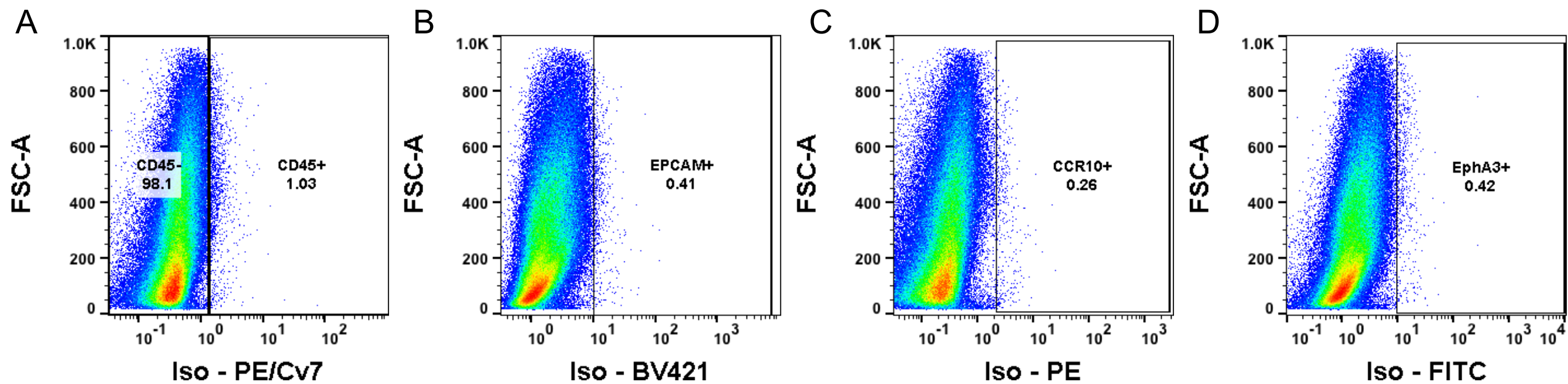


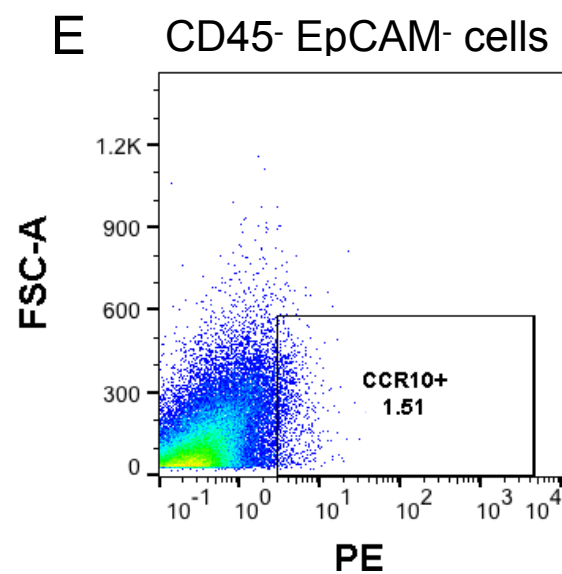
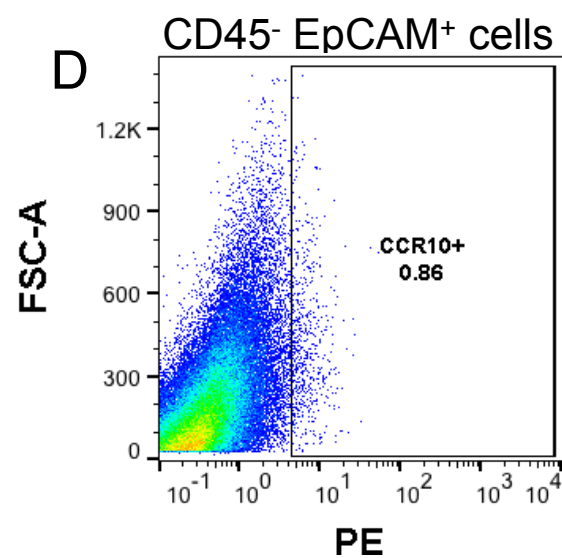
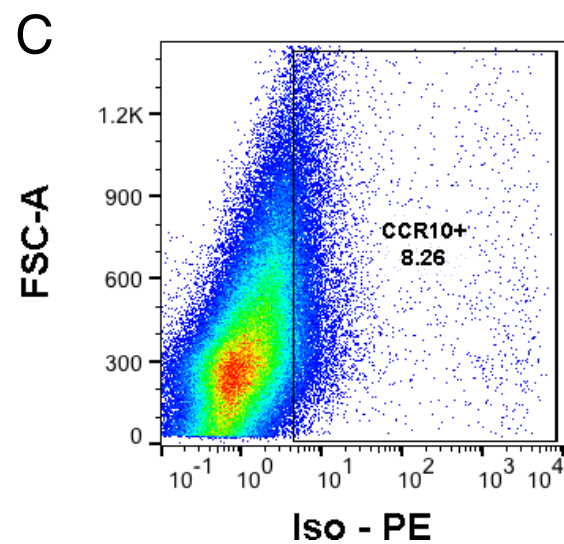
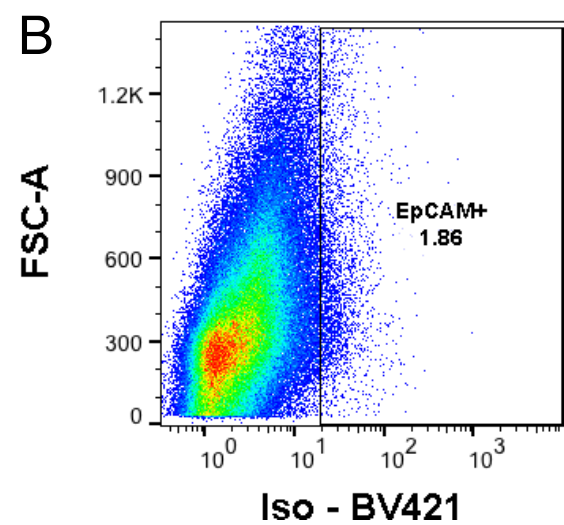
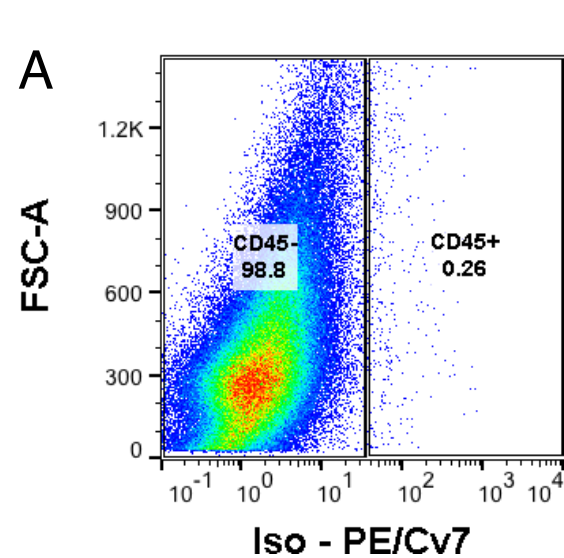
EpCAM FMO

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CCR10 FMO





CCR10 FMO

