Supporting Information

SI Materials and Methods

Materials

Agarose, HBSS, and RPMI 1640 were obtained from Thermo Scientific (Waltham, MA, USA). Receptor antagonists, molecule inhibitors and a phospho-MAPK array were obtained from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum (FBS), LPS, ATP, ATP-γ-S, ADO, AMP, a protease inhibitor cocktail, Ficoll-Paque, Dextran and RIPA buffer were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for western blots and LSCM were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies for FCM and apoptosis kits were obtained from BD Bioscience (San Jose, CA, USA). Antibody for blocking β1 integrin was obtained from EMD Millipore (Darmstadt, Germany). Antibody for blocking β2 integrin was obtained from BioLegend (San Diego, CA, USA).

Ethics statement

The Medical Ethical Committee of Jiangsu University approved this study. After written informed consent was obtained, blood specimens were extracted from the cubital veins of healthy drug-free donors. Consent for the use of these samples was given by the Medical Ethical Committee of Jiangsu University. All of the experiments were performed in accordance with the approved guidelines.

Preparation of human neutrophils

Venous blood was obtained from healthy volunteers. Neutrophils were isolated as previously described (1). Briefly, 3% dextran was used to sediment erythrocytes followed by Ficoll-Paque density centrifugation to remove peripheral blood mononuclear cells. Remaining erythrocytes were removed by two rounds of hypotonic lysis using ddH_2O. Neutrophils were ~95% viable and ~97.5% pure as determined by FCM. Neutrophils were suspended in HBSS with Ca^{2+} and Mg^{2+} and 1% FBS at 1.0 ×

10^7 cells/mL.

Neutrophils were administered with the indicated doses of LPS, and chemotaxis was assessed. Antagonists or inhibitors were pre-incubated for 30 min in the indicated experiments.

Under agarose neutrophil chemotaxis model

The under agarose neutrophil chemotaxis model was performed as previously described (2, 3). Briefly, a 1.2% agarose solution was boiled and mixed 1:3 with heated medium containing 50% HBSS with Ca^{2+} and Mg^{2+} and 50% RPMI 1640 (20% heat-inactivated FBS). Approximately 3 mL of above-described solution was poured into a 35-mm culture dish and cooled gently. After the agarose cooled and solidified, three wells, 3.5 mm in diameter and 2.8 mm apart, were cut into a straight line in the gel. The middle well was filled with chemoattractants and the side wells were filled with neutrophils treated as indicated. Gels were incubated for 2 h in a 37 ℃/5% CO_2 incubator. Chemotaxis distances were observed using a microscope.

To eliminate the extracellular calcium in the chemotaxis assay, Ca^{2+} and Mg^{2+}-free HBSS was used, and the agarose solution contained 2 mmol/L EGTA. In the experiments using a low concentration of agarose, we decreased the agarose concentration from 1.2% to 0.8%. For measuring chemotaxis distances of dHL-60 cells, the agarose concentration was 0.8%.

Flow cytometry

Neutrophils were washed twice with ice-cold PBS and resuspended in PBS containing 5% FBS. Antibodies, annexin V or 7-AAD were added according to the manufacturer's protocol. After incubating for 45 min at 4 ℃ in the dark, the neutrophils were assessed.

Neutrophil phagocytosis assay

Neutrophil phagocytic activity was detected using the pHrodo E. coli BioParticles Phagocytosis kit (Life Technologies, CA, USA). Neutrophils were incubated with the pHrodo E. coli particles for 1 h, and the fluorescence intensity was determined by FCM.

Generation and differentiation of P2X1 knockout or overexpressed and MK2 knockout HL-60 cell lines

Lenti-CAS9-sgRNA system for P2X1 and MK2 knockout were constructed by GeneChem (Shanghai, China). Lentiviral vectors for P2X1 overexpression was constructed by Hanbio Biotechnology (Shanghai, China). Stably knocked out and overexpressing cell lines were generated as previously described (1). HL-60 cells were transduced with lentiviral vectors at an MOI of approximately 30 in the presence of 5 μg/ml polybrene. After 24 hours, the culture medium was removed, and fresh medium was added to the HL-60 cells. After 72 hours of transduction, puromycin was added to the medium at a concentration of 5 μg/ml for stable cell line selection. The relevant empty lentivectors were used as negative controls. The expression of P2X1 and MK2 was determined by western blot analysis.

HL-60 cells were differentiated into a neutrophil-like phenotype as previously described (1). Briefly, DMSO was added to the medium at a final concentration of 1.3%. After a 6-d incubation, cells were harvested and replanted in fresh medium.

Electron microscopy

Chemotactic neutrophils in the under agarose chemotaxis model were fixed with 2.5% glutaraldehyde. Fixed samples were dehydrated in an ascending series of ethyl alcohols, critical-point-dried and subsequently sputtered with gold. Morphological characteristics of the chemotactic neutrophils in wells were observed with a LEO 440 scanning electron microscope.

Atomic force microscopy imaging

AFM imaging of neutrophils was performed as previously described (4). Briefly, chemotactic neutrophils were fixed with 2% paraformaldehyde. Following dehydration in an ascending series of ethyl alcohols, a tapping mode of AFM was used to acquire topographic and phase images of the neutrophils in an atmospheric environment. The procedure was repeated for five cells, and each cell was scanned at least three times.

Laser scanning confocal microscopy

Staining of P2X1 receptor was performed as previous described (5). Cells in culture medium were washed twice with ice-cold PBS and fixed with 2% paraformaldehyde for 10 min at room temperature. Staining of P-MLC or F-actin in migrating neutrophils was performed previously described (2). Neutrophils were first fixed with 2% paraformaldehyde to preserve the morphological characteristics and then washed twice with ice-cold PBS. Triton X-100 (0.01%) was used for permeabilizing. During P2X1 or P-MLC staining, cells were blocked with 5% goat serum for 1 h followed by incubation with a 1:200 primary antibody and a 1:2000 secondary antibody. During F-actin staining, cells were blocked with 5% goat serum for 1 h followed by incubation with CytoPainter Phalloidin-iFluor 647 for 30 min. Neutrophils were observed using the Leica SP8 microscope.

Phospho-MAPK proteome array

The phospho-MAPK proteome array was performed according to manufacturer's instructions. Neutrophils were treated for 30 min, and 300 μg of cell lysates were prepared for the phospho-MAPK proteome array assay. The pixel density in each spot of the array was determined by ImageJ software.

Western blotting

Western blot analysis was performed as described previously (4). RIPA buffer containing protease and phosphatase inhibitor cocktails was used for lysis of the cells. The lysates were incubated with 3 ×SDS buffer, boiled and loaded on 10% SDS–PAGE gels. Approximately 20 μg of protein was subjected to electrophoresis on 10% SDS polyacrylamide gels with the use of the discontinuous system and transferred onto nitrocellulose membranes. The membranes were incubated with primary antibody and followed by secondary antibody conjugated to horseradish peroxidase (3:5000). An ECL reagent was used to visualize the bands with FluorChem FC3 (ProteinSimple, San Jose, CA, USA), and AlphaView 3.4.0 software was used for quantification.

Phosphorylated p38 (p-p38) was normalized to total p38 (t-p38) and presented as the percent of control.

Measurement of the Calcium Influx

Intracellular calcium was measured according to the manufacturer's instructions. Briefly, neutrophils were preloaded with Fluo-4/AM (10 μmol/L) and pluronic acid (0.02%) at room temperature in HBSS. After incubation for 30 min, cells were washed twice and incubated in HBSS for an additional 15 min. Neutrophils were transferred to a 96-well plate and treated with either fMLP or LPS. Calcium signals were observed and recorded using LSCM.

Adhesion Assay

96-well plates were pre-coated with 20 μL FBS for 2 h. 10^5 neutrophils in 100 μL for each group were added and incubated in 37 ℃/5% CO_2 incubator for 1 h. Then, neutrophils were fixed with 2% paraformaldehyde for 15 min in room temperature. Unbound cells were then removed by washing and vigorous shaking. DAPI (blue) was used to stain neutrophils and observed by fluorescence microscope. Mean fluorescence intensity was detected.

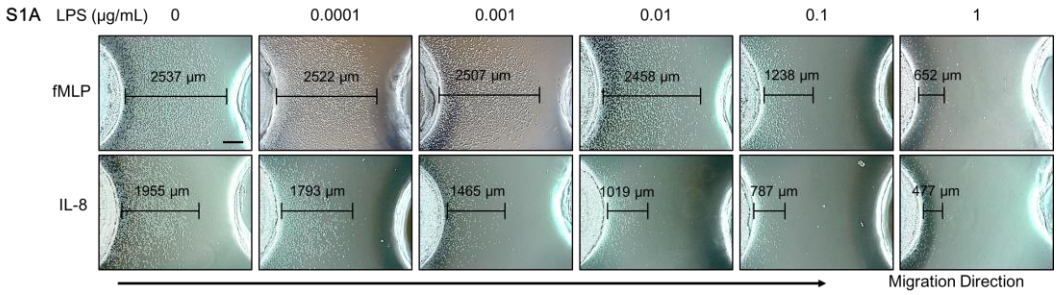
Statistical analyses

All of the data are presented as the mean ± standard deviation. Student's t test was used to compare the differences between two groups. One-way analysis of variance (ANOVA) and Tukey's test were used for the three or more groups comparisons. A value of $P < 0.05$ was considered to be statistically significant.

References

1. Liu X*, et al.* (2012) Bidirectional regulation of neutrophil migration by mitogen-activated protein kinases. *Nat Immunol* 13(5):457-464.
2. Heit B*, et al.* (2008) PTEN functions to 'prioritize' chemotactic cues and prevent 'distraction' in migrating neutrophils. *Nat Immunol* 9(7):743-752.

3. Wang X, Qin W, Zhang Y, Zhang H, & Sun B (2016) Endotoxin promotes neutrophil hierarchical chemotaxis via the p38-membrane receptor pathway. *Oncotarget* 7(45):74247-74258.
4. Liu D*, et al.* (2016) Suppressive effect of exogenous carbon monoxide on endotoxin-stimulated platelet over-activation via the glycoprotein-mediated PI3K-Akt-GSK3beta pathway. *Sci Rep* 6:23653.
5. Chen Y*, et al.* (2006) ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 314(5806):1792-1795.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0.001
0.01
0.1
1
fMLP
IL-8
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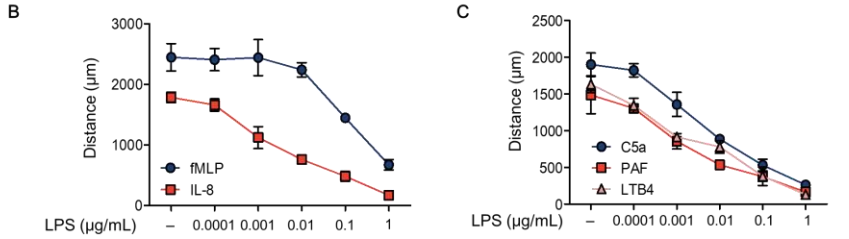
2507 μm
2458 μm
1238 μm
652 μm
1955 μm
1793 μm
1465 μm
1019 μm
787 μm
477 μm
Migration Direction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IL-8
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C
Distance (μm)
C5a
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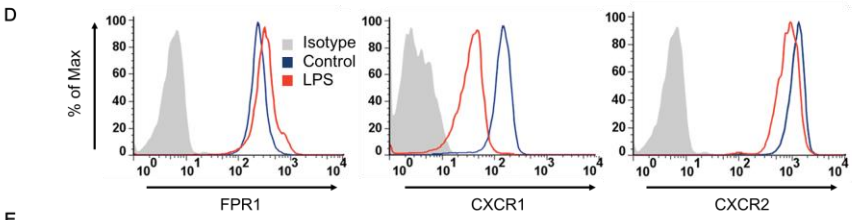
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Isotype
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LPS
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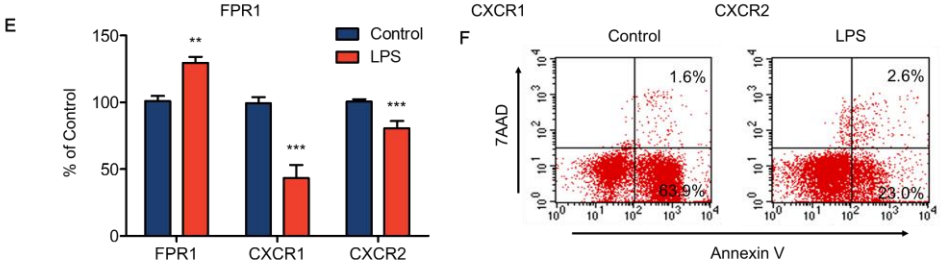
CXCR2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Control
LPS
FPR1
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F
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LPS
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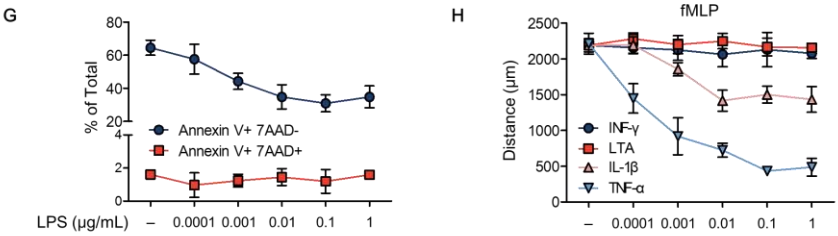
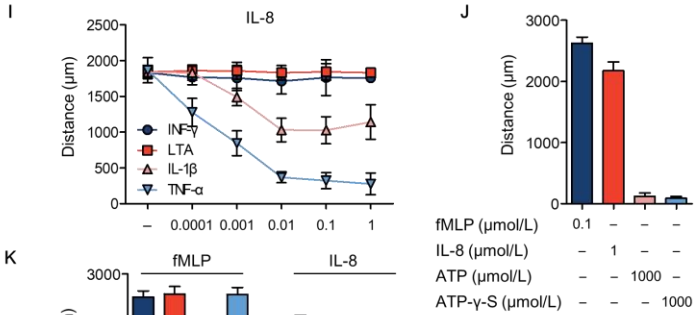
63.9%
2.6%
23.0%
Annexin V


G
% of Total
Annexin V+ 7AAD-
Annexin V+ 7AAD+
LPS (μg/mL)
H
fMLP
Distance (μm)
INF-γ
LTA
IL-1β
TNF-α


I
IL-8
Distance (μm)
INF-γ
LTA
IL-1β
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J
Distance (μm)
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IL-8 (μmol/L)
ATP (μmol/L)
ATP-γ-S (μmol/L)

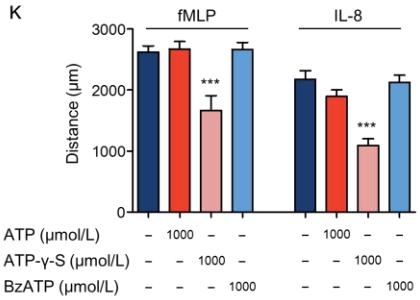

K
fMLP
IL-8
Distance (μm)
ATP (μmol/L)
ATP-γ-S (μmol/L)
BzATP (μmol/L)

Fig. S1 (A-B) Under agarose chemotaxis assays of neutrophils. Neutrophils were treated with gradient concentrations of LPS. After migrating for 2 h, the chemotaxis distances to fMLP (0.1 μmol/L) and IL-8 (1 μmol/L) were observed and analyzed. Scale bar, 500 μm. (C) Under agarose chemotaxis assays of neutrophils. Neutrophils were treated with gradient concentrations of LPS. After migrating for 2 h, the chemotaxis distances to C5a (1 μmol/L), PAF (1 μmol/L) or LTB4 (0.1 μmol/L) were detected. (D-E) FCM of membrane FPR1, CXCR1 and CXCR2 expression on neutrophils treated with 1 μg/mL LPS for 30 min. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group (Student t test). (F-G) Neutrophils were treated with the indicated concentration of LPS for 4 h. Apoptosis was assayed by flow cytometry. Early apoptosis was indicated as annexin V+/7-AAD-, while late apoptosis was indicated as annexin V+/7-AAD+. A scatter diagram of neutrophils treated for 1 μg/mL LPS is shown. (H-I) Neutrophils were treated with IFN-γ, LTA, IL-1β or TNF-α. Chemotaxis toward either fMLP or IL-8 was detected. (J) Under agarose chemotaxis assays of neutrophils. Chemotaxis distances to fMLP (0.1 μmol/L), IL-8 (1 μmol/L), ATP (1000 μmol/L) or ATP-γ-S (1000 μmol/L) were observed and analyzed. (K) Neutrophils were treated with ATP, ATP-γ-S and BzATP. Chemotaxis distances to fMLP (0.1 μmol/L) and IL-8 (1 μmol/L) were observed and analyzed. $^{***}P < 0.001$, compared with control group (ANOVA with Tukey's test). Data are representative of six (A-C, H-K) or four (D-G) independent experiments. Mean and SD are presented.

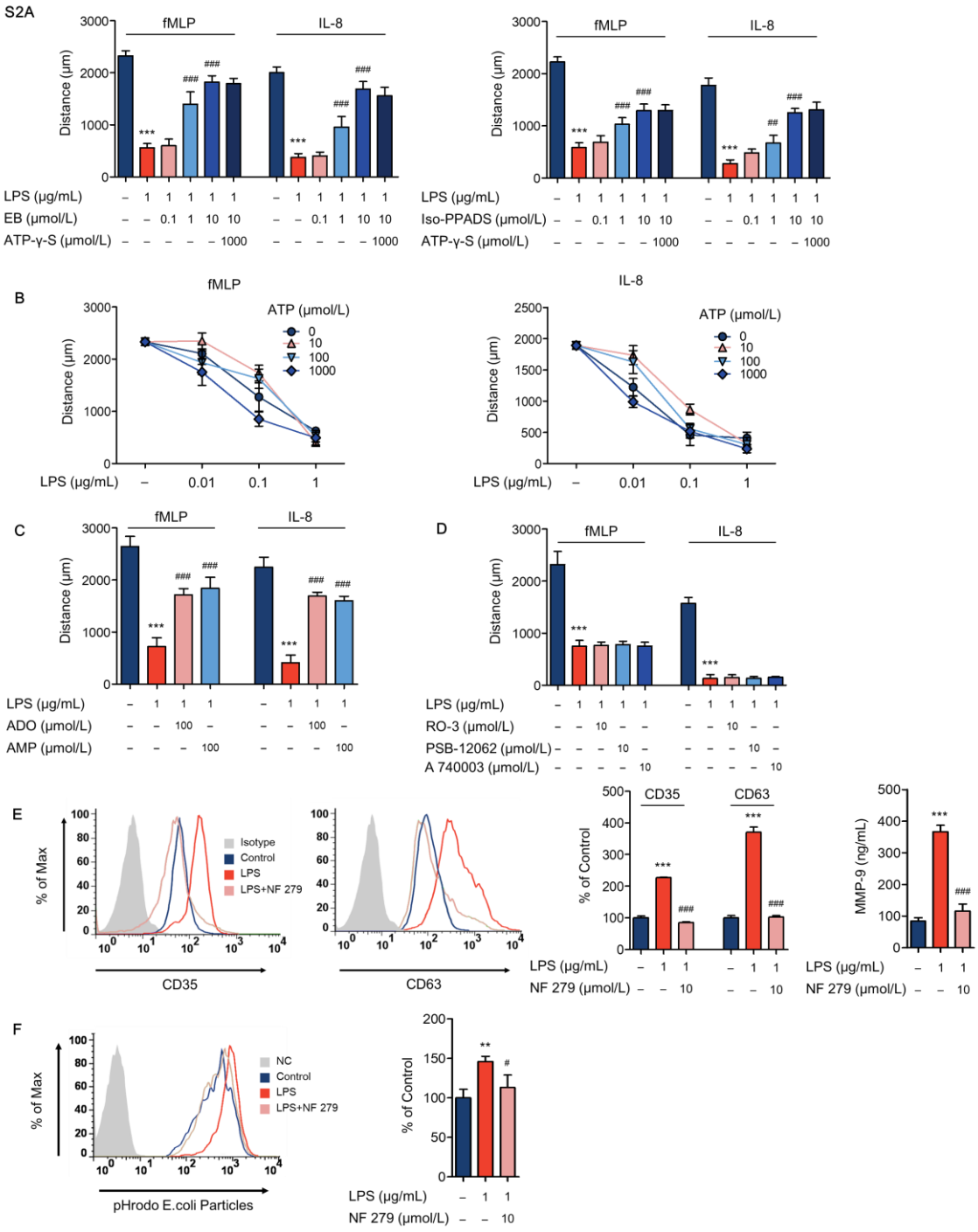


Fig. S2. (A) Neutrophils were pre-incubated with two pan-P2X purinergic antagonists, Evans Blue and iso-PPADS for 30 min. Subsequently, 1 μg/mL LPS was introduced, and neutrophil chemotaxis toward either fMLP or IL-8 was detected. (B) LPS combined with gradient concentrations of ATP was administered to neutrophils. The chemotaxis distances toward either fMLP or IL-8 were assayed. (C) LPS combined with ADO or AMP was administered to neutrophils. The chemotaxis distances toward either fMLP

or IL-8 were assayed. (D) Neutrophils were pre-incubated with P2X3 (RO-3), P2X4 (PSB-12062) or P2X7 (A 740003) receptor antagonists for 30 min. Subsequently, 1 μg/mL LPS was introduced, and neutrophil chemotaxis toward either fMLP or IL-8 was detected. (E) Neutrophils pre-treated with the P2X1 receptor antagonist (10 μmol/L) were stimulated with 1 μg/mL LPS for 30 min. Neutrophil exocytosis of secretory vesicles, gelatinase and specific granules were evaluated by their specific markers, CD35, MMP9 and CD63. CD35 and CD63 were detected by FCM, and MMP9 was detected by ELISA. (F) Neutrophils pre-treated with the P2X1 receptor antagonist (10 μmol/L) were stimulated with 1 μg/mL LPS for 30 min. Neutrophil phagocytic activity was detected by FCM using pHrodo-labeled E. coli particles. Data are representative of six (A-D) or four (E and F) independent experiments. Mean and SD are presented. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$, compared with LPS group (ANOVA with Tukey's test).

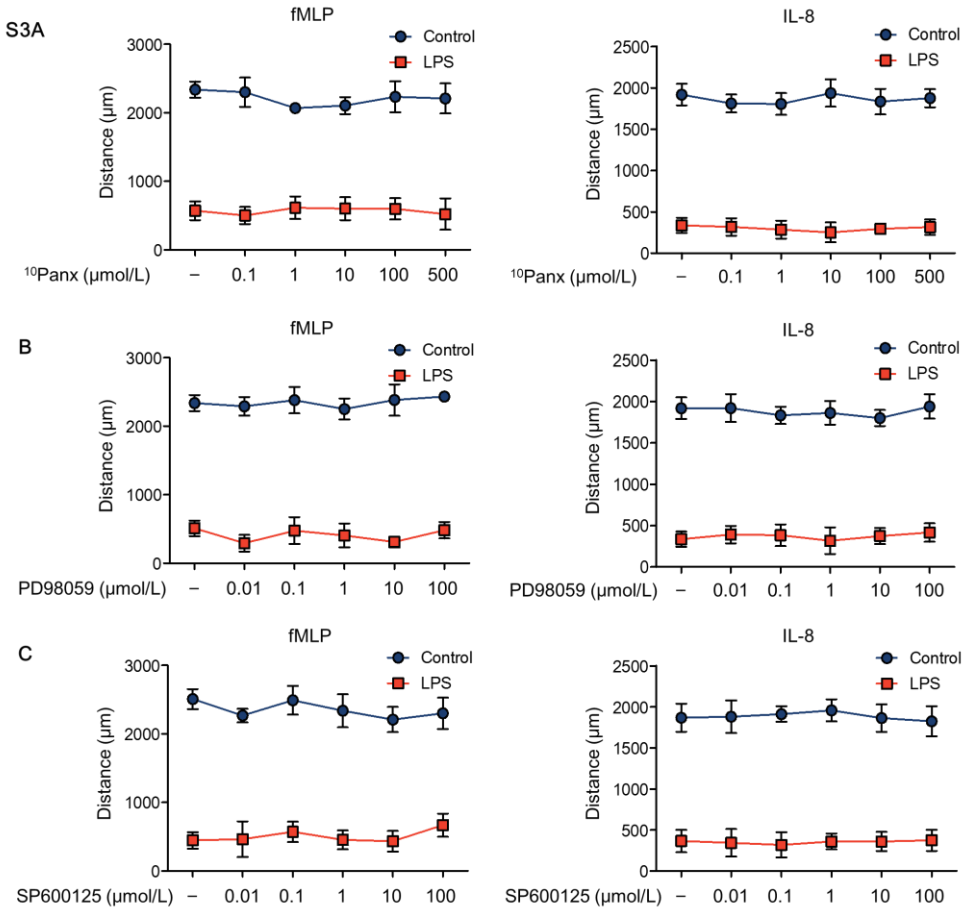


Fig. S3. Neutrophils were pre-incubated with Panx1 inhibitor ([10]Panx), ERK1/2 inhibitor (PD98059) or JNK inhibitor (SP600125). Subsequently, 1 μg/mL LPS was introduced, and neutrophil chemotaxis toward either fMLP or IL-8 was detected. Data are representative of six independent experiments. Mean and SD are presented.

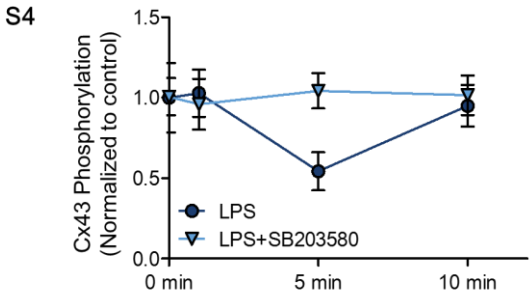


Fig. S4. Neutrophils were loaded with a p38 inhibitor (10 μmol/L) for 30 min followed by 1 μg/mL LPS. Dephosphorylation of Cx43 was observed by western blot. Data are representative of four independent experiments. Mean and SD are presented.

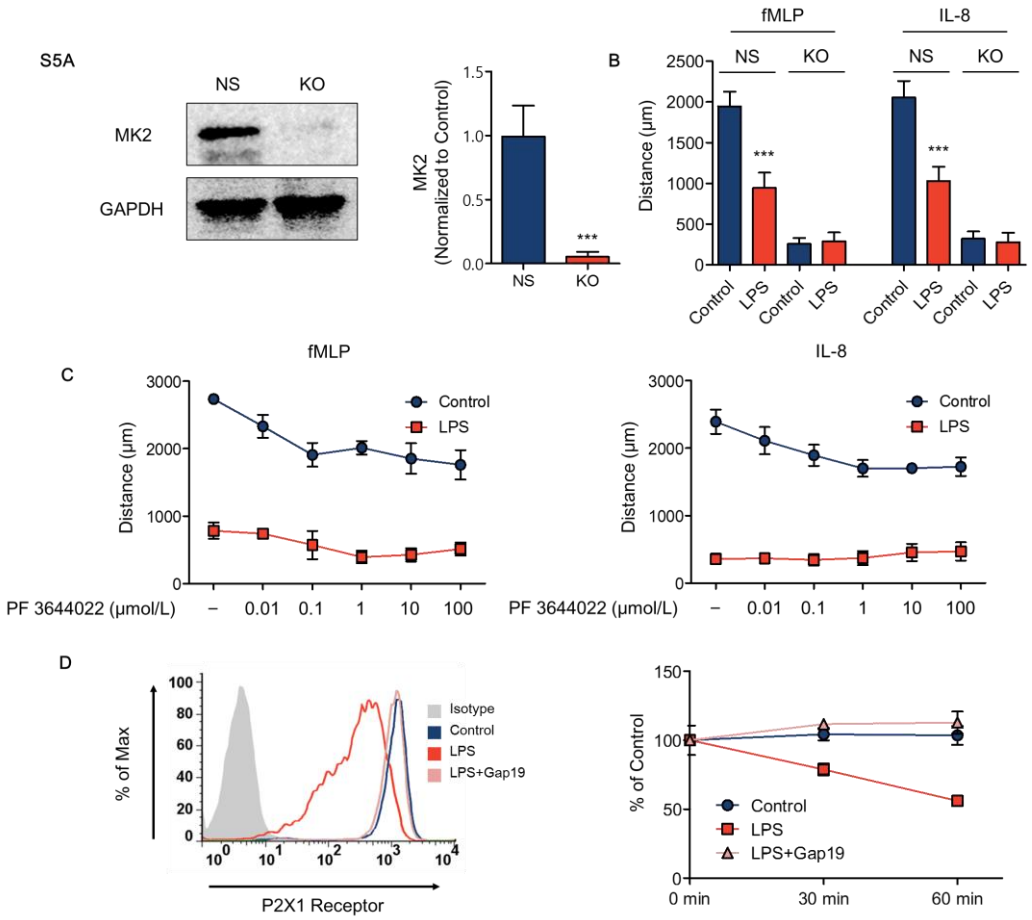


Fig. S5. (A) MK2 was knocked out (KO) in dHL-60 cells. The relevant empty lentivectors were used to induce control nonspecific (NS) expressing cells. Expression of the MK2 was detected by western blot. (B) dHL-60 cells were treated with 10 μg/mL LPS, and chemotaxis toward fMLP or IL-8 was determined. (C) Human neutrophils were pre-treated with a MK2 inhibitor (PF 3644022) followed by 1 μg/mL LPS. Neutrophil chemotaxis toward either fMLP or IL-8 was assayed. (D) Neutrophils were pre-treated with a Cx43 inhibitor (500 μmol/L) for 30 min followed by 1 μg/mL LPS. Membrane P2X1 receptor expression levels were observed by FCM after the neutrophils were stimulated with LPS for 30 min and 60 min. A FCM histogram of neutrophils treated for 60 min is shown. Data are representative of six (B-C) or four (A, D) independent experiments. Mean and SD are presented. $^{***}P < 0.001$, compared with control group (Student t test).

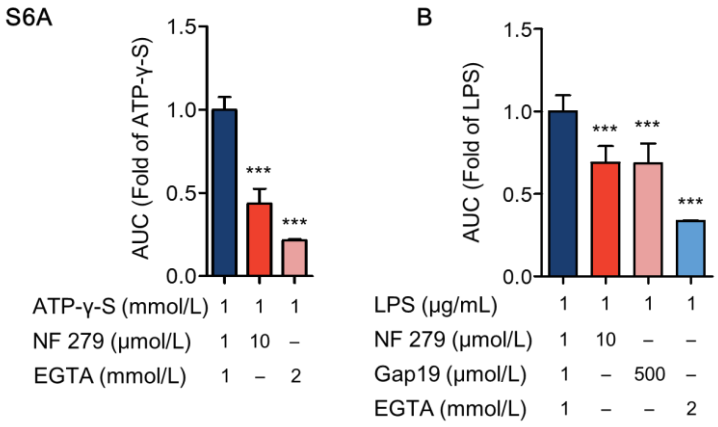


Fig. S6. The P2X1 receptor antagonist (10 μmol/L) was pre-loaded for 30 min. EGTA (2 mmol/L) was introduced to eliminate calcium in the medium. ATP-γ-S (1 mmol/L)-induced neutrophil calcium influx was assayed using LSCM with the fluorescence dye Fluo-4/AM. The quantitative data are shown. (B) Either the P2X1 receptor antagonist (10 μmol/L) or Cx43 inhibitor (500 μmol/L) was pre-incubated for 30 min. EGTA (2 mmol/L) was introduced to eliminate calcium in the medium. LPS-induced calcium influx into neutrophils was assayed using LSCM with the fluorescence dye Fluo-4/AM. Data are representative of six (B) or four (A) independent experiments. Mean and SD are presented. $^{***}P < 0.001$, compared with control group (ANOVA with Tukey's test).

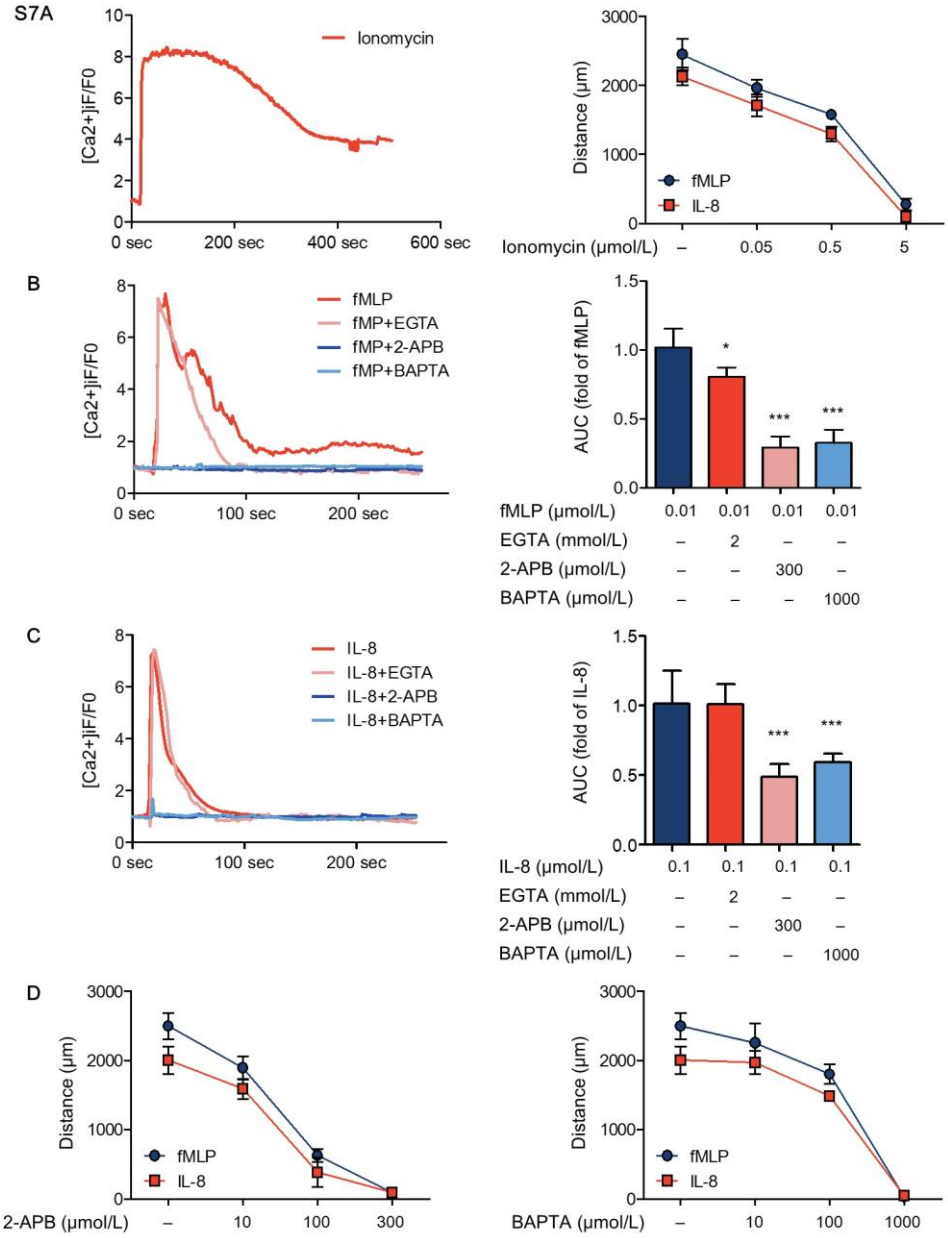


Fig. S7. (A) Left: Neutrophils were stimulated with 5 μmol/L ionomycin and calcium influx was detected by the fluorescence dye Fluo-4/AM. Right: Neutrophils were pre-treated with ionomycin for 10 min. Neutrophil chemotaxis toward either fMLP or IL-8 was assayed. (B, C) EGTA, 2-APB or BAPTA was pre-treated with neutrophil for 30 min. Neutrophils were stimulated with 0.01 μmol/L fMLP or 0.1 μmol/L IL-8 and calcium mobilization was detected by the fluorescence dye Fluo-4/AM. (D) 2-APB or BAPTA was pre-treated with neutrophil for 30 min. Neutrophil chemotaxis toward either fMLP or IL-8 was assayed. Data are representative of six (D) or four (A, B and

C) independent experiments. Mean and SD are presented. $^{*}P < 0.05$ and $^{***}P < 0.001$, compared with control group (ANOVA with Tukey's test).

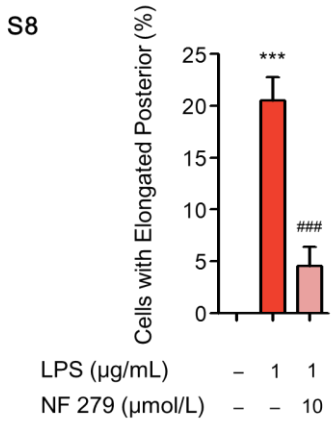


Fig. S8. The P2X1 receptor antagonist (10 μmol/L) was pre-incubated for 30 min, and 1 μg/mL LPS was used to stimulate the neutrophils. Morphological characteristics of neutrophil migration toward fMLP were observed by SCM. Percent of cells with elongated posterior ($n > 60$) was calculated. Data are representative of four independent experiments. Mean and SD are presented. $^{***}P < 0.001$, compared with control group; $^{###}P < 0.001$, compared with LPS group (ANOVA with Tukey's test).

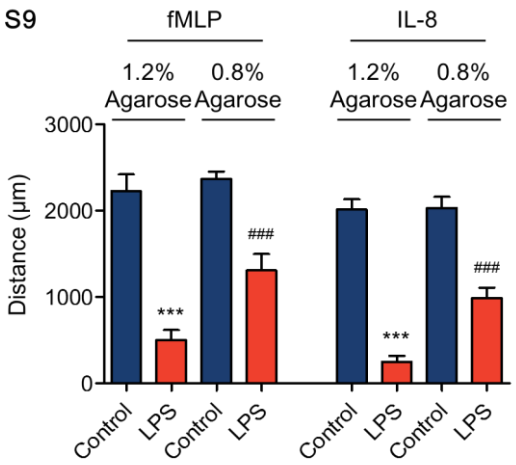


Fig. S9. Under agarose neutrophil chemotaxis assays were performed using normal agarose concentration (1.2%) or low agarose concentration (0.8%), respectively. Neutrophils were stimulated with 1 μg/mL LPS, and migration toward either fMLP or IL-8 was detected. Data are representative of six independent experiments. Mean and SD are presented. $^{***}P < 0.001$, compared with 1.2% agarose control group; $^{###}P < 0.001$, compared with 1.2% agarose LPS group (ANOVA with Tukey's test).

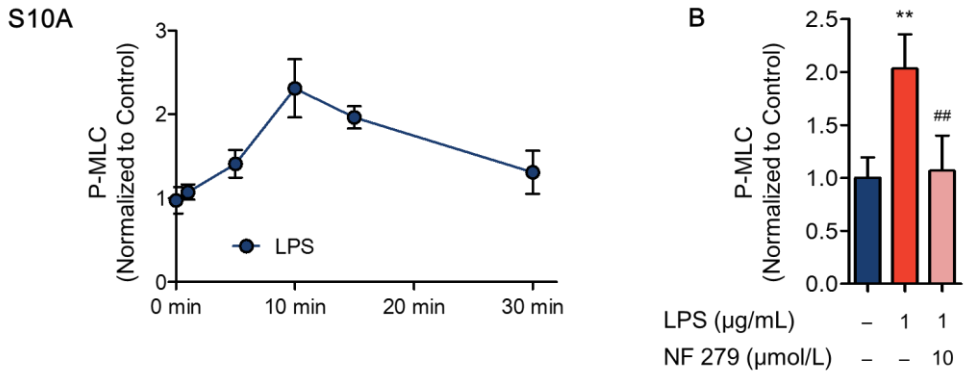


Fig. S10. Phosphorylation of MLC was determined by western blot. (A) LPS (1 μg/mL) was used to stimulate the neutrophils for indicated minutes. (B) The P2X1 receptor antagonist (10 μmol/L) was pre-incubated for 30 min, and 1 μg/mL LPS was used to stimulate the neutrophils for 10 min. Data are representative of three independent experiments. Mean and SD are presented. $^{**}P < 0.01$, compared with control group; $^{\#\#}P < 0.01$, compared with LPS group (ANOVA with Tukey's test).

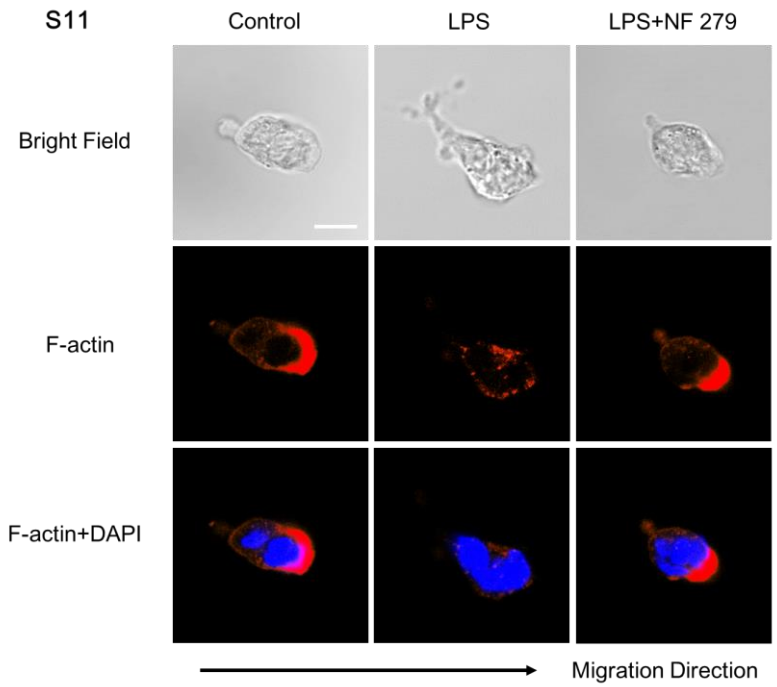


Fig. S11. The P2X1 receptor antagonist (10 μmol/L) was pre-incubated for 30 min, and 1 μg/mL LPS was used to stimulate the neutrophils. The distribution of F-actin (Red) during neutrophil migration toward fMLP was stained with CytoPainter Phalloidin-iFluor 647 and observed by LSCM. Nuclei were counterstained with DAPI (blue). Images are representative of 20 cells. Scale bar, 5 μm. Data are representative of four independent experiments.

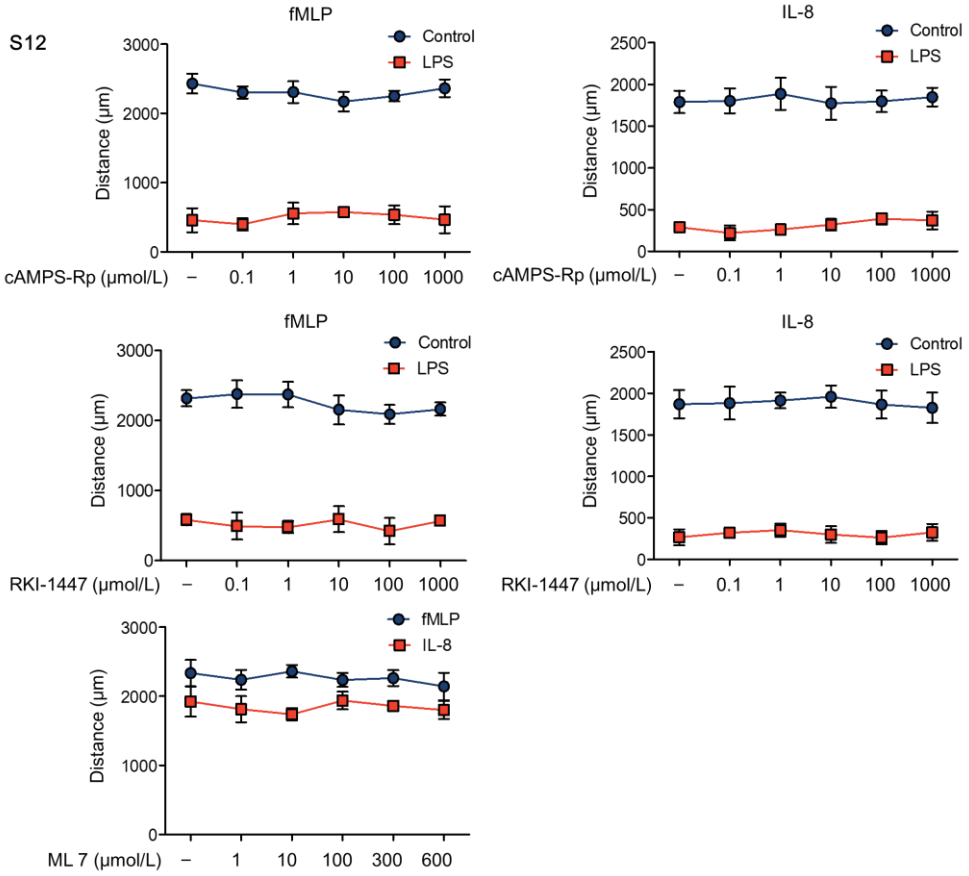


Fig. S12. cAMP inhibitor (cAMPS-Rp), ROCK inhibitor (RKI-1447) or MLCK inhibitor (ML 7) was pre-incubated with neutrophils for 30 min followed by 1 μg/mL LPS. Neutrophil migration toward either fMLP or IL-8 was detected. Data are representative of six independent experiments. Mean and SD are presented.

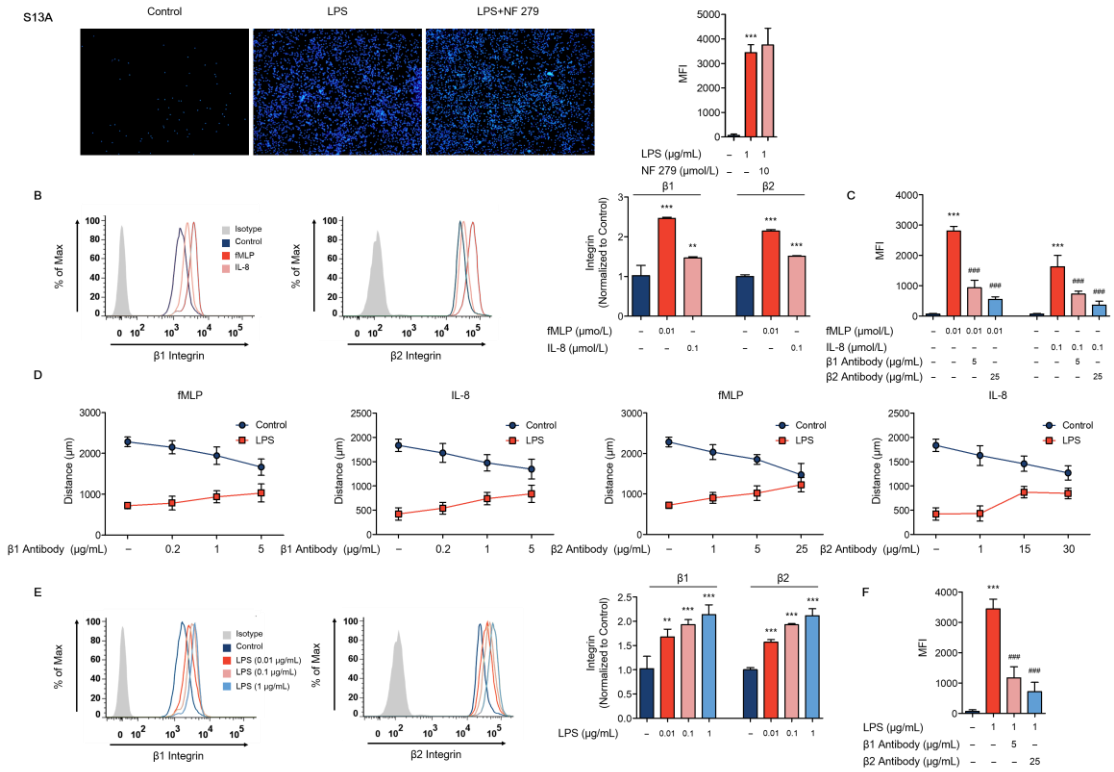


Fig. S13. (A) The P2X1 receptor antagonist (10 μmol/L) was pre-loaded for 30 min followed by 1 μg/mL LPS for 1 h. Unbound cells were washed and adhered cells were stained with DAPI (blue). Mean fluorescence intensity (MFI) was assessed using fluorescence microscope. (B) Neutrophils were stimulated with 0.01 μmol/L fMLP or 0.1 μmol/L IL-8 for 1 h. Membrane expression of β1 and β2 integrins was detected by FCM. (C) Blocking antibody of β1 or β2 integrin was pre-incubated for 1 h followed by 0.01 μmol/L fMLP or 0.1 μmol/L IL-8 for 1 h. Unbound cells were washed and adhered cells were stained with DAPI. MFI was assessed using fluorescence microscope. (D) Blocking antibody of β1 or β2 integrin was pre-incubated for 1 h. Neutrophil chemotaxis toward fMLP or IL-8 was assayed in the absence or presence of 1 μg/mL LPS. (E) Neutrophils were stimulated with 1 μg/mL LPS for 1 h. Membrane expression of β1 and β2 integrins was detected by FCM. (F) Blocking antibody of β1 or β2 integrin was pre-incubated for 1 h followed by 1 μg/mL LPS for 1 h. Unbound cells were washed and adhered cells were stained with DAPI. MFI was assessed using fluorescence microscope. Data are representative of six (A, C, D and F) or four (B, E) independent experiments. Mean and SD are presented. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with fMLP, IL-8 or LPS group

(ANOVA with Tukey's test).

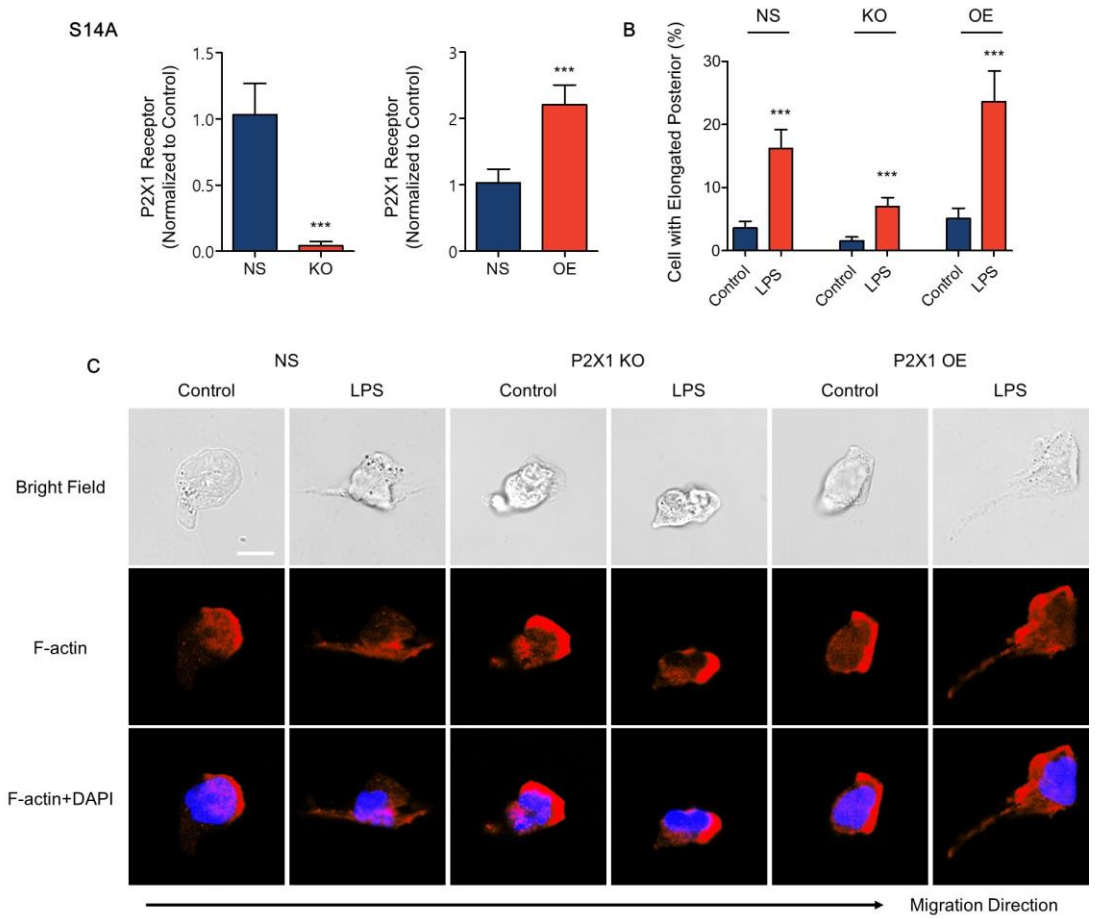


Fig. S14. (A) The P2X1 receptor was either knocked out (KO) or overexpressed (OE) in dHL-60 cells. The relevant empty lentivectors were used to induce control nonspecific (NS) expressing cells. Expression of the P2X1 receptor was detected by western blot. (B) 10 μg/mL LPS was used to stimulate dHL-60 cells. The morphological characteristics of dHL-60 cell migration toward fMLP were observed by SCM. Percent of cells with elongated posterior ($n > 60$) was calculated. (C) 10 μg/mL LPS was used to stimulate dHL-60 cells. The distribution of F-actin (Red) during neutrophil migration toward fMLP was stained with CytoPainter Phalloidin-iFluor 647 and observed by LSCM. Nuclei were counterstained with DAPI (blue). Images are representative of 20 cells. Scale bar, 5 μm. Data are representative of four independent experiments. Mean and SD are presented. $^{***}P < 0.001$, compared with control group (Student t test).

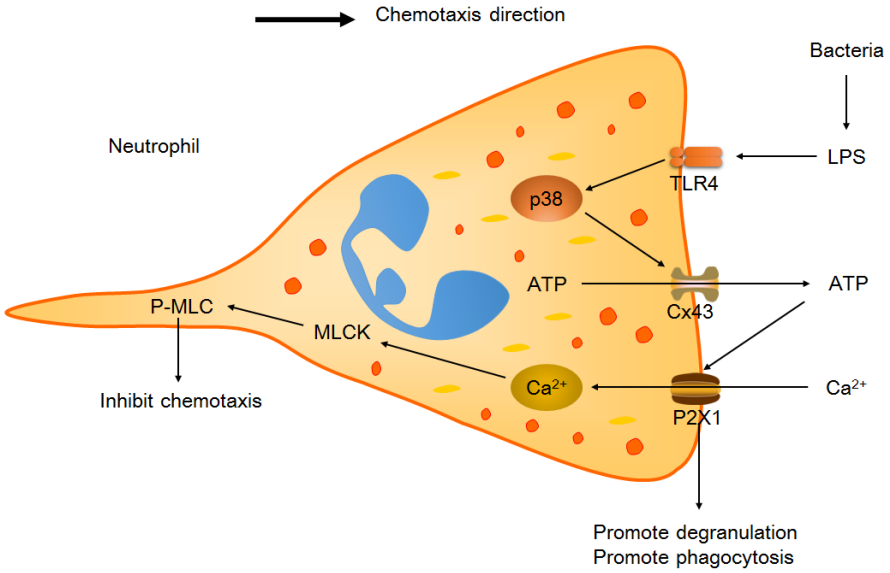
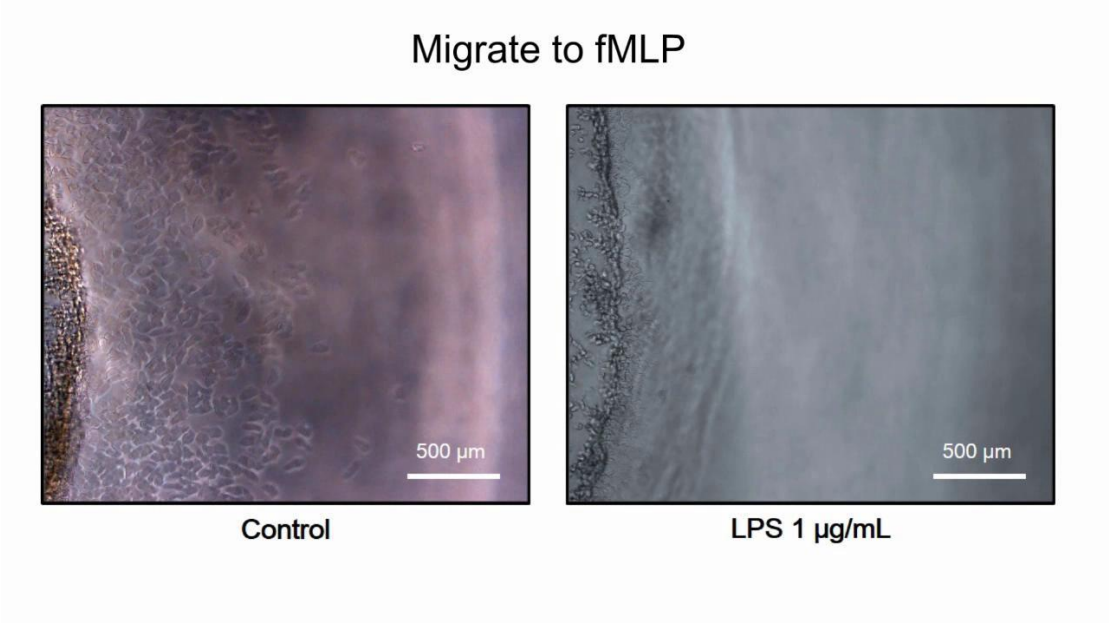
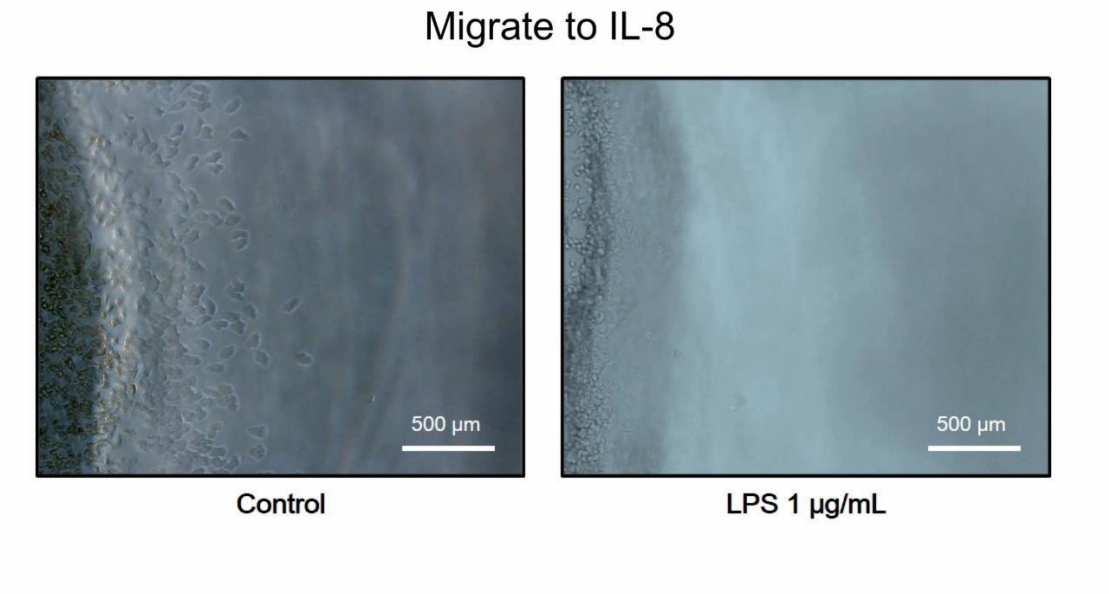


Fig. S15. Schematic illustration of the mechanism of the inhibitory effect of Endotoxin on neutrophil chemotaxis. LPS-induced autocrine ATP signaling inhibits neutrophil chemotaxis by enhancing MLC phosphorylation, which provides insight into the role of autocrine ATP signaling in the host innate immune response.



Movie S1：

Neutrophils were treated with 1μg/mL LPS. Chemotaxis distances to fMLP (0.1 μmol/L) were observed for 50 min using a microscope. Scale bar, 500 μm.



Movie S2:

Neutrophils were treated with 1 μg/mL LPS. Chemotaxis distances to IL-8 (1 μmol/L) were observed for 50 min using a microscope. Scale bar, 500 μm.