TITLE: Blockade of p38 kinase impedes the mobilization of pro-tumorigenic myeloid populations to impact breast cancer metastasis.

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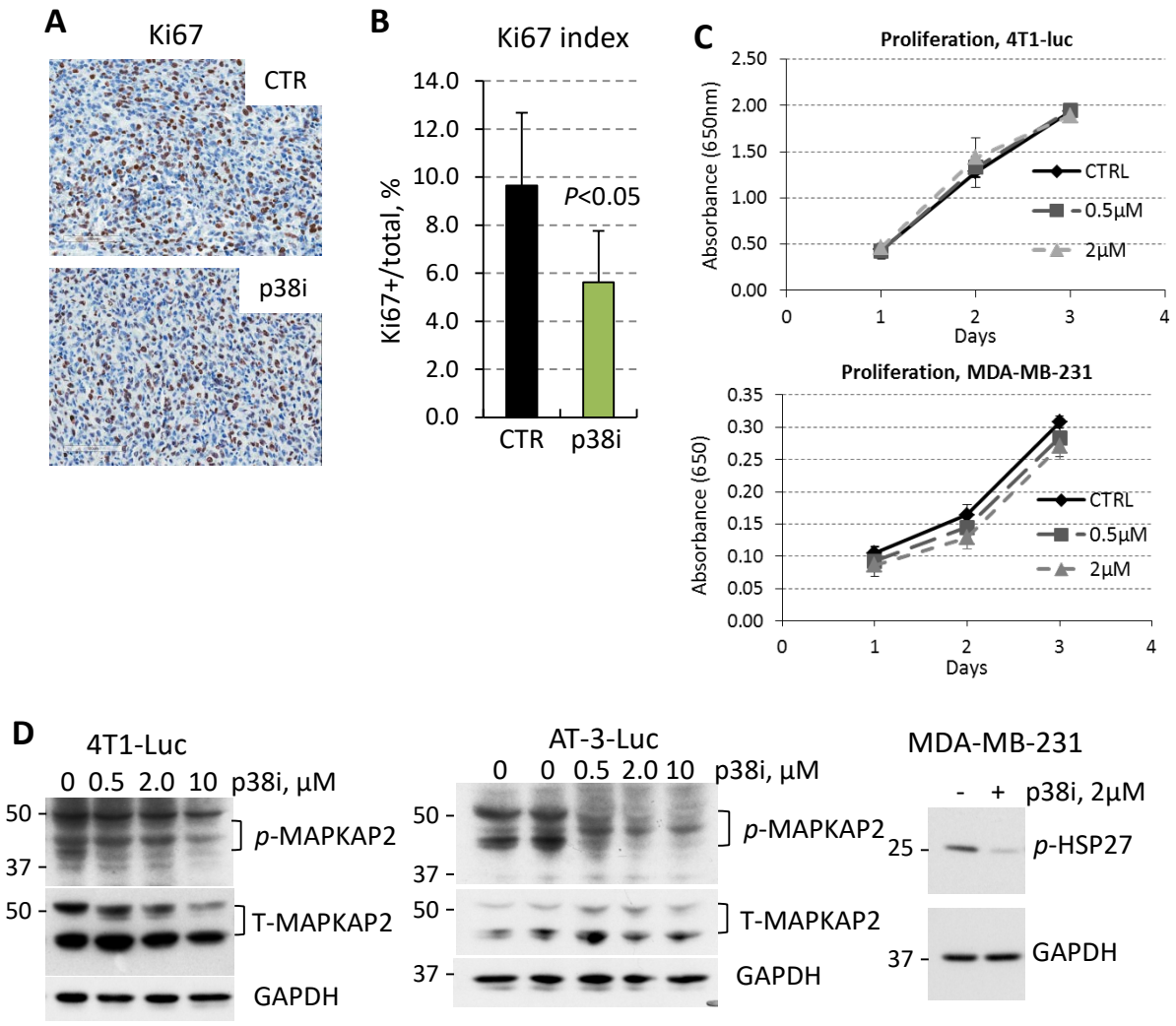
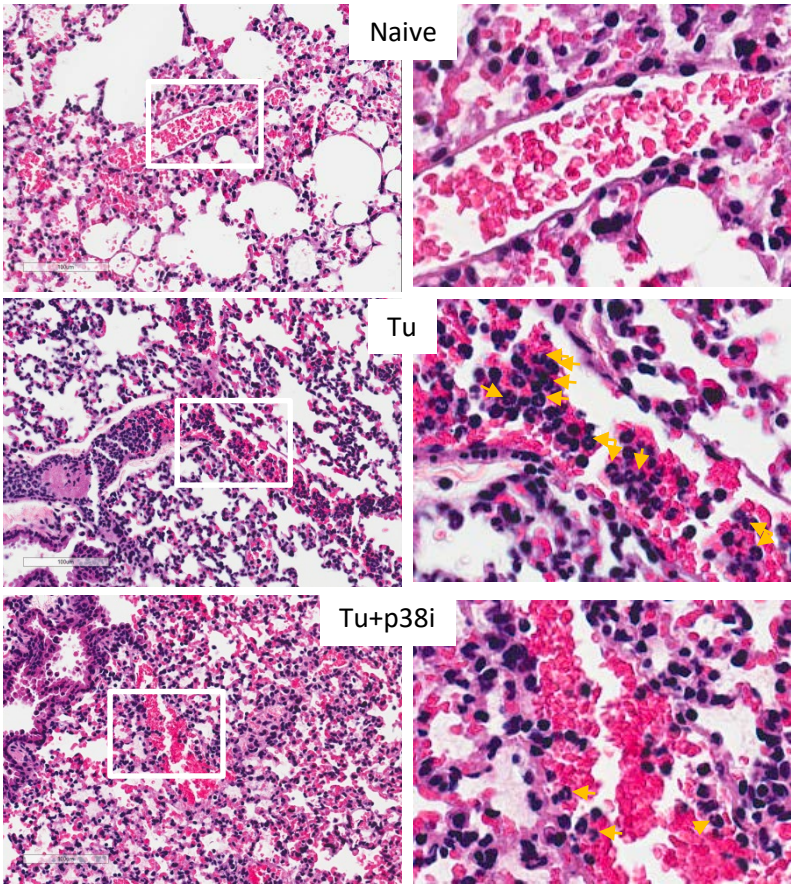
Reagent or Resource	Source	Identifier
Antibodies		
anti-GAPDH	Santa Cruz Biotechnology	Cat# sc-25778; RRID: AB_10167668
anti-p38	Santa Cruz Biotechnology	Cat# sc-81621; RRID: AB_1127392
anti-IκBα	Santa Cruz Biotechnology	Cat# sc-371; RRID: AB_2235952
anti-phospho-HSP27 (Ser82)	Cell Signaling Technology	Cat# 2401; RRID: AB_331644
anti-phospho-p38	Cell Signaling Technology	Cat# 9211; RRID: AB_331641
anti-phospho-MAPKAPK2 (Thr222; 9A7)	Cell Signaling Technology	Cat# 3316, RRID:AB_2141311
anti-phospho-MAPKAPK2 (Thr334; 27B7)	Cell Signaling Technology	Cat# 3007, RRID:AB_490936
anti-MAPKAPK-2 (D1E11) Rabbit mAb antibody	Cell Signaling Technology	Cat# 12155, RRID:AB_2797831
anti-alpha-Tubulin mouse mAb, Clone B-5-1-2	Sigma-Aldrich	Cat# T6074, RRID:AB_477582
anti-FLAG, monoclonal M2 antibody	Sigma-Aldrich	Cat# F3165, RRID:AB_259529
Goat Anti-Rabbit IgG (H L)-HRP Conjugate antibody	Bio-Rad	Cat# 170-6515, RRID:AB_11125142
Goat Anti-Mouse IgG (H L)-HRP Conjugate antibody	Bio-Rad	Cat# 170-6516, RRID:AB_11125547
anti-Ly6G antibody, InVivoMab anti-mouse clone1A8	Bio X Cell, West Lebanon	Cat# BE0075-1, RRID:AB_1107721
InVivoMab rat IgG2a isotype control antibody	Bio X Cell, West Lebanon	Cat# BE0089, RRID:AB_1107769
Rat anti-mouse CD31 (PECAM) antibody	BD Biosciences	Cat#550274, RRID:AB_393571
Biotinylated secondary anti-rat antibody (goat)	BD Biosciences	Cat#559286, RRID:AB_397214
anti-CD 163, macrophage-associated antigen antibody	Novocastra	Cat#NCL-CD163, RRID:AB_563510
Rat anti-mouse F4/80 mAb (clone CI:A3-1)	Bio-Rad	Cat#MCA497RT, RRID:AB_1102558
Rabbit monoclonal anti-Ki67 (clone SP6)	ThermoFisher	Cat#RM-9106-S1, RRID:AB_149792
anti-CD45 antibody	BD Biosciences	Cat# 553080, RRID:AB_394610
anti-CD11b antibody	BD Biosciences	Cat# 563553, RRID:AB_2738276
anti-CD8 antibody	BD Biosciences	Cat# 553033, RRID:AB_394571
anti-Ly6C antibody	Biolegend	Cat# 128032, RRID:AB_2562178
anti-Ly6G antibody	Biolegend	Cat# 127607, RRID:AB_1186104
anti-CD4 antibody	Biolegend	Cat# 100552, RRID:AB_2563053

Chemicals, Peptides and Cytokines		
D-luciferin	Gold Biotechnology, St Louis	LUCK-1G
Ralimetinib (LY2228820)	Selleck Chemicals	Cat# S1494
Recombinant human TNF alpha	ProSpec-Tany TechnoGene Ltd.	CYT-223
TRIzol Reagent	Invitrogen	Cat# 15596-026
ELISA assays for mouse Ccl2/MCP1	BioLegend	Cat#432704, lot#B257711
Collagenase/Hyaluronidase (10X in DMEM)	StemCell Technologies	Cat#07912
G-CSF	Peprotech	Cat#250-05
GM-CSF	Peprotech	Cat#315-03
CellTrace Violet Dye	Invitrogen	Cat#C34557

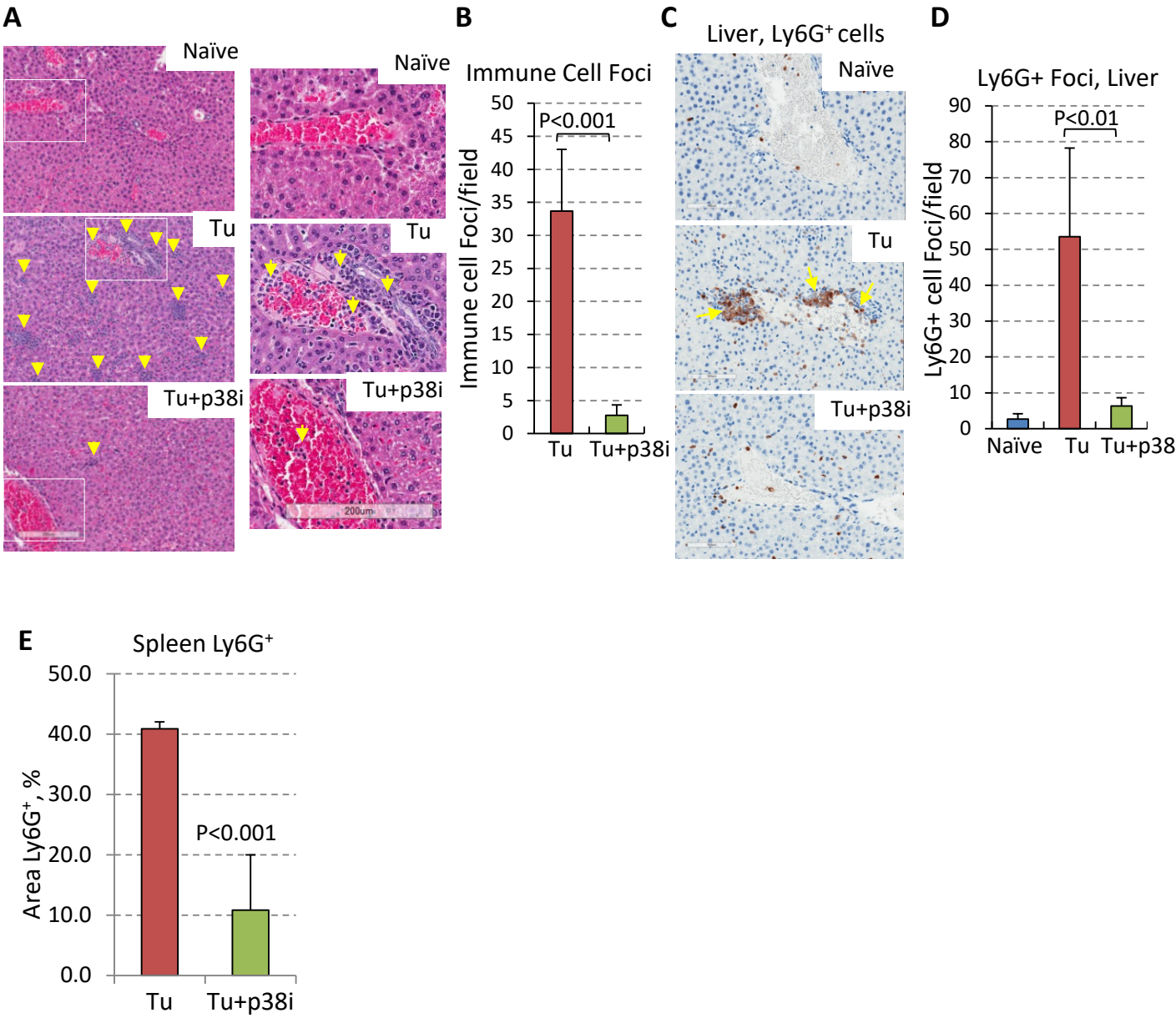
Special Instruments and Reagents		
Luminex 200 system	Luminex	
Human Cytokine/Chemokine Panel	EMD Millipore	Milliplex Magnetic Bead 41-Plex: EGF, Eotaxin, FGF-2, Flt-3L, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNg, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IL-1α, IL-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MCP-3, MDC, MIP-1α, MIP-1β, PDGF, PDGF-AA, RANTES, sCD40L, TGFα, TNFα, TNFβ, and VEGF
Human Primers	*Sequences*	
CCL2	Forward: GCCTCCAGCATGAAAGTCTC	
	Reverse: AGGTGACTGGGGCATTGAT	
5S rRNA	Forward: GGCCATACCACCCTGAACGC	
	Reverse: AGCCTACAGCACCCGGTATT	
Mouse Primers	*Sequences*	
S100a8	Forward: CCGTCTTCAAGACATCGTTTGA	
	Reverse: GTAGAGGGCATGGTGATTTCCT	
Mmp8	Forward: TGCCACGATGGTTGCAGAG	
	Reverse: AGGCATTTCCATAATCCCCATTG	
Cxcr2	Forward: ATGCCCTCTATTCTGCCAGAT	
	Reverse: GTGCTCCGGTTGTATAAGATGAC	
Csf2rb	Forward: GTGGAGCGAAGAGTACACTTG	
	Reverse: CCAAAGCGAAGGATCAGGAG	
5S rRNA	Forward: GGCCATACCACCCTGAACGC	
	Reverse: AGCCTACAGCACCCGGTATT	



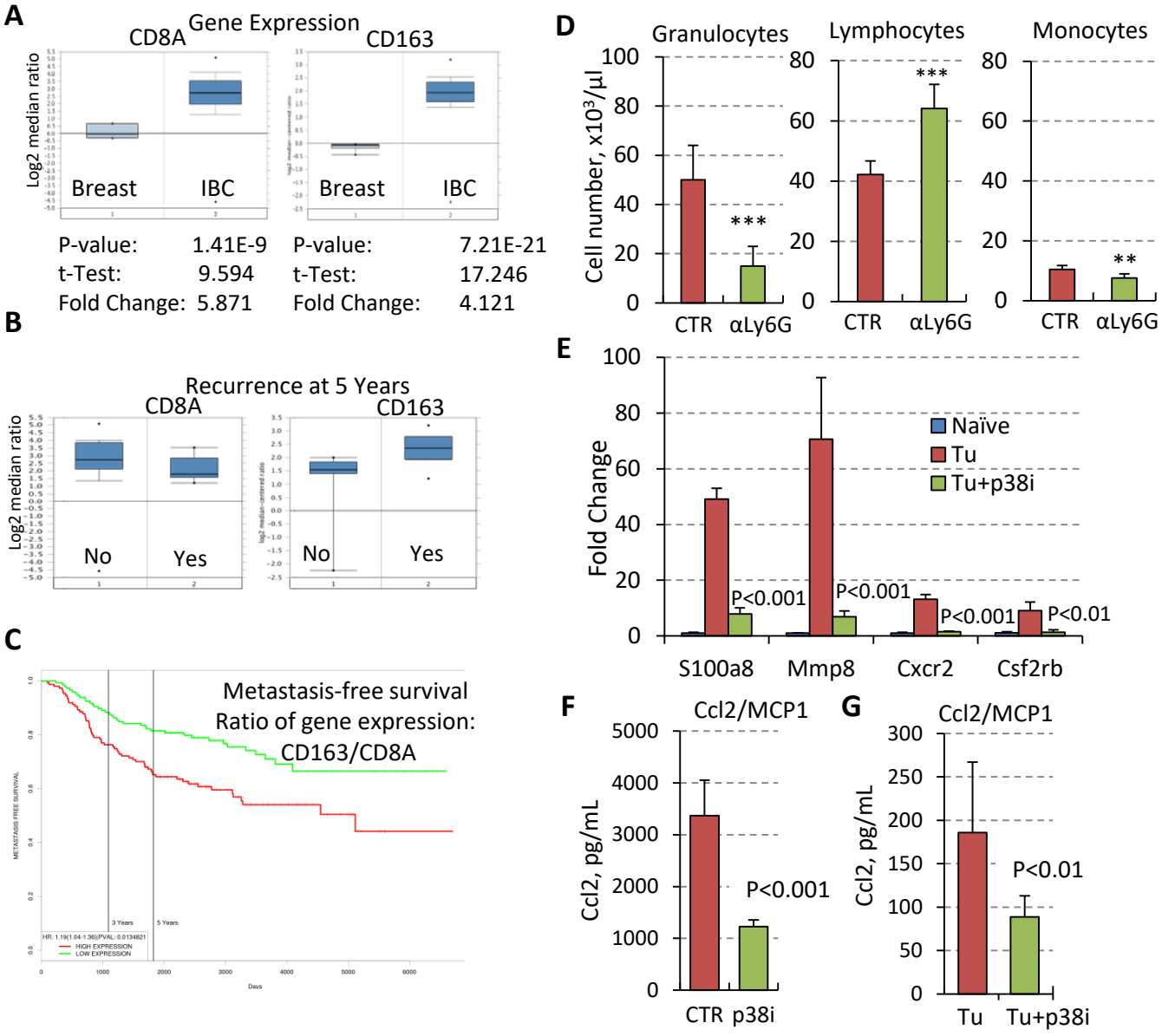
Supplementary Figure 1. (**A**) Images of Ki67-stained tumor sections from control and Ralimetinib-treated tumor-bearing animals. (**B**) Quantification of the proliferation index using Ki67-stained tumor tissues, 3 sections per tumor, 3 tumors per group. (**C**) Proliferation of cancer cell lines in the absence or presence of p38i (Ralimetinib). (**D**) Immunoblotting of whole-cell extracts from tumor cells treated with various doses of p38i Ralimetinib for 6 hours.



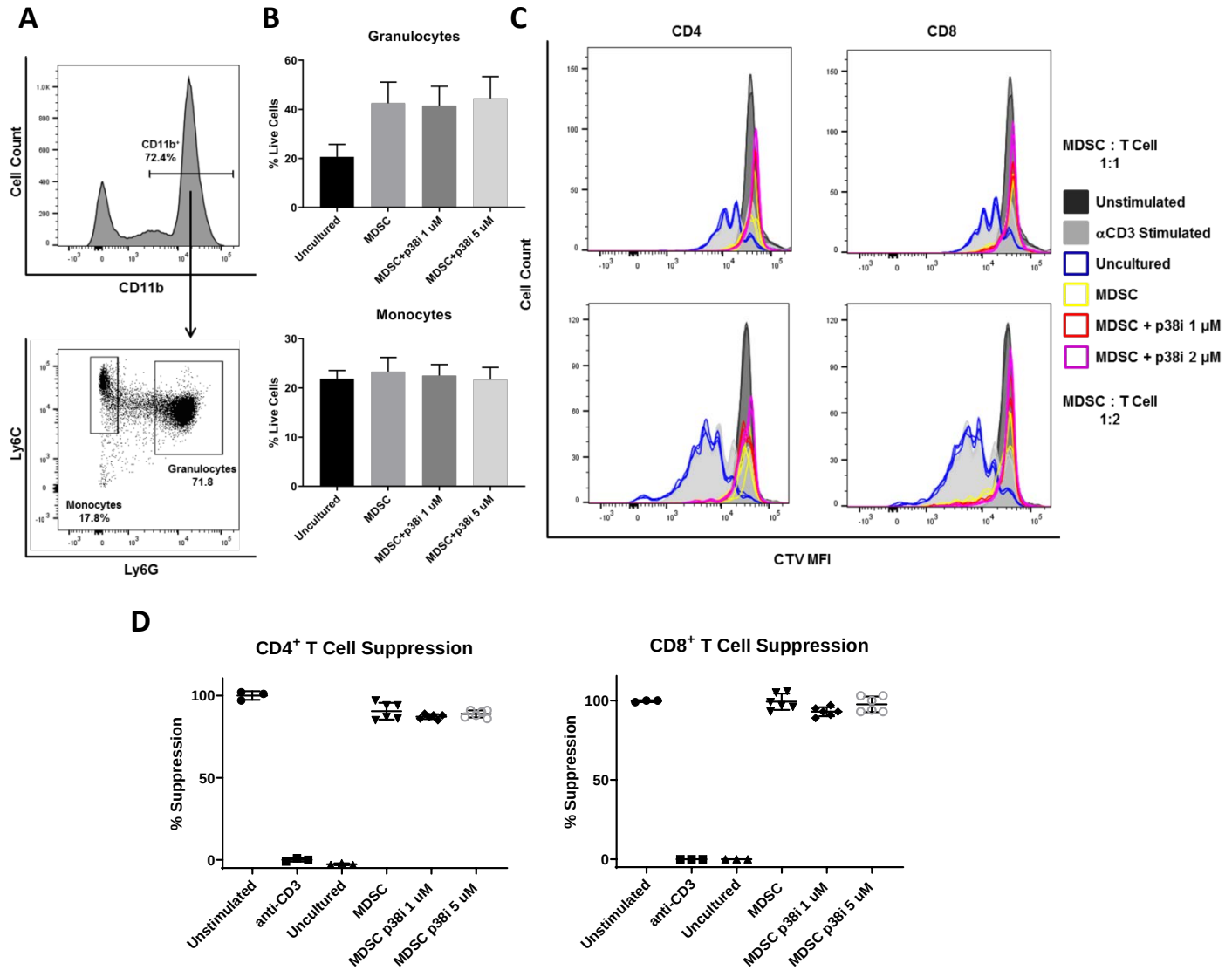
Supplementary Figure 2. **Blockade of p38 reduces the accumulation of MDSCs in the lungs of tumor-bearing mice**. Images of H&E-stained lung tissues from tumor-free (naïve), 4T1-Luc tumor-bearing (Tu) and tumor-bearing mice treated with p38i at study endpoint, bar=100μm. Panels on the right show enlarged images of the outlined areas with myeloid cells within blood vessels.



Supplementary Figure 3. **Blockade of p38 reduces accumulation of PMN-MDSCs in the liver and spleen of tumor-bearing mice**. (**A**) Images of H&E-stained liver sections from tumor-free (naïve), 4T1-Luc tumor-bearing (Tu) and tumor-bearing mice treated with p38i at study endpoint (14 days). Enlarged images of the outlined areas show the presence of myeloid cells within and in the proximity of blood vessels. Yellow arrows show clusters of myeloid cells, bar=200µm. (**B**) Quantification of myeloid cell foci in the liver tissues reflecting: the number of foci per field, 3 fields per section, 3 mice per group. (**C**) Detection of Ly6G$^+$ cell foci in liver tissues. Note, these foci are localized in the proximity of blood vessels, bar=100µm. (**D**) Quantification of Ly6G$^+$ cell foci in the liver sections reflecting 3 fields per section, 3 mice per group. (**E**) Quantification of Ly6G$^+$ cells in the spleen sections (IHC, Ly6G), 3 fields per section, 3 mice per group. Comparisons were done with material from naïve and comparable-size tumor groups and statistical significance was determined using the two-tailed unpaired t-test.



Supplementary Figure 4. **The role of TAMs and PMN-MDSCs**. (**A**) Expression of CD8A and CD163 in stromal compartments of human breast cancers. The data are generated with *Oncomine* web-tool using the dataset of the Finak Breast Cancer Stroma Study [Finak et al., 2008, Nature Medicine **14**(5): 518-527]. (**B**) Breast cancer recurrence at 5 years using *Oncomine* and the Finak Breast Cancer Stroma Study. (**C**) Kaplan-Meier estimation of the metastasis-free survival of breast cancer patients and CD163/CD8A expression ratio using a dataset [Drukker et al.. 2014, Breast Cancer Research and Treatment **143**(3): 587-592] and a prognostic *PROGgeneV2* tool. (**D**) Complete Blood Counts (CBCs) were performed on peripheral blood at endpoint of the anti-Ly6G study. Blood was collected by cardiac puncture and assayed using the HemaTrue Analyzer and HeskaView Integrated Software version 2.5.2. (**E**) Quantitative RT-PCR of S100a8, Mmp8, Cxcr2, and Csf2rb mRNA expression in the lungs of naïve (tumor-free control), tumor-bearing (Tu), or Tu mice treated with Ralimetinib. The assays were done in triplicates using two lungs per group from the study described in Figure 2. (**F**) Evaluation of Ccl2/MCP1 by ELISA assays in conditioned media from 4T1 cells treated with vehicle-control (DMSO) or 2 µM of p38i Ralimetinib for 48 hours. (**G**) ELISA assays for Ccl2/MCP1 in 4T1 tumor extracts from mice treated by p38i at day 14. Comparisons were done with material from comparable-size tumor groups and statistical significance was determined using the two-tailed unpaired t-test (**P<0.01; ***P<0.001).



Supplement Figure 5. Effect of p38 blockade on the immune suppressive activity of MDSCs generated *in vitro*. (**A**) Evaluation of the indicated cell population in mouse bone marrow from naïve BALB/c mice. (**B**) Frequency of monocytic $CD11b^{+}Ly6C^{hi}Ly6G^{-}$ and granulocytic $CD11b^{+}Ly6C^{lo}Ly6G^{+}$ populations before and after incubation of bone marrow cultures for 4 days with recombinant mouse G-CSF and GM-CSF with or without 1-5 μM Ralimetinib (n=3). (**C**) Mean fluorescence intensity (MFI) of Cell Trace Violet (CTV)-labeled $CD4^{+}$ and $CD8^{+}$ T cell populations incubated alone or in culture with MDSCs. (**D**) Relative suppression of $CD4^{+}$ and $CD8^{+}$ T cells by MDSCs.