Supplementary Figures

The generation of neutrophils in the bone marrow is controlled by autophagy

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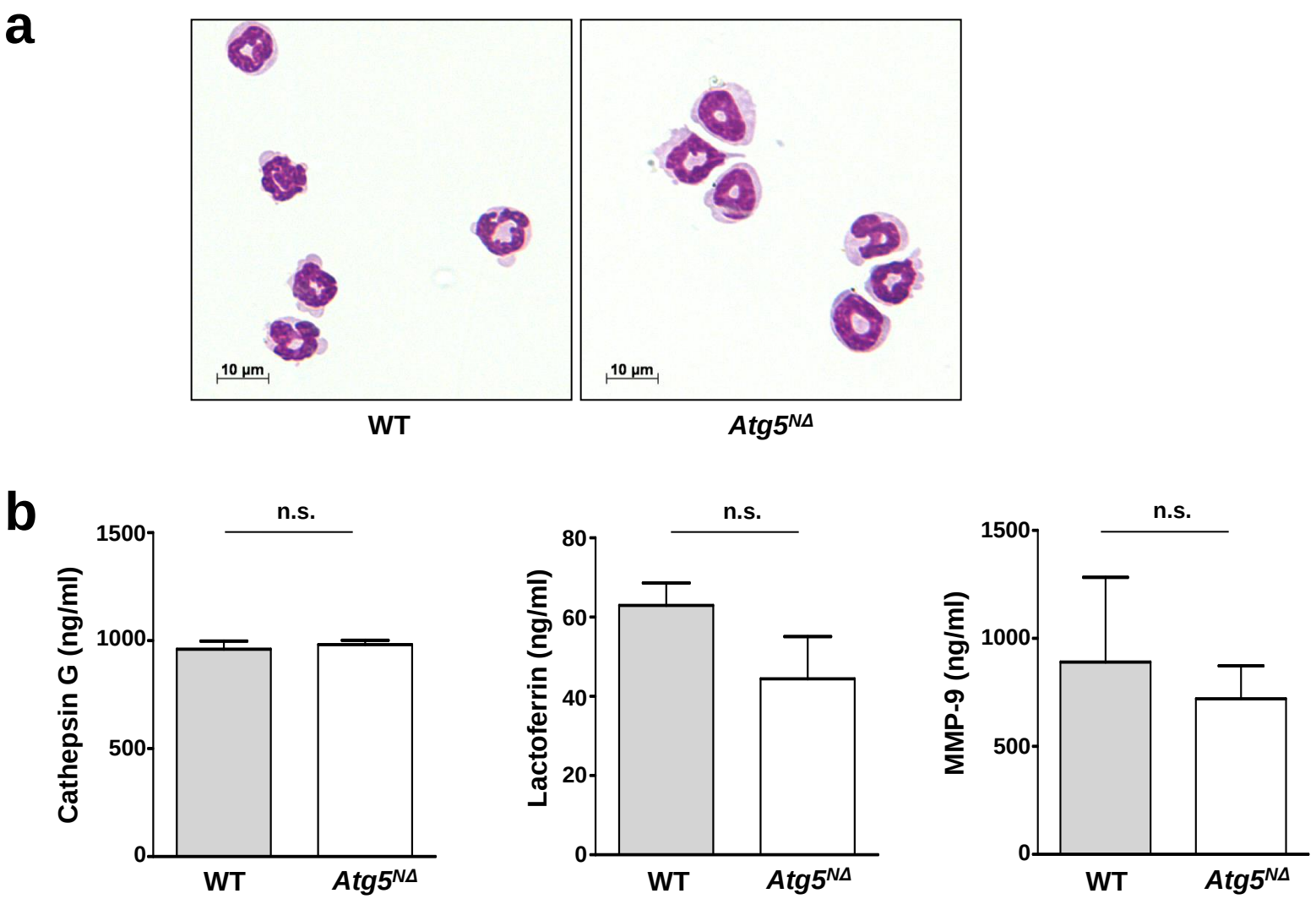

Figure S1 ATG5-deficient neutrophils exhibit no abnormalities in morphology and degranulation. (**a**) Light microscopy. Cytospins of freshly purified mature bone marrow-derived neutrophils of WT and *Atg5*$^{N\Delta}$ mice were stained with the Hematocolor Set and cell morphology was evaluated. Bars, 10 µm. (**b**) Freshly purified mature bone marrow-derived neutrophils of WT and *Atg5*$^{N\Delta}$ mice were primed with cytochalasin B and subsequently stimulated with fMLP. Supernatant samples were acquired after 1 h and analyzed by ELISA for the presence of cathepsin G (released from primary granules), lactoferrin (released from secondary granules), and MMP-9 (released from tertiary granules). Values are means ± SEM (n=3).

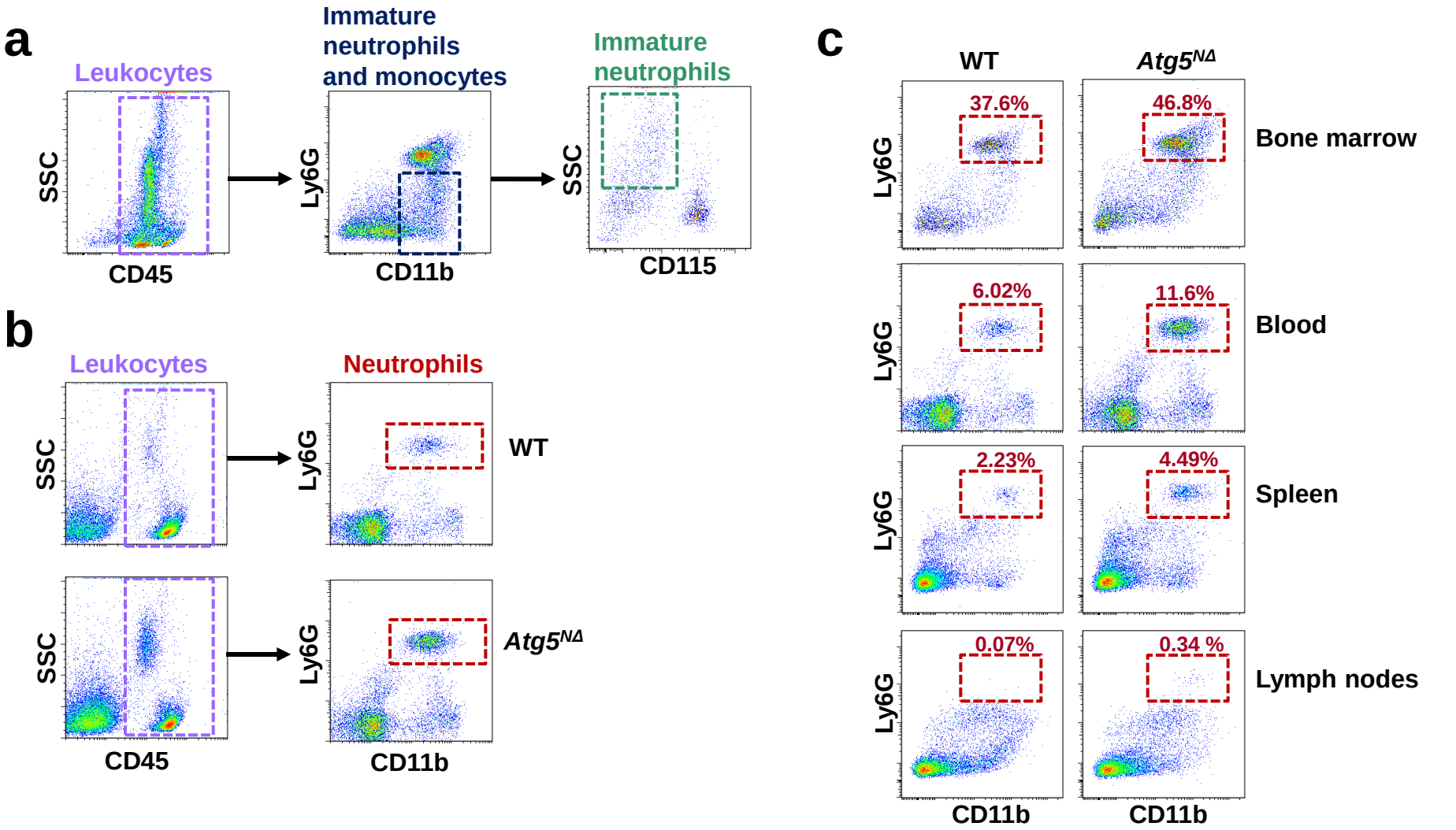

Figure S2 Neutrophil numbers are increased in *Atg5*$^{N\Delta}$ mice. (**a**) Flow cytometry. Representative flow cytometry dot plots of a mouse bone marrow cell suspension. Immature neutrophil precursors were defined as a CD45^{+}CD11b^{+}Ly6G^{-}CD115^{-}SSC^{high} cell population. (**b**) Flow cytometry. Representative flow cytometry dot plots of whole blood cell suspensions of WT and *Atg5*$^{N\Delta}$ mice. Mature neutrophils were defined as a CD45^{+}CD11b^{+}Ly6G^{+} population. The same gating strategy was applied when determining neutrophil numbers in the bone marrow, spleen, and lymph nodes. (**c**) Flow cytometry. Representative flow cytometry dot plots of bone marrow, blood, spleen, and lymph node cell suspensions in WT and *Atg5*$^{N\Delta}$ mice. The dot plots depict the gating of neutrophils for measuring the relative numbers of neutrophils in these organs. *Atg5*$^{N\Delta}$ mice exhibit consistently higher neutrophil numbers in each organ compared to WT mice (see Figure 2a).

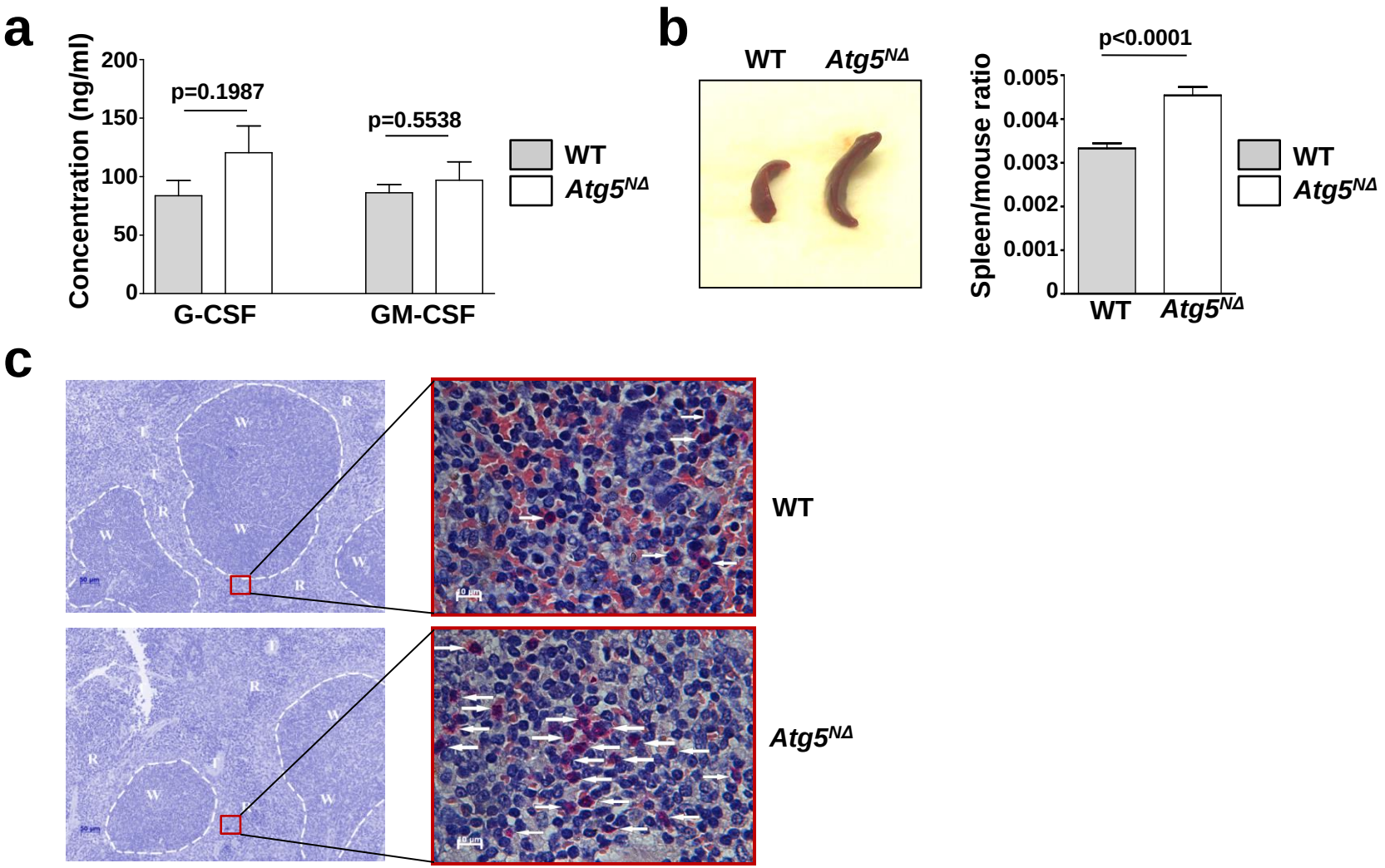

Figure S3 Neutrophil numbers in *Atg5*$^{N\Delta}$ mice are not related to increased G-CSF or GM-CSF levels in serum, but are associated with splenomegaly. (**a**) Magnetic bead-based immunoassay. G-CSF and GM-CSF concentrations in the serum of WT and *Atg5*$^{N\Delta}$ mice did not differ significantly. Values are means ± SEM (n=5). (**b**) The spleen is significantly enlarged in *Atg5*$^{N\Delta}$ mice. (Right) Statistical analysis of spleen/mouse body weight ratios. Values are means ± SEM (n=3). (**c**) Histology. Spleen sections were stained with naphthol AS-D chloroacetate and counterstained with hematoxylin solution. Lower magnifications (left panel) show trabeculae (T), white pulp (W), and red pulp (R) areas of the spleen. The red squares designate the area analyzed with the higher magnification. Higher magnification images (right panel; Bars, 10 μm) demonstrate the pink-stained neutrophils (white arrows) in the red pulp area.

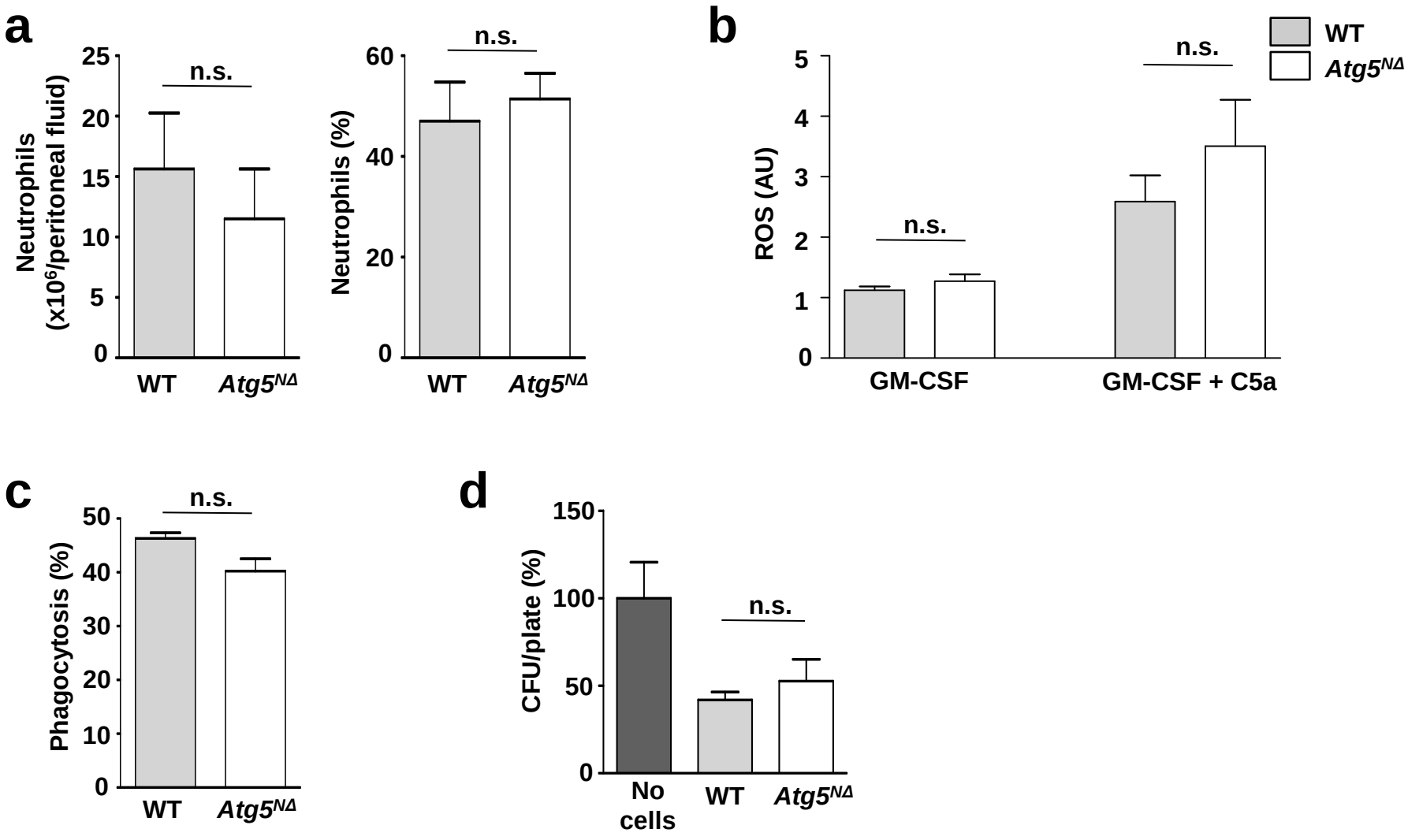
a
Neutrophils (x10^6/peritoneal fluid)
25
20
15
10
5
0
n.s.
WT
Atg5NΔ
Neutrophils (%)
60
40
20
0
n.s.
WT
Atg5NΔ
b
ROS (AU)
5
4
3
2
1
0
n.s.
n.s.
GM-CSF
GM-CSF + C5a
WT
Atg5NΔ
c
Phagocytosis (%)
50
40
30
20
10
0
n.s.
WT
Atg5NΔ
d
CFU/plate (%)
150
100
50
0
n.s.
No cells
WT
Atg5NΔ

Figure S4 Neutrophils of *Atg5$^{N\Delta}$* mice are normally attracted by IL-8 *in vivo* and exhibit normal effector functions *in vitro*. (**a**) *In vivo* recruitment assay. WT and *Atg5$^{N\Delta}$* mice were injected intraperitoneally with IL-8 (or PBS). Recruited neutrophils in the intraperitoneal cavity were counted. Values are means ± SEM following IL-8 injection (n=3). PBS treatment was not followed by any neutrophil recruitment. (**b**) Flow cytometry. The detection of ROS production was based on the oxidation of dihydrorhodamine 123. The indicated mature neutrophil populations were stimulated with GM-CSF either alone or together with C5a. Values are means ± SEM (n=3). (**c**) Flow cytometry. Red fluorescent beads were opsonized with mouse serum (30 min, 37°C) and then incubated with the indicated stimulated mature neutrophil populations for 1 h. Values are means ± SEM (n=3). (**d**) Bacterial killing assay. Primary bone marrow-derived neutrophils were incubated with opsonized bacteria (45 min, 37°C), rotating end-over-end. Following the incubation, supernatants were collected, diluted 1:200 and plated on agar. Bacteria were grown overnight and the colonies counted the next day. Tubes containing bacteria alone (no cells) were treated the same way and used as controls. Values are means ± SEM (n=4) and represent the percentage of colony-forming units in a sample in relation to the colony-forming units in controls without neutrophils.

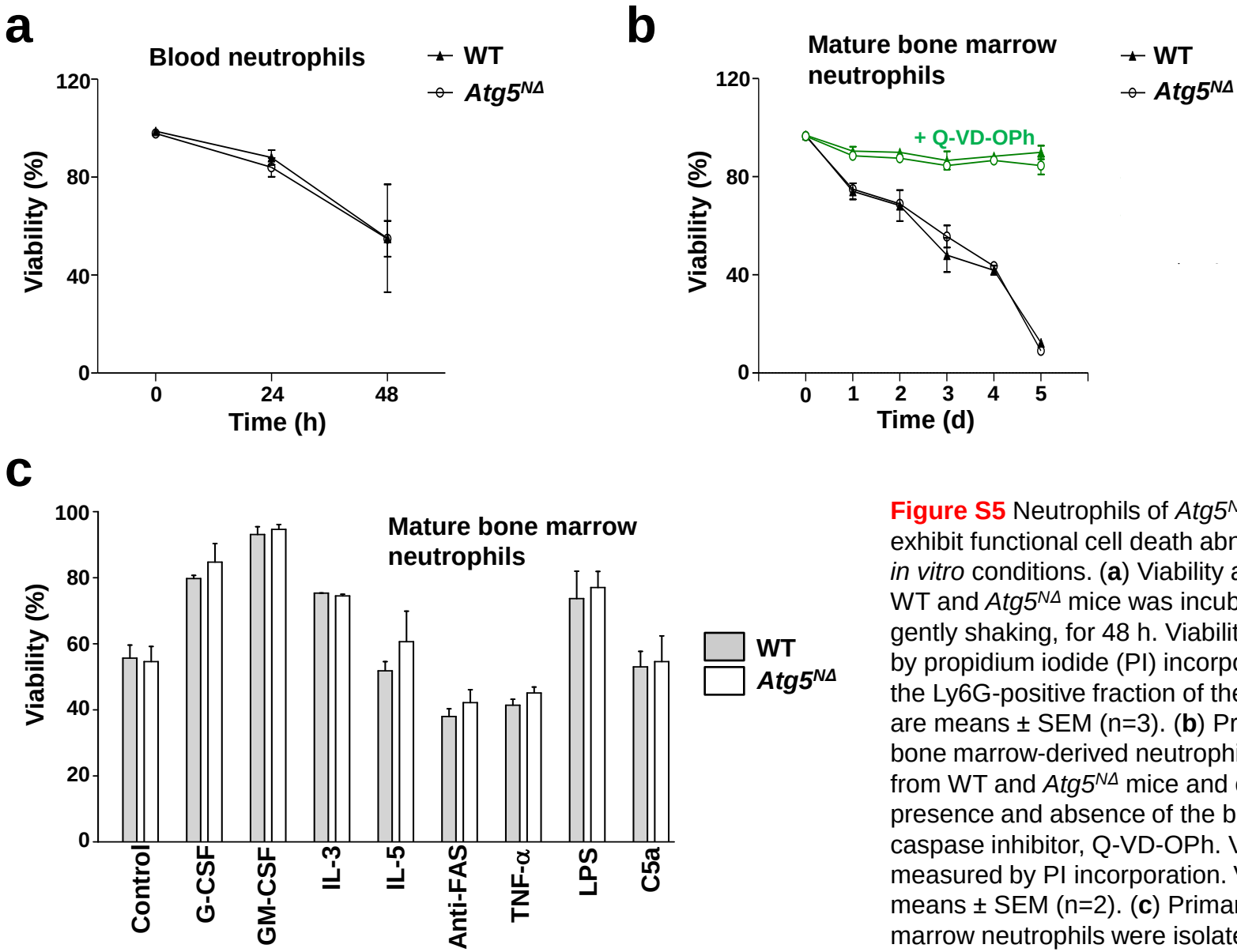

Figure S5 Neutrophils of *Atg5*$^{N\Delta}$ mice do not exhibit functional cell death abnormalities under *in vitro* conditions. (**a**) Viability assay. Blood from WT and *Atg5*$^{N\Delta}$ mice was incubated at 37°C, gently shaking, for 48 h. Viability was measured by propidium iodide (PI) incorporation in cells of the Ly6G-positive fraction of the blood. Values are means ± SEM (n=3). (**b**) Primary mature bone marrow-derived neutrophils were isolated from WT and *Atg5*$^{N\Delta}$ mice and cultured in the presence and absence of the broad spectrum caspase inhibitor, Q-VD-OPh. Viability was measured by PI incorporation. Values are means ± SEM (n=2). (**c**) Primary mature bone marrow neutrophils were isolated from WT and *Atg5*$^{N\Delta}$ mice and cultured in the presence and absence of the indicated ligands for 24 h. Viability was measured by PI incorporation. Values are means ± SEM (n=2).

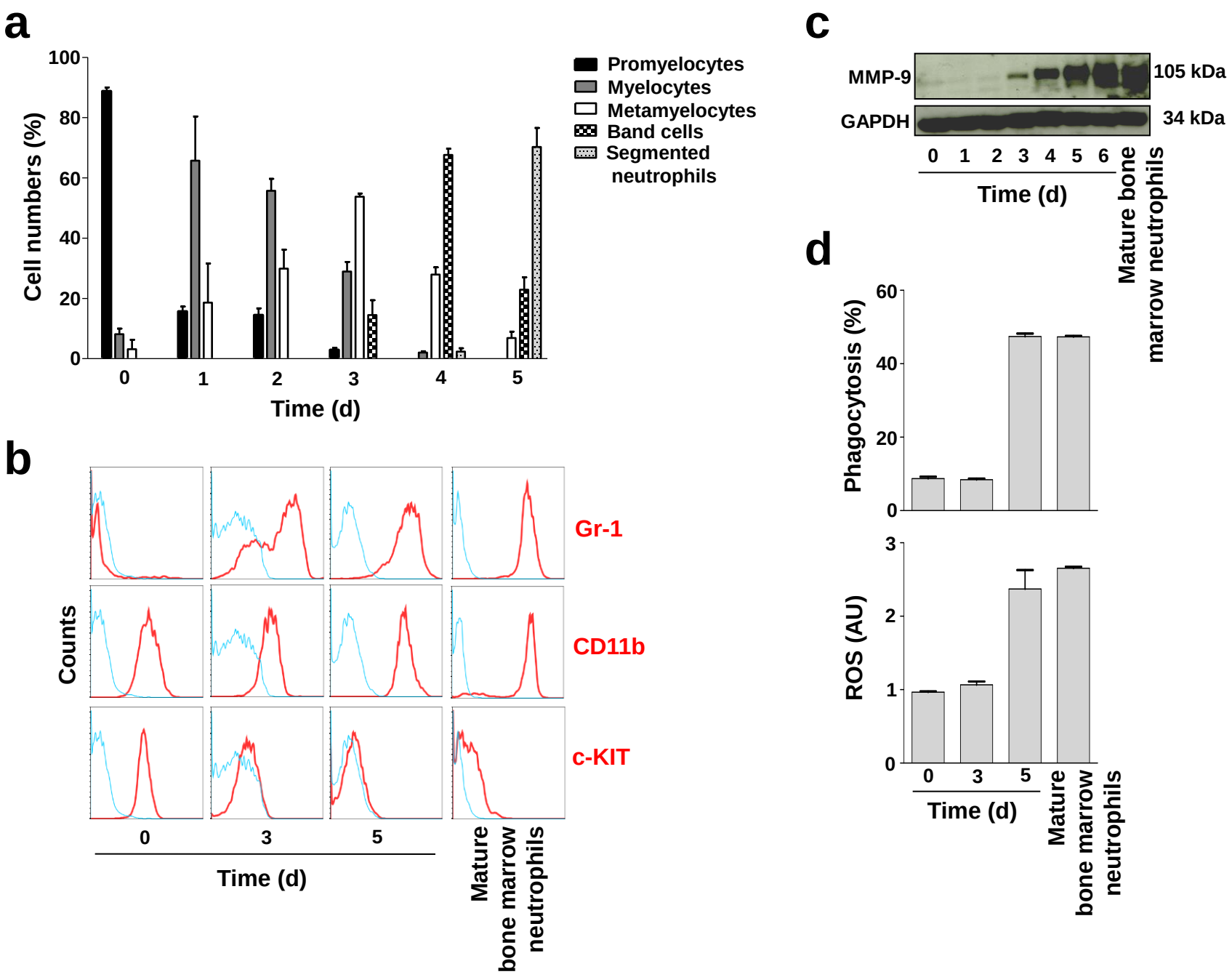
a
Cell numbers (%)
100
80
60
40
20
0
0
1
2
3
4
5
Time (d)
Promyelocytes
Myelocytes
Metamyelocytes
Band cells
Segmented neutrophils
b
Counts
Gr-1
CD11b
c-KIT
0
3
5
Time (d)
Mature bone marrow neutrophils
c
MMP-9
105 kDa
GAPDH
34 kDa
0 1 2 3 4 5 6
Time (d)
Mature bone marrow neutrophils
d
Phagocytosis (%)
60
40
20
0
ROS (AU)
3
2
1
0
0
3
5
Time (d)
Mature bone marrow neutrophils

Figure S6 Hoxb8 neutrophils represent a suitable model to study neutrophil differentiation. (a) Light microscopy. Hoxb8 neutrophil progenitors were allowed to differentiate and analyzed daily. Cytospins were stained with the Hematocolor Set and quantified. Based on cell size and morphology of the nuclei, cells were categorized as promyelocytes, myelocytes, metamyelocytes, band cells, and segmented neutrophils. Values are means ± SEM (n=3). (b) Flow cytometry. Hoxb8 neutrophil progenitors were allowed to differentiate for the indicated times, and Gr-1, CD11b, and c-KIT surface expression analyzed. Primary mature bone marrow-derived neutrophils served as a control. Representative original flow cytometric data are shown (n=3). Red, specific marker expression as indicated. Blue, isotype-matching control antibody. (c) Immunoblotting. Hoxb8 neutrophil progenitors were allowed to differentiate for the indicated times and MMP-9 expression analyzed. Primary mature bone marrow-derived neutrophils served as a control. Results are representative of three independent experiments. (d) Flow cytometry. (Upper panel) Red fluorescent beads were opsonized with mouse serum (30 min, 37°C) and then incubated with the indicated neutrophil populations for 1 h. Values are means ± SEM (n=3). (Lower panel) The detection of ROS production was based on the oxidation of dihydrorhodamine 123. The indicated neutrophil populations were primed with GM-CSF and subsequently stimulated with C5a. Values are means ± SEM (n=3).

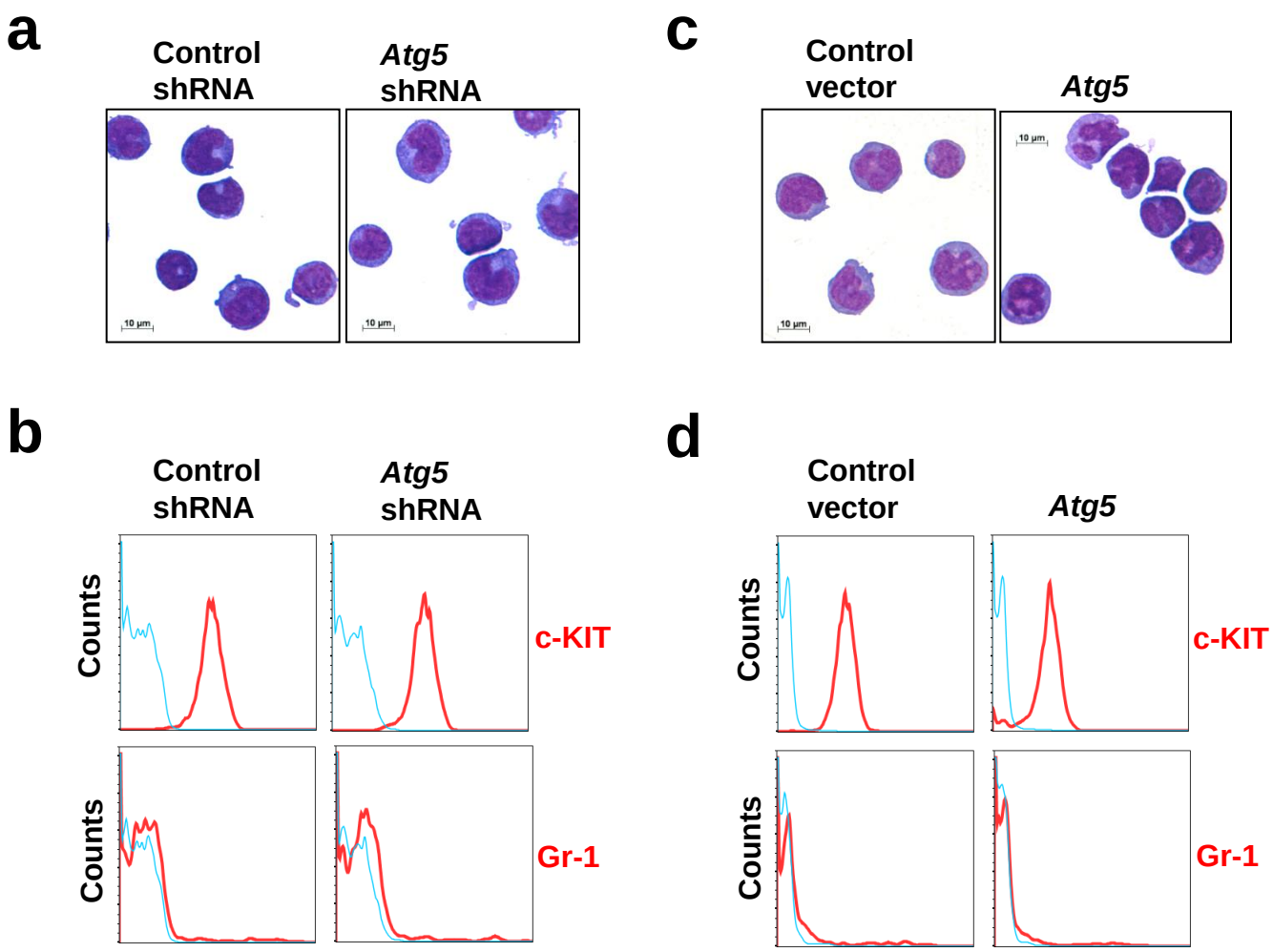

Figure S7 Morphological and biochemical characteristics of undifferentiated Hoxb8 neutrophils. (**a**) and (**c**) Light microscopy. Cytospins of the indicated Hoxb8 neutrophil progenitor populations were stained with the Hematocolor Set and their morphology evaluated. All immature neutrophil populations had the morphology of promyelocytes. Bars, 10 μm. (**b**) and (**d**) Flow cytometry. The indicated Hoxb8 neutrophil progenitor populations fully expressed the c-Kit surface marker and lacked expression of the Gr-1 differentiation marker. The results are representative of three independent experiments.

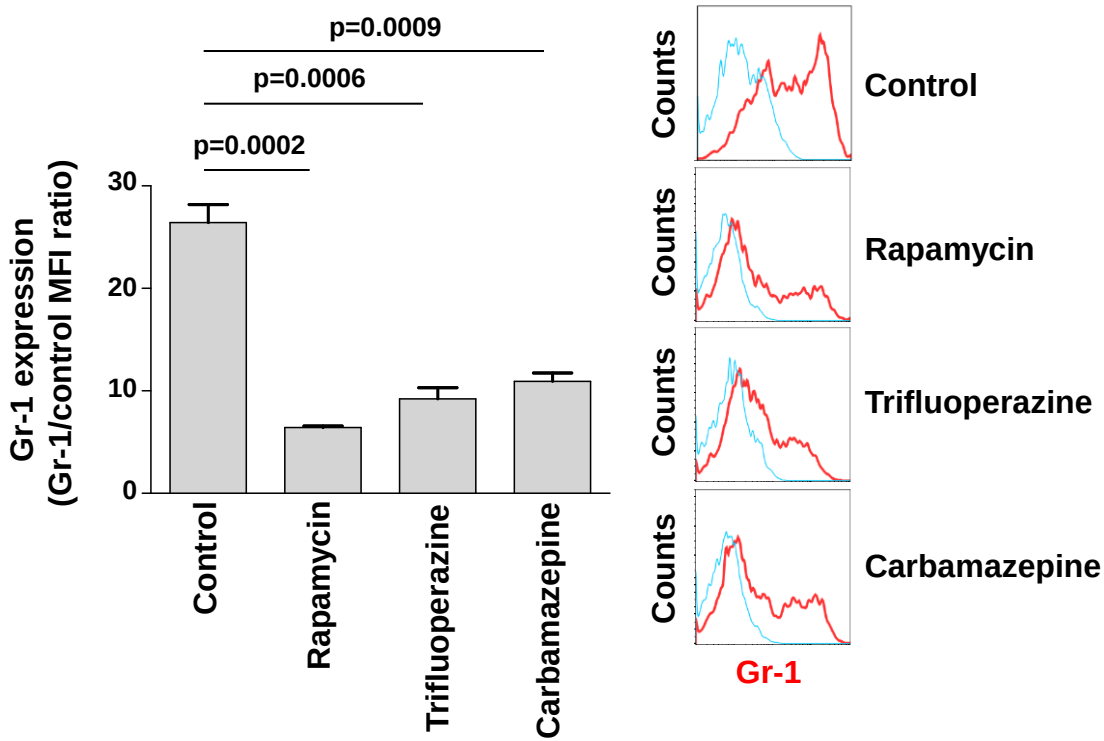

Figure S8 Pharmacological induction of autophagy prevents proper neutrophil differentiation as assessed by flow cytometric Gr-1 surface expression analysis. Undifferentiated Hoxb8 neutrophils were treated with 100 nM rapamycin, 500 nM trifluoperazine, or 10 μM carbamazepine. Analysis was performed on day 3 after the initiation of differentiation. Values are means ± SEM (n=3). (Right) Representative original flow cytometric data are shown. Red, Gr-1 expression. Blue, isotype-matching control antibody.

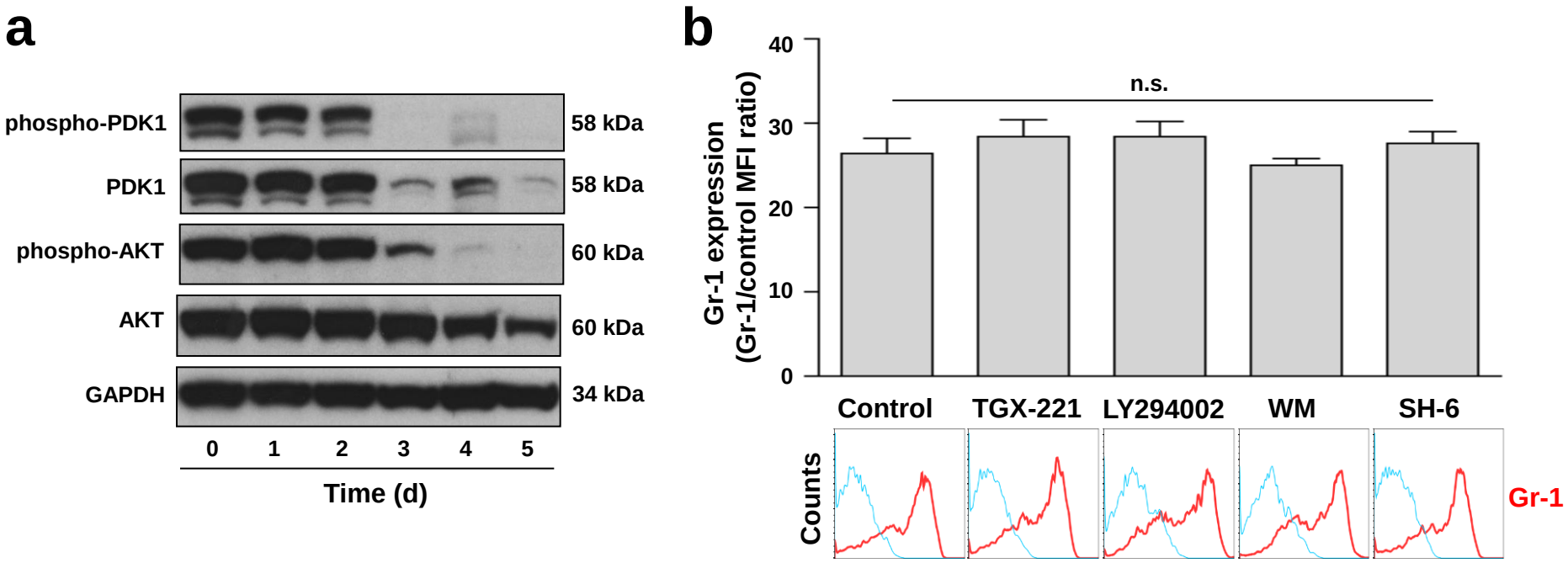

Figure S9 The PI3K pathway is not crucial for neutrophil differentiation. (**a**) Immunoblotting. Hoxb8 neutrophil progenitors were allowed to differentiate and analyzed at the indicated times. Phosphorylation (Ser241) of PDK1 decreased during differentiation and was undetectable in mature Hoxb8 neutrophils. The same observation was performed for AKT Thr308 phosphorylation. (**b**) Flow cytometry. Hoxb8 neutrophil progenitors were allowed to differentiate in the presence and absence of 50 nM TGX-221 (inhibitor of type I PI3K), 100 nM LY294002 (unspecific PI3K inhibitor), 30 nM wortmannin (WM; unspecific PI3K inhibitor) or 5 μM SH-6 (AKT inhibitor), and analyzed for Gr-1 expression on day 3 after the initiation of differentiation. Values are means ± SEM of two independent experiments. Representative original flow cytometric data are shown below. Red, Gr-1 expression. Blue, isotype-matching control antibody.