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T cells Instruct Immune Checkpoint Inhibitor Therapy Resistance in Tumors Responsive to IL-1 and TNF α Inflammation

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Abstract

Resistance to immune checkpoint inhibitors (ICIs) is common, even in tumors with T cell infiltration. We thus investigated consequences of ICI-induced T cell infiltration in the microenvironment of resistant tumors. T cells and neutrophil numbers increased in ICI-resistant tumors following treatment, in contrast to ICI-responsive tumors. Resistant tumors were distinguished by high expression of IL-1 Receptor 1 (IL1R1), enabling a synergistic response to IL-1 and TNF α to induce G-CSF, CXCL1, and CXCL2 via NF- κ B signaling, supporting immunosuppressive neutrophil accumulation in tumor. Perturbation of this inflammatory

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resistance circuit sensitized tumors to ICIs. Paradoxically, T cells drove this resistance circuit via TNF α both *in vitro* and *in vivo*. Evidence of this inflammatory resistance circuit and its impact also translated to human cancers. These data support a mechanism of ICI resistance, wherein treatment-induced T cell activity can drive resistance in tumors responsive to IL-1 and TNF α , with important therapeutic implications.

Keywords

Cancer Immunotherapy; Immune Checkpoint Inhibitors; Immunotherapy Resistance; MDSCs; IL-1 Receptor Signaling in Cancer; TNF α Signaling; NF- κ B Signaling; T cell-mediated Immunosuppression

Introduction

Immune checkpoint inhibitors (ICIs) are not effective in many patients, and principles determining treatment response versus failure remain incompletely understood. Among immunologic programs governing response to ICIs is T cell infiltration into the tumor microenvironment (TME), where “inflamed” or “hot” tumors, such as those with microsatellite instability-high (MSI-H) status, exhibit higher response rates (1). Nevertheless, resistance to ICIs occurs commonly even in T cell-infiltrated tumors (2,3); yet there is a limited understanding of the underlying mechanisms. For example, MSI-H tumors demonstrate increased neoantigen load and T cell infiltration, yet not all respond to ICIs (2,4,5). We reasoned that the development of T cell-infiltrated tumor models would enable the study of T cell states and their interactions with the TME during ICI resistance, despite T cell infiltration.

Inflammation is a broad term encompassing multiple processes that can promote or prevent tumor progression. For instance, inflammation can be driven by the transcription factor nuclear factor- κ B (NF- κ B), which is activated in multiple cell types in the TME by stimuli including the cytokines TNF α , IL-1 α , and IL-1 β (6). NF- κ B signaling can recruit and activate immunosuppressive myeloid cells to suppress T cell cytotoxicity (7) (8). Accordingly, its inhibition in the TME can improve immunologic control of tumors (9). Of note, T cells and NF- κ B-mediated inflammation can co-exist in the TME, yet their relationship is incompletely defined. Much of the recent research efforts have focused on T cell exhaustion from chronic antigen stimulation and immunosuppressive signals, including those provided by myeloid cell subsets (10). However, it is unclear whether conventional CD4⁺ or CD8⁺ T cells can themselves regulate inflammation in the TME and whether the interplay between these factors affects immunotherapy response.

Here, we use T cell-infiltrated mouse syngeneic tumor models of ICI response and resistance to discover that T cells can drive a TNF α and IL-1-mediated immunosuppressive inflammatory response from IL1R1-expressing tumor cells, mediating ICI resistance. These findings refine the paradigm of T cell responses to ICI therapy with substantial clinical implications, revealing an opportunity to dissociate the tumor-promoting aspects of T cell activity from their tumoricidal functions for therapeutic gain.

Materials and Methods

Cell lines

Parental LLC cells (RRID:CVCL_4358, sex: male) were gifted by Dr. Ross Levine in 2015 (Memorial Sloan Kettering Cancer Center). B16F10 cells (RRID:CVCL_0159, sex: male) were gifted by Dr. Jeffrey Bluestone in 2018 (University of California, San Francisco (UCSF)). EO771 cells (RRID:CVCL_GR23, sex: female) were gifted from Dr. Robin Anderson in 2015 (Peter MacCallum Cancer Center). EMT6 cells (RRID:CVCL_1923, sex: female) were purchased from ATCC in 2023. LLC, B16F10, and EO771 parental and derivative cell lines were cultured in DMEM (UCSF Media Production CCFAA005) supplemented with 10% fetal calf serum (Omega Scientific, FB-02), 10mM HEPES (UCSF Media Production CCFGL002), and 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (UCSF Media Production CCFGK004). EMT6 cell lines were cultured in Waymouth's MB 752/1 medium (Fisher Scientific, 11-220-035) with 2mM L-glutamine (UCSF Media Production CCFGB002), 15% fetal calf serum and 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (UCSF Media Production CCFGK004). For generation of MSH2 and IL1R1 knock out cell lines, a combination of two targeting guide crRNAs (Dharmacon), or scrambled nontargeting control, were mixed with tracrRNA and recombinant Cas9 protein (QB3 Macrolab, UC Berkeley) at 1:1:2:2 molar ratio and incubated at 37°C to form the ribonucleoprotein complex. This was then mixed with 1ul of 100uM single-stranded oligodeoxynucleotides (ssODN) enhancer (IDT), and 3 × 10⁵ trypsinized cells in electroporation buffer and electroporated using the Lonza 4D 96-well electroporation system (V4SC-9096). Nucleic acid sequences, electroporation buffer type and electroporation code are found in Supplementary Table S1. After a 15 minute recovery in a 37°C CO₂ incubator, cells were expanded in culture at subculture ratios of 1:10–1:15 every 48 hours. MSI-H cell lines used have undergone 60 passages in total. All cell lines underwent mycoplasma testing through ATCC with negative results as of November 1, 2023. Cells were used for experiments within 2–3 passages of thaw.

Following the initial experiments included in this manuscript, cell line verification testing was performed via short tandem repeat (STR) analysis on the syngeneic mouse tumor cell lines used, via ATCC. All cell lines were successfully matched, but it revealed that a cell line we had previously attributed to be the AT3 breast cancer cell line (RRID:CVCL_VR89) was in fact the LLC cell line (RRID: CVCL_4358), along with its MSS and MSI-H derivative lines (11). To further ensure that the cell line was correctly identified as LLC, we performed a PCR assay for PyMT transgene detection, which should be present in the AT3 line because it was derived from an MMTV-PyMT mouse. We confirmed this to be true in an independently purchased reference AT3 cell line, now available from Millipore, but not in our LLC cell lines (Supplementary Fig. S1). Furthermore, we used exome sequencing data from our cell line to verify the presence the of two point mutations reported in the literature to be found in the LLC line (12,13) (Supplementary Table S2).

Animals and *in vivo* treatments

All mice were housed in an American Association for the Accreditation of Laboratory Animal Care- accredited animal facility and maintained in specific pathogen-free

conditions with typical light/dark cycles and standard chow. Animal experiments were approved by and conducted in accordance with UCSF Institutional Animal Care & Use Program protocol AN184195. Female C57BL/6J (RRID:IMSR_JAX:000664) and BALB/cJ (RRID:IMSR_JAX:000651) mice between 8 and 12 weeks old were purchased from the Jackson Laboratory. B16F10 (1×10^5 cells per 100 μ L), LLC (5×10^5 cells per 100 μ L), EO771 (8×10^6 cells per 100 μ L), or EMT6 (5×10^5 cells per 100 μ L) cells in serum-free DMEM were transplanted into the right subcutaneous flank for B16F10 melanoma, the left 4th mammary fat pad for EO771 and EMT6, and either the flank or the mammary fat pad for LLC. Unless indicated as in Figure 1 and Supplementary Fig. S2 for subcutaneous implantation, LLC cells were implanted into the mammary fat pad for all experiments. Different cell numbers for inoculation were chosen for each model to address disparities in *in vivo* tumor growth rates. TCR transgenic OT-I CD45.1 mice (RRID:IMSR_JAX:003831) were bred at our facility. Female B6.129S-*Tnf*^{tm1Gkl/J} mice (RRID:IMSR_JAX:005540, *Tnf*^{-/-} in manuscript), and B6.129X1(Cg)-*Csf3r*^{tm1Link/J} mice (RRID:IMSR_JAX:017838, *Csf3r*^{-/-} in manuscript) between 7–8 weeks of age were purchased from the Jackson Laboratory. For T cell-specific *Tnf* knockout mice, *Tnf*(*fl/fl*) mice(14) crossed to the Ai14 Cre reporter (RRID:IMSR_JAX:007914) were obtained (gift from Dr. Ophir Klein, UCSF); these mice were crossed to *Cd4*-cre mice purchased from the Jackson Laboratory (RRID:IMSR_JAX:022071) to obtain T cell-specific *Tnf* knockout mice. Control *Cd4-cre*(*WT*) mice included mice with *Tnf*(*fl/fl*) or *Tnf*(*fl/+*) genotypes. All mice were heterozygotes or homozygotes for the Ai14 Cre reporter allele.

For all anti-PD-1 and anti-CTLA4 treatments, 250 μ g of anti-PD-1 clone RMP1–14 (Bio X Cell BE0146, RRID:AB_10949053) and 200 μ g of anti-CTLA4 clone 9H10 (Bio X Cell BE0131, RRID: AB_10950184) in 100 μ L PBS (Corning, 21–031-CV) were injected intraperitoneally at days 3, 6, 9 and 12 post tumor injection. For IL1R1 inhibition, 200 μ g of antibody clone JAMA-147 (Bio X Cell BE0256, RRID: AB_2661843) was injected intraperitoneally every 3 days starting at day 3 post tumor injection. For Thy1 depletion, 200 μ g of antibody clone M5/49.4.1 (Bio X Cell BE0076, RRID: AB_1107681) was injected intraperitoneally at days –2, 0, 7 and 14 relative to tumor injection. For anti-CD4 (Bio X Cell BP0003–1, RRID: AB_1107636) and anti-CD8 (Bio X Cell BP0061, RRID: AB_1125541) treatments, 400 μ g of each antibody was injected intraperitoneally on days –1, 3, 7, 10, 13, and 16 post tumor injection. Where indicated by “IgG”, isotype-matched control IgG antibodies were used at the same concentrations as experimental antibodies (Bio X Cell BE0089, RRID: AB_1107769; BE0087, AB_1107782). For G-CSF neutralization, 10 μ g of antibody (R&D Systems MAB414, RRID: RRID:AB_2085954) was injected intraperitoneally starting at day 5 post tumor implantation.

MSI PCR quantification

Microsatellite instability in the mouse cell lines was quantified in triplicate using a panel detecting four microsatellite markers (15). Genomic DNA from cell pellets was isolated using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, K0722) according to manufacturer protocol. 20 ng of genomic DNA was used as input for a PCR reaction using Platinum Taq (Thermo Fisher Scientific, 10–966-018) with 2mM MgCl₂, 0.2 μ M each primer (Supplementary Table S3), and 200 μ M dNTP (New England Biolabs,

N0447L). The cycling profile included: 1 cycle at 94 °C for 4 minutes, 94 °C for 30 seconds, 56 °C for 45 seconds and 72 °C for 30 seconds for a total of 35 cycles, and a final extension at 72 °C for 6 minutes (MJ Research PTC-225 thermocycler). Samples were submitted for fragment analysis by Genewiz, and analyzed using PeakScanner 2 (Thermo Fisher Scientific).

Next generation sequencing and neoantigen priority score analysis

Whole exome and bulk RNA sequencing were performed with flash frozen cell pellets submitted to MedGenome (Foster City, CA). For whole exome sequencing, the library was prepared using the Agilent SureSelectXT Mouse All Exon Kit (G7550) from 3 µg of gDNA, and sequenced on NovaSeq 6000 with paired-end 100 read length. Data was processed using Fastq preprocessing using FastQC v0.11.8 and adapter trimming with fastq-mcf v1.05. 99.66% of the total reads aligned to the reference genome. 87.77% of the aligned reads passed the alignment filter (mapping quality $\geq$ 29 and insert size between 100–1000). Reads after duplicate removal, indel realignment and recalibration featured more than 98.49% coverage and 86.67–114.47X average depth. Somatic variant calling was performed using Strelka (v2.9.10). Default settings were used for variant calling. For realignment and base-recalibration, dbsnp150 variants were used. The identified somatic variants were further filtered and only passed and on-target variants were considered for downstream analysis. Default parameters provided by Strelka were used to filter passed somatic variants. The on-target variants were filtered based on the coordinates of the target regions provided by the vendor. RNA sequencing was performed using two biological replicates per cell line using Illumina TruSeq Stranded Total RNA library preparation, and sequenced using NovaSeq with target of 50 million total reads per sample. 86.18–91.46% of total reads for each sample had Quality scores of > 30 . Kallisto (v0.46.1) was used to quantify abundances of the transcripts in transcripts per million (TPM) with the strand specific read processing argument ‘--rf-stranded’.

For neoantigen priority score analysis, the MuPeXI pipeline was used as described (16). The somatic allele frequency estimate was extracted from the strelka vcf records following the Strelka user guide and added to the vcf files. The vcf files with allele frequency, the mean expression output files from Kallisto and the GRCm38 references were given as input to the MuPeXI pipeline (v1.2.0) with the species option set to mouse and the HLA alleles set to ‘H-2-Kb,H-2-Db’.

Mass cytometry antibodies

All mass cytometry antibodies and staining concentrations are listed in Supplementary Table S4. Primary conjugates of mass cytometry antibodies were prepared using the Maxpar antibody conjugation kit (MCP9 for Cadmium, 201110A or X8 for non-Cadmium metals, 201300; Fluidigm) according to the manufacturer’s recommended protocol. After labeling, antibodies were diluted in HRP-Protector Peroxidase Stabilizer (Candor, 222 050) for Cadmium conjugated antibodies, and Candor PBS Antibody Stabilizer (131 050) supplemented with 0.02% sodium azide for non-Cadmium conjugated antibodies. Antibodies were stored at 4°C long term.

Cell preparation

Tissue preparations from tumor, tumor-draining lymph node, spleen and lymph node were performed simultaneously. After euthanasia by CO₂ inhalation, peripheral blood was collected via cardiac puncture and transferred into heparin-coated vacuum tubes. Blood was diluted into ACK lysis buffer (Gibco A1049201), incubated for 3 minutes on ice then quenched with 10 mL PBS with 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma EDS-100G) and 0.5% bovine serum albumin (BSA, Sigma A7030–500G) (PBS/EDTA/BSA). Spleens and tumor-draining inguinal lymph nodes were homogenized with a syringe pusher over 70 µm filter (Celltreat, 229483) with PBS/EDTA. Tumors were finely minced and digested in RPMI-1640 (UCSF Media Production, CCFAE001) with 4 mg ml⁻¹ collagenase IV (Worthington, LS004189) and 0.1 mg ml⁻¹ DNaseI (Sigma, DN25–1G) in a scintillation vial with a micro stir bar (Thermo Scientific, 1451363) for 30 minutes at 37 °C. After digestion, cells were filtered twice over 70 µm filters with PBS/EDTA. All tissue samples were centrifuged at 500 *g* for 5 minutes at 4°C, resuspended in PBS/EDTA, and mixed 1:1 with PBS/EDTA containing 100 mM cisplatin (Sigma, P439425MG) for 60 seconds before quenching 1:1 with PBS/EDTA/BSA. Cells were centrifuged again, resuspended in PBS/EDTA/BSA at a density between 1 × 10⁶ and 1 × 10⁷ cells per mL, fixed for 10 minutes at room temperature using 1.6% formaldehyde final (Pierce, 28908), washed twice with PBS/EDTA/BSA and frozen in 100ul buffer at –80°C. All samples were processed on ice with buffers at 4 °C.

Mass cytometry

2 × 10⁶ cells from tumor and 1 × 10⁶ cells from other organs from each animal were barcoded with distinct combinations of stable Pd isotopes in 0.02% saponin (Sigma, SAE0073) in PBS as previously described (17). Cells were washed twice with cell-staining media (PBS with 0.5% BSA (Sigma A7030) and 0.02% NaN₃ (Sigma 712895G) and combined into a single 15 mL conical tube. For staining, cells were resuspended in cell-staining media, blocked with FcX (Biolegend 101320, RRID:AB 1574975) for 5 minutes at room temperature, then incubated for 30 minutes at room temperature on a shaker, with surface marker antibody cocktail in 1 mL total volume for tumor, and 500 µL volume for blood, spleen, and tumor-draining lymph node. Cells were washed in cell-staining media then permeabilized with methanol (Sigma, 179337–2.5L) for 10 minutes at 4 °C, then washed again in cell-staining media. Cells were then incubated with the intracellular antibody cocktail in the same volume as surface markers for 30 minutes at room temperature on a shaker. Cells were washed in cell-staining media then incubated with 1 mL PBS containing 1:5000 Iridium intercalator (Fluidigm 201192A) and 4% final formaldehyde (Pierce, 28908). Cells were maintained in this final buffer for 2 days prior to sample acquisition on a CyTOF 2 mass cytometer (Fluidigm) using Maxpar Cell Acquisition Solution (Fluidigm 201241). 1–5 × 10⁵ cells were acquired per each sample. Data normalization was performed using EQ Four Element Calibration Beads (Fluidigm, 201078) included during sample acquisition, using Normalizer v0.3 (Nolan lab). Debarcoding was performed using Single Cell Debarcoder v0.2 (Nolan lab).

Mass cytometry data analysis

Each antibody clone and lot were titrated to optimal staining concentrations using primary mouse samples containing positive and control cell populations. Immune cell subsets were manually gated using the strategy shown in Supplementary Fig. S3. Input cells for DAsq were exported from manually gated cells, with the same number of cells imported per sample. UMAP dimensionality reduction using the package “uwot” (v.0.1.16) was performed using all available protein markers except Ki-67. Differential abundance (DA) analysis was performed using the package “DAsq” (v.1.0.0) using the top 10 principal components determined from the hyperbolic arcsine (asinh) expression with a cofactor 5 for each marker for each cell. Top DA cells were determined by thresholding cells with positive or negative DA measures beyond the extent of random distribution.

For pairwise correlation analysis involving neutrophil clusters, clusters were first identified using UMAP and Phenograph (18) using cells from each organ, with manual annotation of clusters representing neutrophils. Cells from LLC MSI-H or B16F10 MSI-H tumor bearing animals were used for further analysis. For each sample, the cluster frequencies were calculated (percent of total singlets for tumor, and percent of live CD45⁺ cells for other organs). Spearman pairwise correlation was performed as previously described (11), and filtered for correlations involving neutrophils with $r > 0.7$ or < -0.7 .

For most analyses, batch correction was not applied as aliquots of the same antibody cocktail were used to acquire the entire dataset for analysis. For experiments acquired separately with antibody cocktails generated at different times, batch correction was applied using the package CyCombine (v. 0.2.16) using an anchor sample common across the two CyTOF runs. The CyCombine batch correction module addresses intra-sample heterogeneity by considering each cell as its own sample and minimizes the batch effects for groups of similar cells, one group at a time. For quantification of immune subsets using subset-specific immune markers, cells were quantified as a percentage of total singlets, including live and dead cells, and CD45⁺ and CD45⁻ cells, to reflect the density of that cell type among cells comprising the harvested organ.

Cytokine quantification

For quantification of cytokines from tumor, minced tumor was lysed using RIPA buffer containing protease inhibitor (Cell Biolabs AKR-190) on ice for 10 minutes, followed by centrifugation at 14,000 rpm for 10 minutes at 4 °C. Supernatant containing cell lysate was collected and normalized to the same A595 using the Bio-Rad Protein Assay (5000006). Samples were frozen and stored at -80 °C until quantification. For quantification of cytokines from plasma, freshly isolated peripheral blood was centrifuged for 10 minutes at 4 °C at 1,000 *g*. 100 µL of supernatant plasma was mixed 1:1 with 100 µL of PBS/EDTA, and frozen until quantification. For multiplex cytokine quantification, lysates or plasma were submitted for mouse discovery assays available through Eve Technologies (Calgary, AB). For G-CSF and CXCL1 ELISA, tumor lysates were analyzed using the mouse G-CSF ELISA Kit (Abcam ab197743) and CXCL1 ELISA Kit (Abcam ab216951) per manufacturer protocol. Principal component analysis was performed using cytokine concentrations as input. Values were log adjusted then analyzed using the function PCA from the R package

FactoMineR (v2.4). Synergy analyses were performed using SynergyFinder Plus web interface (synergyfinder.org (19)), following normalization of G-CSF concentrations with 100% representing the maximum value for each cell line.

Flow cytometry and cell sorting

All flow cytometry antibodies and concentrations used for analysis can be found in Supplementary Table S5. Tissue samples were initially processed as for CyTOF, and cells were stained for viability with Zombie-NIR stain (Biolegend 423106). Cell surface staining was performed in cell-staining media (PBS with 0.5% BSA and 0.02% NaN_3) for 20 minutes at room temperature. For intracellular staining, cells were permeabilized and fixed using eBioscience Foxp3/Transcription Factor Staining Buffer Set prior to staining in 1X permeabilization buffer (Invitrogen 00-5523-00). Stained cells were analyzed with a CytoFLEX flow cytometer (Beckman Coulter) or an LSR II flow cytometer (BD Biosciences). Stained live cells were sorted using FACSAria cell sorters (BD Biosciences) using a 100 μm nozzle and event rate of 3000–4000 per second into collecting vessels kept at 4 °C until downstream use. Cell sorting gating strategies are shown in Supplementary Fig. S4. Flow cytometry data analysis was performed using CellEngine using automatically updated release versions (cellengine.com).

In vitro cellular assays

For tumor-T cell co-culture, CD8^+ T cells and tumor cells were sorted as above. Prior to plating, 96 well flat bottom plate wells were coated with anti-CD3 (clone 145–2C11, 10 $\mu\text{g}/\text{mL}$ final in PBS, UCSF Monoclonal Antibody Core) for 4–6 hours at 37°C and washed twice with PBS. For each cell population in the co-culture, 3.5×10^4 cells were plated together with anti-CD28 (clone 37.51, 3 $\mu\text{g}/\text{mL}$ final, UCSF Monoclonal Antibody Core) and any neutralizing antibodies (25 $\mu\text{g}/\text{mL}$ final; anti-TNF α : Bio X Cell BE0058 RRID: AB_1107764; anti-GM-CSF: Bio X Cell BE0259 RRID: AB_2687738; anti-FasL: Bio X Cell BE0319 RRID: AB_2819046) in 200 μL final volume of T cell media (RPMI 1640 (UCSF Media Production, CCFAE001) with 10% FCS, 25mM HEPES (UCSF Media Production CCFGL002), 2mM L-glutamine (UCSF Media Production CCFGB002), 33 μM 2-mercaptoethanol (MP Biomedicals ICN19024283), 100 U mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin (UCSF Media Production CCFGK004)). After 24 hours of culture in a 37 °C incubator, the supernatant was collected for G-CSF ELISA.

For OT-I T cell MDSC assay, $\text{CD45}^+ \text{CD11b}^+ \text{Ly6G}^+$ neutrophils were sorted as above from LLC MSI-H tumor, while OT-I cells were harvested from spleen of OT-I CD45.1 mice (RRID:IMSR_JAX:003831) by processing over a 70 μm filter. In 96 well U-bottom plates, 1×10^5 OT-I splenocytes and 2.5×10^4 sorted neutrophils were combined with or without SIINFEKL peptide (Genscript RP10611) at 0.4 ng/mL in 200 μL T cell media. Cells were incubated in a 37 °C CO_2 incubator for 42 hours, then BrdU was added to the wells at 10 μM final concentration for an additional 6 hours (Invitrogen 8811–6600-42). Cells were collected and stained with Zombie NIR, anti-TCR β , anti-CD45.1, and anti-CD8a then anti-BrdU as per manufacturer protocol for the eBioscience BrdU Staining Kit for Flow Cytometry (Invitrogen 8811–6600-42). Cells were analyzed using an LSR II flow cytometer.

For assessment of G-CSF and CXCL1 by ELISA following TNF α , IL-1 α , or IL-1 β stimulation, 10 ng/ml of cytokine (Biolegend 575202, 575002, 575102) was added (unless otherwise stated) to cell culture media 24 hours prior to collection of supernatant for analysis using ELISA kits for G-CSF (Abcam, ab197743), or CXCL1 (Abcam, ab216951). For analysis of I κ B α by flow cytometry, TNF α and IL-1 α (1 ng/ml) were added to culture media 20 minutes prior to cell harvest and processing for staining and flow cytometry as above. For NF- κ B inhibition, QNZ (Selleck Chemicals S4902) was added at 0.5 μ M final concentration 1 hour prior to addition of TNF α and IL-1 α .

For quantification of H-2Kb or H-2Db expression on tumor cell lines by flow cytometry, cells were treated with or without IFN γ (62.5 ng/mL for 48 hours, Biolegend 575302).

Quantitative reverse transcription PCR

Cells were sorted into 500 μ L TRIzol reagent (Thermo Fisher Scientific). RNA was isolated using Direct-zol 96 MagBead RNA kit (Zymo) per manufacturer protocol. cDNA was generated using Iscript Reverse Transcription Supermix (Bio-Rad) per manufacturer protocol. Real-time PCR was performed using Taqman primers for *Csf3* (Thermo Fisher Scientific Mm00438334_m1), *Cxcl1* (Mm04207460_m1), *Il1a* (Mm00439620_m1), *Il1b* (Mm00434228_m1) and *Gapdh* (Mm99999915_g1) on a Bio-Rad C1000 Thermal Cycler. For breast cancer organoid RT-qPCR, Taqman primers for *IL1R1* (Hs00991010_m1), *TNFRSF1A* (Hs01042313_m1), *CXCL8* (Hs00174103_m1), and *UBC* (Hs05002522_g1) were used. $\Delta\Delta C_q$ values were calculated against *Gapdh* or *UBC* then against biological control. In addition to biological replicates indicated, quantification for each replicate was performed in technical triplicate.

Western blot

Protein lysates were generated using RIPA buffer containing protease inhibitor (Cell Biolabs) on ice for 10 minutes, followed by centrifugation at 14,000 rpm for 10 minutes at 4 °C. Supernatant containing cell lysate was collected, and 20 μ g of lysate was loaded onto NuPAGE 4–12% Bis-Tris protein gel (Thermo Fisher Scientific), with even loading assessed by Ponceau S staining (Cell Signaling Technology 59803). Gels were run in NuPAGE MOPS SDS running buffer, then transferred to a nitrocellulose blot in NuPAGE Transfer Buffer. Blots were incubated with PBS-T (0.1% Tween 20) with 5% BSA, then incubated with primary antibody against MSH2 (clone FE11, Thermo Fisher Scientific, 1:500 dilution, RRID: AB_2533139) overnight at 4 °C. After washing in PBST, HRP goat anti-mouse IgG secondary antibody (RRID:AB_562588) was added in PBS-T and incubated for 1 hour. Blot was developed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) and acquired using a Bio-Rad ChemiDoc system.

Organoid culture

Organoid cultures were generated from breast tumor samples collected at Brigham & Women's Hospital and processed on the day of surgery. This study was reviewed by the Harvard Medical School Institutional Review Board and deemed not human subjects research, and patients gave their informed consent to have tissue used for scientific research. Viable tissue was minced and placed in a 50 mL conical tube that contained: 18 mL

AddF+++ (Advanced DMEM/F12 containing Glutamax (Invitrogen 12634–034), HEPES (Invitrogen 15630–056), and antibiotics (Invitrogen 15140–122) + 2 mL collagenase (10 mg/ml, Sigma, C9407). The tube was placed into an orbital shaker at 37 °C for 30 min. After digestion, 30 mL AddF+++ plus 2% FBS was added to the tube and the pellet was collected, resuspended in BME type 2 (R&D Systems, 353300502), and cultured as previously described (20). Briefly, a drop of 50 μ L of this suspension was placed in the center of a well in a 24-well non-treated culture plate (Corning, 3738) and transferred to a 37°C, 5% CO₂ incubator to harden for 20 min. Subsequently, 500 μ L Type 1 breast cancer organoid culture medium (21) was added to the well. Medium was changed every 2–3 days. TrypLE Express (Gibco, 12604013) was used for passaging approximately every 1–2 weeks. Organoids were viably frozen by Recovery™ Cell Culture Freezing Medium (Gibco, 12648010). TORG40 was generated from a triple-negative inflammatory breast cancer, and TORG139 was generated from a triple-negative breast cancer.

Human breast cancer single-cell RNAseq analysis

Data was obtained from <https://lambrechtslab.sites.vib.be/en/single-cell> and is deposited at the European Genome-phenome Archive (EGA) under study no (EGAS00001004809) and under data accession no. EGAD00001006608. Data was imported to Seurat (v. 4.1.0) as a Seurat Object. For quality control, the data was previously filtered by excluding all cells expressing <200 or >6,000 genes, cells that contained <400 unique molecular identifiers (UMIs), and cells with >15% mitochondrial counts. Raw counts were normalized using SCTransform (v. 0.3.3) and glmGamPoi (v. 1.2.0) with regression of cell cycle genes, percent mitochondrial counts, and number of UMIs (counts). Principal Component Analysis (PCA) was performed using variable genes. Cell clusters were defined using Seurat's FindNeighbors (dim 1–20) and FindClusters (resolution 0.5) and were visualized using Uniform Manifold Approximation and Projection (UMAP). Cluster annotations for major cell types were provided in the metadata and aligned well with cluster assignment. The T cell cluster was fine annotated into CD4⁺, CD8⁺, and Tregs based on expression of *CD4*, *CD8A*, *CD8B*, and *FOXP3*. The data was then filtered to CD8⁺ T cells, tumor cells, CD4⁺ T cells, and myeloid cells.

CD8⁺ T cells were reclustered using the above parameters. Clusters were defined using the FindAllMarkers function in Seurat which identified differentially expressed genes (DEGs). Genes must be expressed in 25% of cells. DEGs between specific clusters, found using FindMarkers, were plotted using EnhancedVolcano (v. 1.8.0). For genes of interest, the density, or for two genes the joint density, was plotted in UMAP space using Nebulosa (v. 1.0.2). DEEnrichRPlot (Seurat and enrichR (v. 3.0)) was run to determine GO terms enriched in specific clusters using the GO_Biological_Process_2021 list.

The proportion of cells in a given cluster from “Pre” or “On” treatment was calculated on a per patient basis by normalizing for the number of CD8⁺ T cells per patient. The frequency of any cluster of CD8⁺ T cells compared “Pre” vs “On” treatment was normalized to the number of cells in the entire tumor. Paired differences in cluster frequency were tested using paired Wilcoxon. Tumor cell scoring for NF- κ B/TNF α signaling was performed using the gene set “HALLMARK_TNFA_SCORING_VIA_NFKB” obtained from Molecular

Signatures Database or MSigDB and the AddModuleScore function in Seurat. Differences in score were calculated “Pre” vs “On” treatment.

CIBERSORTx analysis

COAD bulk RNA sequencing data from TCGA (GDC Data Portal, Project ID TCGA-COAD, Data type=RNAseq) was analyzed using the CIBERSORTx platform (cibersortx.stanford.edu). Proportion of neutrophils as percentage of immune cells in sample was deconvoluted using the LM22 signature matrix and default parameters. Patients were stratified as MSS or MSI-H using a published dataset (22). A total of 152 MSS and 36 MSI-H patient samples were analyzed. Neutrophil proportions from the CIBERSORTx output was compared across the two patient groups using a two-tailed Mann-Whitney test using GraphPad Prism (v10.3.1).

Human peripheral blood neutrophil to lymphocyte ratio analysis

Data from patients with MSI-H tumors from our institutional database of tumors submitted for next generation sequencing were identified (UCSF IRB 18–24633, conducted in accordance with the Belmont Report, patients provided written informed consent for research). The dataset was further filtered to non-central nervous system tumors and patients receiving ICI therapy (included single or combinations of pembrolizumab, nivolumab, atezolizumab, or ipilimumab) during their course of treatment. Among this group of patients, those who experienced radiologist-confirmed imaging-based progression were identified, with the date of progression defined as the date of imaging. 6-week averaged values were derived from all available complete blood count with differential test results (allowing quantification of neutrophils) from prior to progression of disease. For each patient and timepoint, the absolute neutrophil count was divided by the absolute lymphocyte count to derive the neutrophil to lymphocyte ratio (NLR).

Human lung cancer dataset analysis

Log2 normalized TPM and relevant clinical data were downloaded from EGA (<https://ega-archive.org/studies/EGAS00001005013>) for the OAK clinical trial (23). Single sample gene signature scores were assigned to bulk RNA-seq samples from OAK by taking the median z-score of the genes that comprise the signature (“*IL1R1*”, “*TNFRSF1A*”, “*IL1A*”, “*IL1B*”, “*TNF*”, “*CD69*”, “*CXCL1*”, “*CXCL2*”, “*CXCL8*”, “*NFKB1*”, “*NFKB2*”, “*CS F3*”). Patients were grouped into “high” or “low” expression based on the cohort-wide median expression of the signature and also CD8A expression. For resultant survival analyses in the atezolizumab arm of OAK, the log-rank test was used to compare Kaplan-Meier survival curves and cox proportional hazards regression models were used to generate hazard ratios and 95% confidence intervals.

Statistical analyses

Unless otherwise indicated, statistical testing was performed using GraphPad Prism v10.3.1. Prior to significance testing for differences among groups, the normality of data distribution for each group being tested was tested via Shapiro-Wilk test, where data with $p > 0.05$ were considered normally distributed. For normal datasets, t test was performed, with

Welch's correction if F test demonstrated unequal SD between groups. For non-normal datasets, Mann-Whitney U test was used. Multiple testing correction was performed using Holm-Sidak method to generate adjusted p values where indicated. Two-tailed tests were performed where direction of change could not be hypothesized a priori, and one-tailed tests were performed to test a specific direction of change. All data points represent biological replicates. For all applicable figure panels, error bars represent mean $\pm$ SD.

Data availability statement

Mass cytometry data have been deposited into Mendeley Data at doi.org/10.17632/mnwfrv4zd6.1. Bulk RNA sequencing data and exome sequencing data are deposited into NCBI SRA database, accession PRJNA1130874. All cell lines are available upon request to the senior author without restrictions.

Results

T Cell-Infiltrated Mouse Syngeneic Tumors Demonstrate Variable Responses to ICIs

To understand mechanisms of ICI response and resistance in T cell-infiltrated tumors, we generated microsatellite instability-high mouse syngeneic tumor cell lines. Only 30–50% of patients with MSI-H tumors respond in pre-treated settings (2,24), and resistance is frequently seen in mouse models (25,26). *Msh2* gene deletion by CRISPR in the polyclonal cell pool nearly depleted MSH2 protein, allowing preservation of any parental tumor heterogeneity, which can impact immunogenicity and ICI sensitivity (27) (Fig. 1A–B; Supplementary Fig. S2A). MSI (28) increased over 4 months of culture (Fig. 1C; Supplementary Fig. S2B), and MSI-H cell lines accumulated single nucleotide polymorphisms and indels (Fig. 1D), consistent with published studies (22,28). Each tumor cell line transcribed neoantigens with a similar distribution and heterogeneity of predicted antigenicity and allele frequency (16) (Fig. 1E; Supplementary Fig. S2C) and induced MHC-I expression upon IFN γ stimulation (Supplementary Fig. S2D).

When implanted into syngeneic mice, the tumor models demonstrated contrasting responses to combination ICI treatment of anti-PD-1 and anti-CTLA4. MSI-H B16F10 melanoma subcutaneous tumors responded better to ICIs compared to MSS tumors despite similar *in vitro* doubling times (Fig. 1F–G; Supplementary Fig. S2E–F). EO771 breast MSS and MSI-H tumors, implanted into the mammary fat pad (MFP), also responded to ICIs, with greater response in MSI-H tumors (Supplementary Fig. S2G–I). However, resistance to ICIs was notable in the MSS and MSI-H LLC tumors in either the subcutaneous space or the MFP, sites of implantation that resulted in similar tumor immune composition (Fig. 1H–I; Supplementary Fig. S2J–N).

To investigate mechanisms responsible for the divergent ICI responses, we used mass cytometry by time-of-flight (CyTOF), analyzing immune infiltrates from tumors on day 13 post-implantation, where tumor sizes were similar across the models. Quantification of major immune cell types in the TME revealed that ICI treatment increased T cell infiltration in both the responsive B16F10 and resistant LLC MSI-H tumors (Fig. 1J–K). ICI resistance in T cell-infiltrated tumors was not exclusive to the MSI-H context or the C57BL/6J

genotype, as EMT6 breast tumors in the MFP of BALB/cJ mice were also resistant, despite increased CD8⁺ T cell infiltration following treatment (Supplementary Fig. S5).

ICI-Resistant Tumors Acquire Unfavorable T cell Activation States After Treatment

To define differences in T cells in ICI-responsive vs. resistant tumors, we analyzed antigen-experienced CD44⁺CD8⁺ and conventional CD4⁺ T cells (CD4⁺ Tconv) by UMAP (29) and differential abundance analysis (30). We identified cell subsets enriched in each condition and defined their differentiation and activation states, focusing on differences between ICI-treated B16F10 MSI-H and LLC MSI-H tumors. CD8⁺ T cells exhibited differentially-abundant (DA) subsets for each condition (Fig. 2A–B) with distinct marker expression (Fig. 2C) and were subsequently grouped by unsupervised clustering. Clusters 4, 5 and 6 were enriched in B16F10 MSI-H tumors and highly expressed markers characteristic of activated effector T cells, including Ki67, granzyme B (GrB), CD39, CD38, and PD1 (Fig. 2D–E). Manual gating confirmed the relative paucity of this subset in LLC MSI-H tumors (Fig. 2F). In contrast, cluster 2, enriched in LLC tumors, had comparatively lower expression of these markers (Fig. 2D–E). LLC tumors were also enriched in cluster 3 cells, which expressed TCF1 but were PD1[−] (Fig. 2D–E), resembling memory precursor-like CD8⁺ T cells that can demonstrate polyfunctionality, including TNFα production (31).

Analysis of CD44⁺CD4⁺ Tconv cells also revealed DA cells (Fig. 2G–I). Clusters 3 and 4, enriched in B16F10 MSI-H tumors, highly expressed Ki67, PD1, CD39, and CD38 (Fig. 2J–K). Manual gating confirmed their relative depletion in LLC MSI-H tumors (Fig. 2L). Similar to the CD8⁺ T cell subsets, cluster 2 cells enriched in LLC MSI-H tumors were TCF1 and PD1[−] (Fig. 2J–K). T cells from the ICI-responsive EO771 MSI-H and ICI-resistant EMT6 models exhibited similar patterns of enrichment as B16F10 MSI-H and LLC MSI-H models, respectively (Supplementary Fig. S6). These data show that, despite increased T cell infiltration, T cells in the ICI-resistant tumors fail to acquire the activation and effector states observed in the ICI-responsive models.

IL-1 and TNFα Synergistically Drive Neutrophil Inflammation via IL1R1-Expressing Tumor Cells

Next, we determined mechanisms responsible for the divergent T cell states. ICI-treated LLC MSI-H tumors contained more Ly6G⁺ neutrophils (gating strategy shown in Supplementary Fig. S3) compared to IgG-treated controls or B16F10 MSI-H tumors (Fig. 3A). Compared to mice with B16F10 MSI-H tumors, those with LLC MSI-H tumors treated with or without ICIs exhibited significantly more positive or negative pairwise correlations between the frequencies of neutrophil cell subsets and other immune cell subsets in the tumor and the periphery (Fig. 3B), suggesting system-wide coordination (11). Neutrophils from ICI-treated LLC MSI-H tumors suppressed OT-I T cell proliferation *in vitro* (Fig. 3C), indicating function as polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) (32). To determine responsible factors, we quantified cytokines in LLC MSI-H tumor lysates. ICI treatment resulted in increased levels of cytokines regulated by the NF-κB pathway, including TNFα, IL-1β, the neutrophil growth factor G-CSF, and neutrophil chemoattractants CXCL1 and CXCL2 (Fig. 3D, E, Supplementary Table S6, and Supplementary Fig. S7A). RT-qPCR of *Csf3* (coding for G-CSF) and *Cxcl1* transcripts

showed that tumor cells increased expression of both transcripts following ICIs (Fig. 3F; Supplementary Fig. S7B and C, and Supplementary Fig. S4A). These data corroborate our prior data and the literature reporting production of G-CSF and CXCL1 from mouse and human tumors (11,33,34) and suggest that ICI treatment amplifies this existing circuit. Fibroblasts in tumors also increased G-CSF transcription following ICIs (Fig. 3F), although tumor cells outnumbered fibroblasts by 10:1 (Supplementary Fig. S7D).

We previously reported that G-CSF production by LLC tumor cells requires IL-1 Receptor 1 (IL1R1) signaling (11). IL-1 α and IL-1 β bind IL1R1 and synergize with TNF α through NF- κ B to upregulate G-CSF, CXCL1 and CXCL2 production, possibly via IL-1-induced increase in TNF receptors (6,35). In LLC tumors, we detected both IL-1 α and IL-1 β , the former being present in larger quantities, while the latter was induced upon ICIs (Supplementary Fig. S7E, F). The transcript for each cytokine increased with ICIs from a mix of tumor and immune cell types (Supplementary Fig. S7G, H). LLC MSI-H and EMT6 cells increased G-CSF and CXCL1 after stimulation by TNF α and IL-1 α *in vitro*, contrasting with minimal responses in B16F10 and EO771 MSI-H tumor cells (Fig. 3G, Supplementary Fig. S8A–D). TNF α synergized with IL-1 α or IL-1 β to induce G-CSF and CXCL1, showing greater than additive responses (Fig. 3G, Supplementary Figs. S8A–G). Interestingly, maximal synergism for G-CSF production was achieved with very low levels of IL-1 β (1ng/mL), whereas increasing amounts TNF α potentiated the synergy (Supplementary Fig. S8E–H). LLC MSI-H cells degraded I κ B more significantly compared to B16F10 MSI-H cells, consistent with greater NF- κ B activation in LLC (Fig. 3H, Supplementary Fig. S8I). The NF- κ B inhibitor QNZ suppressed G-CSF and CXCL1 expression from LLC cells (Fig. 3I).

To understand why LLC and EMT6 cells were more sensitive to stimulation by IL-1 and TNF α , we quantified IL1R1 and TNFR1 by flow cytometry. LLC MSI-H and EMT6 cells highly expressed IL1R1 in contrast to minimal expression in B16F10 MSI-H and EO771 MSI-H cells, while all lines expressed TNFR1 (Fig. 3J–K; Supplementary Fig. S9A–E). There was no difference in IL1R1 expression between LLC MSS and MSI-H cells (Supplementary Fig. S9F). Bulk RNA sequencing of the cell lines showed that *Il1r1* was one of the most differentially regulated genes within the pathway (Supplementary Fig. S9G). For TNF pathway genes, we confirmed similar high expression of *Tnfrsf1a*, coding for TNFR1, between these cells (Supplementary Fig. S9H). *Tnfrsf1b* (coding for TNFR2), which can contribute to CXCL1 inflammation (36), was expressed at low levels but was slightly increased in LLC cells (Supplementary Fig. S9H). These data implicate an inflammatory immunosuppressive circuit supporting ICI resistance in tumor types responsive to IL-1 and TNF α , notable for their expression of IL1R1.

IL1R1 Perturbation Limits Immunosuppressive Inflammation and Restores Favorable T Cell States to Sensitize Resistant Tumors to ICIs

We next perturbed this inflammatory axis *in vivo*. IL1R1-neutralizing antibody reduced G-CSF and CXCL1 in LLC MSI-H tumors (Supplementary Fig. S10A), decreased neutrophils in tumor (Supplementary Fig. S10B), and sensitized LLC MSI-H tumors to ICIs (Fig. 4A–B; Supplementary Fig. S10C). Importantly, tumor-specific deletion of IL1R1 via CRISPR also

sensitized LLC MSI-H tumors to ICIs, implicating tumor-intrinsic IL1R1 signaling during ICI resistance (Fig. 4C–D; Supplementary Fig. S10D). We additionally tested the effect of antagonizing G-CSF via tumor implantation in G-CSF receptor-null mice or injection of G-CSF neutralizing antibody, which depleted neutrophils from tumors and improved tumor control with ICIs (Supplementary Fig. S10E–J).

We determined changes in T cell states upon IL1R1 blockade in ICI-treated LLC MSI-H tumors by CyTOF. DA analysis identified enriched CD8⁺ T cells with IL1R1 blockade, highly expressing effector markers such as GrB and CD39, akin to those more frequently found in B16F10 tumors (Fig. 4E–G; Fig. 2E). Clustering of DA cells highlighted this phenotype, identifying cluster 1, highly expressing Ki67, GrB, CD39, and CD38 (Fig. 4H–I). Manual gating confirmed an increase in this subset with IL1R1 blockade (Fig. 4J). Among CD44⁺ CD4⁺ Tconv cells, clusters 1 and 2 were enriched in the IL1R1 blockade group, highly expressing Ki67, CD39, CD38, PD1, and GrB (Fig. 4K–O). Manual gating of this subset confirmed relative enrichment with IL1R1 blockade (Fig. 4P). In contrast, TCF1⁺ PD1[−] CD8⁺ cluster 5 and CD4⁺ cluster 4 T cells, previously found to be enriched in LLC MSI-H tumors in comparison to B16F10 MSI-H tumors (Fig. 2E–K), were reduced after IL1R1 blockade. Although tumor-associated macrophages and endothelial cells can express IL1R1 (37,38), we did not observe significant changes in CD11b⁺ F4/80⁺ macrophages or CD31⁺ endothelial cell frequencies nor significant changes in expression of macrophage markers, including PD-L1, CD206, CD38, CD39, iNOS, and MHCII, following anti-IL1R1 treatment (Supplementary Fig. S10K–M). Thus, perturbation of the inflammatory circuit via IL1R1 inhibition sensitized resistant tumors to ICIs and shifted CD8⁺ and CD4⁺ T cells to cytotoxic effector-like states.

T Cells Drive the Inflammatory ICI Resistance Circuit via TNFα

Because activated T cells often produce TNFα, we hypothesized that T cells may paradoxically exacerbate the inflammatory resistance program. Depleting T cells in LLC MSI-H tumors using an anti-Thy1 significantly decreased G-CSF, CXCL1, and neutrophil infiltration in the TME (Fig. 5A–C). Although fibroblasts can express Thy1, they were not significantly depleted (Supplementary Fig. S11A). Anti-CD4 plus anti-CD8 for T cell depletion also reduced G-CSF, CXCL1, and neutrophils, requiring elimination of both T cell types (Supplementary Fig. S11B–C). CD4⁺ and CD8⁺ T cell depletion in the EMT6 model also reduced tumor-infiltrating neutrophils (Supplementary Fig. S11D).

We hypothesized that T cell-derived TNFα might stimulate tumor cells to produce inflammatory cytokines that support neutrophils. Co-culturing CD3/CD28-stimulated CD8⁺ T cells from LLC MSI-H tumors with LLC MSI-H tumor cells increased G-CSF production in the supernatant more than five-fold, indicating that CD8⁺ T cells were sufficient to drive this circuit (Fig. 5D–E; Supplementary Fig. S4B). Importantly, the increase in G-CSF was largely dependent on TNFα (Fig. 5E). Neutralization of other candidate factors, such as FasL or GM-CSF, did not reduce G-CSF (Supplementary Fig. S11E). *In vivo*, treatment with ICIs increased TNFα-expressing CD4⁺ and CD8⁺ T cells in LLC MSI-H tumors (Fig. 5F). This was also accompanied by increases in TNFα⁺ tumor-infiltrating neutrophils, a cell type also known to express TNFα (14) (Supplementary Fig. S11F). Importantly, CD4⁺

and CD8⁺ T cell depletion decreased TNFα⁺ neutrophils in the TME (Supplementary Fig. S11G), demonstrating dependence on T cell activity. TNFα⁺ CD8⁺ T cells were less likely to express Ki67 or GrB in comparison to TNFα⁻ cells (Fig. 5G–H), segregating these key effector functions into different T cell subsets. TNFα⁺ CD4⁺ T cells also showed lower expression of Ki67 (Supplementary Fig. S11H).

We implanted LLC MSI-H tumors in *Tnf* knockout mice lacking host-derived TNFα, which sensitized the tumors to ICI treatment, accompanied by a decrease in neutrophils (Fig. 5I–J; Supplementary Fig. S11I–J). Furthermore, we generated T cell-specific *Tnf* knockout mice as previously described (*Cd4-cre Tnf^{fl/fl}*) (14) (Supplementary Fig. S11K–M). Tumor growth was also restricted following ICIs in mice specifically lacking T cell-derived TNFα (Fig. 5K–L; Supplementary Fig. S11N). The combination of IL1R1 blockade and T cell TNFα knockout did not result in significantly different tumor control compared to single perturbations alone in keeping with synergy between the two pathways (Supplementary Fig. S11O–Q). These data support a mechanism whereby ICI-induced infiltration of T cells producing TNFα exacerbates NF-κB-driven transcription of G-CSF and CXCL1 in tumor cells to create a paradoxical circuit of immunosuppression.

T cell, Tumor, and Neutrophil Inflammation Circuit Is Active in Human Cancer and Is Associated with Poor Clinical Outcome

We interrogated our model of T cell-exacerbated tumor inflammation in human cancer. First, we evaluated breast cancer organoids derived from two different resected primary tumors (TORG40, from inflammatory triple-negative breast cancer, and TORG139, from triple-negative breast cancer). Stimulation with IL-1α and TNFα induced production of G-CSF and CXCL1 from TORG40 organoids, while the TORG139 organoids produced no G-CSF and little CXCL1 (Fig. 6A–B). TORG40 also had a higher induction of *CXCL8* transcripts (Supplementary Fig. S12A). Consistent with the mouse models, TORG40 cells expressed more *IL1R1* but similar levels of *TNFR1* compared to TORG139 cells (Fig. 6C; Supplementary Fig. S12B).

To understand the relationship between human T cells and tumor cells in the context of ICI treatment, we analyzed a single-cell RNA-sequencing dataset of paired tumor samples from non-metastatic breast cancer patients before and after pembrolizumab (39) (Fig. 6D). 7 clusters were identified across CD8⁺ T cells (Fig. 6E; Supplementary Fig. S12C). Notably, CD8⁺ T cells expressing *TNF* were largely distinct from those expressing GrB (*GZMB*) or Ki67 (Fig. 6F; Supplementary Fig. S12C), paralleling results from our mouse models (Fig. 5G–H). Comparison of differentially expressed genes in the two *TNF*-expressing clusters to the *GZMB*⁺ exhausted cluster revealed higher expression of activation markers including CD69 (Supplementary Fig. S12D–E). While the *TNF*-expressing clusters significantly increased in patients following pembrolizumab, the *GZMB*⁺ Exhausted cluster was not significantly changed (Fig. 6G–I, Supplementary Fig. S12F). Increases in the *TNF*-expressing clusters were positively correlated with each other (Supplementary Fig. S12G). A variety of Th0, Th1, and Th17 CD4⁺ T cell subsets expressed *TNF* (Supplementary Fig. S13A–C). Among these, *TNF*⁺ Th1 subsets increased following pembrolizumab, characterized by expression of *ENTPD1* (coding for CD39), similar to TNFα⁺ mouse CD4⁺

T cells (Supplementary Figs. S13D and E, and S11H). A portion of C1q⁺ macrophages expressing *TNF* also increased following treatment (Supplementary Fig. S13F–I).

We hypothesized that an increase in *TNF*-expressing cells in *IL1R1*-expressing tumors would activate tumor-intrinsic NF- κ B inflammation. We determined the level of *IL1R1* expression in tumor cells and their NF- κ B activity from an established gene set. Tumor NF- κ B scores were higher in the IL1R1-high group of patients following ICIs (Fig. 6J). Furthermore, CD8⁺ *TNF*⁺ *GZMK*⁺ and *TNF*⁺ *IFNG*⁺ exhausted cluster frequencies were positively correlated with tumor NF- κ B scores only in the IL1R1-high group (Supplementary Fig. S13J). Thus, ICI treatment can induce expansion of *TNF*⁺ inflammatory leukocytes associated with increased tumor-intrinsic NF- κ B signaling when the tumor cells express *IL1R1*.

Because droplet-based single-cell RNA-sequencing may not efficiently capture neutrophils, we leveraged additional human datasets containing neutrophil quantification. Recently, an inflammatory hub composed of NF- κ B transcription factors, inflammatory tumor cells, lymphocytes, neutrophils, and chemokines has been described in colorectal cancer (COAD) (40). Inferring immune composition using CIBERSORTx on the Cancer Genome Atlas (TCGA) RNA-sequencing dataset for COAD patients showed an increase in neutrophils in MSI-H tumors compared to MSS tumors (Supplementary Fig. S13K). We reasoned that ICI resistance in patients with MSI-H cancers may correlate with exacerbated neutrophilia, similar to the systemic mobilization of neutrophils during ICI resistance in mice. We identified MSI-H cancers in our institutional next-generation sequencing database and analyzed ICI failures. When compared to a 6-week period prior to initiation of ICIs, the peripheral blood neutrophil to lymphocyte ratio (NLR) was increased prior to disease progression (Fig. 6K). On the other hand, for patients without progression, NLR did not change over the same interval of time.

We determined the significance of the inflammatory circuit regarding ICI treatment outcomes, analyzing RNA sequencing data from the atezolizumab arm of OAK (NCT02008227), a randomized phase III trial comparing atezolizumab vs. docetaxel in locally advanced or metastatic non-small cell lung cancer (23). Following stratification of patients into CD8A high vs. low groups, we determined the impact of a custom gene set score “inflamm” consisting of genes involved in the ICI resistance circuit: *IL1R1*, *TNFRSF1A*, *IL1A*, *IL1B*, *TNF*, *CD69*, *CSF3*, *CXCL1*, *CXCL2*, *CXCL8*, *NFKB1*, *NFKB2*. Patients in the CD8A high but “inflamm” low group had superior overall survival compared to the other groups in aggregate (p=0.00124; HR=1.667 (1.222– 2.278)) and to each individual group (Fig. 6L). Notably, those patients with CD8A high and “inflamm” high tumors did not experience a survival benefit, similar to our resistant mouse models. Similar trends were seen for progression-free survival (Supplementary Fig. S13L). These data support the proposed ICI resistance mechanism in tumors with T cell infiltration (Fig. 6M).

Discussion

In this study, we define a mechanism by which T cells exacerbate an inflammation circuit and paradoxically drive resistance to ICI therapy. TNF α produced by T cells after treatment

synergized with IL-1 to activate the NF- κ B pathway in IL1R1⁺ tumor cells, promoting neutrophil recruitment. Inhibition of TNF α was recently shown to improve ICI efficacy and protect against autoimmune adverse effects in mouse models (9,41). However, TNF α can be produced by many cell types (14). Our data show that T cell-derived TNF α promotes adaptive resistance to therapy in IL1R1-expressing tumors.

Prior data highlight an immunosuppressive axis involving PMN-MDSCs critical for treatment outcome (32). IL-1 and TNF can promote CXCL1, CXCL2 and IL-8 from tumor cells and fibroblasts, culminating in PMN-MDSC recruitment to the TME (36,42). We provide new functional data demonstrating a paradoxical role for effector T cells in exacerbating this inflammatory resistance circuit after ICI treatment and provide an important mechanistic insight that this pathway is activated in IL1R1⁺ tumor cells. Various T cell subsets have been implicated in tumor development and progression, most notably Tregs, which classically suppress effector T cell responses. Th17 and $\gamma\delta$ T cells can also drive pro-tumor inflammation via IL-17 production (43), while CD8⁺ T cells have been previously shown to promote chemical carcinogenesis (44). Interestingly, in the context of autoimmunity and immune-related adverse events, effector memory CD4⁺ T cells can promote NF- κ B inflammation in dendritic cells in a TNFR- and CD40-dependent manner (45). We now show that ICI treatment can result in CD4⁺ and CD8⁺ T cell production of TNF α that exacerbates immunosuppressive inflammation and limits therapeutic efficacy. This adds nuance to the prevailing view that T cell effector functions are beneficial in the TME and for response to immunotherapy, distinguishing productive from detrimental effector functions depending on tumor IL1R1 expression. Our results provide a new explanation for why patients may experience poor responses to ICIs despite predicted or confirmed T cell infiltration and refine the current models of ICI response to include the active role for tumor-infiltrating T cells in shaping the inflammatory tone of the TME.

There may be a number of additional, complementary mechanisms through which IL-1 and TNF α amplify immune suppression in the TME. IL-1 signaling in fibroblasts has been shown to promote MDSC accumulation and activity (46). TNF α can also promote T cell activation-induced cell death (47), tumor cell dedifferentiation (48), and stabilization of immunosuppressive molecules such as PD-L1 and TGF- β , which can potentiate Treg function (49). Additional experiments are needed to dissect the contribution and interaction of each mechanism. Furthermore, although we have leveraged T cell-infiltrated MSI-H tumors, the mechanism presented was not specific to MSI-H tumors. The common denominators for engaging the resistance circuit are recruitment of TNF-expressing T cells, presence of IL-1, and tumor IL1R1 expression, which collectively result in activation of the NF- κ B pathway to drive neutrophil recruitment.

IL-1/IL1R1/TNF axis inhibition is being tested in clinical trials. Based on the finding that IL-1 β inhibition via Canakinumab may reduce lung cancer incidence and mortality, phase III CANOPY trials were launched, albeit demonstrating no significant impact on survival with inhibition of IL-1 β alone ([NCT03447769](#), [NCT03631199](#), [NCT03626545](#)). A phase I-II trial is testing an IL1R1 inhibitor Isuanakinra in combination with ICIs in patients with advanced solid tumors ([NCT04121442](#)). The combination of TNF inhibitors Infliximab or Certolizumab with Nivolumab and Ipilimumab was tested in the TICIMEL

phase Ib trial in patients with advanced or metastatic melanoma, demonstrating safety and promising efficacy (50). Lastly, an inhibitor of CXCR1 and CXCR2, receptors for neutrophil chemokines including CXCL1, CXCL2, and CXCL8, is being tested in combination with ICIs (NCT03161431, NCT05570825, NCT04599140). Our results suggest that patients with T cell-infiltrated tumors and IL1R1⁺ tumor cells may be most likely to benefit. Here, we provide evidence for G-CSF/G-CSFR inhibition as a potential therapeutic strategy in combination with ICIs.

In summary, our data elucidate a mechanism by which T cells can unexpectedly promote ICI resistance depending on the tumor context. These results can inform new strategies for therapy selection, immune monitoring, and therapeutic strategies to convert inflammation-promoting T cell activity into productive anti-tumor immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Authors' Disclosures

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Synopsis

Mechanisms of resistance to immune checkpoint inhibitor therapies remain unclear. Here, we reveal the functional significance of tumor-infiltrating T cells in resistant tumors, which instruct immunosuppressive inflammation in mouse and human cancers responsive to IL-1 and TNF α .

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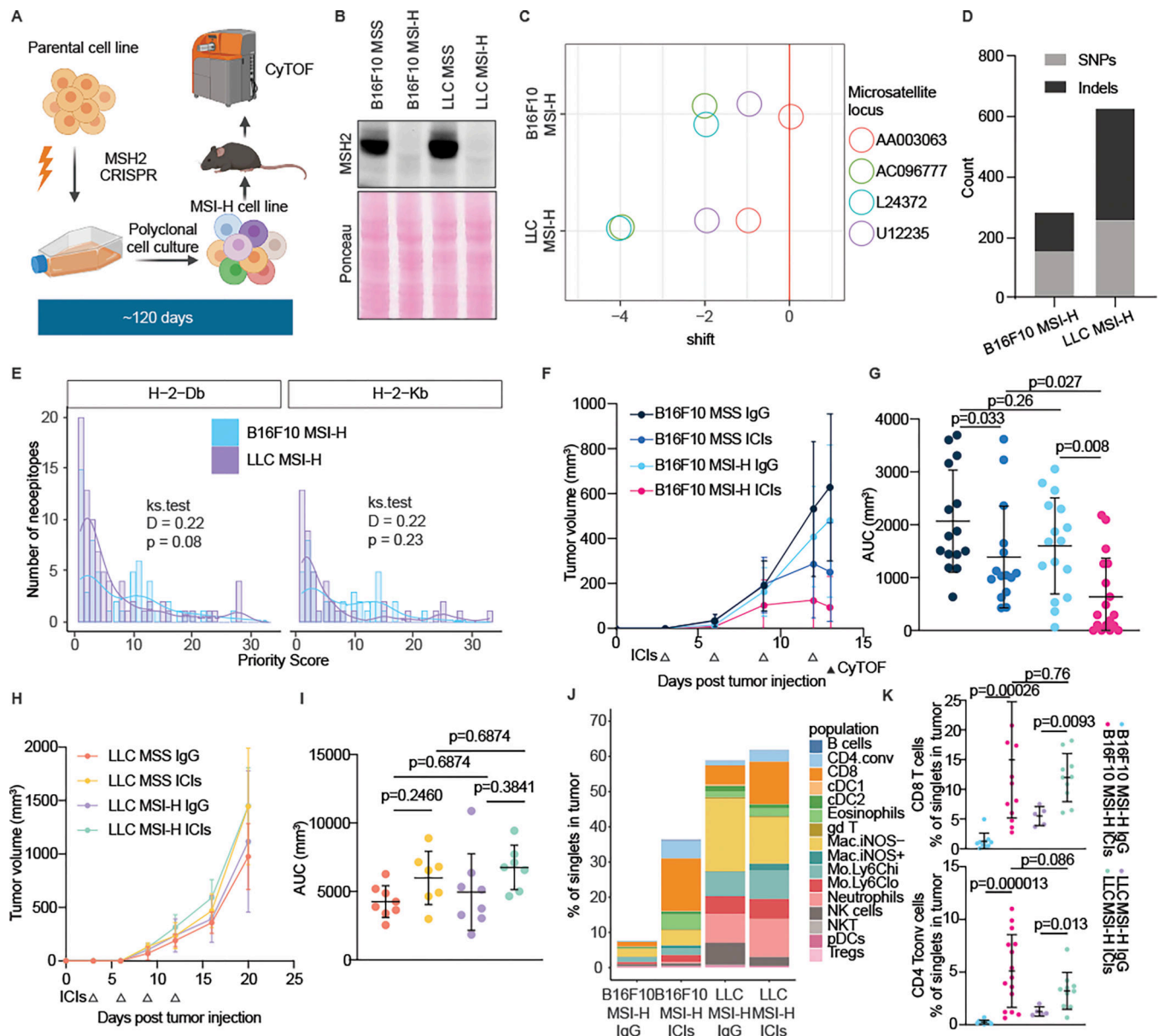


Figure 1: T cell-infiltrated tumors demonstrate variable responses to ICIs.

A, Outline for generation of MSI-H syngeneic tumors. **B**, Western blot of whole cell lysates. Representative of 3 independent experiments. **C**, Targeted microsatellite PCR assay comparing amplicon size at microsatellite loci for MSI-H vs. MSS cell line. Representative of two independent experiments. **D**, Whole exome sequencing quantification of single nucleotide polymorphisms (SNPs) and insertion-deletion mutations (indels) for indicated MSI-H cell lines compared to MSS lines. **E**, MuPeXI neopeptide affinity scores for indicated cell lines and HLA class. D statistic and p values calculated using a two-sided Kolmogorov-Smirnov test. **F and G**, Mean volumes and area under curves (AUC) for B16F10 subcutaneous tumors treated with or without anti-PD1 and anti-CTLA4 antibodies (ICIs) at indicated timepoints (white arrowhead). Black arrowhead denotes timepoint for sample collection for CyTOF analysis. n=15–18 mice per condition from two independent

experiments. Two-tailed adjusted p value by Mann-Whitney tests. **H and I**, Analysis as in **(F)** and **(G)** for subcutaneous LLC tumors. n=7–8 mice per condition from one experiment. Two-tailed adjusted p values by Welch's t tests. **J**, Immune subsets defined by manual gating of CyTOF data from B16F10 subcutaneous or LLC MFP tumors harvested at day 13 post implantation. Conv=conventional; Mac=macrophages; Mo=monocytes. Bar height for each population represents mean of biological replicates (n=5–15 mice) from three independent experiments. **K**, Quantification of CD8⁺ T cells (top panel) and CD4⁺ Tconv (bottom panel) from **(J)**. Two-tailed adjusted p value by Mann-Whitney tests.

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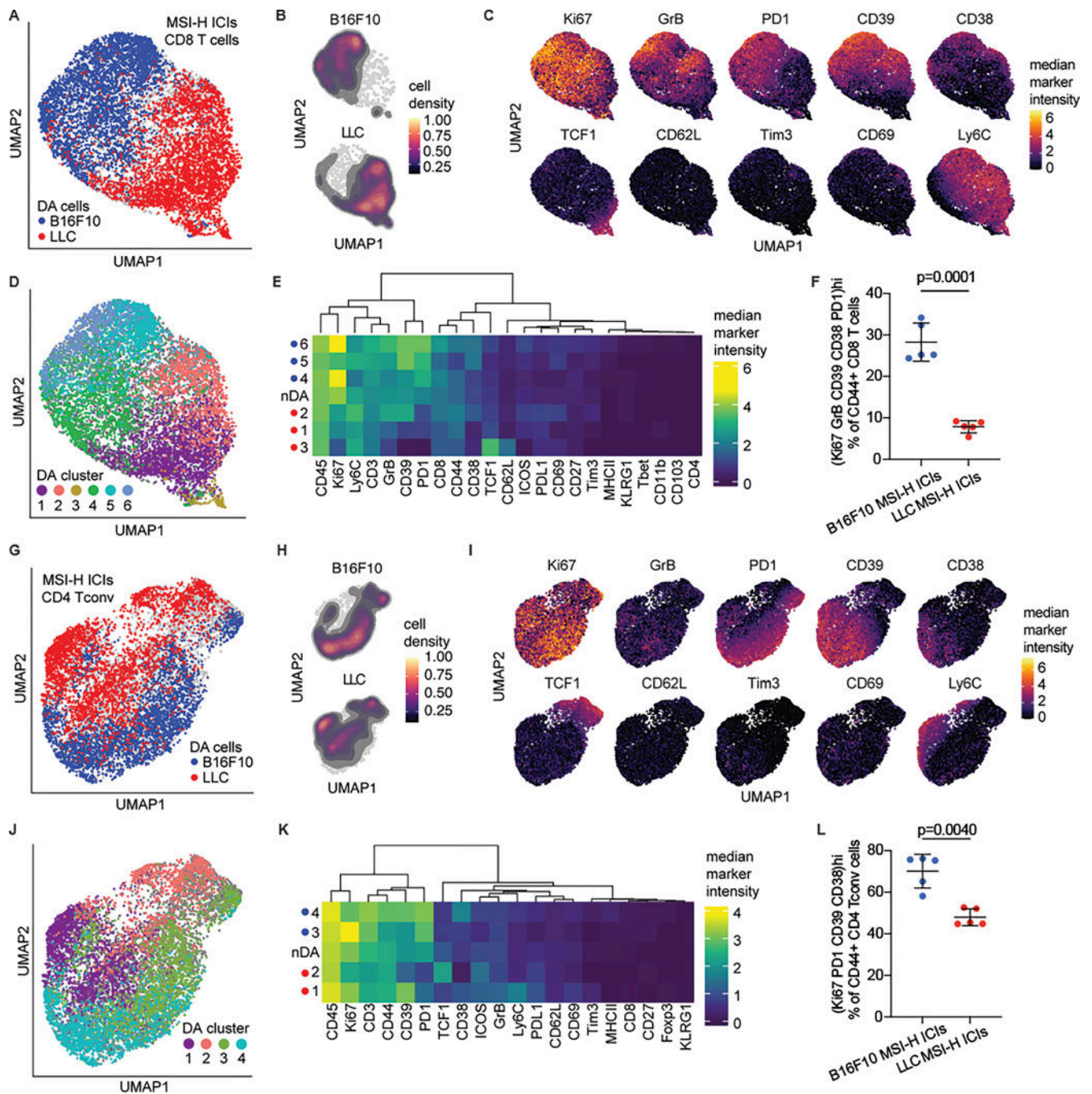


Figure. 2: Distinct effector protein expression in T cells in ICI-responsive vs. ICI-resistant tumors.

A, Differential abundance (DA) analysis of 10,000 CD44⁺ CD8⁺ T cells from ICI-treated B16F10 MSI-H and LLC MSI-H tumors harvested at day 13. CyTOF data acquired with n=5 mice each from one representative experiment of three independent *in vivo* tumor growth experiments. **B**, Density plots for cells in each tumor type from (**A**). **C**, Feature plots for selected markers colored by asinh transformed staining intensity. **D**, Clustering of DA cells from (**A**). **E**, Heatmap of median marker intensities for cells in each DA cluster.

nDA, non-DA cells. Colors indicate clusters more abundant in B16F10 (blue) or LLC (red) tumors. **F**, Manually gated CD8⁺ T cell subset in B16F10 and LLC tumors. P value by one-tailed Mann-Whitney test. **G**, DA analysis as in **(A)** for CD44⁺ CD4⁺ Tconv cells. **H**, Density plots for cells in each tumor type. **I**, Feature plots for selected markers colored by asinh transformed staining intensity. **J**, Clustering of DA cells from **(G)**. **K**, Heatmap of median marker intensities for cells in each DA cluster. Colors indicate clusters more abundant in B16F10 (blue) or LLC (red) tumors. **L**, Manually gated CD4⁺ Tconv cell subset in B16F10 and LLC tumors. P value by one-tailed Mann-Whitney test.

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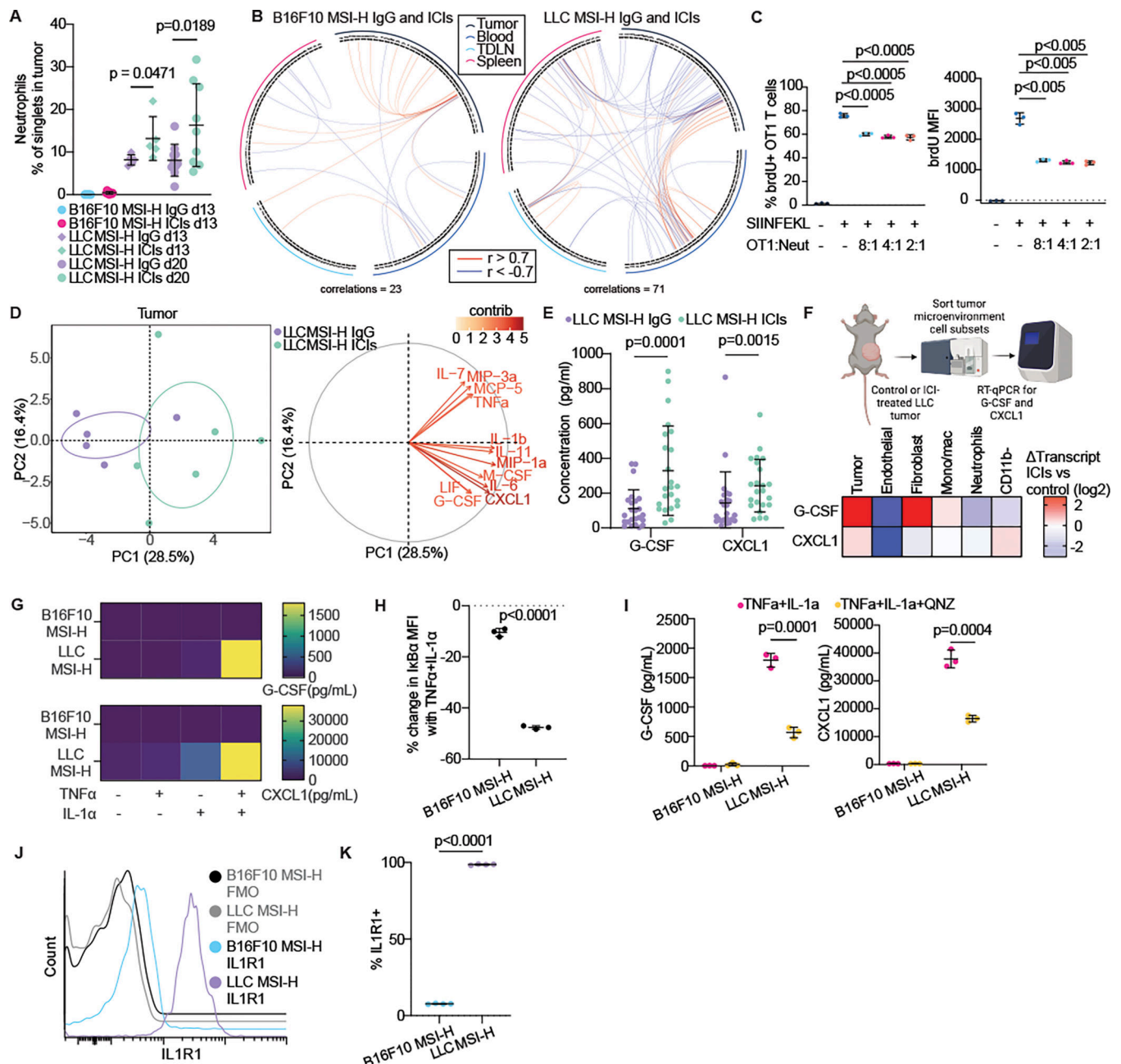


Figure 3: IL-1 and TNF α synergistically drive immunosuppressive neutrophil inflammation from IL1R1-expressing tumor cells.

A, Neutrophil quantification in B16F10 and LLC tumors at indicated timepoints and treatment conditions (n=5–10 mice) from two independent experiments. P values by one-tailed Welch's t test. **B**, Pairwise Spearman correlations between unsupervised immune cell clusters across tumor, blood, tumor-draining lymph node, and spleen from CyTOF analysis of one representative experiment. Connecting lines indicate pairs of correlated clusters of which at least one cluster is a neutrophil, indicating positive (red) or negative (blue) correlations with $r > 0.7$ or $r < -0.7$. Samples harvested at day 13 from n=20 mice for B16F10 and at day 20 from n=10 mice for LLC. **C**, brdU median fluorescence intensity (MFI) and

% positive splenic OT-I T cells cultured with SIINFEKL peptide and/or sorted neutrophils from day 20 LLC MSI-H tumors, from n=3 mice per condition in one experiment. Adjusted p values by Welch's t tests. **D**, Left panel, principal component (PC) analysis of cytokines in day 20 tumor lysates by multiplexed bead assay in one representative experiment, n=5 per condition. 95% confidence ellipse is shown. Right panel, loadings plot of contribution by the top 12 cytokines. **E**, Quantification of indicated cytokines in day 20 tumor lysates by multiplexed bead assay in 5 independent experiments. Two-tailed p values by Mann-Whitney test. **F**, RT-qPCR of cytokine transcripts from sorted cells from day 20 LLC MSI-H ICI-treated tumors, with expression normalized to control treated tumors. Mean of n=3–6 biological replicates from two independent experiments. **G**, ELISA of G-CSF in cell culture supernatant of indicated cell lines treated with or without indicated cytokines for 24h in three biological replicates. One representative experiment of five independent experiments. **H**, I κ B α MFI in indicated cell lines with or without TNF α and IL-1 α from three biological replicates. One representative experiment of two independent experiments. P value by two-tailed t test. **I**, ELISA of G-CSF and CXCL1 in cell culture supernatant of indicated cell lines following indicated treatment conditions from three biological replicates. One representative experiment of two independent experiments. P value by two-tailed t test. **J**, Representative histogram of IL1R1 fluorescence staining intensity for indicated cell lines compared to fluorescence-minus-one controls. **K**, % IL1R1⁺ cells in indicated cell lines from one representative experiment with four biological replicates. Two independent experiments were performed. P value by two-tailed t test.

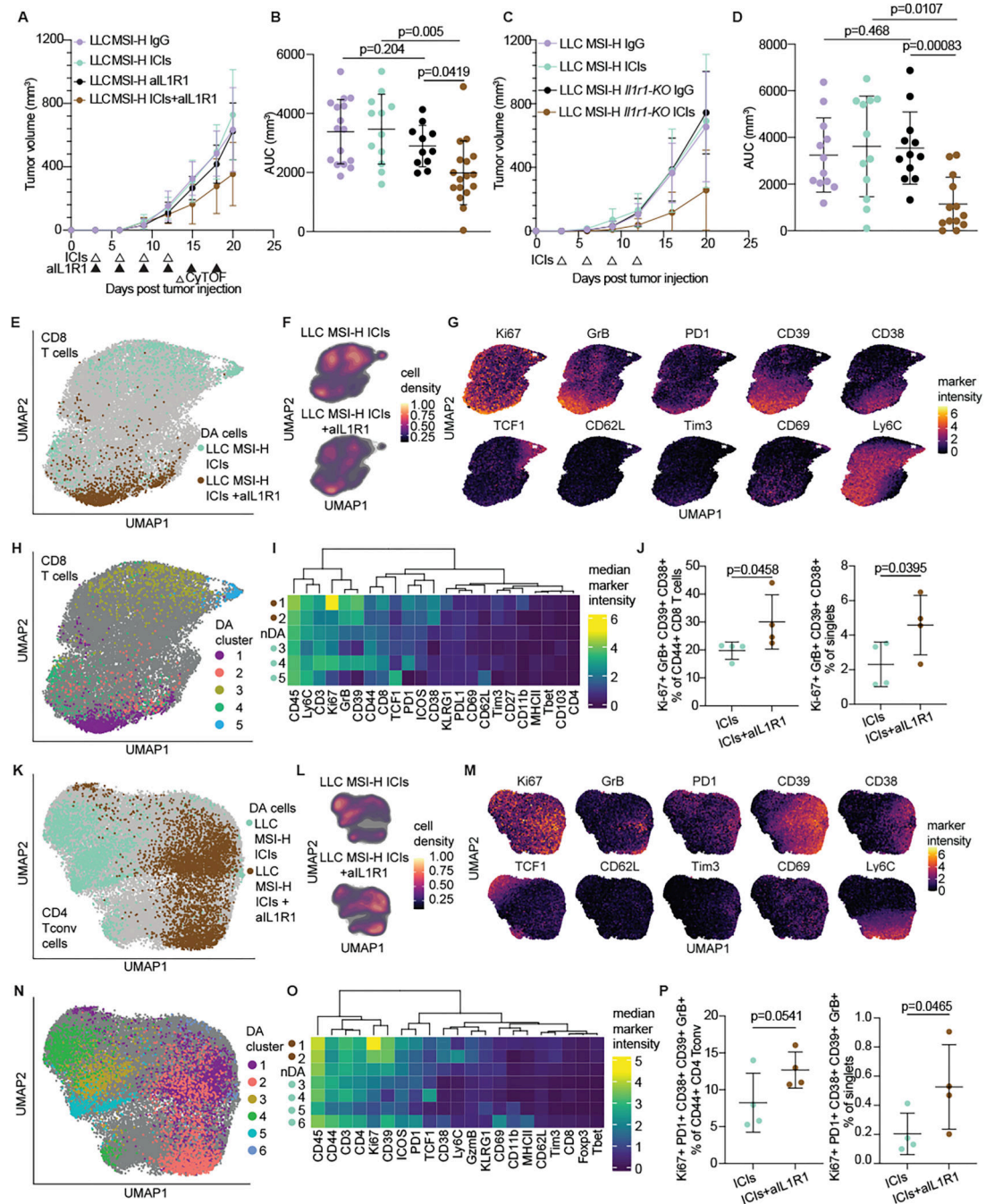


Figure 4: IL1R1 perturbation limits immunosuppressive inflammation to sensitize resistant tumors to ICIs.

A and B, Mean volumes and AUC for LLC MSI-H tumors treated with or without ICIs and IL1R1-neutralizing antibodies at the indicated timepoints (arrowheads). n=11–17 mice per condition from three independent experiments. Two-tailed adjusted p value by Mann-Whitney test. **C and D**, Mean volumes and AUCs for LLC MSI-H tumors with or without tumor *Il1r1* knock out (*Il1r1*-KO) treated with or without ICIs. n=12–13 mice per condition from two independent experiments. Two-tailed adjusted p value by Mann-Whitney test. **E**,

Differential abundance (DA) analysis of 26,400 CD44⁺ CD8⁺ T cells from ICI-treated LLC MSI-H tumors +/- aIL1R1 treatment (n=4 mice each from one representative experiment analyzed by CyTOF) harvested at day 13. Colored dots indicate thresholded top DA cells increased in the indicated condition. **F**, Cell density plots for each condition. **G**, Feature plots for selected markers colored by asinh transformed staining intensity. **H**, Clustering of DA cells from (**E**). **I**, Heatmap of median marker intensities for cells in each DA cluster. nDA, non-DA cells. Colored dots indicate clusters enriched in the aIL1R1 condition (brown) vs. control (teal). **J**, Manually gated CD8⁺ T cell subset in each condition, quantified as % of parent (left panel) and of singlets in tumor (right panel). P value by one-tailed t test. **K**, DA analysis as in (**E**) for CD44⁺ CD4⁺ Tconv cells. **L**, Density plots for cells in each condition. **M**, Feature plots for selected markers colored by asinh transformed staining intensity. **N**, Clustering of DA cells from (**K**). **O**, Heatmap of median marker intensities for cells in each DA cluster. nDA, non-DA cells. Colored dots indicate clusters enriched in the aIL1R1 condition (brown) vs. control (teal). **P**, Manually gated CD4⁺ Tconv cell subset in each condition, quantified as % of parent (left panel) and of singlets in tumor (right panel). P value by one-tailed t test.

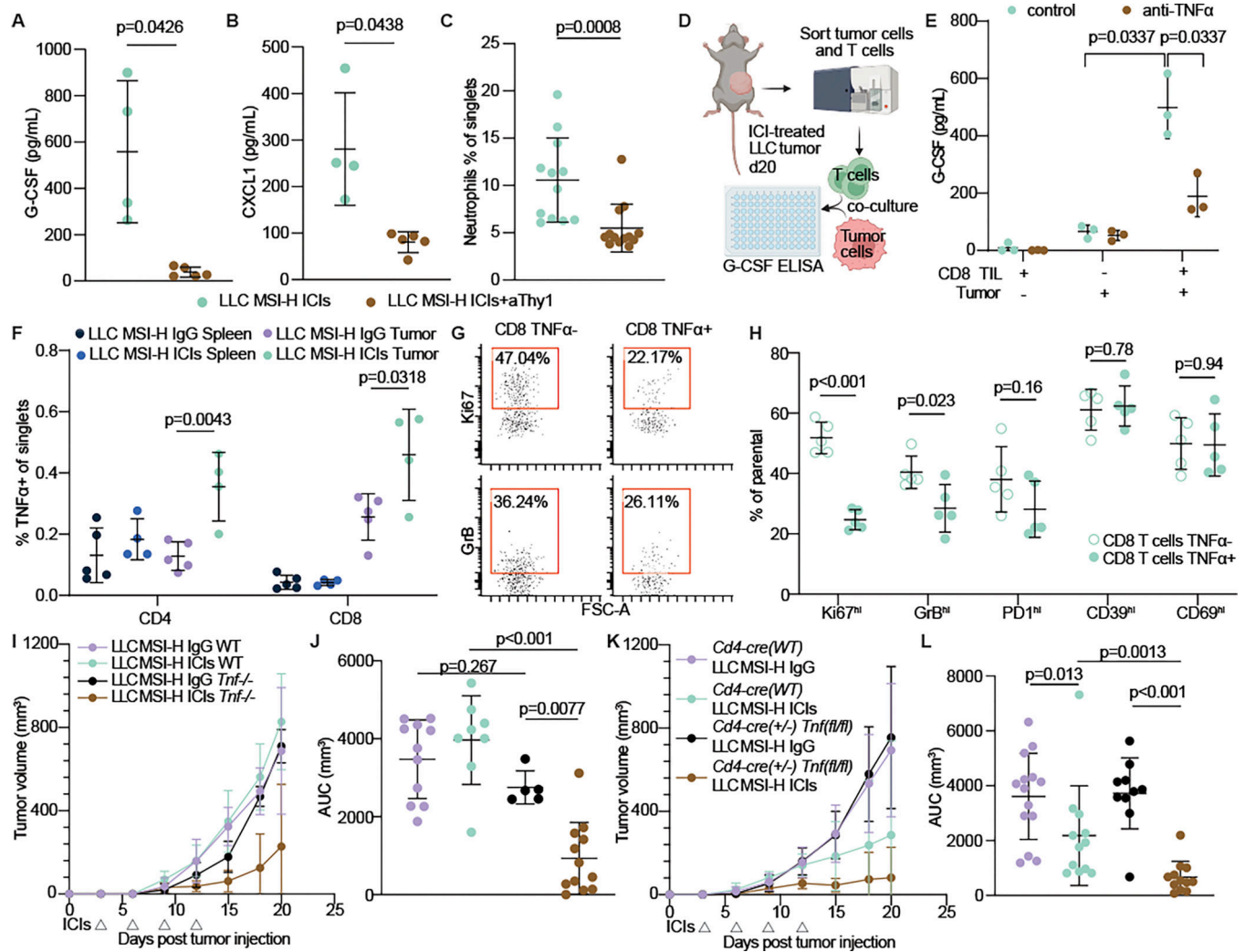


Figure 5: T cells drive the inflammatory ICI resistance circuit via TNF α .

A, G-CSF concentration in day 20 tumor lysates with $n=4-5$ mice per condition from one experiment. P value by two-tailed Welch's t test. **B**, Quantification as in (**A**) for CXCL1. **C**, Quantification of neutrophils in day 20 tumors in two independent experiments, $n=12-13$ mice per condition. P value by two-tailed Welch's t test. **D**, Schematic for T cell-tumor coculture. **E**, G-CSF concentration in supernatant of 24h culture containing indicated sorted cell types from day 20 LLC MSI-H tumors, with or without TNF α neutralizing antibody, from one representative experiment with $n=3$ mice. Two independent experiments were performed. Adjusted p values by two-tailed Welch's t test. **F**, Quantification of TNF α ⁺ CD4⁺ or CD8⁺ T cells in tumor or spleen of day 20 LLC MSI-H tumor bearing mice treated with indicated antibodies, $n=4-5$ mice from one representative experiment. Three independent experiments were performed. P value by two-tailed t test. **G**, Ki-67 and GrB fluorescence intensity in TNF α ⁻ or TNF α ⁺ CD8⁺ T cells. **H**, % of TNF α ⁻ and TNF α ⁺ CD8⁺ T cells in high expression gates for each marker. P value by two-tailed t tests. $n=5$ mice from one representative experiment. Two independent experiments were performed. **I and J**, Mean volumes and AUC for LLC MSI-H tumors treated with IgG or ICIs in

wild-type C57BL/6J or *Tnf*-null mice. Adjusted p values by Mann-Whitney test. n=5–12 from two independent experiments. **K and L**, Mean volumes and AUC for LLC MSI-H tumors treated with IgG or ICIs, implanted in *Cd4-cre(WT) Tnf(fl/fl or fl/+)* control mice or *Cd4-cre(+/-) Tnf(fl/fl)* mice. Adjusted p values by Mann-Whitney test. n=10–14 mice from two independent experiments.

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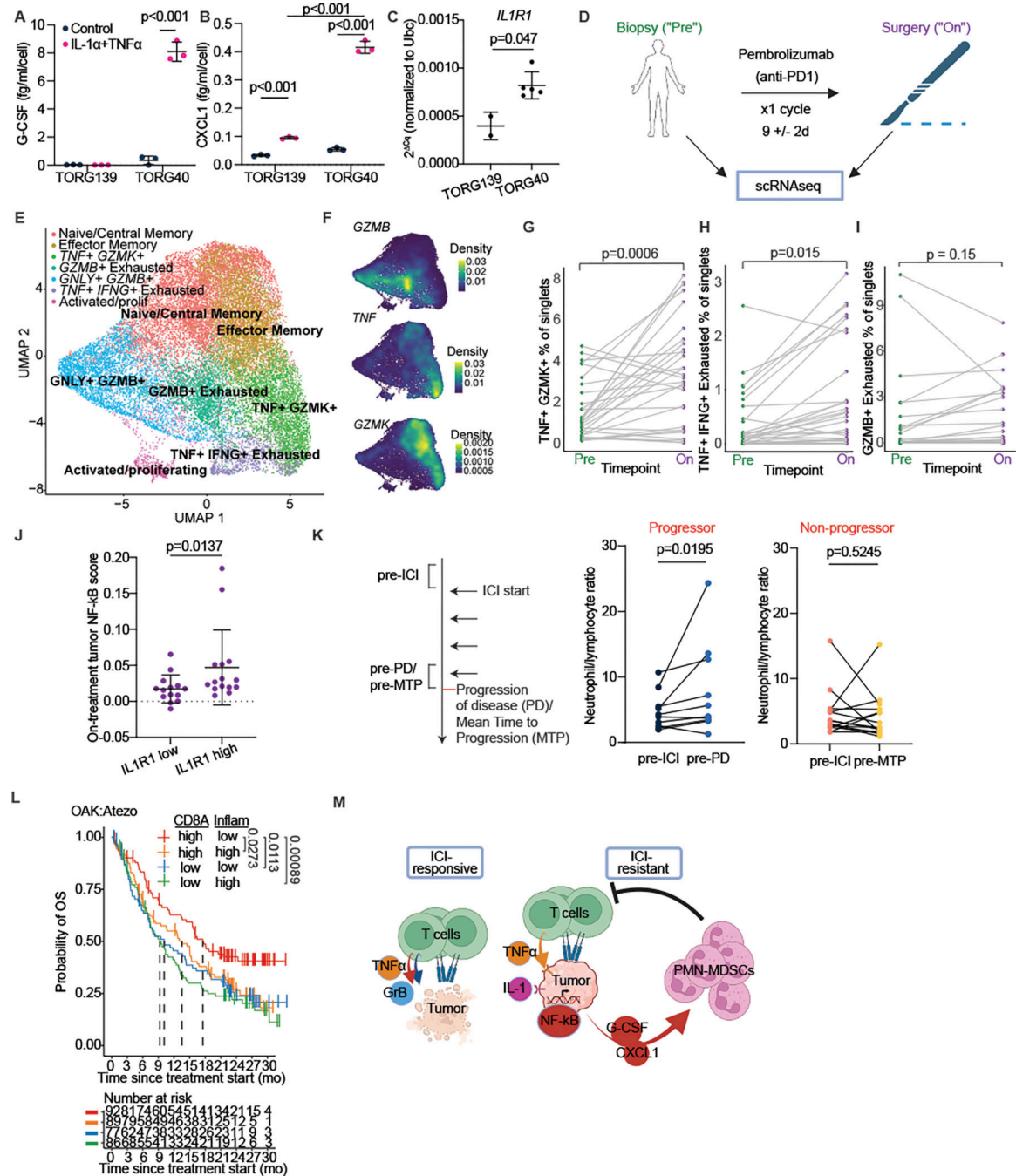


Figure 6: T cell, tumor, and neutrophil inflammation circuit in human cancer.

A, ELISA of G-CSF in supernatant of breast cancer organoid culture following indicated treatments for 24h, from one representative experiment with n=3 biological replicates. Two independent experiments were performed. P value by two-tailed t test. **B**, Analysis as in **(A)** for CXCL1. Adjusted p values by t tests. **C**, *IL1R1* RT-qPCR for breast organoids from two independent experiments. P value by one-tailed Mann-Whitney test. **D**, BIOKEY study schematic (EGAS00001004809) analyzed in **(E-J)**. **E**, UMAP of 24,349 CD8⁺ T cells color coded by CD8⁺ T cell phenotype. **F**, Cell density plots of *GZMB*, *TNF*, and *GZMK*

expressing cells. **G-I**, % cells in indicated cluster out of singlet cells in tumor for pre vs. on-treatment samples for each patient. P value by two-tailed Wilcoxon matched-pairs signed rank test. **J**, Quantification of on-treatment NF- κ B scores in tumor cells for each patient, stratified by *IL1R1* expression ($\geq 1\%$ (high) or $< 1\%$ (low) of tumor cells). **K**, Schema and plots of neutrophil/lymphocyte ratios in peripheral blood of patients with MSI-H tumors treated with ICI therapy, stratified by pregressor vs. non-progressor. P value by two-tailed Wilcoxon matched-pairs signed rank test. **L**, Kaplan-Meier curves from the OAK trial ([NCT02008227](#)), stratified by *CD8A* and inflammation gene score (“inflamm”). P values by log-rank test. **M**, Mechanistic model.

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