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IL1β promotes immune suppression in the tumor microenvironment independent of the inflammasome and gasdermin D

Máté Kiss[1,2], Lieselotte Vande Walle[3,4], Pedro H.V. Saavedra[3,4], Els Lebegge[1,2], Helena Van Damme[1,2], Aleksandar Murgaski[1,2], Junbin Qian[5,6], Manuel Ehling[7,8], Samantha Pretto[7,8], Evangelia Bolli[1,2], Jiri Keirsse[1,2], Pauline M. R. Bardet[1,2], Sana M. Arnouk[1,2], Yvon Elkrim[1,2], Maryse Schmoetten[1,2], Jan Brughmans[1,2], Ayla Debraekeleer[1,2], Amelie Fossoul[3,4], Louis Boon[9], Geert Raes[1,2], Geert van Loo[3,10], Diether Lambrechts[5,6], Massimiliano Mazzone[7,8], Alain Beschin[1,2], Andy Wullaert[3,4,10], Mohamed Lamkanfi[3,4,#], Jo A. Van Ginderachter[1,2,#,*], Damya Laoui[1,2,#,*]

[1] Myeloid Cell Immunology Lab, VIB Center for Inflammation Research, Brussels, Belgium
[2] Lab of Cellular and Molecular Immunology, Vrije Universiteit Brussel, Brussels, Belgium
[3] VIB Center for Inflammation Research, Ghent, Belgium
[4] Department of Internal Medicine and Pediatrics, Ghent University, Ghent, Belgium
[5] Laboratory of Translational Genetics, VIB Center for Cancer Biology, Leuven, Belgium.
[6] Laboratory for Translational Genetics, Department of Human Genetics, KU Leuven, Leuven, Belgium
[7] Laboratory of Tumor Inflammation and Angiogenesis, VIB Center for Cancer Biology, Leuven, Belgium
[8] Laboratory of Tumor Inflammation and Angiogenesis, Department of Oncology, KU Leuven, Leuven, Belgium
[9] Polpharma Biologics, Utrecht, the Netherlands
[10] Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium
# Equal contribution

***Corresponding authors:**
Damya Laoui
E-mail: dlaoui@vub.be; Mailing address: Cellular and Molecular Immunology, Vrije Universiteit Brussel, Building E, Floor 8, Pleinlaan 2, 1050 Brussels, Belgium; Phone: +32 2 629 1969; Fax: +32 2 629 1981
Jo A. Van Ginderachter
E-mail: jo.van.ginderachter@vub.be; Mailing address: Cellular and Molecular Immunology, Vrije Universiteit Brussel, Building E, Floor 8, Pleinlaan 2, 1050 Brussels, Belgium; Phone: +32 2 629 1979; Fax: +32 2 629 1981

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Synopsis: IL1β is implicated in cancer progression. The authors show that IL1β promotes neutrophil accumulation in tumors and suppresses antitumor immunity in an inflammasome-independent manner, suggesting that therapeutic inhibition of the inflammasome will not limit IL1β production in certain cancer types.

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ABSTRACT

Interleukin-1β (IL1β) is a central mediator of inflammation. Secretion of IL1β typically requires proteolytic maturation by the inflammasome and formation of membrane pores by gasdermin D (GSDMD). Emerging evidence suggests an important role for IL1β in promoting cancer progression in patients, but the underlying mechanisms are ill-defined. Here, we have shown a key role for IL1β in driving tumor progression in two distinct mouse tumor models. Notably, activation of the inflammasome, caspase-8 as well as the pore-forming proteins GSDMD and mixed lineage kinase domain–like protein (MLKL) in the host were dispensable for the release of intratumoral bioactive IL1β. Inflammasome-independent IL1β release promoted systemic neutrophil expansion and fostered accumulation of T-cell suppressive neutrophils in the tumor. Moreover, IL1β was essential for neutrophil infiltration triggered by antiangiogenic therapy, thereby contributing to treatment-induced immunosuppression. Deletion of IL1β allowed intratumoral accumulation of $CD8^+$ effector T cells that subsequently activated tumor-associated macrophages. Depletion of either $CD8^+$ T cells or macrophages abolished tumor growth inhibition in IL1β–deficient mice, demonstrating a crucial role for $CD8^+$ T cell–macrophage crosstalk in the antitumor immune response. Overall, these results support a tumor-promoting role for IL1β through establishing an immunosuppressive microenvironment and show that inflammasome activation is not essential for release of this cytokine in tumors.

INTRODUCTION

Chronic inflammation can promote tumor development and progression through various means, such as providing survival signals, suppressing T-cell function, inducing angiogenesis and enabling invasion and metastasis via tissue remodeling (1). In addition, immune cells recruited to the tumor as part of the inflammatory response often antagonize anticancer therapies (2). Hence, counteracting tumor-promoting inflammation appears to be key to improving disease outcomes in many cancer types (3). This, however, requires a more complete understanding of the mechanisms driving tumor-associated inflammation.

Interleukin-1β (IL1β) is a pro-inflammatory cytokine whose role in cancer is increasingly recognized (4,5). In a recent study, long-term treatment with a neutralizing IL1β-specific antibody led to a dose-dependent reduction of lung cancer incidence and mortality in a large cohort of atherosclerosis patients with a history of myocardial infarction (6). The importance of chronic IL1β-driven inflammation in cancer is further supported by the identification of an IL1β-induced transcriptional signature in the peripheral immune cells of renal cell cancer and breast cancer patients (7,8). Moreover, p53 mutations, late-stage disease and the basal-like subtype in breast cancer are all associated with significantly increased *IL1B* expression and may further augment systemic inflammation (8,9). IL1β also promotes tumor angiogenesis and the recruitment of myeloid cells (5,10,11). In contrast, it supports antitumor T-cell responses and suppresses metastatic outgrowth in mice, suggesting its role may be context dependent (5,12,13).

IL1β is produced as a biologically inactive precursor (pro-IL1β). The cleavage of this inactive precursor to generate the active form is typically mediated by caspase-1 (14). Activation of caspase-1 is triggered by the inflammasome, a multiprotein complex that assembles upon activation of intracellular receptors such as NLRP3, AIM2 and NLRC4. These receptors are activated by distinct danger- or pathogen-associated molecular patterns, such as extracellular ATP, double-stranded DNA and bacterial flagellin (14). Once cleaved, IL1β follows an unconventional secretory pathway that typically requires membrane pores composed of gasdermin D (GSDMD), activation of which is also induced by the inflammasome (5,14).

Despite emerging interest in IL1β as an oncology target, several questions regarding IL1β signaling in the context of cancer remain unanswered. First, although IL1β secretion is elevated in breast and lung tumors compared with adjacent non-involved tissues (8,15), the exact cellular source of increased IL1β production within these tumors remains poorly characterized. In fact, malignant cells, fibroblasts and immune cells are all considered potential sources of IL1β release in various tumor types (16-19). Second, the inflammasome is dispensable for IL1β

release in several types of sterile inflammation (20-22), raising the question whether caspase-1 is critically required for the proteolytic maturation and release of active IL1β in the tumor microenvironment (TME). GSDMD is essential for *in vivo* IL1β release in several mouse models of inflammation, but it has not yet been determined whether it plays a similar role in tumors (23-26). In addition, we still have limited understanding about how IL1β release impacts the complex TME and to what extent its effect is conserved across different tumor types. To address these knowledge gaps, we set out to examine the source of IL1β in tumors, the requirement of inflammasomes and GSDMD for its release and its impact on the TME in mouse models of non-small cell lung cancer (NSCLC) and triple-negative breast cancer (TNBC).

MATERIALS AND METHODS

Mice

All experiments were performed with age-matched female mice. C57BL/6 mice were from Janvier, *Il1b*$^{-/-}$ mice were provided by François Huaux (UCL, Belgium), UBI-GFP mice were from Jackson. *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$ and *Gsdmd*$^{-/-}$ mice were provided by Vishva M. Dixit (Genentech, USA). *Ripk3*$^{-/-}$*Casp8*$^{-/-}$, *Casp1/11*$^{-/-}$ and *Mlkl*$^{-/-}$ mice were from Thirumala-Devi Kanneganti (St Jude Children's Research Hospital, USA). *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice were generated by crossing the *Casp1/11*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ strains. *Gsdmd*$^{-/-}$*Mlkl*$^{-/-}$ mice were generated by crossing the *Gsdmd*$^{-/-}$ and *Mlkl*$^{-/-}$ strains. Both *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Gsdmd*$^{-/-}$*Mlkl*$^{-/-}$ mice were generated in the VIB Center for Inflammation Research, Ghent, Belgium. In all experiments involving knock-out mice, wild-type (+/+) or heterozygote (+/-) littermate mice were used as controls as specified in the figures and figure legends.

All procedures followed the guidelines of the Belgian Council for Laboratory Animal Science and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (licenses 14-220-26, 16-220-3, 19-220-35).

Cell lines

LLC cells (ATCC, cat# CRL-1642) were purchased in 2017. E0771 cells (CH3 Biosystems, cat# 94A001) were received from Prof. Massimiliano Mazzone in 2016. B16F10 cells (ATCC, cat# CRL-6475) were purchased in 2012. EG7 cells were received from Prof. Karine Breckpot in 2007. LLC, E0771 and B16F10 cells were maintained in DMEM (Gibco, cat# 41965-039) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Capricorn Scientific, cat# FBS-12A), 300 μg/ml L-glutamine (Sigma, cat# G8540), 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco, cat# 15140122). For EG7 cells, the medium was RPMI (Gibco, cat# 52400-025) and the supplements were the same. HEK293-IL1R1 cells (Invivogen, cat# hkb-il1r, purchased in 2019) were cultured in DMEM with 10% (v/v) heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Normocin (Invivogen, cat# ant-nr-1) and HEK-Blue Selection antibiotics (Invivogen, cat# hb-sel). All cell lines used in experiments were cultured for 7-14 days and for 3-6 passages. Test for Mycoplasma infection was performed in 2019 and all cell lines were negative. The cell lines were not authenticated in the past year.

Tumor models

For tumor implantation, 3×10^6 LLC cells, 1×10^6 B16F10 cells or 3×10^6 EG7 cells were injected subcutaneously into the right flank of mice in 200 μl of HBSS. For orthotopic breast tumor

implantation, 5×10^5 E0771 cells were injected into the left 4th mammary fat pad in 50 µl of HBSS mixed with Growth Factor Reduced Matrigel (Corning, cat# 356230) in a 1:1 ratio. Tumor volumes were determined by caliper measurements and calculated using the formula: $V = \pi \times (d^2 \times D)/6$, where d is the shortest diameter and D is the longest diameter.

Treatments

Anti-VEGFR2 (clone DC101, BioXCell, cat# BE0060) or isotype control antibody (clone HRPN, BioXCell, cat# BE0088) was administered intraperitoneally every 3 days starting from day 4 of tumor growth at a dose of 40 mg/kg body weight.

For macrophage depletion, CSF1R inhibitor PLX5622 was administered via rodent chow (1200 mg PLX5622/kg chow) starting from day 6 of tumor growth. PLX5622 was provided by Plexxikon. Control and PLX5622-containing AIN-76A rodent chow was prepared by Research Diets.

For $CD8^+$ T–cell depletion, 200 µg CD8-specific antibody (clone YTS169, provided by Polpharma Biologics) was injected intraperitoneally every 2–3 days starting 1 day prior to tumor inoculation.

For neutrophil depletion, mice received daily intraperitoneal injections of 200 µg CXCR2 inhibitor (SB225002, Selleckchem, cat# S7651) dissolved in saline with 5% DMSO and 8% Tween-80 starting from the day of tumor implantation. In addition, starting from day 12 of tumor progression, mice received 75 µg Ly6G-specific antibody (clone 1A8, BioXcell, cat# BE0075-1) intraperitoneally every second day, followed by 150 µg anti-rat immunoglobulin (clone MAR 18.5, BioXCell, cat# BE0122) intraperitoneally 24 h later. The anti-Ly6G treatment was only maintained for 6 days due to the development of anti-rat antibodies in treated mice after one week which limit its efficacy (27).

Blood collection and tissue dissociation

Blood was collected from mice in 1 ml syringes containing 0.5 M EDTA (Duchefa, cat# E0511). Tumors were excised, cut in small pieces, incubated with 10 U/ml collagenase I (Worthington, cat# CLSS-1), 400 U/ml collagenase IV (Worthington, cat# CLSS-4) and 30 U/ml DNase I (Worthington, cat# DCLS) in RPMI for 30 min at 37°C, squashed and filtered. Spleens were mashed through a cell strainer, bone marrow was flushed out from the femurs into RPMI. All single-cell suspensions were treated with ACK (Ammonium-Chloride-Potassium) erythrocyte lysis buffer.

Flow cytometry and cell sorting

Single-cell suspensions were resuspended in HBSS and samples for flow cytometry analysis were incubated with Fixable Viability Dye eFluor 506 (1:1000, eBioscience, cat# 65-0866-14) for 30 min at 4°C. Next, cell suspensions were washed with HBSS and resuspended in HBSS with 2 mM EDTA and 1% (v/v) FCS. To prevent nonspecific antibody binding to Fcγ receptors, cells were pre-incubated with CD16/CD32-specific antibody (clone 2.4G2, BD Biosciences, cat# 553142). Cell suspensions were then incubated with fluorescently labelled antibodies diluted in HBSS with 2 mM EDTA and 1% (v/v) FCS for 20 min at 4°C and then washed with the same buffer.

For intracellular staining of IFNγ, tumor single-cell suspensions were incubated in *ex vivo* culture medium (see *Ex vivo* cell culture below) containing 50 ng/ml PMA, 500 ng/ml ionomycin and Golgiplug (1:1000, BD Biosciences, cat# 555029) for 4 hours at 37°C. After washing the samples with HBSS, surface proteins were stained first as described above, followed by fixation and permeabilization using the Cytofix/Cytoperm kit (BD Biosciences, cat# 554714), then staining of intracellular IFNγ.

The following fluorochrome-conjugated antibodies were used in the study: CD45 (clone 30-F11, Biolegend, cat# 103116), CD11b (clone M1/70, Biolegend, cat# 101216 and 101228), Ly6G (clone 1A8, Biolegend, cat# 127616 and 127608), SiglecF (clone E50-2440, BD Biosciences, cat# 552126), MHC-II (clone M5/114.15.2, Biolegend, cat# 107632 and 107626), Ly6C (clone HK1.4, Biolegend, cat# 128010), F4/80 (clone CI:A3-1, Bio-Rad, cat# MCA497A488), CD11c (clone HL3, BD Biosciences, cat# 553802), CD24 (clone M1/69, Biolegend, cat# 101822), NK1.1 (clone PK136, Biolegend, cat# 108728), CD19 (clone 1D3, BD Biosciences, cat# 553786 and clone B4, Biolegend, cat# 115538), TCRβ (clone H57-597, Biolegend, cat# 109212 and 109229), CD4 (clone GK1.5, Biolegend, cat# 100434), CD8 (clone 53-6.7, Biolegend cat# 100712 and 100738), FoxP3 (clone FJK-16s, eBioscience, cat# 45-5773-82), CD44 (clone IM7, Biolegend, cat# 103006), CD62L (clone MEL-14, eBioscience, cat# 12-0621-83), CD69 (clone H1.2F3, Biolegend, cat# 104505), GZMB (clone GB11, Biolegend, cat# 515400), CD31 (clone 390, eBioscience, cat# 46-0311-82), CD64 (clone X54-5/7.1, Biolegend, cat# 139306), CD40 (clone 3/23, Biolegend, cat# 124609), IFNγ (clone XMG1.2, BD Biosciences, cat# 554412), IL1R1 (clone 35F5, BD Biosciences, cat# 557489).

For neutrophil cell death analysis, tumor and spleen cell suspensions were resuspended in HBSS with 2 mM EDTA and 1% (v/v) FCS, stained for surface proteins, washed with the same buffer and resuspended in Annexin V binding buffer (Biolegend, cat# 422201) containing Pacific Blue Annexin V (1:20, Biolegend, cat# 640918). After 15 min incubation at room

temperature in the dark, cells were washed with Annexin V binding buffer and resuspended in the same buffer. 7-AAD (Biolegend, cat# 420404) was added 10 min before analysis.

Flow cytometry data were acquired using a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo software. Samples with less than 10% viable cells and tumor samples with cell contamination from the tumor-draining lymph node (identified as outliers in B-cell and naive T-cell abundance) were excluded from further analyses.

Surface protein expression was quantified by calculating the Δ median fluorescence intensity (MFI) value: ΔMFI = MFI surface protein – MFI isotype control.

For fluorescence-activated cell sorting of myeloid-cell populations, tumor single cell suspensions were enriched for $CD11b^+$ cells using magnetic cell separation according to the manufacturer's protocol (Miltenyi, cat# 130-049-601). 7-AAD staining was used to exclude dead cells. Cell subsets were then sorted into RPMI with 10% (v/v) FCS, 300 µg/ml L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) MEM non-essential amino acids (Gibco, cat# 11140050), 1 mM sodium pyruvate (Gibco, cat# 11360070) and 0.02 mM 2-mercaptoethanol (Sigma, cat# M6250). Fluorescence-activated cell sorting was performed using a BD FACSAria II (BD Biosciences).

Ex vivo cell culture

The viability of cells after cell sorting was confirmed using trypan blue staining. For *ex vivo* culture, 3×10^5 cells/well were cultured at 37°C for 24 h in flat-bottom 96 well plates in 200 µl/well RPMI containing 10% (v/v) FCS, 300 µg/ml L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 1% (v/v) MEM non-essential amino acids, 1 mM sodium pyruvate and 0.02 mM 2-mercaptoethanol.

Adoptive transfer of neutrophils

Neutrophils from the spleen of LLC tumor-bearing UBI-GFP mice were isolated by magnetic cell separation using anti-Ly6G microbeads according to the manufacturer's protocol (Miltenyi). 5×10^6 GFP-expressing neutrophils in 100 µl HBSS were injected through the tail vein into recipient LLC tumor-bearing mice, which were sacrificed 24 h later.

T-cell suppression assays

2×10^5 neutrophils or monocytes sorted from tumors were added to 2×10^5 naïve C57BL/6 splenocytes stimulated with anti-CD3 (1 µg/ml, clone 145-2C11, produced in-house) and anti-CD28 (2 µg/ml, clone 37.51, eBioscience, cat# 16-0281-85) and cultured in flat-bottom 96-well

plates in culture medium for *ex vivo* cell culture described above. After 24 h of culture at 37°C, 1 μCi (0.037 MBq) ^{3}H-thymidine (Perkin-Elmer, cat# NET027A005MC) was added and after another 18 h of culture T-cell proliferation was measured as count per minute in a liquid scintillation counter.

For measuring T-cell proliferation via flow cytometry, splenocytes were labelled with CellTrace Violet dye (ThermoFisher, cat# C34571) following the manufacturer's instructions and co-cultured with tumor-derived neutrophils as described above. To inhibit potential T-cell suppressive pathways, 0.5 mM Nω-Nitro-L-arginine methyl ester (L-NAME, Sigma, cat# N5751), 0.5 mM Nω-Hydroxy-nor-L-arginine (Nor-NOHA, Sigma, cat# 399275) or 200 U/ml superoxide dismutase (Sigma, cat# S5395) was added to the co-cultures. After 42 h of culture at 37°C, the frequency of CellTrace^{low} proliferating T-cells was determined via flow cytometry.

RNA extraction, cDNA preparation, and quantitative real-time PCR

Tumor tissue was snap-frozen in liquid nitrogen and homogenized in 1 ml TRIzol (Invitrogen, cat# 15596026) in gentleMACS M tubes (Miltenyi, cat# 130-093-236 using the gentleMACS Dissociator (Miltenyi, cat# 130-093-235). RNA was extracted using TRIzol and 1 μg RNA was reverse-transcribed with oligo(dT) and SuperScript II RT (Invitrogen, cat# 18064022) following the manufacturers' protocols. Quantitative real-time PCR was performed in triplicates for each sample in the CFX Connect Real-Time System (Bio-Rad) using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, cat# 1725274) and the following primers: *Rps12*-F: GGAAGGCATAGCTGCTGGAGGTGT, *Rps12*-R: CCTCGATGACATCCTTGGCCTGAG; *Il1b*-F: GTGTGGATCCCAAGCAATAC, *Il1b*-R: GTCTGCTCATTCACGAAAAG; *Cxcl1*-F: GCTTGAAGGTGTTGCCCTCAG, *Cxcl1*-R: AAGCCTCGCGACCATTCTTG; *Cxcl2*-F: TGGAAGGAGTGTGCATGTTC, *Cxcl2*-R: CACGAAAAGGCATGACAAAA; *Cxcl3*-F: CACCCAGACAGAAGTCATAGCCAC, *Cxcl3*-R: TGGTGAGGGGCTTCCTCCTTT; *Cxcl5*-F: CTCGCCATTCATGCGGAT, *Cxcl5*-R: CTTCAGCTAGATGCTGCGGC; *Cxcl7*-F: CTCAGACCTACATCGTCCTGC, *Cxcl7*-R: GTGGCTATCACTTCCACATCAG; *Cxcl12*-F: TCATCCCCATTCTCCTCATC, *Cxcl12*-R: ATAAAGGAGCCTCCCTCTGC; *Cxcl9*-F: CCTCCTTGCTTGCTTACCAC, *Cxcl9*-R: TTTTCACCCTGTCTGGCTCT; *Cxcl10*-F: AATTGCCCTTGGTCTTCTGA, *Cxcl10*-R: CCTTGGGAAGATGGTGGTTA; *Cxcl16*-F: GTCTCCTGCCTCCACTTTCT, *Cxcl16*-R: CTAAGGGCAGAGGGGCTATT; *Ccl5*-F: GTGCCCACGTCAAGGAGTAT, *Ccl5*-R: CGAGTGGGAGTAGGGGATTA; *Il12b*-F: TCAGGGACATCATCAAACCA, *Il12b*-R: CTACGAGGAACGCACCTTTC; *Cd40*-F: GCTGTGAGGATAAGAACTTGGAGG, *Cd40*-

R: GCATCCGGGACTTTAAACCACA; *Cd86*-F: CCTCCAAACCTCTCAATTTCA, *Cd86*-R: TCGGCTTCTTGTGACATACAAT. The following program was used for real-time PCR: 95 °C 3 min, 40×(94 °C 30 s, 54 °C 30 s, 72 °C 45 s). Expression values were calculated using the ΔCt method as follows: $2^{-(CtA-CtB)}$, where CtA is the Ct value of the gene of interest and CtB is the Ct value of the house-keeping gene *Rps12*.

Cytokine and nitrite measurements

IL1β, IFNγ and CXCL9 were measured using ELISA (IL1β from cell culture supernatants: R&D Systems cat# MLB00C; IL1β from serum: R&D Systems cat# MHSLB00; IFNγ: R&D Systems cat# DY485; CXCL9: R&D Systems cat# DY492. ELISA measurements were obtained using a VersaMax microplate reader (Molecular Devices) set to 450 nm. Measurements at 540 nm were used for background correction. G-CSF, CXCL10, CXCL16 and CCL5 were measured using multiplex immunoassay (Bio-Rad, cat# 171G5015M and 12009159) according to the manufacturers' protocols.

Nitrite levels were determined by adding 50 µl Griess reagent (5% phosphoric acid containing 0.2% naphthylethylenediamine dihydrochloride and 2% sulphanilamide) to 50 µl culture supernatant in a 96 well plate and measuring the optical density at 548 nm a VersaMax microplate reader.

Western blotting

Cell lysates for immunoblots were prepared by resuspending cells in a lysis buffer containing 20 mM pH 7.4 Tris HCl (Sigma, cat# T3253), 200 mM NaCl and 1% (v/v) NP-40 (Sigma, cat# I8896). Cell lysates and cell culture supernatants were denatured in 4x Laemmli buffer (250 mM Tris HCl, 8% (w/v) SDS [Sigma, cat# L3771], 40% (v/v) Glycerol [Sigma, cat# G5516], 0.02% (w/v) Bromophenol blue, 5% (v/v) 2-mercaptoethanol) at 95°C for 10 min. Proteins were separated using SDS-PAGE gels (for IL1β: 16% gel [Invitrogen, cat# P00160BOX] 200 V; for caspase-1/8: 12% gel [Invitrogen, cat# P00120BOX] 164 V) were transferred to PVDF membranes (Bio-Rad, cat# 1704273). Blocking, incubation with antibody, and washing of the membrane were done in PBS supplemented with 0.05% (v/v) Tween 20 and 3% (v/v) non-fat dry milk. Immunoblots were incubated overnight with primary antibodies against caspase-1 (Adipogen, cat# AG-20B-0042-C100), IL-1β (Genetex, cat# GTX74034) caspase-8 (full length: Enzo Life Sciences, cat# ALX-804-447-C100; cleaved: Cell Signaling Technology, cat# 8592S) and β-actin (Santa Cruz Biotechnology, cat# sc-47778-HRP). Horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, cat# 115-035-

146), anti-rabbit (Jackson ImmunoResearch Laboratories, cat# 111-035-144), or anti-rat (Jackson ImmunoResearch Laboratories, cat# 112-035-143) secondary antibody was used to detect proteins by enhanced chemiluminescence (Thermo Scientific, cat# 34578). Mouse bone marrow-derived macrophages (BMDMs) treated with 0.5 μg/ml LPS (Invivogen, cat# tlrl-smlps,) for 3 h followed by 5 mM ATP (Roche, cat# 10519987001) for 45 min were used as positive controls for IL-1β blots and *Casp1/11*$^{-/-}$ BMDMs treated with 1 μg/ml anthrax protective antigen (Quadratech, cat# 171E) and 500 ng/ml anthrax lethal factor (Quadratech, cat# 172B) for 2 h were used as positive controls for caspase-8 blots. BMDMs were generated by culturing bone marrow cells in IMDM (Lonza, cat# 12-722F) containing 10% (v/v) FCS, 30% (v/v) L929 cell-conditioned medium, 1% (v/v) MEM non-essential amino acids (Lonza, cat# BE13-114E), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2 for 6 days.

Histology

For the assessment of tumor blood vessel perfusion, mice were injected intravenously with 0.05 mg FITC-conjugated lectin (Vector Laboratories, cat# / FL-1171). After 10 minutes, mice were sacrificed and tumors were harvested.

Tumor hypoxia was detected via intraperitoneal injection of 60 mg/kg body weight pimonidazole hydrochloride (Hypoxyprobe, cat# HP3-100Kit) into tumor-bearing mice. After 1 h, mice were sacrificed and tumors were harvested.

Tumor samples were fixed in 2% PFA overnight at 4°C, then dehydrated and embedded in paraffin. Serial sections of 7 μm thickness were made. Slides were first rehydrated to further proceed with antigen retrieval in citrate solution (DAKO, cat# S1699) at 100°C for 20 min. Slides were then incubated in 0.3% hydrogen peroxide in methanol for 20 min to block endogenous peroxidases. The sections were blocked with donkey serum (Sigma, cat# D9663) for 45 min and incubated overnight at room temperature with the following antibodies: anti-CD31 (BD Biosciences, cat# 550274), anti-FITC (Serotec, cat# 4510-7604), anti-αSMA-Cy3 (Sigma, cat# C6198), anti-pimonidazole (Hypoxyprobe, cat# HP3-100Kit). Next, appropriate secondary Alexa Fluor 488/647-conjugated antibodies (Invitrogen,cat# A-21206, A-21110, A-31573) or biotin-labeled antibodies (Jackson Immunoresearch, cat# 712-065-153) were applied. After biotin-labelled antibodies, TSA Cyanine 3 or Cyanine 5 amplification kits (Perkin Elmer, cat# NEL704A001KT and NEL705A001KT) were used according to the manufacturer's instructions. Hoechst solution was used to stain nuclei. Mounting of slides was done with ProLong Gold mounting medium without DAPI (Invitrogen, cat# P36931). Imaging

and microscopic analysis was performed with an Olympus BX41 microscope and CellSense imaging software. Slides were scanned using Zeiss AxioScan Z.1 slide scanner. $CD31^+$ blood vessel density and the proportion of FITC-$lectin^+$ (perfused) and αSMA^+ (pericyte-covered) blood vessels were determined by manual counting in 6 representative microscopic images/tumor. The proportion of $pimonidazole^+$ hypoxic areas were determined in whole tumor cross-sections using ImageJ.

Analysis of single-cell RNA-seq data from human tumors

The droplet-based scRNA-seq data of 8 untreated lung cancer patients (28) (10x Genomics 3' RNA library kit, ArrayExpress:E-MTAB-6149 and E-MTAB-6653) were processed and clustered using Seurat (v2.3.4) package. Cell matrix was filtered (nUMI > 400, 200 < nGene < 6000, mitochondrial RNA < 25%), normalized, regressed for confounding factors (nUMI, patient, mitochondrial RNA and cell cycle) and scaled. The variable genes (normalized expression between 0.125 and 3, quantile-normalized variance > 0.5) were used to construct principal components (PCs), followed by graph-based clustering (tSNE and Louvain algorithm). Cell type annotation was based on the expression of established marker genes. pDCs were initially co-clustered with B-cells, and then annotated back to myeloid population, where most other DCs were co-clustered with. Then the myeloid cells were subclustered to identify monocytes (*SELL*, *CDKN1C*, *MTSS1*), macrophages (*CD68, CD163, MCR1*), DCs (*CLEC9A*, *XCR1*, *CD1C*, *CD1A, LILRA4*) and neutrophils (*FCGR3B*). Similar analysis was performed for 5'-scRNA-seq data from 14 treatment-naïve breast cancers (29) (ArrayExpress: E-MTAB-8107), and the myeloid cells were further subclustered and annotated.

Statistical analysis

Statistical analyses were performed in GraphPad Prism software. For relevant pairwise comparisons, unpaired two-tailed t-test was used to calculate the *P* value. Tumor growth curves were compared by 2-way ANOVA with Holm-Sidak multiple comparisons test. To assess correlation, Pearson correlation coefficient was calculated. A P value < 0.05 was considered statistically significant. For statistically significant differences, the P value is indicated in graphs as the following: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P < 0.0001$. Comparisons found to be nonsignificant are not shown.

RESULTS

Myeloid cells are the primary source of IL-1β in lung and breast tumors

To determine the cellular sources of IL1β in lung and breast tumors with an unbiased approach, we analyzed single-cell RNA-seq datasets from human NSCLC and breast cancer. Unsupervised clustering of the data followed by identification of known cell lineages based on marker gene expression revealed 13 and 10 major cell types in lung and breast tumors, respectively (**Figure 1A**, **Supplementary Figure 1A**). We found that the cell populations with the highest average expression levels of *IL1B* in both tumor types were myeloid cells—neutrophils, monocytes, dendritic cells (DCs) and macrophages—whereas other cell populations showed considerably (>10-fold) lower or no expression (**Figure 1B,C**). Of note, only a small number of neutrophils could be detected in these datasets, presumably due to their low transcript counts, while these cells are known to be well represented in both tumor types based on flow cytometry (30,31).

To investigate IL1β production in more detail, we turned to mouse models of NSCLC and breast cancer: Subcutaneous Lewis lung carcinoma (LLC), a p53-mutant lung adenocarcinoma, and orthotopic E0771, a p53-mutant TNBC model with basal-like characteristics (32,33). Mice with LLC or E0771 tumors showed significantly elevated IL1β levels in the serum compared with naive mice, indicating the presence of tumor-induced IL1β-driven inflammation in these models (**Figure 1D**). To assess the contribution of myeloid cells to intratumoral IL1β release, we separated the $CD11b^+$ and $CD11b^-$ fractions of tumors and measured the expression of *Il1b* mRNA in freshly isolated cells as well as the secretion of the cytokine following 24 h of *in vitro* culture. We found that both mRNA expression and protein secretion of IL1β were almost exclusively restricted to the $CD11b^+$ fraction of tumors (**Figure 1E,F**). Importantly, the LLC and E0771 cell lines did not produce bioactive IL-1β (**Supplementary Figure 1B,C**). The majority of the $CD11b^+$ fraction in LLC and E0771 tumors consisted of neutrophils, monocytes and tumor-associated macrophages (TAMs) (**Figure 1G,** for gating strategies, see **Supplementary Figure 2**). Consistent with published reports, the TAM population included MHC-II^{high} and MHC-II^{low} subsets, which possess immunostimulatory and anti-inflammatory gene expression profiles, respectively (34,35). We then isolated these cell populations from tumors and assessed their IL1β secretion *in vitro*. In LLC tumors, monocytes and neutrophils showed the highest secretion levels, followed by MHC-II^{high} TAMs and MHC-II^{low} TAMs (**Figure 1H**). In contrast, IL1β secretion was comparable across the different myeloid cell types isolated from E0771 tumors (**Figure 1H**).

Altogether, these results demonstrate that myeloid cells are the primary source of IL1β in human and mouse lung and breast tumors.

IL1β deletion inhibits systemic expansion and intratumoral accumulation of neutrophils

To investigate the impact of IL1β release on tumor progression, we implanted LLC or E0771 tumors in IL1β-deficient (*Il1b*$^{-/-}$) mice and their wild-type (*Il1b*$^{+/+}$) littermates. Loss of IL1β delayed tumor growth in both tumor models with a more pronounced effect in E0771 breast tumors, where IL1β-deficiency was, in some cases, associated with regression or durable tumor control (**Figure 2A, Supplementary Figure 3A**). Notably, subcutaneous implantation of E0771 tumors in *Il1b*$^{-/-}$ mice did not result in significant growth inhibition, suggesting that the effect of IL1β may depend on the tissue microenvironment (**Supplementary Figure 3B**).

IL1β release induces neutrophilia during systemic inflammation and this may have an influence on tumor progression due to the wide range of tumor-promoting activities linked to neutrophils (36). For this reason, we analyzed the frequency of circulating CD11b^{+}Ly6G^{+} neutrophils in naive and tumor-bearing *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice. Both LLC and E0771 tumors induced expansion of circulating neutrophils, and this was abrogated in the absence of IL1β (**Figure 2B**). These changes were mirrored by G-CSF levels in the blood, suggesting that tumor-induced, IL1β-dependent systemic neutrophil expansion is driven by G-CSF (**Figure 2C**). Loss of IL1β prevented the LLC-induced expansion of bone marrow neutrophils, while tumor-induced accumulation of splenic neutrophils was prevented by IL1β-deletion in both tumor models (**Figure 2D**). Next, we assessed whether IL1β-deficiency has an influence on neutrophils infiltrating primary tumors. We found that loss of IL1β strongly reduced the abundance of neutrophils in both LLC and E0771 tumors (**Figure 2E**). The reduced abundance of neutrophils observed in tumor-bearing IL1β-deficient mice was likely not due to increased neutrophil cell death, as the proportions of necrotic/apoptotic neutrophils were not elevated in the spleens and tumors of *Il1b*$^{-/-}$ mice compared to wild-type controls (**Supplementary Figure 3C,D**). To test whether the reduced abundance of tumor-infiltrating neutrophils is solely due to their decreased levels in the circulation or also due to altered recruitment, we adoptively transferred equal numbers of GFP-expressing splenic neutrophils into LLC tumor-bearing *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice and assessed their frequency in the tumor after 24 hours. As shown in **Figure 2F**, recruitment of GFP^{+} neutrophils to the tumor was strongly reduced in *Il1b*$^{-/-}$ mice even though their frequency in the circulation remained comparable to wild-type controls. Since CXCR2 ligands, particularly CXCL1 and CXCL2, have been shown to be critical for neutrophil extravasation (37), we investigated whether these chemokines are affected by IL1β release in

the tumor. We found that all CXCR2 ligands, including *Cxcl1*, *Cxcl2*, *Cxcl3*, *Cxcl5* and *Cxcl7*, but not the CXCR4 ligand *Cxcl12*, showed strongly reduced expression in the absence of IL1β (**Figure 2G**).

Of note, the effect of IL1β on the expansion and recruitment of neutrophils was not restricted to the LLC and E0771 tumor models. We also observed a significant reduction of circulating and tumor-infiltrating neutrophils in IL1β-deficient mice with EG7 lymphoma and B16-F10 melanoma tumors, which show greatly differing levels of neutrophil abundance (**Supplementary Figure 3E,F**).

Neutrophil recruitment to the tumor has been shown to drive therapy resistance and immunosuppression during treatment with antiangiogenic agents targeting VEGF signaling (38,39). Hence, we analyzed whether IL1β is required for neutrophil infiltration during antiangiogenic therapy and examined the effect of VEGFR2-specifc antibody treatment in *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice in the LLC tumor model. While anti-VEGFR2 treatment did not affect the levels of circulating neutrophils (**Supplementary Figure 3G**), we observed a 103% increase in the abundance of tumor-infiltrating neutrophils in treated wild-type mice, and this therapy-induced neutrophil recruitment was completely abrogated in IL1β-deficient animals (**Figure 2H**). This was associated with a significantly reduced tumor burden in anti-VEGFR2-treated *Il1b*$^{-/-}$ mice compared to *Il1b*$^{+/+}$ mice with the same treatment (**Figure 2I**).

Collectively, these results indicate that loss of IL-1β delays tumor progression in mouse models of NSCLC and TNBC and this is accompanied by reduced systemic expansion and tumor infiltration of neutrophils. In addition, IL1β-deletion prevents accumulation of neutrophils in the tumor triggered by antiangiogenic therapy.

The inflammasome and GSDMD are dispensable for IL-1β-mediated neutrophil mobilization

Next, we determined whether or not the delayed tumor progression and strong reduction of neutrophil recruitment to tumors in *Il1b*$^{-/-}$ mice can be recapitulated in mice lacking various inflammasome components, which would indicate their requirement for bioactive IL1β production in tumors. Deficiency of NLRP3 and NLRC4, two caspase-1–activating NOD-like receptors, did not affect *in vitro* IL1β release of tumor-derived myeloid cells (**Figure 3A; Supplementary Figure 4A**). Consistent with this, deletion of these inflammasome components did not alter tumor progression or neutrophil recruitment in mice with LLC and E0771 tumors as opposed to IL1β deficiency (**Figure 3B,C; Supplementary Figure 4B,C**). To more directly assess the potential role of canonical and non-canonical inflammasome pathways, we analyzed tumors in mice with combined deletion of inflammatory caspases 1 and 11 (*Casp1/11*$^{-/-}$).

Deletion of caspase-1/11 led to a partial reduction in IL1β secretion levels by LLC tumor-derived myeloid cells (**Figure 3D**). However, this was not sufficient to alter tumor progression or neutrophil recruitment in LLC tumors (**Figure 3E,F**). IL1β release by E0771 tumor-derived myeloid cells was not reduced in *Casp1/11*$^{-/-}$ mice and, correspondingly, tumor growth and neutrophil infiltration remained unaltered in these tumors (**Figure 3D-F**). An IL1β immunoblot on the culture supernatants of tumor-derived *Casp1/11*$^{-/-}$ myeloid cells confirmed the inflammasome-independent production of mature IL1β in both tumor models (**Figure 3G**).

Caspase-8 has shown redundancy with caspase-1 in producing active IL1β in some cases, cleaving pro-IL1β at the same site (40,41). Active caspase-8 could be detected by immunoblot in sorted tumor-infiltrating myeloid cells but not in their circulating precursors (**Supplementary Figure 4D**). Hence, we assessed the contribution of caspase-8 to IL1β release and neutrophil recruitment in tumors by using *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice, in which *Ripk3* deletion rescues embryonic lethality caused by caspase-8-deficiency (42). We also generated *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice to evaluate the potential redundant roles of caspase-1/11 and -8. Caspase-8 deletion in both the *Ripk3*$^{-/-}$ and *Casp1/11*$^{-/-}$*Ripk3*$^{-/-}$ backgrounds led to partial blockade of *in vitro* IL1β release in myeloid cells derived from LLC tumors but not from E0771 tumors (**Supplementary Figure 4E,H**). However, this was not sufficient to affect tumor progression and neutrophil recruitment (**Supplementary Figure 4F,G,I,J**). Together, these data suggest slightly different mechanisms of IL1β production by myeloid cells in LLC and E0771 tumors, but an overall independence of tumor growth and neutrophil recruitment from inflammasomes and caspase-8.

Membrane pore formation by GSDMD is critical for IL1β release in mouse models of autoinflammation, steatohepatitis, disseminated intravascular coagulation and sepsis (23-26). To investigate a potential role for GSDMD in IL1β release, LLC and E0771 tumors were implanted in *Gsdmd*$^{-/-}$ mice and *Gsdmd*$^{+/+}$ littermates. However, GSDMD-deficiency did not reduce IL1β release of tumor-derived CD11b^{+} myeloid cells, tumor growth, and neutrophil recruitment (**Figure 3H-J**). Alternatively, necroptosis induced by membrane pores composed of MLKL has been suggested to mediate IL1β release independently of GSDMD-dependent pyroptosis *in vitro* (43). However, neither MLKL-deficiency nor GSDMD/MLKL double-deficiency had a significant effect on myeloid cell IL1β release, tumor progression and neutrophil recruitment (**Supplementary Figure 5A-F**).

Overall, these data from two distinct mouse tumor models demonstrate that activation of the inflammasome and caspase-8 as well as the formation of membrane pores by GSDMD and

MLKL are dispensable for the release of bioactive IL-1β by tumor-associated myeloid cells and consequential neutrophil recruitment.

IL1β release is not essential for tumor angiogenesis

To investigate the mechanism of reduced tumor growth in *Il1b*$^{-/-}$ mice, we studied tumor angiogenesis, which has been described as being potentially IL1β-regulated (10,11). However, we did not find any major differences in tumor blood vessel density, pericyte coverage and vessel perfusion between *Il1b*$^{-/-}$ and *Il1b*$^{+/+}$ mice (**Supplementary Figure 6A-G**). Slightly less hypoxic areas were observed in the tumors of *Il1b*$^{-/-}$ mice, however, this was likely due to the smaller average tumor size since volume-matched tumors did not show such difference (**Supplementary Figure 6H**). These observations suggested that IL1β is not essential for tumor angiogenesis and reduced tumor growth in IL1β-deficient animals may be explained by immune-mediated mechanisms.

Tumor-infiltrating neutrophils suppress T-cell activation via nitric oxide production

Next, we examined whether neutrophils recruited by IL1β to LLC or E0771 tumors were able to suppress T-cell proliferation. CD11b^{+}Ly6G^{+} neutrophils isolated from primary tumors inhibited proliferation of splenocytes stimulated with anti-CD3 and anti-CD28 (**Figure 4A**). Tumor-infiltrating neutrophils from *Il1b*$^{-/-}$ mice showed similar T-cell suppressive activity to neutrophils from *Il1b*$^{+/+}$ controls, suggesting that IL1β dominantly affects their recruitment rather than their immunosuppressive activity (**Figure 4B**).

To identify the mechanism by which neutrophils recruited to the tumor are able to suppress T-cell activation, we inhibited three key T-cell suppressive mechanisms described previously: Nitric oxide synthesis, arginase activity and superoxide production (44). Neutrophils isolated from LLC tumors were co-cultured with activated splenocytes in the presence of L-NAME (nitric oxide synthase inhibitor), Nor-NOHA (arginase inhibitor) or superoxide dismutase. Only L-NAME restored T-cell proliferation and IFNγ production in the presence of tumor-derived neutrophils (**Figure 4C-D**). Measurement of nitrite, a stable breakdown product of nitric oxide, in the supernatants of co-cultures confirmed NO production by tumor-derived neutrophils and its reduction upon inhibition of nitric oxide synthase (**Figure 4E**).

Overall, these results indicate that neutrophils recruited to the tumor can suppress T-cell proliferation and activation via nitric oxide production.

IL1β deletion relieves immune suppression in the tumor microenvironment

Consistent with the immunosuppressive phenotype of tumor-infiltrating neutrophils, impaired neutrophil recruitment in *Il1b*$^{-/-}$ mice (**Figure 2E**) was accompanied by an elevated abundance of cytotoxic CD8^{+} T cells, whereas the infiltration of CD4^{+} T cells and FoxP3^{+} regulatory T cells remained unaltered (**Figure 5A-C**). In addition, a higher proportion of tumor-infiltrating CD4^{+} and CD8^{+} T cells showed an effector T-cell phenotype in *Il1b*$^{-/-}$ mice, as indicated by the increased CD44^{+}CD62L^{-} effector vs. CD44^{-}CD62L^{+} naive T-cell ratio (**Figure 5D,E, Supplementary Figure 7A**). Upregulation of the activation marker CD44 and expansion of IFNγ^{+} cells among CD8^{+} T cells further indicated an enhanced cytotoxic T-cell response in the absence of IL1β (**Figure 5F**). Correspondingly, enhanced infiltration of neutrophils upon anti-VEGFR2 therapy in wild-type mice (**Figure 2H**) was associated with impaired effector T-cell differentiation and this was counteracted by IL1β deletion (**Figure 5G, Supplementary Figure 7B,C**). Among the immune cells possessing T-cell stimulatory potential, conventional DCs did not show altered infiltration in tumors of *Il1b*$^{-/-}$ mice (**Supplementary Figure 7D**). In LLC tumors of IL1β-deficient mice, we observed reduced infiltration of monocytes that possessed potent T-cell suppressive capacity (**Figure 5H,I**). However, TAM abundance was not reduced in LLC and E0771 tumors (**Figure 5J**). Among TAMs, we found higher abundance of MHC-II^{high} TAMs (**Figure 5K**), a phenotype that has been shown to be driven by effector T cells (45,46), and, in turn, possesses the capacity to stimulate T-cell responses (34,35).

These changes in the TME were likely indirect effects of IL1β-deficiency rather than the lack of direct IL1β effects on tumor-infiltrating immune cells, as we could not detect IL1R1 expression on T cells, monocytes, macrophages and neutrophils in either model (**Supplementary Figure 8**).

Reducing the abundance of immunosuppressive neutrophils in the TME administering both a CXCR2 inhibitor and a Ly6G-specific antibody did not fully mirror the changes observed in *Il1b*$^{-/-}$ mice (**Supplementary Figure 9**). Similar to IL1β-deficiency, neutrophil depletion led to a reduction in LLC tumor growth and upregulation of the activation marker CD44 on CD8^{+} T cells (**Supplementary Figure 9A-C**). In contrast to IL1β deletion, however, neutrophil depletion caused an 89% increase in immunosuppressive monocyte infiltration into tumors and did not significantly enhance the abundance of tumor-infiltrating effector CD8^{+} T cells and immunostimulatory TAMs (**Supplementary Figure 9D-H**).

In summary, these data indicate that IL1β promotes the establishment of an immunosuppressive tumor microenvironment characterized by impaired accumulation of effector T cells and suppression of TAM activation. This is partly driven by IL1β-dependent recruitment of

immunosuppressive neutrophils, but likely requires additional, yet unknown, IL1β-activated immunosuppressive pathways in the TME.

CD8$^+$ T cells drive antitumor immunity in IL1β-deficient mice

Next, we set out to determine whether the increased abundance and activation state of tumor-infiltrating cytotoxic CD8$^+$ T cells is responsible for the inhibition of tumor progression in IL1β-deficient mice. Systemic depletion of CD8$^+$ T cells using a CD8-specific antibody fully restored LLC tumor growth in *Il1b*$^{-/-}$ mice to wild-type levels, while it had no effect in wild-type mice (**Figure 6A, B**). In addition, CD8$^+$ T-cell depletion reduced the activation of tumor-infiltrating CD4$^+$ T cells in *Il1b*$^{-/-}$ mice, indicated by the decreased frequency of cells showing the effector phenotype and IFNγ production (**Figure 6C, D, Supplementary Figure 10A**). Analogously, acquisition of an immunostimulatory MHC-II^{high} phenotype by TAMs in *Il1b*$^{-/-}$ mice required the presence of CD8$^+$ T cells (**Figure 6E**). Furthermore, elevated expression of CD40 on MHC-II^{high} TAMs in *Il1b*$^{-/-}$ mice was reduced to wild-type levels upon CD8$^+$ T-cell depletion (**Figure 6F**). Consistent with these observations, presence of MHC-II^{high} TAMs but not MHC-II^{low} TAMs showed a strong positive correlation with effector CD8$^+$ T-cell infiltration in both LLC and E0771 tumors (**Figure 6G, Supplementary Figure 10B**).

Overall, these results show that CD8$^+$ cytotoxic T cells are required for the inhibition of tumor progression and are key drivers of CD4$^+$ T-cell and TAM activation in IL1β-deficient mice.

Macrophages are required for the antitumor CD8$^+$ T-cell response in IL1β-deficient mice

Last, we wanted to assess whether macrophages participate in amplifying the antitumor T-cell response in the absence of IL1β. To test this hypothesis, we depleted macrophages in tumor-bearing mice using the CSF1R inhibitor PLX5622. This small-molecule inhibitor is highly specific for CSF1R and has been successfully used before to deplete TAMs (47). Administration of PLX5622 reduced TAM infiltration in LLC tumors by 89%, whereas it only caused a 28% reduction in E0771 tumors (**Supplementary Figure 11A**). Based on these results, we examined the impact of macrophage depletion in the LLC tumor model. PLX5622 treatment in *Il1b*$^{-/-}$ mice restored tumor growth to wild-type levels, while it did not have an effect in wild-type mice (**Figure 7A**). Analysis of the tumor immune cell composition confirmed that CSF1R inhibition efficiently eliminated TAMs in both *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice whereas it did not deplete neutrophils, monocytes and DCs in tumors (**Figure 7B,C, Supplementary Figure 11B**). Analysis of tumor-infiltrating T cells revealed that macrophage depletion in *Il1b*$^{-/-}$ mice reduced CD8$^+$ T-cell abundance to the wild-type level (**Figure 7D**). Similarly, macrophage depletion in

Il1b$^{-/-}$ mice restored the ratio of tumor-infiltrating effector vs. naive CD8^{+} T cells to levels similar to *Il1b*$^{+/+}$ mice (**Figure 7E, Supplementary Figure 11C**). In addition, the proportions of CD8^{+} T cells expressing the activation markers CD69 and granzyme B were reduced by macrophage depletion in both *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (**Figure 7F,G**). Consistent with these results, TAM depletion led to lower intratumoral expression of chemokines commonly associated with T-cell trafficking, including CXCL9, CXCL10, CXCL16 and CCL5 (**Figure 7H**) (48). Depletion of TAMs in *Il1b*$^{-/-}$ mice also reduced the intratumoral expression of the costimulatory molecules *Cd40* and *Cd86* as well as the Th1 stimulatory cytokine *Il12b*, further supporting that TAMs in LLC tumors are an important source of T-cell stimulatory signals (**Figure 7I**).

Together, these results indicate that macrophages support the accumulation and activation of CD8^{+} T cells in tumors and play a critical role in tumor control in the absence of IL1β.

DISCUSSION

In this study, we demonstrate in two distinct mouse models that IL1β, released mainly by neutrophils, monocytes and macrophages, plays a key role in systemic neutrophil expansion during tumor progression and in promoting neutrophil infiltration into tumors. Our results are consistent with previous reports demonstrating a role for IL1β in systemic neutrophil expansion in breast cancer, however, these studies primarily focused on its consequences on the metastatic environment and not the primary tumor (13,17). Earlier studies utilizing IL1β blockade or IL1β-overexpressing cancer cells have demonstrated that this cytokine promotes infiltration of myeloid cells into tumors, but the exact identity of these cells remained ill-defined (10,49,50). More recently, IL1β was reported to induce CCL2 and promote the recruitment of monocytes and subsequent accumulation of macrophages in the 4T1 mouse model of breast carcinoma (51). We observed a similar impairment of monocyte recruitment in the absence of IL1β in LLC tumors but not in E0771 breast tumors, suggesting that the link between IL1β release and monocyte recruitment may not be a general phenomenon.

We also show in this study that the canonical and non-canonical inflammasomes are dispensable for the production of bioactive IL1β in LLC and E0771 tumors, and combined deletion of caspase-1/11 was not sufficient to recapitulate the *in vivo* phenotype observed in *Il1b*$^{-/-}$ mice. Although caspase-8 was activated in tumor-infiltrating myeloid cells, deletion of this enzyme was not sufficient to completely block bioactive IL1β release and neutrophil infiltration. A diverse range of additional enzymes have been shown to cleave pro-IL1β, including proteinase 3, neutrophil elastase, cathepsin G, granzyme A, chymase, matrix metalloproteinases and meprins (41,52,53). Several of these enzymes may be active and play redundant roles in the TME, therefore it might not be possible to pinpoint a single enzyme that is responsible for IL1β production in tumors. Since several reports have demonstrated the beneficial effect of genetic or pharmacological inhibition of NLRP3 and caspase-1 on tumor progression in mice, it is likely that requirement of the inflammasome for IL1β release depends on the availability of alternative cleavage pathways determined by the immune microenvironment (5). Alternatively, decreased tumor progression observed in some of these studies may also be explained by IL1β-independent effects such as inhibition of inflammasome-mediated IL18 release (14).

To our knowledge, the contribution of GSDMD and MLKL to IL1β release in tumors had not been evaluated before. In LLC and E0771 tumors, these pore-forming proteins were not required for IL1β release and neutrophil recruitment. This suggests the existence of alternative

release mechanisms that may include passive release through myeloid cell necrosis, which has been linked to IL1β release *in vitro* and is likely to occur in the TME (54).

Inflammasome-independent IL1β release promoted tumor progression and immunosuppression in both LLC and E0771 tumor models. Interestingly, tumor control upon IL1β deletion was much more pronounced in the E0771 model compared to LLC. The mechanisms underlying the different tumor growth inhibition in the two models remain to be identified and may relate to differences in implantation sites and immunogenicity.

This study adds further support to previous observations linking IL1β release to immune suppression in the TME (19,51,55). Our results provide novel insights primarily about the impact of IL1β release on the phenotype of tumor-infiltrating T-cells and macrophages in two distinct mouse models. Importantly, our results indicate that the immunosuppressive activity of IL1β is only partly mediated by neutrophil recruitment and likely requires additional IL1β-activated immunosuppressive pathways. IL1β-dependent recruitment of immunosuppressive monocytes observed in LLC tumors is possibly one of these. Nevertheless, IL1β may have immunosuppressive effects on additional cell types in the TME and/or systemically that were not examined in the current study, such as cancer cells, endothelial cells and fibroblasts. Further research is needed to better understand how these cells are impacted by IL1β release in lung and breast cancer.

A notable observation in this study is that macrophages were required for LLC tumor control in *Il1b*$^{-/-}$ mice. These results are consistent with previous reports showing the requirement of macrophages for an effective antitumor cytotoxic T-cell response following therapies that reduce immune suppression in the TME (56,57). There are several potential mechanisms by which macrophages can participate in tumor control in IL1β-deficient mice and these are mutually nonexclusive. First, macrophages may be important for T-cell recruitment. Although intratumoral expression of T-cell chemoattractants was not elevated in *Il1b*$^{-/-}$ mice, macrophage-depletion strongly reduced their levels. It is conceivable that signals maintaining T-cell exclusion are suppressed in *Il1b*$^{-/-}$ mice, allowing macrophage-derived T-cell chemoattractants to exert their function. Second, immunostimulatory TAMs may promote the intratumoral priming and expansion of antitumor CD8^{+} T cells and/or survival of tumor-infiltrating CD8^{+} T cells primed in the lymph node. Third, TAMs activated by CD8^{+} effector T cells may have a direct cytotoxic effect on cancer cells. Fourth, extratumoral macrophages, such as the ones residing in the tumor-draining lymph node, may also contribute to the priming of antitumor CD8^{+} T cells (58). Further studies are needed to elucidate the relative contribution of these mechanisms to the macrophage-dependent tumor control in *Il1b*$^{-/-}$ mice.

We found that immunostimulatory MHC-II^{high} TAMs released large amounts of IL1β. It will be important to consider the detrimental effects of IL1β on antitumor immunity in the context of therapies that aim to reprogram TAMs towards a proinflammatory state which often involves the upregulation of IL1β. In light of our results, it is possible that addition of IL1β blockade would improve the efficacy of such TAM-reprogramming therapies.

In conclusion, this study provides support for the role of IL1β as a tumor-promoting factor whose inactivation results in an immune permissive TME. We suggest that the existence of inflammasome-independent IL1β release and neutrophil recruitment demonstrated here will have to be taken into consideration when applying the growing range of inflammasome inhibitors for cancer therapy (5).

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REFERENCES

1. Grivennikov SI, Greten FR, Karin M. Immunity, Inflammation, and Cancer. Cell. 2010;140:883–99.

2. Coffelt SB, de Visser KE. Immune-mediated mechanisms influencing the efficacy of anticancer therapies. Trends in Immunology. 2015;36:198–216.

3. Coussens LM, Zitvogel L, Palucka AK. Neutralizing tumor-promoting chronic inflammation: a magic bullet? Science. 2013;339:286–91.

4. Van Gorp H, Lamkanfi M. The emerging roles of inflammasome-dependent cytokines in cancer development. EMBO Rep. 2019;20:71–15.

5. Karki R, Kanneganti T-D. Diverging inflammasome signals in tumorigenesis and potential targeting. Nat Rev Cancer. 2019;19:197–214.

6. Ridker PM, MacFadyen JG, Thuren T, Everett BM, Libby P, Glynn RJ. Effect of interleukin-1beta inhibition with canakinumab on incident lung cancer in patients with

atherosclerosis: exploratory results from a randomised, double-blind, placebo-controlled trial. The Lancet. 2017;390:1833–42.

7. Chittezhath M, Dhillon MK, Lim JY, Laoui D, Shalova IN, Teo YL, et al. Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. Immunity. 2014;41:815–29.

8. Wu T-C, Xu K, Martinek J, Young RR, Banchereau R, George J, et al. IL1 Receptor Antagonist Controls Transcriptional Signature of Inflammation in Patients with Metastatic Breast Cancer. Cancer Res. 2018;78:5243–58.

9. Wellenstein MD, Coffelt SB, Duits DEM, van Miltenburg MH, Slagter M, de Rink I, et al. Loss of p53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis. Nature. 2019;572:538–42.

10. Schmid MC, Avraamides CJ, Foubert P, Shaked Y, Kang SW, Kerbel RS, et al. Combined blockade of integrin-α4β1 plus cytokines SDF-1α or IL-1β potently inhibits tumor inflammation and growth. Cancer Research. 2011;71:6965–75.

11. Carmi Y, Dotan S, Rider P, Kaplanov I, White MR, Baron R, et al. The Role of IL-1 in the Early Tumor Cell-Induced Angiogenic Response. The Journal of Immunology. 2013;190:3500–9.

12. Lee P-H, Yamamoto TN, Gurusamy D, Sukumar M, Yu Z, Hu-Li J, et al. Host conditioning with IL-1β improves the antitumor function of adoptively transferred T cells. J Exp Med. 2019;216:2619–34.

13. Castaño Z, Juan BPS, Spiegel A, Pant A, DeCristo MJ, Laszewski T, et al. IL-1β inflammatory response driven by primary breast cancer prevents metastasis-initiating cell colonization. Nat Cell Biol. 2018;20:1084–21.

14. Van Gorp H, Van Opdenbosch N, Lamkanfi M. Inflammasome-Dependent Cytokines at the Crossroads of Health and Autoinflammatory Disease. Cold Spring Harbor Perspectives in Biology. 2019;11:a028563–19.

15. Colasante A, Mascetra N, Brunetti M, Lattanzio G, Diodoro M, Caltagirone S, et al. Transforming growth factor beta 1, interleukin-8 and interleukin-1, in non-small-cell lung tumors. Am J Respir Crit Care Med. 1997;156:968–73.

16. Okamoto M, Liu W, Luo Y, Tanaka A, Cai X, Norris DA, et al. Constitutively active inflammasome in human melanoma cells mediating autoinflammation via caspase-1 processing and secretion of interleukin-1beta. J Biol Chem. 2010;285:6477–88.

17. Coffelt SB, Kersten K, Doornebal CW, Weiden J, Vrijland K, Hau C-S, et al. IL-17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis. Nature. 2015;522:345–8.

18. Ershaid N, Sharon Y, Doron H, Raz Y, Shani O, Cohen N, et al. NLRP3 inflammasome in fibroblasts links tissue damage with inflammation in breast cancer progression and metastasis. Nature Communications. 2019;:1–15.

19. Das S, Shapiro B, Vucic EA, Vogt S, Bar-Sagi D. Tumor Cell–Derived IL1β Promotes Desmoplasia and Immune Suppression in Pancreatic Cancer. Cancer Res. 2020;80:1088–40.

20. Fantuzzi G, Ku G, Harding MW, Livingston DJ, Sipe JD, Kuida K, et al. Response to local inflammation of IL-1 beta-converting enzyme- deficient mice. J Immunol. 1997;158:1818–24.

21. Guma M, Ronacher L, Liu-Bryan R, Takai S, Karin M, Corr M. Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. Arthritis Rheum. 2009;60:3642–50.

22. Cassel SL, Janczy JR, Bing X, Wilson SP, Olivier AK, Otero JE, et al. Inflammasome-independent IL-1 mediates autoinflammatory disease in Pstpip2-deficient mice. Proc Natl Acad Sci USA. 2014;111:1072–7.

23. Kanneganti A, Malireddi RKS, Saavedra PHV, Vande Walle L, Van Gorp H, Kambara H, et al. GSDMD is critical for autoinflammatory pathology in a mouse model of Familial Mediterranean Fever. J Exp Med. 2018;215:1519–29.

24. Luan J, Chen W, Fan J, Wang S, Zhang X, Zai W, et al. GSDMD membrane pore is critical for IL-1β release and antagonizing IL-1β by hepatocyte-specific nanobiologics is a promising therapeutics for murine alcoholic steatohepatitis. Biomaterials. 2019;227:119570.

25. Wu C, Lu W, Zhang Y, Zhang G, Shi X, Hisada Y, et al. Inflammasome Activation Triggers Blood Clotting and Host Death through Pyroptosis. Immunity. 2019;50:1401–4.

26. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S, et al. Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling. Nature. 2015;526:666–71.

27. Boivin G, Faget J, Ancey P-B, Gkasti A, Mussard J, Engblom C, et al. Durable and controlled depletion of neutrophils in mice. Nature Communications. 2020;:1–9.

28. Lambrechts D, Wauters E, Boeckx B, Aibar S, Nittner D, Burton O, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. Nature Medicine. 2018;24:1277–89.

29. Qian J, Olbrecht S, Boeckx B, Vos H, Laoui D, Etlioglu E, et al. A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling. Cell Research. 2020;352:1–18.

30. Ruffell B, Au A, Rugo HS, Esserman LJ, Hwang ES, Coussens LM. Leukocyte composition of human breast cancer. Proceedings of the National Academy of Sciences. 2012;109:2796–801.

31. Kargl J, Busch SE, Yang GHY, Kim K-H, Hanke ML, Metz HE, et al. Neutrophils dominate the immune cell composition in non-small cell lung cancer. Nature Communications. 2017;8:1–11.

32. Rizzo MG, Soddu S, Tibursi G, Calabretta B, Sacchi A. Wild-type p53 differentially affects tumorigenic and metastatic potential of murine metastatic cell variants. Clin Exp Metastasis. 1993;11:368–76.

33. Crosby EJ, Wei J, Yang XY, Lei G, Wang T, Liu C-X, et al. Complimentary mechanisms of dual checkpoint blockade expand unique T-cell repertoires and activate adaptive anti-tumor immunity in triple-negative breast tumors. OncoImmunology. 2018;7:e1421891–17.

34. Movahedi K, Laoui D, Gysemans C, Baeten M, Stange G, Van den Bossche J, et al. Different Tumor Microenvironments Contain Functionally Distinct Subsets of Macrophages Derived from Ly6C(high) Monocytes. Cancer Research. 2010;70:5728–39.

35. Georgoudaki A-M, Prokopec KE, Boura VF, Hellqvist E, Sohn S, Östling J, et al. Reprogramming Tumor-Associated Macrophages by Antibody Targeting Inhibits Cancer Progression and Metastasis. CellReports. 2016;15:2000–11.

36. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. Nat Rev Cancer. 2016;16:431–46.

37. Girbl T, Lenn T, Perez L, Rolas L, Barkaway A, Thiriot A, et al. Distinct Compartmentalization of the Chemokines CXCL1 and CXCL2 and the Atypical Receptor ACKR1 Determine Discrete Stages of Neutrophil Diapedesis. Immunity. 2018;49:1062–6.

38. Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M, et al. G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. Proceedings of the National Academy of Sciences. 2009;106:6742–7.

39. Rivera LB, Meyronet D, Hervieu V, Frederick MJ, Bergsland E, Bergers G. Intratumoral Myeloid Cells Regulate Responsiveness and Resistance to Antiangiogenic Therapy. CellReports. 2015;11:577–91.

40. Lukens JR, Gurung P, Vogel P, Johnson GR, Carter RA, McGoldrick DJ, et al. Dietary modulation of the microbiome affects autoinflammatory disease. Nature. 2014;516:246–9.

41. Afonina IS, Müller C, Martin SJ, Beyaert R. Proteolytic Processing of Interleukin-1 Family Cytokines: Variations on a Common Theme. Immunity. 2015;42:991–1004.

42. Oberst A, Dillon CP, Weinlich R, McCormick LL, Fitzgerald P, Pop C, et al. Catalytic activity of the caspase-8-FLIPL complex inhibits RIPK3-dependent necrosis. Nature. 2011;:1–6.

43. Gutierrez KD, Davis MA, Daniels BP, Olsen TM, Ralli-Jain P, Tait SWG, et al. MLKL Activation Triggers NLRP3-Mediated Processing and Release of IL-1β Independently of Gasdermin-D. J Immunol. 2017;198:2156–64.

44. Molinier-Frenkel V, Castellano F. Immunosuppressive enzymes in the tumor microenvironment. FEBS Letters. 2017;591:3135–57.

45. Corthay A, Skovseth DK, Lundin KU, Røsjø E, Omholt H, Hofgaard PO, et al. Primary Antitumor Immune Response Mediated by CD4+ T Cells. Immunity. 2005;22:371–83.

46. Spear P, Barber A, Rynda-Apple A, Sentman CL. Chimeric Antigen Receptor T Cells Shape Myeloid Cell Function within the Tumor Microenvironment through IFN-γ and GM-CSF. J Immunol. 2012;188:6389–98.

47. Peranzoni E, Lemoine J, Vimeux L, Feuillet V, Barrin S, Kantari-Mimoun C, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti–PD-1 treatment. Proceedings of the National Academy of Sciences. 2018;115:E4041–50.

48. Franciszkiewicz K, Boissonnas A, Boutet M, Combadière C, Mami-Chouaib F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. Cancer Research. 2012;72:6325–32.

49. Carmi Y, Rinott G, Dotan S, Elkabets M, Rider P, Voronov E, et al. Microenvironment-Derived IL-1 and IL-17 Interact in the Control of Lung Metastasis. The Journal of Immunology. 2011;186:3462–71.

50. Lu T, Ramakrishnan R, Altiok S, Youn J-I, Cheng P, Celis E, et al. Tumor-infiltrating myeloid cells induce tumor cell resistance to cytotoxic T cells in mice. J Clin Invest. 2011;121:4015–29.

51. Kaplanov I, Carmi Y, Kornetsky R, Shemesh A, Shurin GV, Shurin MR, et al. Blocking IL-1β reverses the immunosuppression in mouse breast cancer and synergizes with anti-PD-1 for tumor abrogation. Proc Natl Acad Sci USA. 2018;4:201812266–9.

52. Schönbeck U, Mach F, Libby P. Generation of biologically active IL-1β by matrix metalloproteinases: A novel caspase-1-independent pathway of IL-1β processing. J Immunol. 1998;161:3340–6.

53. Schett G, Dayer J-M, Manger B. Interleukin-1 function and role in rheumatic disease. Nat Rev Rheumatol. 2016;12:14–24.

54. Cullen SP, Kearney CJ, Clancy DM, Martin SJ. Diverse Activators of the NLRP3 Inflammasome Promote IL-1b Secretion by Triggering Necrosis. CellReports. 2015;11:1535–48.

55. Tian T, Lofftus S, Pan Y, Stingley CA, King SL, Zhao J, et al. IL1α Antagonizes IL1β and Promotes Adaptive Immune Rejection of Malignant Tumors. Cancer Immunology Research. 2020;8:660–71.

56. van der Sluis TC, Sluijter M, van Duikeren S, West BL, Melief CJM, Arens R, et al. Therapeutic Peptide Vaccine-Induced CD8 T Cells Strongly Modulate Intratumoral Macrophages Required for Tumor Regression. Cancer Immunology Research. 2015;3:1042–51.

57. Etzerodt A, Tsalkitzi K, Maniecki M, Damsky W, Delfini M, Baudoin E, et al. Specific targeting of CD163+ TAMs mobilizes inflammatory monocytes and promotes T cell–mediated tumor regression. J Exp Med. 2019;216:2394–411.

58. Asano K, Nabeyama A, Miyake Y, Qiu C-H, Kurita A, Tomura M, et al. CD169-Positive Macrophages Dominate Antitumor Immunity by Crosspresenting Dead Cell-Associated Antigens. Immunity. 2011;34:85–95.

FIGURE LEGENDS

Figure 1. Myeloid cells are the primary source of IL1β in lung and breast tumors

(A) tSNE plot of cells pooled from human lung tumors (n=8) color-coded based on cell clusters or *IL1B* expression level. (RBCs=Red blood cells, DCs=dendritic cells)

(B) Average *IL1B* expression in different cell populations in human lung tumors based on pooled scRNA-seq data from 8 tumors.

(C) Average *IL1B* expression in different cell populations in human breast tumors based on pooled scRNA-seq data from 14 tumors.

(D) IL1β concentration in the serum of naive mice (n=12) and mice with LLC (n=20) and E0771 (n=10) tumors. Data pooled from two independent experiments. Unpaired two-tailed t-test. ** P<0.01.

(E) Expression of *Il1b* measured by qPCR in the $CD11b^+$ and $CD11b^-$ fractions isolated by magnetic cell separation from LLC (n=4) and E0771 (n=7) tumors. Representative data from two independent experiments.

(F) IL1β secretion of the $CD11b^+$ and $CD11b^-$ fractions isolated by magnetic cell separation from LLC (n=3) and E0771 (n=5) tumors measured by ELISA after 24 h *in vitro* culture. Representative data from two independent experiments.

(G) Relative frequency of major $CD11b^+$ cell populations in LLC (n=10) and E0771 (n=11) tumors measured by flow cytometry. Representative data from four independent experiments. For gating strategies, see **Supplementary Figure 2**.

(H) IL1β secretion of the major $CD11b^+$ cell populations isolated by cell sorting from LLC (n=4) and E0771 (n=3) tumors measured by ELISA after 24 h *in vitro* culture. Data pooled from four and three independent experiments, respectively.

Bar graphs show mean and SEM.

Figure 2. IL1β deletion inhibits tumor growth and reduces both systemic expansion and intratumoral accumulation of neutrophils

(A) LLC (n=9-10) and E0771 tumor growth (n=11) in $Il1b^{+/+}$ and $Il1b^{-/-}$ mice. Representative data from two independent experiments.

(B) Frequency of neutrophils in the blood of naive (n=14-15) and tumor-bearing $Il1b^{+/+}$ and $Il1b^{-/-}$ mice with LLC (n=5) and E0771 (n=10-11) tumors. Data pooled from two independent experiments.

(C) G-CSF levels measured via cytokine multiplex in the serum of naive (n=10) and tumor-bearing *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice with LLC (n=10) and E0771 (n=9-11) tumors. Data pooled from two independent experiments.

(D) Frequency of neutrophils in the bone marrow and spleen of naive and tumor-bearing *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice with LLC (n=4-5) and E0771 (n=5-9) tumors. Data pooled from two independent experiments.

(E) Frequency of neutrophils in LLC (n=7-9) and E0771 (n=8) tumors in *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice. Representative data from two independent experiments.

(F) Frequency of GFP^{+} neutrophils in LLC tumors and the blood 24 h after adoptive transfer in *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=3-4). Data from one independent experiment.

(G) Expression of neutrophil chemoattractants normalized to *Rps12* measured by qPCR in LLC tumor lysates of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=6-8). Data from one independent experiment.

(H) Frequency of neutrophils in LLC tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with anti-VEGFR2 or IgG isotype control antibody normalized to the IgG isotype-treated control group within each genotype (n=7-10). Data from one independent experiment.

(I) LLC tumor growth in *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with anti-VEGFR2 or IgG isotype control antibody (n=8-10). Data from one independent experiment.

Cell type frequencies were determined by flow cytometry. Graphs show mean and SEM. Panels A, I: two-way ANOVA with Holm-Sidak multiple comparisons test, Panels B-H: unpaired two-tailed t-test. * P<0.05, ** P<0.01, *** P<0.001, **** P < 0.0001.

Figure 3. Activation of the inflammasome and gasdermin D are dispensable for IL1β-mediated neutrophil infiltration in tumors

(A) IL1β secretion of the CD11b^{+} fraction of LLC (n=7) and E0771 tumors (n=5-6) from *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ mice measured by ELISA following 24 h *in vitro* culture.

(B) LLC (n=7) and E0771 (n=6-7) tumor growth in *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ mice.

(C) Frequency of neutrophils in LLC (n=7) and E0771 (n=5-7) tumors in *Nlrp3*$^{+/+}$ and *Nlrp3*$^{-/-}$ mice.

(D) IL1β secretion of the CD11b^{+} fraction of LLC (n=7-8) and E0771 tumors (n=6-8) from *Casp1/11*$^{+/+}$ and *Casp1/11*$^{-/-}$ mice measured by ELISA following 24 h *in vitro* culture. Unpaired two-tailed t-test. **** P < 0.0001.

(E) LLC (n=5-6) and E0771 (n=8-9) tumor growth in *Casp1/11*$^{+/+}$ and *Casp1/11*$^{-/-}$ mice.

(F) Frequency of neutrophils in LLC (n=6-10) and E0771 (n=7-8) tumors in *Casp1/11*$^{+/+}$ and *Casp1/11*$^{-/-}$ mice.

(G) Immunoblots of caspase-1 and IL-1β on cell extracts and supernatants of *Casp1/11*$^{+/+}$ and *Casp1/11*$^{-/-}$ CD11b^{+} myeloid cells isolated from LLC and E0771 tumors and cultured for 24 h *in vitro*. Mouse bone-marrow derived macrophages (BMDM) treated with LPS+ATP were used as positive controls.

(H) IL1β secretion of the CD11b^{+} fraction of LLC (n=4) and E0771 tumors (n=7-8) from *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice measured by ELISA following 24 h *in vitro* culture.

(I) LLC (n=7) and E0771 (n=7) tumor growth in *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice.

(J) Frequency of neutrophils in LLC (n=6-7) and E0771 (n=7) tumors in *Gsdmd*$^{+/+}$ and *Gsdmd*$^{-/-}$ mice.

Neutrophil frequencies were determined by flow cytometry. All panels show representative data from two or three independent experiments. Graphs show mean and SEM.

Figure 4. Tumor-infiltrating neutrophils suppress T-cell activation via nitric oxide production

(A) T-cell proliferation following co-culture of splenocytes with LLC or E0771 tumor-derived neutrophils in a 1:1 ratio, measured via ^{3}H-thymidine incorporation (c.p.m.: count per minute; n=3, data pooled from three independent experiments).

(B) T-cell proliferation following co-culture of splenocytes with LLC tumor-derived *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ neutrophils in a 1:1 ratio, measured via ^{3}H-thymidine incorporation (c.p.m.: count per minute; n=3, data pooled from three independent experiments).

(C) T-cell proliferation following co-culture of splenocytes with LLC tumor-derived neutrophils in a 1:1 ratio in the presence of NOS inhibitor (NOS inh.; L-NAME), arginase inhibitor (Arg. inh.; Nor-NOHA), and superoxide dismutase (SOD), measured via CellTrace dilution (n=4, data pooled from four independent experiments).

(D) IFNγ secretion in the supernatants of splenocyte-neutrophil co-cultures from (C), measured via ELISA (n=4, data pooled from four independent experiments).

(E) NO production in the supernatants of splenocyte-neutrophil co-cultures from (C), based on nitrite levels measured via Griess test (n=4, data pooled from four independent experiments).

Graphs show mean and SEM. Unpaired two-tailed t-test. * P<0.05, ** P<0.01, *** P<0.001, **** P < 0.0001.

Figure 5. IL1β deletion relieves immune suppression in the TME

(A)-(C) Frequency of the indicated cell populations in LLC (n=7-9) and E0771 (n=6-7) tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice.

(D) Representative flow cytometry plot of CD44 and CD62L expression on CD8^{+} T cells in LLC tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice.

(E) Ratio of effector (CD44^{+}CD62L^{-}) and naive (CD44^{-}CD62L^{+}) T cells in LLC (n=6-8) and E0771 (n=6-7) tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice. For frequencies of effector and naive T cells see **Supplementary Figure 7A**.

(F) Frequency of IFNγ^{+} cells and expression levels of CD44 within the CD8^{+} T-cell population in LLC tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=9-10).

(G) Ratio of effector (CD44^{+}CD62L^{-}) and naive (CD44^{-}CD62L^{+}) T cells in LLC tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with anti-VEGFR2 or IgG isotype control antibody normalized to the IgG isotype-treated control group within each genotype (n=7-9). Data from one independent experiment. For frequencies of naive and effector T cells, see **Supplementary Figure 7C**.

(H) Frequency of the monocytes in LLC (n=7-10) and E0771 (n=6-7) tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice.

(I) T-cell proliferation following co-culture of splenocytes with LLC tumor-derived monocytes in a 1:1 ratio, measured via ^{3}H-thymidine incorporation (c.p.m.: count per minute; n=3, data pooled from three independent experiments).

(J) Frequency of TAMs in LLC (n=7-10) and E0771 (n=6-7) tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice.

(K) Frequency of MHC-II^{high} TAMs in LLC (n=7-10) and E0771 (n=6-7) tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice.

Cell-type frequencies and surface protein expression were determined by flow cytometry. For gating strategies see **Supplementary Figure 2**. All panels show representative data from two independent experiments unless otherwise noted. Graphs show mean and SEM. Unpaired two-tailed t-test. * P<0.05, ** P<0.01, *** P<0.001.

Figure 6. CD8^{+} T cells drive antitumor immunity in IL1β-deficient mice

(A) LLC tumor growth in anti-CD8-treated and untreated *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=9-10).

(B)-(E) Frequency of indicated cell populations in LLC tumors in anti-CD8–treated and untreated *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=9-10).

(F) CD40 expression on MHC-II^{high} TAMs in LLC tumors in anti-CD8-treated and untreated *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice (n=9-10).
(G) Correlation of CD44^{+}CD62L^{-} effector CD8^{+} T-cell and MHC-II^{high} TAM abundance in LLC (n=9) and E0771 (n=8) tumors. Pearson *r* values and *P* values are indicated in the graphs.
Cell type frequencies and surface protein expression were determined by flow cytometry. Data from one independent experiment. Graphs show mean and SEM. Panel A: two-way ANOVA with Holm-Sidak multiple comparisons test, Panels B-F: unpaired two-tailed t-test, Panel G: Pearson correlation. * P<0.05, ** P<0.01, *** P<0.001, **** P < 0.0001.

Figure 7. Macrophages are required for the antitumor CD8^{+} T-cell response in IL1β-deficient mice
(A) LLC tumor growth in *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with PLX5622 or control diet (n=7-8).
(B)-(G) Frequency of indicated cell populations in LLC tumors of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with PLX5622 or control diet measured by flow cytometry (n=7-8).
(H) Expression of selected chemokines normalized to *Rps12* measured by qPCR in LLC tumor lysates of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with PLX5622 or control diet (n=7-8) and concentration of the same chemokines measured by ELISA/multiplex immunoassay in total tumor supernatants after 24 h *in vitro* culture.
(I) Expression of selected immunostimulatory genes normalized to *Rps12* measured by qPCR in LLC tumor lysates of *Il1b*$^{+/+}$ and *Il1b*$^{-/-}$ mice treated with PLX5622 or control diet (n=7-8).
Data from one independent experiment. Graphs show mean and SEM. Panel A: two-way ANOVA with Holm-Sidak multiple comparisons test, Panels B-I: unpaired two-tailed t-test. * P<0.05, ** P<0.01, *** P<0.001, **** P < 0.0001.