MATERIALS AND METHODS

Mice

All animal experiments were performed in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) of the Center for Excellence in Molecular Cell Science, Chinese Academy of Sciences. *CD45-Dre*, *Gata6-iCreER*, *R26-tdTomato* (*R26-tdT*), and Gata6-flox mouse lines have been previously described. *Gata6-RSR-tdT-DTR* mouse line was generated by Shanghai Biomodel Organism Science and Technology Development. Mice used in this study were maintained in a specific pathogen-free (SPF) facility with a standard 12-hour light/dark cycle and fed a standard chow diet (Jiangsu Xietong, 1010085).

Cell Culture

B16F10, Hepa1-6, and LLC cells were purchased from the National Collection of Authenticated Cell Cultures. B16F10 cells were cultured in RPMI-1640 medium (Thermo, 22400089) supplemented with 10% FBS (Thermo, A5669701) and 1% antibiotic antimycotic (Thermo, 15240062). Hepa1-6 and LLC cells were cultured in DMEM (Thermo, 11965092) supplemented with 10% FBS and 1% antibiotic antimycotic.

Cell isolation and flow cytometry

For pleural and peritoneal cavity cells: Mice were anesthetized by subcutaneous injection of 1% pentobarbital sodium. Peritoneal cells were collected by flushing the peritoneal cavity with 8 ml of sterile, cold PBS in a single injection. For pleural cavity cells, 4 ml of sterile, cold PBS was used. The recovered lavage fluid was centrifuged at 500 × g for 5 minutes at 4 °C, and the cell pellet was washed with PBS prior to staining.

For lung tissue: After euthanasia, mice were perfused via the right ventricle with 10 ml of cold PBS to remove blood from the lungs. Subsequently, lungs were inflated through the trachea with 10 ml of digestion solution containing Collagenase IV (5 mg/ml), 5% FBS, Dispase (0.72 U/ml) and DNase I (1 U/ml) in RPMI-1640 medium (FBS: Gibco 10099141; RPMI-1640: Invitrogen 22400089). The lungs were excised, minced into small pieces, and incubated in 10 ml digestion solution at 37 °C for 60 minutes with

shaking and frequent agitation. Following digestion, the cell suspension was filtered through a 70 μm strainer and centrifuged at 500 × g for 15 minutes at 4 °C. The supernatant was discarded, and the cells were then incubated with 1 ml Red Blood Cell lysis buffer (eBioscience, 00-4333-57) at room temperature for 5 minutes. After adding 9 ml PBS, the suspension was centrifuged again at 500 × g for 5 minutes at 4 °C to remove the supernatant. Finally, cells were washed twice with PBS before staining.

For cavity cells, blood cells, liver, and lung single-cell suspension, the cells were stained with primary antibodies containing CD45 FITC (eBioscience, 11-0451, 1:200), F4/80 PE-Cy7 (Biolegend, 123114, 1:200), CD11b APC (eBioscience, 17-0112-81, 1:200), CD11B FITC (eBioscience, 11-0112-85, 1:200), LY6G APC (eBioscience, 17-9668-80, 1:100), LY6C BV605 (Biolegend, 128035, 1:100), CD45 APC-eFlour780 (eBioscience, 47-0451-82, 1:400), CD3 FITC (eBioscience, 11-0031-82, 1:200), NK1.1 APC (eBioscience, 17-5941-8, 1:200), CD19 PE-Cy7(eBioscience, 25-0193, 1:200), CD4 eFluor450 (eBioscience, 17-8898-82, 1:200), CD8 PE-Cy7 (eBioscience, 17-8898-82, 1:200) at 4 °C for 30 min. Next, the cells were washed and re-suspended by PBS, and then stained with DAPI (Vector Laboratories) at 4 °C for 5 min before FACS. The cells were analyzed using Attune NxT Flow Cytometer (Thermo Fisher Scientific). For Granzyme B APC (eBioscience, 17-8898-82, 1:200), staining was performed using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (00-5523-00) according to the manufacturer's instructions. Data were generated using FlowJo (Tree Star).

Tamoxifen Administration

Tamoxifen (Sigma, T5648) was dissolved in corn oil at a concentration of 20 mg/mL and administered via oral gavage at 0.2 mg/g body weight daily for five consecutive days to induce Cre-mediated recombination.

Tumor Models

To establish lung metastases, 2×10^5 B16F10 and 2×10^6 B16F10 Hepa1-6, or LLC tumor cells were injected into the tail vein of tamoxifen-treated mice. For primary pleural tumor formation, 2×10^5 B16F10-Luc cells were directly injected into the pleural cavity.

Tissue Preparation and Immunofluorescence

Pleural and peritoneal cavity cells were collected by lavage with cold PBS and fixed in 4% paraformaldehyde (PFA). Lung and visceral organs were dissected, rinsed in PBS, and fixed in 4% PFA at 4°C for 1 hour, then dehydrated overnight in 30% sucrose. Samples were embedded in OCT and sectioned at 10 μm thickness. Sections were blocked with 2.5% donkey serum and DAPI in PBST (0.2% Triton X-100) for 30 minutes, followed by incubation with primary antibodies overnight at 4°C. Alexa-conjugated secondary antibodies were applied for 30 minutes at room temperature. Nuclei were counterstained with DAPI, and slides were mounted in antifade medium. Imaging was performed using Nikon A1 FLIM and Olympus FV4000 confocal microscopes. Primary antibodies used: F4/80 (Abcam, ab6640, 1:500), GATA6 (CST, 5851T, 1:500), DTR (R&D, AF-259-NA, 1:500), tdTomato (Rockland, 200-101-379, 1:1000). Secondary antibodies: Alexa Fluor 488/555/647 donkey anti-rat, anti-rabbit, anti-goat (Invitrogen, Jackson ImmunoResearch).

Whole-Mount Imaging

Lungs and tumor-bearing organs were placed in 1% agarose with PBS and imaged using a Zeiss AxioZoom V16 stereomicroscope for bright-field and fluorescence whole-mount visualization.

Histology

Masson–Fontana staining of lung sections was performed using a commercial melanin staining kit (BP-DL371-50 mL) according to the manufacturer's instructions to assess tumor burden. Tumor area and the number of lung surface tumors were quantified with ImageJ software.

Statistical Analysis

All experiments were repeated independently at least three times. Data are presented as mean ± standard deviation (SD). For comparisons between two groups, unpaired two-

tailed Student's t-tests were used. Kaplan-Meier survival curves were analyzed using the log-rank test. Statistical significance was set at $p < 0.05$. Analyses were performed using GraphPad Prism 10.4.2.

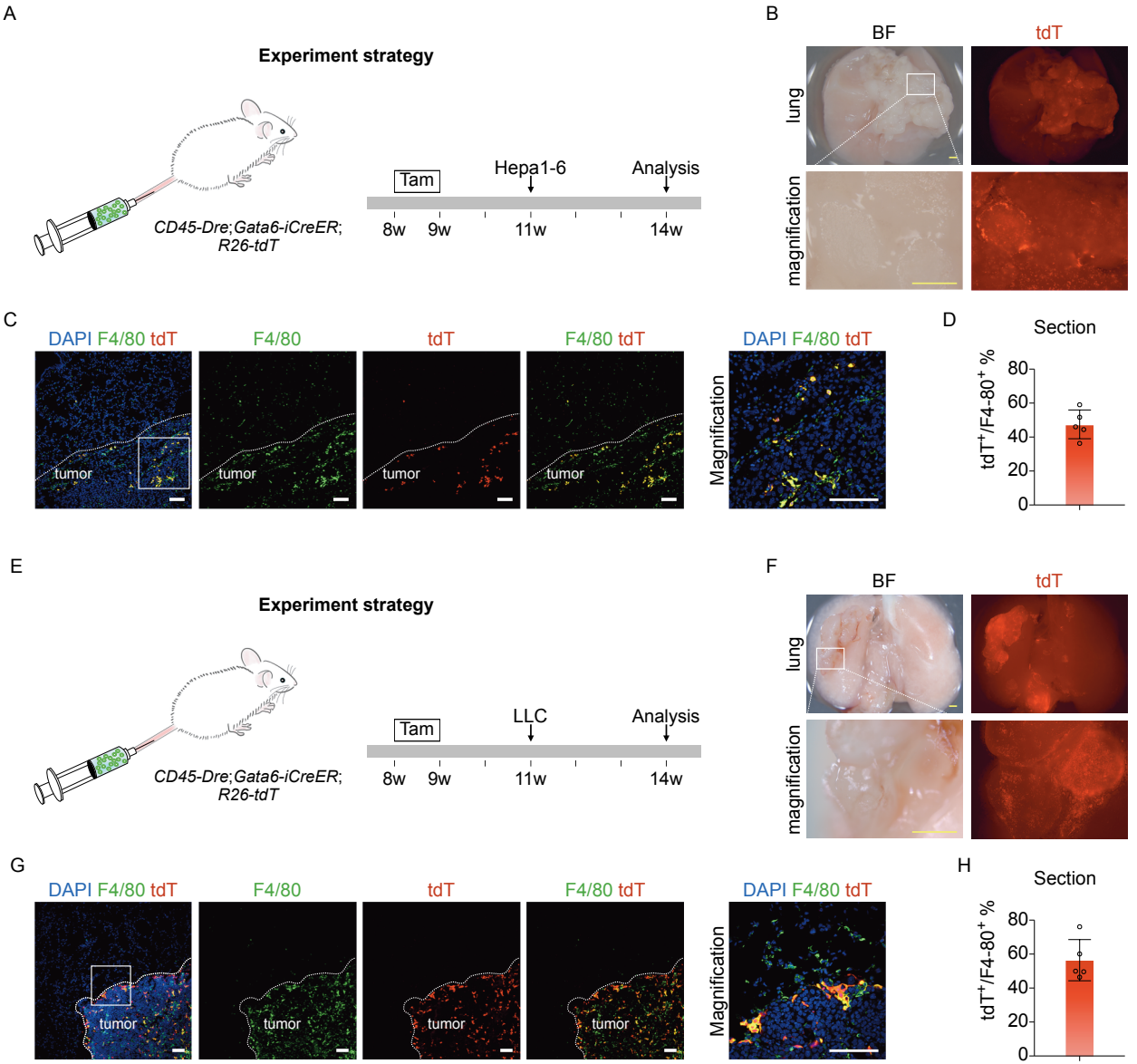

Figure S1. **Pleural cavity macrophages invade into lung tumors**. (A) A schematic showing the experimental strategy. (B) Whole-mount bright-field and fluorescence images of lungs from mice inoculated with Hepa1-6 cells. (C) Immunostaining for tdT and F4/80 on lung sections. (D) Quantification of the percentage of F4/80^{+} macrophages expressing tdT in tumors located at the lung peripheral. Data are the mean ± SD; n = 5 mice per group. (E) A schematic showing the experimental strategy. (F) Whole-mount bright-field and fluorescence images of lungs from mice inoculated with LLC cells. (G) Immunostaining for tdT and F4/80 on lung sections. (H) Quantification of the percentage of F4/80^{+} macrophages expressing tdT in tumors located at the lung peripheral. Data are the mean ± SD; n = 5 mice per group. Scale bar: 1mm (yellow), 100μm (white).

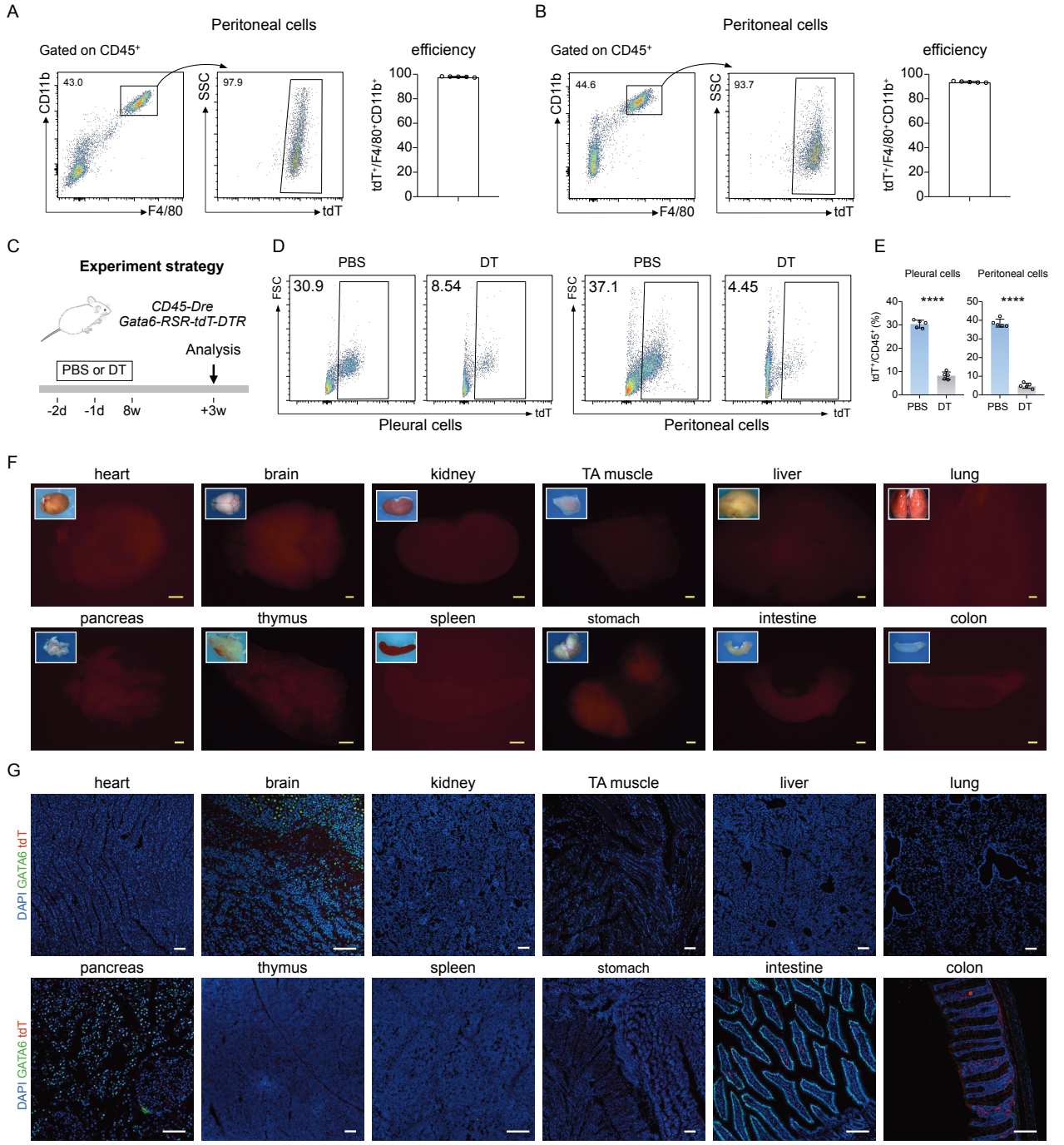

Figure S2. Characterization of *Gata6-RSR-tdT-DTR* knock-in allele. (A-B) FACS gating strategy for analyzing the percentage of peritoneal or pleural cavity macrophages expressing tdT. (C) Schematic showing the experimental design. (D) Flow cytometry analysis of the percentage of tdT$^+$ cavity macrophages from mice treated with PBS or DT. (E) Quantification of the percentage of tdT$^+$ cavity macrophages by FACS. (F) Whole-mount bright-field and fluorescence images of visceral organs from *CD45-Dre*;*Gata6-RSR-tdT-DTR* mice. (G) Immunostaining for tdT and GATA6 on organ sections. Scale bar: 1mm (yellow), 100μm (white).

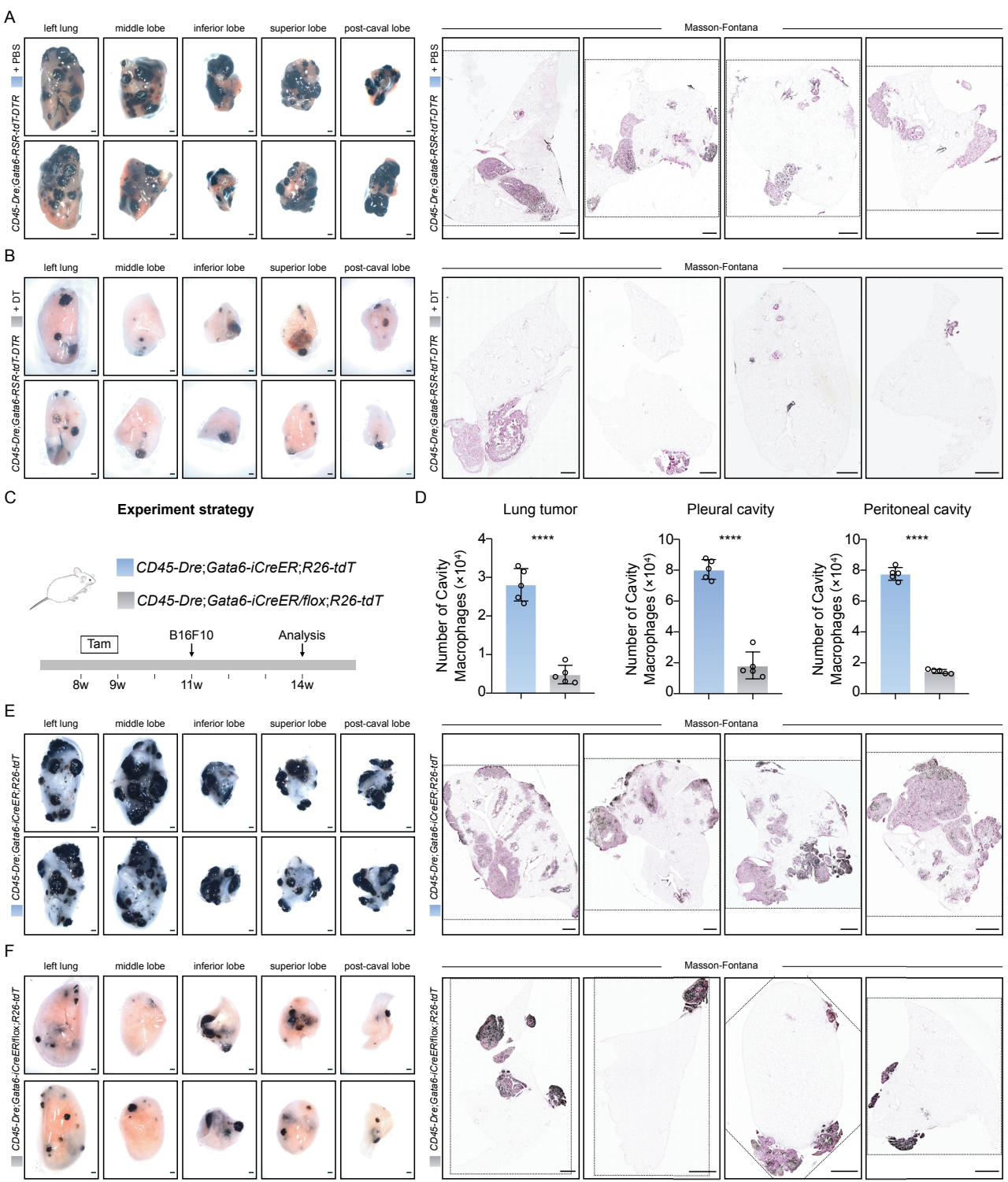

Figure S3. Genetic ablation of pleural cavity macrophages inhibits tumor growth. (A) Whole-mount bright-field view of lungs from indicated mice at 21 days after inoculation with B16F10 cells. (B) Masson-Fontana staining of lung sections from indicated mice at 21 days after inoculation with B16F10 cells. (C) Schematic showing the experimental design. (D) Quantification of cavity macrophages in peritoneal and pleural spaces and tumor. Data are the mean ± SD; n = 5 mice per group. (E) Whole-mount bright-field view of lungs from indicated mice at 21 days after inoculation with B16F10 cells. (F) Masson-Fontana staining of lung sections from indicated mice at 21 days after inoculation with B16F10 cells. Scale bar: 1 mm.

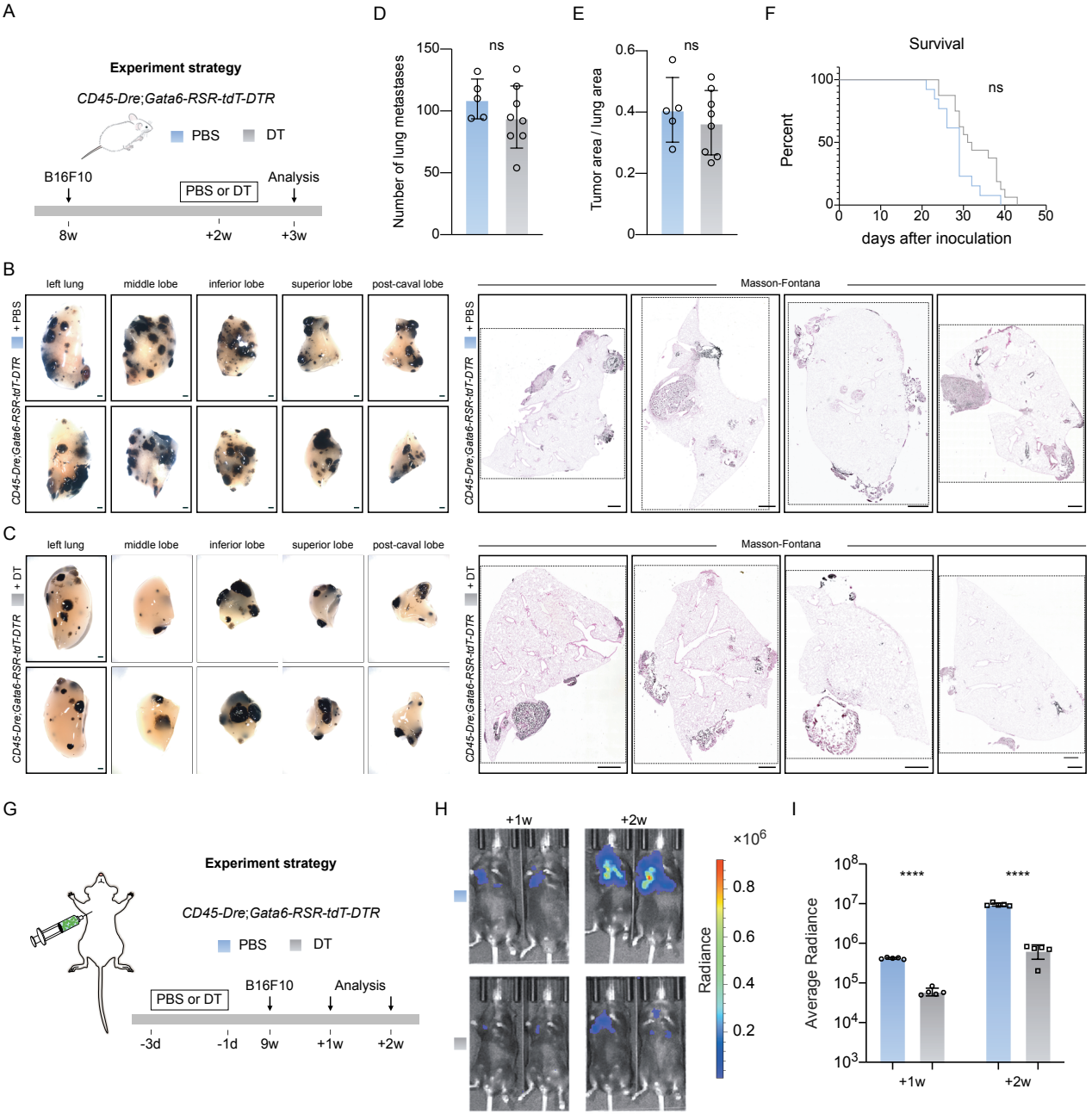

Figure S4. The function of pleural cavity macrophages in established tumors and the primary tumors. (A) Schematic illustrating the genetic ablation of cavity macrophages. (B-C) Whole-mount bright-field view of lungs and Masson-Fontana staining of lung sections from indicated mice at 21 days after inoculation with B16F10 cells. (D) Quantification of the number of B16F10 lung metastases. Data are the mean ± SD; n = 5 mice per group. (E) Quantification of the ratio of tumor-covered area to total lung area. Data are the mean ± SD; n = 5 mice per group. (F) Kaplan-Meier plot illustrating the survival rates of mice in the study. n = 5 mice per group. (G) Schematic showing the experimental strategy for inducing primary tumors by melanoma cell inoculation. (H) Bioluminescence imaging of the indicated mice from 1 to 2 weeks after B16F10-Luc cell inoculation. (I) Quantification data showing luciferase activity. Data are means ± SD; n = 5. Scale bar: 1mm (black).

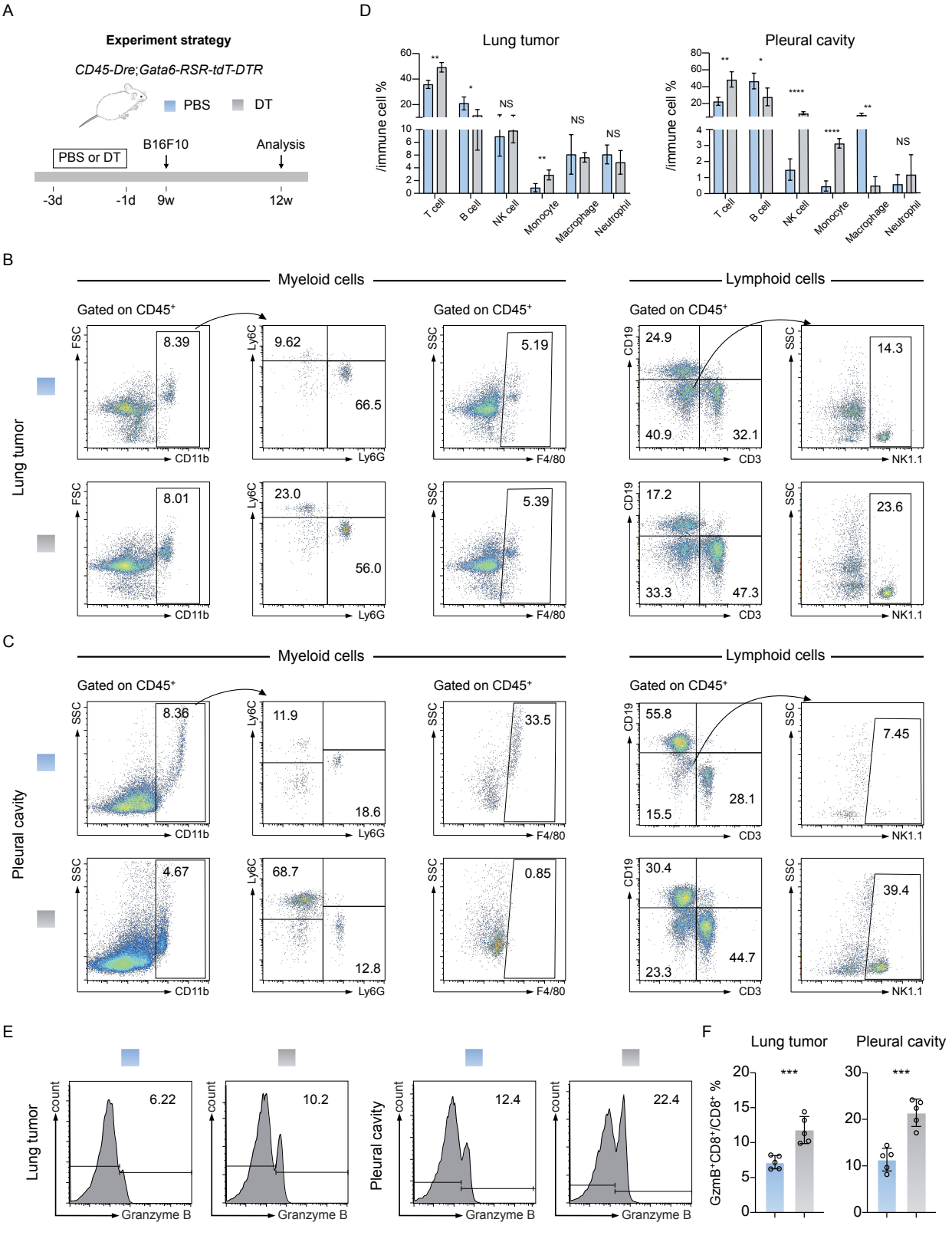
A
Experiment strategy
CD45-Dre;Gata6-RSR-tdT-DTR
PBS
DT
PBS or DT
B16F10
Analysis
-3d
-1d
9w
12w
D
Lung tumor
Pleural cavity
/immune cell %
T cell
B cell
NK cell
Monocyte
Macrophage
Neutrophil
NS
B
Myeloid cells
Lymphoid cells
Gated on CD45+
Lung tumor
FSC
CD11b
Ly6C
Ly6G
SSC
F4/80
CD19
CD3
NK1.1
8.39
9.62
66.5
5.19
24.9
40.9
32.1
14.3
8.01
23.0
56.0
5.39
17.2
33.3
47.3
23.6
C
Pleural cavity
8.36
11.9
18.6
33.5
55.8
15.5
28.1
7.45
4.67
68.7
12.8
0.85
30.4
23.3
44.7
39.4
E
count
Granzyme B
6.22
10.2
12.4
22.4
F
GzmB+CD8+/CD8+ %
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Figure S5. The immune profile of lung tumors and pleral cells. (A) Schematic figure showing the experimental strategy. (B) Flow cytometric analysis of the proportions of myeloid and lymphoid cells in lung tumors from mice treated with PBS or DT. (C) Flow cytometric analysis of the percentages of myeloid and lymphoid cells among pleural cells from mice treated with PBS or DT. (D) Quantification of myeloid and lymphoid cell subsets in by flow cytometry. Data are the mean ± SD; n = 5 mice per group. (E) Flow cytometric analysis of the percentages of Granzyme B^+CD8^+ T cells in lung tumors and pleural cells from mice treated with PBS or DT. (F) Quantification of $GzmB^+CD8^+$ T cells by flow cytometry. Data are the mean ± SD; n = 5 mice per group.