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Neutralizing neutrophil destruction

Although neutrophils rapidly respond to clear pathogens, they can also mediate tissue destruction in the process. Hou and colleagues used mouse pulmonary bacterial infection models to see how inositol hexakisphosphate kinase 1 (IP6K1) mediates protective and detrimental responses. This kinase was important for destructive neutrophil-platelet aggregates in the lungs; inhibition of IP6K1 enhanced bacterial clearance and reduced lung damage. They further outline the mechanism behind these observations and suggest that IP6K1 inhibition could be a useful host-directed antibacterial therapy.

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Supplementary Materials for

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/10/435/eaal4045/DC1)

Table S1 (Microsoft Excel format). Primary data.

Materials and Methods

Mice

Ip6k1-knockout (KO) mice were generated by targeted deletion of the *Ip6k1* exon 6 coding sequence as previously described (*1*). Corresponding wild-type (WT) littermates were used as paired controls for *Ipk61*-KO mice. Mice aged 10–14 weeks were used. Mouse bone marrow neutrophils were prepared as described by Zhu *et al.* (*31*). The Children's Hospital Animal Care and Use Committee approved and monitored all animal procedures.

Bacteria or LPS-induced acute pneumonia

After anesthesia with ketamine hydrochloride (100 mg/kg intraperitoneally (i.p.)) and xylazine (10 mg/kg i.p.), mouse tracheas were surgically exposed and a total volume of 40 μl of saline, a dose of $2x10^6$ cfu of *E. coli* (strain 19138; American Type Culture Collection), a dose of $5x10^8$ cfu of *Stapyhlococcus aureus* (strain 10390; American Type Culture Collection), or the indicated amount of LPS (LPS from *E. coli* O111:B4 or LPS from *E.coli* O157:H7; Sigma-Aldrich was instilled intratracheally via an angiocatheter inserted through the trachea and into the left bronchus. Colloidal carbon (1%) was included in the instillate to indicate deposition. After surgery and wound closure, mice were suspended by their front legs to help deliver the instillate deep into the left lobe before being placed back into the cage with soft and warm bedding for recovery. Mice were euthanized by CO_2 at each time point. For polyP treatment, Medium Chain Polyphosphate (p100) (Kerafast Inc) was injected intraperitoneally (50μg/g body weight) 10 min after the instillation of LPS. For treatment with IP6K1 inhibitor TNP (N2-(m-(trifluoromethyl)benzyl) N6-(p-nitrobenzyl)purine), mice were injected intraperitoneally with

TNP once a day (20 mg/kg body weight) for 10 days before the induction of lung inflammation. TNP (Tocris Bioscience) was dissolved in DMSO/Tween80/water (1/1/8) at a concentration of 2 mg/ml and was injected directly without dilution.

Immunohistochemistry

Freshly deparaffinized and rehydrated sections were permeabilized with 0.1% Tx-100 in PBS and blocked with PBS containing 10% goat serum. Sections were incubated with rabbit anti-mouse CD41 (Abcam) and rat anti-mouse Ly6G antibody (Abcam) at 4°C overnight followed by 2 h 37°C incubation with Alexa 555-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) and Alexa 488-conjugated goat anti-rat IgG secondary antibody (Invitrogen), respectively. Nuclei were labeled by DAPI counterstaining.

Neutrophil accumulation

WT and *Ip6k1*-KO mice were anesthetized and instilled with bacteria or LPS as described above. After 24 h, mice were euthanized by CO_2. The chest cavity was opened and a catheter was tied to the trachea. Bronchoalveolar lavage (BAL) was performed (1 mL PBS/15mM EDTA ×10) in each group. The BAL fluid (BALF) was centrifuged at 450 × g for 10 min. The total number of cells in the BALF was counted by hemocytometry. Differential cell counts were conducted on cytospin preparations stained with a modified Wright-Giemsa stain (Volu-Sol, Inc.). Neutrophils were identified by their lobular or segmented nuclei. The percentage of neutrophils in the whole population (%PMN) was determined accordingly. Total numbers of neutrophils (#PMN) was calculated as follows: [#PMN] = [cell density] × volume × [%PMN]. Emigrated neutrophils in the alveolar air spaces were also quantified by morphometric analysis of tissue sections.

Emigrated neutrophils in lung sections (5 mice/group) 24 h after infection were quantified using standard point-counting morphometry (*7*). Briefly, mice were euthanized and their hearts tied off to maintain pulmonary blood volume. Lungs were then excised and fixed by intratracheal instillation of Bouin's solution at a pressure of 23 cm H_2O and embedded in paraffin. Five to 6 μm-thick sections were cut and stained with hematoxylin and eosin (H&E). Neutrophils could be easily recognized by their lobular or segmented nuclei under high magnification. A counting grid (10 × 10, covering 70,000 μm^2 of the magnified field) was reflected onto the field of view using a drawing tube. Randomly selected fields of pneumonic peripheral lung largely free of non-capillary blood vessels and bronchioles or larger airways were examined. At least three grids (300 points) were counted for each lung. The relative volumes of the parenchymal regions occupied by emigrated neutrophils were calculated by investigators blinded to the identities of the mice and were expressed as a percentage of the total parenchymal region volume (including both tissue and air spaces).

BALF total protein levels and cytokine/chemokine levels

BALF samples were obtained from mice 24 h after *E. coli* challenge. BAL was performed with 1 ml cold PBS/15mM EDTA flushed in and out three times. Protein concentrations were measured in BALF using the Bio-Rad protein assay reagent. A standard curve was constructed using BSA. Cytokine levels in BALF were measured with ELISA kits according to the manufacturer's protocol (R&D Systems).

In situ detection of apoptosis

Lung sections were stained using a TACS TdT Kit following the manufacturer's protocol (R&D Systems).

Bacterial burden

Lung tissues were washed three times with cold sterile PBS and cut into small pieces. The tissue was then homogenized in sterile PBS on ice using the Tissue-Tearor (Model 9853370, BioSpec Products, Inc.). The probe was moved up and down in the tube for 30 s (1 cycle) with 6 homogenization cycles and 1 min incubation on ice between cycles. Lung homogenates were then serially diluted in ice-cold sterile PBS and aliquots were spread on Luria broth (LB) agar plates. After overnight incubation at 37°C, colonies were counted and bacterial viability was expressed as cfu per lung calculated based on the dilution factor.

Mortality induced by bacteria pneumonia

Due to animal welfare concerns and as requested by the Institutional Animal Care and Use Committee (IACUC), we used hypothermia (reduced body temperature) as an indicator of pneumonia-induced mortality in mice. This method can provide an earlier and more humane experimental endpoint. We first determined the fatal hypothermia temperature (FHT, the temperature at which mice will inevitably die) under our experimental condition. We found that when body temperature reached ≤29 °C, 100% (n=12) of mice instilled with bacteria died. Thus, in this experiment, mice with a body temperature below 29°C were counted as dead mice, and were euthanized immediately by asphyxiation due to inhalation of CO_2. To compare the body temperature of bacteria challenged wild-type and IP6K1 knockout mice during the course of

pneumonia, the mice were checked every 6 hours for 7 days (or until the mice died). Since more frequent monitoring of body temperature was not practically feasible, some mice would die in the 6 hour interval without being detected to be hypothermic.

Cyclophosphamide-induced mouse neutropenia

Cyclophosphamide powder (Cytoxan®, Bristol-Myers Squibb) was dissolved in distilled water for injection at a final concentration of 20 mg/ml. Cyclophosphamide was injected i.p. at a total dose of 250 mg/kg (two 0.5 mL injections on day 1 (150 mg/kg) and day 4 (100 mg/kg)). Blood samples (~30 µL) were taken from the retro-orbital sinuses of anesthetized uninfected mice using heparinized capillary tubes (Modulohm A/S) on days 1, 4, 5, 6, and 7. Total and differential white blood cell counts (neutrophils, lymphocytes, and monocytes) were performed using a Hemavet 850 hematology system (Drew-Scientific Inc).

In vitro killing of bacteria by neutrophil-platelet co-culture

Fresh overnight culture of *Escherichia coli* (strain 19138; ATCC) and *Staphylococcus aureus* (strain 10390; ATCC) were suspended in PBS at an OD600 of 0.20 and opsonized with 10% mouse serum for 1 hr at 37ºC in a water bath. Purified WT or *Ip6k1*-KO neutrophils (1×10^6) and WT or *Ip6k1*-KO platelets (2×10^8) were incubated with *E. coli* or *S. aureus* (5×10^6 cfu) for 1 hr. with intermittent shaking. After each time period, cells were lysed by adding distilled H_2O 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 37ºC. Bacterial suspension without any cells was used as input control. In vitro bacterial killing capabilities were reflected by the decrease of bacteria colony forming units after the incubation.

Flow cytometry

Murine neutrophils and platelets were incubated as described above. Cells were then harvested, washed with ice-cold PBS, and stained with APC0-CD11b (eBioscience), PE-CY7-Ly6G (BD Bioscience), FITC-CD41 (eBioscience), and isotope controls to detect leukocyte and platelet antigens. Samples were examined with a FACSCanto II flow cytometer (Becton Dickinson). Neutrophils were gated by their forward- and side-scatter characteristics and by their Ly-$6G^+$/$CD11b^+$ (neutrophil) expression pattern (*30*). Platelets were detected by CD41 staining. NPAs were Ly-$6G^+$ $CD41^+$. All data were analyzed using FlowJo software (TreeStar; FlowJo LLC).

Assessment of pulmonary capillary permeability

Evans blue dye (EBD, 40 mL/kg) was injected into mouse tail veins 30 min before termination of the experiment to assess vascular leak. Following euthanasia, lungs were perfused free of blood with Dulbecco's phosphate-buffered saline (DPBS) before being excised en bloc. The lungs were then homogenized in DPBS (1 mL/0.1 mg of tissue), incubated with two volumes of formamide (18 h, 56°C), and centrifuged at 5,000 × g for 30 min. The optical density of the supernatant was determined by spectrophotometry at 620 nm. Extravasated EBD concentration (microgram EBD per lung) in lung homogenates was calculated against a standard curve.

PolyP extraction and determination

PolyP was extracted from platelets using perchloric acid and quantified as the amount of orthophosphate residues (Pi) released upon sample treatment with recombinant

exopolyphosphatase (PPX; from yeast *Saccharomyces cerevisiae*) as described by Müller *et al.* (*14*). Briefly, platelets were pelleted from platelet-rich plasma (PRP) and subjected to acid extraction (0.5 M perchloric acid for 30 min on ice) and neutralized with 1 M potassium carbonate. The neutralized extract was clarified (900 × g, 5 min) and the supernatant was subjected to overnight incubation with or without recombinant *S. cerevisiae* exopolyphosphatase PPX (2 µg) to completely hydrolyse polyP. The released orthophosphate (Pi) was estimated using malachite green reagent (33.75 mg malachite green, 105 mg ammonium molybdate in 100 mL of 1 N hydrochloric acid) at a 1:4 ratio of sample to reagent by incubating the reaction for 10 min at room temperature and reading the absorbance at 650 nm (EnSpire multimode plate reader, PerkinElmer). The Pi contributed by polyP was determined by calculating the difference in Pi content between the PPX digested and undigested platelet extracts from the same sample. To isolate polyP from the supernatant of neutrophil-platelet co-culture, the supernatants were first incubated with proteinase K (750 mg/ml, 37ºC, 1 hr) and extracted with a 1:1 phenol/chloroform mixture. The aqueous phase was then chloroform extracted. PolyP was precipitated from the extracts with barium acetate (0.1 M, pH 4.5) and quantified as described above.

Accumulation of adoptively transferred neutrophils at inflammatory sites

Bone marrow–derived neutrophils were labeled with CFSE (final concentration, 5µM) or Snarf-1 (final concentration, 5 µM) at 37°C for 10 min and then washed twice with PBS. Labeled cells were mixed (1:1) as indicated and injected intravenously (via the tail vein) into neutropenic mice challenged with LPS for 2.5 h. BALF was harvested 1.5 h after granulocyte transfusion. The number of adoptively transferred neutrophils recruited to the lung was analyzed using a FACSCanto II flow cytometer and FACSDiva software (BD Biosciences). Relative

accumulation of WT and *Ip6k1*-KO neutrophils was calculated as the ratio of indicated populations in the lung.

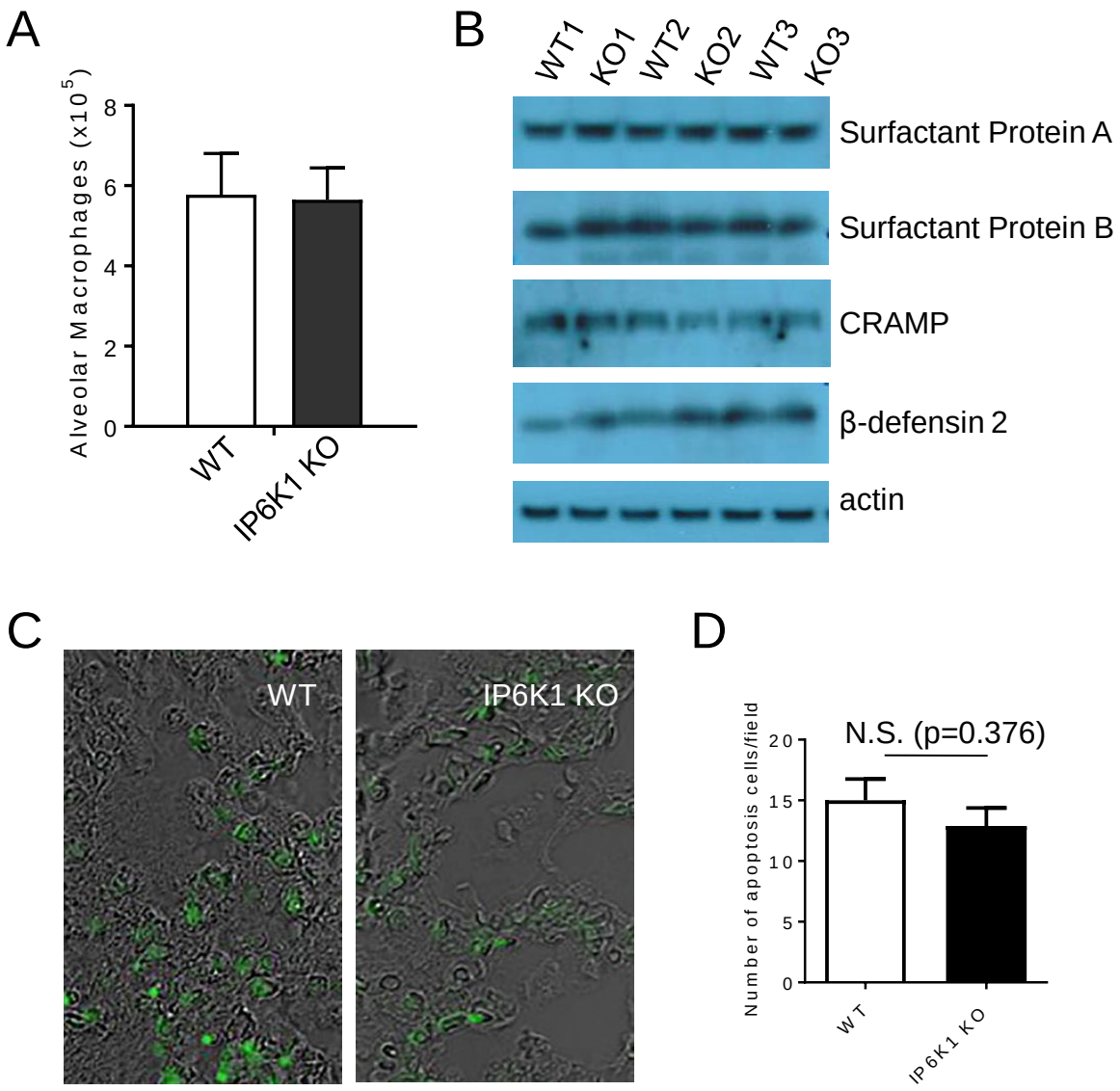

Fig. S1. Disruption of InsP6K1 does not alter alveolar macrophage number, expression of surfactant proteins/antimicrobial peptides, and neutrophil apoptosis during lung inflammation. (A) The number of alveolar macrophages in BALF of unchallenged mice. Data shown are means ±SEM (n=4 mice per group). **(B-D)** Mice were challenged by intratracheal instillation of LPS (5 mg/kg body weight) and sacrificed 24 h later. **(B)** Whole lungs were homogenized. The levels of indicated surfactant proteins/antimicrobial peptides were assessed by western blotting with surfactant protein A (Abcam), surfactant protein B (Abbiotec), CRAMP (cathelin-related antimicrobial peptide) (Santa Cruz), or beta-defensin 2 (Abbiotec) antibodies. **(C)** The viability of accumulated neutrophils was determined by the TUNEL assay. Green cells indicate apoptotic neutrophils. **(D)** Neutrophil apoptosis was expressed as the number of green cells per field of view. At least 10 fields of view were randomly picked for each experiment and

the averages were used for the calculation. Data shown are means ±SEM (n≥9 mice per group). $p<0.05$ was defined as significant.

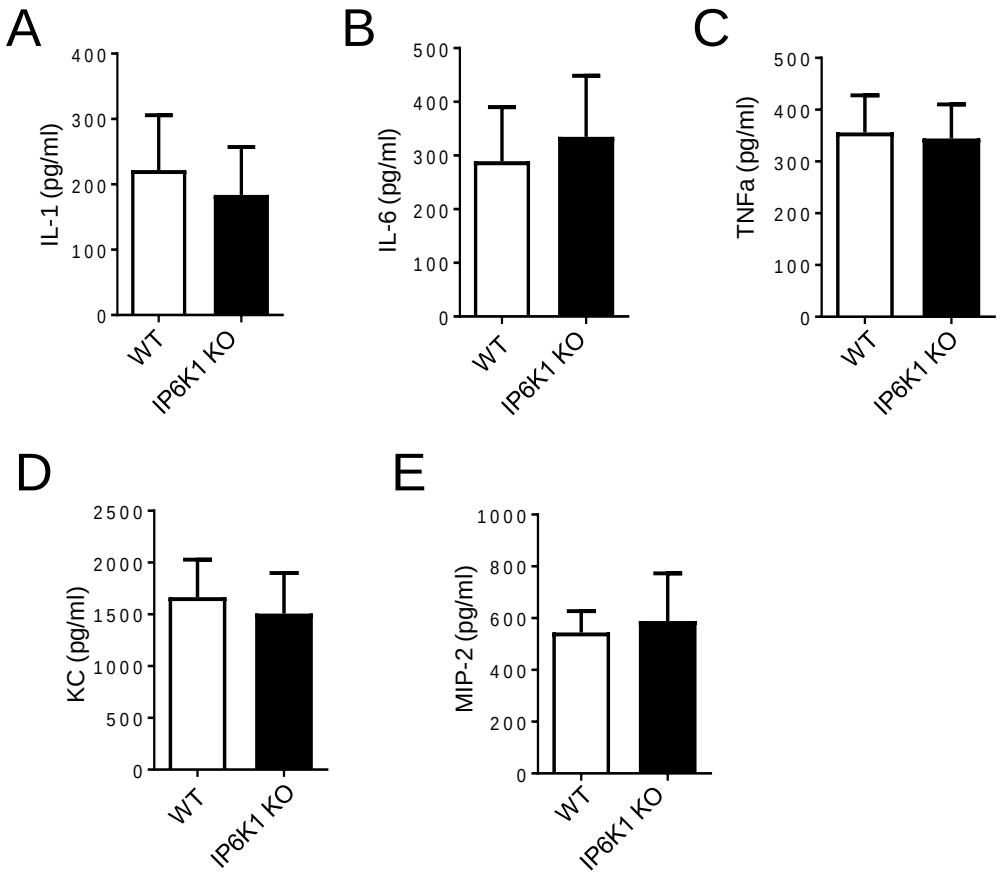

Fig. S2. Disruption of InsP6K1 does not affect the production of proinflammatory cytokines/chemokines. Mice were intratracheally instilled with $2x10^6$ CFU of *E coli* and sacrificed after 24 h. BALF was collected using ice-cold PBS/15 mM EDTA. Cytokines/chemokine (IL-1, IL-6, TNF-α, KC and MIP-2) levels were determined using specific enzyme-linked immunosorbent assay (ELISA) kits. Data shown are means ±SEM of ≥5 experiments. $p<0.05$ was defined as significant.

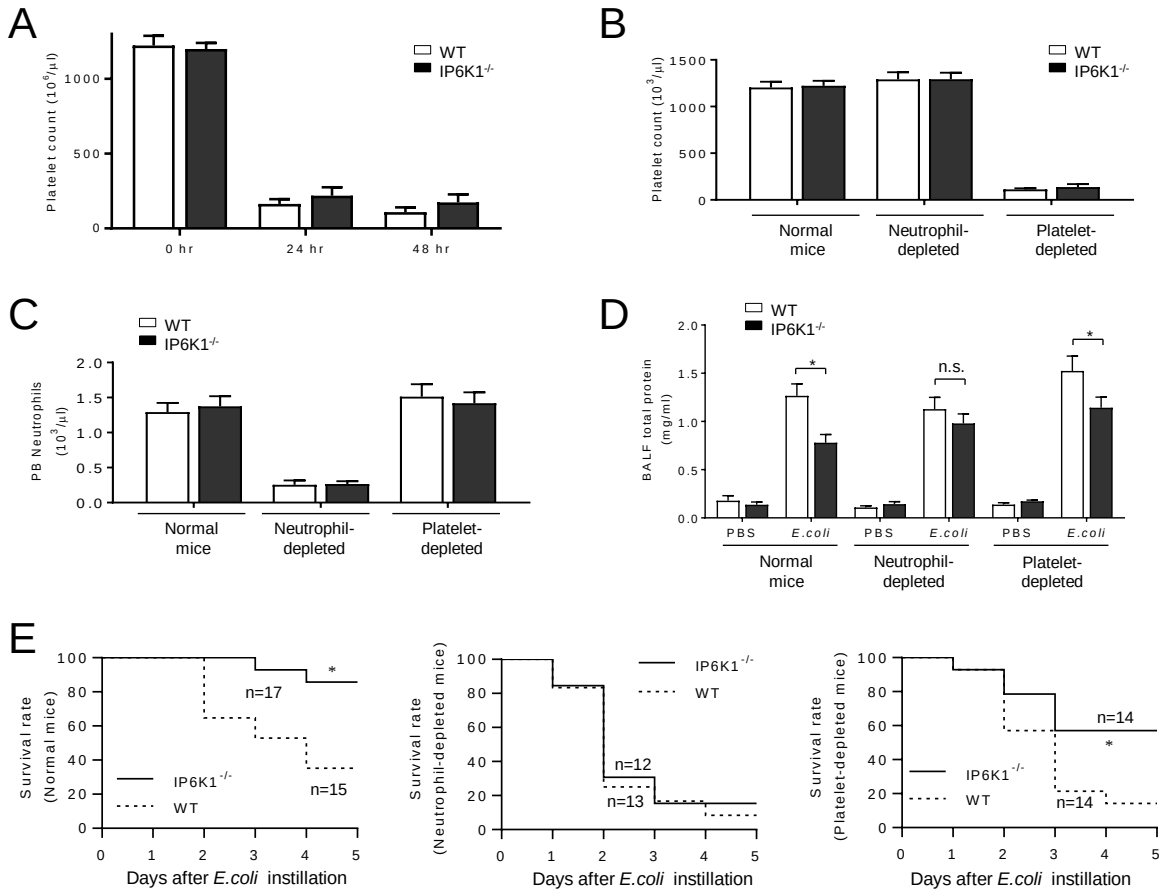

Fig. S3. *E. coli*–induced pneumonia in untreated, neutrophil-depleted, and platelet-depleted mice. (A) Platelet depletion in WT and IP6K1-deficient mice. Mice were intravenously injected with a single dose of platelet depletion (anti-GPIb/CD42b) antibody. The peripheral blood platelet counts were assessed at indicated time points. Data shown are means ±SD (n=3 mice per group). **(B)** Peripheral blood platelet count in untreated, neutrophil-depleted, and platelet-depleted mice. Data shown are means ±SD (n=5 mice per group). **(C)** Peripheral blood neutrophil count in untreated, neutrophil-depleted, and platelet-depleted mice. Data shown are means ±SD (n=5 mice per group). **(D)** BALF total protein level. The experiment was conducted as described in Figure 1I-L. Data shown are means ±SD (n=5 mice per group). **(E)** Survival rates of *E. coli*-challenged WT and IP6K1-deficient mice. Age- and sex-matched (10-week old male) wild-type and IP6K1-deficient mice were intratracheally challenged with 5×10^6 (for untreated and platelet-depleted mice) or 5×10^5 (for neutrophil-depleted mice) live *E. coli* and monitored for 5 days. Survival rates were analyzed using the Kaplan-Meier survival curves and log-rank test. *$p < 0.05$ *vs* WT.

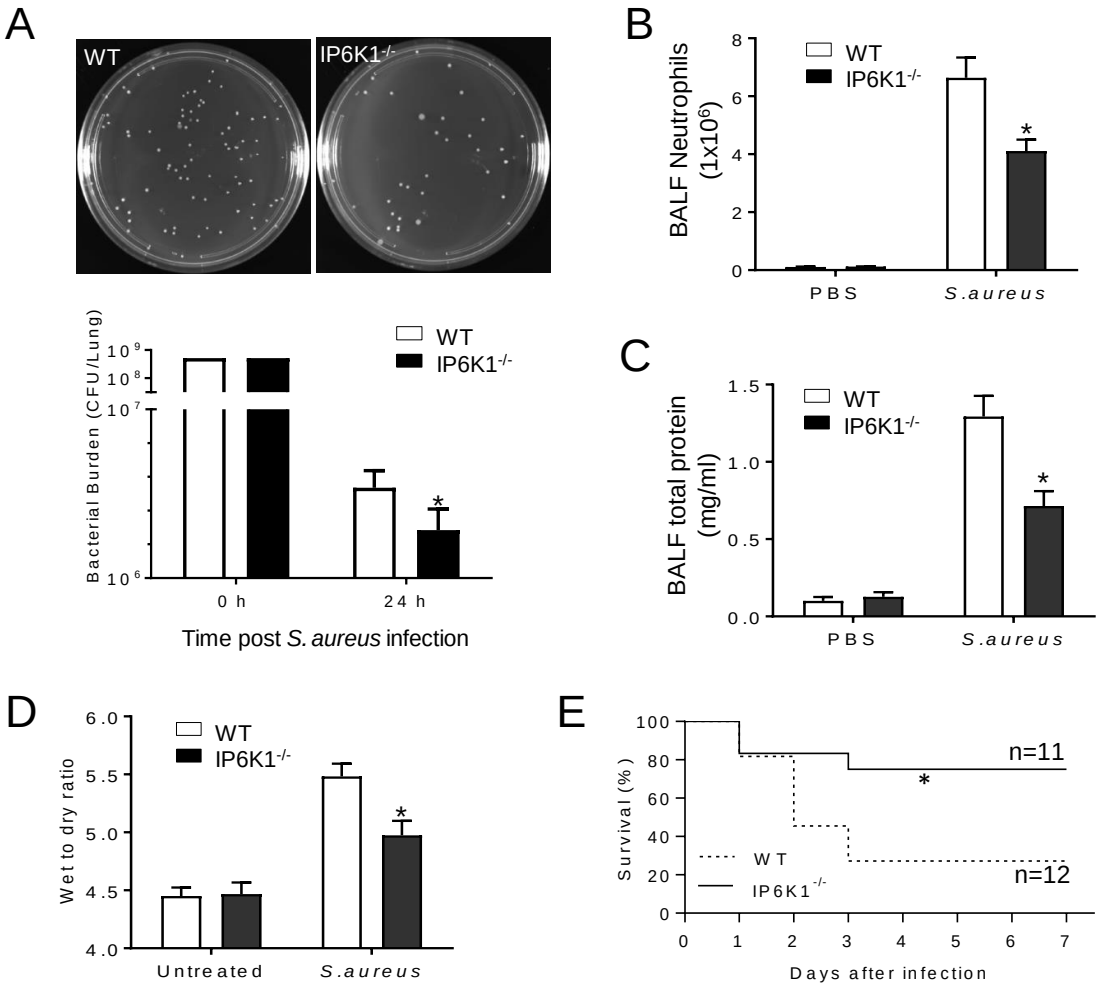

Fig. S4. Disrupting IP6K1 enhances bacterial killing and reduces lung damage in *S. aureus*–induced pneumonia. Mice were intratracheally instilled with 5.13 x 10^8 cfu of *S. aureus* and euthanized at indicated time points. (**A**) Bacterial killing in inflamed lungs. Live bacteria were quantified as cfu per lung. Data shown are means ±SEM (n=4 mice). **(B)** The numbers of neutrophils in bronchoalveolar lavage fluid (BALF). All data are presented as mean ±SEM (n=4 mice). (**C**) BALF total protein level. Protein accumulation in the inflamed lung was measured using a Bio-Rad protein assay kit. Data shown are means ±SEM (n=4 mice). **(D)** Lung wet weight-to-dry weight ratio was measured at 24 hr after *S. aureus* instillation. Values are means ± SEM; n = 5 mice/group. **(E)** Survival rates of *S. aureus*-challenged WT and IP6K1-deficient mice. Age- and sex-matched (10-week old male) wild-type and IP6K1-deficient mice were intratracheally challenged with 1×10^9 live *S. aureus* and monitored for 7 days. Survival rates were analyzed using the Kaplan-Meier survival curves and log-rank test. * $p < 0.05$ *vs* WT.

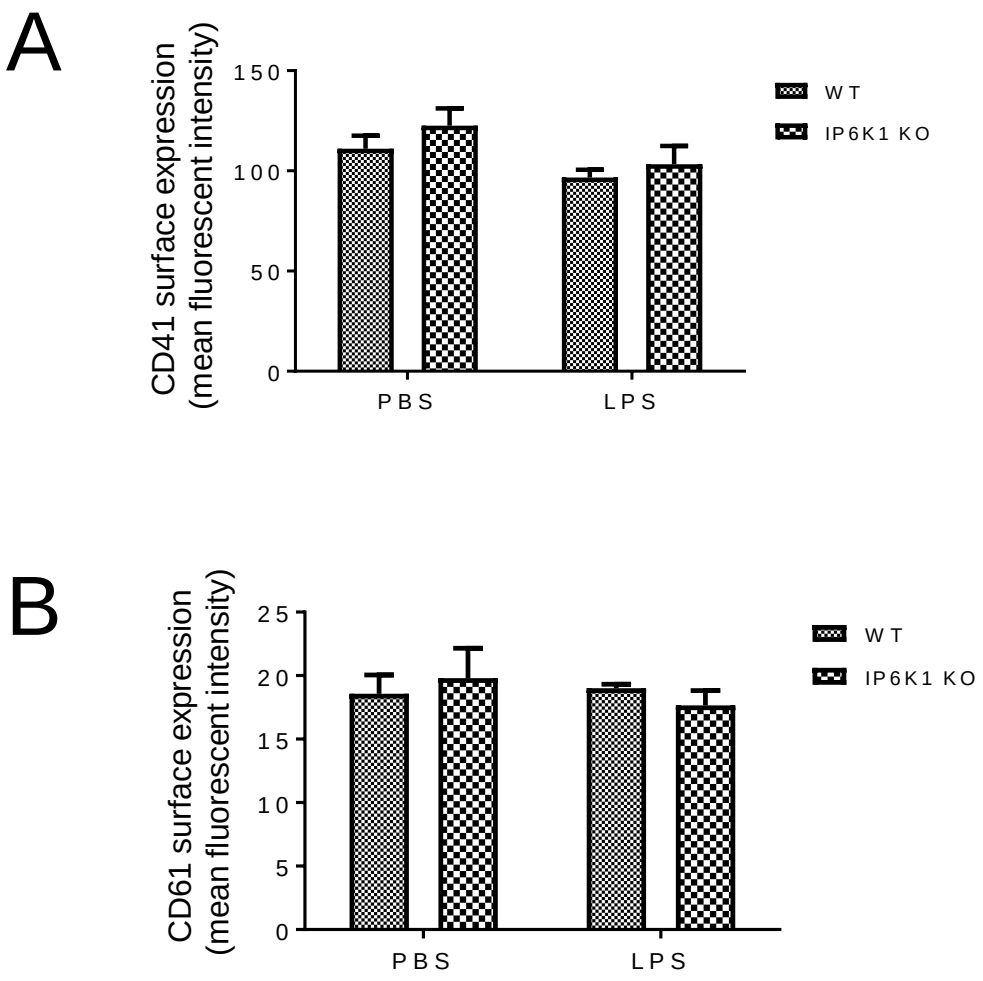

Fig. S5. InsP6K1-deficient platelets expressed the same amount of platelet markers CD41 and CD61. Mice were intratracheally instilled with 5 mg/kg LPS (*E. coli* O111:B4) and euthanized at 24 hr after LPS instillation. Surface expression of CD41 **(A)** and CD61 **(B)** on the peripheral blood platelets was analyzed using FACS. Data shown are means ±SEM of four experiments.

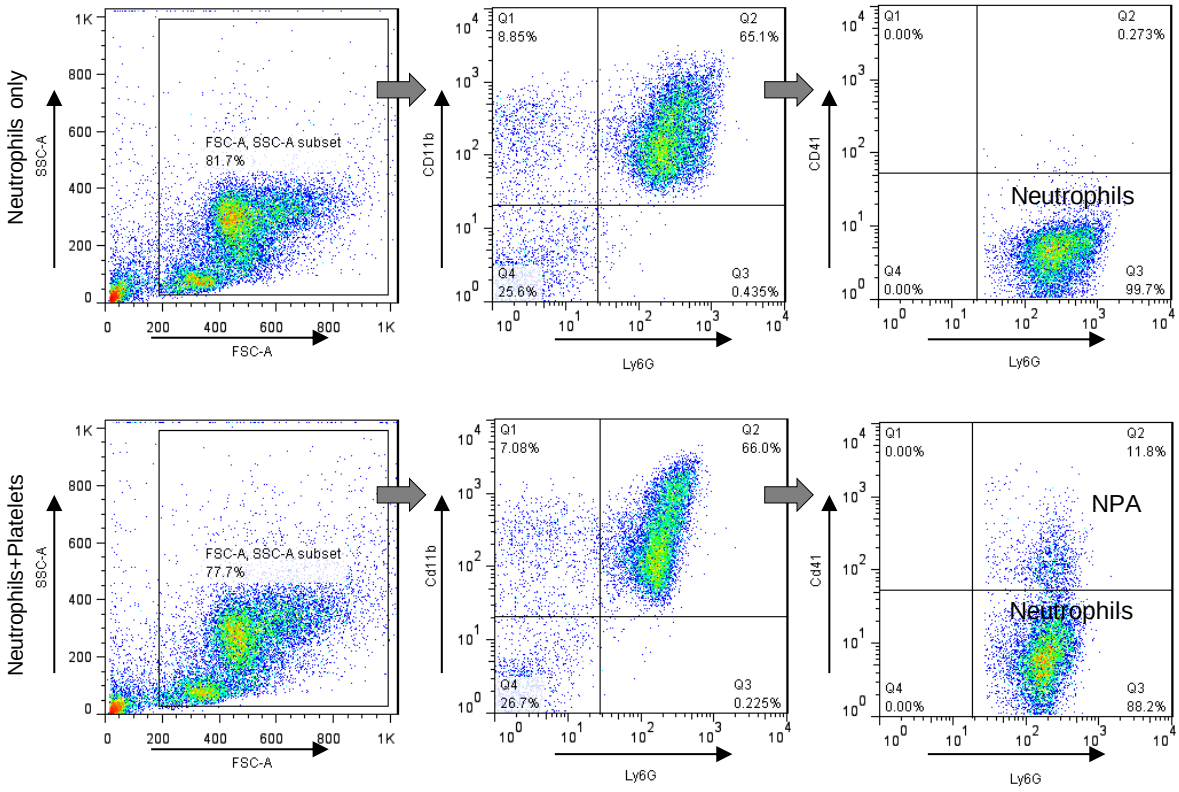

Fig. S6. Flow cytometry analysis of ex vivo NPA formation. Neutrophils and platelets were isolated from WT mice. Neutrophils were incubated with LPS (5 μg/mL or 1 μg/mL) for 2 h at 37°C in the presence or absence of platelets. After incubation, cells were stained with CD11b, CD41, and Ly6G and analyzed by flow cytometry to detect NPA. NPAs were CD41 and Ly6G double-positive on FACS.

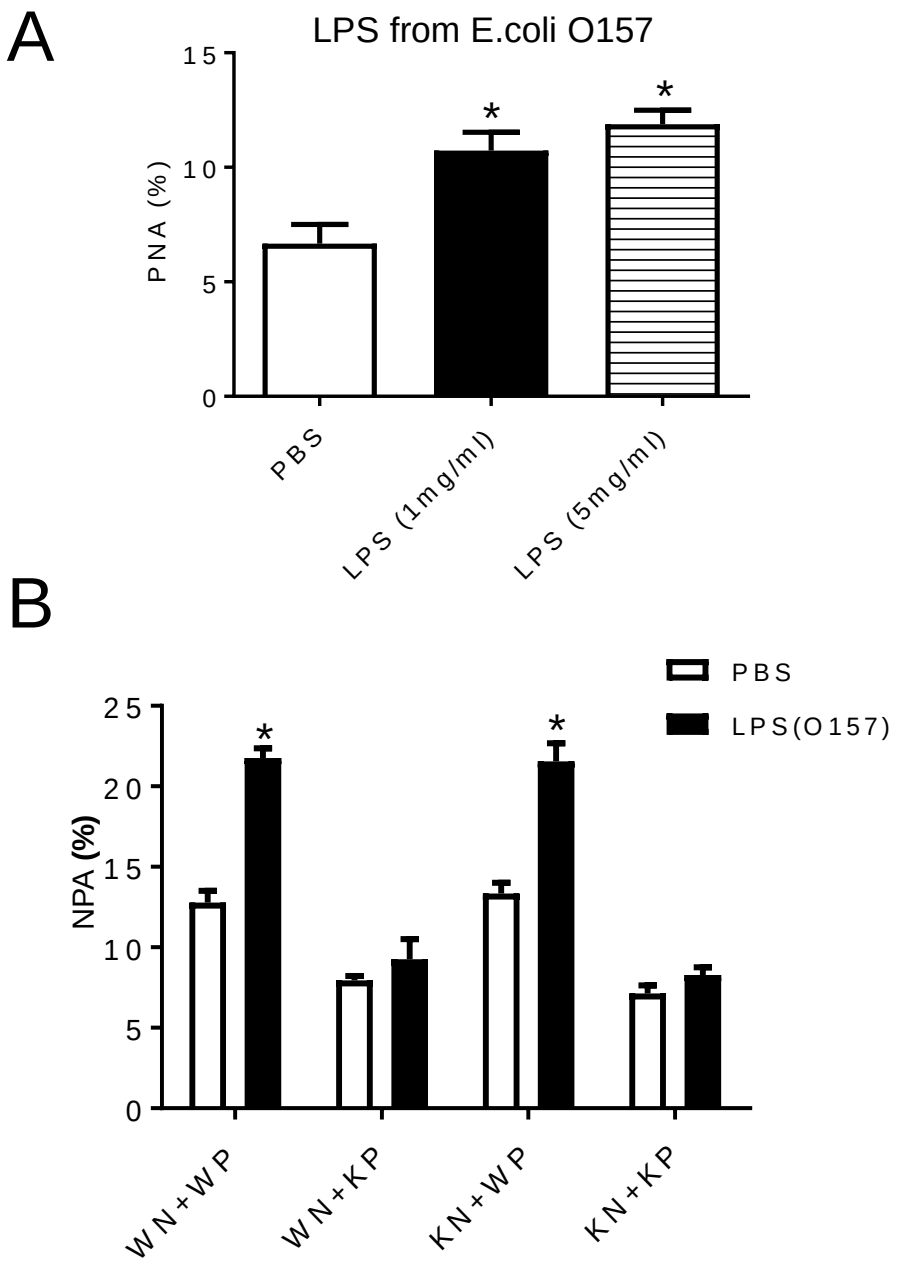

Fig. S7. NPA formation triggered by LPS from *E. coli* O157:H7. (A) Neutrophil-platelet aggregates can be induced by LPS O157:H7. Neutrophils and platelets were isolated from WT mice and incubated with LPS O157:H7 (1 mg/ml or 5 mg/ml) for 2 h at 37°C to induce NPAs ex vivo. After incubation, cell mixtures were stained with CD11b, CD41, and Ly6G and analyzed by flow cytometry to detect NPAs. NPA formation was calculated at the indicated LPS concentrations. Data shown are means ±SEM of four experiments. **(B)** NPA formation between WT or *Ip6k1*$^{-/-}$ neutrophils and platelets. The experiment was conducted using LPS O157:H7 as described in Figure 3G. Data shown are means ±SEM of four experiments. *p<0.05 *vs.* cells treated with PBS.

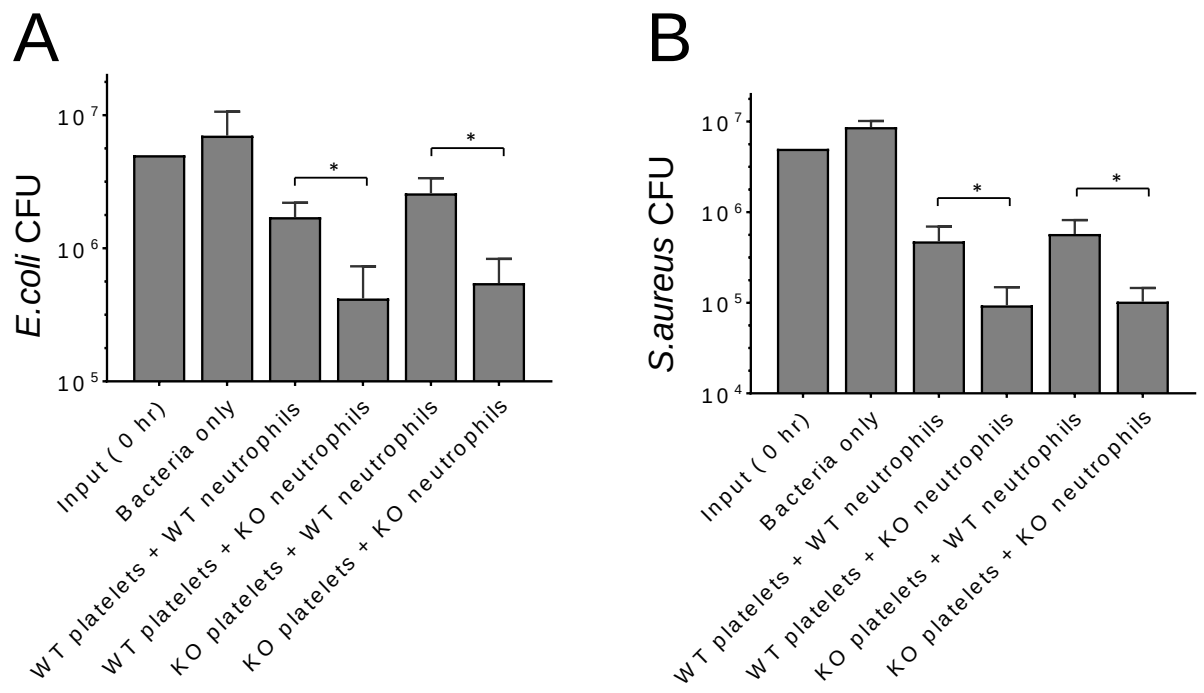

Fig. S8. In vitro killing of bacteria by neutrophil-platelet coculture. Purified WT or *Ip6k1*-KO neutrophils (1 x 10^6) and WT or *Ip6k1*-KO platelets (2x 10^8) were incubated with **(A)** *E. coli* or **(B)** *S. aureus* for 1 hr. Diluted aliquots were spread on agar plates and incubated overnight at 37°C. In vitro bacterial killing capabilities were reflected by the decrease of bacteria colony forming units (CFU) after the incubation. Data shown are means ±SD (n=5 mice per group). **p*<0.05.

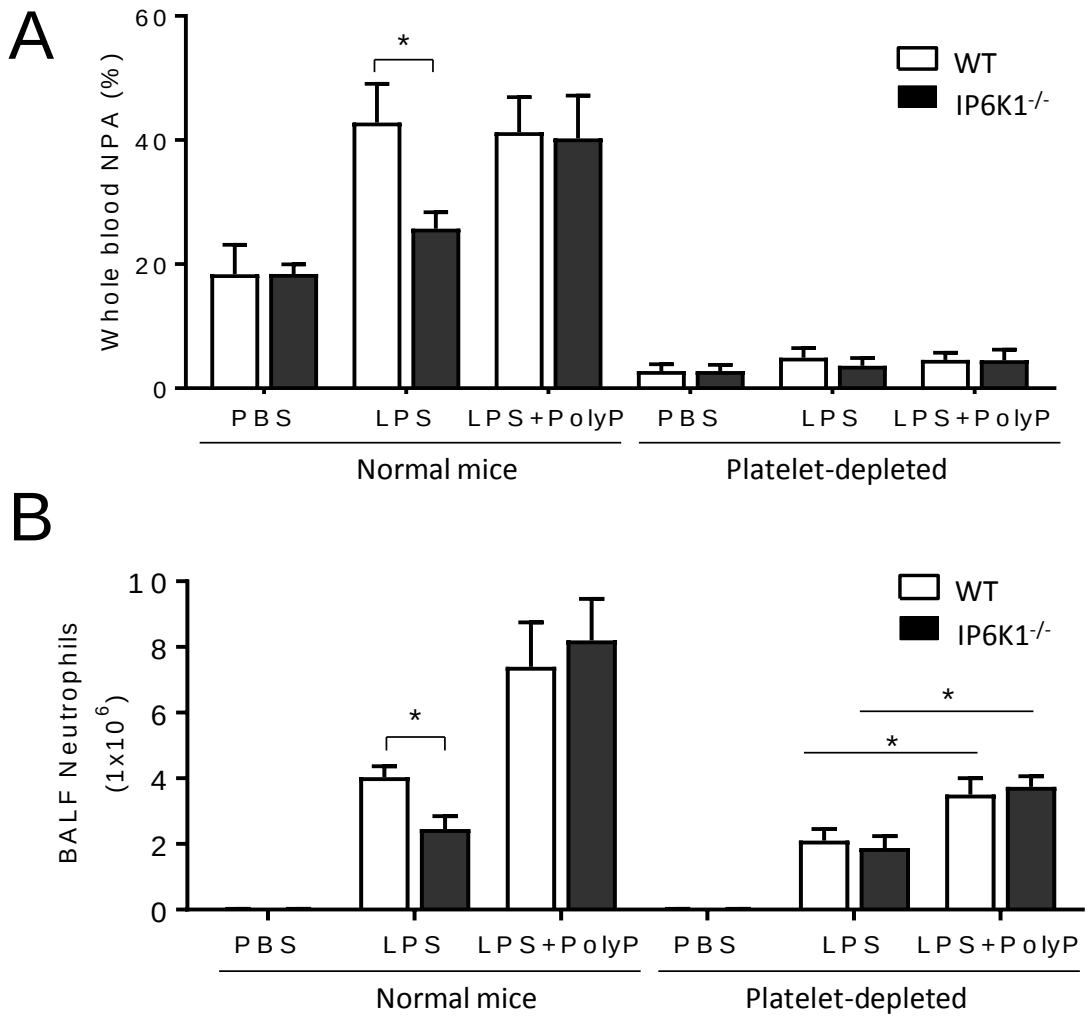

Fig. S9. PolyP can also enhance neutrophil accumulation via an NPA-independent mechanism. (A) NPA formation in the presence or absence of polyP. FACS analysis of NPA formation in peripheral blood was conducted as described in Figure 4E. Both untreated and platelet-depleted mice were used in this experiment. Shown are percentage of NPAs in whole blood. Data shown are means ±SD (n=5 mice per group). **(B)** Neutrophil accumulation to the inflamed lungs in untreated or polyP-treated normal mice or platelet-depleted mice. The numbers of neutrophils in BALF were determined as described in Figure 4F. Data shown are means ±SD (n=5 mice per group). *p<0.05.

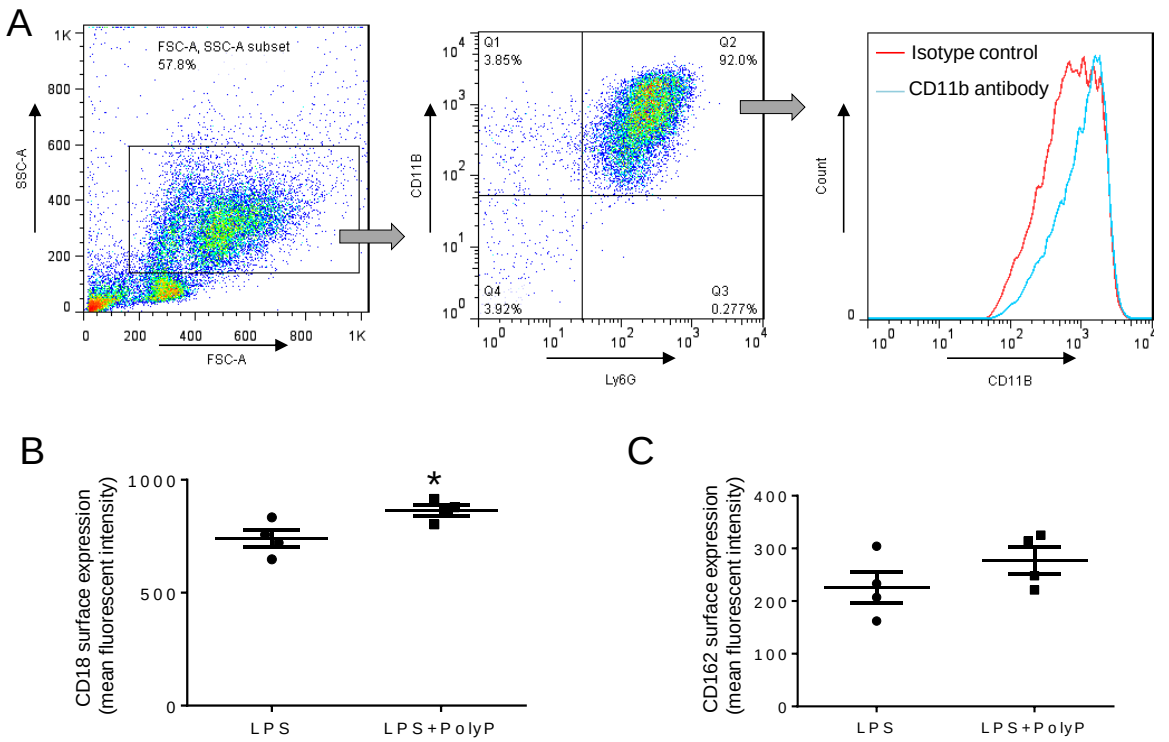

Fig. S10. Surface expression of adhesion molecules on neutrophils. (A) WT neutrophils and platelets were treated with polyP for 2 h. CD11b surface levels were detected by FACS. **(B-C)** Surface expression of adhesion molecules CD18 and Cd162 on neutrophils. WT neutrophils and platelets were treated with polyP for 2 h. CD18 and CD162 surface levels were detected by FACS. Data shown are means ±SEM of four experiments. **p*<0.05 *vs.* cells treated with LPS alone.

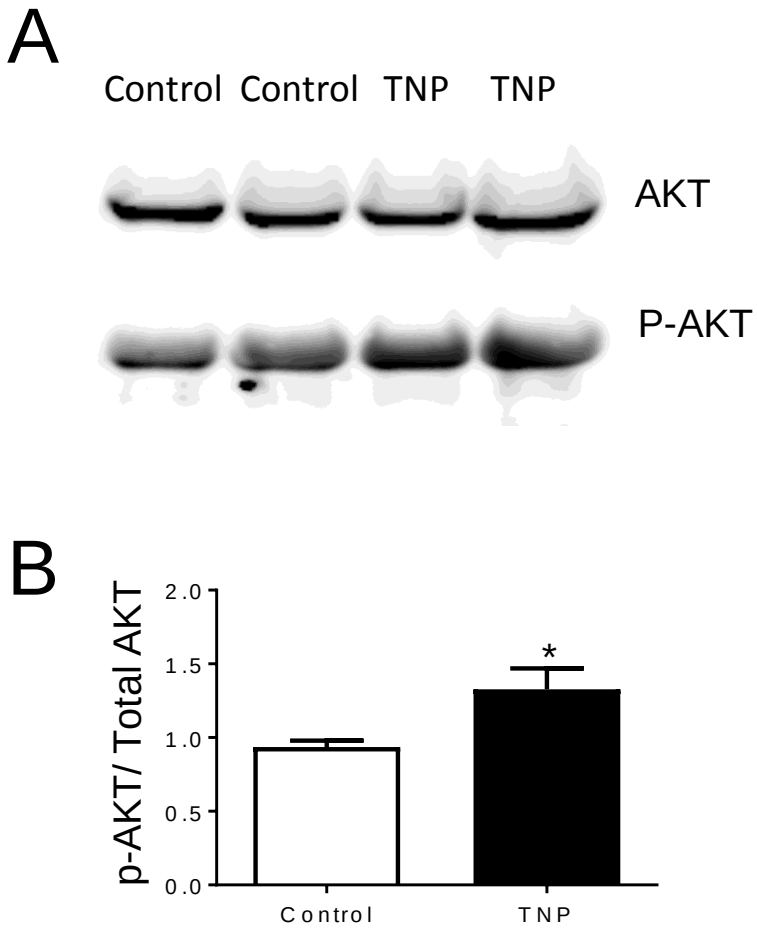

Fig. S11. The level of PtdIns(3,4,5)P3 signaling, assessed by phospho-Akt, is elevated in TNP-treated mice. Mice were treated with TNP for 10 days (20 mg/kg body weight, once a day). Bone marrow neutrophils were isolated and lysed. Total/phosphorylated Akt in cell lysates was detected by western blotting. Relative amounts of phosphorylated Akt were quantified with NIH ImageJ software. Data shown are means ±SEM of four experiments. *p<0.05 *vs.* neutrophils isolated from mice treated with DMSO alone.