JEM

Supplemental material

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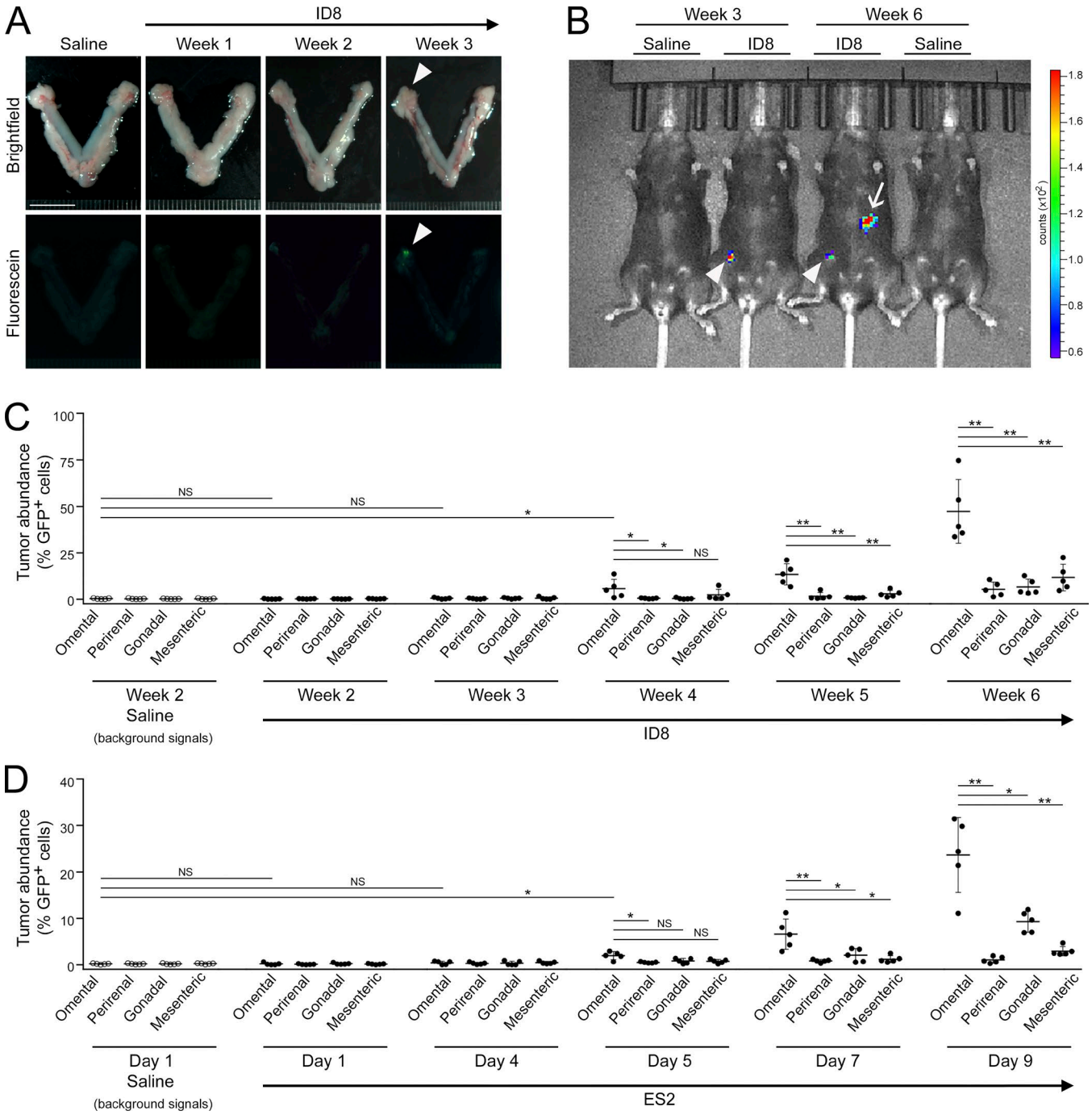

Figure S1. **Kinetics of tumor implant formation in ID8 orthotopic and ES2 i.p. models. (A–C)** Immunocompetent C57BL/6 mice were injected i.b., between the left ovary and oviduct and contralateral to the omentum, with ID8 cells that express GFP and luciferase and thereafter evaluated at the indicated times (n = 5 mice at each time point). **(A)** Representative images of the reproductive organs viewed under light and fluorescence microscopy are shown. Bar, 10 mm. Primary tumor formation at the injection site is denoted by arrowhead. **(B)** Representative images of mice with primary tumors (arrowhead) and omental implants (arrow) detected by bioluminescence imaging are shown. **(C)** The abundance of GFP$^+$ tumor cells in omental (lesser and greater combined), perirenal (contralateral to injection site), gonadal, and mesenteric fat tissues quantified by flow cytometry is shown. *, P < 0.05; **, P < 0.01 (paired t test, between matched tissues at a given time point; unpaired t test, between different groups). **(D)** Abundance of tumor cells in visceral fat tissues at the indicated times following i.p. injection of female nude mice with GFP-expressing ES2 cells (n = 5 mice at each time point). *, P < 0.05; **, P < 0.01, (paired t test, between matched tissues at a given time point; unpaired t test, between different groups). Nontumor control mice were injected with saline either i.b. (C) or i.p. (D) to distinguish background signals (n = 5 mice per group). Error bars in C and D represent SD.

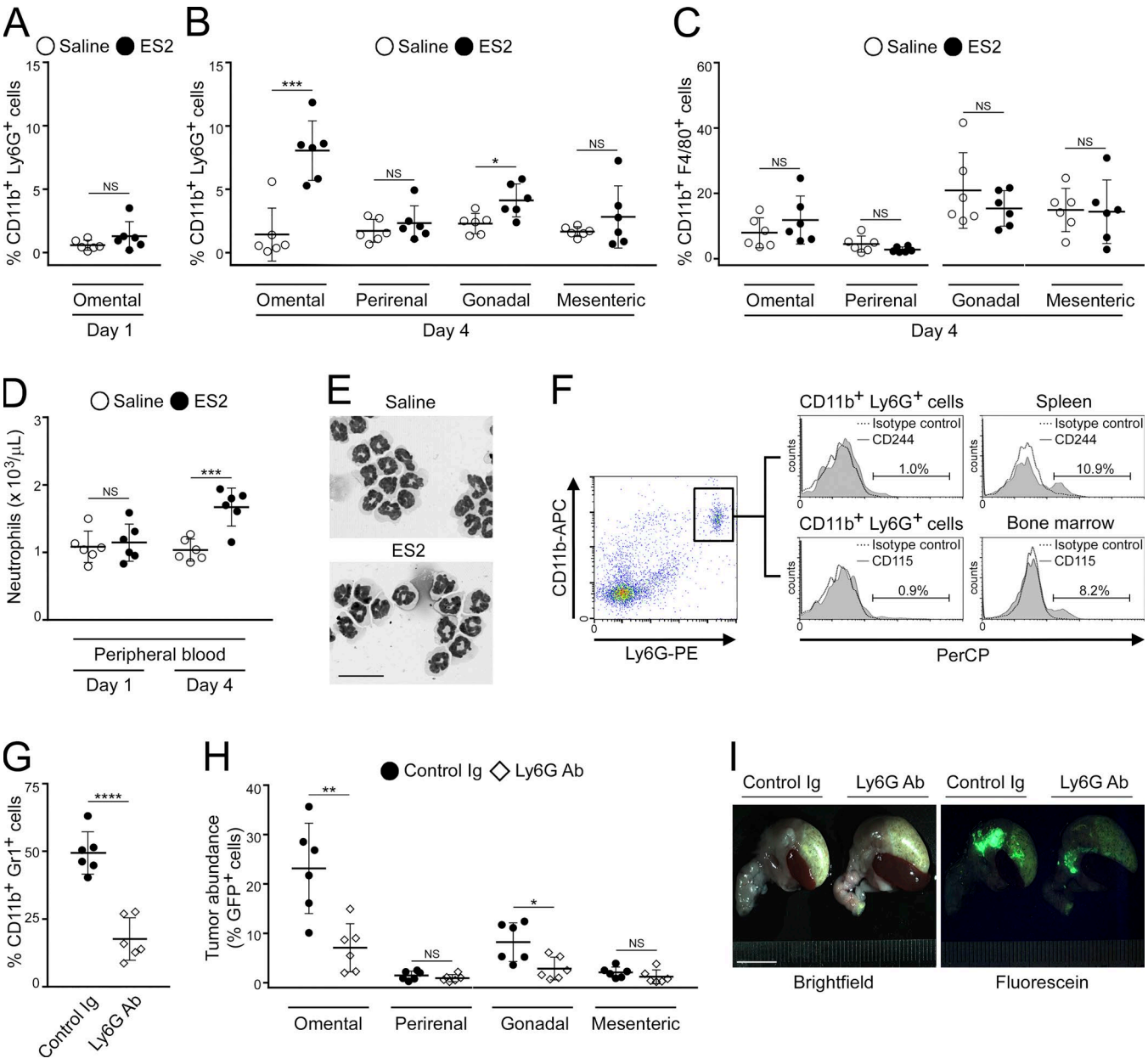

Figure S2. **Abundance, characteristics, and depletion of neutrophils in ES2 i.p. xenograft models. (A–C)** Female nude mice were injected i.p. with GFP-expressing ES2 cells or with saline and euthanized at 1 d or at 4 d thereafter (n = 6 mice per group at each time point). Fat tissues of each mouse were analyzed by flow cytometry to confirm the absence of GFP$^+$ tumor cells and to determine the abundance of CD11b$^+$Ly6G$^+$ cells (neutrophils) and CD11b$^+$F4/80$^+$ cells (macrophages). Shown are the abundance of neutrophils in omental tissues at day 1 (A) and of neutrophils (B) and macrophages (C) in omental and other fat tissues at day 4. *, P < 0.05; ***, P < 0.001 (unpaired t test). **(D–F)** Peripheral blood was collected from nude mice at 1 and 4 d after i.p. injection of ES2 cells or saline (n = 6 mice per group at each time point). **(D)** Neutrophil counts were determined by CBC. ***, P < 0.001 (unpaired t test). **(E)** Ly6G$^+$ cells were isolated from blood drawn at day 4 and stained with Giemsa dye. Bar, 20 µm. Shown are representative examples of staining. **(F)** CD244 and CD115 expression was evaluated by flow cytometry in circulating CD11b$^+$Ly6G$^+$ cells of mice in the ES2 group at day 4. Normal spleen and bone marrow of C57BL/6 mice were stained as controls for CD244 and CD115 expression, respectively. Shown are gating strategy and representative analyses. **(G–I)** Nude mice were administered either Ly6G Ab or control Ig (n = 6 mice per group) at 3 and 1 d before i.p. injection of ES2 cells and then evaluated at 9 d thereafter. **(G)** Abundance of neutrophils in peripheral blood quantified by flow cytometric analysis. ****, P < 0.0001 (unpaired t test). **(H)** Abundance of GFP$^+$ tumor cells in fat tissues. *, P < 0.05; **, P < 0.01 (unpaired t test). **(I)** Representative images of omental tumors viewed under light and fluorescence microscopy. Bar, 10 mm. Error bars in A–D, G, and H represent SD.

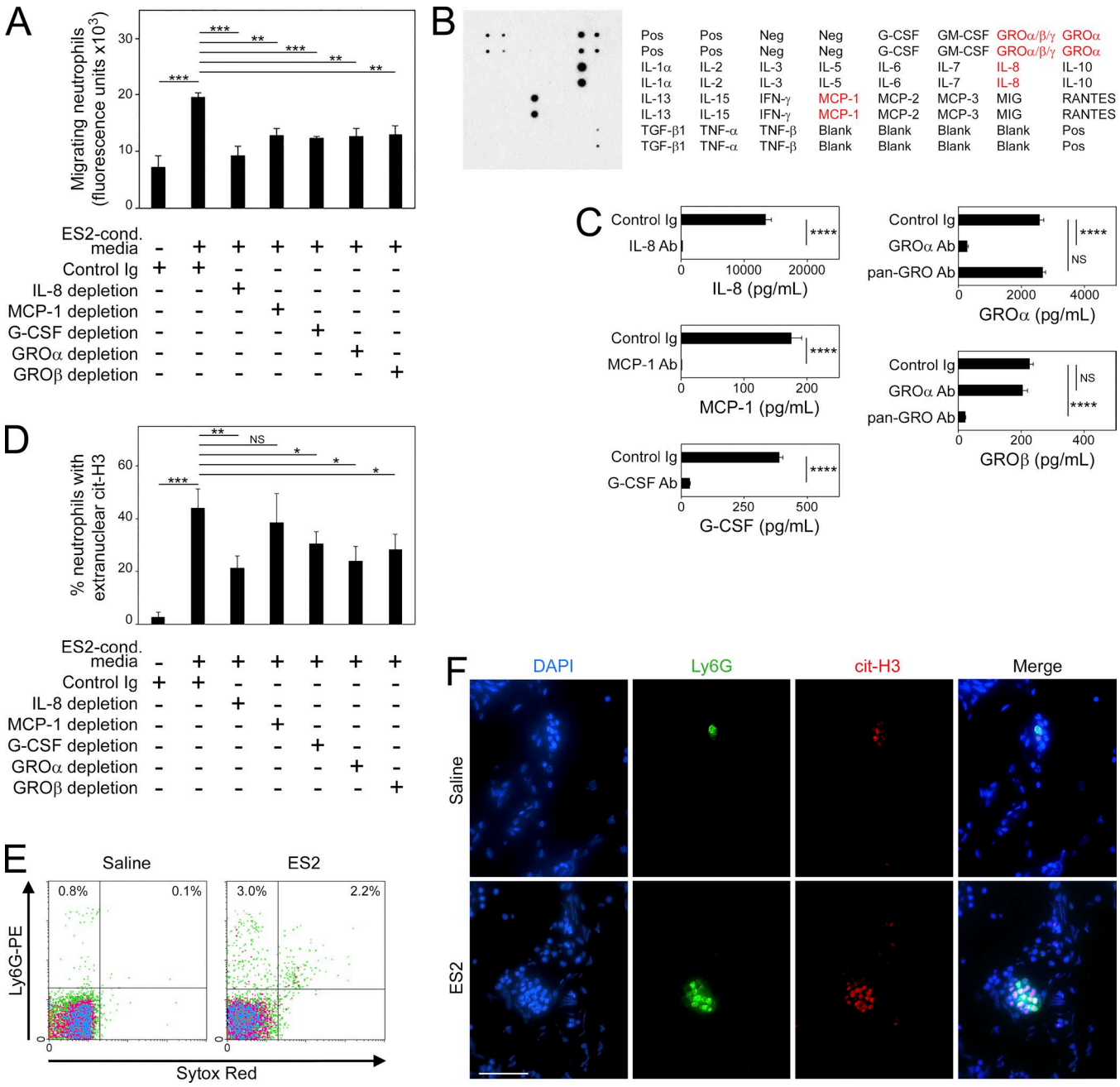

Figure S3. **Ovarian cancer cells secrete factors that induce neutrophils to mobilize and form NETs. (A)** Normal human peripheral blood neutrophils were stimulated with nonconditioned media or with ES2-conditioned media that had been depleted of the indicated cytokines or was nondepleted (i.e., treated with control Ig. See C for depletion details). At 2 h following stimulation, chemotaxis was assayed in migration chambers. Migrating neutrophils were stained with CyQuant dye and quantified by measuring fluorescence. Shown are means ± SD of three independent experiments. Migrating cells were evaluated in four replicate wells in each experiment. Each experiment used neutrophils of a different healthy donor. **, P < 0.01; ***, P < 0.001 (unpaired *t* test). **(B)** Detection of cytokines in ES2-conditioned media by Ab array. Data were verified in two independent experiments. **(C)** ES2-conditioned media was depleted of the indicated cytokines by IP using neutralizing Abs. Efficacy of depletion of each cytokine in conditioned media was confirmed by ELISA. Means ± SD of three independent assays are shown. ****, P < 0.0001 (unpaired *t* test). Because GROα and GROβ have extensive homology, several commercially available Abs were tested at a range of concentrations for specificity in preliminary studies. As shown, IP using GROα Ab (1.5 µg/ml) reduced GROα levels by 90% without significantly reducing GROβ. IP using pan-GRO Ab (0.2 µg/ml) reduced GROβ levels by 90% without significantly reducing GROα. Concentrations of Abs to other cytokines were as follows: IL-8 (0.3 µg/ml), MCP-1 (4.5 µg/ml), and G-CSF (0.3 µg/ml). **(D)** Neutrophils were stimulated for 4 h, as in A, and then stained with cit-H3 Ab and DAPI. In each experiment, the average percentage of neutrophils with extranuclear cit-H3 staining was calculated by scoring five random ×400 microscopic fields. An average of 50 neutrophils per field were scored. Shown are means ± SD of three independent experiments. Each experiment used neutrophils of a different donor. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (unpaired *t* test). **(E and F)** Nude mice were injected i.p. with nonfluorescent ES2 cells or with saline (n = 5 mice per group) and euthanized at 4 d thereafter. Shown are representative examples of flow cytometric analysis of Ly6G and Sytox Red staining in fresh omental tissues (E) and Ly6G (green) and cit-H3 (red) staining in frozen omental tissue sections (F). Bar, 50 µm.

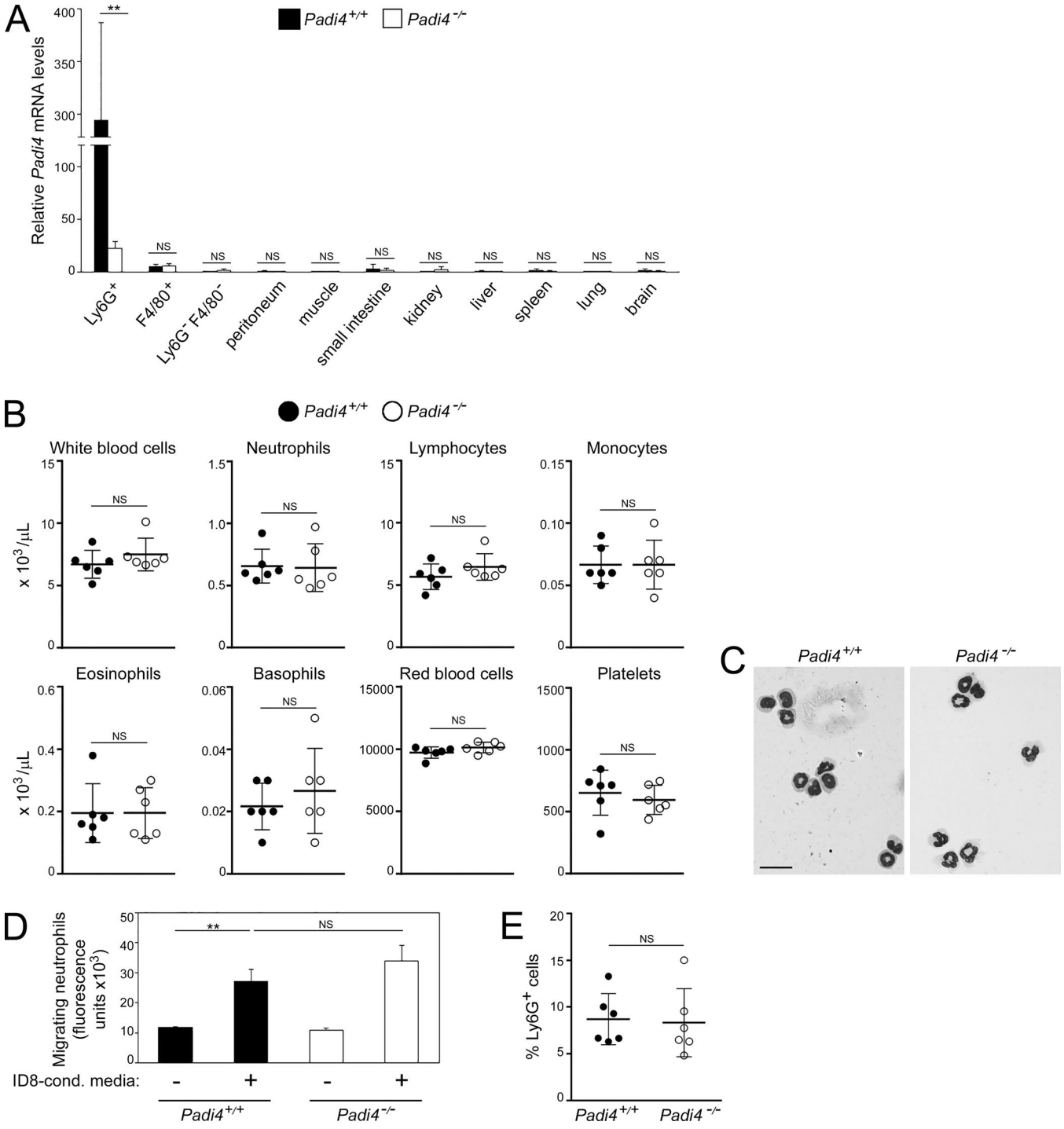

Figure S4. **Characterization of neutrophil-specific *Padi4*-deficient mice. (A)** qRT-PCR analysis of *Padi4* mRNA levels in tissues of 6–7-wk-old female neutrophil-specific *Padi4*$^{-/-}$ mice and control siblings (n = 3 mice per group). Neutrophils and macrophages were isolated from peripheral blood by positive selection using anti-Ly6G and anti-F4/80 microbeads, respectively. Ly6G$^-$F4/80$^-$ cells refer to the pool of nonselected blood cells. **, P < 0.01 (unpaired t test). **(B)** CBC analysis of neutrophil-specific *Padi4*$^{-/-}$ mice and control siblings (n = 6 mice per group; unpaired t test). **(C)** Morphology of peripheral blood neutrophils visualized by Giemsa staining. Bar, 20 µm. Three neutrophil-specific *Padi4*$^{-/-}$ mice and three control siblings were analyzed. Shown are representative images. **(D)** Chemotaxis of neutrophils of neutrophil-specific *Padi4*$^{-/-}$ and control mice was assayed in migration chambers at 2 h following stimulation with nonconditioned media or ID8-conditioned media. Migrating neutrophils were stained with CyQuant dye and quantified by measuring fluorescence. Shown are means ± SD of three independent experiments. **, P < 0.01 (unpaired t test). **(E)** Neutrophil-specific *Padi4*$^{-/-}$ mice and control siblings were injected i.b. with ID8 cells and euthanized at 3 wk thereafter when primary tumors, but no metastasis, had formed in both groups. Shown are the abundance of Ly6G$^+$ cells in omental tissue of each mouse in each of the groups (n = 6 mice per group), expressed as a percentage of the nonfat cellular content (unpaired t test). Error bars in B and E represent SD.

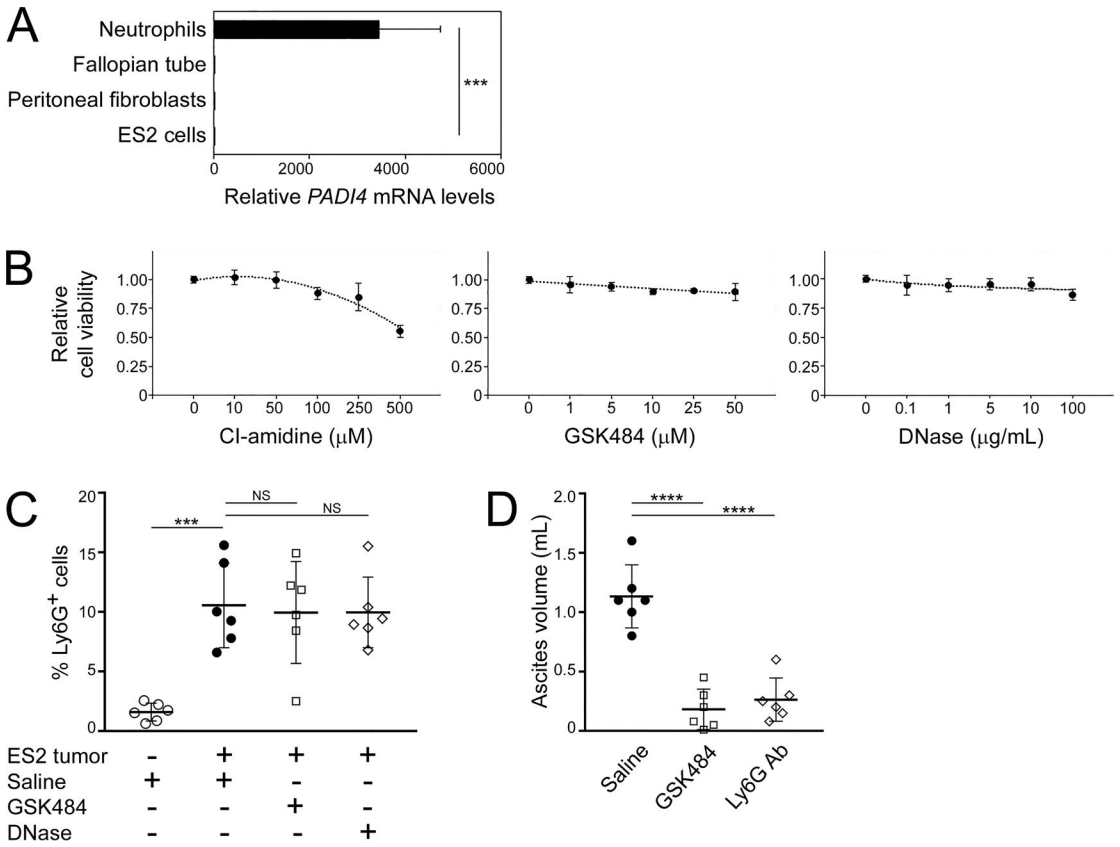

Figure S5. **Effects of NET-inhibiting agents on ovarian cancer cell viability, neutrophil abundance, and ascites. (A)** qRT-PCR analysis of relative *PADI4* mRNA levels in ES2 cells and in normal human tissues/cells. Shown are means ± SD of three independent experiments. ***, P < 0.001 (unpaired *t* test). **(B)** ES2 cells were incubated in suspension cultures without or with the addition of CI-amidine, GSK484, or DNase at the indicated concentrations and evaluated for viability at 2 d thereafter by MTT assay. Shown are means ± SD of three independent experiments. Viability of drug-treated cells is expressed relative to that of untreated cells. **(C)** Female nude mice were injected i.p. with ES2 cells. At 1 d thereafter, mice were randomized into groups and then administered saline, GSK484 (20 mg/kg), or DNase (5 mg/kg) daily for 9 d. A nontumor control group of mice was administered saline daily. Shown are the abundance of $Ly6G^+$ cells in omental tissue of each mouse in each of the groups (n = 6 mice per group). ***, P < 0.001 (unpaired *t* test). **(D)** Nude mice were injected i.p. with ES2 cells, administered saline, GSK484 (20 mg/kg daily), or Ly6G Ab (200 µg per mouse, twice a week) for 18 d (n = 6 mice per group). Mice were then euthanized, and ascites was collected from the abdominal cavity. Shown are the volume of ascites in each mouse in each of the groups. ****, P < 0.0001 (unpaired *t* test). Error bars in C and D represent SD.