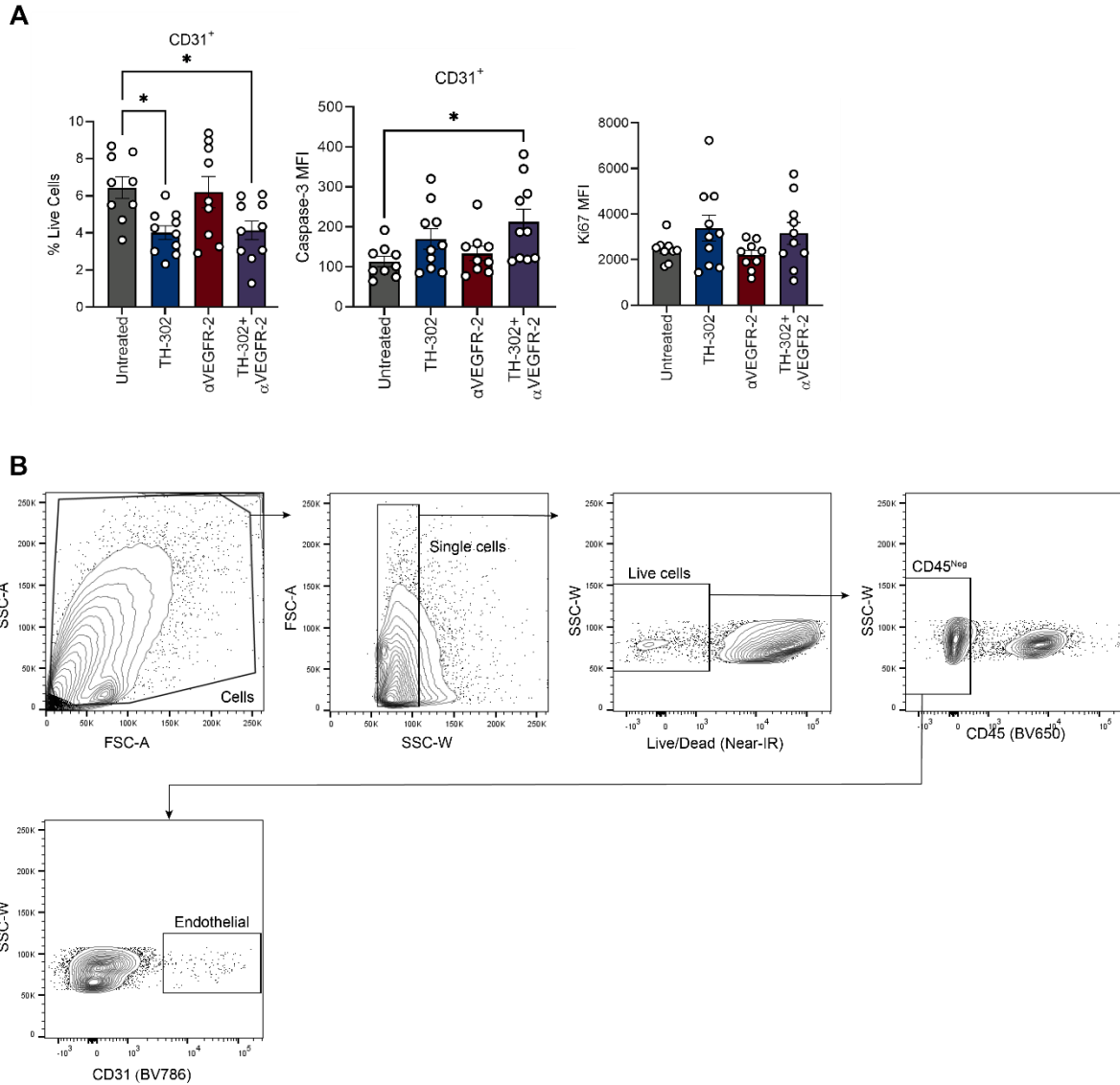
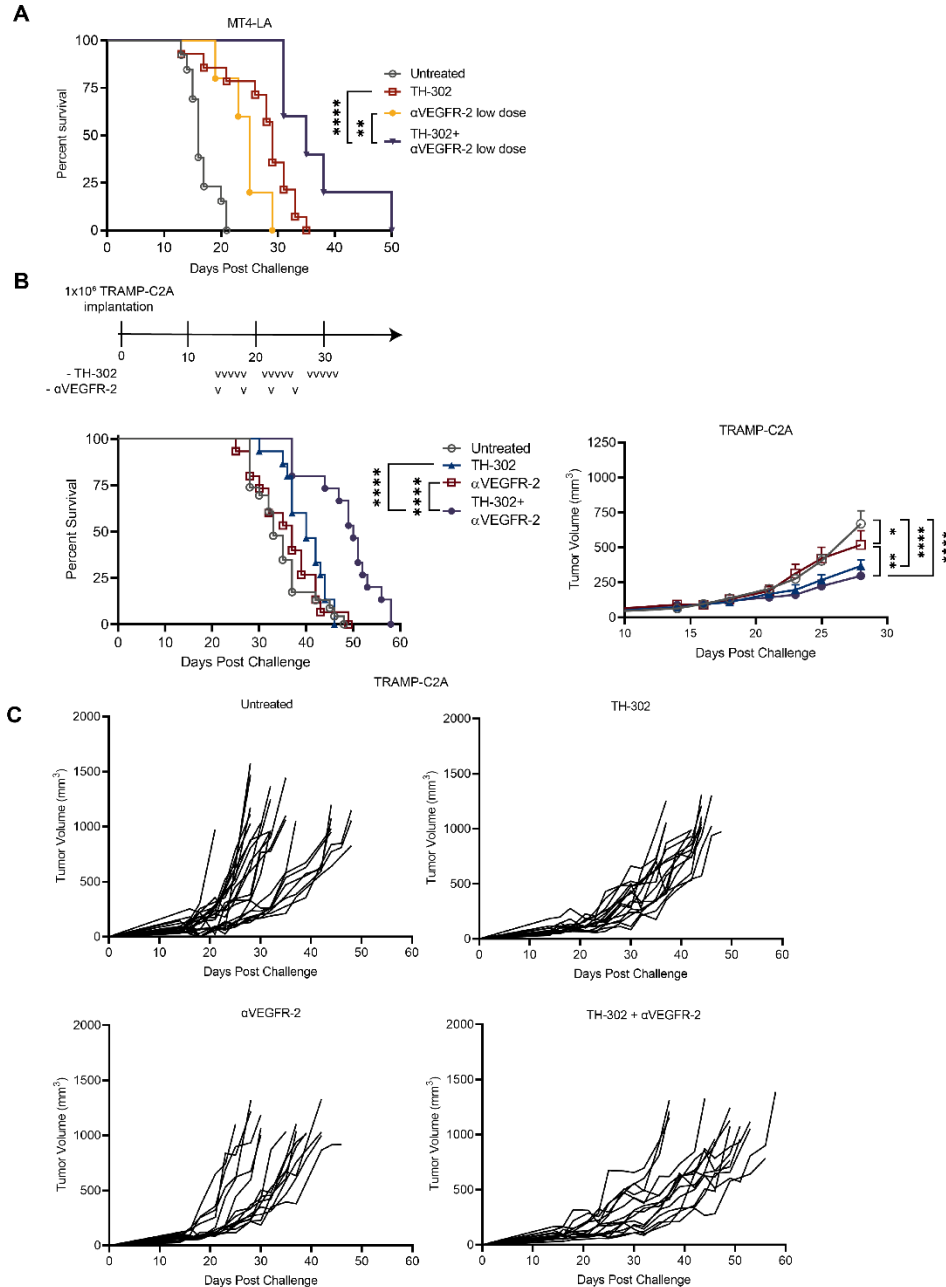


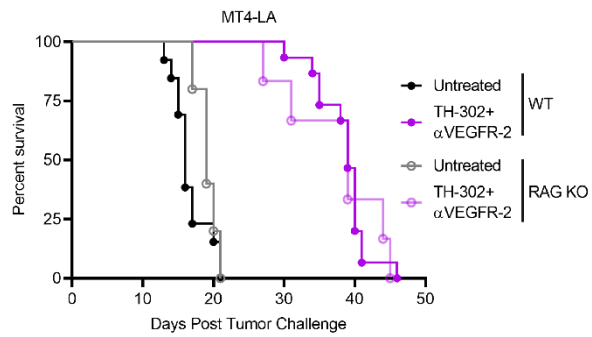
Supplementary Figures and Legends
JCI Insight (Revisions 2023) – Arthur Liu *et al.*



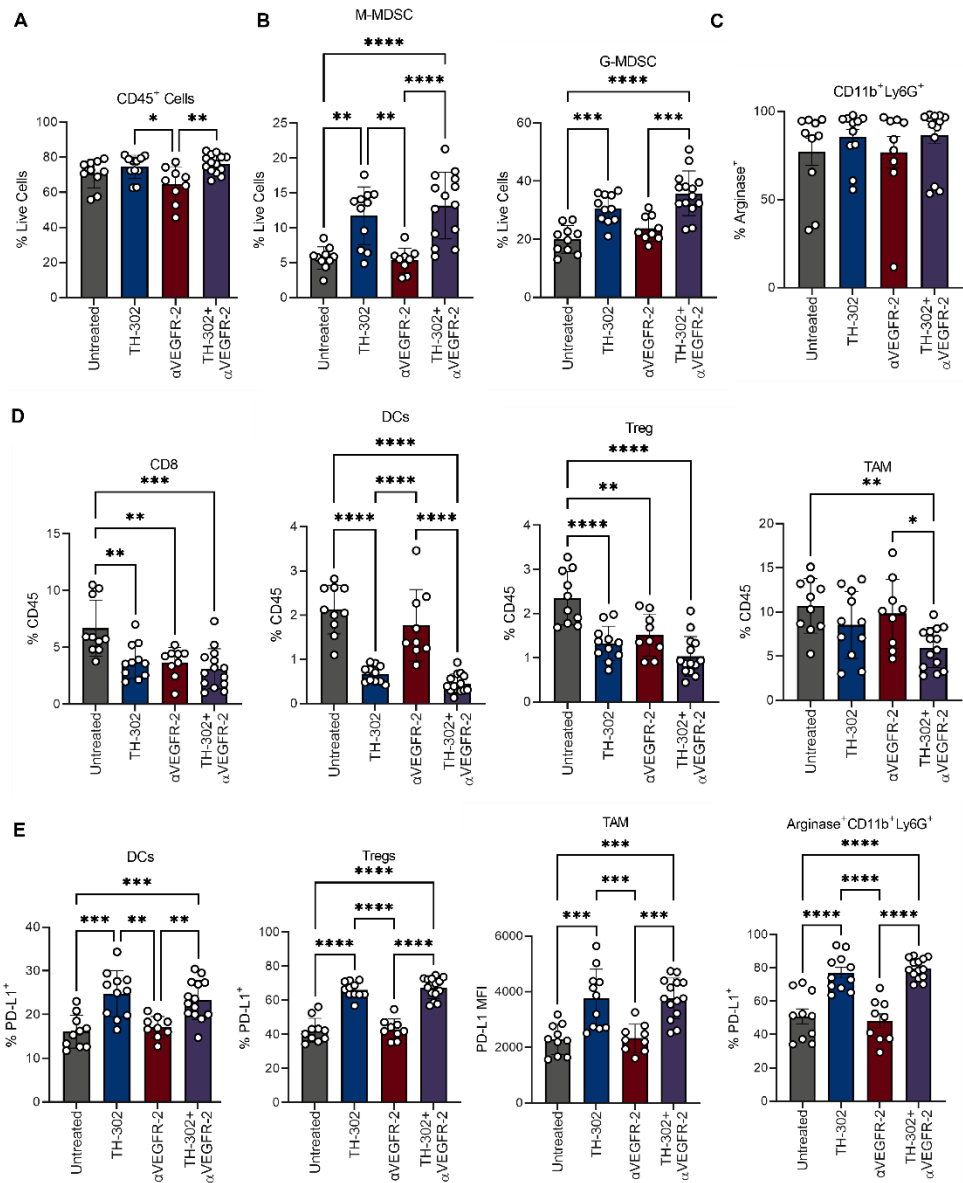
Supplementary Figure 1. Combination TH-302 and α VEGFR-2 results in endothelial cell pruning. 10-day established MT4-LA tumors were treated for five consecutive days of TH-302 and two doses of α VEGFR-2 prior to resection and processing for flow cytometry analysis. For endothelial cell analysis, the pellet fraction from the Ficoll density gradient separation was collected. **(A)** CD31⁺ cells were assessed for their total frequency and expression of Active Caspase-3 and Ki67 ($n = 9-10$ mice per group). **(B)** Representative gating strategy for CD31⁺ endothelial cells. One-way ANOVA followed by Tukey's correction for multiple comparisons. * P $adj < 0.05$, ** P $adj < 0.01$, *** P $adj < 0.001$, **** P $adj < 0.0001$.



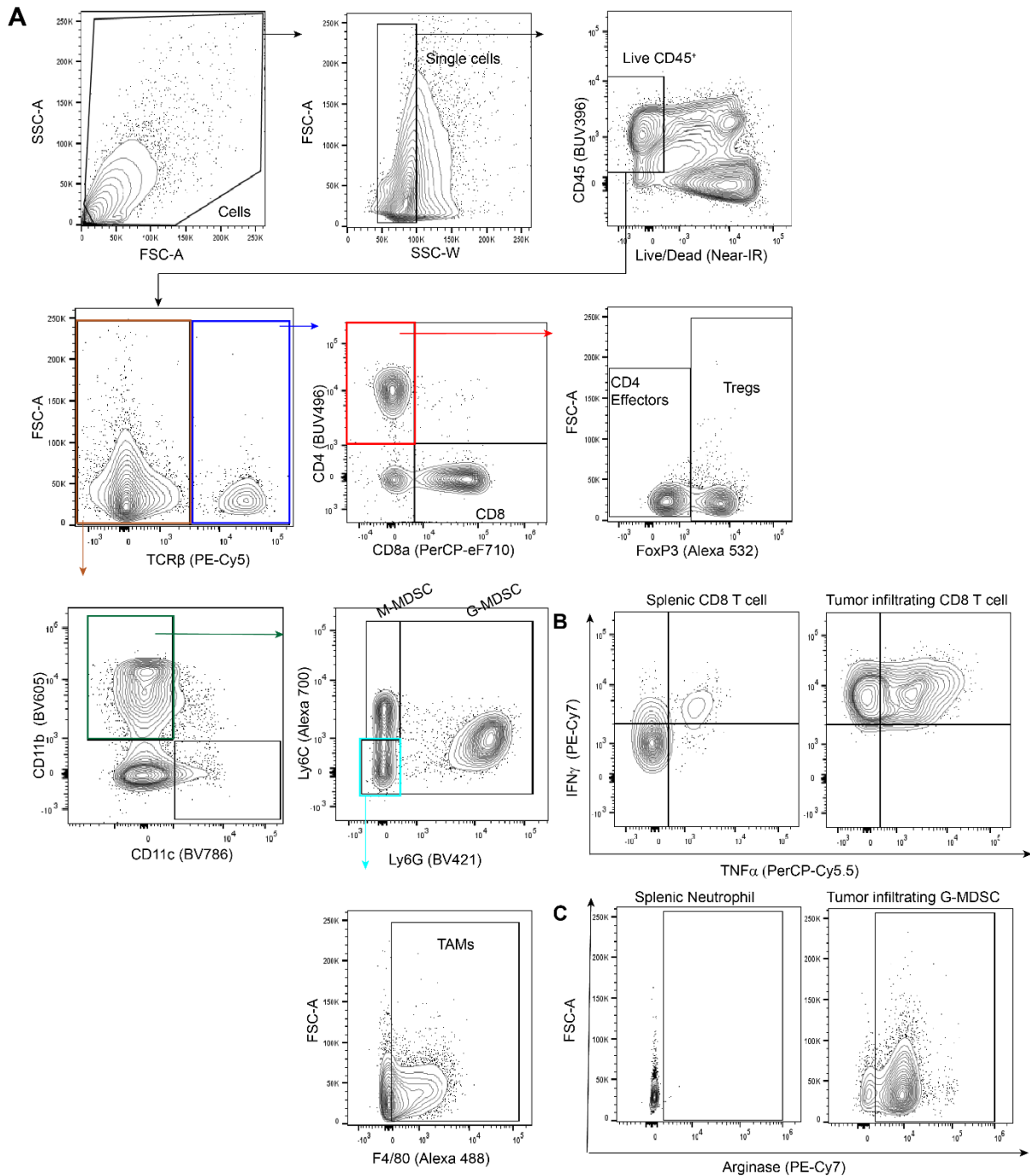
Supplementary Figure 2. Low dose α VEGFR-2 treatment of orthotopic pancreatic tumors and survival of mice challenged with TRAMP-C2A tumors. (A) Survival of mice treated with 10 mg/kg α VEGFR-2 ($n = 5-10$ mice per group). Untreated and TH-302 treated mice data is first presented in Figure 2. (B) Mice bearing 14-day pre-implanted subcutaneous TRAMP-C2A prostate tumors were treated as described and monitored for survival and tumor growth ($n = 15-23$ mice per group). (C) Individual tumor growth curves of TRAMP-C2A shown in (B). Log-rank (Mantel-Cox) test for survival and two-way ANOVA followed by Tukey's correction for multiple comparisons for tumor growth. * $P_{adj} < 0.05$, ** $P_{adj} < 0.01$, *** $P_{adj} < 0.001$, **** $P_{adj} < 0.0001$; data are mean \pm SEM.



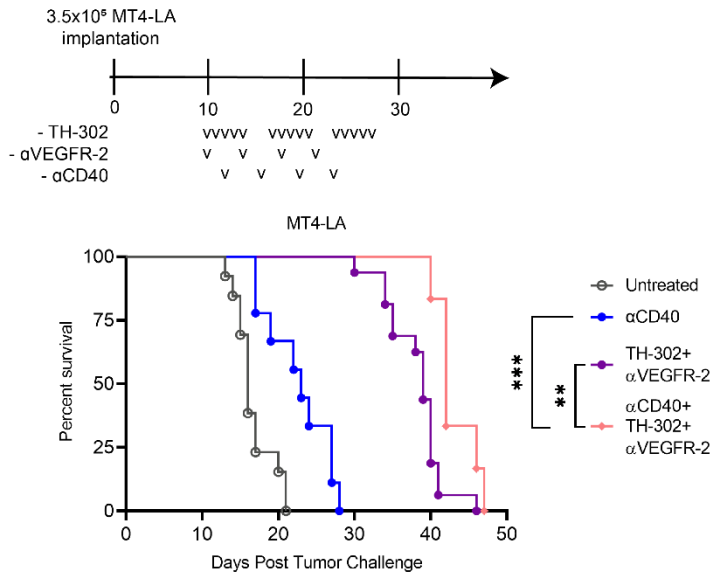
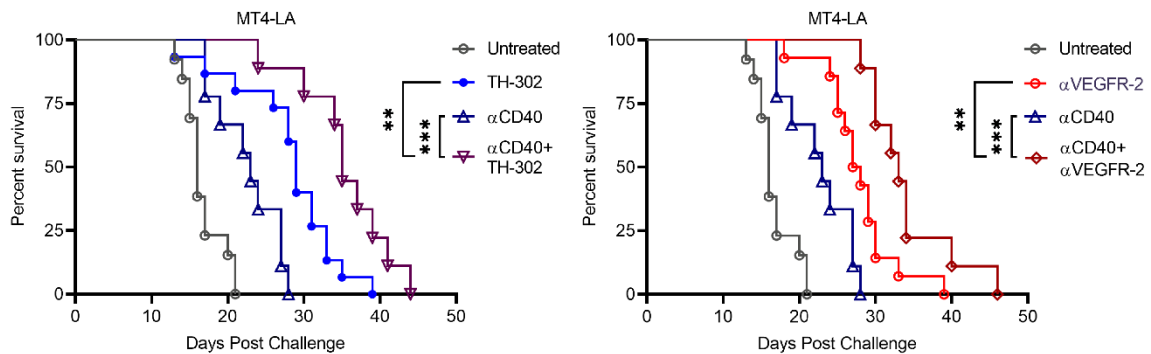
Supplementary Figure 3. Combination TH-302 and VEGFR2 blockade is not dependent on the adaptive immune response. 10 day established MT4-LA tumors in wild type or *Rag*-knockout mice were treated with TH-302 and VEGFR-2 as described in Figure 2 (n = 5-15 per group. Wild type mouse data is first presented in Figure 2. Log-rank (Mantel-Cox) test for survival. **P* *adj* < 0.05, ***P* *adj* < 0.01, ****P* *adj* < 0.001, *****P* *adj* < 0.0001; data are mean ± SEM.



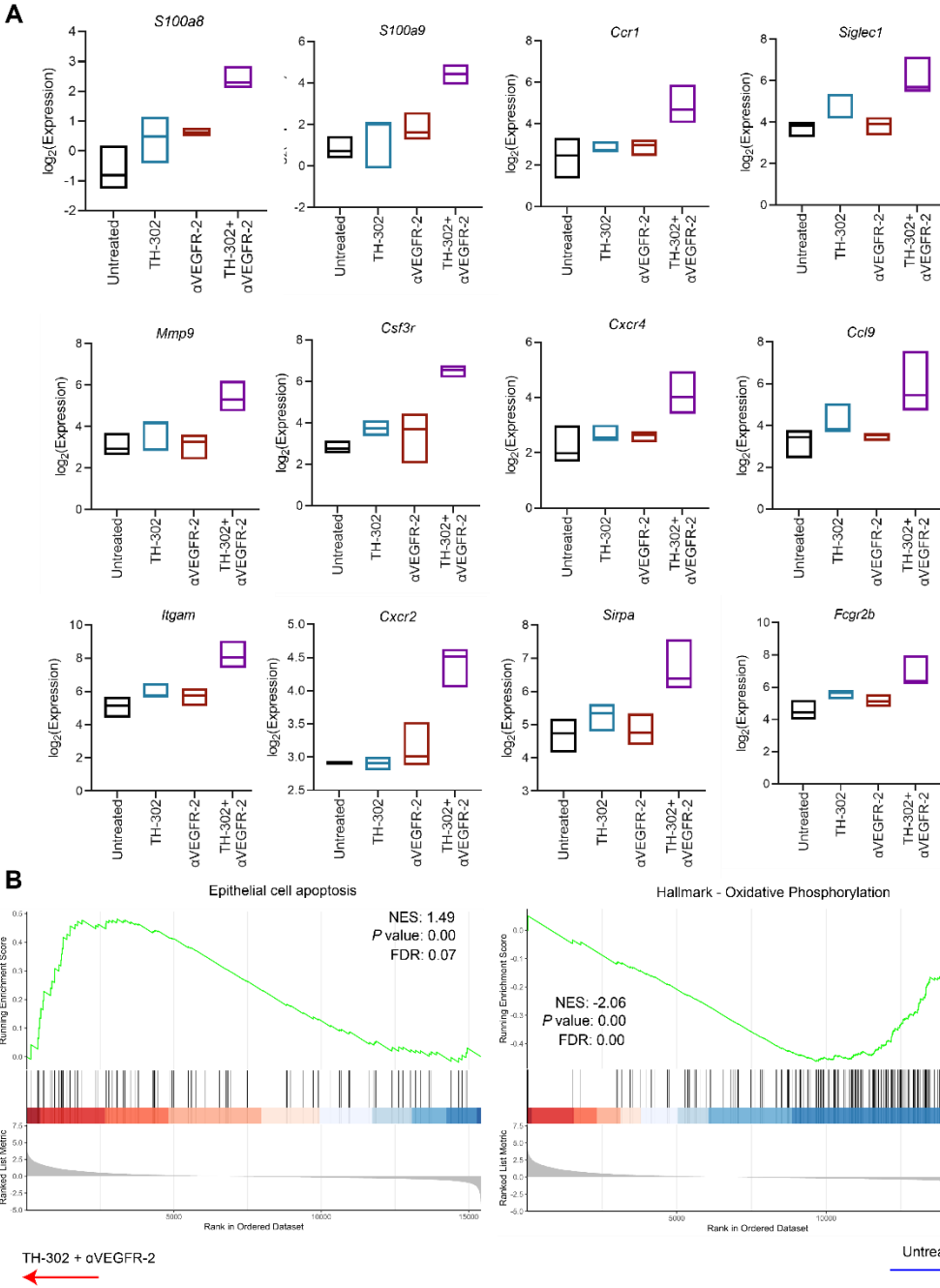
Supplementary Figure 4. Effect of TH-302 and VEGFR-2 blockade on tumor infiltrating immune cells. 10-day established MT4-LA tumors were treated for five consecutive days of TH-302 and two doses of VEGFR-2 blockade prior to resection for flow cytometry analysis. **(A)** Percent of live, CD45⁺ cells. **(B)** Percentage of live, M-MDSC and G-MDSC. **(C)** Proportion of Ly6G positive cells that express Arginase 1. **(D)** Proportion of CD8 T cells, dendritic cells (DCs), CD4 T regulatory cells (Treg), and tumor associated macrophages (TAMs). **(E)** Frequency of PD-L1 expressing DCs, Tregs, and TAMs **(A-E)** n = 10-14 per group. One-way ANOVA followed by Tukey's correction for multiple comparisons. **P* *adj* < 0.05, ***P* *adj* < 0.01, ****P* *adj* < 0.001, *****P* *adj* < 0.0001; data are mean ± SEM.



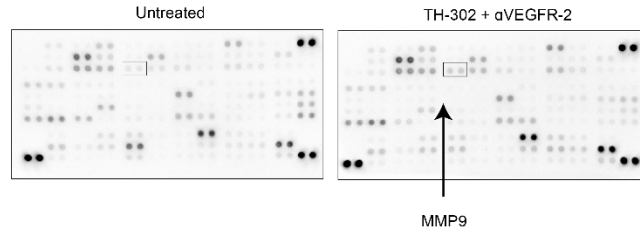
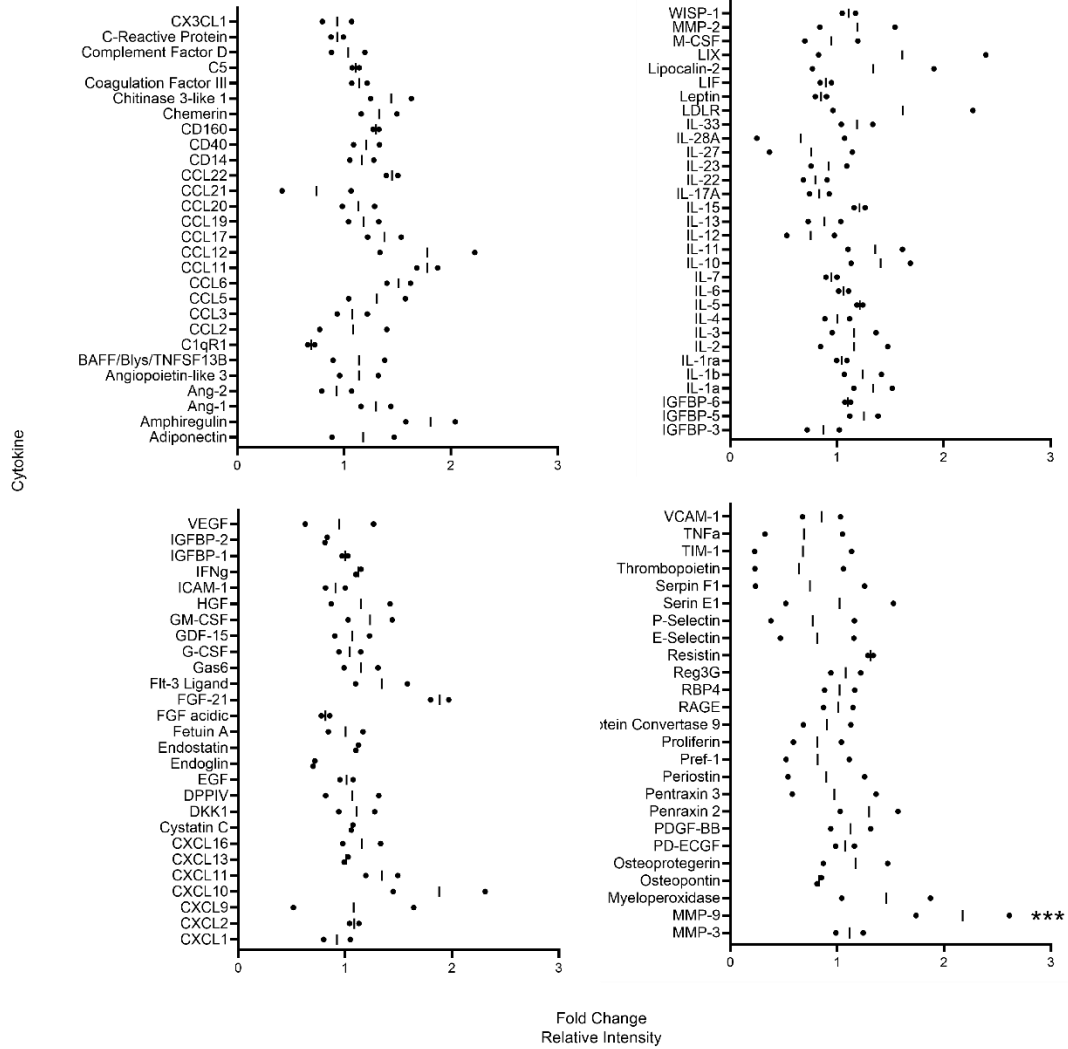
Supplementary Figure 5. Tumor infiltrating immune cell gating schema. (A) Representative gating for live, tumor infiltrating immune cells and representative gating for **(B)** dual expressing IFN γ and TNF α T cells and **(C)** arginase gating on Ly6G⁺ cells.

A**B**

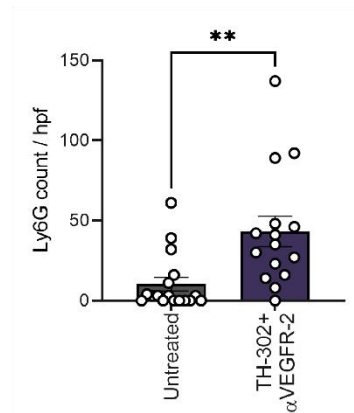
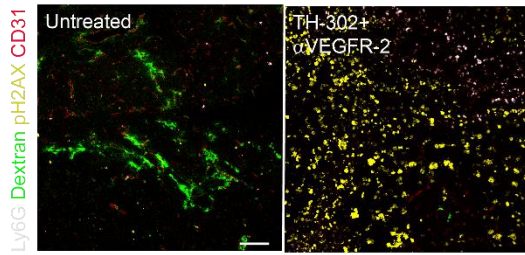
Supplementary Figure 6. αCD40 provides an additive benefit when combined with either TH-302 or αVEGFR-2. (A) αCD40 used in combination with TH-302 and αVEGFR-2 or (B) with each monotherapy ($n = 9-15$ mice per group). αCD40 survival curves presented in (B) are the same but presented in two separate graphs for clarity. Untreated and TH-302 treated mice are first presented in Figure 2. Log-rank (Mantel-Cox) test. * $P_{adj} < 0.05$, ** $P_{adj} < 0.01$, *** $P_{adj} < 0.001$, **** $P_{adj} < 0.0001$; data are mean \pm SEM.



Supplementary Figure 7. Normalized expression levels of selected neutrophil-related genes expressed in treated tumors. (A) Genes enriched in combination treated tumors over untreated. (B) Leading edge analysis of epithelial cell apoptosis and hallmark oxidative phosphorylation ($n = 3$ mice per group). NES, normalized enrichment score. FDR, false discovery rate.

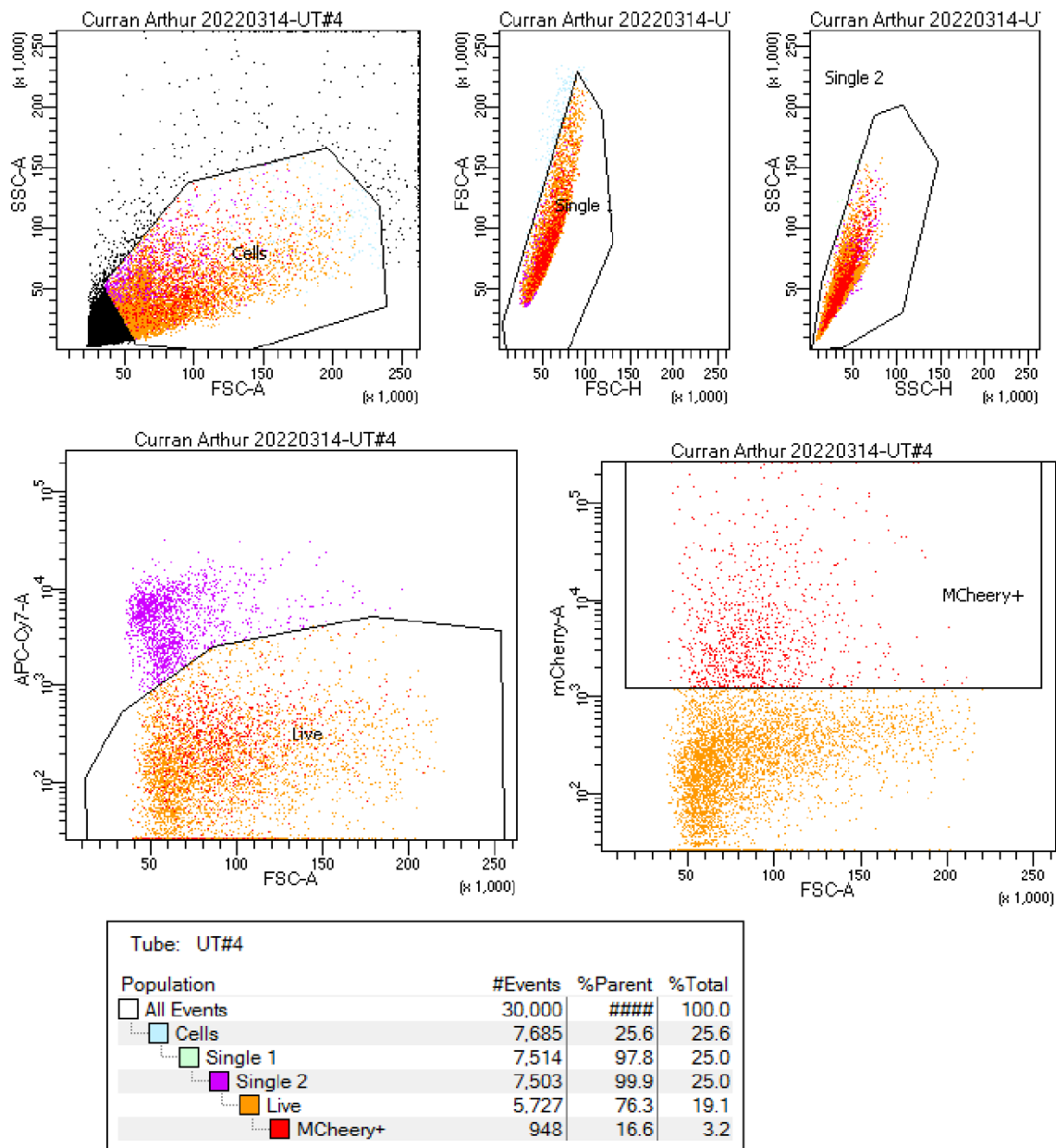
A**B**

Supplementary Figure 8. MMP9 is enriched in combination TH-302 and αVEGFR-2 treated tumors. (A) Representative proteome Profiler Mouse XL Cytokine Array membranes and (B) associated quantification ($n = 2-3$ pooled tumors per group, 2 independent experiments). One-way ANOVA followed by Tukey's correction for multiple comparisons. $*P_{adj} < 0.05$, $**P_{adj} < 0.01$, $***P_{adj} < 0.001$, $****P_{adj} < 0.0001$; data are mean \pm SEM.

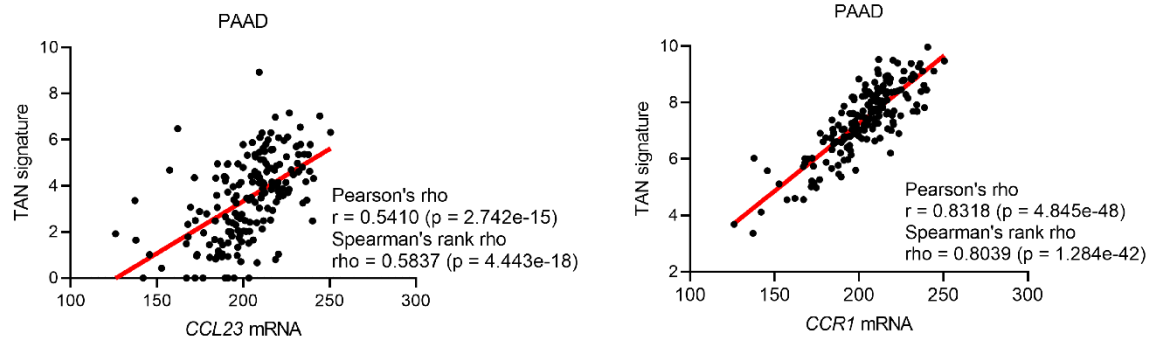


Supplementary Figure 9. Quantification of Ly6G positive cells in the tumor microenvironment.

Left: Mice with untreated tumors or those that received one cycle of TH-302 and α VEGFR-2 treatment were injected with dextran prior to sacrifice. Tumors were resected, embedded in OCT for sectioning. Slides were stained with antibodies against Ly6G, pH2AX, and CD31. Right: Quantification of Ly6G counts per high power field. Unpaired two-tailed Student's *t* test. **P* adj < 0.05, ***P* adj < 0.01, ****P* adj < 0.001, *****P* adj < 0.0001; data are mean \pm SEM.



Supplementary Figure 10. Gating scheme for sorting mCherry positive pancreatic tumor cells for RNA extraction. Implanted pancreatic tumors were treated and processed as described in Figure 6 and Methods section before fluorescence-activated cell sorting on a BD FACSaria



Supplementary Figure 11. Pearson's rho correlation between a TAN gene signature and *CCL23* or *CCR1* transcript in The Cancer Genome Atlas pancreatic adenocarcinoma (PAAD) dataset ($n = 183$).

Supplementary Table 1. List of antibodies and reagents used for flow cytometry and immunofluorescence

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Flow cytometry		
CD45.2 – clone 104	BD Biosciences	564616
CD4 – clone GK1.5	BD Biosciences	612952
PD-1 – clone RMP1-30	BD Biosciences	748267
LAG-3 – clone C9B7W	BioLegend	125227
CD11c – clone HL3	BD Biosciences	563735
Ly6G – clone 1A8	BD Biosciences	562737
Ki67 – clone B56	BD Biosciences	563462
CD11b – clone M1/70	BD Biosciences	563015
IA/IE – clone M5/114	BD Biosciences	563415
F4/80 – clone BM8	Invitrogen	MF48020
CD8a – clone SK1	Invitrogen	46-0087-42
FoxP3 – clone FJK-16s	Invitrogen	58-5773-82
Tox – clone REA473	Miltenyi Biotec	130-120-716
TCRbeta – clone H57-597	Invitrogen	15-5961-82
Arginase – clone A1exF5	Invitrogen	25-3697-82
Active Caspase-3 – clone C92-605	BD Biosciences	564094
Ly6C – clone AL-21	BD Biosciences	561237
Near-IR Live/Dead	Invitrogen	L34975
CD31 – clone 390	BioLegend	102435
PD-L1 – clone MIH5	BD Biosciences	563369
CCR1 – clone S15040E	BioLegend	152507
CXCR4 – clone 2B11	BD Biosciences	551966
CXCR2 – clone V48-2310	BD Biosciences	747812
CSF3R – clone N/A	R&D Systems	FAB6039G
IFN γ - clone XMG1.2	Invitrogen	25-7311-41
Sirp α - clone P84	Invitrogen	25-1721-82
TNF α - clone MP6-XT22	BioLegend	506321
Immunofluorescence		
Anti-pimonidazole – clone 4.3.11.3	Hypoxyprobe	HP6-1000Kit
DAPI – clone N/A	Invitrogen	D1306
CD31 – clone 390	BD Biosciences	563608
Ki67 – clone D3B5	Cell Signaling Technology	9129
NG2 – clone N/A	Millipore	AB5320
pH2AX (Ser139) – clone 20E3	Cell Signaling Technology	9718
Ly6G – clone 1A8	BD Biosciences	562737
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	Thermo Fisher Scientific	A-21206

Goat anti-Rat IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 405	Thermo Fisher Scientific	A48261
Cleaved Caspase-3 (Asp175) Antibody #9661	Cell Signaling Technology	9661S

sSupplementary Table 2. List of critical resources and reagents

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fetal Bovine Serum	Gibco	26140079
DMEM	Gibco	11995065
Penicillin-Streptomycin	Thermo Fisher Scientific	15140122
Polybrene	Sigma-Aldrich	#TR-1003-G
Corning® Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEV-free	Corning	356231
Insulin solution from bovine pancreas	Sigma-Aldrich	I0516-5ML
Standard Insulin Syringe with Needle EasyTouch™ 0.5 mL 1/2 Inch 29 Gauge NonSafety Thin Wall	MHC Medical	829555
Phosphate Buffered Saline, pH 7.2	Gibco	20012050
InVivoMAb anti-mouse VEGFR-2 Antibody	Bio X Cell	BE0060
Anti-Mouse CTLA-4 (Clone 9H10) – Purified in vivo PLATINUM™ Functional Grade	Leinco Technologies	C2841
Anti-Mouse CD279 (PD-1) (Clone RMP1-14) – Purified in vivo PLATINUM™ Functional Grade	Leinco Technologies	P372
InVivoMAb anti-mouse Ly6G	Bio X Cell	BE0075-1
InVivoMAb anti-rat Kappa Immunoglobulin Light Chain	Bio X Cell	BE0122
InVivoMAb anti-mouse CD40	Bio X Cell	BE0016-2
Foxp3 / Transcription Factor Fixation/Permeabilization	Invitrogen	00-5521-00
Anti-Mouse CD32/CD16 – Purified in vivo PLATINUM™ Functional Grade	Leinco Technologies	C681
Fisherbrand™ Color-Coded Capillary Tubes	Fisher Scientific	22-260950
BD Vacutainer™ Plastic Blood Collection Tubes with Sodium Heparin: Hemogard™	Fisher Scientific	23-021-017
Red Blood Cell Lysing Buffer Hybri-Max	Sigma-Aldrich	R7757-100ML
Tris Buffered Saline with Tween® 20	Cell Signaling Technology	9997
Bovine Serum Albumin	Sigma-Aldrich	A2934-100G
Hypoxyprome Plus Kit	Hypoxyprome	HP2-200Kit

Fluorescein isothiocyanate–dextran average mol wt 2,000,000	Sigma-Aldrich	FD2000S
Histopaque-1119	Sigma-Aldrich	11191- 6X100ML
CD8 T cell isolation Kit	Miltenyi-Biotec	130-104-075
eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (500X)	Thermo Fisher Scientific	00-4975-93
RNeasy Mini Kit	Qiagen	74104
SuperScript™ IV First-Strand Synthesis System	Thermo Fisher Scientific	18091200
CD11b MicroBeads, human and mouse - lyophilized	Miltenyi-Biotec	130-097-142
CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry	Thermo Fisher Scientific	C34554
Proteome Profiler Mouse XL Cytokine Array	R&D Systems	ARY028
Proteome Profiler Mouse Chemokine Array Kit	R&D Systems	ARY020
Stainless Steel Beads, 5 mm	Qiagen	69989
Tissue Lyser II	Qiagen	85300
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific	23225
12-well Corning® Costar® Transwell®, diam. 6.5 mm, pore size 8.0 µm	Corning	3422
CountBright™ Absolute Counting Beads	Thermo Fisher Scientific	C36950
BX471	MedChemExpress	HY-12080
Mouse CCL9/10/MIP-1 gamma DuoSet ELISA	Biotechne R&D Systems	DY463