Supplemental Material

Polymorphonuclear myeloid derived suppressor cells and phosphatidylinositol-3 kinase gamma are critical to tobacco-mimicking oral carcinogenesis in mice

Supplemental Data Table of Contents

Supplemental Table S1: Taqman gene expression assays (from ThermoFisher)

Gene of interest	Assay ID
Gapdh	Mm99999915_g1
Cxcl1	Mm04207460_m1
Cxcl2	Mm00436450_m1
Cxcl3	Mm01701838_m1
Cxcl5	Mm00436451_g1
Csf2	Mm01290062_m1
Csf3	Mm00438334_m1

Supplemental Table S2: Antibodies used for flow cytometry (FC), multispectral imaging (MSI); Immunohistochemistry (IHC)

Antibody Target	Vendor (Catalog/clone)	Dilution	Use
E-Cadherin	BD Bio (610181)	1:200	MSI
F4/80	BioRad (A3-1)	1:500	MSI
B220	BD Bio (550286)	1:100	MSI
GR1	BD Bio (550291)	1:50	MSI
CD3ε	BD Bio (550275)	1:100	MSI
Ly6g	Cell Signaling (87048)	1:200	IHC
CD45	Biolegend (103133)	0.25 ug/test	FC
CD11b	eBioscience (17-0112-83)	1.25 ug/test	FC
Ly6G	Biolegend (127623)	1.3 ug/test	FC
Ly6C	Biolegend (128005)	0.5 ug/test	FC
CD16/32 (FC block)	Biolegend (101302)	2 ul/million cells	FC

Abbreviations: R&D: R&D Systems; BD Bio: BD Biosciences

Supplemental Table S3: Antibodies used for mass cytometry. All antibodies were purchased via University of Colorado Cancer Center Flow Cytometry Shared Resource Antibody Bank

Antibody Target (clone) Conjugate	Vendor (Catalog #)	Panel
CD45 (30-F11) 89Y	F (3089005B)	1 & 2
Ly-6G (1A8) 141Pr	F (3141008B)	1 & 2
CD11c (N418) 142Nd	F (3142003B)	1 & 2
TCRβ (H57-597) 143Nd	F (3143010B)	1 & 2
CD69 H1.2F3 145Nd	F (3145005B)	1 only
CD4 (RM4-5) 145Nd	F (3145002B)	2 only
F4/80 (BM8) 146Nd	F (3146008B)	1 & 2
CD11b (M1/70) 148Nd	F (3148003B)	1 & 2
CD19 (6D5) 149Sm	F (3149002B)	1 & 2
Ly-6C (HK1.4) 150Nd	F (3150010B)	1 & 2
CD25 (3C7) 151Eu	F (3151007B)	1 & 2
CD3ε (145-2C11) 152Sm	F (3152004B)	1 & 2
CD274/PD-L1 (10f.9G2) 153Eu	F (3153016B)	1 & 2
CD324/E-Cadherin (DECMA-1) 158Gd	F (3158018B)	1 & 2
CD279 (RMP1-30) 159Tb	F (3159006B)	1 & 2
CD62L/L-selectin (MEL-14) 160Gd	F (3160008B)	1 & 2
iNos (CXNFT) 161Dy mouse	F (3161011B)	1 & 2
CD366 (Tim-3)(RMT3-23) 162Dy	F (3162029B)	1 only
TNFα (MP6-XT22) 162Dy	F (3162002B)	2 only
CD326/EpCAM (G8.8) 165Ho	F (3165014B)	1 only
IFNγ (XMG1.2) 165Ho	F (3165003B)	2 only
Arginase 1 (polyclonal) 166Er	R&D (AF5868)	1 & 2
CD8α (53-6.7) 168Er	F (3168003B)	1 & 2
CD161/NK1.1 (PK136) 170Er	F (3170002B)	1 & 2
CD44 (IM7) 171Yb	F (3171003B)	1 only
Granzyme B (GB11) 171Yb	F (3171002B)	2 only
CD4 (RM4-5) 172Yb	F (3172003B)	1 only
Perforin (OMAK-D) 172Yb	F (3172018B)	2 only
MHC Class II (M5/114.15.2) 174Yb	F (3174003B)	1 & 2
CD45R/B220 (RA3-6B2) 176Yb	F (3176002B)	1 & 2

Abbreviations: F: Fluidigm; R&D: R&D Systems

Supplemental Materials and Methods

Cell line generation, transplantation and daughter cell line generation

Tongues of mice 26 weeks post-4NQO initiation were split in half longitudinally, with half fixed in 10% neutral buffered formalin for paraffin embedding and histology analyses and the other half placed into DMEM. For generating cell lines, half tongues were individually minced with a sterile scalpel then transferred to a gentleMACS C Tube (Miltenyi Biotec) in 2.5 mL filter sterilized Tumor Dissociation Kit enzyme mix (Miltenyi Biotec) and processed on a gentleMACS Dissociator following manufacturer's protocol (Miltenyi Biotec). After 40 min incubation at 37^{o} with gentle rotation, the components were centrifuged 5 min at 300 xg. The pelleted cells and tissue were washed twice with 10 mL complete media (DMEM/F12 containing 10% FBS and 1x Primocin). Cells/tissue (unstrained) were plated to a 10 cm tissue culture plate in 10 mL complete media for 4-7 days to initiate cultures. To discourage fibroblast growth, cell monolayers were washed in PBS and switched to "Ker media" (Keratinocyte media containing 2 ng/mL EGF and 1x Priomocin). Cells were expanded in Ker media for one to two months, discarding early-lifting fibroblasts after trypsinization, until pure epithelial populations were expanded and fibroblast-appearing cells were depleted after which cells were maintained in Ker media and a subculture expanded in complete media. For transplantation, cell lines established from 4NQO-treated mouse A1088 and A1089 were trypsinized and resuspended in cold 50% Matrigel, 50% PBS at a concentration of 10 million cells/mL and 100 µL (1 million) backloaded to 29-gauge insulin syringes on ice. Female athymic nude mice and female C57BL/6J mice were anesthetized with vaporized isoflurane then transplanted with 100 µL (1 million cells) to the flank. Nude mice, but not C57BL/6J mice, developed tumors that were harvested and used to generate daughter tumor cell lines in the same manner as the parental tongue cell lines. 4NQO tongue cell line from mouse A1088 gave rise to daughter A1206 tumor cells; 4NQO tongue cell line from mouse A1089 gave rise to daughter A1207 tumor cells.

Histology, Immunohistochemistry (IHC) and Multispectral imaging (MSI)

5 µm tissue sections on glass slides were dewaxed and hydrated using xylenes and graded alcohols. IHC was performed on rehydrated tissue sections using citrate antigen retrieval buffer for 5 minutes at 93 °C in a pressure cooker, then incubated in 3% hydrogen peroxide to quench endogenous peroxidase activity. Slides were washed in TBS with 0.1% tween-20 (TBST), blocked in TBST with 10% BSA, then incubated overnight with the primary Ab (Supplementary Table S1). After washing in TBST, slides were incubated in SignalStain Boost Detection Reagent (Cell signaling #8114), washed again, and incubated in SignalStain DAB Substrate Kit according to manufacturer's protocol (Cell signaling #8059) until brown staining could be detected. Slides were counterstained with hematoxylin, dehydrated, and coverslipped with CytoSeal 60 mounting medium (Epredia #831016).

MSI staining and image capture was performed at Human Immune Monitoring Shared Resource within the University of Colorado Human Immunology and Immunotherapy Initiative. For MSI staining, rehydrated mouse tongue samples were stained using the Opal 7-Color Automation IHC Kit-50 Slide according to manufacturer's instructions (Akoya Biosciences) using the antibodies listed in Supplemental Table S1. Sections on glass slides were heat treated in citrate antigen retrieval buffer for 20 min at 93 °C (Leica), blocked in Antibody Diluent (Akoya Biosciences), incubated for 30 min with the primary Ab, 10 min with horseradish peroxidase (HRP)-conjugated secondary polymer (anti-rabbit, Akoya Biosciences), and 10 min with HRP-reactive OPAL fluorescent reagents (Akoya Biosciences). Slides were washed between staining steps with Bond Wash (Leica) and stripped between each round of staining with heat treatment in antigen retrieval buffer. After the final heat treatment in antigen retrieval buffer, the slides were stained with spectral DAPI (Akoya Biosciences), and coverslipped with Prolong Diamond mounting media (Thermo Fisher). Whole slide scans were imaged on the Vectra 3.0 Automated Quantitative Pathology Imaging System (Akoya) using the 20× objective. We selected multiple regions of interest with immune cell staining and dysplasia in each section using Phenochart software (Akoya Biosciences) at 40× magnification, which were then analyzed with inForm software (v2.4.10, Akoya) to unmix adjacent fluorochromes, subtract autofluorescence, segment the tissue into tumor and stroma regions, segment the cells into nuclear, cytoplasmic, and membrane compartments, and phenotype the cells according to morphology and cell marker. Independent projects were created to phenotype each cellular marker, then merged, consolidated, and analyzed in R Studio using the phenoptrReports plug-in (Akoya Biosciences) to quantify the total number of each cell type as a percentage of the total number of cells per image and averaged across images for each mouse. Dysplasia in MSI-stained sections was scored by a treatment blinded pathologist.

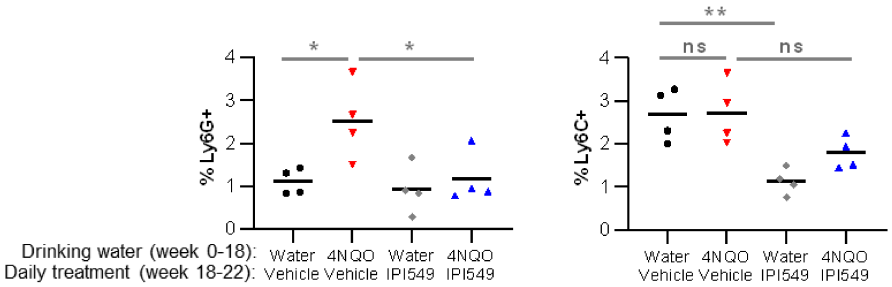
Gene expression analyses

RNA was harvested from tumor cell lines using Qiagen RNeasy Plus kit following manufacturer's instructions. Gene expression was assessed by RT-qPCR using Taqman Assays (ThermoFisher) detailed in Supplementary Table S1, 50 ng RNA and iTaq Universal Probes One-Step Kit (BioRad) following manufacturer's instructions on a 7500 Fast Real-Time PCR System (ThermoFisher). Expression of each gene of interest was normalized to the expression of reference gene *Gapdh* using the $2^{\Delta CT}$ method.

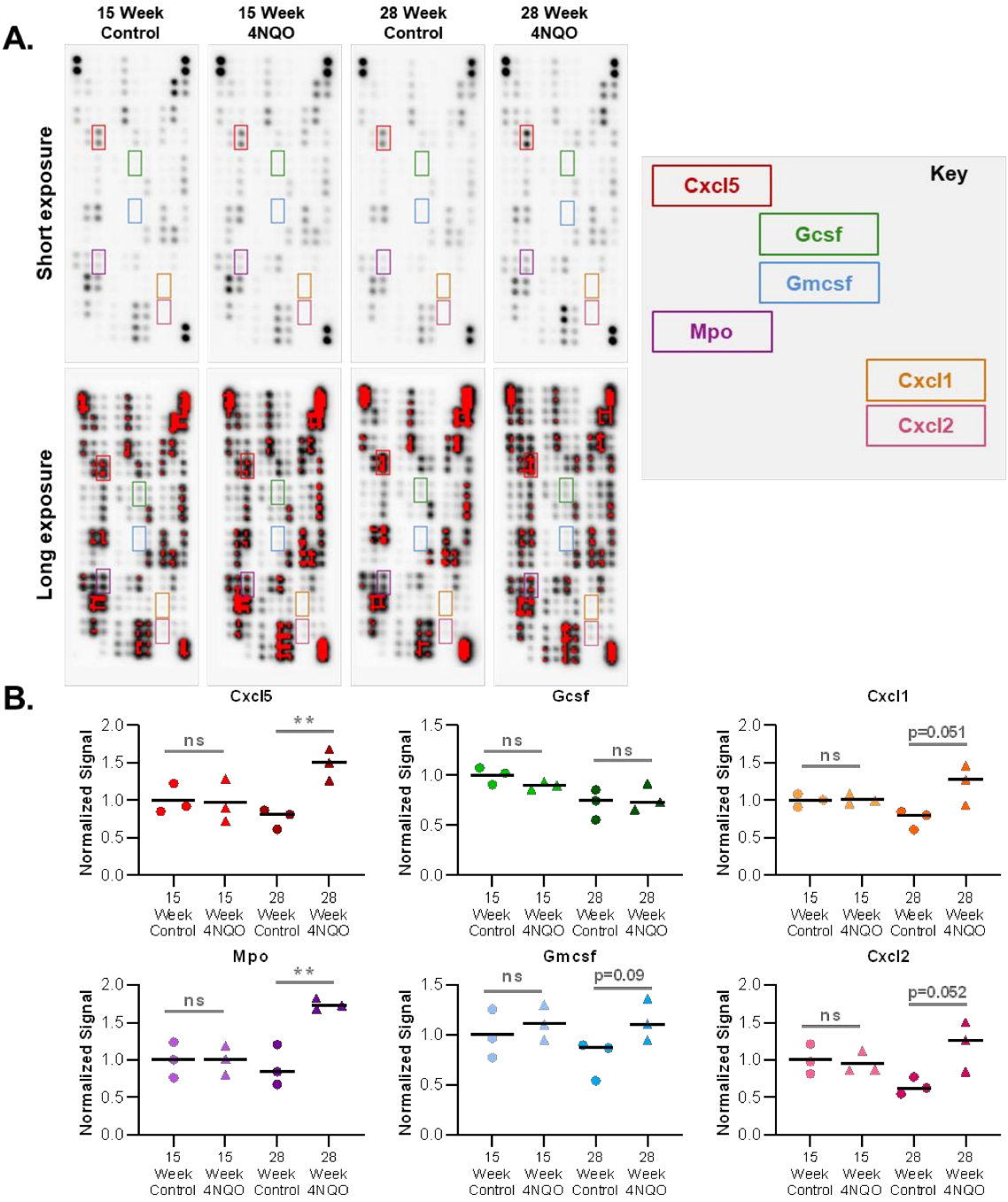
Tumor harvest, single cell dissociation and Mass cytometry

Tumors were harvested from euthanized mice, minced with razor blades and then dissociated using gentleMACS C Tube (Miltenyi Biotec) and Tumor Dissociation Kit enzyme mix (Miltenyi Biotec) as described above for cell line generation. Cell suspensions were strained through a 100 µm strainer, pelleted by centrifugation at 300 xg, then red blood cells (RBCs) were lysed with 1x RBC Lysis Buffer (eBioscience)

following manufacturer's protocol. Neutralized cell suspensions were pelleted by centrifugation at 300 xg then resuspended in PBS with 40 µg/mL DNase and incubated 15 min at 37 degrees, then strained through a 40 µm strainer and diluted with Maxpar staining buffer to obtain cell counts. 10 million cells were incubated in FC block (Biolegend) for 10 minutes, then stained with Fluidigm antibodies (Supplementary Table S2, panel 1) according to manufacturer protocol for surface epitopes, fixed/permeabilized and stained for intracellular epitopes (Fluidigm). For detection of intracellular cytokines and other antigens, single cell suspensions were prepared the same, then incubated with BD GolgiPlug™ Protein Transport Inhibitor containing Brefeldin A at 1:1000 concentration for 4 hours at 37^{o}, with gentle shaking every 30 minutes, then FC blocked and stained with antibodies in Supplementary Table S2, panel 2. Cells were analyzed on a Helios mass cytometer (Fluidigm) at the University of Colorado Flow Cytometry Shared Resource, and normalized FCS files were uploaded to Cytobank (Cytobank, Inc.) for statistical analysis. Positive staining for cisplatin was used to exclude dead cells, and nucleated cells were confirmed with IR-Dye (Fluidigm). Live, nucleated CD45+ cells were clustered and gated based on marker expression.

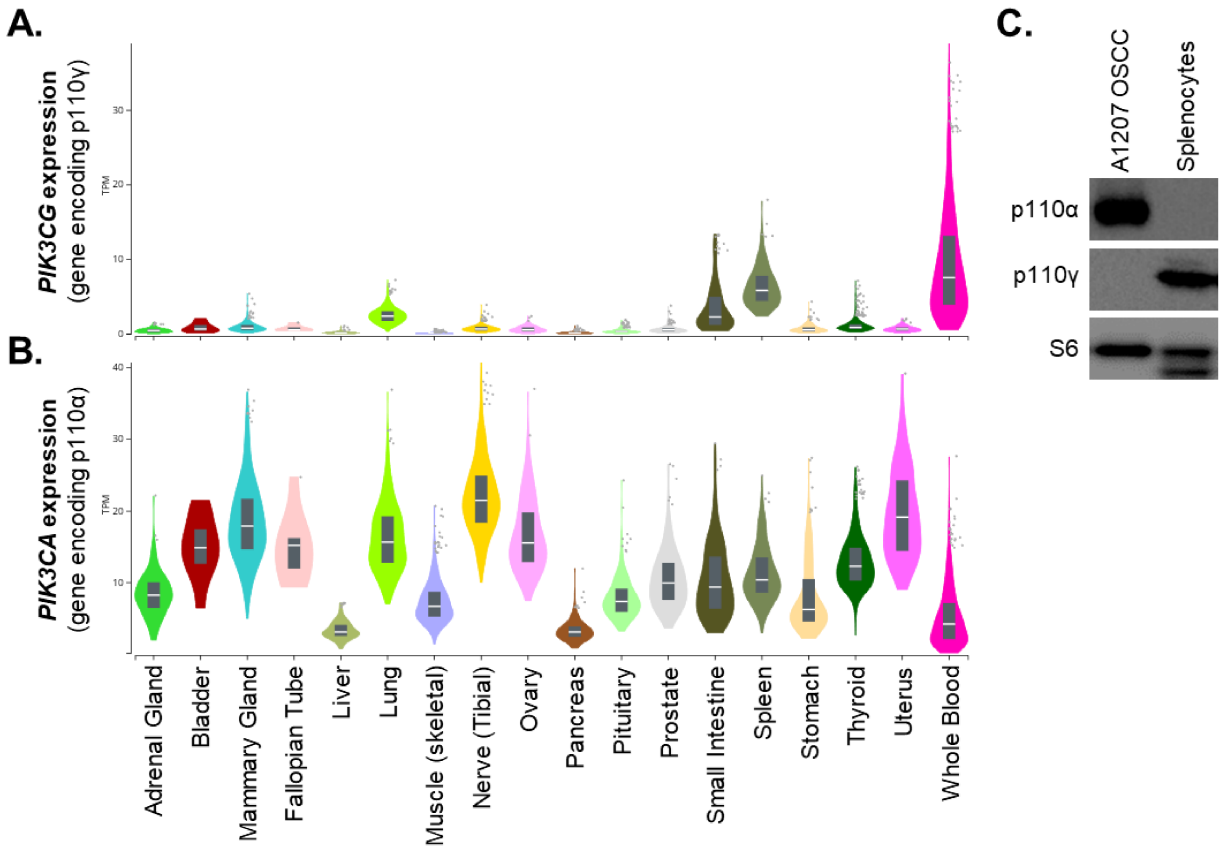


Supplementary Figure S1: Ly6G+ granulocyte increase in the tongues of mice undergoing 4NQO carcinogenesis is reversed by IPI549 treatment . Mice were provided regular drinking water or water containing 50 µg/mL 4NQO for 18 weeks. At 18 weeks, mice were treated daily with vehicle or IPI549 for another 4 weeks and tongues harvested on week 22. Tongues were dissociated, stained and analyzed by mass cytometry to determine the relative abundance of Ly6G+ granulocytes and Ly6C+ monocytes (n=4 tongues/group; *p<0.05; **p<0.01 determined by unpaired Student's T test)

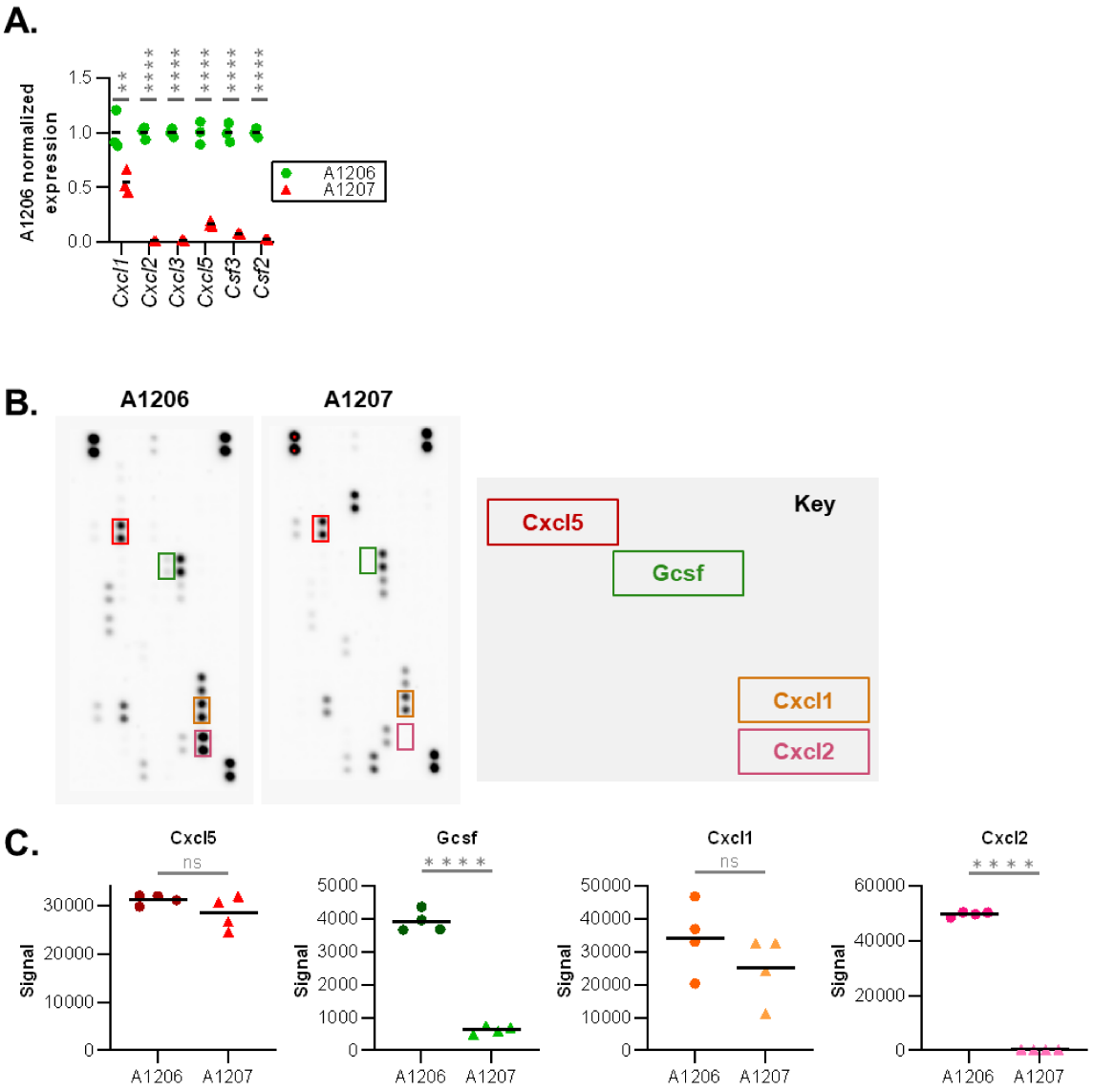


Supplementary Figure S2: Mice undergoing tongue carcinogenesis have increased granulocyte-recruiting chemokines in their plasma. Mice were provided regular drinking water (control) or water containing 50 µg/mL 4NQO for 18 weeks then all received regular drinking water thereafter. The plasma of mice sacrificed 15- or 28 weeks-post-initiation of water or 4NQO treatment were harvested and analyzed by cytokine array to determine the relative expression of a panel of chemokines, growth factors and cytokines. A) Representative cytokine array images of both short and long exposure times. Red are saturated pixels. B) Quantification of the levels chemokines related to granulocyte recruitment

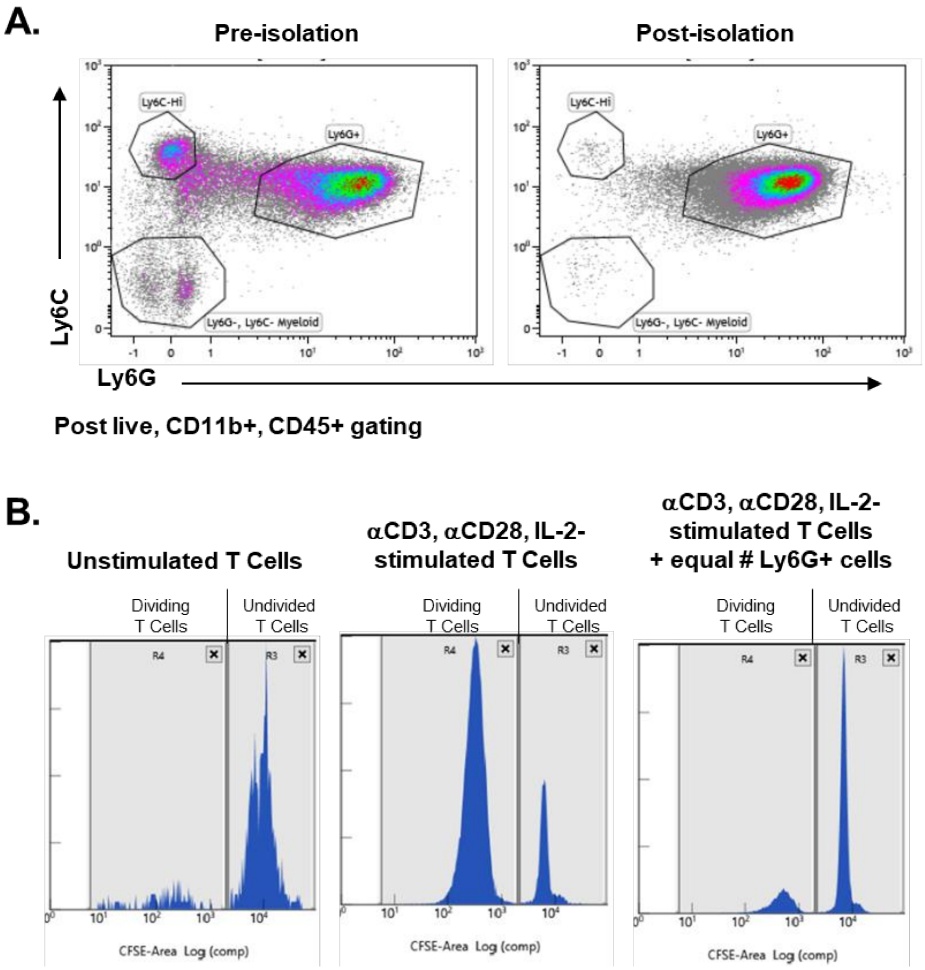
and growth factors related to granulopoiesis are presented (3 mice/group; **p<0.01 determined by unpaired Student's T test).



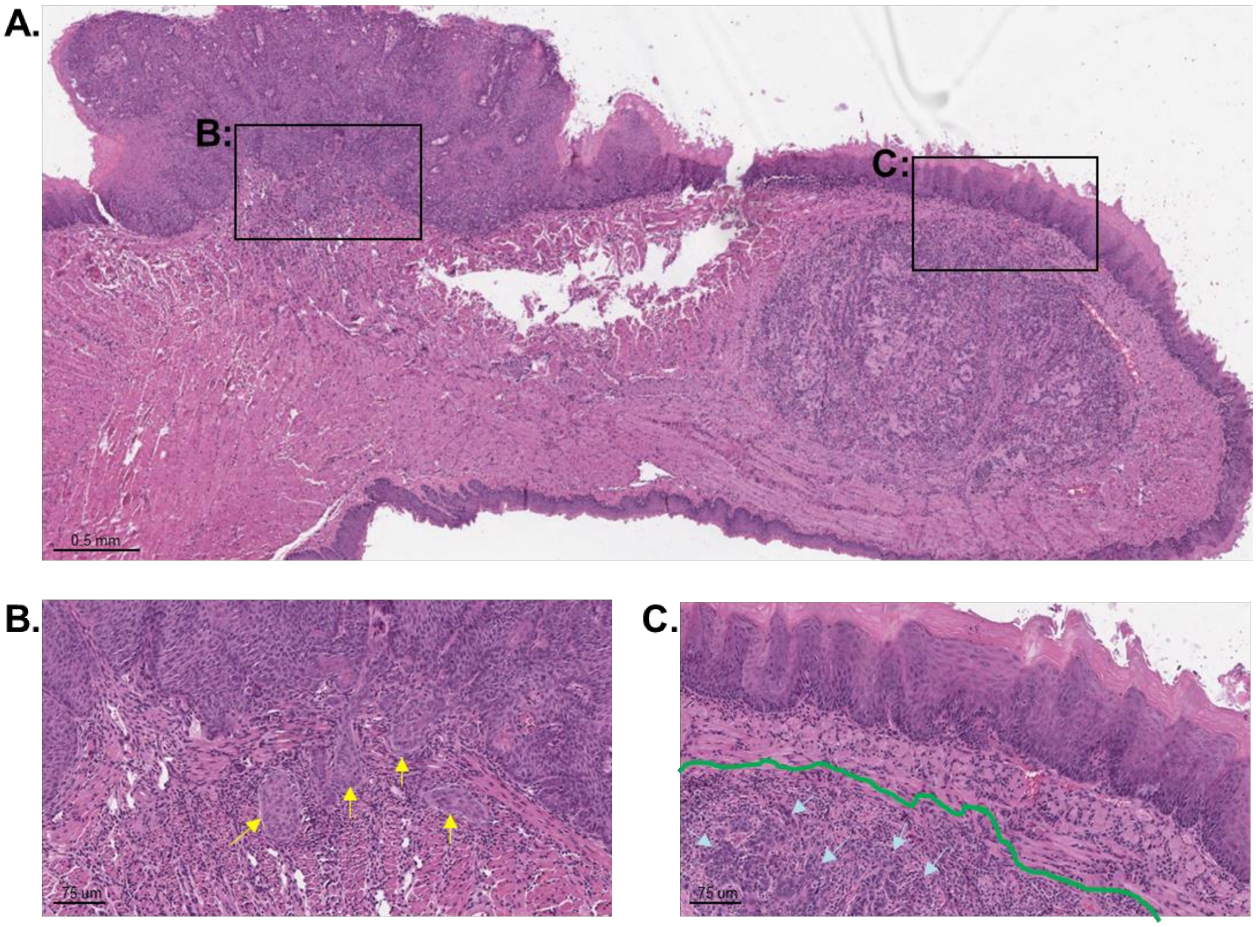
Supplementary Figure S3: Leukocytes, but not epithelial tissues or SCC, express p110γ. A) The expression of *PIK3CG*, the gene encoding p110γ, and B) the expression of *PIK3CA*, the gene encoding p110α, was examined using GTExPortal (evaluating bulk tissue expression, GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2)). C) The expression of p110γ, p110α, and S6 (loading control) was evaluated in the lysates of A1207 SCC cells and mouse splenocytes using immunoblot analysis.



Supplementary Figure S4: A1206 cells have elevated levels of granulopoiesis-stimulating growth factors and granulocyte-recruiting chemokines compared to A1207. A) RNA was harvested from A1206 and A1207 cells and used to evaluate the expression of the indicated genes (normalized to the expression of *GAPDH*) using RT-qPCR then A1207 expression was normalized to the expression observed in A1206 cells. B-C) The conditioned media of A1206 and A1207 cells was evaluated for the levels of chemokines and cytokines using antibody based "cytokine array". Representative images are presented in panel B and the expression of four replicate samples of each cell type is presented in panel C. ****p<0.0001 as determined by Student's t test



Supplementary Figure S5: Examples of Ly6G+ cell magnetic bead capture and CD8+ T cell proliferation analysis. A) Ly6G+ cells were isolated using magnetic beads. The purity of Ly6G+ cells was evaluated in pre- and post-isolation samples using flow cytometry gated on live, CD11b+, CD45+ events. Representative biaxial Ly6G vs Ly6C plots are presented. B) CD8a+ T lymphocytes were isolated from spleens of wild type mice using magnetic beads, labelled with CFSE, left unstimulated or stimulated with IL-2 and anti-CD3/anti-CD28 microbeads and cultured alone or with an equal number of Ly6G+ cells isolated from the spleens of A1206 tumor bearing mice for three days. Representative CFSE depletion (T cell proliferation) plots are presented.



Supplementary Figure S6: Distinguishing SCC induced by 4NQO in the endogenous tongue epithelium from SCC induced by A1207 tumor cell transplantation. A) Representative low power image of a tongue from a 4NQO-treated mouse then transplanted with A1207 tumor cells allowed to grow for 4 weeks. Boxes in this lower power image outline the location of the high-power images in panels B-C. B) Tongue epithelium SCC invading into the underlying muscle are pointed out with yellow arrows. C) A transplanted A1207 tumor is circumscribed with a green line (including immune cell infiltration) and tumor nests are pointed out with blue arrows.