Immune Netw. 2023 Jun;23(3):e23
https://doi.org/10.4110/in.2023.23.e23
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Original Article

Check for updates

Extracellular Acidification Augments NLRP3-Mediated Inflammasome Signaling in Macrophages

Byeong Jun Chae [1,2], **Kyung-Seo Lee** [1,2], **Inhwa Hwang** [1,2], **Je-Wook Yu** [1,2,*]

[1]Department of Microbiology and Immunology, Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, Seoul 03722, Korea
[2]Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 03722, Korea

OPEN ACCESS

Received: Sep 27, 2022
Revised: Jan 9, 2023
Accepted: Jan 12, 2023
Published online: Feb 14, 2023

***Correspondence to**
Je-Wook Yu
Department of Microbiology and Immunology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Email: jewookyu@yuhs.ac

ORCID iDs
Byeong Jun Chae
https://orcid.org/0000-0003-4162-7814
Kyung-Seo Lee
https://orcid.org/0000-0001-7823-0381
Inhwa Hwang
https://orcid.org/0000-0001-5235-3519
Je-Wook Yu
https://orcid.org/0000-0001-5943-4071

Conflicts of Interest
The authors declare no potential conflicts of interest.

ABSTRACT

Inflammation is a series of host defense processes in response to microbial infection and tissue injury. Inflammatory processes frequently cause extracellular acidification in the inflamed region through increased glycolysis and lactate secretion. Therefore, the immune cells infiltrating the inflamed region encounter an acidic microenvironment. Extracellular acidosis can modulate the innate immune response of macrophages; however, its role for inflammasome signaling still remains elusive. In the present study, we demonstrated that macrophages exposed to an acidic microenvironment exhibited enhanced caspase-1 processing and IL-1β secretion compared with those under physiological pH. Moreover, exposure to an acidic pH increased the ability of macrophages to assemble the NLR family pyrin domain containing 3 (NLRP3) inflammasome in response to an NLRP3 agonist. This acidosis-mediated augmentation of NLRP3 inflammasome activation occurred in bone marrow-derived macrophages but not in bone marrow-derived neutrophils. Notably, exposure to an acidic environment caused a reduction in the intracellular pH of macrophages but not neutrophils. Concordantly, macrophages, but not neutrophils, exhibited NLRP3 agonist-mediated translocation of chloride intracellular channel protein 1 (CLIC1) into their plasma membranes under an acidic microenvironment. Collectively, our results demonstrate that extracellular acidosis during inflammation can increase the sensitivity of NLRP3 inflammasome formation and activation in a CLIC1-dependent manner. Thus, CLIC1 may be a potential therapeutic target for NLRP3 inflammasome-mediated pathological conditions.

Keywords: Acidosis; NLR family, pyrin domain-containing 3 protein (NLRP3); Inflammasome; Chloride channels; Inflammation

INTRODUCTION

Inflammation is an immune cell-mediated host defense response against microbial infection, tissue damage, or cellular stress. The inflammatory response ultimately neutralizes the threat and restores homeostasis (1). Despite its essential role in infection and repair, inflammation is often accompanied by increased acidity in the affected region. The reduction in extracellular pH is caused by the extensive infiltration and activation of

IMMUNE NETWORK

Abbreviations

AIM2, absent in melanoma 2; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester; BMDMs, bone marrow-derived macrophages; BMDNs, bone marrow-derived neutrophils; CLIC1, chloride intracellular channel protein 1; NLRP3, NLR family pyrin domain containing 3; PI, propidium iodide.

Author Contributions

Conceptualization: Chae BJ, Yu JW; Data curation: Chae BJ; Formal analysis: Lee KS, Hwang I; Funding acquisition: Yu JW; Investigation: Chae BJ; Methodology: Lee KS, Hwang I; Supervision: Yu JW; Writing - original draft: Chae BJ, Lee KS; Writing - review & editing: Chae BJ, Lee KS, Hwang I, Yu JW.

immune cells in the inflamed region, consequently forming a hypoxic microenvironment. The glycolytic metabolism rate in inflammatory cells is enhanced with the increased demand for energy and oxygen. Consequently, the secreted lactic acid accumulates in the extracellular space, leading to an acidic environment, a condition called acidosis (2-4). Although body fluids possess efficient pH-buffering mechanisms, local or systemic pH fluctuations frequently occur under inflammatory conditions. Tissue acidosis has been observed in patients with infections, ischemia, or autoimmune diseases (5-8). This acidic microenvironment may modulate the degree of the immune response by the surrounding immune cells (9); however, the processes involved in acidosis-mediated immune modulation require additional clarification.

During inflammation, the recruited and infiltrated immune cells in the inflamed region encounter this abnormal extracellular acidosis, which modulates the potential of the immune response. Immune cells can sense alterations in the extracellular pH via proton-recognizing surface receptors, including G protein-coupled receptors, acid-sensing ion channels, and transient receptor potential channel vanilloid subfamily 1 (9,10). This pH-sensitive receptor signaling might affect the immune cells in a proton receptor-dependent manner. Numerous contradictory studies have demonstrated that extracellular acidosis exerts immune stimulatory or inhibitory effects on macrophages and neutrophils, which are pivotal in propagating inflammation and its resolution, respectively. Mildly acidic conditions can enhance the production of TNF-α and the expression of the inducible form of nitric oxide synthase in peritoneal macrophages (11). In contrast, tumor-associated macrophages exhibit M1 to M2 phenotypic switching upon exposure to acidosis (12). Likewise, neutrophils undergo morphological and immunological changes in acidic environments, characterized by delayed apoptosis, reduced phagocytosis, and decreased ability to produce neutrophil extracellular traps (13,14). These findings emphasize the significance of acidosis-induced immunomodulation of inflammatory cells.

The inflammasome is a caspase-1-activating intracellular protein complex that induces the maturation and release of critical proinflammatory cytokines IL-1β and IL-18 (15,16). Inflammasome formation is initiated by several pattern recognition receptors, among which NLR family pyrin domain containing 3 (NLRP3) can be activated by diverse abnormal pathogen- or host-derived factors, such as microbial components or endogenous metabolites and stress signals, respectively (17,18). An acidic environment acts as an inflammasome-stimulating danger signal, and this effect may be dampened by inhibiting potassium channels (19). However, the mechanisms underlying the direct effect of an acidic pH on inflammasome assembly and activation remain poorly understood.

Potassium efflux is crucial for NLRP3 inflammasome assembly and activation; however, the underlying mechanism remains elusive (20-22). In this context, chloride efflux was proposed as a downstream event of potassium efflux and an essential step in activating the NLRP3 inflammasome (23-26). Chloride intracellular channels (CLICs) are responsible for chloride efflux by NLRP3 agonists due to a translocation of CLICs into the plasma membrane (23). CLIC1 is one of the Cl^- channels for Cl^- efflux that contributes to NLRP3 activation and translocates to the plasma membrane in a pH-dependent manner (27,28). Considering that both potassium and chloride ion levels play a central role in inflammasome assembly, we hypothesized that acidosis might affect the activity of ion channels and subsequent inflammasome activity. Moreover, as circulating neutrophils are recruited rapidly to the sites of inflammation, their inflammatory role, along with that of macrophages, is critical for

https://doi.org/10.4110/in.2023.23.e23

regulating and resolving inflammation (29,30). However, the precise immunomodulatory effects of acidic environments on the inflammasome activity of neutrophils require further investigation. In the present study, we investigated whether extracellular acidification can modulate NLRP3 inflammasome activity in macrophages and neutrophils.

MATERIALS AND METHODS

Mice

C57BL/6 (Orient Bio, Seongnam, Korea) and *Nlrp3*$^{-/-}$ (Jackson Laboratory, Bar Harbor, ME, USA) mice were bred at Yonsei University College of Medicine. All mice were maintained under specific pathogen-free conditions, and 8–12-week male mice were used for the experiments. Protocols for the animal experiments were approved by the Institutional Ethical Committee, Yonsei University College of Medicine. All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

Reagents and Abs

LPS, ATP, nigericin, poly dA:dT, IAA94, and Duolink *In Situ* PLA kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ac-YVAD-cmk was obtained from Bachem (Torrance, CA, USA). The dye, 2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM), MitoSOX, MQAE (N-(Ethoxycarbonylmethyl)-6-Methoxyquinolinium Bromide) and DAPI were obtained from Invitrogen (Waltham, MA, USA). Flagellin (FLA-ST Ultrapure) was obtained from InvivoGen (San Diego, CA, USA). Alexa fluor 488™ Phalloidin was purchased from ThermoFisher Scientific. Abs were purchased for the detection of NLRP3 (Adipogen, San Diego, CA, USA), ASC (Adipogen), IL-1β (R&D systems, Minneapolis, MN, USA), caspase-1 (Adipogen), β-actin (Santa-Cruz Biotechnology, Dallas, TX, USA), and CLIC1 (Proteintech, Rosemont, IL, USA) Na^+-K^+ ATPase (Cell Signaling Technology, Danvers, MA, USA), and α-tubulin (Santa-Cruz Biotechnology).

Cell culture

Mouse bone marrow cells were isolated from the femurs and differentiated into bone marrow-derived macrophages (BMDMs) by culturing in L929-conditioned DMEM for 5–7 days. Until use, BMDMs were maintained in L929-conditioned DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Bone marrow-derived neutrophils (BMDNs) were isolated using an EasySep™ mouse neutrophil enrichment kit (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's protocols (31). Isolated BMDNs were cultured in RPMI1640 supplemented with 10% FBS, 1% antibiotics, and 4 μg/ml aprotinin. Immortalized NLRP3-GFP BMDMs and ASC-GFP THP-1 cells were a gift from Dr. E.S. Alnemri (Thomas Jefferson University, Philadelphia, PA, USA). ASC-GFP THP-1 cells were maintained in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate (Gibco), 0.15% sodium bicarbonate, 10 mM HEPES, 0.05 mM β-mercaptoethanol (Gibco), and 1% antibiotics. ASC-GFP THP1 cells were stimulated with PMA (0.4 μM, 2 h), and grown for 18–24 h before treatments. Mouse peritoneal macrophages were collected from the peritoneal lavage fluid four days after intraperitoneal injection with 3% thioglycollate medium (1.5 ml). After removing non-adherent cells, peritoneal macrophages were cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics. To mimic extracellular acidosis, Opti-MEM or DMEM was titrated to desire pH of 6.0–8.0 using 5N sodium hydroxide and hydrochloric acid. Then, cells were incubated in pH-adjusted Opti-MEM or DMEM for 10 min before further treatment.

https://doi.org/10.4110/in.2023.23.e23

Immunoblot analysis

Cells were lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were separated by SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes. Cell culture supernatants were precipitated by methanol/chloroform as described previously before immunoblotting (32). All blot images are representative of at least three independent experiments and have been cropped for presentation.

mRNA quantification

Total RNA was extracted using Trizol reagent (Invitrogen) and reverse transcribed using a Primscript RP Master Mix (Takara, Tokyo, Japan), according to the manufacturer's protocols. Quantitative real-time PCR was performed using SYBR premix Ex Taq (Takara). The following primers were used (mouse): *Il-1β* (5′-GCC CAT CCT CTG TGA CTC AT-3′ and 5′-AGG CCA CAG GTA TTT TGT CG-3′), *Il-6* (5′-AGT TGC CTT GGG ACT GA-3′ and 5′-TCC ACG ATT TCC CAG AGA AC-3′), *Nlrp3* (5′-TAC GGC CGT CTA CGT CTT CT-3′ and 5′-CGC AGA TCA CAC TCC TCA AA-3′), β-actin (5′-CCT TCC TGG GCA TGG AGT CCT G-3′ and 5′-GGA GCA ATG ATC TTG ATC TTC-3′).

Inflammasome activation assay

To activate the NLRP3 inflammasome, cells were primed with LPS (0.25 μg/ml, 3 h) for transcription of NLRP3 and pro-IL-1β, followed by treatment with ATP (1–5 mM, 0–30 min) or nigericin (1–3 μM, 0–30 min). Cells were transfected with poly dA:dT (0.7 μg/ml, 3 h) and flagellin (300 μg/ml, 3 h) using Lipofectamine 2000 (2 μg/ml) or N-(2,3-dioleoyloxy-1-propyl) trimethylammonium methyl sulfate (DoTAP, 3 μg/ml) to activate the absent in melanoma 2 (AIM2) and NLRC4 inflammasome, respectively. Inflammasome activation was determined by the presence of cleaved caspase-1 and IL-1β in immunoblots from culture supernatants and by quantifying extracellular IL-1β in culture supernatants using ELISA (R&D Systems). To evaluate cell death, cells were stained with propidium iodide (PI, 1 μg/ml), and the uptake of PI was assayed using flow cytometry (FACSVerse, BD).

Inflammasome assembly assay

To measure the oligomerization of NLRP3, speck-like aggregates of NLRP3-GFP were assessed in NLRP3-GFP-expressing BMDMs using confocal microscopy. To determine the oligomerization of ASC, the number of ASC-GFP specks in ASC-GFP-expressing THP-1 cells was counted in several fields using fluorescence microscopy. To visualize the molecular interaction of NLRP3 with ASC, a proximity-ligation assay was performed using a Duolink *In Situ* Red starter kit (Sigma) with anti-ASC or anti-NLRP3 Abs according to the manufacturer's protocol. The relative proximity ligation signals (PL signals/DAPI signals) were quantified using Image J software and calculated as a relative fold-change compared with that in untreated controls.

Determination of intracellular ion concentration

Cells were treated in a 100-mm petri dish and lysed with 7.5% HNO_3. Intracellular K^+, Na^+, and Ca^{2+} concentrations were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES) using an OPTIMA 8300 ICP spectrometer (Perkin Elmer). To assess the intracellular Cl^- level, cells were suspended in cell culture grade water and lysed by pipetting and centrifuged at 10,000 *g* for 5 min. Then, supernatants (50 μl) were mixed with MQAE (1 mM, 50 μl) and the fluorescence was measured at excitation 350 nm and emission 460 nm.

https://doi.org/10.4110/in.2023.23.e23

Determination of intracellular pH

Cells were stained with 1 μM BCECF-AM and maintained at 37°C for 30 min. Stained cells were washed and incubated with pH-adjusted Opti-MEM or calibration solution for 10 min at 37°C. BCECF-AM fluorescence was detected with FITC using flow cytometry (FACSVerse, BD). The pH calibration solutions were used to establish a standard curve and were composed of 145 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 20 μM nigericin, and the pH was adjusted to 6.0, 6.5, 7.0, 7.5, or 8.0 using 11 N HCl and 5 N NaOH.

Measurement of mitochondrial ROS production

Following appropriate treatments, cells were suspended in Hank's balanced salt solution and stained with MitoSOX (2.5 μM) at 37°C for 30 min. The fluorescence of the cells were monitored and analyzed by flow cytometry (FACSVerse, BD).

Subcellular fractionation

Cells were lysed in a buffer containing 20 mM HEPES, 40 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, and protease inhibitors by passing through 21 gauge needle. The lysates were centrifuged at 800 *g* for 10 min to pellet the nucleus and unbroken cells. The supernatants were centrifuged at 5,000 *g* for 10 min, and the resultant supernatants were used for cytosolic fraction and the pellets were used as membrane-containing fraction.

Statistical analysis

All values are expressed as the mean ± SEM of individual samples. Data were analyzed using one-way ANOVA followed by Dunnett's post hoc test or two-way ANOVA with the Bonferroni post hoc test. Statistical significance was set at $p \leq 0.05$. Analyses were performed using GraphPad Prism.

RESULTS

Extracellular acidification enhances the inflammasome response and pyroptosis in macrophages in response to NLRP3-activating stimulation

To investigate the effect of extracellular pH on NLRP3 inflammasome activation, BMDMs were first primed with LPS and incubated in pH-adjusted media (pH 6.0–8.0) for 10 min and then stimulated with ATP for 30 min. Inflammasome activation was determined by measuring IL-1β secretion using ELISA and detecting the presence of active caspase-1 (p20) in the culture supernatant. At physiological pH (pH 7.5), LPS/ATP stimulation caused a robust extracellular release of IL-1β from BMDMs (**Fig. 1A**). Notably, acidic pH (6.0 and 6.5) significantly increased LPS/ATP-induced IL-1β secretion in BMDMs compared with that at pH 7.5 (**Fig. 1A**). However, exposure to acidic pH alone failed to induce IL-1β secretion in BMDMs (**Fig. 1A**). Similarly, the use of nigericin as an NLRP3 activator was accompanied by a robust IL-1β release at pH 6.4 than at pH 7.4 (**Fig. 1B**), indicating that extracellular acidosis can augment NLRP3 inflammasome activation. Macrophages exposed to acidic pH exhibited enhanced caspase-1 processing in response to LPS/ATP and LPS/nigericin stimulation compared with that at physiological pH (**Fig. 1C**). This acidic microenvironment-mediated augmentation of NLRP3 activation was also observed in peritoneal macrophages (**Supplementary Fig. 1A**).

As inflammasome activation is followed by pyroptosis, a form of inflammatory cell death (33), we examined LPS/ATP-induced pyroptosis of macrophages exposed to acidic or neutral

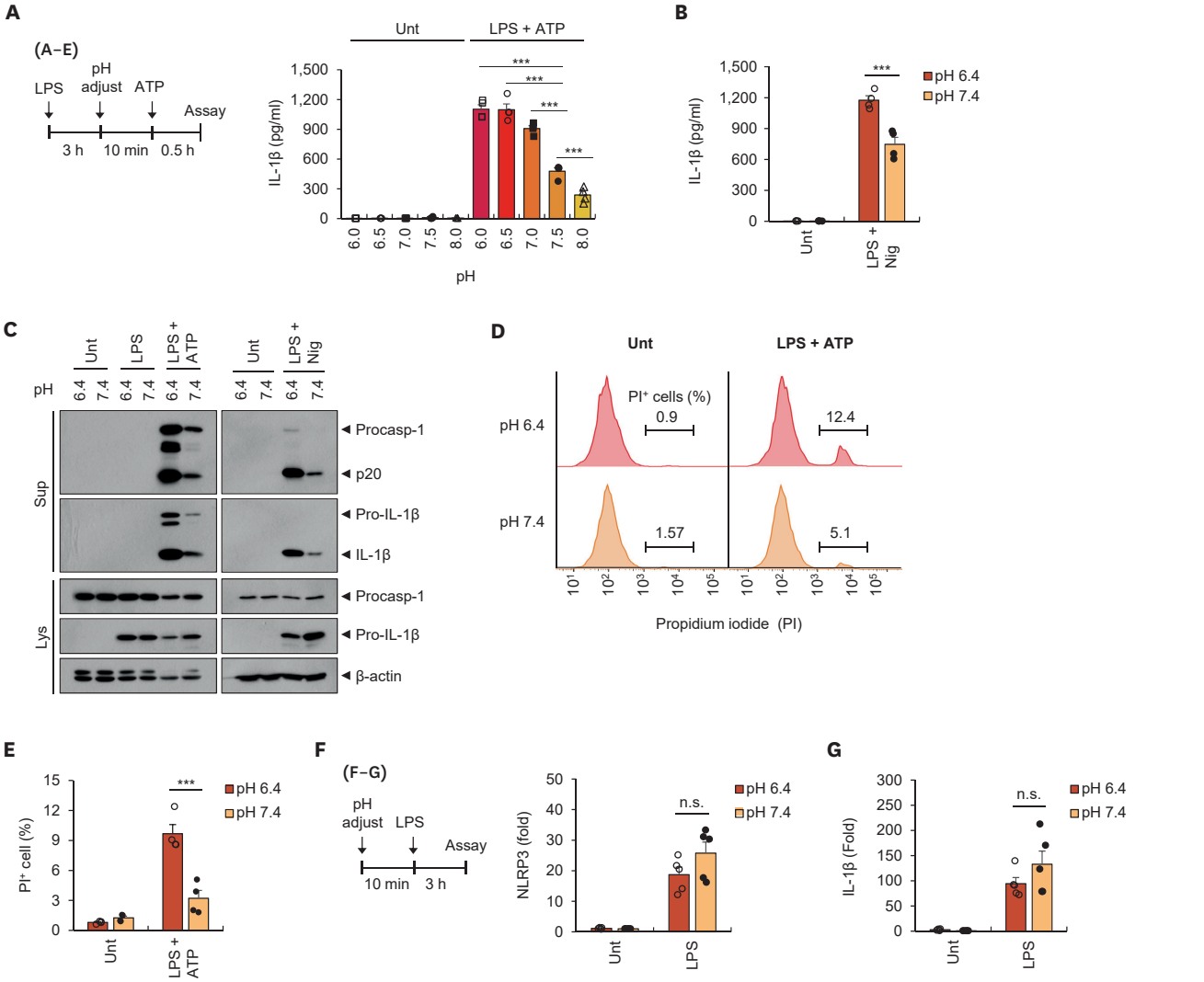

Figure 1. Acidic pH enhances inflammasome activation in macrophages in response to NLRP3 agonists. (A–E) BMDMs were treated with LPS (0.25 μg/ml, 3 h) and incubated in pH-adjusted medium (pH 6.0–8.0) for 10 min. Cells were then stimulated with NLRP3 agonists ATP (3 mM, 30 min) or nigericin (Nig, 3 μM, 30 min). (A) Quantification of IL-1β in the supernatant of BMDMs primed with LPS, incubated in pH 6.0–8.0 medium followed by ATP treatment (n=4). (B) Quantification of IL-1β in BMDMs treated with LPS, incubated at pH 6.4 or pH 7.4, followed by nigericin treatment (n=4). (C) Immunoblots of BMDMs primed with LPS, incubated at pH 6.4 or 7.4, followed by ATP or nigericin treatment. Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated Abs. (D) BMDMs were primed with LPS and incubated at pH 6.4 or 7.4, followed by ATP treatment. Cells were then stained with propidium iodide (PI) and analyzed by flow cytometry. Data are depicted as a representative histogram (D) and quantification of PI-positive BMDMs (E) (n=4). (F–G) Quantification of *Nlrp3* (F) and *Il1b* (G) mRNA levels in BMDMs exposed to pH 6.4 or pH 7.4 medium for 10 min, followed by LPS (0.25 μg/ml for 3 h) treatment (n=5). Data represent the mean ± SEM. Asterisks indicate significant differences between sample means (***p<0.001, n.s., not significant).

pH by measuring PI uptake. Consistent with inflammasome activation, LPS/ATP treatment enhanced pyroptotic cell death at pH 6.4, indicated by the presence of more PI-positive cells than at pH 7.4 (**Fig. 1D and E**). Then, we evaluated LPS-induced transcription in BMDMs exposed to acidic or neutral pH to provide molecular insights into acidosis-mediated enhanced inflammasome activation. The levels of LPS-induced transcription of *Nlrp3*, *Il1b*, and *Il6* mRNA were not different in macrophages under acidic and neutral environments (**Fig. 1F, G**, and **Supplementary Fig. 1B**). These observations indicate that extracellular acidosis can increase the intensity of NLRP3 inflammasome signaling, but not of the Toll-like receptor-mediated response, in macrophages.

https://doi.org/10.4110/in.2023.23.e23

Extracellular acidification specifically enhances the NLRP3-mediated inflammasome response in macrophages

Next, we examined the environmental pH-dependent response of other inflammasome signaling mediated by AIM2 and NLRC4. We first primed BMDMs with LPS and incubated the cells in pH-adjusted media (pH 6.4 or 7.4). Cells were then transfected with poly dA:dT and flagellin to activate the AIM2 and NLRC4 inflammasome, respectively. No significant differences in IL-1β release were observed following AIM2 or NLRC4 activation between macrophages exposed to acidic and neutral pH-adjusted media (**Fig. 2A**). Consistently, AIM2- and NLRC4-mediated processing of caspase-1 and IL-1β was similar in BMDMs regardless of the environmental pH (**Fig. 2B**).

Collectively, these results indicate that extracellular acidosis augments NLRP3-mediated inflammasome activation but does not affect TLR and other inflammasome signaling

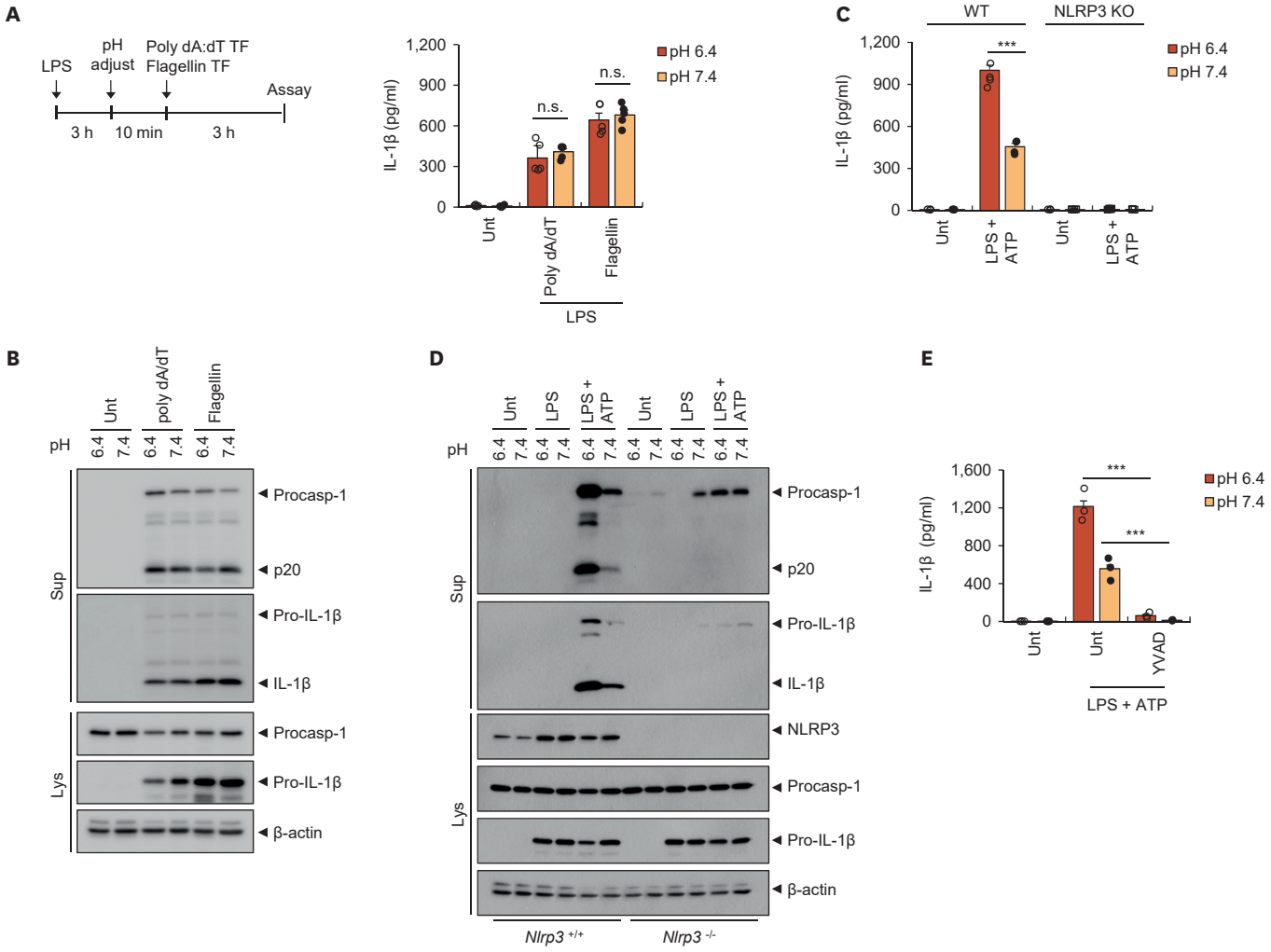

Figure 2. Acidic pH augments the NLRP3-dependent inflammasome, but not the AIM2- or NLRC4-dependent inflammasome. (A) Quantification of IL-1β in the supernatant of BMDMs treated with LPS, exposed to pH 6.4 or pH 7.4 medium, and transfected with poly dA:dT (0.7 μg/ml for 3 h) or flagellin (300 μg/ml for 3 h) (n=5). (B) Immunoblots of BMDMs treated as in (A). (C) Quantification of IL-1β in the supernatant of wild-type (WT) and *Nlrp3*-deficient BMDMs primed with LPS and incubated in pH-adjusted medium, followed by ATP stimulation (3 mM for 30 min) (n=4). (D) Immunoblots of WT and *Nlrp3*-deficient BMDMs stimulated as in (C). (E) Quantification of IL-1β in the supernatants of BMDMs primed with LPS and incubated in the pH-adjusted medium in the presence of YVAD (20 μM for 15 min), followed by ATP (3 mM for 30 min) treatment (n=3). (B, D) Culture supernatants (Sup) or cellular lysates (Lys) were immunoblotted with the indicated Abs. Data represent the mean ± SEM. Asterisks indicate significant differences between sample means (***p<0.001, n.s., not significant).

https://doi.org/10.4110/in.2023.23.e23

pathways in macrophages. We, therefore, examined whether acidification can mediate NLRP3-independent caspase-1 and IL-1β activation in macrophages upon LPS/ATP stimulation by evaluating inflammasome activation in *Nlrp3*-deficient macrophages. Unlike wild-type BMDMs, LPS/ATP-induced IL-1β secretion (**Fig. 2C**) and caspase-1 processing (**Fig. 2D**) were abolished in *Nlrp3*-knockout BMDMs. Furthermore, the caspase-1-specific inhibitor YVAD completely inhibited LPS/ATP-induced IL-1β release in BMDMs regardless of exposure to acidic or neutral medium (**Fig. 2E**). These observations suggest that extracellular acidification specifically increases the responsiveness of NLRP3-caspase-1 inflammasome signaling.

Extracellular acidification increases the sensitivity of NLRP3 for detecting agonists

To examine whether extracellular acidification affects the sensitivity of NLRP3 activation, LPS-primed BMDMs were incubated in a pH-adjusted medium and then stimulated with ATP in a time-dependent manner. Extracellular acidic pH caused more rapid and pronounced IL-1β release from macrophages than physiological pH (**Fig. 3A**). Furthermore, more IL-1β was secreted by BMDMs under acidic pH than by macrophages under neutral pH following stimulation with a lower concentration of ATP (1.2 mM) or nigericin (1 μM) (**Fig. 3B**). Consistently, BMDMs stimulated by lower concentrations of ATP or nigericin at pH 6.4 processed more caspase-1 and IL-1β than cells at pH 7.4 (**Fig. 3C**). These findings demonstrate that extracellular acidification renders the NLRP3 inflammasome more susceptible to environmental stress.

We next examined whether extracellular acidosis can increase the sensitivity of NLRP3 inflammasome assembly in macrophages. We measured NLRP3 oligomerization in NLRP3-GFP stably-expressing macrophages. Compared with the neutral pH, an acidic environment significantly increased NLRP3 speck-like aggregates in macrophages upon LPS/ATP stimulation (**Fig. 3D and E**). Given that the active form of NLRP3 might associate with ASC, a proximity ligation assay was used to measure the protein interaction between NLRP3 and ASC in BMDMs cultured in a pH-adjusted medium. BMDMs exhibited a significantly increased PL signal at pH 6.4 compared with those at pH 7.4 in response to LPS/ATP stimulation (**Fig. 3F** and **Supplementary Fig. 2**). Then, the effect of extracellular acidification on the ASC oligomerization using ASC-GFP-expressing THP-1 cells was examined. In accord with the above observations, LPS-nigericin-induced ASC speck formation was significantly increased at pH 6.4 compared with that at physiological pH (**Fig. 3G** and **Supplementary Fig. 3**). These results indicate that extracellular acidification increases the danger-sensing potential and sensitivity of NLRP3 in macrophages.

Extracellular acidification reduces the intracellular pH of macrophages but not that of neutrophils

Next, we determined the effect of extracellular acidosis on the NLRP3-activating potential of neutrophils, a major cell type infiltrating into inflamed regions (30,34). We prepared neutrophils from mouse bone marrow cells. BMDNs were primed with LPS and incubated in a pH-adjusted medium for 10 min before stimulating with ATP for 30 min. Unlike BMDMs, no significant differences between acidic and neutral pH were observed in caspase-1 processing (**Fig. 4A**) and IL-1β release (**Fig. 4B and C**) in BMDNs upon LPS/ATP stimulation.

To provide molecular insights into these cell-specific pH-dependent inflammasome responses, we measured the fluctuation of the intracellular pH of BMDMs and BMDNs under different environmental pHs. The intracellular pH was measured by BCECF-AM dye

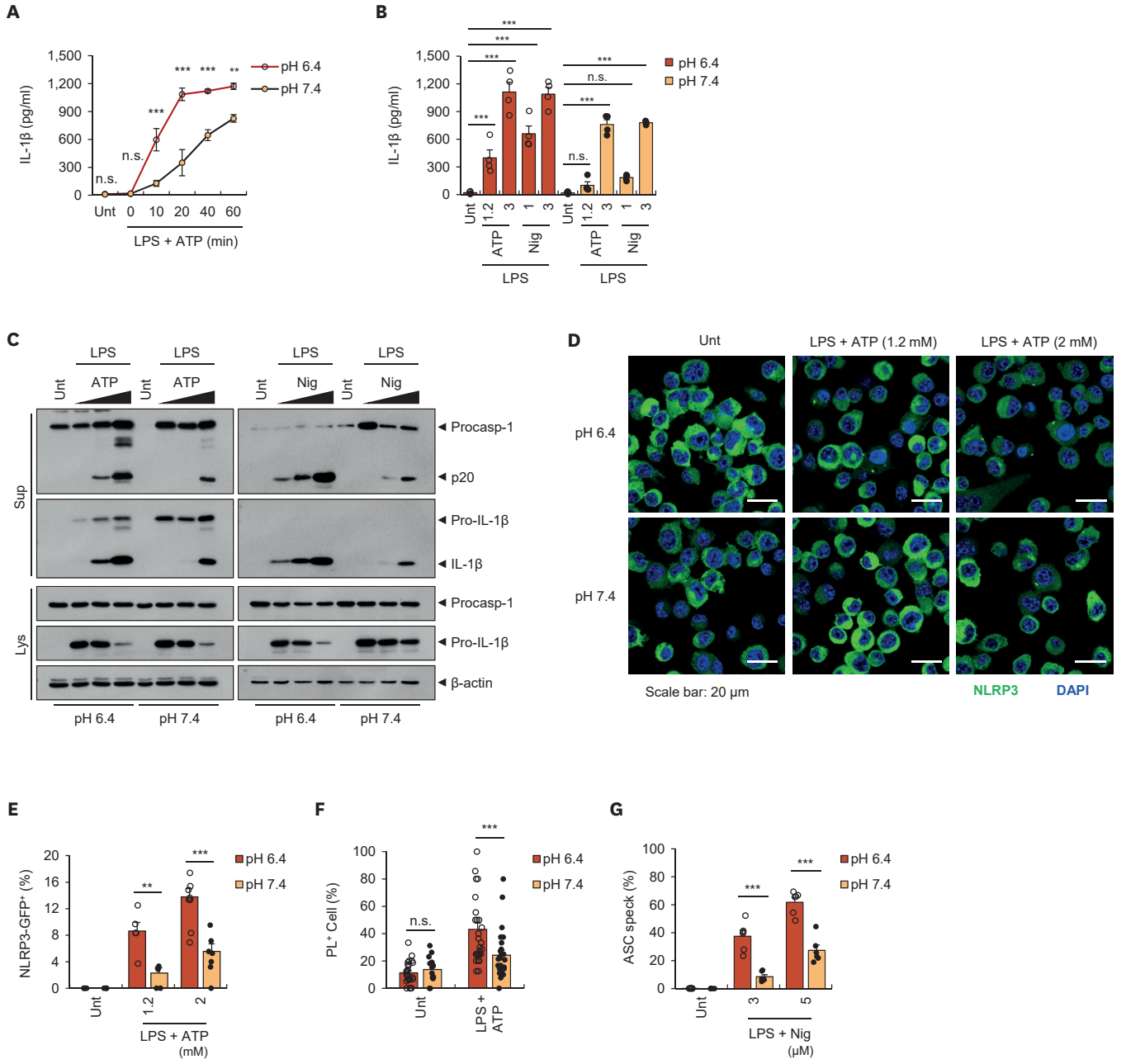

Figure 3. Acidic pH increases the ability of NLRP3 to sense agonists and assemble the inflammasome complex. (A) Quantification of IL-1β in the supernatant of LPS-primed BMDMs incubated in pH-adjusted medium and stimulated with ATP (3 mM) for the indicated times (n=3). (B) Quantification of IL-1β in the supernatant of LPS-primed BMDMs incubated in pH-adjusted medium and then stimulated with ATP (1.2 or 3 mM for 40 min) or nigericin (1 or 3 μM for 40 min) (n=4). (C) Immunoblots of culture supernatants and cellular lysates from LPS-primed BMDMs incubated in the pH-adjusted medium and then stimulated with ATP (0.5, 1, or 2 mM for 30 min) or nigericin (0.75, 1.5, or 3 μM for 30 min). (D) Representative immunofluorescence images of NLRP3-GFP-expressing BMDMs treated with LPS, incubated in pH-adjusted medium, then stimulated with ATP (1.2 or 2 mM for 30 min). Nuclei were stained with DAPI (blue). Scale bars are 20 μm. (E) Quantification of NLRP3-GFP speck-like aggregates of BMDMs treated as in (D) (n=4–7). (F) Quantification of proximity ligation (PL) signal-positive BMDMs primed with LPS, incubated in pH-adjusted medium, then stimulated with ATP (2.5 mM for 30 min). PL signals represent the molecular association of NLRP3 and ASC (n=26). (G) Quantification of speck-containing THP-1-ASC-GFP cells treated with LPS, incubated in pH-adjusted medium, and then stimulated with nigericin (3 or 5 μM, 30 min) (n=6). Data are expressed as the mean ± SEM. Asterisks indicate significant differences (**p<0.01, ***p<0.001, n.s., not significant).

https://doi.org/10.4110/in.2023.23.e23

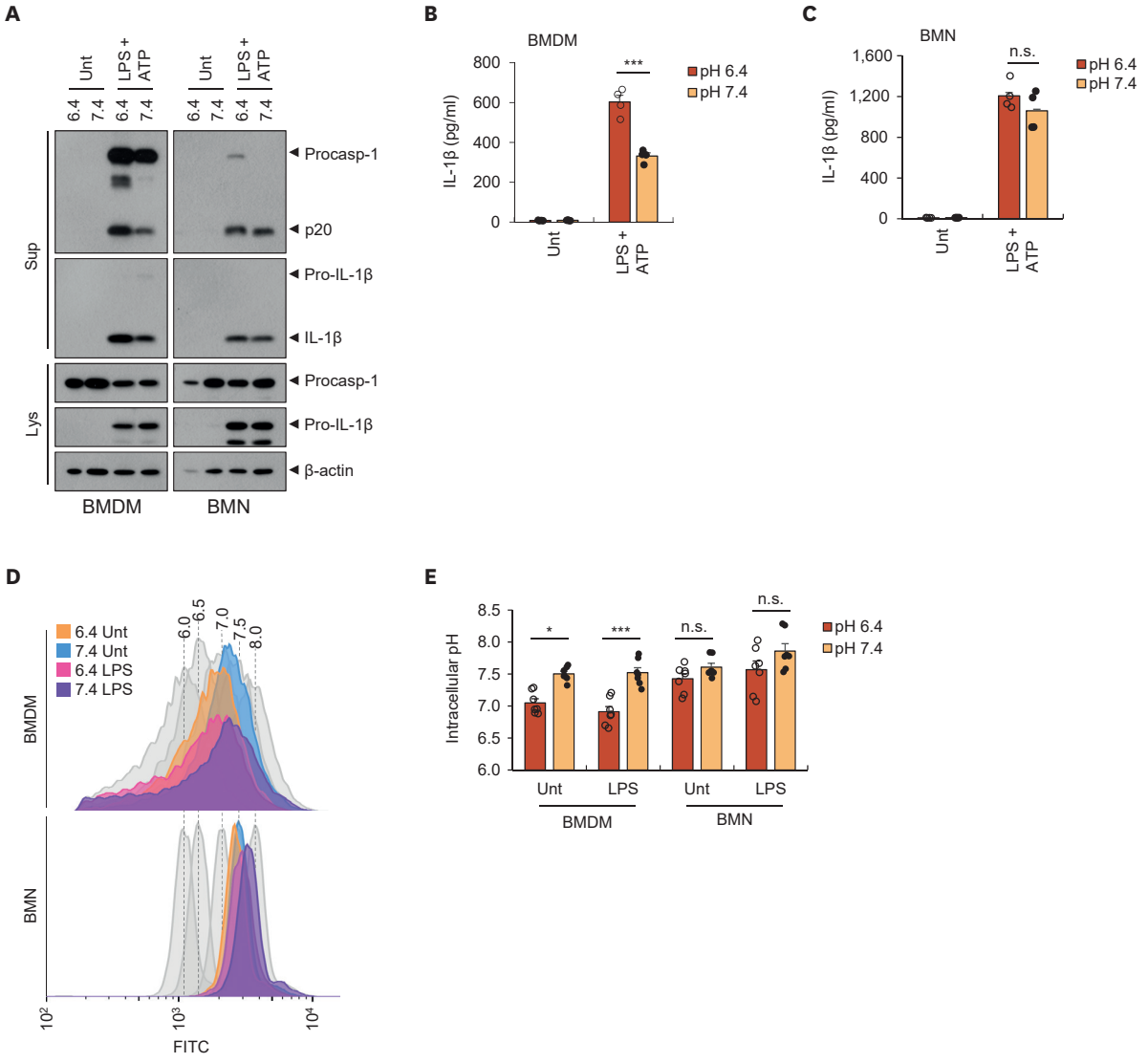

Figure 4. Acidic pH does not affect NLRP3 inflammasome activation in neutrophils. (A) Immunoblots of BMDMs or BMDNs primed with LPS, incubated in the pH-adjusted medium, and stimulated with ATP (3 mM for 30 min). (B–C) Quantification of IL-1β in the supernatant of BMDMs (B) or BMDNs (C) treated as in (A) (n=4). (D–E) Quantification of the intracellular pH in BMDMs or BMDNs treated with LPS and stained with BCECF-AM, followed by incubation in the pH-adjusted medium for 10 min. The mean fluorescence intensity of BCECF-AM was determined by flow cytometry analysis. Data are displayed as a representative histogram (D), and intracellular pH was calculated using the calibration curve (E) (n=7). Asterisks denote statistical significance (*p<0.05, ***p<0.001, n.s., not significant).

and quantified based on calibration buffers (pH 6.0–8.0) (**Fig. 4D**). LPS stimulation did not influence the internal pH in both cell types (**Fig. 4E**). At physiological pH, intracellular pH was neutral (around pH 7.5) in BMDMs and BMDNs (**Fig. 4E**). Notably, exposure to acidic pH rapidly decreased intracellular pH in macrophages; however, no significant differences in intracellular pH were observed in neutrophils upon exposure to acidic pH (**Fig. 4E**). These observations indicate that acidosis decreases intracellular pH in macrophages, but not in neutrophils, which could determine the inflammasome potential in macrophages.

Extracellular acidification facilitates chloride efflux in macrophages upon stimulation with NLRP3 agonist

Intracellular ion concentration is critical for NLRP3 activation (21,22,35,36); therefore, we determined cytosolic ion concentration ($[K^+]_{in}$, $[Na^+]_{in}$, $[Ca^{2+}]_{in}$ and $[Cl^-]_{in}$) in BMDMs under

https://doi.org/10.4110/in.2023.23.e23

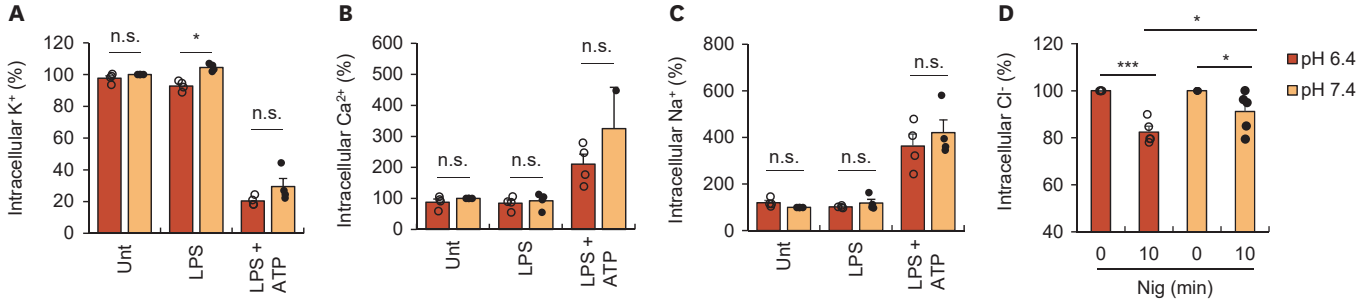

Figure 5. Acidic pH does not alter intracellular ion levels in BMDMs upon NLRP3-activating stimulation. (A-C) The intracellular concentration of K^+ (A), Na^+ (B), and Ca^{2+} (C) in BMDMs treated with LPS, incubated in the pH-adjusted medium, and then stimulated with ATP (3 mM for 10 min). Ion concentration was measured by ICP-OES, and each group was qualitatively compared with untreated BMDMs exposed to pH 7.4 (n=4). (D) Determination of intracellular $[Cl^-]$ in BMDMs primed with LPS, incubated in pH-adjusted medium, then stimulated with nigericin (3 μM, 0 or 10 min). $[Cl^-]$ was assessed by MQAE fluorescence (n=5). Asterisks denote statistical significance (*p<0.05, ***p<0.001, n.s., not significant).

inflammasome-activating condition in pH-adjusted medium. ATP stimulation caused a rapid decrease in internal $[K^+]$ in macrophages; however, no significant difference was observed in the levels of internal $[K^+]$ between macrophages cultured in acidic and neutral pH (**Fig. 5A**). Unlike $[K^+]$, ATP stimulation led to an increase in intracellular sodium $[Na^+]$ and calcium $[Ca^{2+}]$ ion levels in macrophages (**Fig. 5B and C**). However, this ATP-driven increase in $[Na^+]$ and $[Ca^{2+}]$ was not significantly altered by the pH of the microenvironment (**Fig. 5B and C**). Then, we checked intracellular $[Cl^-]$ in the presence or absence of NLRP3 agonist nigericin. Consequently, nigericin treatment caused a decrease in internal $[Cl^-]$ in macrophages (**Fig. 5D**). Of notice, this nigericin-induced Cl^- efflux was significantly stronger at pH 6.4 than that at pH 7.4. These observations suggest that intracellular $[Cl^-]$ might be a critical factor for the enhanced inflammasome sensitivity in acidification.

Extracellular acidification induces the translocation of chloride efflux channel CLIC1 into the plasma membrane in macrophages

Next, we examined whether an acidified intracellular pH can affect the transport of CLIC1, one of the Cl^- channels for Cl^- efflux. At the resting state, the majority of CLIC1 was detected in the cytosol of BMDMs at both acidic and neutral pH (**Fig. 6A**). Interestingly, LPS/nigericin stimulation caused an increase in the plasma membrane-associated CLIC1 at acidic pH, but not at physiological pH (**Fig. 6A and B**). An acidic environment significantly increased CLIC1-F-actin colocalization upon LPS/nigericin stimulation in BMDMs compared with the increase at neutral pH (**Fig. 6B**). Consistently, LPS/ATP stimulation led to an increased localization of CLIC1 in the membrane fraction at pH 6.4, but did not induce at pH 7.4 (**Fig. 6C**). However, the total amount of CLIC1 was not altered by acidosis (**Fig. 6D**). Unlike BMDMs, acidosis-triggered CLIC1 transport was not apparent in BMDNs upon LPS/nigericin stimulation (**Fig. 6E, F** and **Supplementary Fig. 4**). These findings indicate that cytosolic acidification facilitates NLRP3 agonist-induced CLIC1 translocation into the plasma membrane in macrophages.

As previous study demonstrated that mitochondrial ROS production is critical for the translocation of CLIC1 (23), we assessed mitochondrial ROS level in macrophages at acidic or neutral pH. LPS/ATP stimulation caused a slight increase in mitochondrial ROS production in BMDMs, but there was no significant difference in mitochondrial ROS level between acidic and neutral medium (**Supplementary Fig. 5**). Then, to examine whether CLIC1-increased Cl^- efflux sensitizes the responsiveness of NLRP3, we pretreated LPS-primed BMDMs with the Cl^- channel inhibitor IAA-94 in the presence of the pH-adjusted medium. Cells were then stimulated with ATP for 30 min. In macrophages, the amount of LPS/ATP-induced IL-1β

https://doi.org/10.4110/in.2023.23.e23

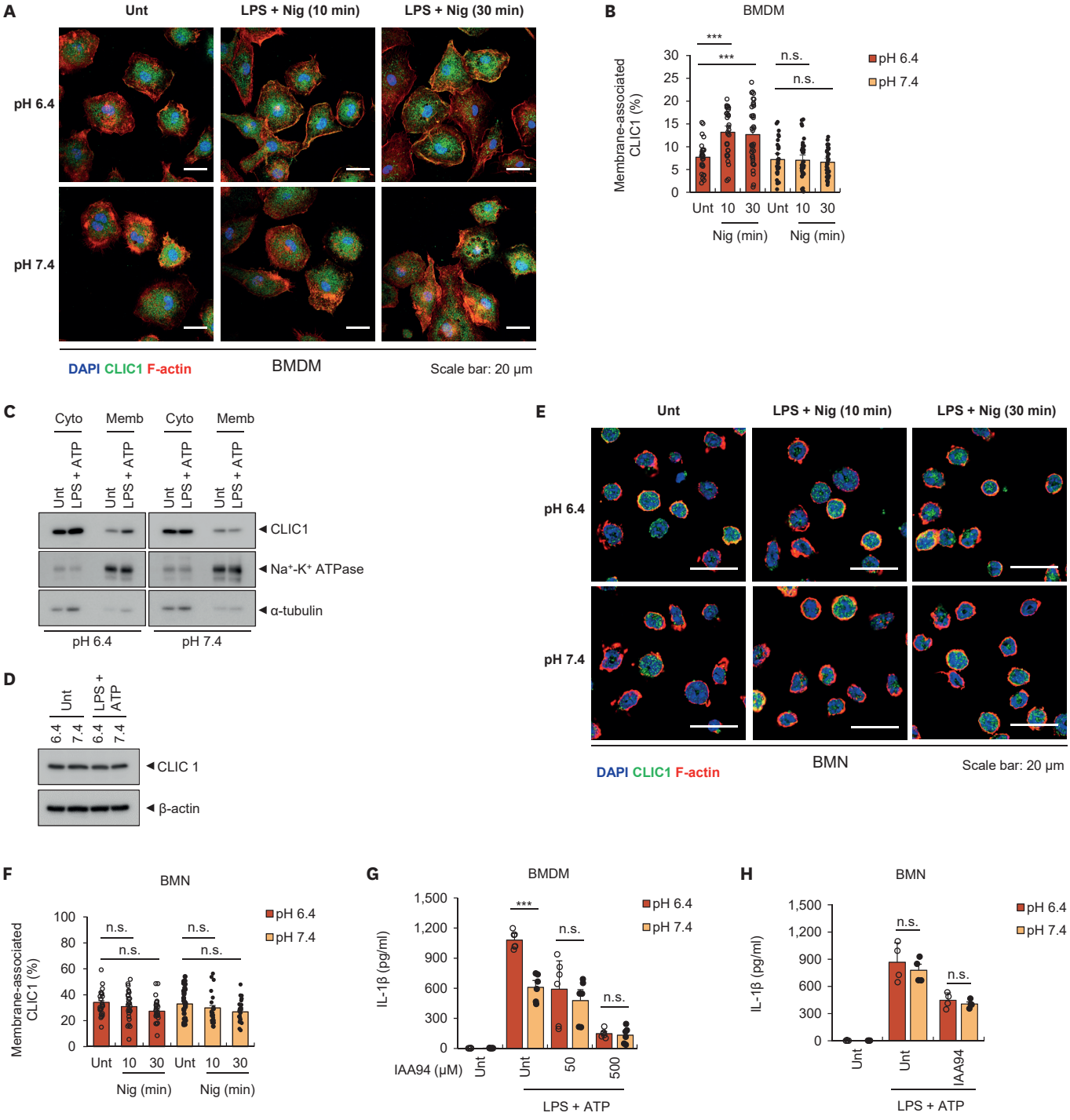

Figure 6. Acidic pH facilitates NLRP3 agonist-induced translocation of CLIC1 to the plasma membrane. (A, B) Determination of CLIC1 association with the plasma membrane in LPS-primed BMDMs incubated in the pH-adjusted medium in the presence of YVAD (20 μM for 15 min), followed by nigericin (3 μM) treatment. YVAD was treated to block caspase-1 activation and pyroptosis of inflammasome-active cells. Cells were stained with anti-CLIC1 (green) and F-actin (red). Nuclei were stained with DAPI (blue). (C) Immunoblots of cytosolic (cyto) and membrane (memb) fraction of BMDMs primed with LPS, exposed to pH-adjusted medium, and then treated with ATP (3 mM, 15 min). (D) Immunoblots of total cell lysates of BMDMs primed with LPS, exposed to pH-adjusted medium, and then treated with ATP. (E, F) Determination of CLIC1 association with the plasma membrane in LPS-primed BMDNs incubated in the pH-adjusted medium in the presence of YVAD (20 μM for 15 min), followed by nigericin (3 μM) treatment. Data are representative immunofluorescence images of BMDMs (A) and BMDNs (E) or as the relative intensity of membrane-associated CLIC1 per total CLIC1 intensity in BMDMs (B) (n=28–40) and BMDNs (n=38–47) (F). (G) Quantification of IL-1β in the supernatant of LPS-primed BMDMs incubated in the pH-adjusted medium in the presence of IAA94 (50 and 500 μM, 10 min), followed by ATP treatment (5 mM, 30 min) (n=6). (H) Quantification of IL-β in the supernatant of LPS-primed BMDNs exposed to pH-adjusted medium in the presence of IAA94 (100 μM, 10 min), followed by ATP treatment (5 mM, 30 min) (n=4). Asterisks denote statistical significance (***p<0.001, n.s., not significant).

https://doi.org/10.4110/in.2023.23.e23

secretion was higher at pH 6.4 than at pH 7.4 (**Fig. 6G**). Notably, treatment with IAA-94 (50 μM) significantly reduced IL-1β secretion at pH 6.4 compared with that in untreated cells. However, the IL-1β secretion was not significantly different at either pH in the presence of IAA-94 (**Fig. 6G**). As acidosis did not affect NLRP3 activation in neutrophils, BMDNs exhibited no significant difference in inflammasome response to IAA-94 at either pH (**Fig. 6H**). These data demonstrate that intracellular acidification facilitates plasma membrane translocation of CLIC1 in the presence of NLRP3 activators. The increased CLIC1 translocation to the plasma membrane of macrophages under an acidic environment might cause excessive chloride ion efflux, leading to the observed increase in NLRP3 activation in macrophages.

DISCUSSION

Extracellular acidosis can independently trigger a pH-dependent secretion of IL-1β via activation of the NLRP3 inflammasome in macrophages (19). However, the molecular mechanism underlying the direct contribution of acidification on NLRP3 activation remains poorly understood. In the present study, we demonstrated that acute exposure to an acidic environment did not induce NLRP3 inflammasome activation, indicating that acidic pH is not a direct danger signal. Conversely, extracellular acidification facilitates intracellular pH reduction and chloride channel translocation into the plasma membrane, leading to an increase in the sensitivity of NLRP3 activation in macrophages.

Our data revealed that acidic pH not only augments NLRP3 inflammasome activation but also enhances the capability of NLRP3 to sense its agonists. A low ATP concentration, which is tolerable at physiological pH, could promote NLRP3-ASC interactions in LPS-primed macrophages under acidic conditions. Mechanistically, an acidic extracellular environment triggers a reduction in the intracellular pH level in macrophages but not in neutrophils. Consequently, this cytosolic acidification increases the translocation of chloride channel CLIC1 into plasma membranes and enhances the sensitivity of NLRP3 activation. A previous study showed that NLRP3 agonist-induced mitochondrial ROS production is critical for the translocation of CLICs to the plasma membrane (23). However, nigericin-induced mitochondrial ROS production was similar in macrophages under acidic and neutral environment in our result. Moreover, to exclude a possibility that the translocation of CLIC1 is the effect of NLRP3 inflammasome activation, we conducted experiments in the presence of YVAD, a caspase-1-specific inhibitor. The specific mechanisms by which acidic pH enhances the rate of CLIC1 translocation require further investigation.

Previous studies showed that other CLICs, including CLIC4 and CLIC5, than CLIC1 are implicated in the activation of NLRP3 inflammasome (23,28). Although CLIC4 and CLIC5 were upregulated by LPS priming, the expression of CLIC1 remained consistent in macrophages even after LPS priming (23). Of note, we focused on CLIC1 in this study to exclude a priming effect of LPS, thus, it will be intriguing to test whether other CLICs can contribute to the enhanced inflammasome sensitivity of extracellular acidification.

As neutrophils are major infiltrating cells from the circulation into the injured or inflamed tissue (26), we conducted experiments to determine if neutrophils are affected by the inflammatory microenvironment. However, unlike macrophages, an acidic environment did not alter the responsiveness of NLRP3 signaling in neutrophils. Instead, neutrophils were resistant to extracellular acidosis-mediated intracellular acidification, which is critical

for the increased responsiveness of the NLRP3 inflammasome. This may be attributed to the abundant secretory granules in neutrophils, which constantly maintain their pH level at 5.5–6.0 by the continuous influx of hydrogen ions into the granules (37,38). A drop in the extracellular pH is accompanied by a subsequent reduction in the intracellular pH, which is rapidly restored by the uptake of hydrogen ions into the secretory granules. This immediate pH-controlling mechanism might help abolish the acidosis-mediated sensitization of the inflammasome in neutrophils. Therefore, although both macrophages and neutrophils are the primary cell types that react against inflammatory insults, only macrophages exhibit a pH-linked NLRP3-activating ability in the area of inflammation.

Collectively, we have provided evidence that extracellular acidosis triggers a rapid reduction in the intracellular pH of macrophages, consequently rendering cytosolic CLIC1 more susceptible to translocation to the plasma membrane by NLRP3 agonists. The increased CLIC1 in the plasma membrane might lead to the sensitization of NLRP3 inflammasome activation, possibly via increased chloride ion efflux. Thus, our data suggest that macrophages might be the core regulatory cells that recognize and respond to pH changes in inflammatory microenvironments. Our results also suggest that CLIC1 may be a potential therapeutic target for NLRP3 inflammasome-mediated pathological conditions.

ACKNOWLEDGEMENTS

This work was supported by the National Research Foundation of Korea Grant, which is funded by the Korean Government (2020R1A2B5B02001823, 2020R1A4A1019009, 2022R1A2C2007569).

SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Extracellular acidification increases LPS/ATP-induced inflammasome activation. (A) Quantification of IL-1β in culture supernatants from peritoneal macrophages treated with LPS and incubated in pH 6.4 or pH 7.4 medium, followed by ATP (1 mM, 30 min) treatment (n=5). (B) Quantification of IL-6 mRNA levels in BMDMs exposed to pH 6.4 or pH 7.4 medium, followed by LPS (0.25 μg/ml, 3h) treatment (n = 4). Asterisks indicate significant differences (***p<0.001, n.s., not significant).

Click here to view

Supplementary Figure 2

Extracellular acidification increases NLRP3-ASC interaction in BMDMs in response to LPS/ATP stimulation. Representative immunofluorescence images of proximity ligation (PL) analysis of NLRP3 and ASC in BMDMs primed with LPS, incubated at pH 6.4 or 7.4, followed by ATP (2.5 mM, 30 min) treatment. PL signals (red) represent the association of NLRP3 and ASC. Nuclei were stained with DAPI (blue). Scale bars, 20 μm.

Click here to view

Supplementary Figure 3

Extracellular acidification increases ASC oligomerization in THP-1 macrophages in response to LPS/nigericin stimulation. Representative fluorescence images of THP-1-ASC-GFP cells treated with LPS, incubated with pH-adjusted medium, and then stimulated with nigericin (3 or 5 μM, 30 min). Speck-like GFP aggregates represent the oligomerized ASC. Scale bars, 100 μm.

Click here to view

Supplementary Figure 4

Effect of acidosis on CLIC1 translocation in bone marrow-derived neutrophils. Representative immunofluorescence images of bone marrow-derived neutrophils after stained with anti-CLIC1 antibody. Cells were treated with LPS, incubated in the pH-adjusted medium in the presence of YVAD (20 μM for 15 min) followed by nigericin (3 μM) treatment. Scale bars, 20 μm.

Click here to view

Supplementary Figure 5

Extracellular acidosis does not affect LPS/ATP-induced mitochondrial ROS production in BMDMs. (A, B) Quantification of mitochondrial ROS in *Nlrp3*-deficient BMDMs primed with LPS, exposed to pH-adjusted medium, followed by ATP (3 mM, 30 min) stimulation. Cells were then stained with MitoSOX and analyzed by flow cytometry. Data were represented as representative histogram (A) or quantification of MitoSOX-positive cells (n=7) (B). Asterisks indicate significant differences ($^{*}p<0.05$, $^{**}p<0.01$, n.s., not significant).

Click here to view

REFERENCES

1. Rathinam VA, Chan FK. Inflammasome, inflammation, and tissue homeostasis. *Trends Mol Med* 2018;24:304-318.
PUBMED | CROSSREF
2. Dubos RJ. The micro-environment of inflammation or Metchnikoff revisited. *Lancet* 1955;269:1-5.
PUBMED | CROSSREF
3. Edlow DW, Sheldon WH. The pH of inflammatory exudates. *Proc Soc Exp Biol Med* 1971;137:1328-1332.
PUBMED | CROSSREF
4. Menkin V, Warner CR. Studies on inflammation: Xiii. Carbohydrate metabolism, local acidosis, and the cytological picture in inflammation. *Am J Pathol* 1937;13:25-44.1.
PUBMED
5. Ricciardolo FL, Gaston B, Hunt J. Acid stress in the pathology of asthma. *J Allergy Clin Immunol* 2004;113:610-619.
PUBMED | CROSSREF
6. Simmen HP, Battaglia H, Giovanoli P, Blaser J. Analysis of pH, pO_2 and pCO_2 in drainage fluid allows for rapid detection of infectious complications during the follow-up period after abdominal surgery. *Infection* 1994;22:386-389.
PUBMED | CROSSREF
7. Nedergaard M, Kraig RP, Tanabe J, Pulsinelli WA. Dynamics of interstitial and intracellular pH in evolving brain infarct. *Am J Physiol* 1991;260:R581-R588.
PUBMED
8. Treuhaft PS, MCCarty DJ. Synovial fluid pH, lactate, oxygen and carbon dioxide partial pressure in various joint diseases. *Arthritis Rheum* 1971;14:475-484.
PUBMED | CROSSREF

9. Erra Díaz F, Dantas E, Geffner J. Unravelling the interplay between extracellular acidosis and immune cells. *Mediators Inflamm* 2018;2018:1218297.
PUBMED | CROSSREF
10. Casey JR, Grinstein S, Orlowski J. Sensors and regulators of intracellular pH. *Nat Rev Mol Cell Biol* 2010;11:50-61.
PUBMED | CROSSREF
11. Bellocq A, Suberville S, Philippe C, Bertrand F, Perez J, Fouqueray B, Cherqui G, Baud L. Low environmental pH is responsible for the induction of nitric-oxide synthase in macrophages. Evidence for involvement of nuclear factor-kappaB activation. *J Biol Chem* 1998;273:5086-5092.
PUBMED | CROSSREF
12. El-Kenawi AE, Ibrahim-Hashim AA, Luddy KA, Pilon-Thomas SA, Gatenby RA, Gillies RJ. Abstract 3213: extracellular acidosis alters polarization of macrophages. *Cancer Res* 2015;75 Suppl 15:3213-3213.
CROSSREF
13. Gabig TG, Bearman SI, Babior BM. Effects of oxygen tension and pH on the respiratory burst of human neutrophils. *Blood* 1979;53:1133-1139.
PUBMED | CROSSREF
14. Faucher N, Naccache PH. Relationship between pH, sodium, and shape changes in chemotactic-factor-stimulated human neutrophils. *J Cell Physiol* 1987;132:483-491.
PUBMED | CROSSREF
15. Schroder K, Tschopp J. The inflammasomes. *Cell* 2010;140:821-832.
PUBMED | CROSSREF
16. Park CH, Lee HS, Kwak MS, Shin JS. Inflammasome-dependent peroxiredoxin 2 secretion induces the classical complement pathway activation. *Immune Netw* 2021;21:e36.
PUBMED | CROSSREF
17. Yu JW, Lee MS. Mitochondria and the NLRP3 inflammasome: physiological and pathological relevance. *Arch Pharm Res* 2016;39:1503-1518.
PUBMED | CROSSREF
18. Swanson KV, Deng M, Ting JP. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nat Rev Immunol* 2019;19:477-489.
PUBMED | CROSSREF
19. Rajamäki K, Nordström T, Nurmi K, Åkerman KE, Kovanen PT, Öörni K, Eklund KK. Extracellular acidosis is a novel danger signal alerting innate immunity via the NLRP3 inflammasome. *J Biol Chem* 2013;288:13410-13419.
PUBMED | CROSSREF
20. Xu Z, Chen ZM, Wu X, Zhang L, Cao Y, Zhou P. Distinct molecular mechanisms underlying potassium efflux for NLRP3 inflammasome activation. *Front Immunol* 2020;11:609441.
PUBMED | CROSSREF
21. Katsnelson MA, Rucker LG, Russo HM, Dubyak GR. K+ efflux agonists induce NLRP3 inflammasome activation independently of Ca2+ signaling. *J Immunol* 2015;194:3937-3952.
PUBMED | CROSSREF
22. Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, Núñez G. K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 2013;38:1142-1153.
PUBMED | CROSSREF
23. Tang T, Lang X, Xu C, Wang X, Gong T, Yang Y, Cui J, Bai L, Wang J, Jiang W, et al. CLICs-dependent chloride efflux is an essential and proximal upstream event for NLRP3 inflammasome activation. *Nat Commun* 2017;8:202.
PUBMED | CROSSREF
24. Daniels MJ, Rivers-Auty J, Schilling T, Spencer NG, Watremez W, Fasolino V, Booth SJ, White CS, Baldwin AG, Freeman S, et al. Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models. *Nat Commun* 2016;7:12504.
PUBMED | CROSSREF
25. Green JP, Yu S, Martín-Sánchez F, Pelegrin P, Lopez-Castejon G, Lawrence CB, Brough D. Chloride regulates dynamic NLRP3-dependent ASC oligomerization and inflammasome priming. *Proc Natl Acad Sci U S A* 2018;115:E9371-E9380.
PUBMED | CROSSREF
26. Verhoef PA, Kertesy SB, Lundberg K, Kahlenberg JM, Dubyak GR. Inhibitory effects of chloride on the activation of caspase-1, IL-1beta secretion, and cytolysis by the P2X7 receptor. *J Immunol* 2005;175:7623-7634.
PUBMED | CROSSREF

27. Warton K, Tonini R, Fairlie WD, Matthews JM, Valenzuela SM, Qiu MR, Wu WM, Pankhurst S, Bauskin AR, Harrop SJ, et al. Recombinant CLIC1 (NCC27) assembles in lipid bilayers via a pH-dependent two-state process to form chloride ion channels with identical characteristics to those observed in Chinese hamster ovary cells expressing CLIC1. *J Biol Chem* 2002;277:26003-26011.
PUBMED | CROSSREF
28. Domingo-Fernández R, Coll RC, Kearney J, Breit S, O'Neill LA. The intracellular chloride channel proteins CLIC1 and CLIC4 induce IL-1β transcription and activate the NLRP3 inflammasome. *J Biol Chem* 2017;292:12077-12087.
PUBMED | CROSSREF
29. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013;13:159-175.
PUBMED | CROSSREF
30. Filep JG. Targeting neutrophils for promoting the resolution of inflammation. *Front Immunol* 2022;13:866747.
PUBMED | CROSSREF
31. Son S, Yoon SH, Chae BJ, Hwang I, Shim DW, Choe YH, Hyun YM, Yu JW. Neutrophils facilitate prolonged inflammasome response in the DAMP-rich inflammatory milieu. *Front Immunol* 2021;12:746032.
PUBMED | CROSSREF
32. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 2009;458:509-513.
PUBMED | CROSSREF
33. McKenzie BA, Dixit VM, Power C. Fiery cell death: pyroptosis in the central nervous system. *Trends Neurosci* 2020;43:55-73.
PUBMED | CROSSREF
34. Karmakar M, Katsnelson MA, Dubyak GR, Pearlman E. Neutrophil P2X7 receptors mediate NLRP3 inflammasome-dependent IL-1β secretion in response to ATP. *Nat Commun* 2016;7:10555.
PUBMED | CROSSREF
35. Lee GS, Subramanian N, Kim AI, Aksentijevich I, Goldbach-Mansky R, Sacks DB, Germain RN, Kastner DL, Chae JJ. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca^{2+} and cAMP. *Nature* 2012;492:123-127.
PUBMED | CROSSREF
36. Schorn C, Frey B, Lauber K, Janko C, Strysio M, Keppeler H, Gaipl US, Voll RE, Springer E, Munoz LE, et al. Sodium overload and water influx activate the NALP3 inflammasome. *J Biol Chem* 2011;286:35-41.
PUBMED | CROSSREF
37. Mollinedo F, Manara FS, Schneider DL. Acidification activity of human neutrophils. Tertiary granules as a site of ATP-dependent acidification. *J Biol Chem* 1986;261:1077-1082.
PUBMED | CROSSREF
38. Paroutis P, Touret N, Grinstein S. The pH of the secretory pathway: measurement, determinants, and regulation. *Physiology (Bethesda)* 2004;19:207-215.
PUBMED | CROSSREF