Supplementary Materials

Inositol hexakisphosphate kinase 1 (InsP6K1) is a key modulator of neutrophil function in innate immunity via negative regulation of PtdIns(3,4,5)P3 signaling.

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Supplementary Methods

Neutrophil isolation

Murine bone-marrow derived neutrophils were isolated using negative depletion of other cell populations using a MidiMACS Separation system (Miltenyi Biotec, Germany). The isolation procedure was conducted at 4 ºC unless specified. Briefly, Bone marrow cells were filtered through a 70 µm cell strainer and suspended in 250 µl of PBS containing 0.5% endotoxin-free BSA and 2 mM EDTA (MACS buffer). Cells were incubated with Rat Anti-Mouse Ter119, B220, CD5, CD4 and CD8 antibodies (BD Biosciences) for 5 min, washed once with 1.5 ml of cold MACS buffer, and then incubated with Goat Anti-Rat IgG Microbeads for 5-10 min, washed with 1.5 ml of MACS buffer and suspended in 250 µl of MACS buffer. Cells were subsequently loaded onto a MACS buffer equilibrated LD column and washed twice with 1 ml of MACS buffer. The flow through was harvested, washed twice with 10 ml of ice-cold RPMI containing 10%FBS/1% Penicillin/Streptomycin and allowed to warm up to room temperature in 10 ml of RPMI medium until used. Human primary neutrophils were isolated from peripheral blood using gradient separation as described previously [1-3].

Measurement of calcium signaling

Calcium flux in response to chemoattractant was measured using flow cytometric analysis. Mouse neutrophils (0.5 x10^6/ml in PBS) were loaded with 1µM Fluo-4 and 2µM fura-red (Molecular Probes) for 45 min at room temperature, washed once with PBS and resuspended in HBSS buffer with Ca^{2+} and Mg^{2+} or calcium-free HBSS as indicated at a density of 1x10^6/ml. Neutrophils were incubated at room temperature for at least 15 min before the analysis on BD FacsCantoII (BD Biosciences) analyzer. Baseline Ca^{2+} was monitored for 30 s, followed by stimulation with 1µM fMLP for 3 min and subsequent addition of 2.5 µM ionomycin for another 3 min. Under the calcium-free condition, extracellular calcium was chelated by incubation of the neutrophils with 5 mM EGTA for 5 min before analysis.

Surface expression of fMLP receptor

The InsP6K1 null and wild-type neutrophils were preblocked for 1 hr in HBSS/1% BSA on ice. Cells were then incubated with indicated amount of FITC-fMLP (Invitrogen, Carlsbad, CA) in the same medium for 1 hr with or without 10 µM unlabeled fMLP (**Supplementary Fig. 3A**). Cells were washed

once, re-suspended in 300 µl of HBSS, and analyzed by flow cytometry. Neutrophils were gated by their characteristic FSC/SSC pattern. Specific binding of FITC-fMLP was calculated by subtracting the geometric mean fluorescence intensity in the presence of 10 µM unlabeled fMLP from the geometric mean fluorescence intensity in the absence of the unlabeled fMLP as previously described [2].

Cell polarization and ruffling assay

Mouse or human neutrophils were isolated and resuspended in RPMI 2% FCS at a concentration of 1x 10^6/ml. Cells were allowed to settle for 5 min on Lab-Tek chambered cover glass coated with fibronectin (10 µg/ml). Images were obtained with an Olympus IX-71 microscope at 40X oil immersion objective for 10 min at 10 sec intervals. Neutrophils were stimulated with 2X concentrated fMLP or C5a as indicated, and the field was changed after every 6-7 frames to capture as many cells as possible. The percentage of polarized neutrophils was determined by calculating the fraction of total cells showing pseudopods or ruffling over the total number of cells present in each frame as previously described [3].

Superoxide production.

Measurement of intracellular or extracellular superoxide production by neutrophils was performed as previously described with minor modifications [3]. To determine extracellular superoxide production, 0.4 x10^6 mouse or human neutrophils were resuspended in HBSS containing 4 U/ml HRP (type XII; Sigma-Aldrich), 5.5 µM isoluminol, and 0.2% BSA for 4 min, and then loaded into each well of a 96-well MaxiSorp plate (Nunc, Rochester, NY). Chemiluminescence was measured using a TriStar LB941 microplate luminometer (Berthold Technologies USA, Oak Ridge, TN). HBSS buffer or indicated amount of fMLP or PMA was injected into the mixture via the injection port of the luminometer. Luminescence was recorded for 2 sec at the fixed time intervals. To measure intracellular superoxide production, the reaction mixture contained 0.4 x10^6 neutrophils, 18kU/ml of SOD, 450U/ml of catalase and 5.5 µM isoluminol. To measure phagocytosis-induced ROS production, mouse bone marrow neutrophils or 5 day differentiated HL60 cells were simulated with opsonized zymosan or *E.coli* bioparticles as indicated (**Fig. 9**). In order to measure the superoxide production from murine inflamed peritoneal cavity, a SOD-inhibitable cytochrome-c assay was conducted as previously described [2]. Peritonitis was induced via i.p injection of 1x10^6 *E.coli*. Peritoneal lavage fluids were collected 4 hours after the injection and used directly for cytochrome C assay. Peritoneal exudate (200µl) was incubated with 1.5 mg/ml Cytochrome c (from equine heart; Sigma) with or without 100 U/mL SOD for 5 minutes at RT. Cytochrome c reduction in each sample was quantified by centrifuging cells and detecting

absorbance (at 550 nm) of the supernatant with a spectrophotometer. Data are represented as the difference in absorbance between samples with and without SOD.

Chemotaxis assay.

The EZ-TAXIScan MIC-1000 (Hirata Corp. of America) was used to investigate real-time horizontal chemotaxis of mouse neutrophils as previously described [4]. The EZ-TAXIScan chamber contains an etched silicon substrate and a flat glass plate. Glass coverslips (Corning) were placed on the glass plate at the bottom of the compartment. Purified bone marrow-derived neutrophils in RPMI-1640 media containing 0.1% BSA ($3x10^6$ cells/ml) were placed into the single hole at the bottom of a 4µm depth and 260µm width microchip. One µl of media with or without 1 µM fMLP was loaded into the contra hole. Images were recorded at 37 °C for 20 min with a 30 sec interval using a CCD camera. Migrating cells were tracked using DIAS software (Solltech, Iowa City, Iowa). Chemotaxis speed and directionality were analyzed from the cell tracks using Matlab as previously described [2].

Transwell migration assay

Neutrophils were suspended in RPMI-1640, 10% FCS at a density of $4x10^6$/ml. The cells were stained with Calcein (5µg/ml, Invitrogen, Carlsbad, CA) for 30 min. The cells were washed twice with HBSS and re-suspended in RPMI-1640, 2% FCS. The bottom wells of the transwell device (Chemotx, Neuroprobe, Gaithersburg,MD) were filled with 29 µl of RPMI1640, 2% FCS containing different concentrations of fMLP. A filter plate was carefully aligned and placed over the bottom wells and a total of 25 µl of the cell suspension was added on top of the filter. The transwell device was incubated for 1 hr at 37ºC in a humidified CO2 incubator. Fluorescence in the lower wells was measured with fluorescence multiwell plate reader. The percentage of cells that migrated was evaluated from the ratio of migrated cells to the total number of cells loaded (from the loading control).

***In vitro* bacterial killing assay**

Fresh overnight culture of *Escherichia coli* (strain 19138; ATCC, Manassas, VA) and *Staphylococcus aureus* (strain 10390; ATCC, Manassas, VA) were suspended in PBS at an OD600 of 0.20 and opsonized with 10% mouse serum for 1 hr at 37 ºC in a water bath. The cells and bacteria were incubated together at 1:5 ratio for *E. coli* and 1:10 for *S. aureus* for 0, 30, 60 and 120 min at 37ºC with intermittent shaking. After each time period, cells were lysed by adding distilled H2O and diluted aliquots were spread on LB

agar (*E. coli*) or Blood agar (*S. aureus*) plates. The CFU were counted after incubating the plates overnight at 37 ºC. Bacterial suspension without any cells was used as input control.

Neutrophil adhesion under shear flow.

To examine neutrophil adhesion under shear flow, a Vena8 chamber (Cellix, Dublin, Ireland) was coated with 10 µl of 10µg/ml human fibronectin for 1h at 37°C and preblocked with 0.5% BSA in HBSS with Ca^{2+} and Mg^{2+} (Invitrogen) until used. Neutrophils ($0.2x10^6$ in 100 µl) were stimulated with 1µM fMLP for 2 min, and loaded into the fibronectin-coated chamber by applying 10 dynes/cm^2 shear flow. Time lapse images were captured by Olympus IX71 microscope with a charge-coupled device (CCD) camera at 5 sec/frame for 5 min with 60 ms exposure. Non-adherent cells floated and appeared elongated due to fast moving, differentiating them from cells which adhered after 5 min under shear flow. For detachment of adherent cells under shear flow, neutrophils ($0.2x10^6$ in 100 µl) were stimulated with 1 µM fMLP, loaded into the Vena8 chamber, and allowed to adhere for 10 min. Increasing shear flow rates of 0. 5, 1, 2, 5, 7.5, 10, 12 dynes/cm^2 at 1 min per flow rate were introduced to detach adhered cells. Time lapse images were taken at 5 sec/frame for 7 min. The percentage of cells detached out of the input cells was plotted over increasing flow rate.

Gentamicin protection assay

Bone marrow–derived neutrophils from WT and InsP6K1 null mice were incubated with mouse serum-opsonized live *E coli* for 1 hour and then with 100 g/mL gentamicin for an additional hour. Viable intracellular bacteria were quantified by subsequent plating the lysed samples on Luria-Bertani agar plates.

In vitro phagocytosis assay

Zymosan BioParticles (Invitrogen, Carlsbad, CA) were opsonized with mouse serum for 1 hr. Neutrophils were suspended in HBSS at a density of $1x10^7$/ml. The cells and opsonized Zymosan particles (1:10 ratio) were incubated at 37 ºC or 4 ºC by end-to-end rotation for 1 hr. Extracellular fluorescence was quenched by trypan blue. Phagocytosis index (PI) was expressed as the number of bioparticles engulfed by 100 neutrophils. Binding index was expressed as the number of bioparticles bound to 100 neutrophils. More than 200 neutrophils were counted in each group.

Measurement of PtdIns(3,4,5)P3 levels in neutrophils

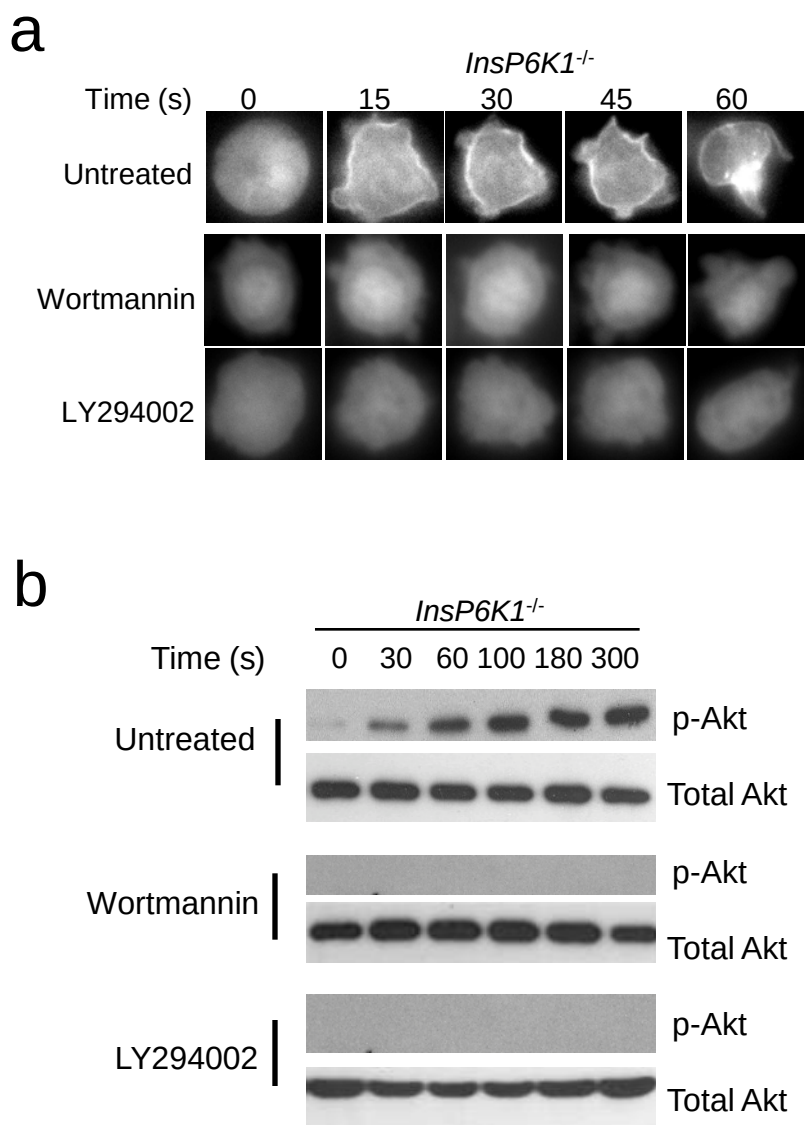
TNP-treated and untreated human neutrophils were stimulated with 1 μM fMLP for 2 min and cellular PtdIns(3,4,5)P3 levels were measured using a Echelon Mass ELISA kit (Echelon Biosciences Inc., Salt Lake City). Briefly, PtdIns(3,4,5)P3 was extracted from 10 million purified neutrophils following manufacturer's lipid extraction protocol. The samples were incubated with a PtdIns(3,4,5)P3 detector protein and then added to a PtdIns(3,4,5)P3-coated microplate for competitive binding. A peroxidase-linked secondary detector and colorimetric detection were used to quantify the PtdIns(3,4,5)P3 detector protein bound to the plate. The colorimetric signal is inversely proportional to the amount of PtdIns(3,4,5)P3 in the sample.

Neutrophil spontaneous death.

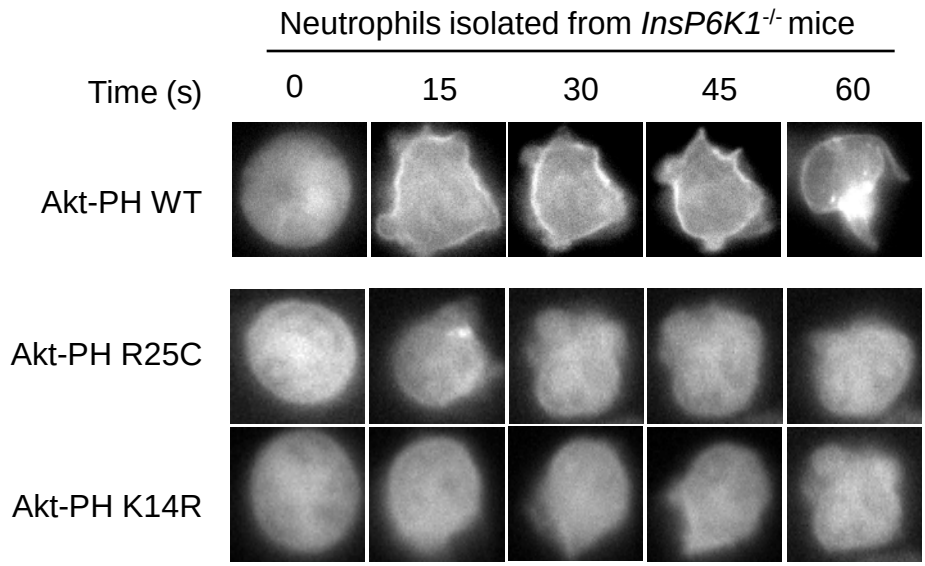
Mouse bone marrow neutrophils were isolated from the femur and tibia of 8-12 week-old mice. Isolated neutrophils were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at a density of $2x10^6$ cells/ml for indicated times. The percentage of dead neutrophils was assessed as previously described[1]. Briefly, apoptotic cells were detected by Annexin V-FITC staining and propidium iodide (PI) staining using an Annexin V Detection Kit (Caltag Laboratories, Burlingame, CA) following a protocol provided by the manufacturer. FACS was performed using a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon laser. Ten thousand cells were collected and analyzed using the BD FACSDiva software (Becton Dickinson). The Annexin V and PI double negative cells were defined as live cells. The percentage of dead cells was calculated as 100 minus the percentage of live cells. The results are the means (± SD) of three independent experiments.

Reference

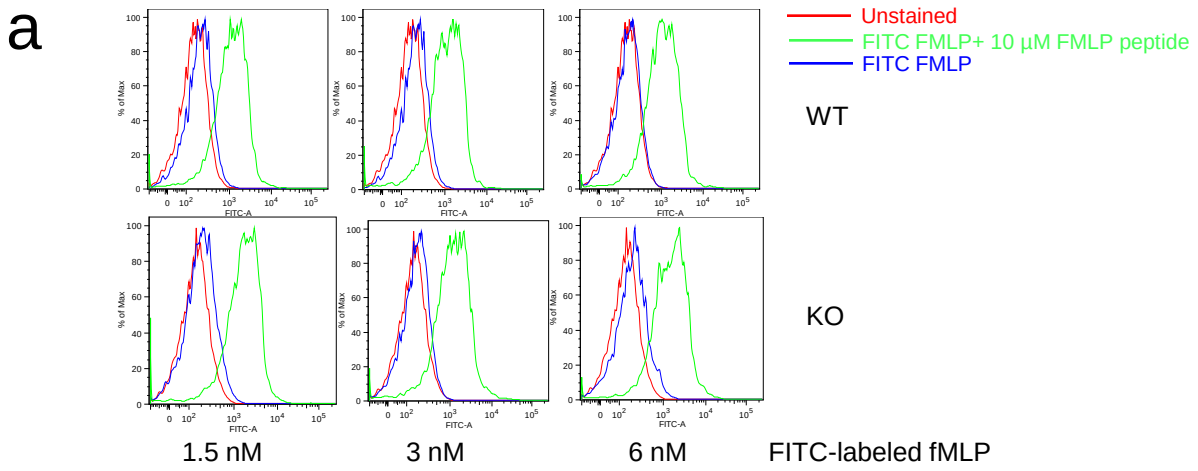
1. Zhu, D. *et al.* Deactivation of phosphatidylinositol 3,4,5-trisphosphate/Akt signaling mediates neutrophil spontaneous death. *Proc Natl Acad Sci U S A* **103**, 14836-14841 (2006).
2. Subramanian, K.K. *et al.* Tumor suppressor PTEN is a physiologic suppressor of chemoattractant-mediated neutrophil functions. *Blood* **109**, 4028-4037 (2007).
3. Jia, Y. *et al.* Inositol 1,3,4,5-tetrakisphosphate negatively regulates phosphatidylinositol-3,4,5-trisphosphate signaling in neutrophils. *Immunity* **27**, 453-467 (2007).
4. Hattori, H. *et al.* Small-molecule screen identifies reactive oxygen species as key regulators of neutrophil chemotaxis. *Proc Natl Acad Sci U S A* **107**, 3546-3551 (2010).

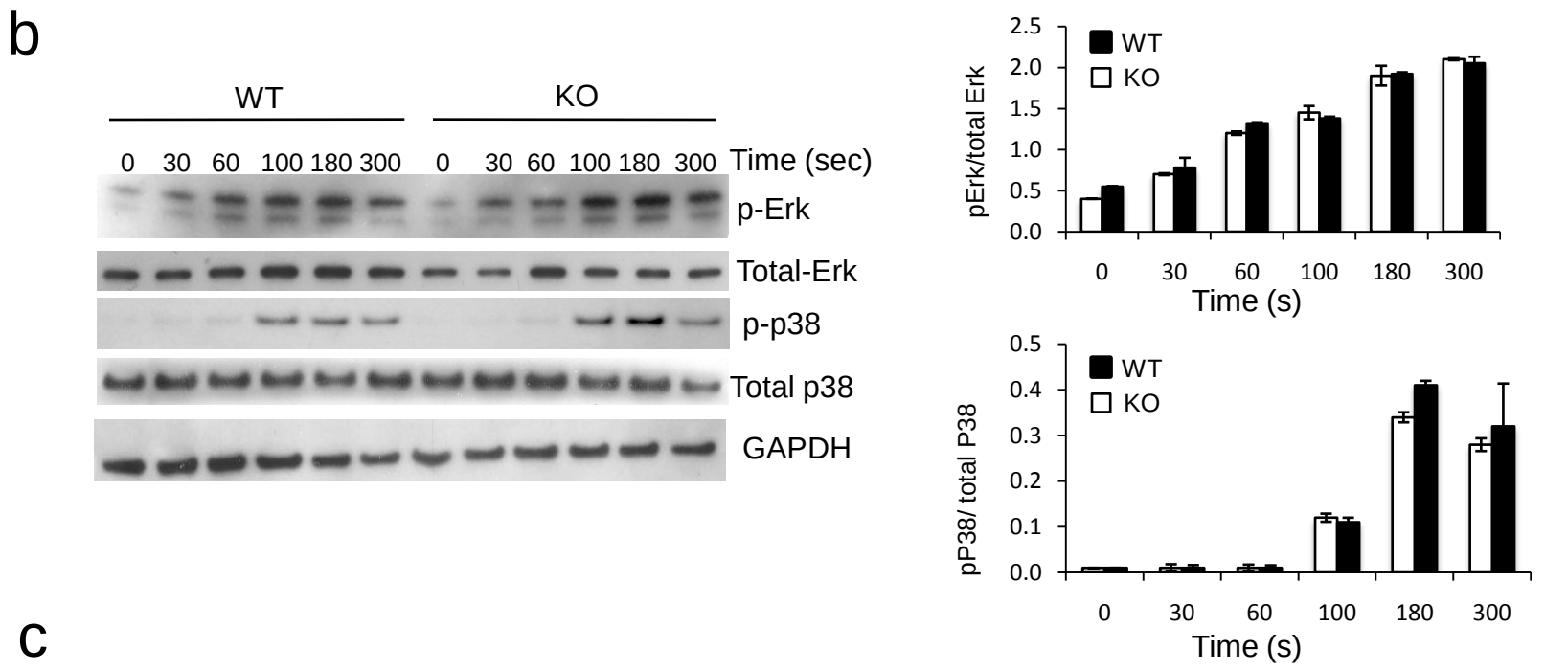


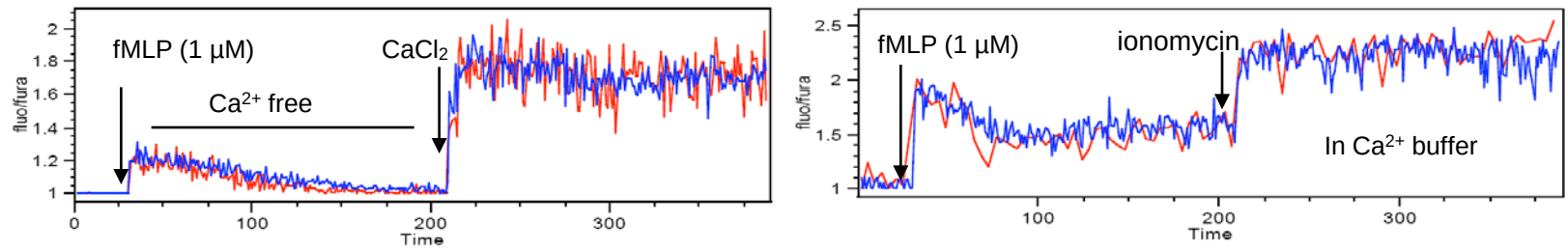
Supplementary Figure 1. InsP6K1 disruption does not enhance Akt plasma membrane translocation and the subsequent Akt activation in the absence of PtdIns(3,4,5)P3. (a) Chemoattractant-elicited plasma membrane translocation of PHAkt-GFP in *InsP6K1*$^{-/-}$ mouse neutrophils. Cells were stimulated with 1 μM fMLP for indicated time and time lapse images were recorded. fMLP-elicited PtdIns(3,4,5)P3 production was blocked by PI3K inhibitors Wortmannin (40 nM) or LY294002 (20 μM). The results shown are representative of three experiments. **(b)** Immunoblot analysis of total and phosphorylated Akt in *InsP6K1*$^{-/-}$ mouse neutrophils stimulated with 1μM fMLP for the indicated time periods.



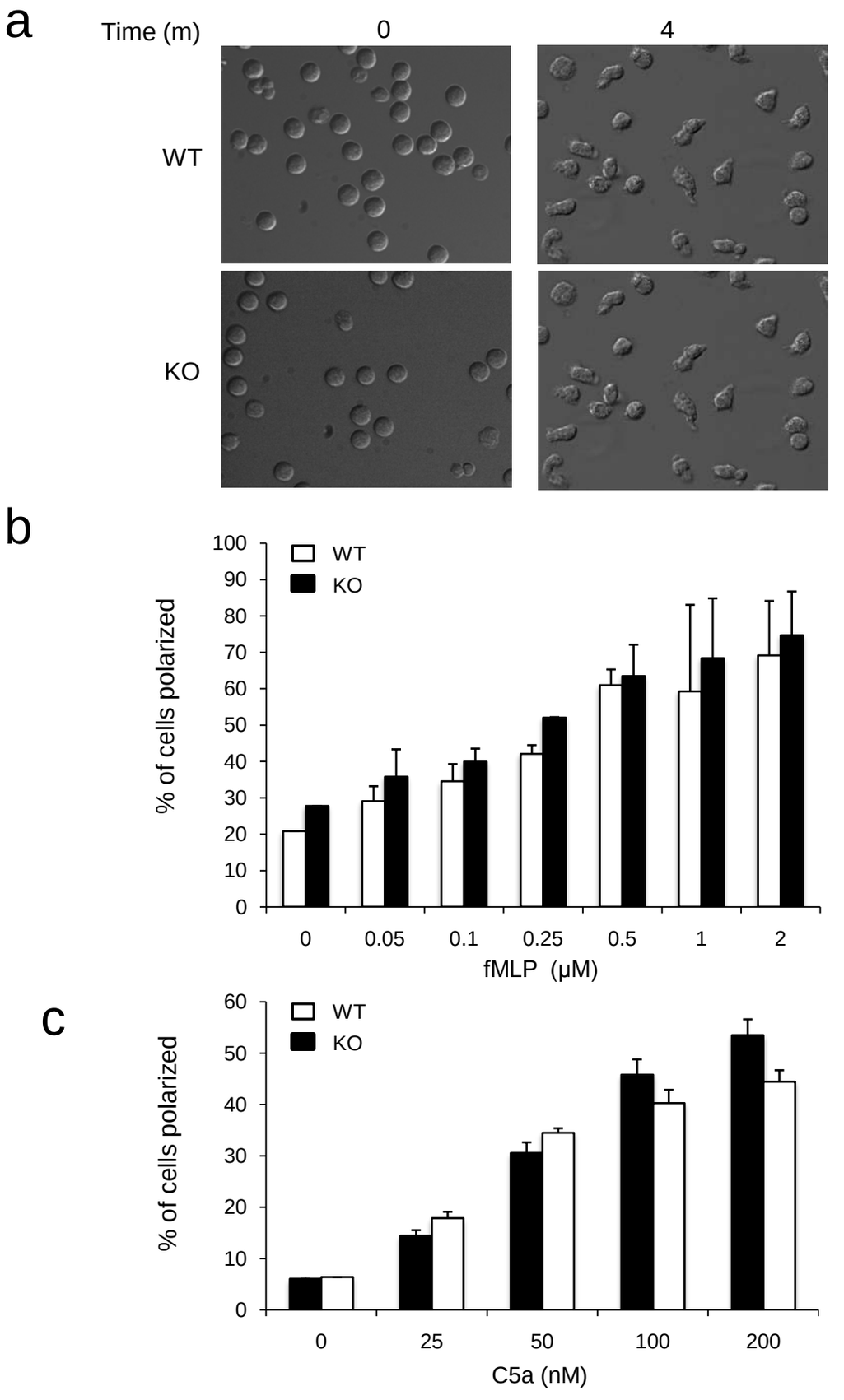
Supplementary Figure 2. InsP6K1 disruption-induced elevation of Akt membrane translocation relies on Akt-PH domain binding to PtdIns(3,4,5)P3. Murine bone marrow derived neutrophils were isolated and transfected with indicated PHAkt-GFP constructs. Chemoattractant-elicited plasma membrane translocation of PHAkt-GFP was analyzed as described in Fig.1e. Akt-PH R25C and Akt-PH K14R are Akt-PH domain mutants that have lost the ability to bind PtdIns(3,4,5)P3.



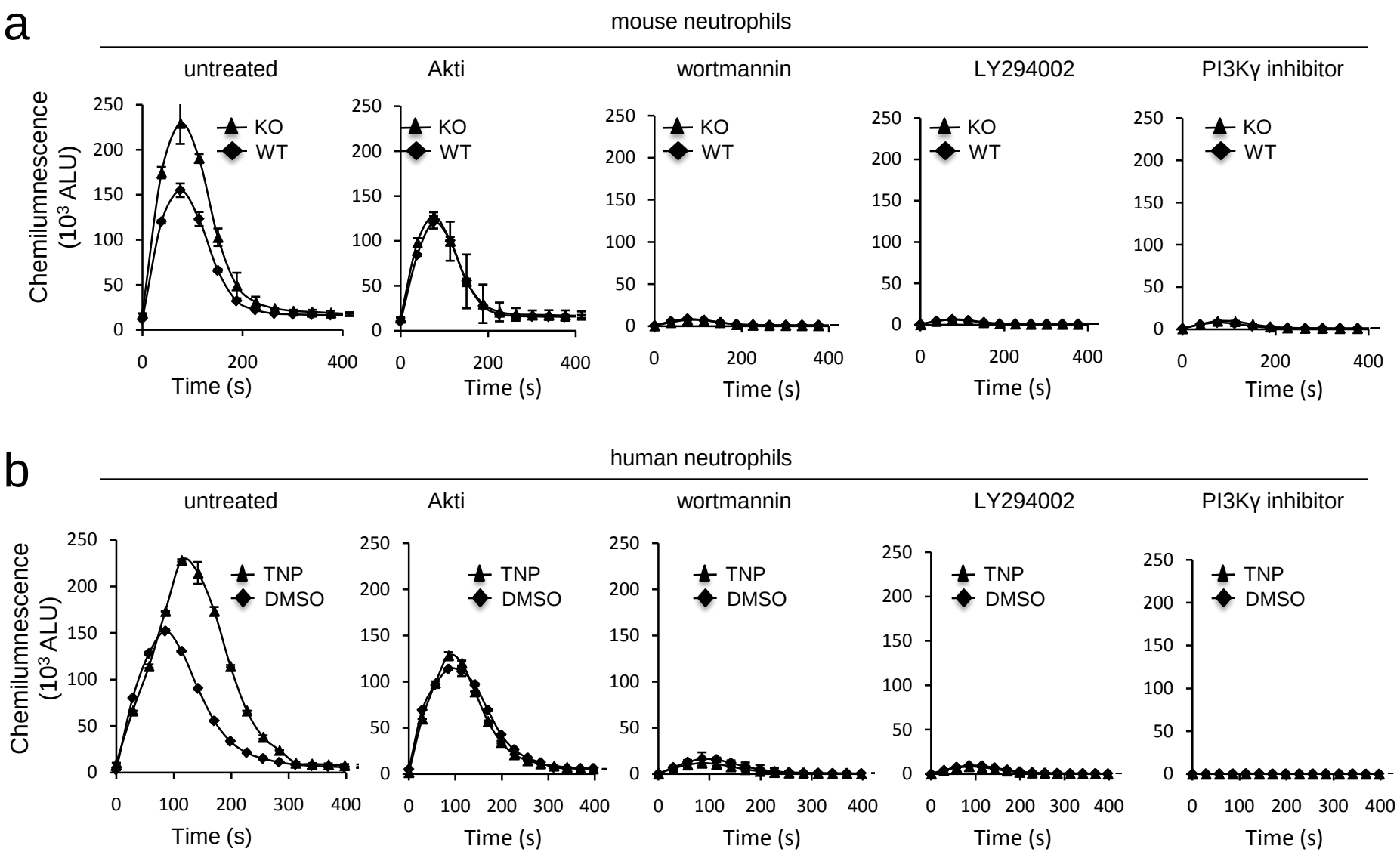




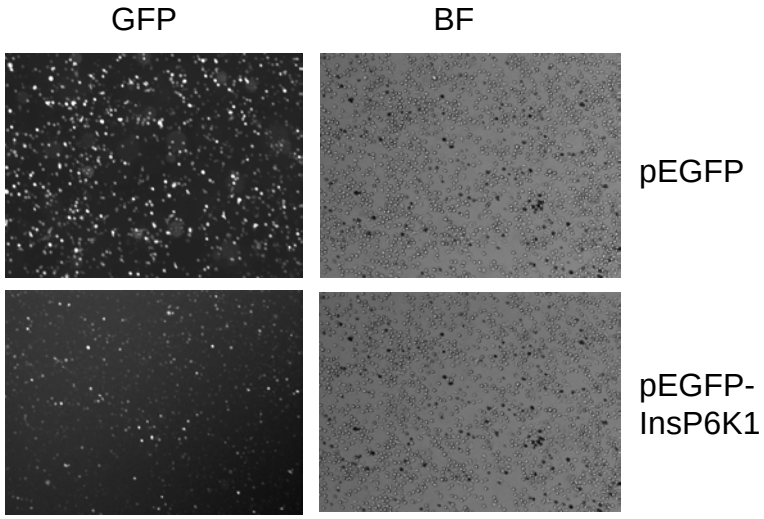
Supplementary Figure 3. Elevated PtdIns(3,4,5)P3 signaling observed in InsP6K1-deficient neutrophils is a specific cellular event caused by InsP7 depletion. (a) fMLP receptor expression on wild-type and InsP6K1-deficient (KO) mouse neutrophils. **(b)** Phosphorylation of Erk and p38 in response to fMLP stimulation in wild-type and InsP6K1-deficient (KO) mouse neutrophils. Relative amounts of phosphorylated protein were quantified with NIH Image software as described in Fig. 1d. Data shown are mean ± SD of 3 experiments. **(c)** fMLP-elicited Ca^{2+} signaling in wild-type (red) and InsP6K1-deficient (blue) mouse neutrophils as calculated with Ca^{2+}-sensitive dyes Fluo-4 and Fura red in calcium-free or calcium-containing buffers.



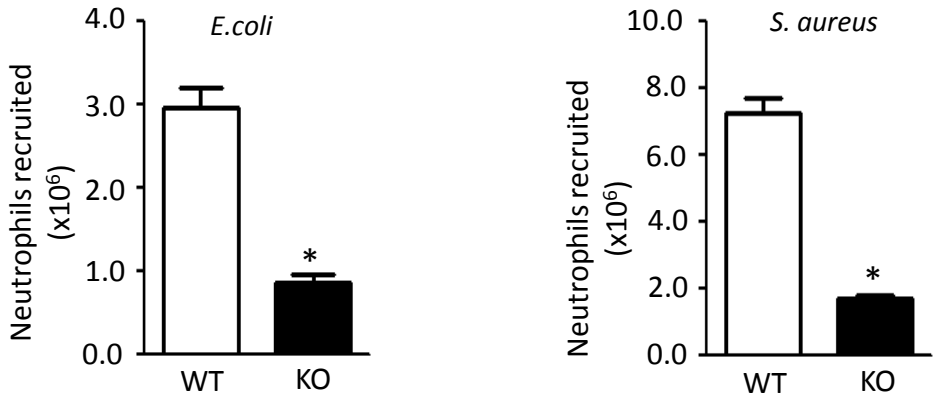
Supplementary Figure 4. InsP6K1-deficient neutrophils possess the same sensitivity to chemoattractant stimulation, compared to wild-type neutrophils. (a) Chemoattractant-elicited polarization of purified wild-type and InsP6K1-deficient neutrophils uniformly stimulated with 50 nM fMLP. Shown are representative images of unstimulated (left) and 4 min-stimulated (right) neutrophils. Two videos of the experiment described in this figure are included in supplemental materials (**Supplementary Movies 1 and 2**). Neutrophils were uniformly stimulated with the indicated concentration of fMLP or C5a. **(b)** Neutrophil polarization elicited by indicated concentration of fMLP. Percentage of polarized cells (extended pseudopods or ruffled) between 4 and 8 min after stimulation was counted. Results are the means (±SD) of three independent experiments. **(c)** Neutrophil polarization elicited by indicated concentration of C5a. Results are the means (±SD) of three independent experiments.



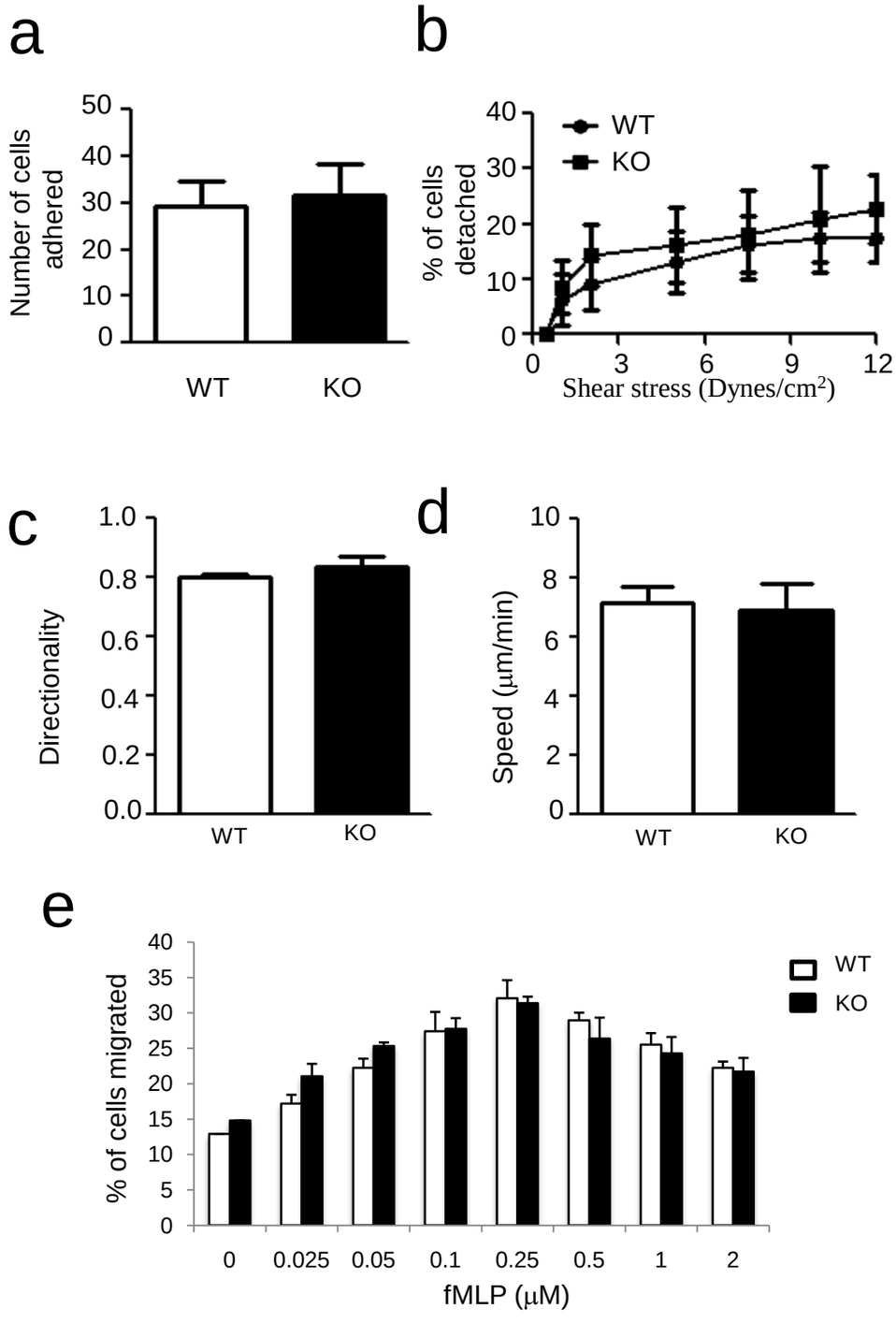
Supplementary Figure 5. InsP6K1 disruption-induced elevation of ROS production in neutrophils requires PtdIns(3,4,5)P3 generation and Akt activation. (a) fMLP-elicited extracellular ROS production by wild-type and InsP6K1-deficient mouse neutrophils in the presence or absence of indicated PI3K and Akt inhibitors. **(b)** fMLP-elicited extracellular ROS production by DMSO and TNP (10 μM, 2 hours)-treated human neutrophils. Cells were stimulated with 1 μM fMLP. fMLP-elicited PtdIns(3,4,5)P3 production was blocked by PI3K inhibitors Wortmannin (40 nM), LY294002 (20 μM), or a PI3Kγ specific inhibitor AS-252424 (50 nM, Cayman Chemical Inc.). fMLP-elicited Akt activation was blocked by Akt Inhibitor VIII (4 μM, EMD Chemicals Inc.).



Supplementary Figure 6. DNA constructs can be transferred efficiently into dHL60 cells with a nucleofection method. Shown are representative fluorescence (GFP) and bright field (BF) images of six-day differentiated HL60 cells expressing EGFP.



Supplementary Figure 7. Disruption of InsP6K1 leads to reduced neutrophil accumulation in the inflamed peritoneal cavity. Shown are total neutrophil numbers in the lavage fluid 4 hours after *E. coli* or *S. aureus injection*. Data shown are mean ± SD of *n*=3 mice. *p < 0.01 by Student's *t* test.



Supplementary Figure 8. InsP6K1-deficient mouse neutrophils exhibit normal adhesion and migration. **(a)** Neutrophil adhesion under shear flow. Two videos of the experiment described in this figure are included in supplemental materials (**Supplementary Movie 3**). **(b)** Detachment of adherent neutrophils under shear flow. Data are presented as % cells detached *vs* input cells (**Supplementary Movie 4**). **(c)** Chemotaxis directionality of wild-type and InsP6K1-deficient mouse neutrophils as measured with a EZ-TAXIScan device and DIAS imaging software. **(d)** Chemotaxis speed. Two videos of the experiment described in figure c and d are included in supplemental materials (**Supplementary Movies 5 and 6**). (**e)** Chemotaxis of wild-type and InsP6K1-deficient mouse neutrophils as assessed using a transwell migration assay. Percentage of cells that migrated into the bottom well was recorded. Results are the means (±SD) of three independent experiments. *$p < 0.01$ (Student's t test).

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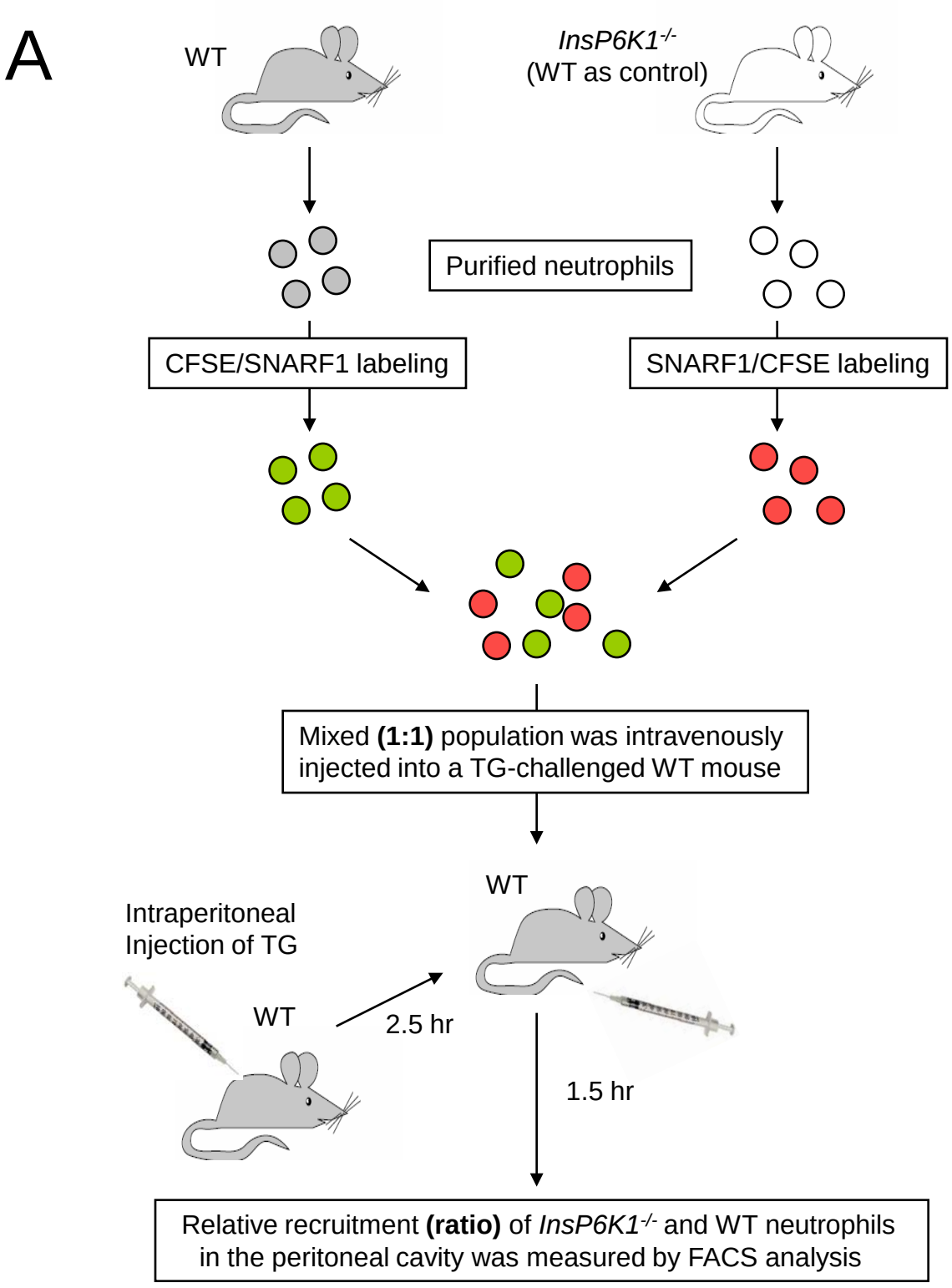

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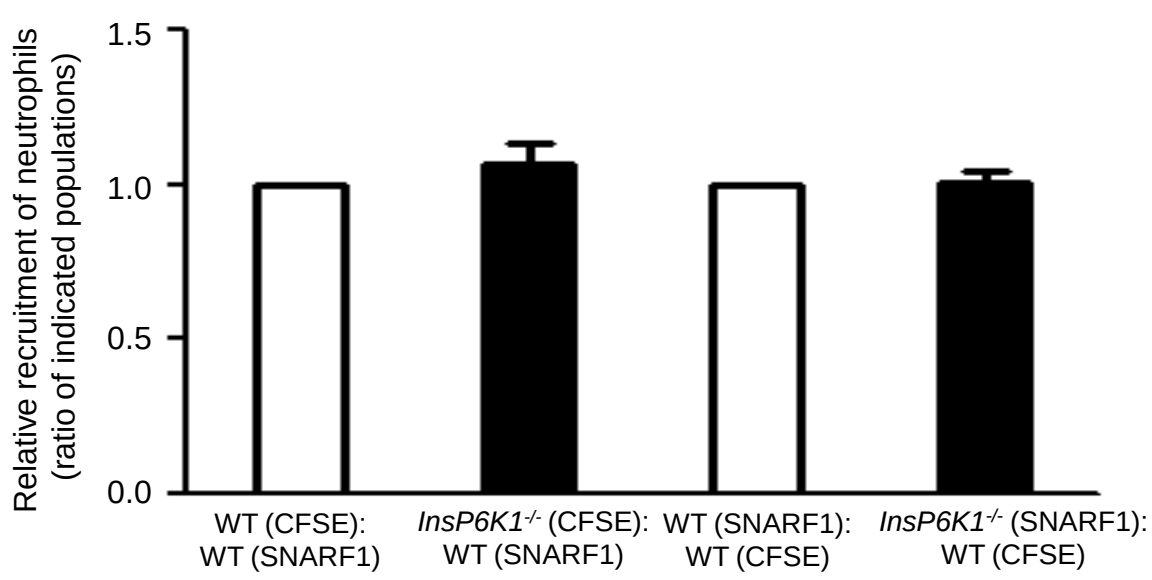
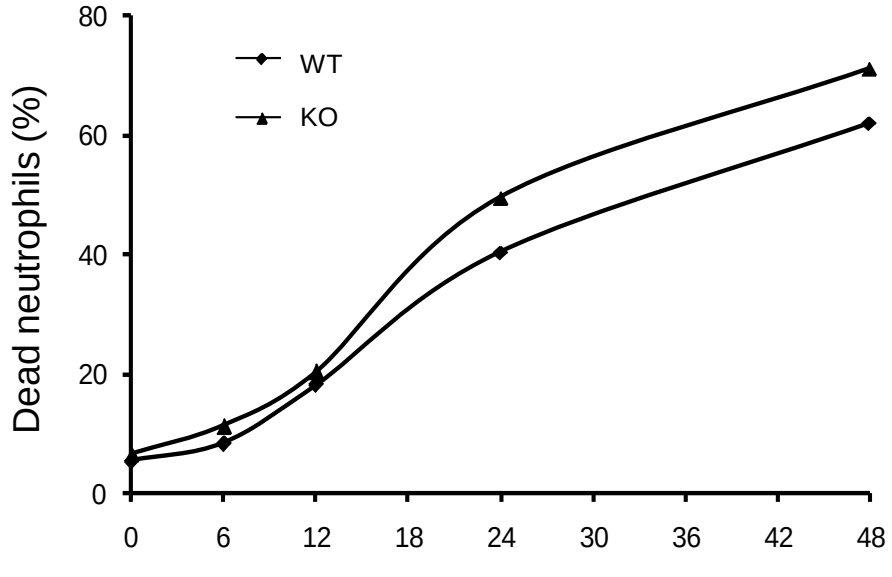


Supplementary Figure 9. The enhanced bacteria-killing capability of InsP6K1-deficient mice is not due to elevated neutrophil recruitment. (a) Schematic diagram of neutrophil adoptive transfer assay. **(b)** Recruitment of adoptively transferred neutrophils in the thioglycolate (TG)-Induced peritonitis model. Neutrophil recruitment was analyzed 1.5 hr after neutrophil adoptive transfer . Data shown are means ± SD of *n*=3 mice.



Supplementary Figure 10. InsP6K1 disruption does not affect spontaneous death of mouse neutrophils. The annexin V and PI double negative cells were defined as live cells. The percentage of dead cells was calculated as 100 minus the percentage of live cells. The results are the means (± SD) of three independent experiments.