

Supplementary Figure 1: subpopulations of cells and their expressed genes based on single-cell RNA-seq and spatial transcriptomics

(A) UMAP of single-cell RNA-seq (scRNA-seq) experiment shown in Figure 1B, including all cell subtypes. (B) UMAP of the spatial transcriptomic experiment shown in Figure 1F, including cell subtypes. (C) Dot plot showing representative marker genes used to annotate cell types in the scRNA-seq dataset. Abundance and average expression scale are shown on the right. (D) Dot plot showing representative marker genes used to annotate cell types in the spatial transcriptomics dataset. Abundance and average expression scale are present on the right.

Supplementary Figure 2: Lymphocyte heterogeneity in the scRNA-seq data

(A) UMAP showing T-cell and NK-cell subsets in the scRNA-seq dataset. (B) Dot plot displaying representative marker genes defining the T-cell and NK-cell subsets identified in the scRNA-seq experiment. The abundance and average expression scales are shown on the right. (C) UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 3: Macrophage heterogeneity in the scRNA-seq data

(A) UMAP showing macrophage subsets in the scRNA-seq dataset. (B) Dot plot displaying representative marker genes defining the macrophage subsets identified in the scRNA-seq experiment. The abundance and average expression scales are shown on the right. (C) UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 4: Dendritic cell heterogeneity in the scRNA-seq data

(A) UMAP showing dendritic cell subsets in the scRNA-seq dataset. (B) Dot plot displaying representative marker genes defining the dendritic cell subsets identified in the scRNA-seq experiment. The abundance and average expression scales are shown on the right. (C) UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 5: T cell heterogeneity in the spatial transcriptomics experiment

(A) UMAP showing T-cell subsets in the spatial transcriptomics dataset.

(B) Dot plot displaying representative marker genes defining the T-cell subsets identified in the spatial transcriptomics experiment. The abundance and average expression scales are shown on the right. **(C)** UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 6: Macrophage heterogeneity in the spatial transcriptomics experiment

(A) UMAP showing macrophage subsets in the spatial transcriptomics dataset.

(B) Dot plot displaying representative marker genes defining the macrophage subsets identified in the spatial transcriptomics experiment. The abundance and average expression scales are shown on the right. **(C)** UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 7: Dendritic cell heterogeneity in the spatial transcriptomics experiment

(A) UMAP showing dendritic cell subsets in the spatial transcriptomics dataset.

(B) Dot plot displaying representative marker genes defining the dendritic cell subsets identified in the spatial transcriptomics experiment. The abundance and average expression scales are shown on the right. **(C)** UMAP showing the expression of representative marker genes. Normalized UMI levels are indicated by the color scale on the right.

Supplementary Figure 8: Cell type distribution across different domains in the section

(A) Spatial domains were generated according to the distribution of cells in the tissue section. **(B)** Stacked bar plot showing the proportional representation of epithelial, endothelial, pericyte, fibroblast, B-cell, T-cell, dendritic-cell, and macrophage populations within each spatial domain defined in (A). **(C)** MERSCOPE image showing the location of epithelial, endothelial, pericyte, fibroblast, B-cell, T-cell, dendritic-cell, and macrophage populations. The bar scale is 1mm. **(D)** MERSCOPE image showing the location of malignant cells and macrophage subsets, M0 (cyan), M1 (red), and M2 (yellow). The bar scale is 1mm. **(E)** Boxplot showing the distribution of

distances between each annotated cell type and the nearest Tregs within the spatial dataset. Cell types are ordered by increasing median distance.

Supplementary Figure 9. Spatial transcriptomic analysis of an independent orthotopic PDAC model. (A) UMAP of spatial transcriptomic data showing the annotated cell populations in tumors generated using PRTP cells. (B) Spatial distribution of the annotated cell populations in the same tumor. (C) H&E staining of an adjacent tumor section. (D) Spatial distribution of the three major tissue hubs enriched in epithelial, fibroblast, or immune cells. (E) Colocalization analysis of the indicated cell populations. Colors represent colocalization z-scores, and asterisks indicate significant enrichment after Benjamini–Hochberg correction ($FDR < 0.05$). Cells with centroids separated by less than 30 μm were considered neighbors. The number of cells in each population is shown on the right. (F) Minimum distance from each cell to the nearest Treg, grouped by cell type and ranked by median distance. Boxes show the median and interquartile range, and whiskers extend to 1.5 times the interquartile range. The red dashed line indicates the lowest median distance (53.51 μm), observed for mregDCs.

Supplementary Figure 10: Regulatory T cells induce the expression of CCR7 and PD-L1 in BMDCs that differentiated with FLT3L. (A) Scheme showing the procedure of generating inducible Tregs. (B) Representative flow cytometry plots showing FOXP3 expression by inducible Tregs. (C) FLT3L-differentiated BMDCs were treated with Treg supernatant or with control media containing only the cytokines (TGF β or IL-2). Representative flow cytometry plots illustrating CCR7 induction are shown (right). The figure includes four technical replicates and was repeated twice, showing similar results. (D) On the left panel, quantification of PD-L1 expression in FLT3L-differentiated BMDCs following co-culture with Tregs. M- millions of cells. On the right panel, treatment with different amounts (ul) of Treg-conditioned supernatant. The figure includes three technical replicates and was repeated three times, showing similar results. (E) MFI of PD-L1 was measured across multiple treatment conditions and cytokines. The figure includes three technical replicates and was repeated twice, showing similar results. (F) The expression level of PD-L1 and CCR7 in BMDCs that were treated with Treg supernatant or co-incubated with iTregs. Co-expression of the two markers is shown. (G) Differential induction of CCR7 in FLT3L-derived bone marrow cells (blue bars) and GM-CSF-derived bone marrow cells (red bars). Fold-change of

CCR7 expression relative to untreated cells (media) is shown. The figure includes three technical replicates and was repeated twice, showing similar results. Sup W/O Treg – only TGF β and IL2. **(H)** PD-L1 expression of GM-CSF-differentiated bone marrow cells. Left: Quantification of PD-L1 expression (fold change relative to media control) in CD11c⁺ BMDCs after treatment with Treg supernatant or co-culture with Treg cells. Representative flow cytometry histograms showing PD-L1 levels across treatment groups on the right. The figure includes three technical replicates and was repeated three times, showing similar results. Statistical significance was determined using an unpaired two-tailed Student's t-test. Data represent mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Supplementary Figure 11: *Tnfsf11* expressed by Tregs induces mregDCs

(A) Overview of the in vitro assay testing the direct effect of IL-15 on iTregs. CD4⁺ T cells were isolated from spleens and differentiated into iTregs for 5 days in the presence of TGF β and IL-2. iTregs were then cultured with either media alone or recombinant IL-15 for 24 hours, followed by flow cytometric analysis to assess IL-15-dependent changes in proliferation and activation marker expression. **(B)** Median distance of each DC subset to Tregs. Box plot showing the median distance from each DC cell type to the nearest Tregs within the spatial transcriptomics dataset. **(C)** Cumulative distribution of distances between DC subsets and Tregs in spatial transcriptomic data. Empirical cumulative distribution functions (ECDFs) show the distribution distance between Tregs and DC subtypes. **(D)** *Ligand–receptor interaction analysis between Tregs and mregDCs using the scRNA-seq data.* The heatmap summarizes the ligand-receptor interaction scores between Treg-derived ligands and mregDCs-expressed receptors. **(E)** Chord diagram summarizing predicted ligand–receptor interactions between Tregs and mregDCs. The ligands are expressed by Tregs, and the receptors are expressed by mregDCs. Lines represent individual ligand–receptor pairs, and line width reflects interaction scores based on the Liana tool. **(F)** Violin plots show *Tnfsf11* and *Tnfrsf11a* transcript levels in Tregs, cDC1, cDC2, mregDCs, and pDCs based the scRNA-seq data. The Y-axis represents the log-normalized expression levels of *Tnfsf11* and *Tnfrsf11a* in each cell. **(G)** Percentage of CCR7⁺ CD11c⁺ cells (left), CCR7 mean fluorescence intensity (middle), and total CCR7⁺ cell counts (right) in BMDCs treated with LPS alone or LPS together with *Tnfsf11*. **(H)** Representative flow cytometry plots showing the level of CCR7⁺ BMDCs following *Tnfsf11* stimulation. **(I)** Quantification of CCR7⁺ CD11c⁺ BMDCs (left) and

CCR7 MFI (right) following co-culture with Tregs transduced with lenti-virus that encodes gRNAs targeting *Tnfrsf11* or non-targeting gRNA (NT). **(J)** Representative flow cytometry histogram overlays illustrating CCR7 induction in BMDCs co-cultured with CRISPR-edited Tregs. (I-J) The figures include three technical replicates and were repeated twice, showing similar results. Statistical significance was determined using an unpaired two-tailed Student's t-test. Data represent mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Supplementary Figure 12. Preferential spatial association of mregDCs and Tregs is linked to an immunoregulatory state in human PDAC.

(A) Spatial transcriptomic maps showing the distribution of Treg score (left) and mregDC score (right) across a PDAC tumor section from patient PA#98 (dataset: GSE278694). Each spot represents a Visium capture location overlaid on the histological image. Color intensity indicates normalized gene signature scores, with higher values reflecting increased enrichment of Treg-associated genes (*FOXP3*, *IL2RA*, *CTLA4*, *TIGIT*, *IKZF2*) or mregDC-associated genes (*FLT3*, *CCR7*, *LAMP3*, *CCL19*, *FSCN1*, *CD274*). **(B)** Spatial transcriptomic spots were categorized based on enrichment of Treg and mregDC gene signatures into four groups: Treg_high (green), mregDC_high (red), Both (Treg and mregDC, blue), and Other (without Treg and mregDC, gray). Each spot represents a Visium capture location overlaid on the corresponding histological section. **(C)** Dot plot showing the expression patterns of canonical Treg markers (*FOXP3*, *IL2RA*, *CTLA4*, *TIGIT*, *IKZF2*) and mregDC markers (*FLT3*, *CCR7*, *LAMP3*, *CCL19*, *FSCN1*, *CD274*) across spatially defined spot categories: Other, mregDC_high, Treg_high, or mregDC_Treg_high. Dot size represents the fraction of spots expressing each gene within a given group, while color intensity indicates the average normalized expression level. **(D)** Number of observed spots with high Treg and mregDC signatures relative to the expected number of spots assuming independent distribution. Each dot represents a patient. **(E)** Dot size indicates the percentage of cells expressing each gene, and color indicates the scaled mean expression within each cell population. **(F)** Heatmaps showing the median within-patient z score for each transcriptional program across the four spatial groups. Rows represent individual patients, and columns represent spatial groups. Red indicates higher relative enrichment, whereas blue indicates lower relative enrichment. **(G)** Forest plot comparing Treg–mregDC and mregDC–CD8 spatial co-occurrence across patients. Squares indicate patient-specific estimates and

horizontal lines indicate 95% confidence intervals. Values greater than 1 indicate a stronger Treg–mregDC association. The vertical dashed line at 1 represents equal association strength. The diamond represents the pooled effect estimated using a random-effects meta-analysis.

Supplementary Figure 13: A model summarizing the interactions between DCs and Tregs

Schematic model summarizing the bidirectional interactions between dendritic cells and Tregs, as identified across scRNA-seq, spatial transcriptomics, and functional assays.

Figure S1

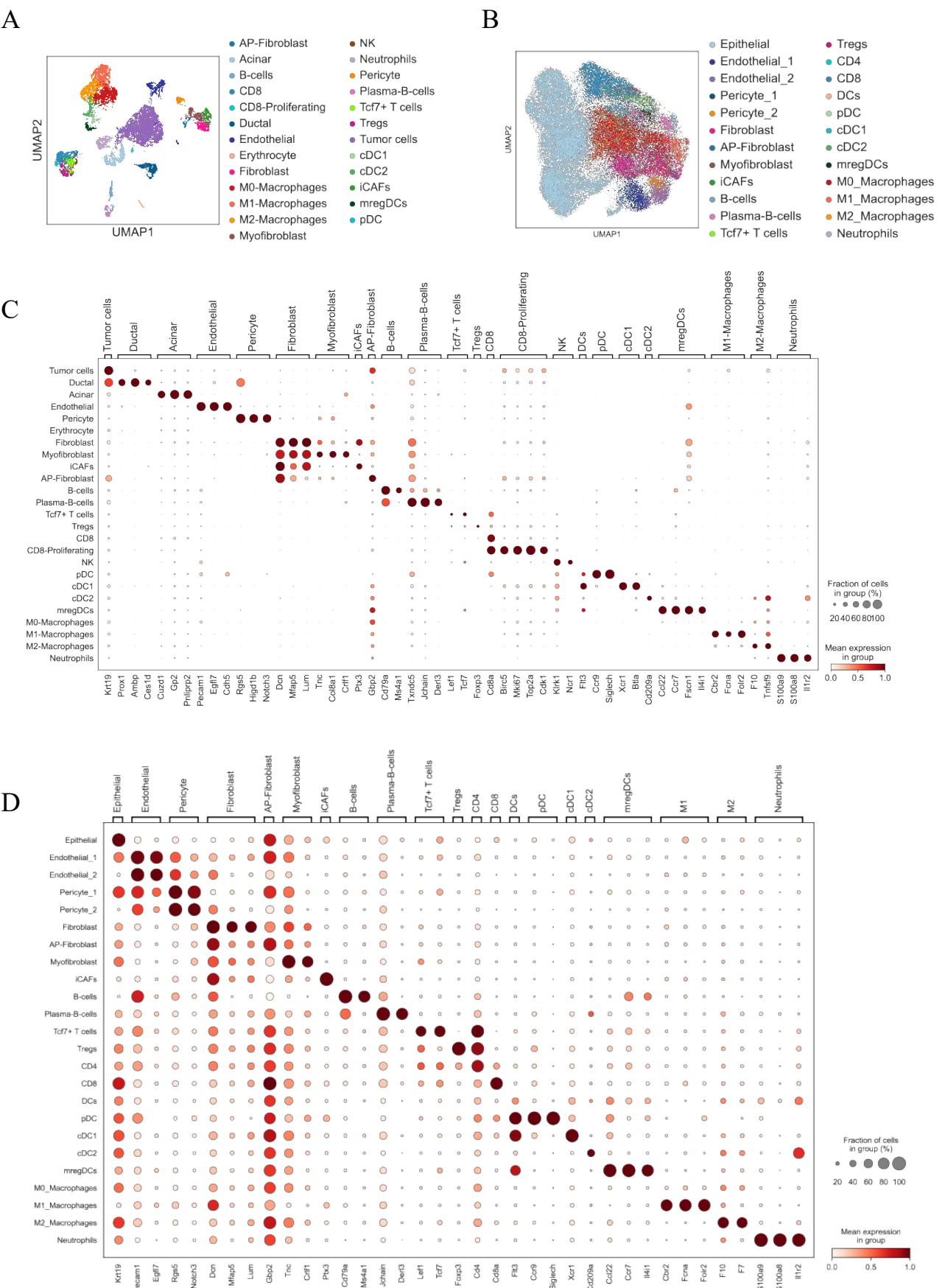


Figure S2

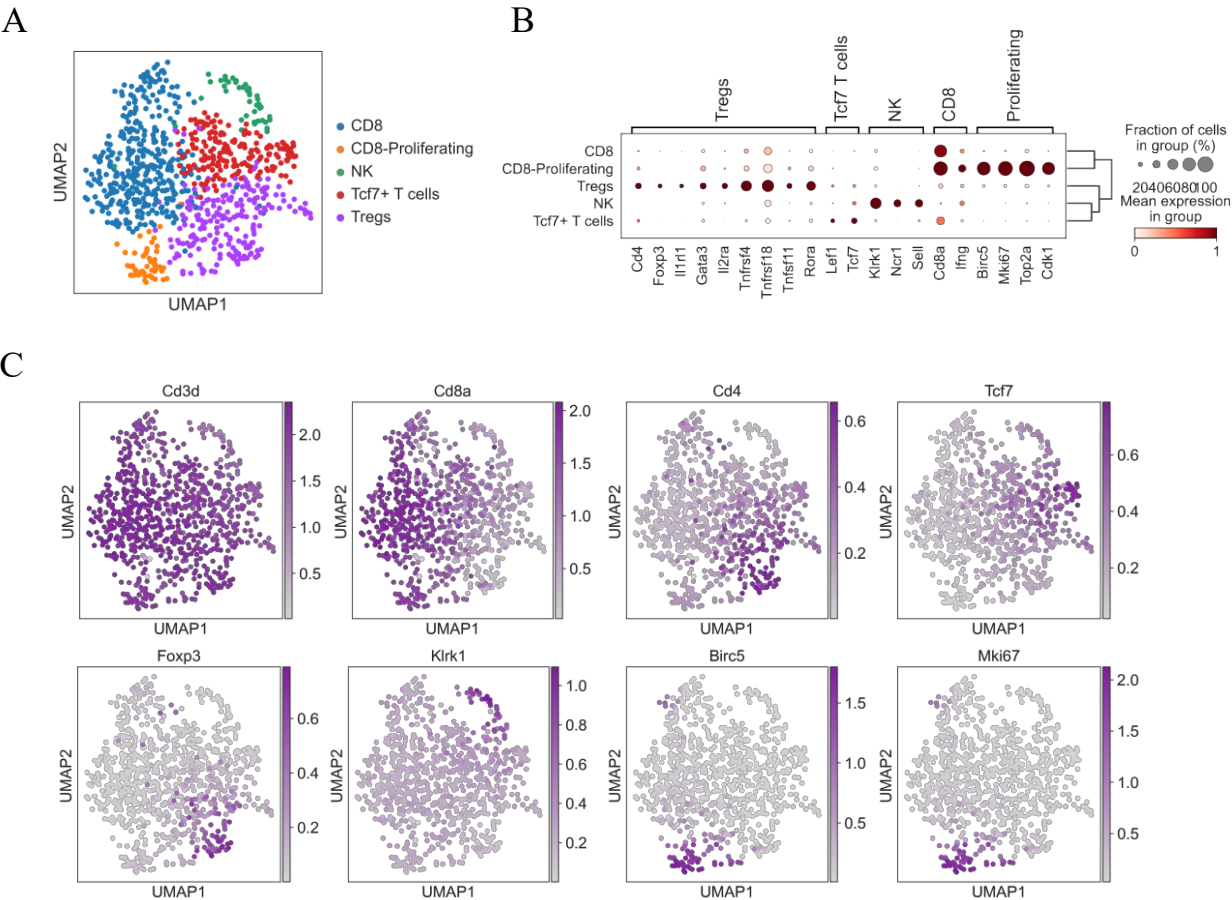
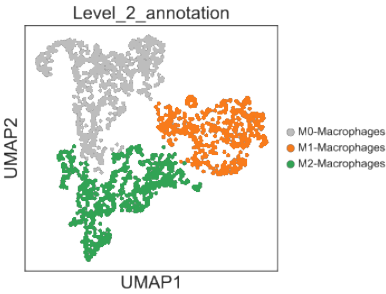
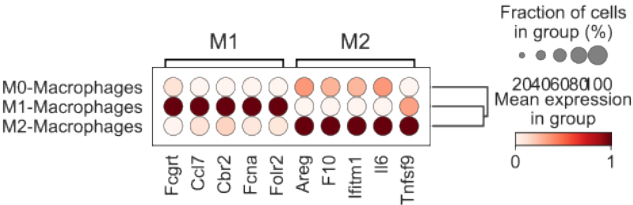


Figure S3

A



B



C

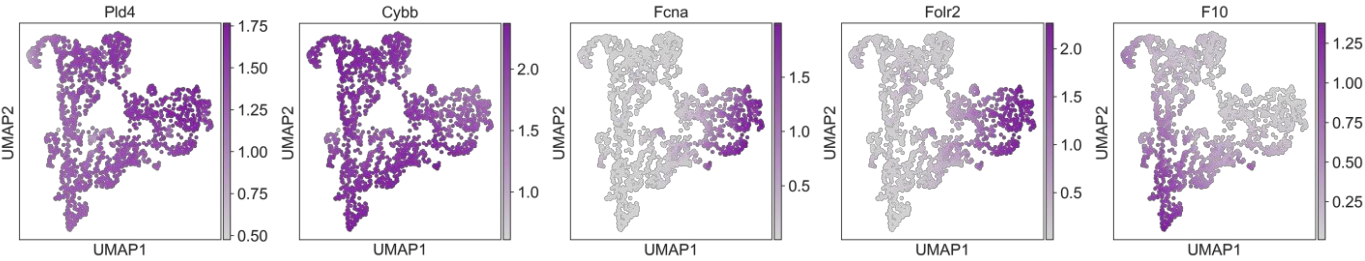


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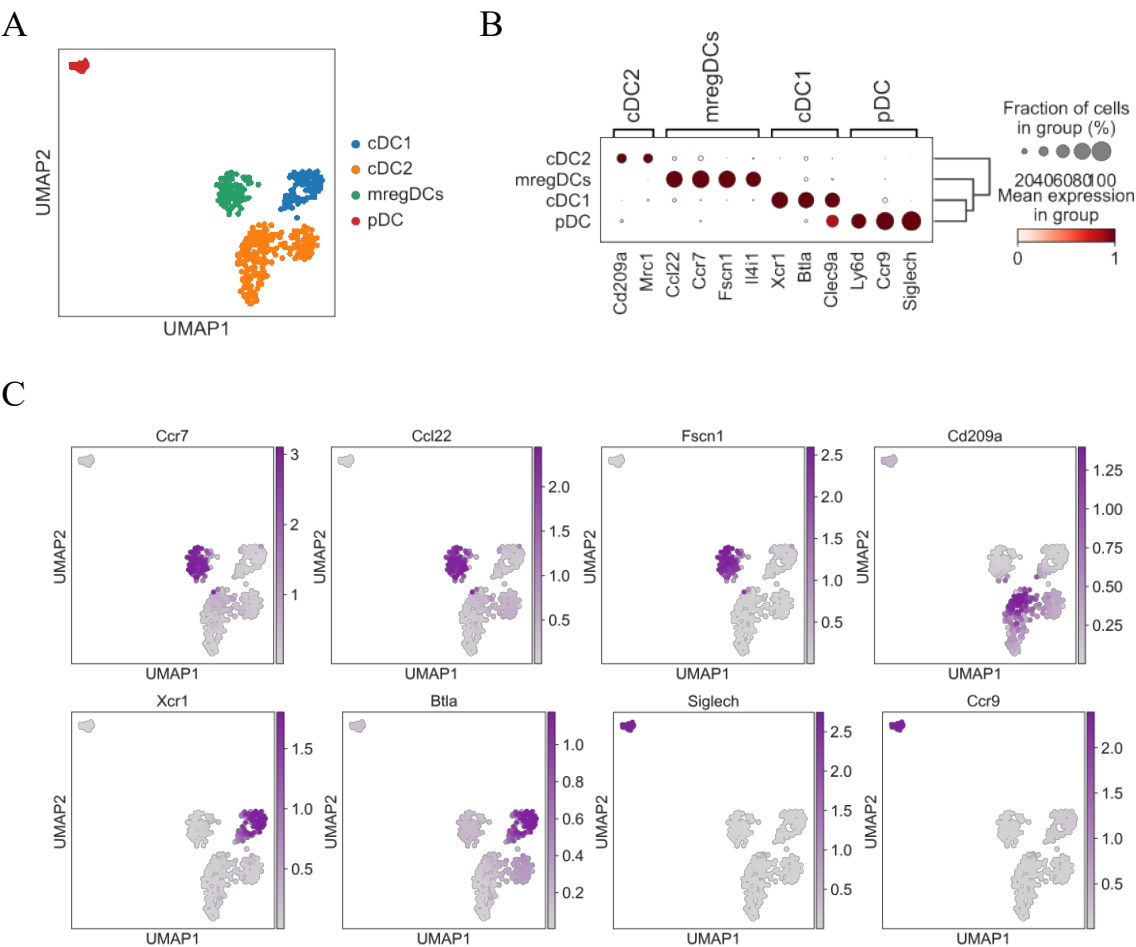


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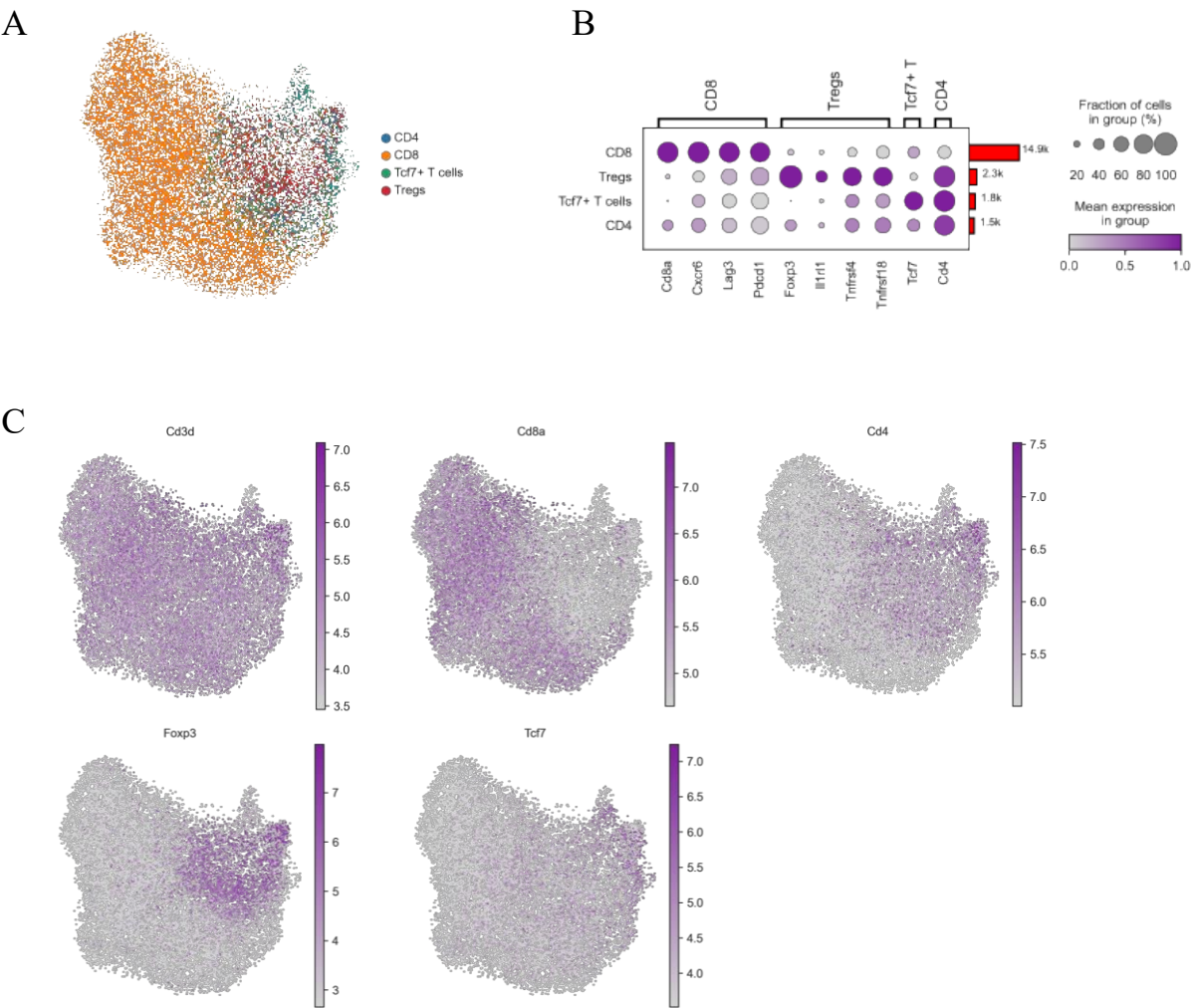


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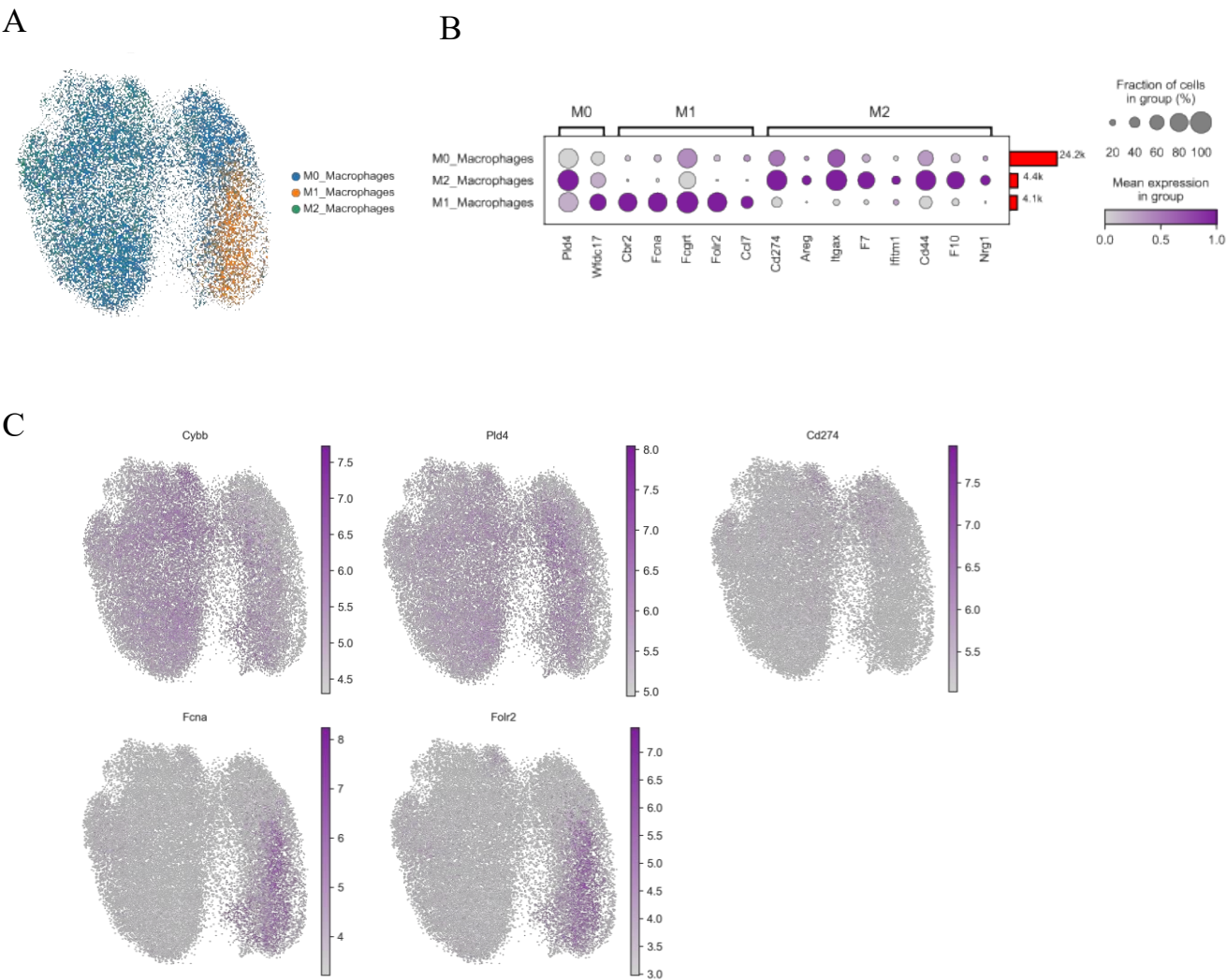


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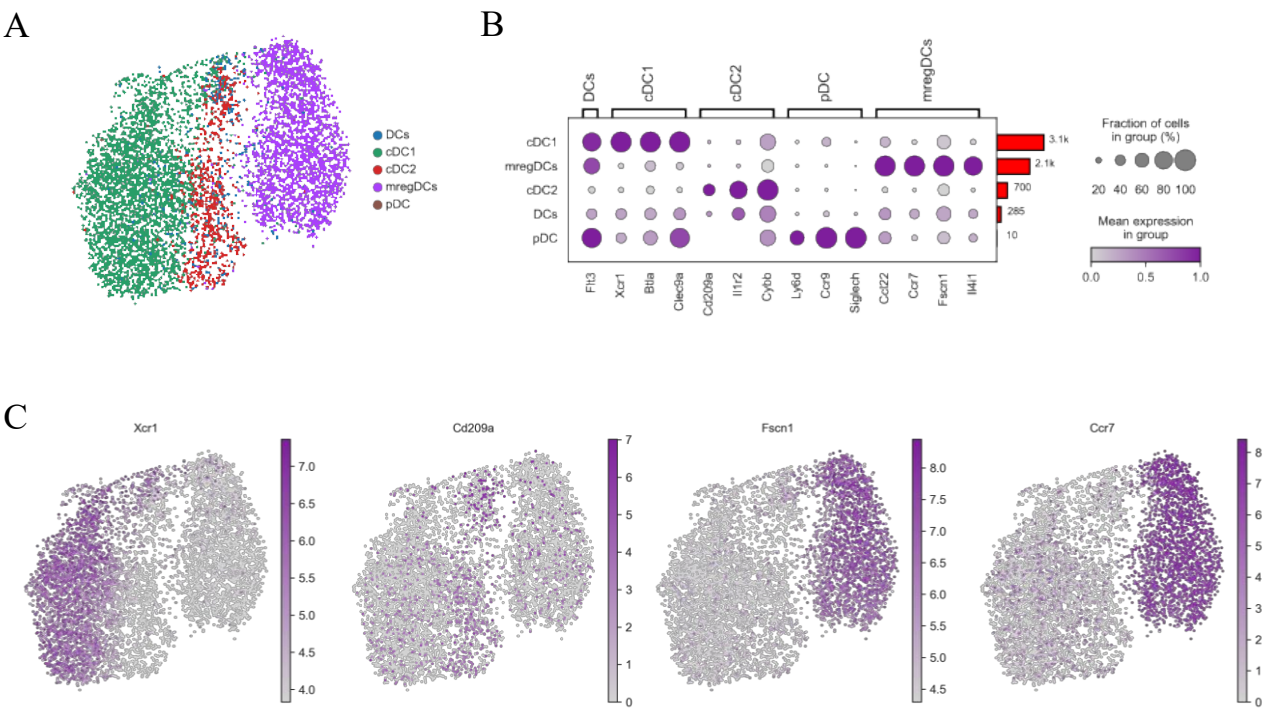


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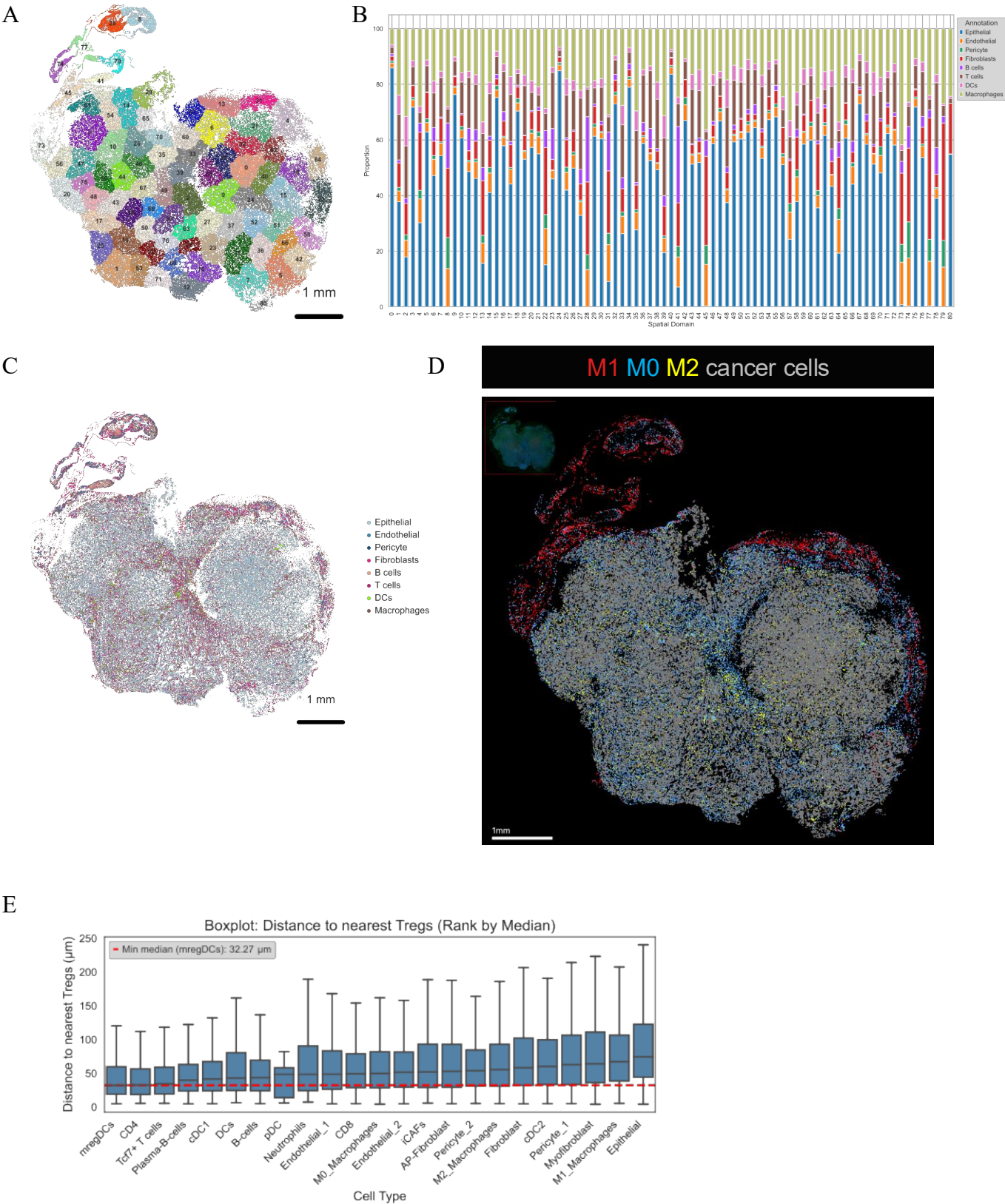


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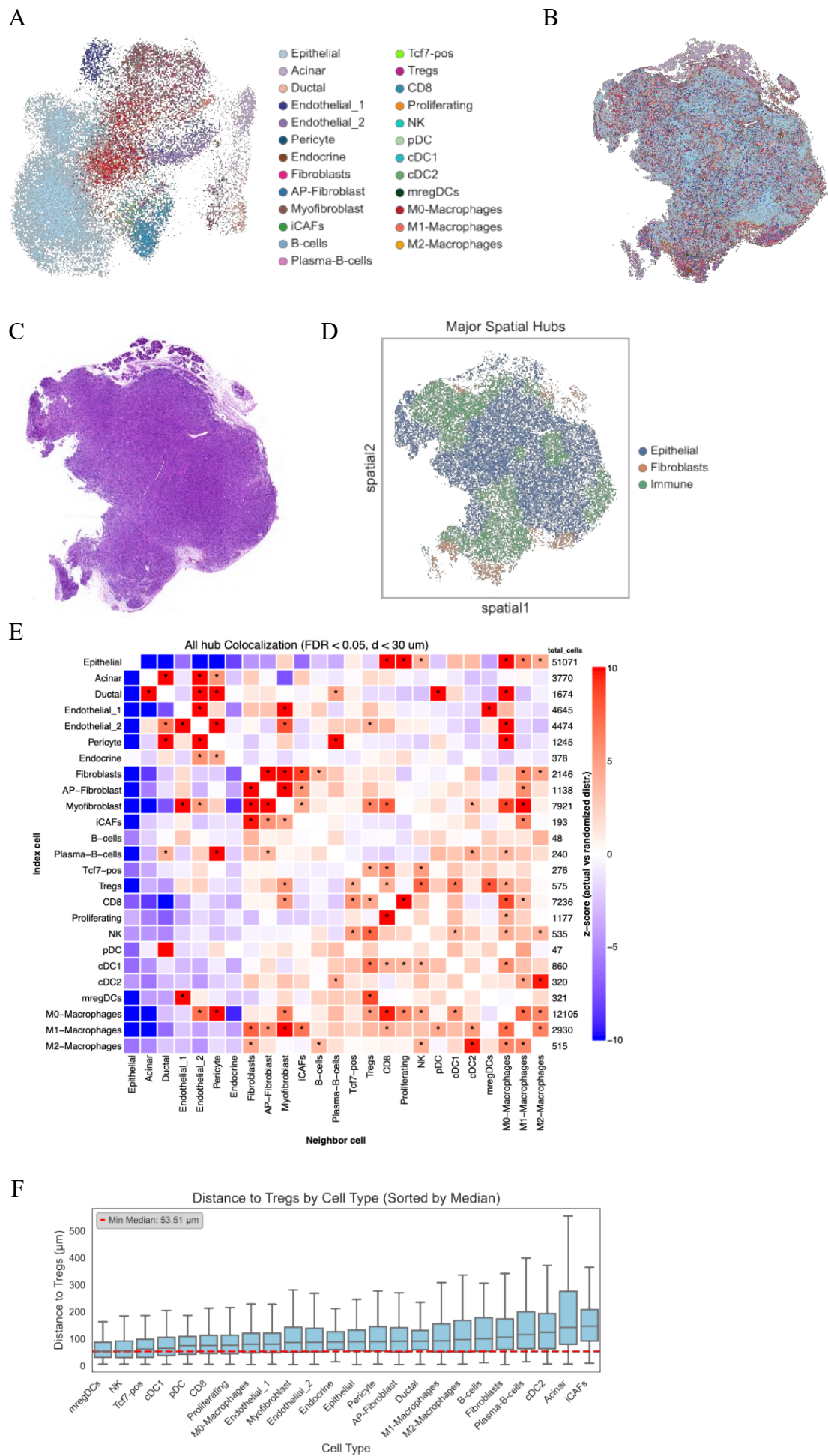


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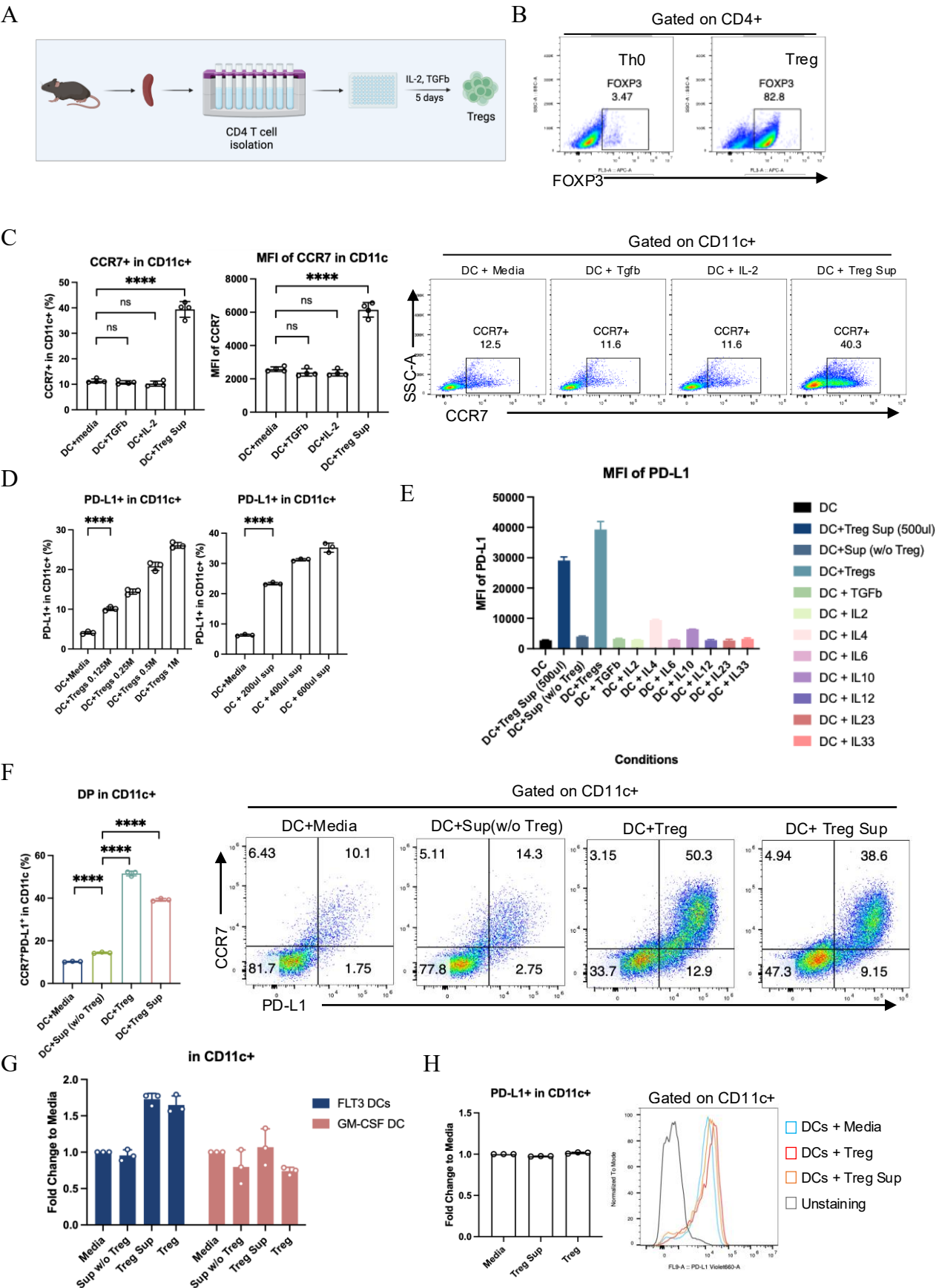
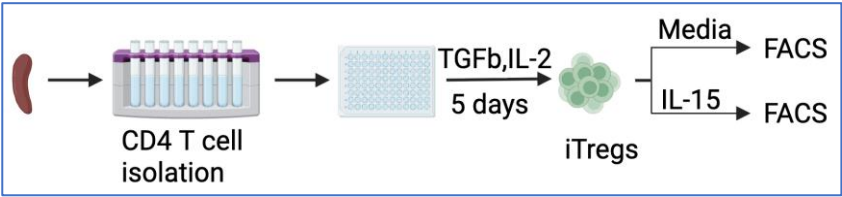
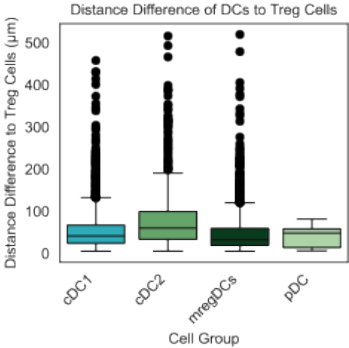


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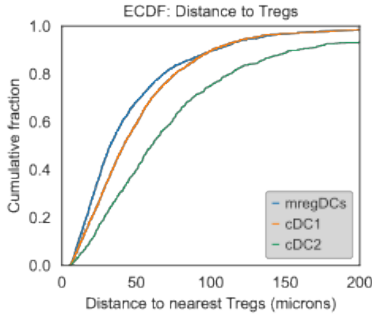
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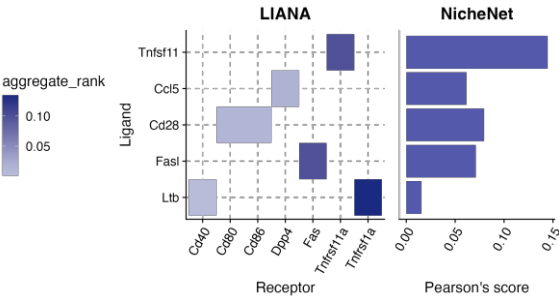
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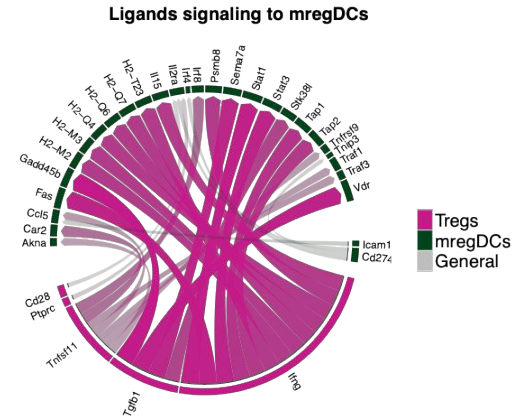
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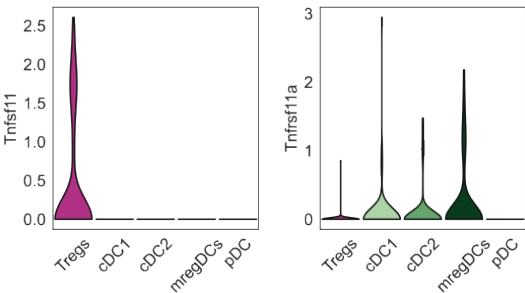
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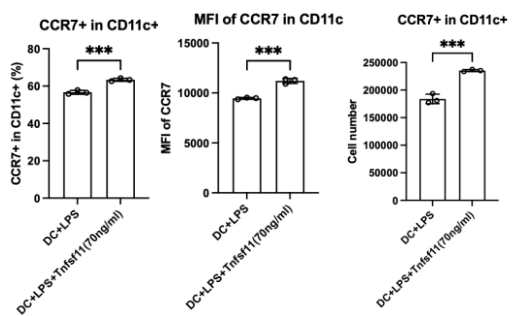
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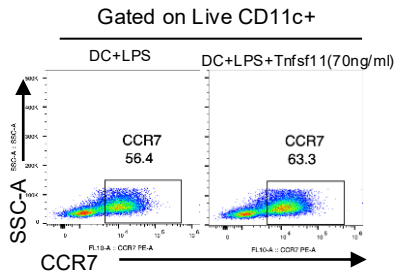
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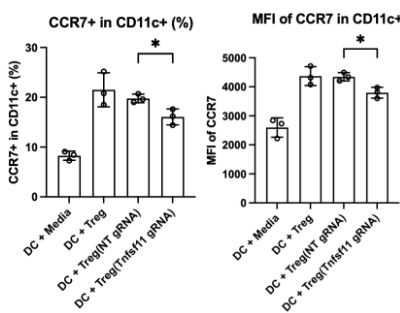
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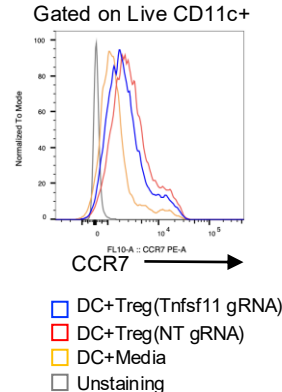


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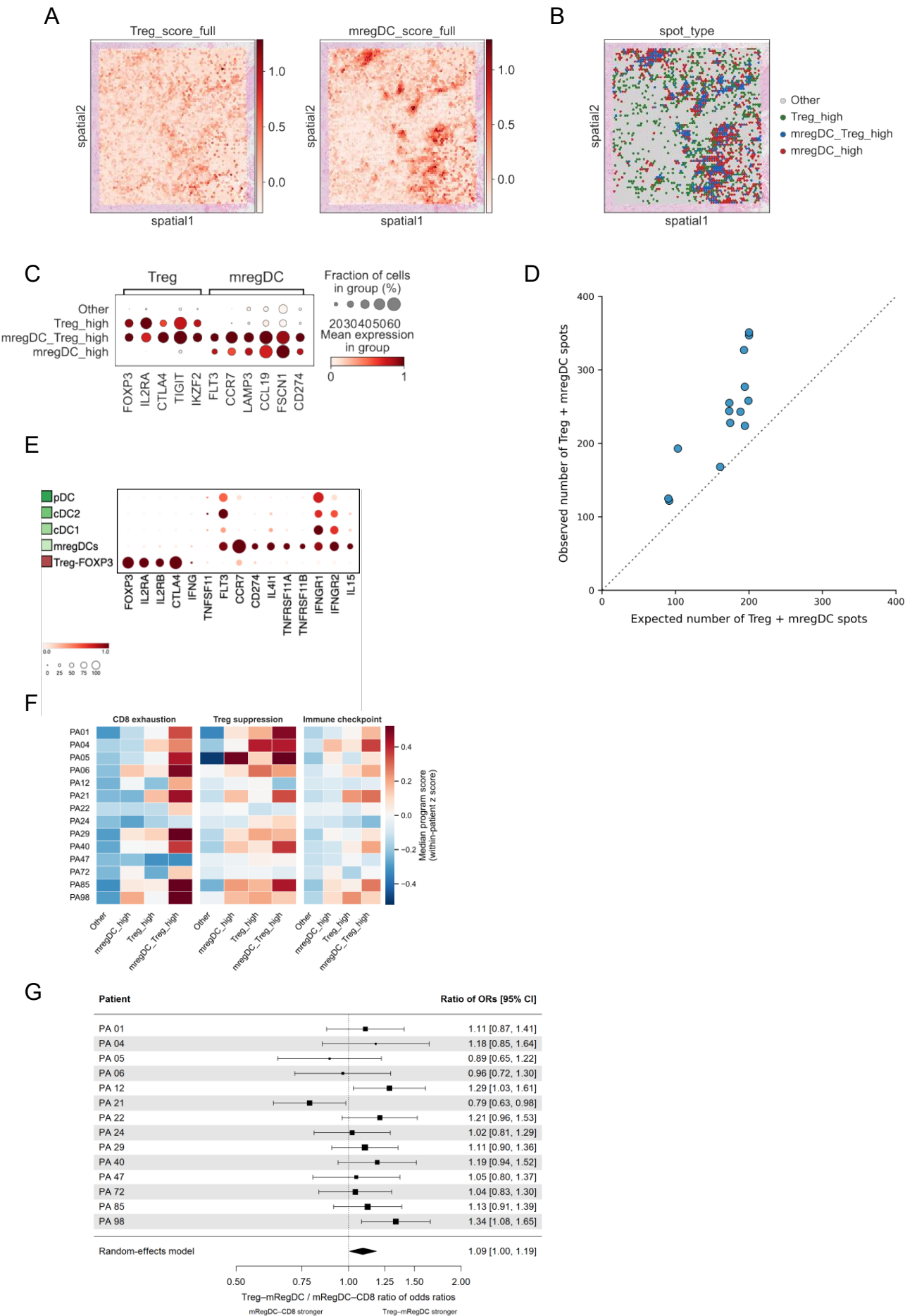
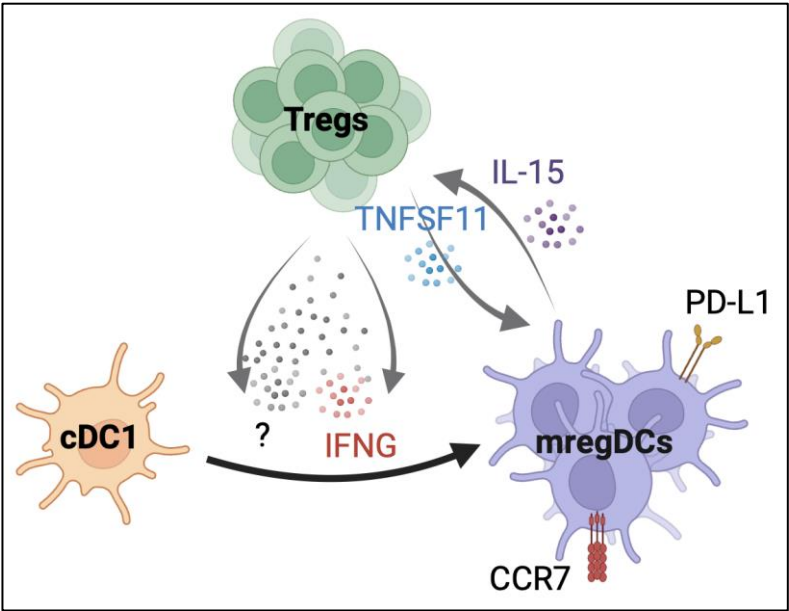


Figure S13



Reagents used in this study

List of plasmids

Lab ID	Plasmid	Source	Backbone
p1	pCMV delta R8.2	Addgene: #12263	NA
p2	pCMV-VSV-G	Addgene: #8454	NA
p408	CRISPseq-BFP-backbone	Addgene: #85707	NA
p411	LentiCRISPRv2-mCherry	Addgene: #99154	NA
p541	Mcherry with crop without puro	made in the lab	p411
p869	Tnfsf11_mCherry	made in the lab	p541
p890	Non-Target 1	made in the lab	p541
p891	Non-Target 2	made in the lab	p541
p908	Ifng_mCherry	made in the lab	p541

List of oligos for gRNA cloning

Lab ID	sgRNA-Forward	sgRNA-Reverse
p869	CACCGAGAGGACAGAGTGACTTTAT	AAACATAAAGTCACTCTGTCCTCTC
p890	CACCGATGTCGGTTTCCGCGAGGTG	AAACCACCTCGCGGAAACCGACATC
p891	CACCGCGCAGGCAGTTCTATGCGGA	AAACTCCGCATAGAACTGCCTGCGC
p908	CACCGTGCTGAAGAAGGTAGTAATC	AAACGATTACTACCTTCTTCAGCAC

List of reagents

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse FOXP3 APC (clone FJK-16s)	Thermo Fisher Scientific	Cat#17-5773-82; RRID: AB_469457
Anti-mouse ST2 Brilliant Violet 421 (clone DIH9)	Biolegend	Cat#145309; RRID: AB_2565634
PE/Cy7 anti-mouse CD4 antibody	Biolegend	Cat# 100421 RRID: AB_312706
FITC anti-mouse CD4 Antibody	Biolegend	Cat# 100406 RRID: AB_312691
APC anti-mouse CD274(PD-L1)	Biolegend	Cat# 124311 RRID: AB_10612935
PerCP/Cyanine5.5 anti-mouse CD25 Antibody	Biolegend	Cat# 101912 RRID: AB_10613643
APC anti-mouse CD152 antibody	Biolegend	Cat# 106310 RRID: AB_2087653
anti-mouse CD209a PE Antibody	Biolegend	Cat# 833003 RRID: AB_2721636

anti-mouse XCR1 Brilliant Violet 421	Biolegend	Cat# 148216 RRID: AB_2565230
PerCP/Cyanine5.5 anti-mouse CD45.2 Antibody	Biolegend	Cat# 109827 RRID: AB_893352
PE anti-mouse/human Ki-67 Antibody	Biolegend	Cat# 151209 RRID: AB_2716014
PE anti-mouse CD197 (CCR7) Antibody	Biolegend	Cat# 120105 RRID: AB_389357
APC/Fire™ 750 anti-mouse CD301 (MGL1/MGL2) Antibody	Biolegend	Cat# 145712 RRID: AB_2819894
Ultra-LEAF™ Purified anti-mouse CD3ε Antibody	Biolegend	Cat#100340 RRID: AB_11149115
Ultra-LEAF™ Purified anti-mouse CD28 Antibody	Biolegend	Cat#102116 RRID: AB_11147170
Purified anti-mouse IL-4 Antibody	Biolegend	Cat#504102 RRID: AB_315316
Purified anti-mouse IFN-γ Antibody	Biolegend	Cat#505802 RRID: AB_315396
Bacterial and virus strains		
Endura™ Competent Cells	BIOSEARCH TECHNOLOGIES	Cat #60242-2
pCMVΔR8.2		Addgene #12263
pCMV-VSV-G		Addgene #8454
LentiCRISPRv2-mCherry		Addgene # 99154
Chemicals, peptides, and recombinant proteins		
DNase I	Sigma-Aldrich	Cat# 10104159001
Collagenase P	Sigma-Aldrich	Cat# 11213865001
RPMI 1640 Medium	Gibco	Cat# 21875-034
DMEM, high glucose	Gibco	Cat# 41965-039
DPBS, no calcium, no magnesium	Gibco	Cat# 14190144
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat# 15140-122
β-Mercaptoethanol (50 mM)	Gibco	Cat# 31350-010
L-Glutamine (200 mM)	Gibco	Cat# 25030-024
HEPES(1M)	Gibco	Cat# 15630056
MEM Non-Essential Amino Acids Solution (100X)	Gibco	Cat# 11140035
Sodium Pyruvate (100 mM)	Gibco	Cat# 11360-039
Fetal Bovine Serum, qualified, Brazil	Gibco	Cat# 10270106
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix	Corning	Cat# CLS354230
Trypsin-EDTA (0.25%)	Gibco	Cat# 25200056
Opti-MEM™ I Reduced Serum Medium 100ml	Gibco	Cat# 31985062
Mouse IL-2 Recombinant Protein	PeproTech	Cat# 212-12-20UG
Recombinant Mouse TGF-beta 1 Protein	R&D	Cat# 7666-MB-005
Mouse IL-15 Recombinant Protein	PeproTech	Cat# 210-15-10UG
Recombinant Murine GM-CSF	PeproTech	Cat# 0315-03-500
Recombinant Murine IL-4	PeproTech	Cat# 214-14-10UG
Recombinant Murine IL-6	PeproTech	Cat# 216-16-10UG
Recombinant Murine IL-10	PeproTech	Cat# 210-10-10UG
Recombinant Murine IL-12	PeproTech	Cat# 210-12-10UG

Recombinant Human IL-23	PeproTech	Cat# 200-23-10UG
Recombinant Murine IL-33	PeproTech	Cat# 210-33-10UG
Mouse Flt-3 Ligand (FLT3L) Recombinant Protein	PeproTech	Cat# 250-31L-1MG
Red Blood Cell Lysing Buffer Hybri-Max	Sigma-Aldrich	Cat# R7757
TRIzol™ Reagent	Thermo Fisher Scientific	Cat# 15596026
OVA 323-339	InvivoGen	Cat# vac-isq
Bovine Serum Albumin (BSA)	warehouse	Cat# 100000042
Critical commercial assays		
True-Nuclear™ Transcription Factor Buffer Set	Biolegend	Cat# 424401
EasySep Mouse CD4+ T Cell Isolation Kit	StemCell	Cat# 19852
PowerUp™ SYBR™ Green Master Mix for qPCR	Applied Biosystems	Cat# A25742
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat# 4368814
HIYIELD Plasmid Mini kit 100 preps (YPD 100)	Biolab	Cat# 9680YPD100
CellTrace™ Far Red Cell Proliferation Kit, for flow cytometry	Thermo Fisher Scientific	Cat# C34572
KAPA Pure Beads	gamidor	Cat# 7983298001
Quick-RNA Miniprep Kit	Zymo Research	Cat# R1054
KAPA Stranded mRNA-Seq Kit	KAPA Biosystems	Cat# KR0960–v6.17
Deposited data		
Raw and analyzed data	This paper	N/A
Experimental models: Cell lines		
KPCY mouse pancreatic cancer cell line (6499c4)	Kerafast, Inc	Cat# EUP015-FP
293T	ATCC	CRL-3216
Experimental models: Organisms/strains		
Mouse: B6.Cg-Tg (Tcra Tcrb) 425Cbn/J	Jackson Laboratory	RRID:IMSR_JAX:004194
Mouse: B6N.129(B6J)-Gt(ROSA)26Sor ^{tm1(CAG-cas9*,-EGFP)Fezh/J}	Jackson Laboratory	RRID:IMSR_JAX:026556
Mouse: B6.129(Cg)-Foxp3 ^{tm3(HBEGF/GFP)Ayr/J}	Dr. Moshe Biton	RRID:IMSR_JAX:016958
Mouse: C57BL/6	Envigo Israel	
Oligonucleotides		
gRNA Primer: Ifng forward: CACCGTGCTGAAGAAGGTAGTAATC	This paper	N/A
gRNA Primer: Ifng reverse: AAACGATTACTACCTTCTTCAGCAC	This paper	N/A
gRNA targeting sequence: Tnfsf11 forward: CACCGAGAGGACAGAGTGACTTTAT	This paper	N/A
gRNA targeting sequence: Tnfsf11 reverse: AAACATAAAGTCACTCTGTCCTCTC	This paper	N/A
gRNA targeting sequence: Non target1 forward: CACCGATGTCGGTTTCCGCGAGGTG	This paper	N/A
gRNA targeting sequence: Non target1 reverse: AAACCACCTCGCGGAAACCGACATC	This paper	N/A

gRNA targeting sequence: Non target2 forward: CACCGCGCAGGCAGTTCTATGCGGA	This paper	N/A
gRNA targeting sequence: Non target2 reverse: AAACTCCGCATAGAACTGCCTGCGC	This paper	N/A
Recombinant DNA		
<i>Ifng</i> -LentiCRISPRv2-mCherry	This paper	N/A
<i>Tnfrsf11</i> -LentiCRISPRv2-mCherry	This paper	N/A
Software and algorithms		
GraphPad Prism (Version 8)	GraphPad software	https://www.graphpad.com/
FlowJo (Version10)	FlowJo	https://www.flowjo.com/
Python (Version 3.9)		
RStudio	RStudio IDE	https://posit.co/download/rstudio-desktop/
R (Version 4.4)		
Suerat (Version 5)		https://github.com/satijalab/seurat