Advanced glycation end products and ATP dependent regulation of innate immune response

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Directed by Professor Je-Wook Yu

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ABSTRACT

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Inflammasome signaling in both macrophages and neutrophils can attribute to host innate immune defense against infection, as well inflammatory disease, such as type 2 diabetes, Alzhimer's disease or atherosclerosis. Risk factors accumulated from our lifestyle regulate our innate immune response, leading to severe diseases, while what factors and how they mediate remain obscure. In this regard, I investigated a novel risk factors from our lifestyle and how they regulate innate immune response, counting on inflammasome activation, in macrophages and neutrophils.

Advanced glycation end products (AGEs), in part 1, are adducts formed on proteins by glycation with reducing sugars, such as glucose, and tend to form and accumulate under hyperglycemic condition. As a risk factor in hyperglycemic condition, AGEs accumulation has been implicated in the pathogenesis of many

degenerative diseases such as diabetic complications. AGEs have also been shown to promote the production of pro inflammatory cytokines, but the roles of AGEs in inflammasome signaling have not been explored in detail. Here I present evidence that AGEs attenuate activation of the NLPR3 inflammasome in bone marrow derived macrophages (BMDMs) as determined by caspase 1 and IL-1β activation. AGEs also impaired the assembly of the NLRP3 inflammasome activation but did not affect the NLRC4 or AIM2 inflammasome activation. Moreover, AGEs treatment inhibited Toll-like receptor (TLR) dependent production of pro inflammatory cytokines in BMDMs. This immunosuppressive effect of AGE was not associated with a receptor for AGEs (RAGE) mediated signaling. Furthermore, AGEs significantly impaired innate immune responses including NLRP3 inflammasome activation and type I interferon production in macrophages upon influenza virus infection. Collectively these observations suggest that AGEs could impair host NLRP3 inflammasome mediated innate immune defenses against RNA virus infection, leading to an increased susceptibility to infection.

ATP, covered in part 2, is a typical damaged associated molecular patterns (DAMPs), elevated locally or systemically upon tissue injury. Macrophages and neutrophils are two major cells of innate immune system and they infiltrate into DAMPs-rich region to initiate inflammation. Both cells can activate inflammasome, while neutrophils do not undergo pyroptosis. However, differences on inflammasome signaling and aftermath from both cells have not been explored in detail. Here I offer evidence that exclusive traits of bone marrow derived neutrophils (BMNs) in inflammasome signaling, including resistance on DAMPs-rich milieu and pyroptosis contribute to the prolonged inflammation. I showed that various inflammasome were activated in neutrophils, while they resist to undergo cell death,

including pyroptosis upon inflammasome activation. Consistently, NLRP3 inflammasome activated neutrophils preserved their functional integrity, encompassing phagocytosis, degranulation and migration. Surprisingly, unlike macrophages, neutrophils under DAMPs-rich condition maintained NLRP3 inflammasome activating potential. Mechanistically, resistance to pyroptosis in neutrophils derived from impaired GSDMD cleavage along with impaired mitochondrial membrane potential loss. Moreover, contrast to macrophages, DAMPs, including ATP exposed neutrophils conserved mitochondrial membrane potential facilitating NLRP3 inflammasome activation. IL-1β processing in neutrophils was NLRP3/ Caspase-1 dependent as macrophages, however secretion was less dependent on GSDMD. Intriguingly, neutrophils secreted IL-1β more rapidly and specifically compared to macrophages upon LPS or LPS/ATP treatment. Furthermore, unlike apoptotic neutrophils, NLRP3 inflammasome activated neutrophils failed to induce efferocytosis leading to resolution of inflammation in peritoneal macrophages. Collectively, these observations firmly suggest that neutrophils under DAMP-rich milieu could preserve their potential to activate NLRP3 inflammasome without pyroptosis, compared to macrophages leading to exacerbated immune response through prolonged inflammation.

Key words: damaged associated molecular patterns, Advanced glycation end products, inflammasome, macrophages, neutrophils, pyroptosis

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I. INTRODUCTION

Our innate immune system comprises the first line defense against invading pathogens. These pathogens are recognized by pattern recognition receptors (PRRs), expressed on the cell surface or in cytoplasm of innate immune cells. There are several types of PRRs and some of them form inflammasome, a protein complex crucial for initiating inflammatory responses, mainly in myeloid cells, including macrophages and neutrophils[1]. Microbial infection or tissue injury could trigger the assembly and activation of inflammasome complex, comprising sensor molecules such as nucleotide binding oligomerization domain like receptor family, pyrin domain containing 3 (NLRP3), or absent in melanoma 2 (AIM2), ASC and procaspase 1, leading to activation of caspase 1[2]. Subsequently, active caspase 1 processes inactive pro IL-1β and gasdermin D (GSDMD) into active form. N-terminus of cleaved GSDMD (GSDMD-NT) forms pores in plasma membrane through oligomerization, which become channel for secretion of mature IL-1β. Furthermore, GSDMD pores promote

plasma membrane rupture and cell death, also known as, pyroptosis, caspase 1/11 dependent cell death[3].

Inflammasome signaling primarily provides a host innate immune defense against wide range of microbial infections, including influenza virus[4]. Accumulating evidence suggests that NLRP3 inflammasome is more focused on detecting DAMPs, especially DAMPs-like molecules from abnormal condition, such as ATP, palmitate and amyloid β. Furthermore, deregulated activation of NLRP3 inflammasome could lead to progression of inflammatory disorders, including type 2 diabetes[5,6]. Indeed, NLRP3 deficient mice showed a remarkable attenuation of disease symptoms associated with type 2 diabetes, Alzhimer's disease and atherosclerosis, demonstrating that uncontrolled activation of NLRP3 inflammasome is critical to the pathogenesis of these metabolic or degenerative disorders[7,8]. However, what risk factors accumulated from our lifestyle and how they regulate NLRP3 inflammasome signaling still remain obscure.

Neutrophils and macrophages are two major cells of our innate immune system. Even though they encompass their own unique traits, they commonly detect pathogens and help to promote inflammation. As both cells infiltrate to DAMPs rich region, their interaction is important for initiation and resolution of inflammation[9,10]. After inflammatory response, apoptotic neutrophils are engulfed by macrophages, leading macrophages to produce anti-inflammatory cytokines, such as IL-10 or TGFβ and to become immunologically silent; the process also known as efferocytosis. Therefore, uncontrolled clearance of apoptotic neutrophils or delayed apoptosis in neutrophils could lead to prolonged inflammation. Indeed, patients with SLE often has defect in efferocytosis[11-13]. Intriguingly, it has been reported that unlike macrophages, neutrophils do not undergo pyroptosis upon inflammasome activation[14-17]. Despite considering importance of neutrophils in inflammatory disease and their cell death for resolution of inflammation, the outcome of inflammasome activation in neutrophils, along with macrophages have not been explored.

Advanced glycation end products (AGEs) belong to a group of heterogeneous compounds formed by non-enzymatic glycation of proteins[18]. AGEs accumulate under hyperglycemic conditions and in the regions associated with diabetic complications. Elevated levels of AGEs were frequently found in samples of patients with type 2 diabetes or aging related diseases[19,20]. By direct binding to cell surface receptors such as receptor for AGEs (RAGE), it can cause host tissue damages, considered to be crucial risk factors for aggravation of age-related degenerative disorders, including diabetic complications[21]. Intriguingly, AGEs were shown to increase the production of pro inflammatory cytokines such as IL-6 in both myeloid and non-myeloid cells[22,23]. The potential impact of AGEs on inflammasome signaling in myeloid cell is unknown, Therefore, here in part 1, I explored potential role of AGEs on inflammasome assembly or activation in macrophage.

ATP is typical damage-associated molecular pattern (DAMPs), released from injured tissue and accumulated in inflammatory or injured site[24,25]. Elevated extracellular ATP is considering to be a risk factor on pathogenesis of various inflammatory disease, as it induces ROS and NLRP3 inflammasome activation by activating P2X7R. Blocking the binding of ATP on P2X7R or decreasing the level of ATP attenuated disease progression, such as RA. In contrast, ATP-P2X7R axis also contributes to proliferation and differentiation of cells, including T cell, by stabilizing mitochondrial metabolism and membrane potential, which is also preserved in M2 macrophage[26]. Indeed, ATP stimulation promotes a preferential M2 differentiation of bone marrow derived macrophage, related anti inflammation[27,28]. Even though both macrophages and neutrophils accumulate in ATP-rich inflammatory site, potential impact of exposure to ATP on those cells is unclear. As a result, in part 2, I examined different effect of extracellular ATP on immune response of neutrophils and macrophages, including inflammasome activation, finally leading to different outcomes.

II. MATERIALS AND METHODS

1. Mice

C57BL/6 mice were obtained from Orient Bio. *Nlrp3*$^{-/-}$ and *GSDMD*$^{-/-}$ mice were purchased from The Jackson Laboratory. All mice were on C57Bl/6 background and 8-10 weeks male mice were used for the experiments. All mice were maintained under specific pathogen-free conditions at Yonsei University College of Medicine. All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

2. Cell cultures

Mouse bone marrow-derived macrophages (BMDMs) were differentiated from bone marrow cells isolated from mouse femurs and tibias of C57BL/6, *Nlrp3*$^{-/-}$ or *GSDMD*$^{-/-}$ mice. Bone marrow progenitor cells were cultured in 5% L929-conditioned Dulbecco's modified Eagle's medium (DMEM, Corning, NY, USA) supplemented with 10% fetal bovine serum (FBS, Corning) and 100 U/ml penicillin and streptomycin (Gibco, Gran Island, NY, USA). After 3 days, non-adherent cells were removed and culture medium was replaced with L929-conditioned DMEM. Then augmented cells were plated in appropriated plate with 5% L929-conditioned DMEM. Mouse peritoneal macrophages were obtained on 5 days after 1.5ml of 3% thioglycolate intraperitoneal injection. Red blood cells were lysed by RBC lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) before plating in Roswell Park Memorial Institute Medium 1640 (RPMI). Next day, non-adherent cells were removed and culture medium was replaced with RPMI. Mouse bone marrow-derived neutrophils (BMNs) were isolated from bone marrow cells in mouse femurs and tibias of C57BL/6, *Nlrp3*$^{-/-}$ or *GSDMD*$^{-/-}$ mice by using EasySep^{TM} mouse neutrophil enrichment kit (Stemcell technologies, USA). After isolation, BMNs were used for experiment immediately, cultured in RPMI 1640 supplemented with 10% FBS and 100 U/ml penicillin and streptomycin or OptiMEM (Life

Technologies). NLRP3-GFP-expressing BMDMs were kindly provided by Dr. E.S. Alnemri (Thomas Jefferson University, Philadelphia, PA, USA). Immortalized BMDMs were maintained in L929-conditioned DMEM. Unless otherwise stated in the following results, 0.5 x 10^6 cell/ml (for BMDMs) or 1 x 10^6 cell/ml (for BMNs) were performed on 12 well plate for experiments.

3. Reagents and antibodies

LPS, nigericin, ATP, Pam3CSK4, poly (dA:dT), poly(I:C), staurosporine, glibenclamide, valiomycin, cytochalasin D, and DOTAP liposomal transfection reagent were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alum was purchased from InvivoGen. Flagellin purified from *P. aeruginosa* was purchased from Invivogen (San Diego, CA, USA). BSA was obtained from Affymetrix. Glucose was obtained from Amresco. The RAGE antagonist FPS-ZM1 was purchased from Merck Millipore. Recombinant mouse S100A8/S100A9 proteins were obtained from R&D Systems. JC-1was purchased from Invitrogen. Zymosan-alexa488 and Annexin V were purchased from ThermoFisher scientific. CytoTox96 non-radioactive cytotoxicity assay kit was obtained from Promega (Madison, WI, USA).

Mouse anti-caspase1 p20 antibody and mouse anti-NLRP3 antibody were obtained from Adipogen (San Diego, CA, USA). Anti-ASC antibody, Anti-Caspase 3 antibody and Anti-b-actin antibody were purchased from Santa Cruz (Dallas, TX, USA). Mouse anti-IL-1β antibody was purchased from R&D Systems (Minneapolis, MN, USA). Mouse GSDMD antibody was purchased from Abcam.

4. Preparation of AGEs

BSA (250 mg/ml) was incubated with glucose (1 M) in phosphate-buffered saline (PBS) under sterile conditions in the dark at 37 °C for 8 weeks. Alternatively, BSA (50 mg/ml) was incubated with glucose (0.5 M) for 24 weeks to induce extensive formation of AGEs. As a control for AGEs, only BSA was incubated in the absence of glucose under the same conditions. After incubation, samples were filtered to remove aggregated particles, normalized, and stored at 20 °C before use. In some experiments, excessive glucose in the AGE preparations was removed by extensive dialysis against PBS. To confirm the formation of AGEs, the fluorescence emission spectrum between 385 and 600 nm (370 nm excitation) was scanned using a Varioskan Flash multimode reader (Thermo Fisher)

5. Assay of inflammasome activation

To stimulate NLRP3 inflammasome activation, mouse BMDMs or BMNs were primed with LPS (0.25 μg/ml) for 3 hr, followed by treatment with ATP (2.5 mM, 30 min), nigericin (5 μM, 45 min), or alum (125 μg/ml, 6 hr). To activate AIM2 inflammasomes, BMDMs or BMNs were transfected with poly(dA:dT) using lipofectamine 2000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. To stimulate NLRC4 inflammasome, BMDMs were infected with P. aeruginosa PAO1. BMNs were transfected with flagellin (0.5 μg) using DOTAP liposomal transfection reagent (2 μl) in Opti-MEM medium (100 μl), incubated at 25 °C for 30 min before treatment. Inflammasome activation was determined by the presence of bands corresponding to active caspase-1 p20 and active IL-1β in immunoblots and was quantified by measuring extracellular IL-1β using a Quantikine IL-1β ELISA Kit (R&D Systems). To measure extracellular levels of IL-6, culture supernatants were assayed using an IL-6-specific ELISA kit according to the manufacturer's instructions (R&D Systems).

6. Assay of inflammasome assembly

To identify ASC oligomerization, treated cells were lysed with 20 mM HEPES (pH 7.5) buffer containing 0.5% Non-idet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA and protease inhibitors and centrifuged at 12,000 rpm for 10 min. The supernatants were used for immunoblotting of soluble lysates and the pellets were resuspended in PBS. For cross-linking of proteins, 0.75 mM disuccinimidyl suberate (DSS, Pierce, Rockford, IL, USA) were added to the resuspended pellets and incubated for 30 min at room temperature in dark and then centrifuged at 12,000 rpm for 10 min. The cross-linked pellets and soluble lysates were separated by SDS-PAGE and immunoblotted with anti-ASC antibody. To determine the formation of NLRP3 speck-like aggregates, NLRP3-GFP-expressing BMDMs were observed under a confocal microscope (LSM 700, Zeiss) after the indicated stimulations. The relative percentage of cells containing NLRP3 specks was then counted.

7. Cell death analysis

Caspase-1-dependent cell death was determined by extracellular release of lactate dehydrogenase (LDH) using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega). The LDH release was calculated as [extracellular LDH/ (intracellular LDH + extracellular LDH) × 100]. For propidium iodide (PI) staining, BMDMs and BMNs were collected using trypsin, then resuspended in PBS containing 2% FBS and 1mM EDTA. 1 mg/ml of PI was added to $1x10^5$ cells in 100 μl of PBS and stained for 5 min on ice. 400 μl of PBS was added before analyzed by flow cytometry. For Annexin V binding assay, 4.5 μl of Annexin V was added to $1x10^5$ cells of BMDMs and BMNs resuspended in PBS (100 μl) for 15 min. Then cells were analyzed by flow cytometry

8. Cell function analysis

To measure phagocytosis ability, BMDMs and BMNs were treated with zymosan (in 1:5 ratio), after indicated stimulation. 30 min after treatment, un-phagocyted zymosan was wash by PBS three times. Then cells were detached by trypsin and resuspended in PBS. Cells were analyzed by flow cytometry (FACSVerse) to analyze engulfed zymosan. To assess *in vitro* migration ability, BMNs were plated in confocal dishes, coated with 10 μg/ml of fibronectin (Gibco). Migration imaging was conducted by Nikon Eclipse Ti2 inverted microscope. DIC and FITC channel (Ex: 475 nm, Em: 530 nm) were captured for every 10 seconds and terminated after 30 minutes. Tracking analysis was performed with 'autoregressive motion' of 'spots' tool.

9. Immunoblotting

Cell culture supernatants were collected after inflammasome stimulations. Proteins in the supernatants were precipitated by methanol/chloroform extraction method. Cell were harvested and then lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% Non-idet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA and protease inhibitors. The soluble lysates were obtained by centrifugation (12,500 rpm for 12 min). All proteins in supernatants and cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to PVDF membrane (Bio-Rad, Hercules, CA, USA) and then immunoblotted. The membranes were blocked with 3% skim-milk in PBS contained 0.1% Tween 20 for 30 min and incubated with appropriate antibodies. All blots shown are representative images of at least three-independent experiments. Images have been cropped for presentation

10. Immunohistochemistry

Cells were fixed in 4% paraformaldehyde for 30 min, and then permeabilized and stained with the primary antibodies targeting Tubulin (Santacruz, SC-32293, 1:500), Tom20 (Santacruz, SC-11415, 1:500) and Rhodamine-Phalloidin (Thermo, R-415, 66 nM) for 1 hr, followed by the appropriate Alexa Fluor 488 secondary antibodies (Molecular Probes) for 1 hr. DAPI counterstaining was used for nuclei visualization. Cell images were acquired and processed with confocal microscopy (LSM 700, Carl Zeiss) and the ZEN2011 software.

11. Preparation of DAMPs containing sup

To obtain DAMPs from dead cells, $1x10^6$ cells of BMDMs were treated with staurosporine (2 μg/ml) for 2 hr in 6 well plate. Then cells were washed with PBS 3 times to remove staurosporine and media was changed to new OptiMEM, incubated for 18 hr. For DAMPs from ruptured cells, $1x10^6$ cells of BMDMs were undergone freeze/thaw cycle 4 times in OptiMEM. Supernatant from both conditions were centrifuged to remove cell debris and stored at 20 °C before use.

12. Influenza A virus infection

Human influenza virus A/Puerto-Rico/8/34 (H1N1) PR8 strains lacking the NS1 open reading frame (delNS1) were provided by Dr. Adolfo Garcia-Sastre (Icahn School of Medicine at Mount Sinai,NewYork) and used. Virus titers were determined by standard plaque assays in Madin-Darby canine kidney cells with a few modifications, as described previously (49). Cells were infected with influenza A virus at a multiplicity of infection (m.o.i.) of 2–4. At 1.5 h post-infection, cells were washed and cultured in regular growth medium for 24 h before being harvested.

13. Cytokine production assay

The levels of IL-1β, IL-6, type-I IFN or MMP9 in the culture supernatants were quantified by mouse IL-1β, IL-6, IFN-γ or MMP9 ELISA (R&D). Inflammatory cytokines in the culture supernatants were also quantified using the Cytometric Bead Array (CBA) Mouse Inflammatory Kit (BD).

14. Measurement of mitochondrial membrane potential

To measure the mitochondrial membrane potential, cells were stained with the membrane potential-sensitive JC-1 dye (1.5 mM for 30 min in 37 °C), which forms red fluorescence-emitting aggregates on polarized mitochondria and green fluorescence-emitting monomers on depolarized mitochondria. Cells were analyzed by flow cytometry using FL1 and FL2 channels

15. Efferocytosis analysis

Untreated, apoptotic and inflammasome activated BMNs were collected by trypsin, followed PBS wash three times. BMNs were resuspended in RPMI and treated to peritoneal macrophage in 1:2.5 ratio, centrifuged in 300 g for 10 min for contact. After 18 hr coculture, un-phagocyted BMNs were washed by PBS three times and media was changed to fresh RPMI. BMNs phagocyted peritoneal macrophages were treated with LPS (0.1 μg/ml) for 6 hr before harvest.

16. *In vivo* model

To induce inflammasome activation in mouse, LPS (1 mg/kg) or PBS (for control) was injected to mouse. 24 hr after LPS injection, mouse was sacrificed, and peritoneal lavage was obtained from 10 ml of PBS. After collecting cells from peritoneal lavage, cells were stained with CD11b, F4/80 and Ly6G antibody for 30 min on ice. PI was added just before analyzing by FACs.

17. Quantification of mRNA production

To measure mRNA production, quantitative real-time PCR assays was performed. Briefly, total cellular RNA was isolated using the TRIzol reagent (Invitrogen) and reverse transcribed using PrimeScriptTM RT Master Mix (Takara) according to the manufacturer's instructions. Template DNA was amplified by quantitative real-time PCR using SYBR Premix Ex TaqTM II (Takara).

18. Statistical analysis

All values were expressed as the mean and SE of three independent experiments unless otherwise indicated. Data were analyzed using one-way analysis of variance followed by Bonferroni *post hoc* test after checking the assumptions of normal distribution by Shapiro test and equal variance by Bartlett or Brown–Forsythe test. Otherwise, Welch's *t*-tests were used for unequal variances. The p values ≤ 0.05 were considered significant. Statistical analyses were carried out using GraphPad Prism and R software.

Table 1. Primers sequence for PCR

Gene	Primer sequence
Mouse IL-1β	Forward: 5'-GCC CAT CCT CTG TGA CTC AT-3' Reverse: 5'-AGG CCA CAG GTA TTT TGT CG-3'
Mouse IL-6	Forward: 5'-AGT TGC CTT CTT GGG ACT GA-3' Reverse: 5'-TCC ACG ATT TCC CAG AGA AC-3'
Mouse NLRP3	Forward: 5'- ATG CTG CTTCGA CAT CTC CT-3' Reverse: 5'-AAC CAA TGC GAG ATC CTG AC-3'
Mouse IFN-β	Forward: 5'-TTC CTG CTG TGC TTC TTC AC-3' Reverse: 5'-CTT TCC ATT CAG CTG CTC CA-3'
Mouse TNFα	Forward: 5'-CGT CAG CCG ATT TGC TAT CT-3' Reverse: 5'-CGG ACT CCG CAA AGT CTA AG-3'
Mouse IL-10	Forward: 5′-CCA AGC CTT ATC GGA AAT GA-3′ Reverse: 5′-TTT TCA CAG GGG AGA AAT CG-3′
Mouse SARM	Forward: 5'-CGC TGC CCT GTA CTG GAG G-3' Reverse: 5'-CTT CAG GAG GCT GGC CAG CT-3'
Mouse GAPDH	Forward: 5'-AAC TTT GGC ATT GTG GAA GG-3' Reverse: 5'-ACA CAT TGG GGG TAG GAA CA-3'
Mouse β actin	Forward: 5'-CCT TCC TGG GCA TGG AGT CCT G-3' Reverse: 5'-GGA GCA ATG ATC TTG ATC TTC-3'
Mouse Rn18s	Forward: 5'-CGC GGT TCT ATT TTG TTG GT-3' Reverse: 5'-AGT CGG CAT CGT TTA TGG TC-3'

III. RESULTS

Part 1. Advanced glycation end products impair NLRP3 inflammasome mediated innate immune response in macrophages

1. AGEs do not trigger, while inhibit TLR-mediated production of pro inflammatory cytokine

To examine the effect of AGEs on macrophages innate immune signaling, I first prepared AGEs by incubating bovine serum albumin (BSA) with a high concentration of glucose for 8 weeks, as described previously[29,30]. Production of AGEs was validated by its unique color and increased protein size (Fig. 1A). Furthermore, unique fluorescence spectrum (excitation 370 nm, emission 44 nm) of AGEs was assessed (Fig. 1B). Then I treated bone marrow-derived macrophages (BMDMs) with AGEs for 6 or 18 h and assessed the secreted pro-inflammatory cytokine. AGEs administration did not trigger the secretion of IL-6 and IL-1β. AGEs treatment also failed to induce IL-1β secretion in the presence of lipopolysaccharide (LPS), but rather suppressed IL-6 secretion (Fig. 1C, D). Focused on unexpected suppression of AGEs on LPS induced IL-6 production, pretreatment with AGEs (10 mg/ml) for 15 hr prior to LPS stimulation significantly attenuated LPS-triggered up-regulation of IL-1β and IL-6 mRNA in BMDMs (Fig. 2A, B). To further examine whether AGEs could dampen the production of pro-inflammatory cytokines in macrophages triggered by other TLR, I stimulated BMDMs with pam4CSK4, a TLR1/2 agonist, or a poly(I:C), a TLR3 agonist. In consistence with LPS stimulation, AGEs pretreatment significantly attenuated pam3CSK4 or poly(I:C)-triggered production of IL-6 and IL-1β mRNA (Fig. 2C, D). These findings clearly demonstrated that AGEs could impair TLR-mediated cytokine production in macrophages

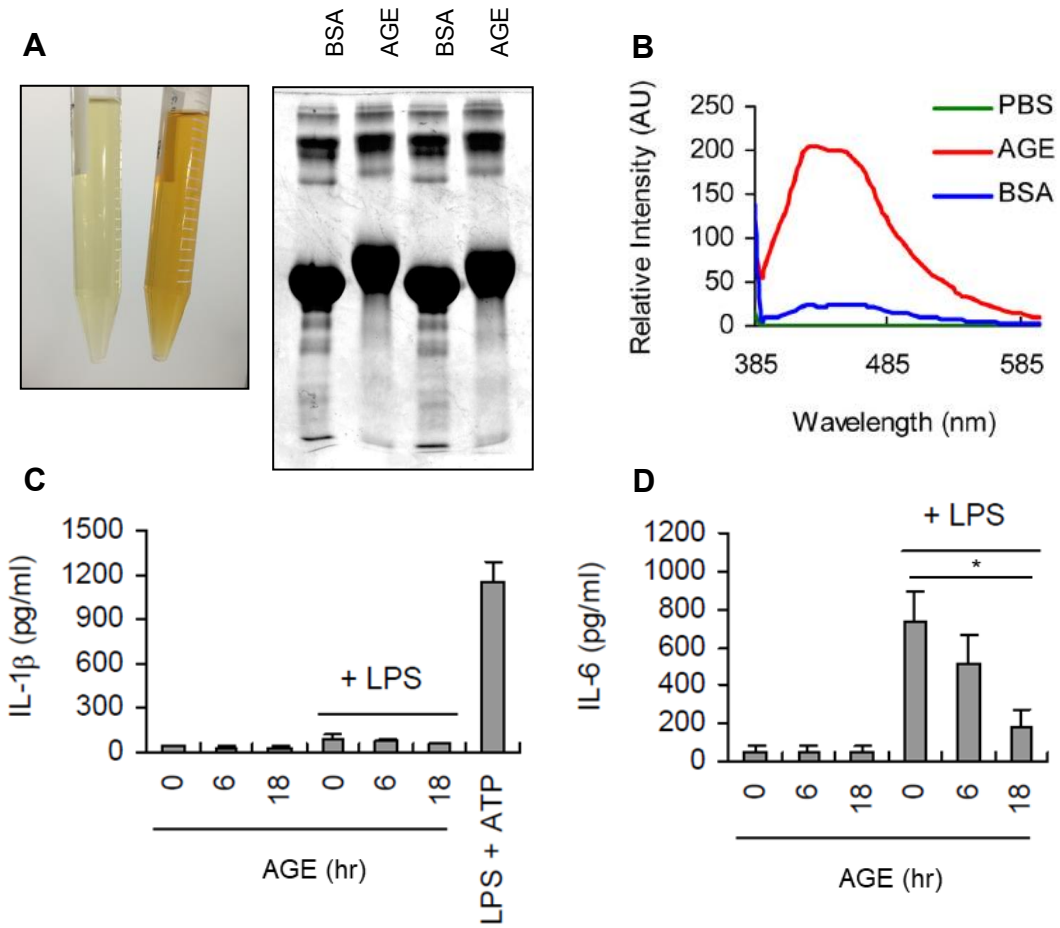


Figure 1. AGEs do not trigger the production of pro-inflammatory cytokines. (A) Color of BSA or AGEs incubated for 8 weeks. (B) The fluorescence emission spectra of PBS (green), 8-week incubated BSA (blue), and 8 wk incubated AGE-BSA (red) at 370 nm excitation. (C, D) Quantification of IL-1β or IL-6 in culture supernatant by ELISA from mouse BMDMs treated AGEs (10 mg/ml, for indicated time) in the presence of LPS (0.25 μg/ml, final 3 hr), or treated with LPS alone (0.25 μg/ml, 3 hr), followed by the treatment with ATP (2.5 mM, 30 min) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (*P<0.01).

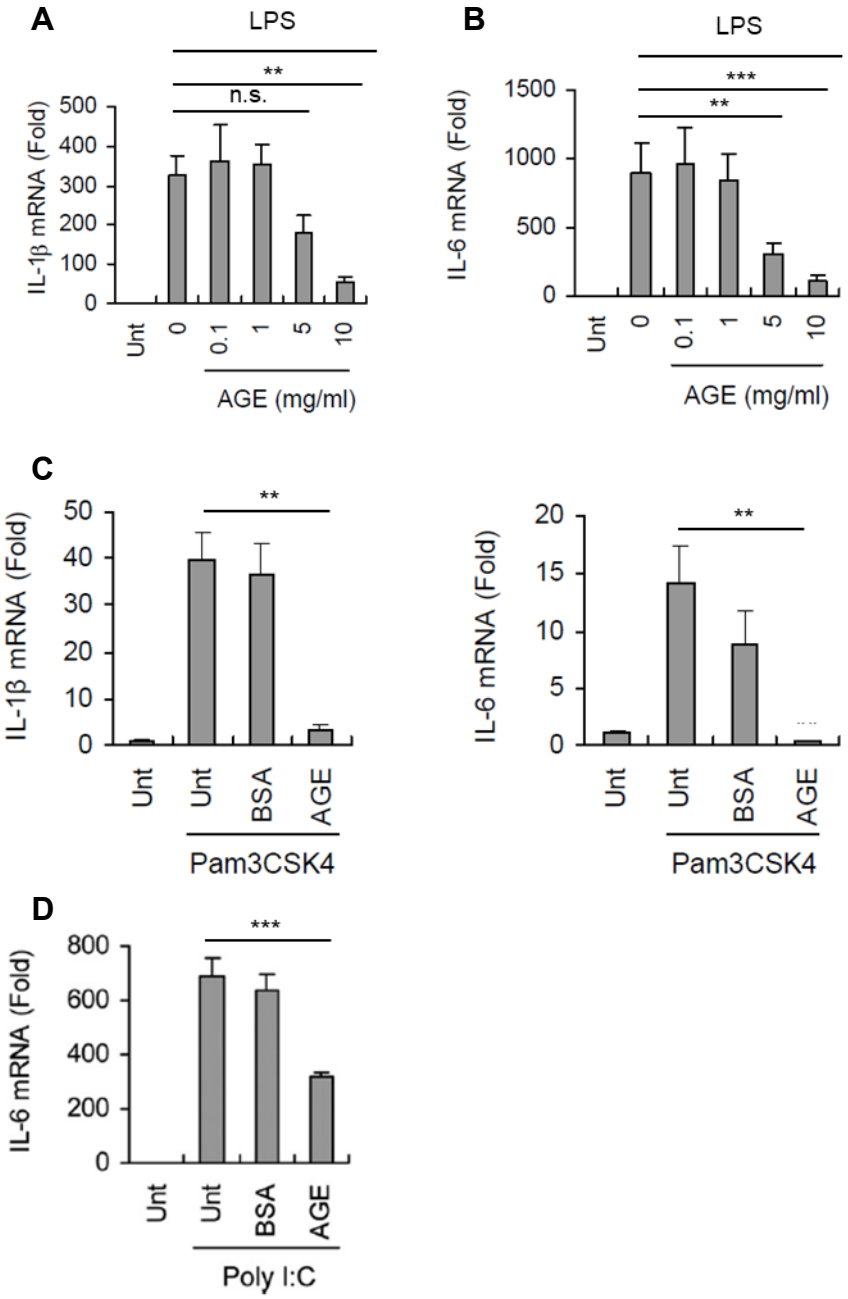
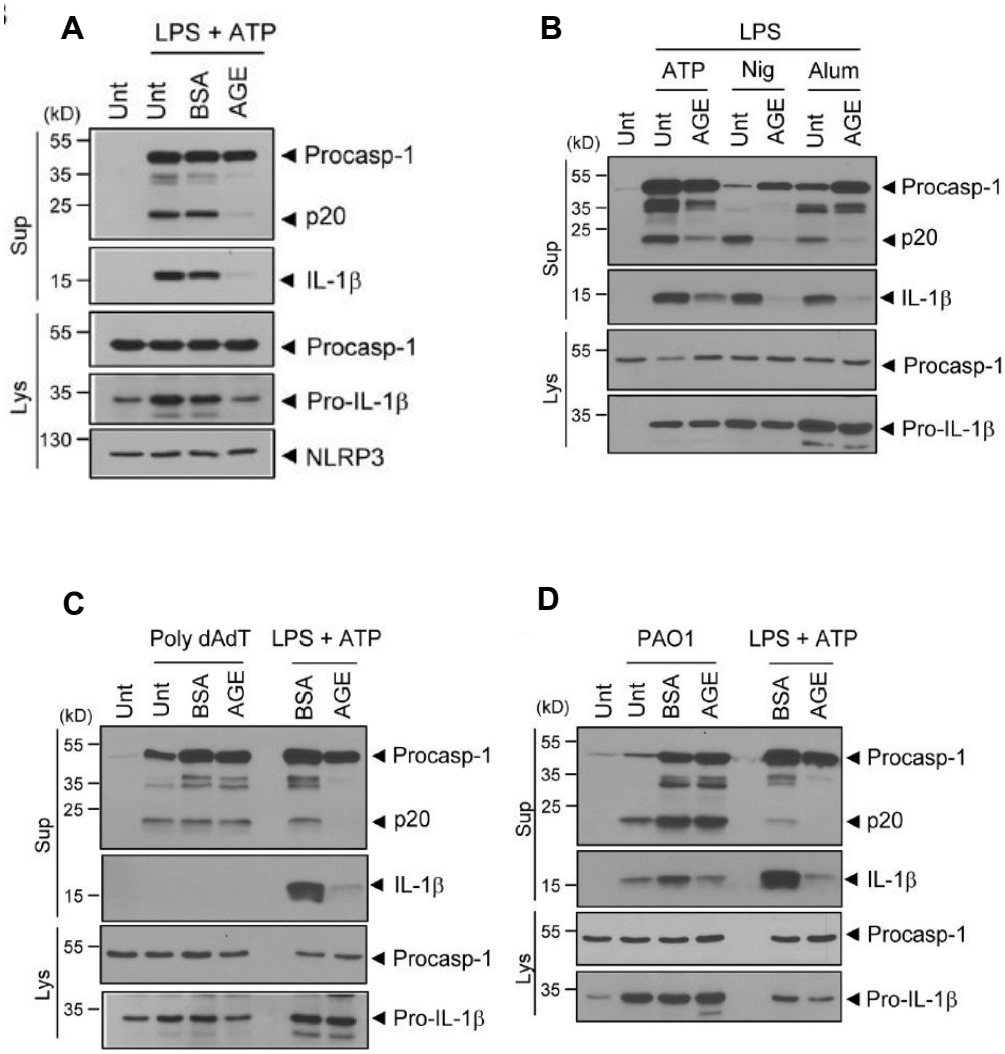
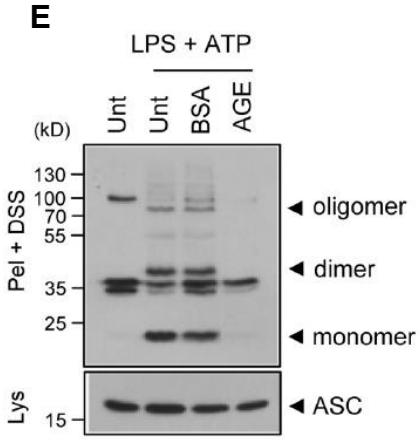


Figure 2. Prolonged AGEs treatment inhibits TLR-mediated production of pro-inflammatory cytokines. (A, B) Quantification of IL-1β or IL-6 mRNA level by qPCR from mouse BMDMs treated with AGEs (0.1~10 mg/ml) for 18hr in the presence of LPS (0.5 μg/ml, final 3hr) (n = 3 or 4). (C) Quantification of IL-1β or IL-6 mRNA level by qPCR from mouse BMDMs treated with AGEs (0.1~10 mg/ml) for 18 hr in the presence Pam3CSK4 (1 μg/ml, final 3 hr) (n = 3). (D) Quantification of IL-6 mRNA level by qPCR from mouse BMDMs were treated with AGEs (10 mg/ml) for 18 hr in the presence of Poly(I:C) (10 μg/ml, final 3h) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; $*P<0.01$, $**P<0.05$, $***P<0.001$).

2. AGEs pretreatment impairs NLRP3 inflammasome assembly and activation

Next, to examine whether AGEs affect caspase 1 and inflammasome signaling, I stimulated BMDMs with AGEs followed by LPS/ATP. Pretreatment with AGEs, but not with BSA, showed a robust reduction in the secretion of active caspase 1 and IL-1β from BMDMs (Fig. 3A). Other NLPR3-mediated inflammasome activation triggered by nigericin or alum was also attenuated by pretreatment with AGEs in LPS primed BMDMs (Fig. 3B). However, poly(dA:dT) transfection-triggered inflammasome activation, mediated by AIM2, was not impaired by AGEs pretreatment (Fig. 3C). Furthermore, NLCR4 inflammasome activation in response to Pseudomonas aeruginosa infection was not reduced by pretreatment with AGEs (Fig. 3D). Moreover, AGEs pretreatment clearly abolished LPS/ATP induced oligomerization of ASC in BMDMs, an essential process of NLRP3 inflammasome signaling (Fig. 3E). In accordance with these data, the formation of speck-like NLRP3 aggregates by nigericin was reduced by AGEs pretreatment (Fig. 3F). These results collectively indicate that AGEs pretreatment specifically impairs the NLRP3 inflammasome activation in macrophages.





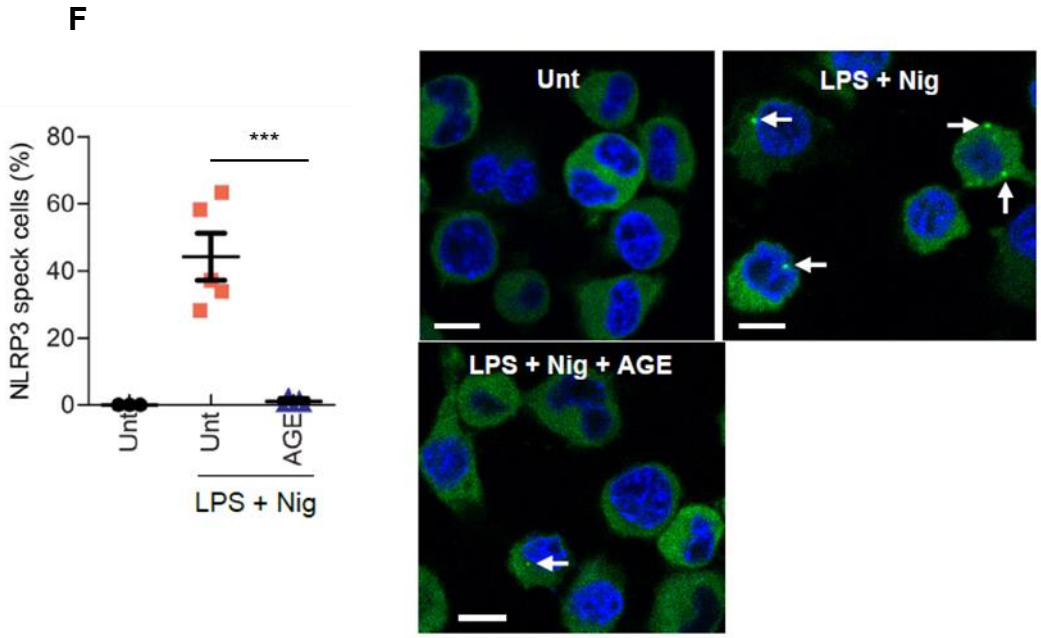


Figure 3. AGEs pretreatment impairs NLRP3 inflammasome assembly and activation. (A) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with BSA or AGEs (10 mg/ml, 18 hr), washed, and treated with LPS (0.25 μg/ml, 3 hr), followed by treatment with ATP (2.5 mM, 30 min). (B) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with BSA or AGEs (10 mg/ml, 18 hr), washed, and treated with LPS (0.25 μg/ml, 3 hr) followed by treatment with ATP (2.5 mM, 30 min), nigericin (Nig, 5 μM, 45 min), or alum (250 μg/ml, 6 hr). (C, D) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with BSA or AGEs (10 mg/ml, 18 hr), washed, transfected with poly(dA:dT) (1 μg/ml, 4 hr) or infected with P. aeruginosa PAO1 (m.o.i. = 3) for 3 hr. (E) Immunoblots of cellular lysates in the succinimidyl suberate (DSS)-derived pellet (pel) fraction from) mouse BMDMs were treated with BSA or AGEs (10 mg/ml) for 18 hr, washed, and treated with LPS (0.25 μg/ml, 3 hr), followed by treatment with ATP (2.5 mM, 30 min). (F) NLRP3-GFP expressing BMDMs were pretreated with AGEs (5 mg/ml) for 18 hr, washed, and treated with LPS (0.25 μg/ml, 3 hr), followed by treatment with nigericin (5 μM, 45 min). Speck-like aggregates of NLRP3 were then counted and displayed as the relative number of NLPR3 speck-containing cells (n = 3) or observed by confocal microscopy. The arrow indicates speck-like aggregates of NLPRP. * Scale bars, 10 μm.

3. RAGE inhibitor fails to block AGEs-mediated impairment of innate immune responses

RAGE is a typical identified receptor for AGEs[21]. Therefore, I examined the potential implication of RAGE in the innate immune-suppressing ability of AGEs. RAGE-specific antagonist FPS-ZM1 failed to affect the AGE-mediated inhibition of LPS/ATP induced caspase 1 cleavage (Fig. 4A). In consistent, RAGE inhibitor failed to affect the AGE-driven suppression of IL-6 mRNA induction in BMDMs by LPS stimulation (Fig. 4B). However, RAGE inhibitor reduced IL-6 mRNA expression triggered by S100 protein, indicating inhibitor is working well (Fig. 4C). In addition, to investigate the mechanism by which AGEs pretreatment could modulate NLRP3 inflammsome signaling, I analyzed TLR4-NFkB signaling axis. AGEs pretreatment considerably inhibited LPS-stimulated phosphorylation and degradation of IkB in BMDMs, indicating that AGEs negatively regulated NF-kB signaling (Fig. 4D). These findings collectively indicated that RAGE signaling is not associated with AGE-driven impairment of innate immune responses.

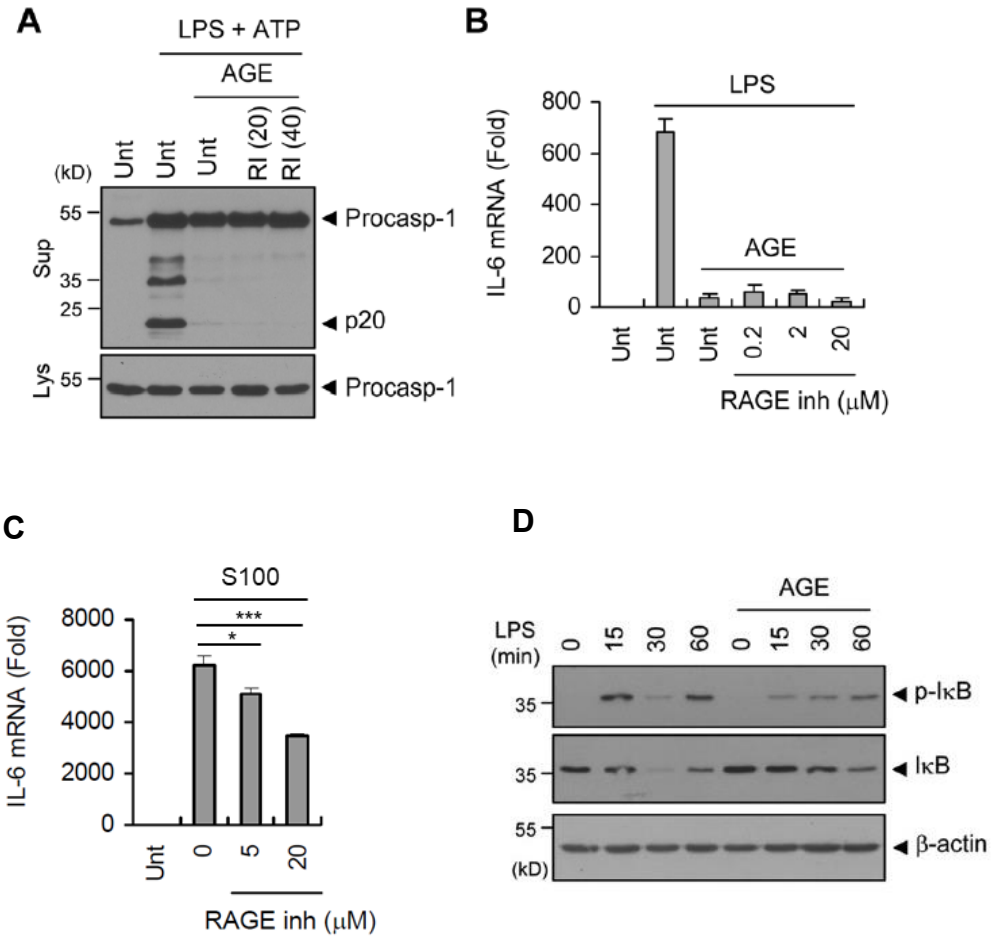


Figure 4. RAGE inhibitor fails to block AGE-mediated impairment of innate immune responses. (A) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with AGEs (10 mg/ml, 18 hr) in the presence of RAGE inhibitor (RI, 20 or 40 μM), washed, and treated with LPS (0.25 μg/ml, 3 hr), followed by treatment with ATP (2.5 mM, 30 min). (B) Quantification of IL-6 mRNA level by qPCR from mouse BMDMs pretreated with AGEs (10 mg/ml, 15 hr) in the presence of RAGE inhibitor (0.2~20 μM), and then further treated with LPS (0.5 μg/ml, for additional 3 hr) (n = 3). (C) Quantification of IL-6 mRNA level by qPCR from mouse BMDMs pretreated with RAGE inhibitor (5 or 20 μM, 30 min), and then further treated with recombinant S100 protein (5 μg/ml, 3 hr) (n = 3). (D) Immunoblots of cellular lysates (Lys) from mouse BMDMs pretreated with AGEs (10 mg/ml, 18 hr), washed, and treated with LPS (0.5 μg/ml, for indicated time). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; $^{**}P<0.05$, $^{***}P<0.001$).

4. AGEs pretreatment inhibits M1 macrophage polarization

It is well-established that macrophage polarization is a finetuning process to adapt to changes in the microenvironment[31,32]. Therefore, I investigated whether AGEs could affect macrophage polarization. AGEs pretreatment inhibited LPS or IFN-γ induced expression of CXCL11, a distinct marker of M1 polarization (Fig. 5A). In addition, LPS-driven elevation in the level of M1 markers, inducible nitric-oxidase synthase (iNOS) was significantly reduced by AGE pretreatment (Fig. 5B). Then, I further explored the effect of AGEs on M2 polarization of macrophages. Unlike M1 polarization markers, AGEs failed to alter the expression level of M2 genes, such as Arg1 and Ym1, in BMDMs upon IL-4 stimulation. Overall, AGEs inhibit M1 polarization of macrophages which is known as pro inflammatory phenotype.

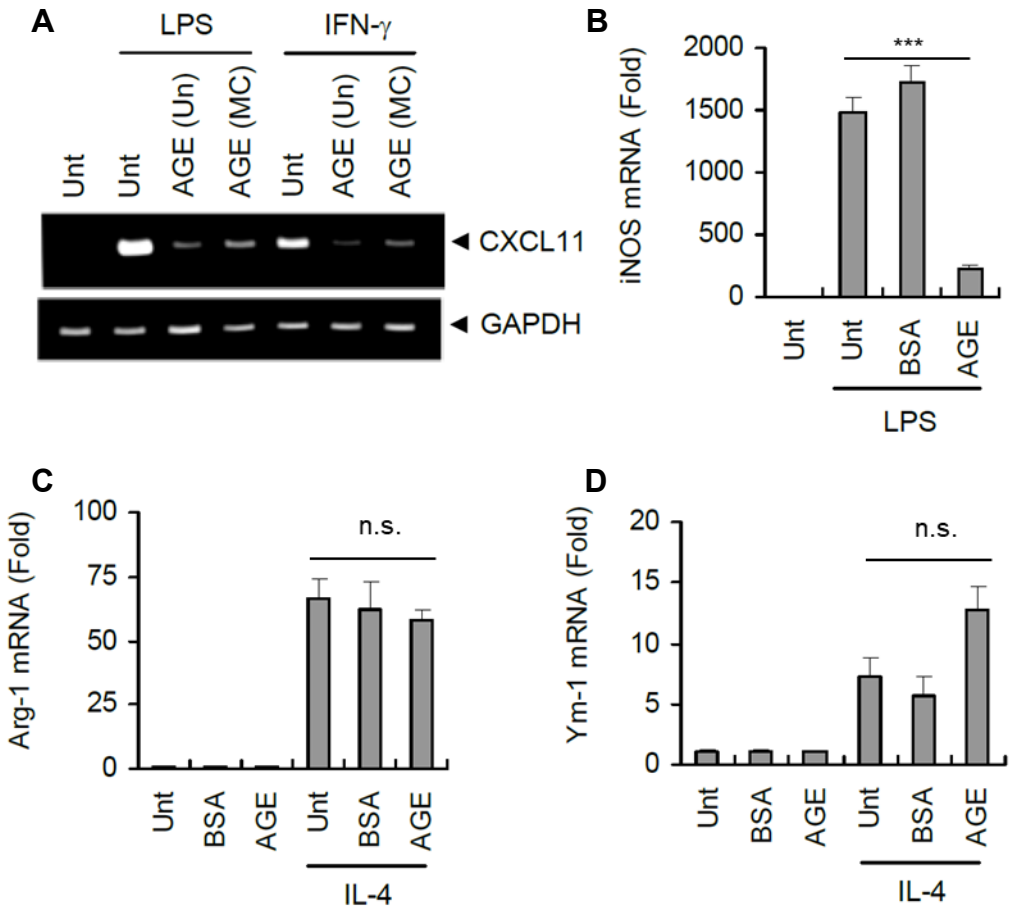


Figure 5. AGEs pretreatment inhibits M1 macrophage polarization. (A) RT-PCR of cxcl11 mRNA from mouse BMDMs pretreated with AGEs (10 mg/ml, 18 hr), washed, and treated with LPS (0.25 μg/ml, 6 hr) or IFNγ (12.5 ng/ml, 6 hr) . (B, C, D) Quantification of inos, arg1 or ym-1 mRNA level by qPCR from mouse BMDMs pretreated with AGEs or BSA (10 mg/ml, 18 hr) in presence of IL-4 (30 ng/ml, final 8 hr) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001).

5. AGEs pretreatment impairs host innate immune defense of macrophages against RNA virus infection

As previous data demonstrated that AGEs pretreatment caused a severe reduction in the TLR induced production of pro-inflammatory cytokines and activation of NLRP3 inflammasome, I further examined whether AGEs could affect host immune response in macrophages upon RNA virus infection. At first, to mimic RNA virus infection, I transfected Poly(I:C) to BMDMs. Consistent with previous data, pretreatment with AGEs, but not with BSA, abolished both caspase 1 activation and IL-1β secretion in BMDMs upon poly(I:C) transfection (Fig. 6A). Next, I measured interferon-β (IFNβ) production by macrophages in response to poly(I:C) transfection. Of note, AGEs pretreatment suppressed IFNβ mRNA production upon poly(I:C) transfection in BMDMs (Fig. 6B). Finally, I infected AGE pretreated BMDMs with influenza A virus. As poly(I:C) infection, AGEs pretreatment impaired influenza A virus induced casasp1 cleavage and IL-1β secretion, as well as IFNβ production and secretion in BMDMs (Fig. 6C-E). These findings suggest that AGEs could impair the host innate immune defense in macrophages against dsRNA-mediated virus infection.

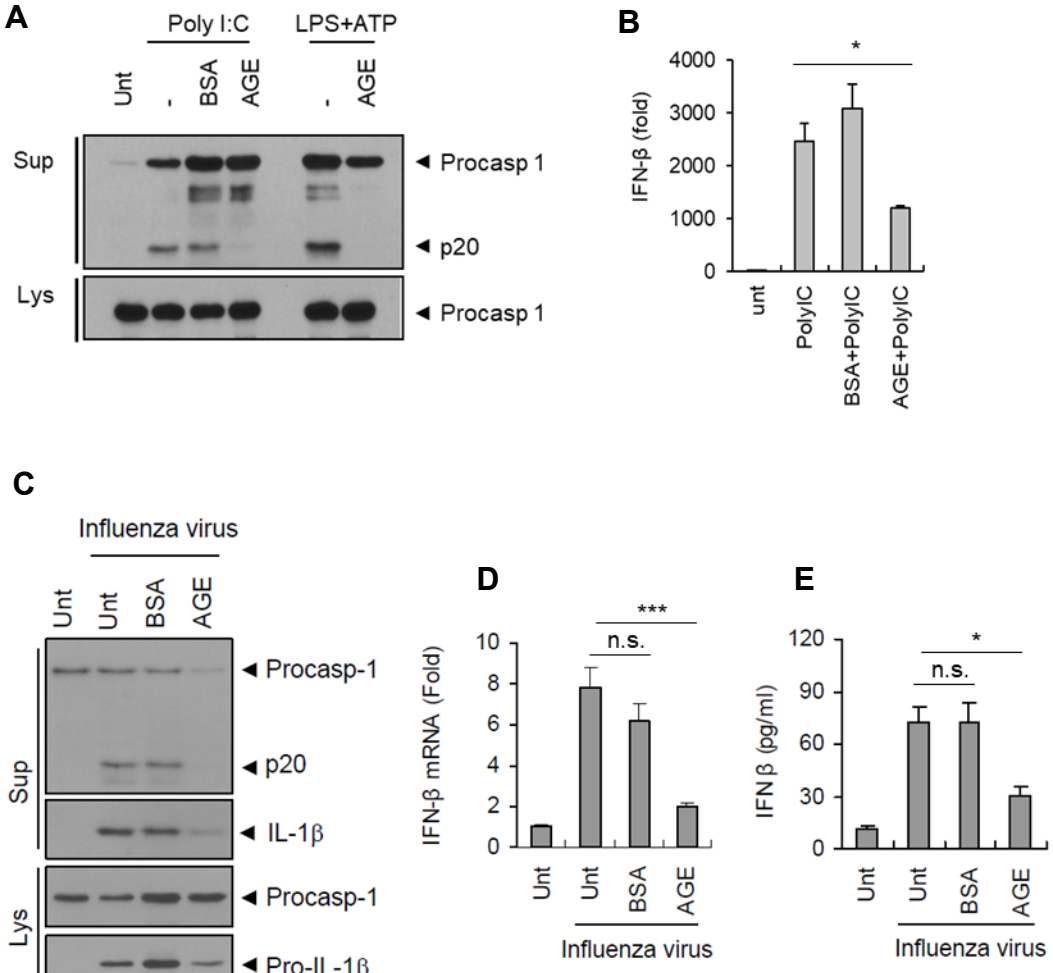


Figure 6. AGEs pretreatment impairs host innate immune defense of macrophages against RNA virus infection. (A) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with AGEs or BSA (10 mg/ml, 18 hr), washed, and transfected with Poly(I:C) (1 μg/ml, 3 hr) or treated with LPS (0.25 μg/ml, 3 hr), followed by treatment with ATP (2.5 mM, 30 min). (B) Quantification of IFN β mRNA level by qPCR from mouse BMDMs pretreated with AGEs or BSA (10 mg/ml, 18 hr), washed, and transfected with Poly(I:C) (1 μg/ml, 3 hr) (n = 3). (C) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMDMs pretreated with AGEs or BSA (10 mg/ml, 18 hr), washed, and infected with influenza A virus (deINS1/ PR8 strain, m.o.i of 3). (D, E) Quantification of IFN β in culture supernatants by ELISA or IFN β mRNA levels by qPCR from mouse BMDMs pretreated with AGEs or BSA (10 mg/ml, 18 hr), washed, and infected with influenza A virus (deINS1/ PR8 strain, m.o.i of 3) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; *$P< 0.01$, **P<0.05, ***P<0.001).

Part 2. Exclusive traits of neutrophils in NLRP3 inflammasome signaling contribute to prolonged inflammation in DAMPs-rich milieu

1. DAMPs-rich condition desensitize NLRP3 inflammasome activation but not in neutrophils

To investigate inflammasome signaling in neutrophils, I first administrated bone marrow-derived neutrophils (BMNs) with various inflammasome activators and assessed activation of inflammasome. LPS primed BMNs followed by ATP, nigericin (NLRP3 activator) treatment and flagellin (NLRC4 activator), polydAdT (AIM2 activator) transfection induced caspase1 cleavage and IL-1β secretion which shows NLRP3, NLRC4 and AIM2 inflammasome are activated in neutrophils, as well macrophages (Fig. 1A-D). Next I compared innate immune response between neutrophils and macrophages in DAMPs abundant milieu, as they migrate to inflammatory site. To mimic DAMPs derived from injured/ dead cells, I collected supernatant from staurosporine-induced apoptotic and freeze/thaw-induced ruptured BMDMs. Then collected supernatant was treated prior to LPS/ATP on BMNs and BMDMs. Unexpectedly, DAMPs containing sup pre-exposure fails to impair LPS/ATP or LPS/Nig induced cleaved caspase 1(p20) and IL-1β secretion in BMNs, in contrast to BMDMs (Fig. 2A-D). Then to mimic ATP rich milieu, I pre-exposed both cells with ATP and then activated NLRP3 inflammasome. Consistently, ATP pre exposure fails to impair LPS/ATP or LPS/Nig induced cleaved caspase 1(p20) and IL-1β secretion in BMNs, in contrast to BMDMs (Fig. 3A-D). This caspase 1 and IL-1β processing inhibiting effect was ATP exposure time dependent and specific to NLRP3 inflammasome in BMDMs (Fig. 3E, F).

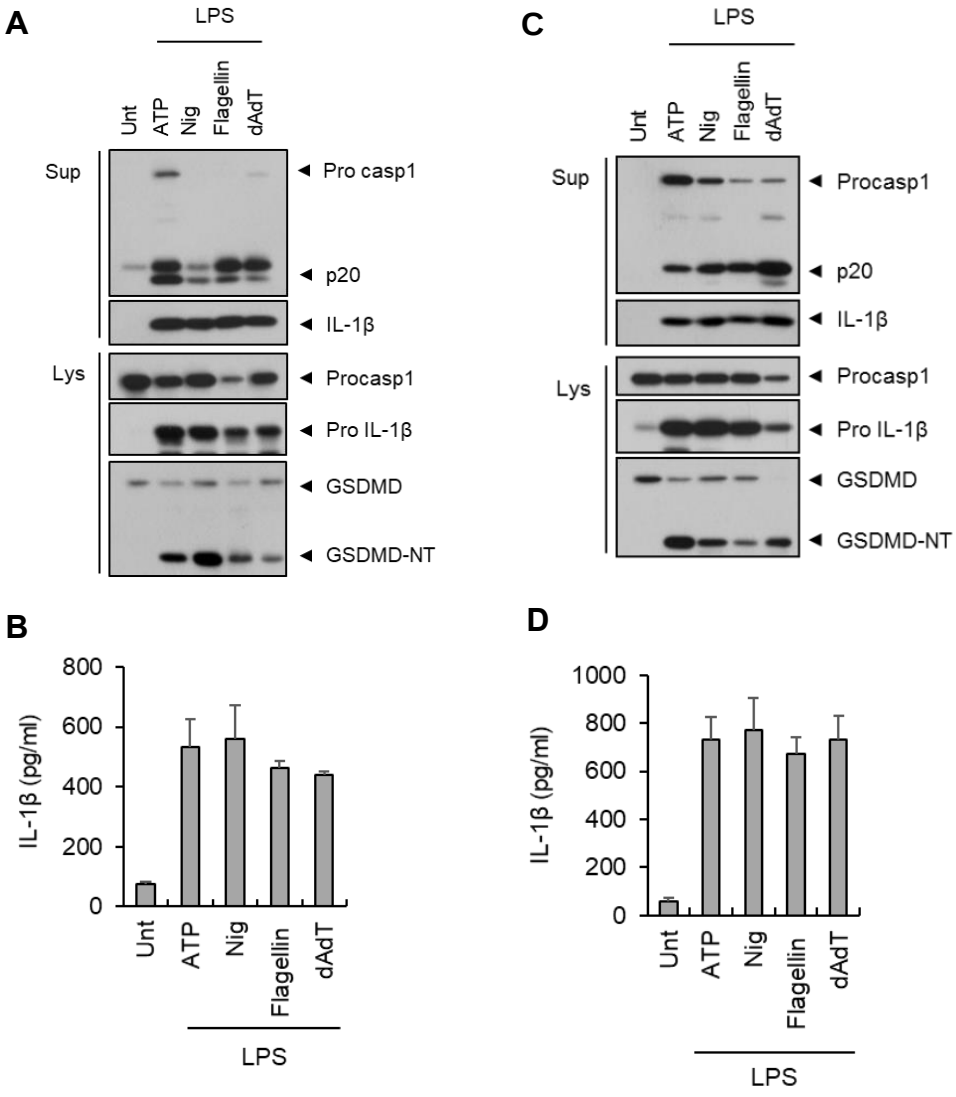


Figure 1. Neutrophils process IL-1β upon various inflammasome stimuli as well macrophages. (A, C) Immunoblots of cultural supernatants (Sup) and cellular lysates (Lys) or (B, D) quantification of IL-1β in culture supernatants from mouse BMNs(A, B) or BMDMs(C, D) treated with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2.5 mM, 30 min), nigericin (*Nig*, 5 μM, 45 min) or transfected with flagellin (500 μg/ml, 6 hr), poly(dA:dT) (1 μg/ml, 6hr) (n = 3).

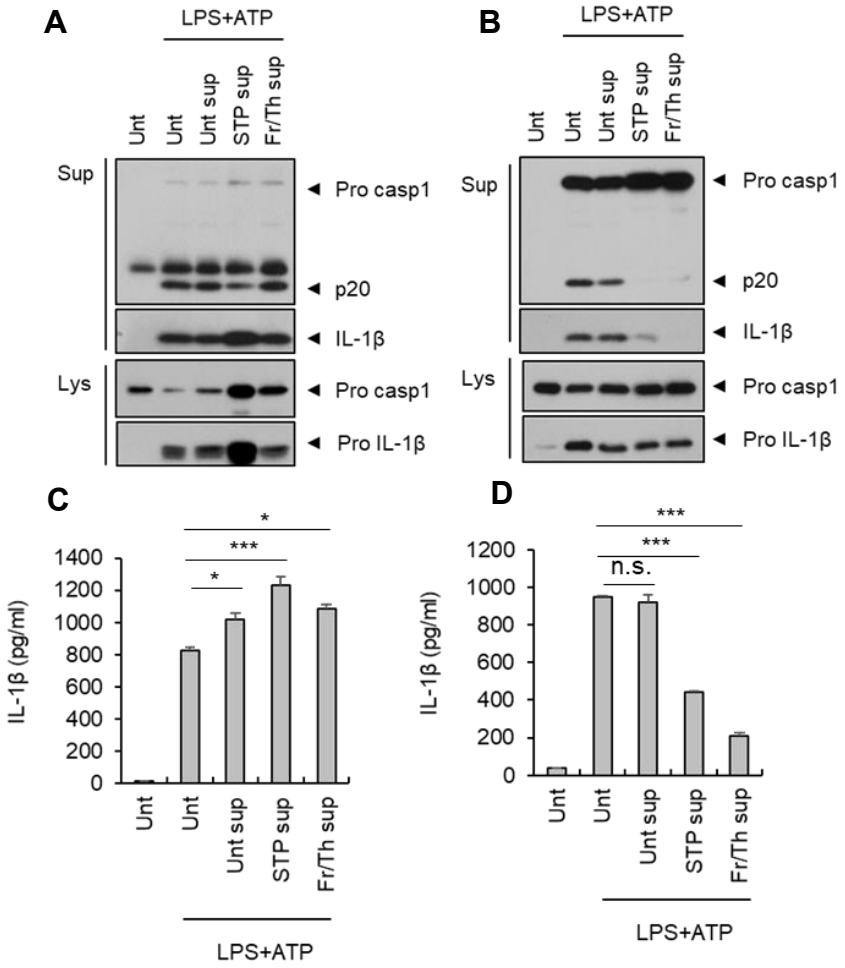
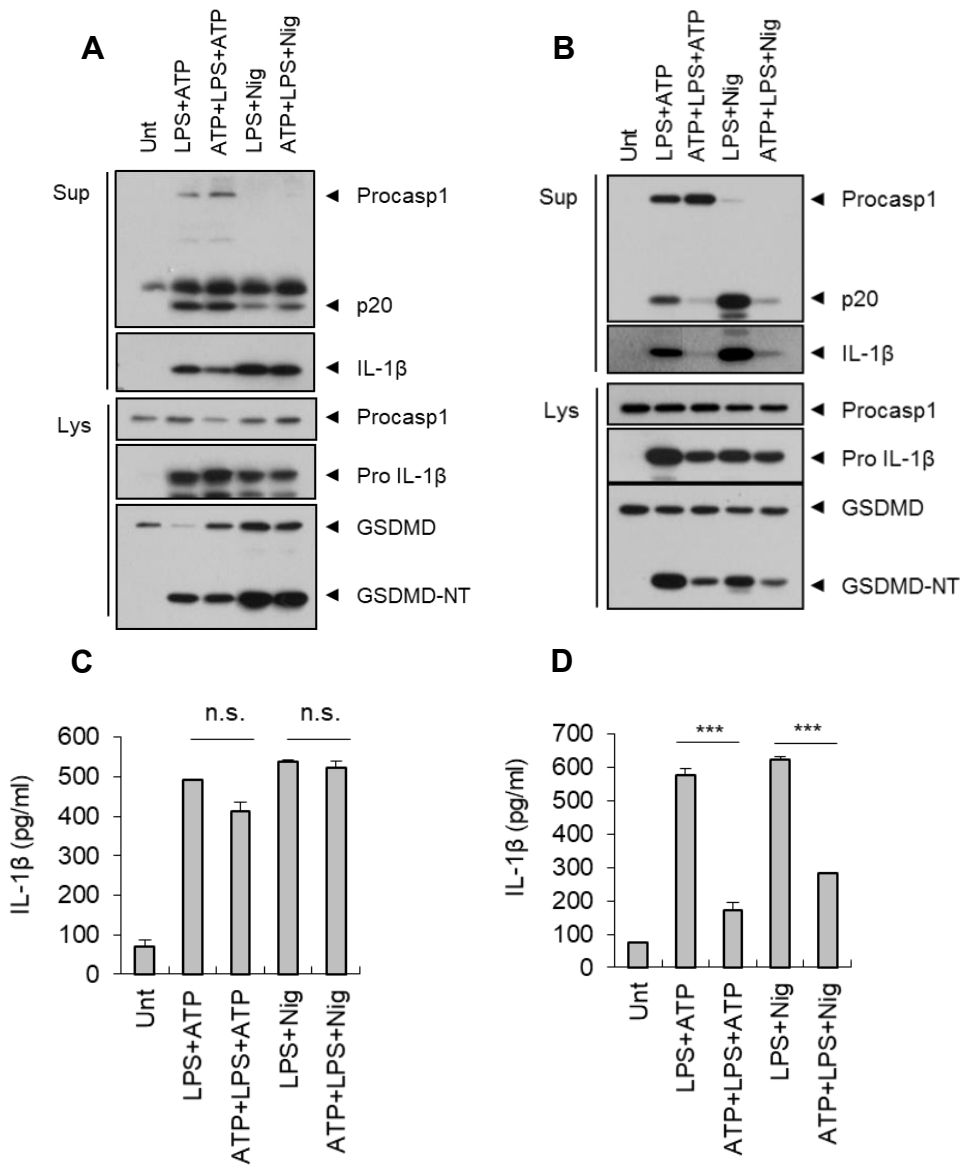


Figure 2. DAMPs containing sup exposure fails to impair NLRP3 inflammasome activation in neutrophils. (A, B) Immunoblots of cultural supernatants (Sup) and cellular lysate (Lys) or (C, D) quantification of IL-1β in culture supernatants from mouse BMNs(A, C) and BMDMs(B, D) pretreated with DAMPs-containing supernatants from BMDMs (1 hr ;Unt: untreated ;STP: staurosporine 2 μg/ml, 2 hr, washed then cultured 18 hr ;Fr/Th: freeze/thaw 4 cycle), washed, and treated with LPS (0.25 μg/ml, 2.5 hr) followed by treatment with ATP (2.5 mM, 30 min) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; *$P<0.01$, ***$P<0.001$).



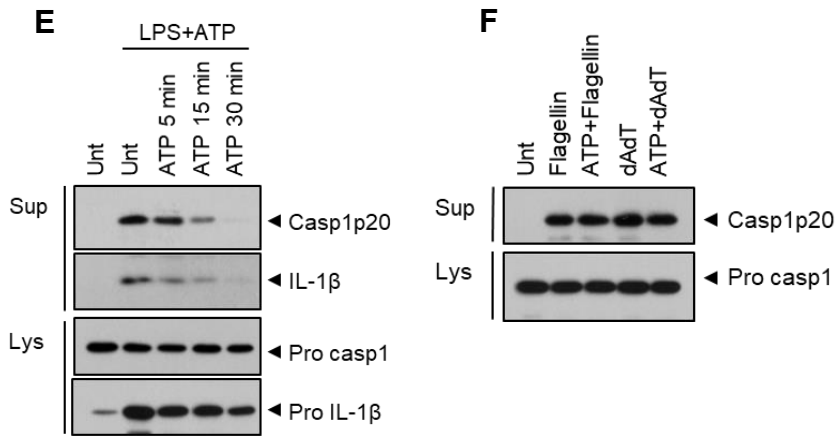
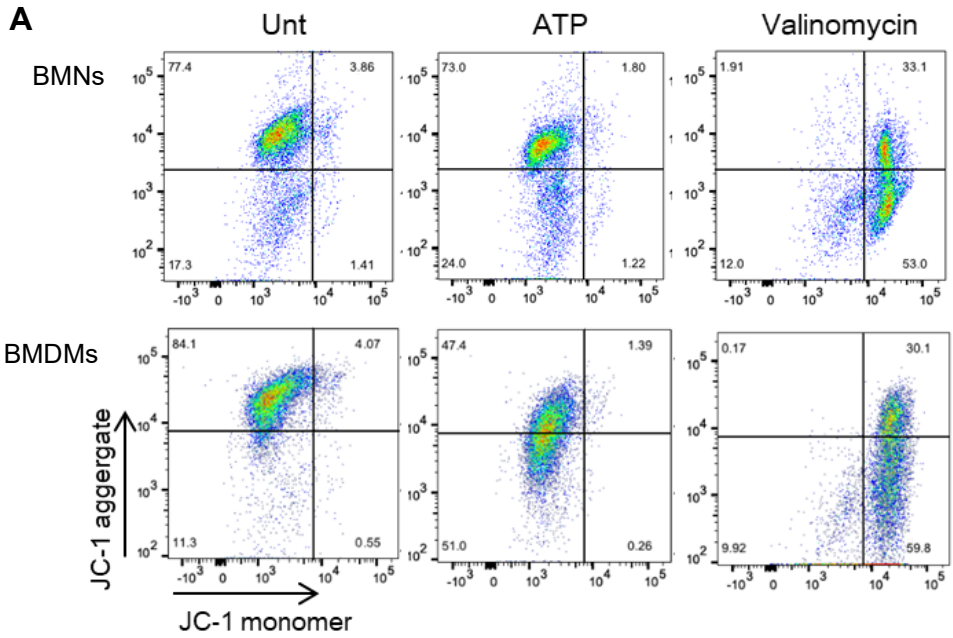
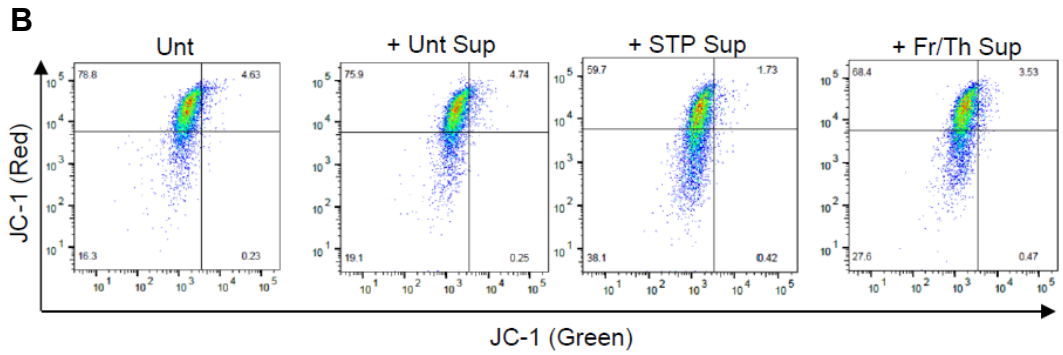


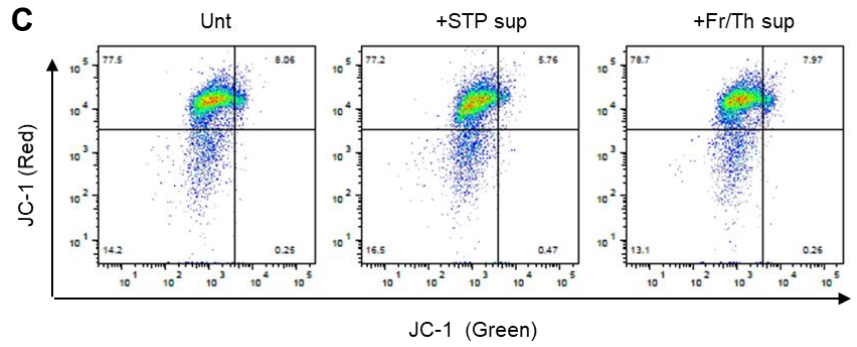
Figure 3. ATP exposure fails to impair NLRP3 inflammasome activation in neutrophils. (A, B, E) Immunoblots of cultural supernatants (Sup) and cellular lysates (Lys) or (C, D) quantification of IL-1β in culture supernatants from mouse BMNs(A, C) and BMDMs(B, D, E) pretreated with ATP (2.5 mM, 30 min or indicated time), washed, and treated with LPS (0.25 μg/ml, 2.5 hr) in fresh optiMEM, followed by treatment with ATP (2.5 mM, 30 min) or nigericin (5 μM, 45 min). (F) Immunoblots of cultural supernatants (Sup) and cellular lystates (lys) from mouse BMDMs pretreated with ATP (2.5 mM, 30 min), washed and transfected with flagellin (500 ng/ml, 4 hr) or polydAdT (2 ug/ml, 4 hr). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001).

2. Intact mitochondrial membrane potential facilitates NLRP3 inflammasome activation in neutrophils

Previously reported that ATP impaired mitochondrial membrane potential and this membrane potential is important for inflammasome activation[33-35]. Therefore, I evaluated mitochondrial membrane potential upon ATP treatment. Valinomycin triggered mitochondrial depolarization in both cells, however, ATP induced it only in BMDMs (Fig. 4A). Furthermore, DAMPs containing sup induced depolarization in BMDMs, but not in BMNs (Fig. 4B, C). Indeed, BMNs undergone mitochondrial depolarization by valinomycin failed to activate LPS/ATP induced NLRP3 inflammasome activation, parallel to BMDMs pretreated with ATP or valinomycin (Fig. 4D, E). Overall, unlike macrophages, neutrophils preserve their ability to activate NLRP3 inflammasome in DAMPs, such as ATP abundant milieu by maintaining mitochondrial membrane potential.







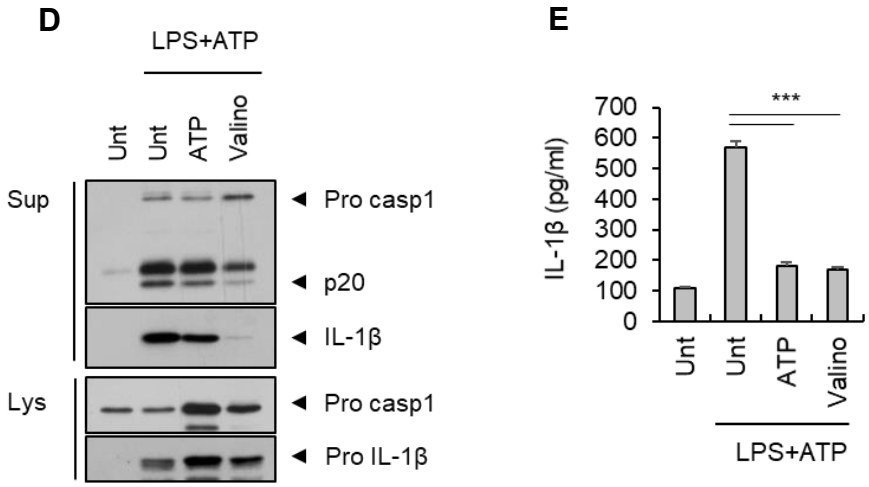
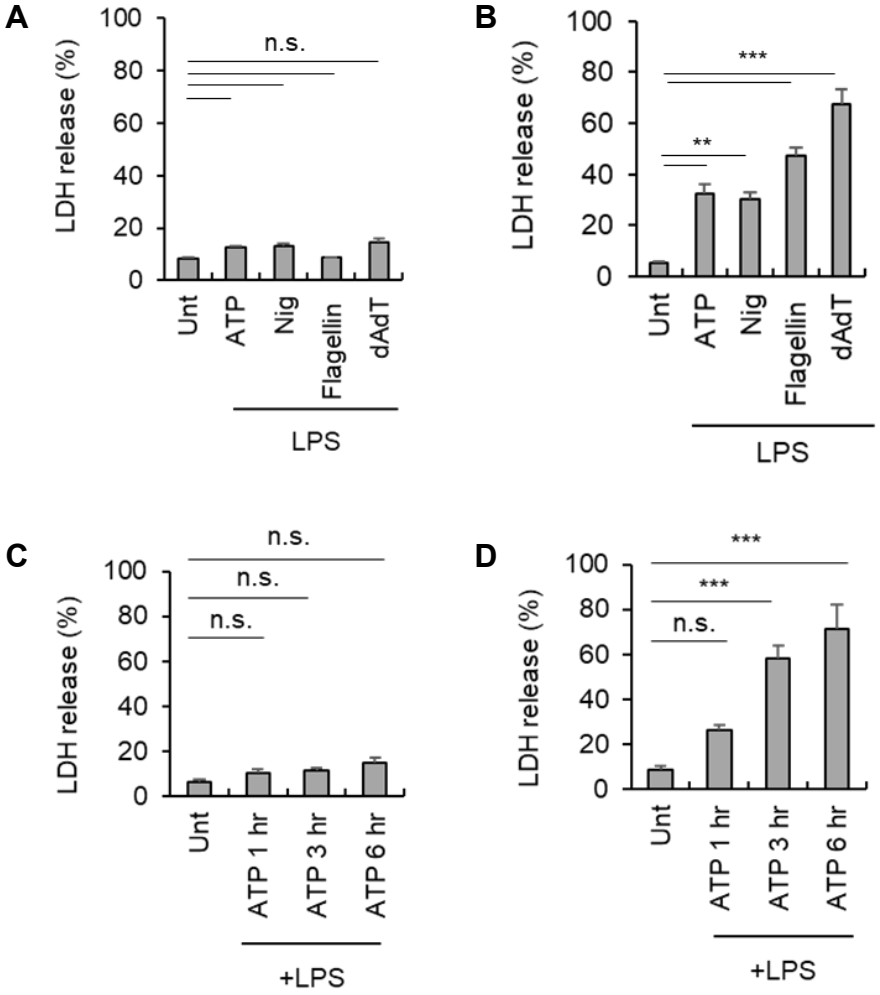


Figure 4. Intact mitochondrial membrane potential facilitates NLRP3 inflammasome activation in neutrophils at DAMPs-rich condition. (A-C) Flowcytometric analysis of mouse BMNs and BMDMs treated with ATP (2.5 mM, 30 min), valinomycine (10 μM, 30 min) or DAMPs-containing supernatants from BMDMs (1 hr ;Unt: untreated ;STP: staurosporine 2 μg/ml, 2 hr, washed then cultured 18 hr ;Fr/Th: freeze/thaw 4 cycle) after staining with JC-1 for mitochondria membrane potential. (D) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMNs or (E) quantification of IL-1β in culture supernatants by ELISA from mouse BMDMs, pretreated with ATP (2.5 mM, 30 min) or valinomycin (Valino; 10 μM, 30 min), washed, and treated with LPS (0.25 μg/ml, 2.5 hr) followed by treatment with ATP (2.5 mM, 30 min) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001).

3. Neutrophils showed impaired pyroptosis

As previous studies reported neutrophils do not undergo pyroptosis after inflammasome activation[14,15,17], I examined indeed neutrophils are lack of pyroptosis. BMNs showed less LDH release to extracellular upon various inflammasome activation compared to BMDMs (Fig. 5A, B). Consistently, BMNs showed less LDH release and propidium iodide staining upon LPS/ATP triggered NLRP3 inflammasome activation compared to BMDMs (Fig. 5C-F). To investigate whether neutrophils resist to lytic cell death, consequence of pyroptosis, released intracellular proteins in supernatant were immunoblotted. Unlike BMDMs, intracellular proteins including B actin, Pro caspase1, NLRP3 weren't released to extracellular in NLRP3 inflammasome activated BMNs (Fig. 6A). Furthermore, compared to BMDMs, BMNs preserved their plasma membrane integrity upon LPS/ATP induced NLRP3 inflammasome activation (Fig. 6B). Previous study reported pyroptotic macrophages also undergo apoptosis[36,37]. Therefore, I evaluated whether neutrophils proceed apoptosis even they resist to pyroptosis upon NLRP3 inflammasome activation. Unlike BMDMs which cleaved caspase 3 was detected, BMNs did not show cleaved caspase 3 after NLRP3 inflammasome activation (Fig. 7A). To examine neutrophils do undergo other types of cell death, I induced apoptosis and necroptosis. As BMDMs, BMNs showed increased LDH release after apoptosis and necroptosis (Fig. 7B). Finally, to investigate neutrophils indeed do not undergo cell death upon inflammasome activation *in vivo*, neutrophils and macrophages were analyzed from LPS injected mouse peritoneal lavage. Indeed, neutrophils didn't show PI staining upon LPS injection, while both resident and infiltrated macrophages show increased PI staining (Fig. 8A-C). Together, these results show that neutrophils resist to pyroptosis under NLRP3 inflammasome activation and resist to cell death is not a common characteristic of neutrophils as they undergo apoptosis and necroptosis.



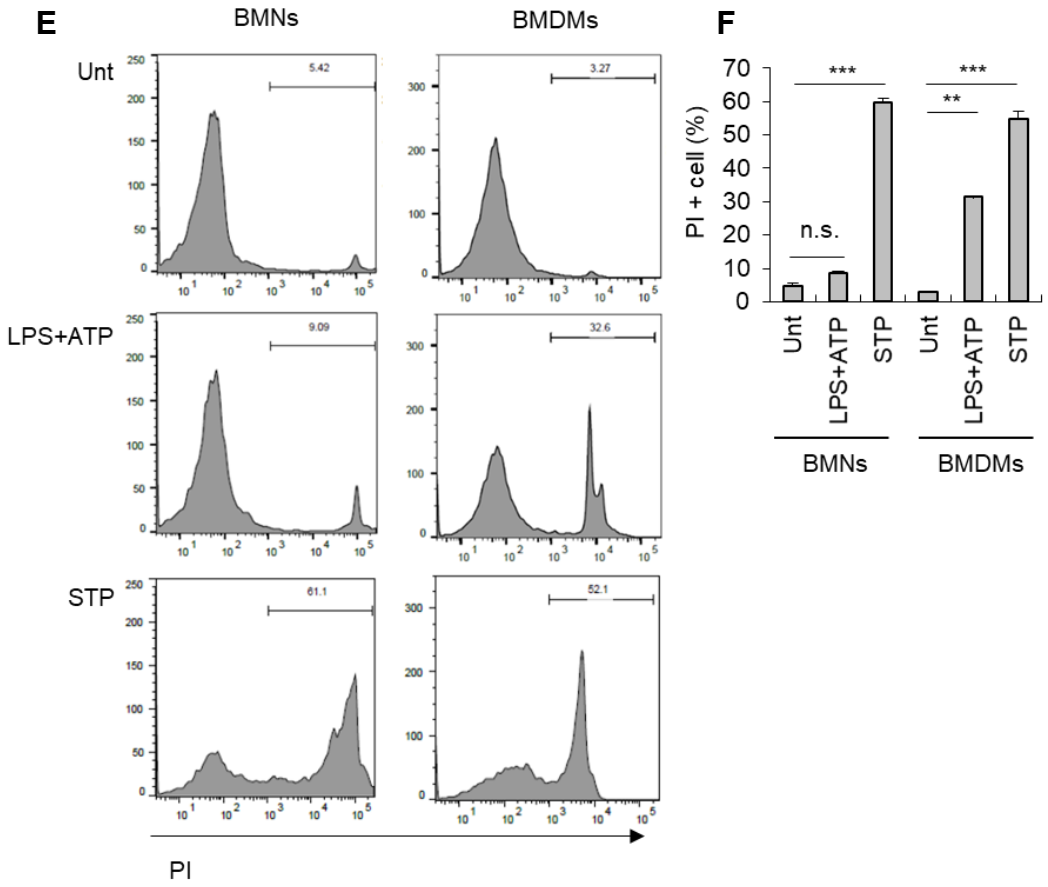


Figure 5. NLRP3 inflammasome activated neutrophils resist to cell death. (A-D) LDH release into culture supernatants of mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2.5 mM, indicated time), nigericin (*Nig*, 5 μM, 45 min) or transfected with flagellin (500 μg/ml, 6 hr), poly(dA:dT) (1 μg/ml, 6hr) (n = 3). (E, F) Flow cytometric analysis of mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2.5 mM, 1 hr) or staurosporine (STP; 2 μg/ml, 6 h), after staining with PI (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ** P<0.05, *** P<0.001).

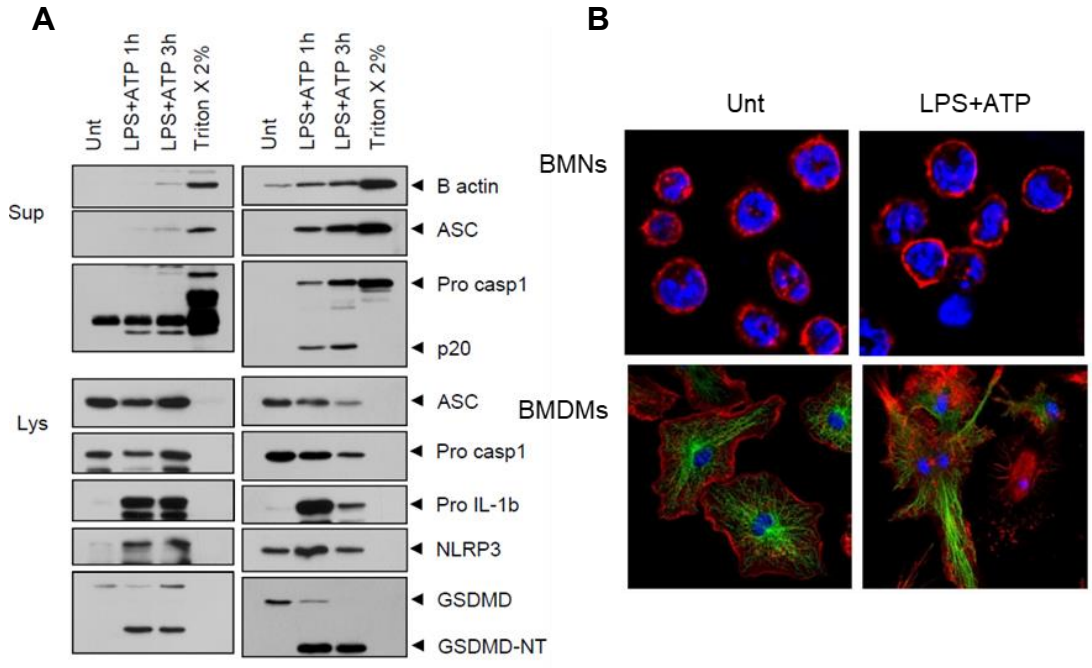


Figure 6. NLRP3 inflammasome activated-neutrophils resist to cell lysis. (A) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2.5 mM, 1 or 3 hr). Triton X (2 %, 30 min) were treated as positive control. (B) Representative immunofluorescence images of mouse BMNs and BMDMs untreated or primed with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2 mM, 1 hr), after staining with anti-F-actin (red), anti-α tubulin (green) and DAPI (blue).

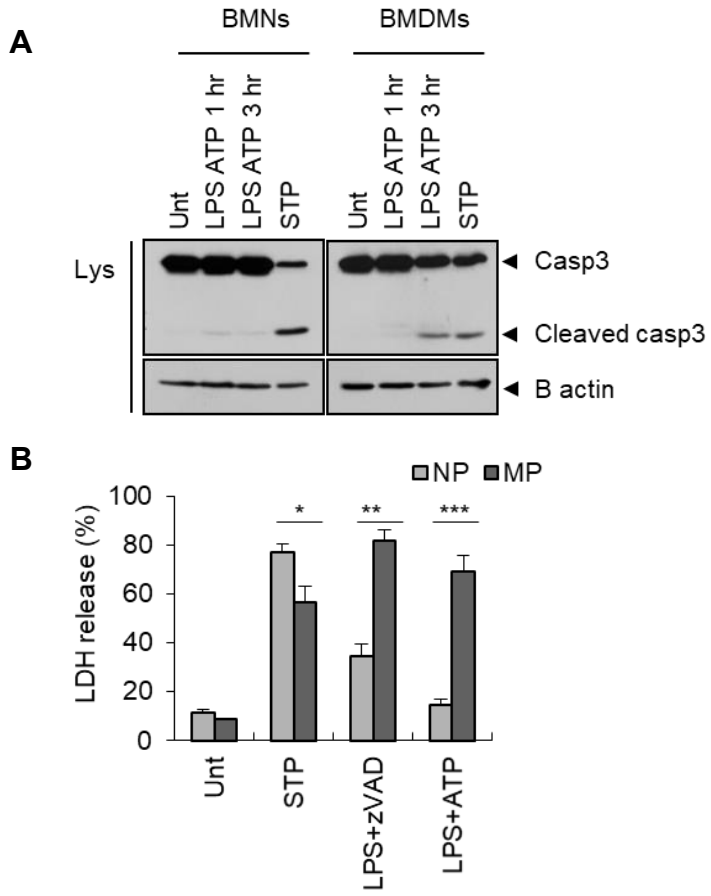
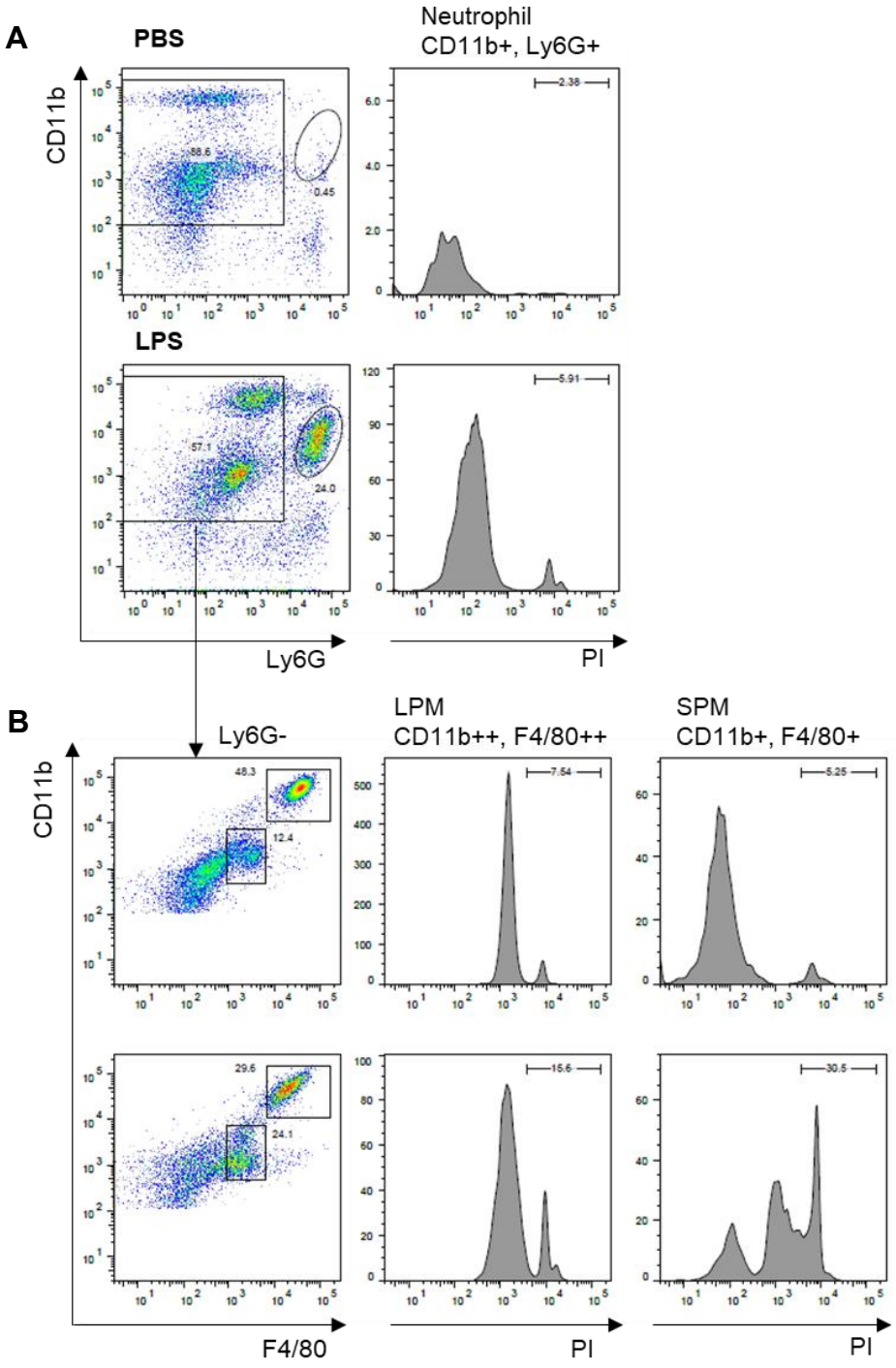


Figure 7. Neutrophils could proceed other types of cell death. (A) Immunoblots of cellular lysates (Lys) from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), followed by treatment with ATP (2.5 mM, 1 or 3 hr) or staurosporine (STP; 2 μg/ml, 6 hr). (B) LDH release into culture supernatants of mouse BMNs and BMDMs treated with staurosporine (STP; 2 μg/ml, 24 hr), untreated (Unt, 24 hr) or LPS (0.25 μg/ml, 24 hr) in the presence of zVAD (20 μM) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test, compared to untreated (compared to Unt, n.s., not significant; *$P<0.01$, **$P<0.05$, ***$P<0.001$).



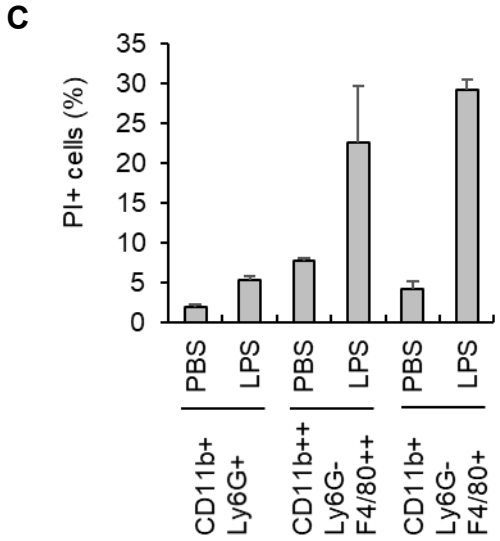
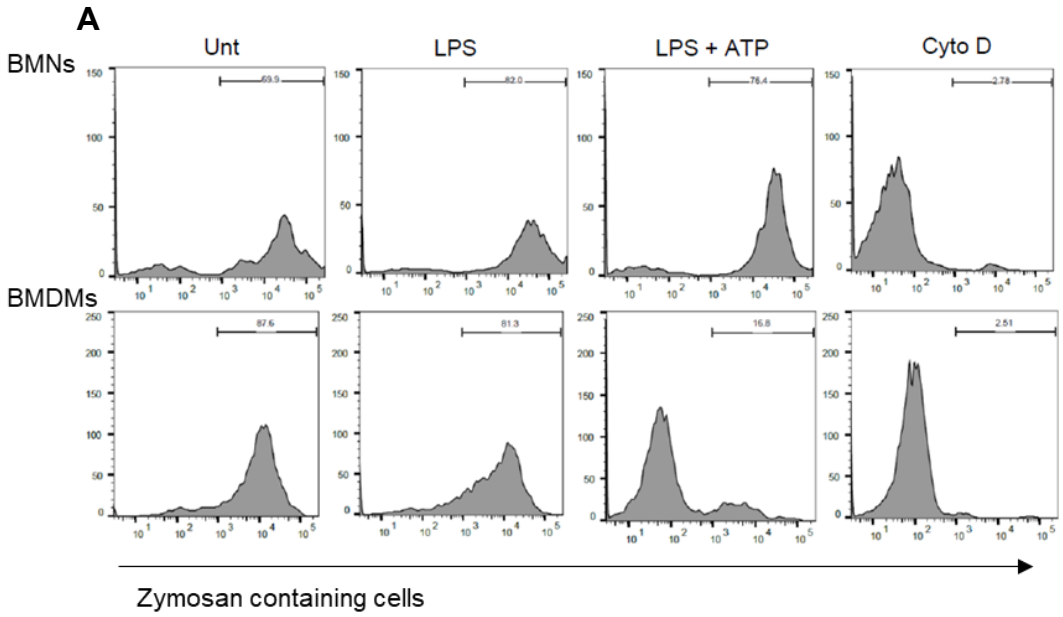
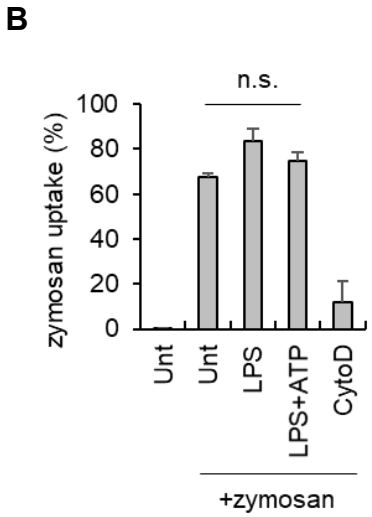


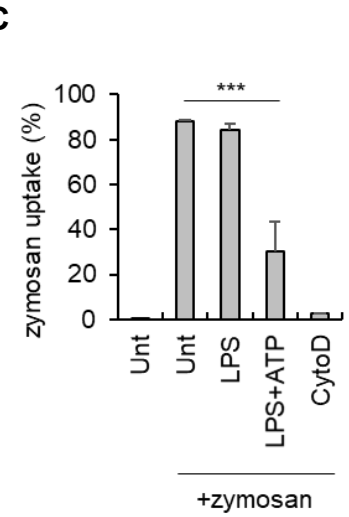
Figure 8. Neutrophils showed impaired cell death in inflammasome activating condition *in vivo*. (A-C) Percentage of PI+ cells among neutrophil ($CD11b^{+}$, $Ly6G^{+}$), Large peritoneal macrophage ($Ly6G^{-}$, $CD11b^{high}$, $F4/80^{high}$) or small peritoneal macrophage ($Ly6G^{-}$, $CD11b^{+}$, $F4/80^{+}$) in the peritoneal exudate of PBS or LPS (1 mg/kg, 24 h, i.p.) injected mouse, analyzed by flowcytometry. (C) Quantification of PI-positive cells in (A, B) (n = 2).

4. Neutrophils maintain their functional integrity upon NLRP3 inflammasome activation

To ascertain whether neutrophils maintain their function after NLRP3 inflammasome activation as they resist pyroptosis, phagocytosis ability of BMNs and BMDMs were examined. Unlike BMDMs, BMNs were able to phagocyte zymosan after LPS/ATP triggered NLRP3 inflammasome activation (Fig. 9A-C). As neutrophils chemotaxis to inflammatory site, migration ability of neutrophils followed by NLRP3 inflammasome activation was investigated. As expected, BMNs maintained migration ability after LPS/ATP triggered inflammasome activation, compared to LPS treatment (Fig. 9D, E). There were no differences in speed, however both LPS and LPS/ATP treated BMNs decreased their straightness. Lastly, as neutrophils contain large amounts of granules, I explored whether degranulation changes upon NLRP3 inflammasome activation. PMA induced degranulation of MMP9 wasn't affected by LPS/ATP treatment in BMNs (Fig. 9F). Overall, unlike macrophages, neutrophils maintain their various function even after NLRP3 inflammasome activation, indirectly implicating their contribution to prolong inflammation.







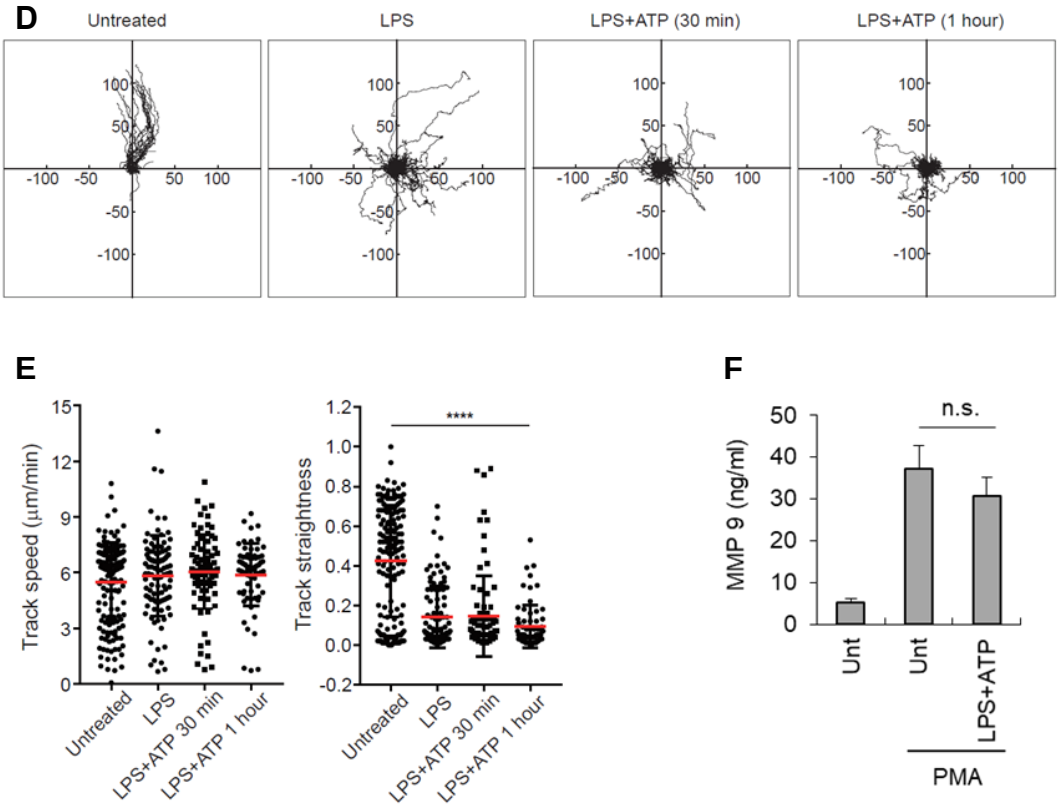


Figure 9. NLRP3 inflammasome activated neutrophils maintain their function. (A-C) Flowcytometric analysis of mouse BMNs(B) and BMDMs(C) treated with LPS (0.25 μg/ml, 3 hr), followed by ATP (2.5 mM, 30 min), or cytochalasin D alone (10 μM, 30 min), after treatment with zymosan-FITC (1:5 ratio, 30 min) for phagocytosis assay (n = 3). (D, E) Migration analysis of BMNs treated with LPS (0.25 μg/ml, 3 hr) alone or followed by ATP (2.5 mM, 30 min), after cells were captured for every 30 sec for 30 min. (F) Quantification of MMP 9 in culture supernatants by ELISA from mouse BMNs treated with LPS (0.25 μg/ml, 3 hr), followed by ATP (2.5 mM, 1 hr) or untreated (4 hr), washed, and then treated with PMA (1 μM, 2 hr) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001).

5. Impaired GSDMD cleavage and mitochondrial membrane potential loss by NLRP3 stimulation could contribute to resistance on pyroptosis

To understand factors manipulating resistance on pyroptosis in neutrophils, I investigated components of NLRP3 inflammasome and pyroptosis. BMNs and BMDMs expressed similar level of GSDMD. However, BMNs showed less procaspase 1 compared to BMDMs, leading to fewer cleavage of GSDMD upon LPS/ATP or LPS/Nig induced NLRP3 inflammasome activation. (Fig. 10A, B). Still, BMNs cleaved comparable IL-1β from similar level of pro form to BMDMs. And these IL-1β cleavage was highly dependent on caspase 1 (Fig. 12B), indicating just relatively low level of caspase 1 is not sufficient for resistance on pyrotosis. To examine GSDMD kinetics in neutrophils, GSDMD-NT was immunoblotted in LPS primed BMNs followed by 1, 3 and 6 hr ATP treatment. Opposed to BMDMs, GSDMD-NT was decreased on ATP 6h in BMNs (Fig. 10C). Finally, recent study reported SARM-dependent mitochondrial membrane potential regulates pyroptosis during inflammasome activation[38]. Consistently, LPS/ATP treated BMDMs showed depolarized mitochondrial membrane potential, while BMNs did not (Fig. 11A). To further investigate the role of mitochondrial membrane potential in resistance on pyrotosis, I depolarized mitochondrial membrane potential with valinomycin administration, after LPS/ATP triggered NLRP3 inflammasome activation in BMNs. Valinomycin treatment didn't increased GSDMD cleavage, while increased LDH release in BMNs (Fig. 11B). In addition, SARM was expressed in BMDMs, while BMNs did not, partly explaining previous data (Fig. 11C). Altogether, neutrophils express relatively low level of caspase 1 compare to macro phages, leading to fewer GSDMD cleavage, an executioner of pyroptosis. Furthermore, GSDMD kinetics along with intact mitochondrial membrane potential could partially contribute to resistance on pyroptosis upon NLRP3 inflammasome activation in neutrophils.

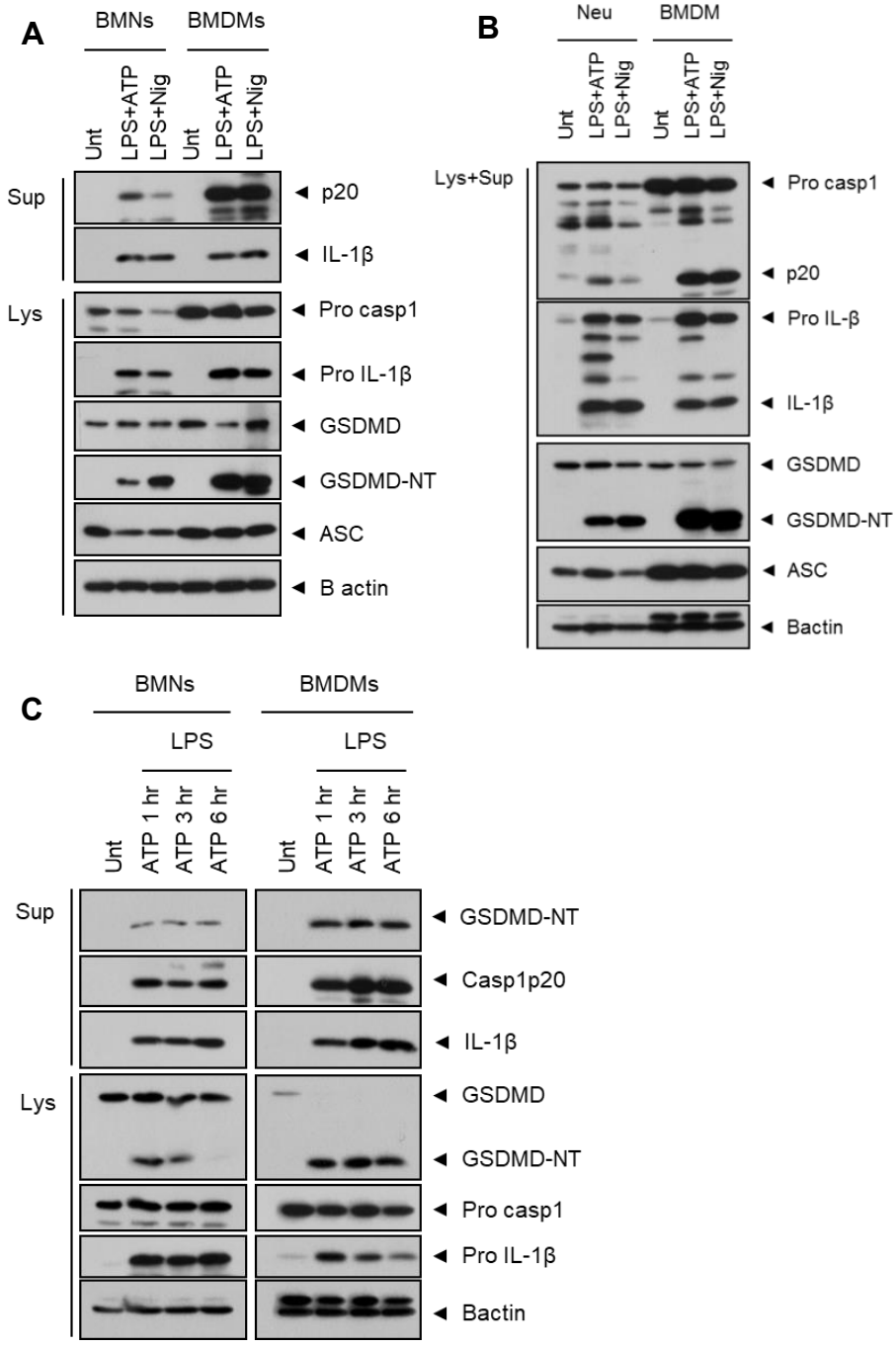


Figure 10. Neutrophils showed impaired GSDMD cleavage by NLRP3 stimulation. (A, B) Immunoblots of cultural supernatants (Sup) and cellular lysates (Lys) together or respectively from mouse BMNs or BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), washed, and then treatment with ATP (2.5 mM, 1 hr) or nigericin (5 μM, 1 hr) in fresh optiMEM. (C) Immunoblots of cultural supernatants (Sup) or cellular lysates (Lys) from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), washed and then treatment with ATP (2.5 mM, 1, 3 or 6 hr).

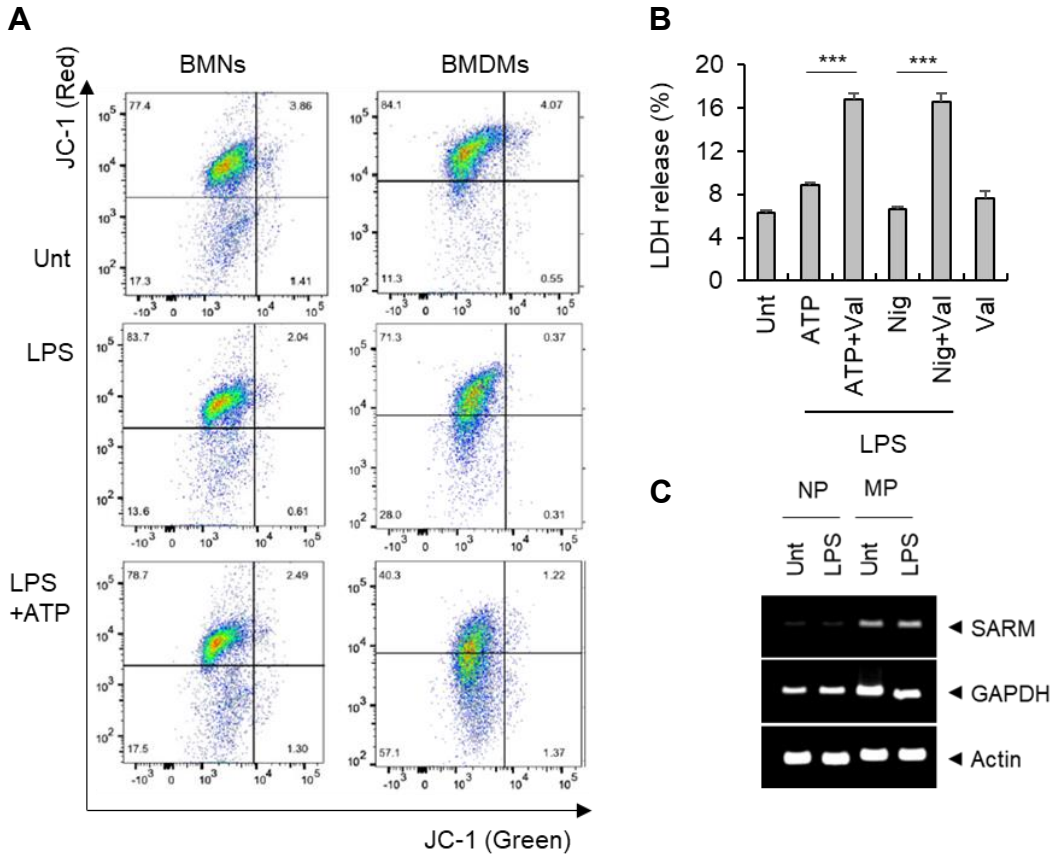
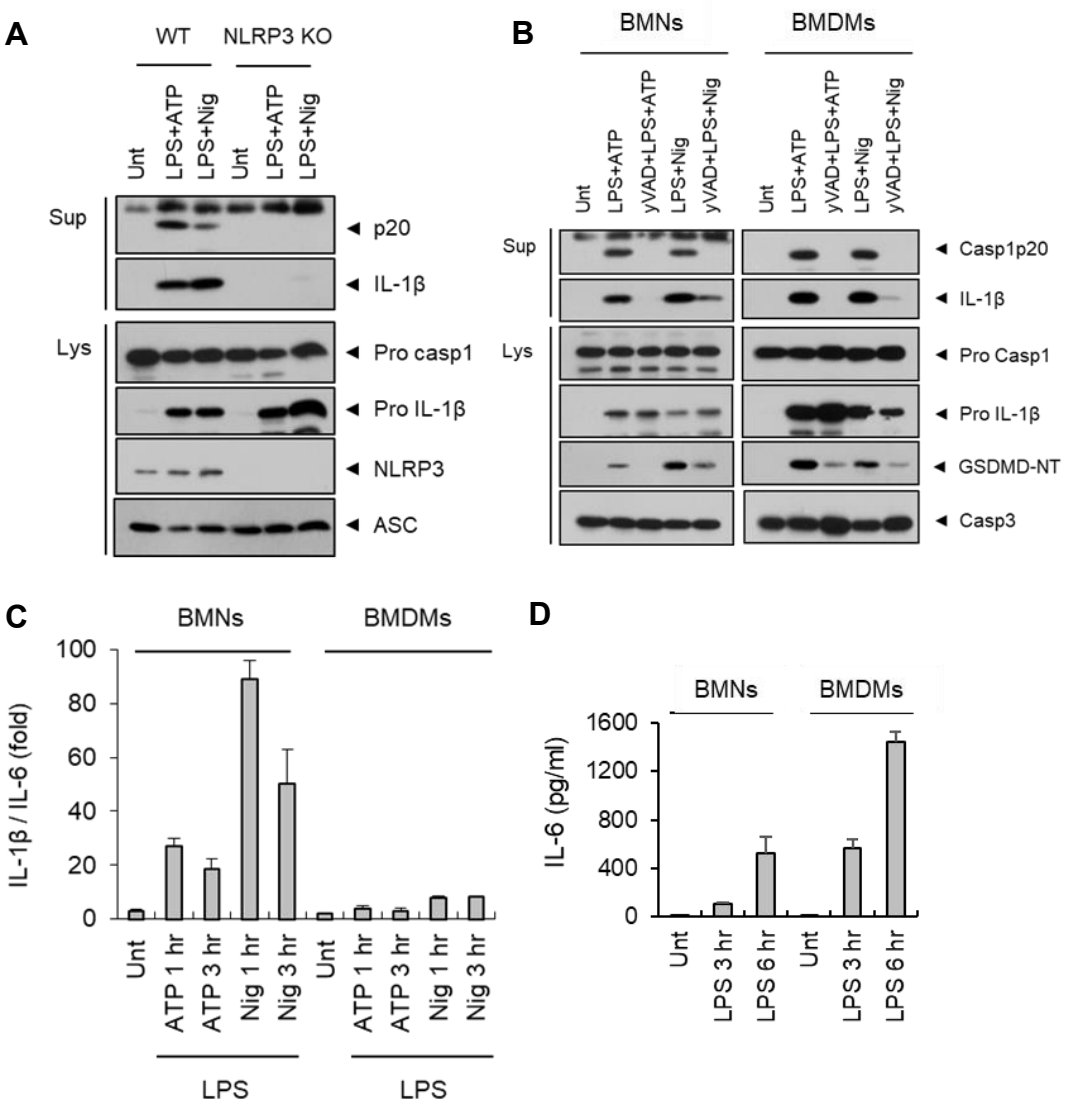


Figure 11. Neutrophils showed impaired mitochondrial membrane potential loss by NLRP3 stimulation. (A) Flowcytometric analysis of mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr), followed by ATP (2.5 mM, 30 min), after staining with JC-1 for mitochondria membrane potential. (B) LDH release into culture supernatants from mouse BMNs treated with LPS (0.25 μg/ml, 2.5 hr) followed by ATP (2.5 mM, 1 hr) in the presence of valinomycin (Val; 10 μM, 10 min after ATP treatment) (n = 3). (C) RT PCR of sarm mRNA of mouse BMNs treated with LPS (0.5 μg/ml, 3 hr). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001).

6. Neutrophils showed distinctive IL-1β secretion patterns

Previously reported that GSDMD pore is important for pyroptosis but also for IL-1β secretion[3,39]. According to data presented above, neutrophils have fewer cleaved GSDMD which could rise possibility that they might have unique IL-1β secretion system. Still, neutrophils processed and secreted IL-1β in NLRP3 and caspase-1 dependent pathway (Fig. 12A, B). To investigate cytokine secretion of neutrophils in detailed, I first compared secreted IL-1β level between macrophages and neutrophils. Interestingly, comparing secreted IL-1β level with IL-6, neutrophils seem to be more focused on IL-1β secretion upon NLRP3 inflammasome activation (Fig. 12C). Indeed, LPS triggered production of various cytokines were lower in BMNs compared to BMDMs (Fig. 12D, F-H), while LPS/ATP induced IL-1β level was comparable (Fig. 12E). Next, to explore neutrophils' dependency on GSDMD in IL-1β secretion, I obtain BMDMs and BMNs from GSDMD deficient mouse. GSDMD deficient BMDMs didn't secrete IL-1β upon inflammasome activation, while BMNs released IL-1β even the amount was decreased, indicating neutrophils are less dependent on GSDMD for IL-1β secretion (Fig. 13A-C). Interestingly, BMNs secreted IL-1β rapidly (within 10 min after ATP treatment) compared to BMDMs upon LPS/ATP stimulation (Fig. 14A, B). In sum, neutrophils seem to secrete IL-1β more specifically and rapidly with less dependency on GSDMD compared to macrophages.



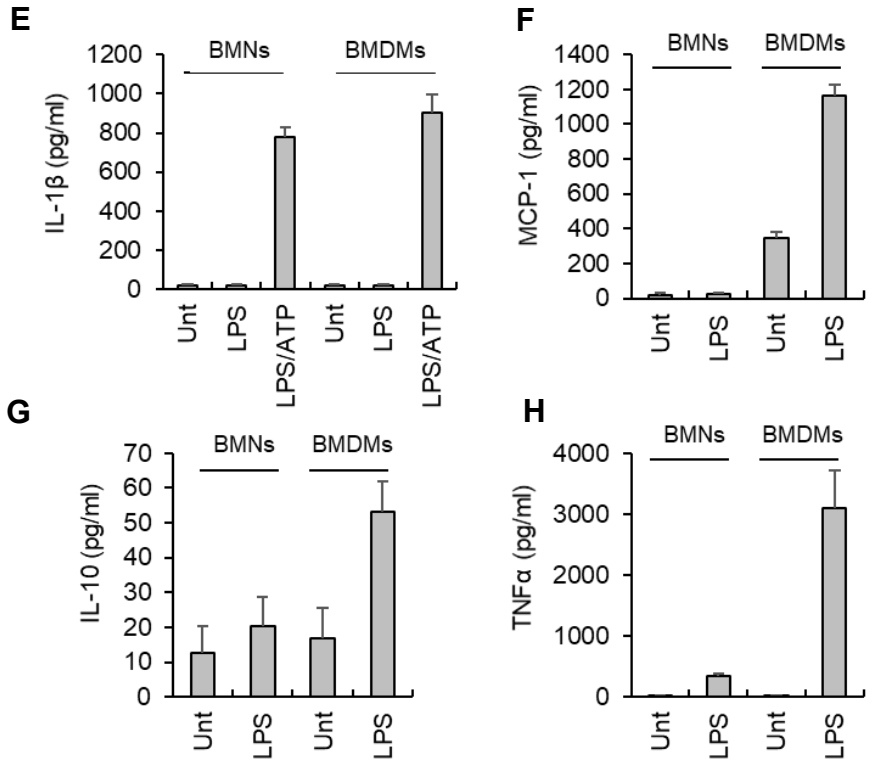


Figure 12. Distinctive IL-1β secretion patterns in neutrophils. (A, B) Immunoblots of cultural supernatants (Sup) and cellular lysates (Lys) together from WT/ NLRP3 KO mouse BMNs(A) or WT BMDMs(B) treated with LPS (0.25 μg/ml, 3 hr) followed by ATP (2.5 mM, 1 hr) or nigericin (5 μM, 1 hr) in presence of yVAD(20 μM). (C) Quantification of IL-1β per IL-6 in culture supernatants from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 3 hr) followed by ATP (2.5 mM, 1, 3 hr) or nigericin (5 μM, 1, 3 hr) (n = 3). (D-H) Quantification of IL-6(D), IL-1β(E), MCP-1(F), IL-10(G) or TNFα(H) in culture supernatants from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, for 3 hr or indicated time) (n = 3).

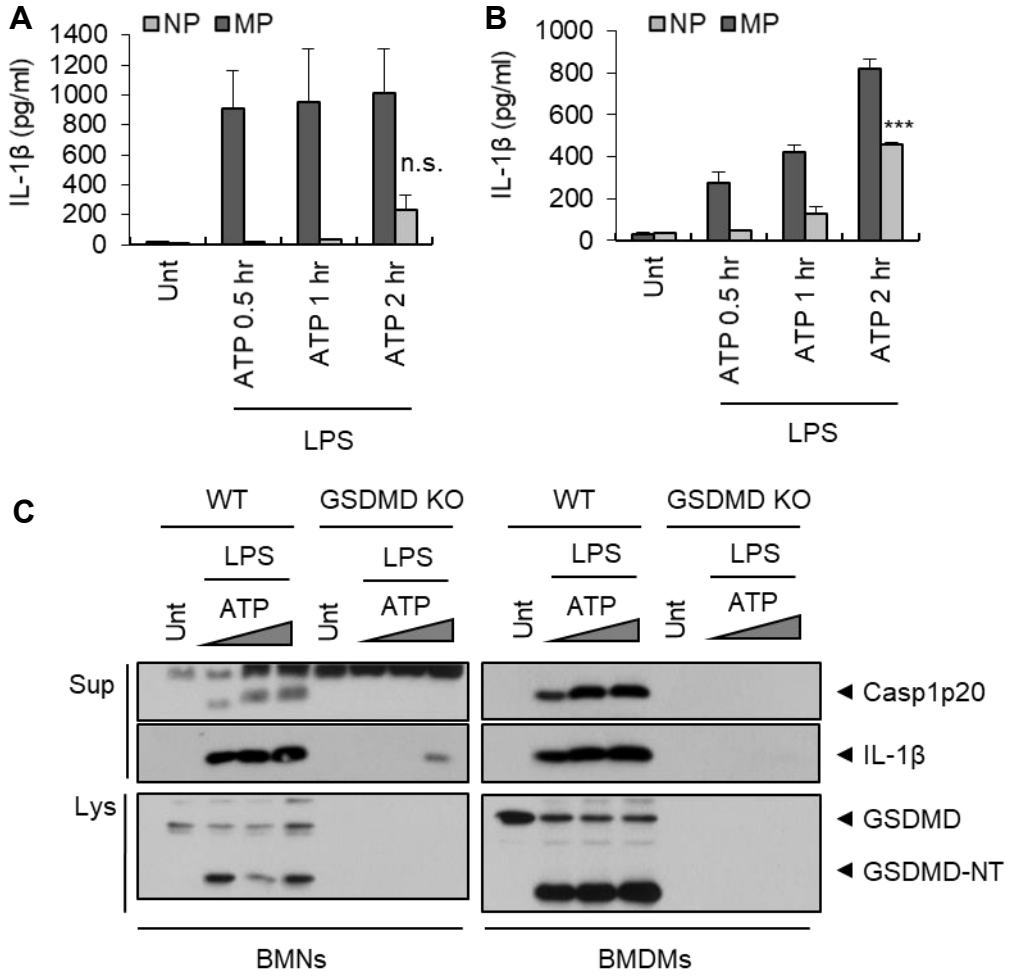


Figure 13. IL-1β secretion is less dependent on GSDMD in neutrophils. (A, B) Quantification of IL-1β in culture supernatants or (C) immunoblots of supernatants (Sup) and cellular lysates (Lys) from WT/ GSDMD KO mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr) followed by ATP (2.5 mM, 0.5 hr, 1 hr, 2 hr) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; ***P<0.001, compared to Unt).

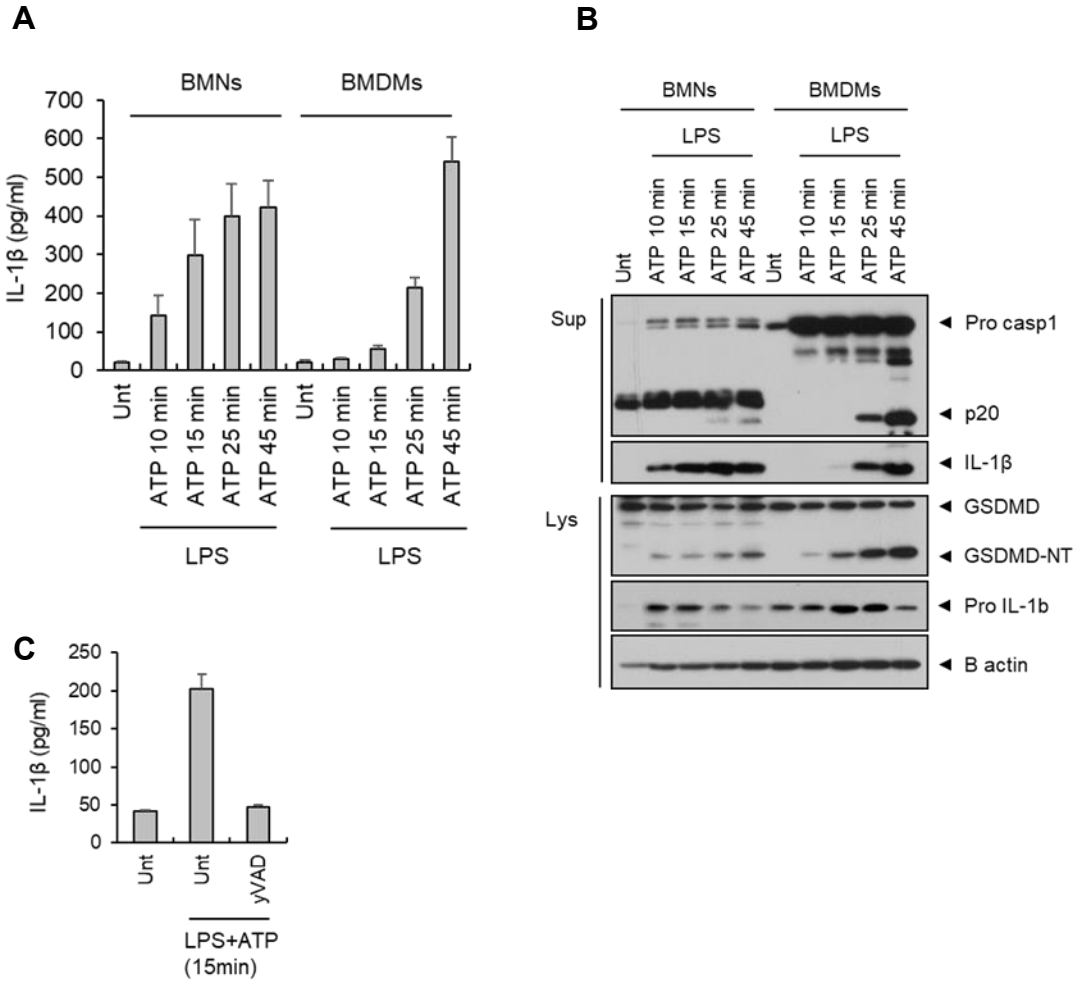


Figure 14. Rapid IL-1β secretion in neutrophils. (A, C) Quantification of IL-1β in culture supernatants or (B) immunoblots of supernatants (Sup) and cellular lysates (Lys) from mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 2.5 hr) followed by ATP (2.5 mM, for indicated time) in presence of yVAD(20 μM) (C) (n = 3).

7. NLRP3 inflammasome activated neutrophils failed to induce efferocytosis

It is well accepted that cell death of neutrophils is important for resolution of inflammation, by inducing efferocytosis.[13] Therefore, I assessed consequences of inflammasome activated neutrophils as they do not undergo cell death. First, exposure of typical eat me signal, phosphatidyl serine was analyzed by binding of annexin V. As reported, LPS/ATP treated BMDMs exposed phosphatidylserine on their surfaces[40,41], while BMNs did not. Still both cells exposed phosphatidylserine upon apoptosis (Fig. 15A). Next, I investigated whether inflammasome activated neutrophils could trigger efferocytosis. Along with previous reports, staurosporine induced apoptotic BMNs induce IL-10 mRNA in peritoneal macrophage (Fig. 15B). Furthermore, they leaded peritoneal macrophage less sensitive to up-regulate IL-6 and TNFα mRNA in response to additional LPS stimulation (Fig. 15C, D). In contrast, NLRP3 inflammasome activated BMNs failed to trigger anti-inflammatory cytokine in peritoneal macrophages (Fig. 15B). Also, they failed to make peritoneal macrophages immunologically silent (Fig. 15C, D). In sum, unlike apoptotic neutrophil, NLRP3 inflammasome activated neutrophil failed to induce efferocytosis, which could contribute to prolonged inflammation.

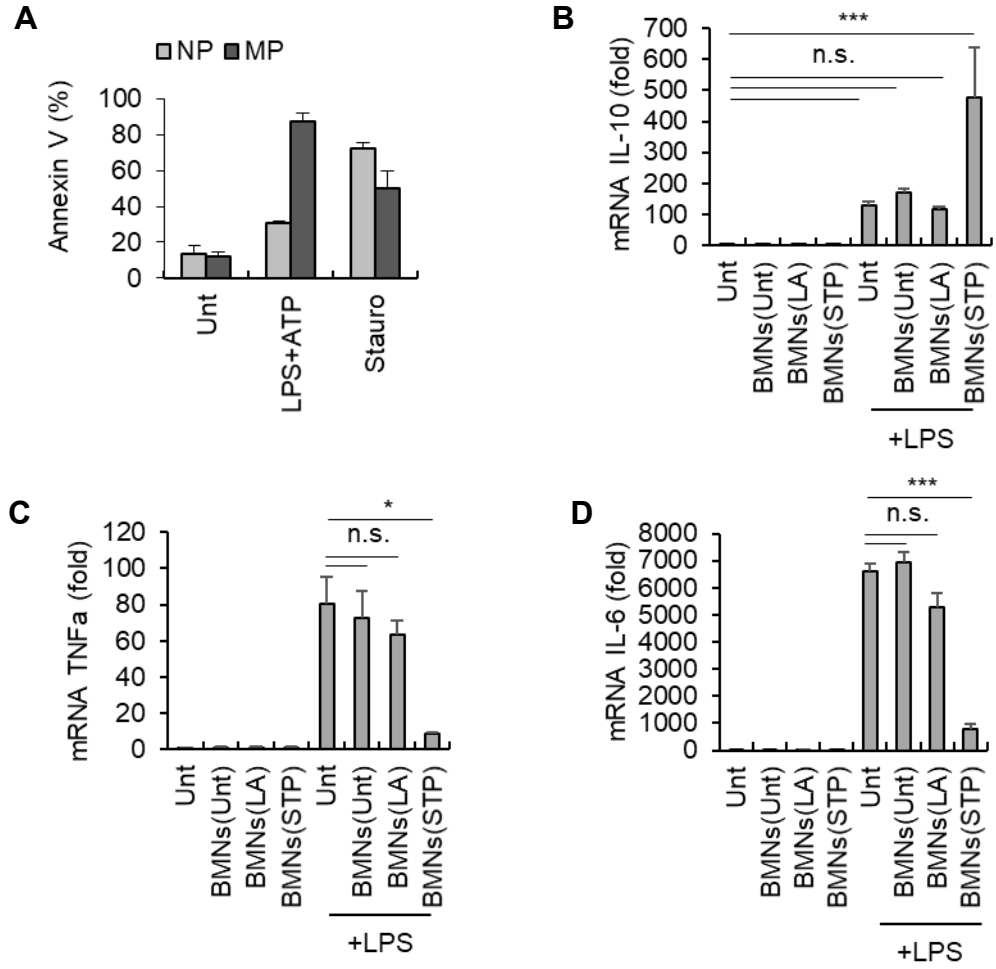


Figure 15. NLRP3 inflammasome activated neutrophils do not induce efferocytosis. (A) Flowcytometric analysis of mouse BMNs and BMDMs treated with LPS (0.25 μg/ml, 3 hr), followed by ATP (2.5 mM, 2 hr) or staurosporine (STP; 2 μg/ml, 5 hr, after staining with Annexin V. (B,C,D) Quantification of cytokines (IL-10, IL-6, TNFα) mRNA level from mouse peritoneal macrophage treated with mouse BMNs (Unt; untreated, LA; LPS+ATP, STP; staurosporine treated cells, 1:3, 18 hr), washed, and then treated with LPS (0.1 μg/ml, 18 hr) (n = 3). Statistical significance was determined by one-way ANOVA with a bonferroni post-test (n.s., not significant; *P<0.01 ***P<0.001).

IV. DISSCUSSION

Our innate immune system recognizes PAMPs from pathogens or DAMPs from injured tissues and proceed immune response under tight regulation. Therefore, when the balance is broken, suppressed or excessive immune response could lead to severe disease.[1] NLRP3 inflammasome can also work in host defense by detecting DAMPs, but its role is more focused on recognizing DAMPs. Accumulating evidences suggest that DAMPs-like molecules from abnormal condition can activate NLRP3 inflammasome, finally leading to metabolic syndrome, such as type 2 diabetes.[4,8] However, what factors and how they are regulated remain largely obscure. Therefore, I started my research to find out novel risk factors accumulated from our lifestyle and how they regulate our innate immune response.

In part 1, I investigated obesity induced risk factors, as obese people have deregulated immune response. AGEs elevated in hyperglycemic condition contribute to pathogenesis of diabetic complications, however their influence on innate immune signaling in macrophages is unclear. In this study, I propose AGEs inhibit NLRP3 inflammasome and TLR signaling, contributing to impaired host defense on obese condition.

My data demonstrated that AGEs failed to trigger the production of pro-inflammatory cytokines in macrophages. Although some previous studies reported that AGEs did not promote the production of cytokines[21,42], many other findings demonstrated the elevated secretion of cytokines by AGEs.[43,44] At present, I cannot fully explain this discrepancy. One possible explanation could be that different cell types were used, as in the case with inflammasome activity. Further detailed investigations will help clarify the potent roles of AGEs in various cell types. In this regard, my data showed novel evidence that AGEs treatment in macrophages could impair LPS-induced upregulation of pro-inflammatory cytokine production, as well as NLRP3 inflammasome activation.

The molecular mechanism underlying the anti-inflammatory function of AGEs is not fully understood at present. However, my observations show that AGEs mediate both TLR-

associated priming step and activation step of NLRP3 inflammasome activation. AGEs treatment remarkably suppressed LPS, Pam3CSK4 or poly I:C induced signaling, related to priming step. In this regard, AGEs failed to block NLRC4 or AIM2 inflammasome signaling, which do not require TLR-mediated priming step for the activation. However, AGEs also attenuated ATP induced caspase 1 activation in NLRP3-reconstituted macrophages. Therefore, further investigations will be required to elucidate the innate immune suppressing mechanism of AGEs in detailed. In addition, RAGE a typical receptor for AGEs, specific inhibitor failed to regulate AGEs mediated suppression of innate immune response, indicating RAGE signaling might not be involved. In addition to RAGE, several other AGE receptors such as AGE-receptor 1 (AGE-R1) are expressed in macrophages.[18,45] Interestingly, previously reported that AGE-R1 has an anti-inflammatory role via the removal of AGEs.[46] Thus, it will be challenging to determine whether AGE-R1 might be involved in the immunosuppressive effects of AGEs. Finally, AGEs treatment did not affect IL-4 driven M2 polarization, they clearly suppressed LPS or IFN γ induced M1 polarization. Even though, AGEs alone did not trigger M1 nor M2 polarization of macrophages, they definitely altered polarization of macrophages under specific conditions, indicating AGEs may induce a distinct phenotypic change in macrophages, leading to the decreased responsiveness.

Considering that inflammasome signaling occurs mainly in myeloid cells such as macrophages, the accumulation of AGEs could have a harmful effect on host innate defense mechanisms involving NLRP3 inflammasome signaling. Indeed, obesity patients, carrying high level of AGEs, are more vulnerable to various infectious disease, such as influenza virus.[47-50] My results suggest that AGEs could impair the host immune defense against a wide range of microbial infections through inhibition of NLRP3 inflammasome activation. AGEs pretreatment significantly dampened innate immune responses such as inflammasome activation and IFN-γ production in response to influenza virus infection, suggesting that AGEs could impair host innate defense mechanisms. Therefore, my results provide a molecular explanation for impaired host immune response against infectious disease.

Collectively, my data suggest that AGE, a risk factor elevated from hyperglycemic condition, could impair NLRP3- and TLR4-mediated innate immune responses, leading to increased susceptibility to infectious diseases.

In part 2, I focused on two major cells of innate immune system, neutrophils and macrophages. Both cells can activate inflammasome, however inflammasome signaling and the outcome of activation in neutrophils remain obscure. In this study, I suggest unlike macrophages, neutrophils contribute to prolonged inflammation through their exclusive traits, including resistance on DAMPs-rich milieu and pyroptosis.

Neutrophils and macrophages migrate to inflammatory site, where DAMPs, such as ATP, are abundant. My results suggest that neutrophils, unlike macrophages preserve capacity to activate NLRP3 inflammasome in ATP rich milieu by maintaining mitochondrial membrane potential, contributing to exacerbated inflammation. The importance of maintained membrane potential, serving as energy source for cells to proceed immune response, including NLRP3 inflammasome activation was recently reported in human monocyte[33] or mouse macrophage.[34] Some suggest mitochondrial depolarization triggers inflammasome activation, however, LPS/peptidoglycan do activate NLRP3 inflammasome without mitochondrial depolarization,[38] which indicates mitochondrial depolarization is not necessary and also result of NLRP3 inflammasome activation in some cases.[35] As neutrophils express comparable level of P2X7R to macrophages[15], difference in mitochondrial membrane potential between two cells upon ATP exposure could be derived from their distinctive pathway on preserving membrane potential. Macrophages retain membrane potential through respiratory chain. However, as neutrophils' mitochondria has limited function[51], they preserve their membrane potential only through complex III of respiratory chain.[52] Since, neutrophils do not require full-functioning respiratory chain for membrane potential, it might be less vulnerable to extracellular ATP compared to macrophages.

Me, and others, previously observed that unlike macrophages, neutrophils resist to caspase 1 dependent pyroptosis[53,54], yet molecular mechanism is not fully understood. My observation supports previous report that neutrophils express less caspase 1, leading to fewer cleaved GSDMD which might contribute to resistant on pyroptosis.[54] However, I showed neutrophils release comparable IL-1β from parallel level of pro-IL-1β to macrophages in caspase 1 dependent pathway, indicating relatively low expression of caspase 1 is insufficient for explanation. Accordingly, mitochondrial depolarization is suggested to contribute to NLRP3 inflammasome dependent pyroptosis[35] and is shown to occur prior to pyroptosis upon caspase 11 activation.[55] Furthermore, recently reported that SARM-dependent mitochondrial depolarization regulates cells to undergo pyroptosis upon NLPR3 activator. SARM deficient macrophages do not undergo pyroptosis as their membrane potential is retained after NLRP3 inflammasome activation.[34] However, inflammasome activators could directly induce early mitochondrial damage, independent of caspase 1 activity, as ATP shown above, caspase 1 activity also contributes to mitochondrial depolarization.[35] Although it is currently difficult to reconcile these observations to proposed mechanism for mitochondrial depolarization upon inflammasome activation, what is clear is that mitochondrial depolarization after NLPRP3 inflammasome activation is important factor for pyroptosis along with GSDMD cleavage. In line with this, my observations propose that neutrophils resist to pyroptosis upon NLPR3 inflammasome activation as they preserve mitochondrial membrane potential, in contrast to macrophages. Indeed, I showed inducing mitochondrial depolarization after NLRP3 inflammasome activation increases LDH release in neutrophils without changes in level of GSDMD cleavage. In addition, possibility of active membrane repair system in neutrophils, as GSDMD pores can be regulated by repair system[56], needed to be investigated further.

Cleaved GSDMD forms pore in plasma membrane, leading to pyroptosis, but also become a channel for IL-1β secretion.[3,57] However, my results of fewer cleaved GSDMD upon inflammasome activation in neutrophils, suggest neutrophils are less dependent on

GSDMD in IL-1β secretion compared to macrophages. Previous studies show that IL-1β can be secreted through microvesicle in microglia, dendritic cells and monocytes.[58-60] Especially rapid IL-1β secretion was highly dependent on microvesicle in monocytes[59], showing the probability that similar mechanism could be proceeded in neutrophils along with my observation. Furthermore, I found that neutrophils secrete IL-1β more specifically rather than other proinflammatory cytokines, including IL-6 and TNFα. Previous study reported that neutrophils produce weak or no proinflammatory cytokine in TLRs activation.[61] In addition, different chromatin organization of the IL-6 gene locus in human neutrophils compared to monocytes results delayed and low level of IL-6 production upon TLR4 signaling.[62] Indeed, I found that LPS triggers weak production of IL-6 on early time (~6 h), while increased on late time (24 hr~). According to recent study, some TLR4 signaling induced cytokines, including IL-6 or IL-12p40, but not IL-1β, require acetyl CoA from citrate to augment histone acetylation, resulting induction of genes.[63] As neutrophils have limited mitochondrial function,[51] lack of citrate, derived from TCA cycle could contribute to low expression of IL-6 and TNFα upon LPS stimulation.

Accordingly, inflammasome activated macrophages proceed various cell death pathway, forcing cells to undergo apoptosis or necrosis when pyroptosis is blocked.[36,37,64] However, my results showed that inflammasome activated neutrophils resist to cell death even pyroptosis doesn't turn out. Still, neutrophils have a capacity to promote other types of cell death, such as apoptosis or necroptosis. These prolonged life span upon inflammasome activation would lead to accumulation of neutrophils at inflammatory site in company with IL-1β, known to recruit neutrophils.[65] I also demonstrated that neutrophils maintain their functions upon NLRP3 inflammasome activation, including migration, phagocytosis and degranulation. Especially degranulated proteases from neutrophils are known to trigger tissue damage during severe inflammation, such as sepsis or lung inflammation, indicating inflammasome activated neutrophils could contribute to tissue damage.[66,67] Finally, I suggest NLRP3 inflammasome activated neutrophils sustain inflammation by delaying resolution of

inflammation. Clearance of neutrophils, known as efferocytosis is important for resolution of inflammation.[65,68] Therefore, delayed cell death or clearance of neutrophils could lead to prolonged inflammation, commonly reported in SLE patients.[69,70] Recently reported that apoptotic cells, besides pyroptotic cells could be engulfed by both professional/ non-professional phagocytes.[71] I found that inflammasome activated neutrophils, unlike apoptotic neutrophils, fail to induce anti-inflammatory cytokine, such as IL-10, and also to promote cell to become immunologically silent, finally leading to sustained inflammation. Collectively, my data suggest that exclusive traits of neutrophils in inflammasome signaling and aftermath of inflammasome activation could contribute to pathogenesis of inflammatory disease, holding great therapeutic potential.

V. CONCLUSION

Here, I demonstrated that risk factors accumulated from our lifestyle, such as AGEs and ATP, can regulate NLPR3 inflammasome in macrophages and neutrophils.

In part 1, AGEs a risk factor elevated in hyperglycemic condition, could impair NLRP3 and TLR4 mediated innate immune responses, leading to increased susceptibility to infectious diseases. AGEs failed to induce the production of pro-inflammatory cytokines in macrophages however, suppressed LPS triggered IL-6 and Pro IL-1β. Consistently, AGEs pretreatment impaired NLRP3-dependent caspase 1 activation. AIM2 and NLRC4 inflammasome was intact from AGEs pretreatment which indicates suppressing effect of AGEs is specific to NLRP3 inflammasome. Furthermore, immunosuppressive effect of AGEs was independent of RAGE-dependent signaling. While, AGEs inhibited NF-kB signaling associated with TLR-dependent priming step of NLRP3 inflammasome activation. Finally, in macrophages, AGEs pretreatment attenuated RNA virus, especially Influenza A virus, induced caspase 1 activation and IL-1β release, as well as IFNβ production and secretion.

In part 2, neutrophils exposed to ATP, a risk factor rich in inflammatory milieu, could preserve their capacity to activate NLRP3 inflammasome without pyroptosis, opposed to macrophages, contributing to aggravated immune response by prolonged inflammation. In contrast to macrophages, pretreatment of DAMP, such as ATP, failed to suppress caspase 1 activation and IL-1β secretion in neutrophils. Maintained mitochondrial membrane potential upon ATP treatment in neutrophils could contribute to this difference. Upon NLRP3 inflammasome activation, neutrophils resist to pyroptosis, which was confirmed by LDH release, PI staining and release of intracellular proteins. Still, neutrophils were possible to undergo apoptosis and necroptosis as macrophages. Even though, mechanisms underline in resistance on pyroptosis after NLRP3 inflammasome activation in neutrophils are unclear, low expression level of caspase 1 leading to less cleavage of GSDMD, as well as GSDMD-

NT kinetics might contribute. In addition, along with recent report, mitochondrial membrane potential could contribute to resistance on pyroptosis in neutrophils. IL-1β secretion was highly and partially dependent on NLRP3 and GSDMD, respectively, in neutrophils upon NLRP3 inflammasome activation. Interestingly, neutrophils seem to be more specific in secreting IL-1β then other pro inflammatory cytokines, including IL-6 and TNFα. As neutrophils do not undergo cell death upon NLRP3 inflammasome activation, they maintain their functions, including phagocytosis, migration and degranulation. Finally, disparate from apoptotic neutrophil, NLRP3 inflammasome activated neutrophil failed to induce efferocytosis on peritoneal macrophage, which is important for resolution of inflammation.

Collectively these data present exposure to risk factors, such as AGEs or ATP, dysregulate immune response in macrophage and neutrophil, which could lead to imbalance in immune system.

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ABSTRACT (IN KOREAN)

Advanced glycation end products와 ATP에 의한 선천 면역 반응 조절 규명

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손 승 환

대식세포와 호중구에서의 인플라마좀 신호는 감염에 대한 숙주의 선천면역 작용, 더 나아가 당뇨, 알츠하이머 병, 동맥경화증과 같은 염증성 질환에 기여한다고 알려져 있다. 우리의 생활 습관으로부터 축적된 위험인자들은 선천면역반응을 조절함으로써 여러 중증 질환을 유발한다고 보고되지만, 어떤 인자들이 어떻게 영향을 주는지는 아직 모호하다. 이러한 이유로 본 연구에서는 우리의 생활 습관으로부터 축적된 위험인자들을 밝히고, 이들이 어떻게 우리의 선천면역계에 영향을 끼치는지, 보다 인플라마좀 활성화에 초점을 두어 연구하였다.

본 연구 1 부에서 다루는 Advanced glycation end products 는 단백질에 당이 결합한 물질로 고혈당 상황에서 형성되고 축적된다. 이는 고혈당 상황에서의 위험인자로 당뇨합병증과 같은 만성 염증성질환의 발병에 중요한 역할을 한다고 알려져 있다. 고혈당 최종 당화물은 염증성 사이토카인의

생성을 유도한다고 보고되었으나 인플라마좀 신호에 대한 영향은 연구가 미흡하다. 본 연구는 고혈당 최종 당화물이 대식세포에서의 인플라마좀 활성화를 억제한다는 사실을 발견하였다. 고혈당 최종 당화물은 NLRP3 인플라마좀의 형성을 억제하였으나 NLRC4, AIM2 인플라마좀 활성에는 영향을 주지 못하였다. 게다가 고혈당 최종 당화물은 대식세포에서 톨 유사 수용체를 통한 염증성 사이토카인의 생성도 감소시켰다. 이러한 면역억제효과는 고혈당 최종 당화물 수용체를 매개하지 않았다. 더 나아가 고혈당 최종 당화물은 인플루엔자 바이러스에 대한 NLRP3 인플라마좀 활성화와 제 1 형 인터페론을 포함하는 선천면역반응을 억제하였다. 결론적으로 위의 결과들은 고혈당 최종 당화물이 RNA 바이러스에 대항하여 발생하는 숙주의 NLRP3 인플라마좀을 통한 선천면역반응을 손상시킴으로써 감염에 더 취약하게 만들 수 있다는 것을 제시한다.

본 연구 2 부에서 다루는 ATP 는 대표적 손상 연관 분자패턴으로 조직 손상에 의해 국소적 혹은 체내 전반적으로 증가된다. 대식세포와 호중구는 대표적인 선천 면역계의 세포로 손상 연관 분자패턴이 많은 부위로 이동하여 염증을 유발한다. 두 세포 모두 인플라마좀을 활성 시킬 수 있지만, 호중구의 경우 파이롭토시스 (pyroptosis)를 유발하지 않는다. 이러한 두 세포간 인플라마좀 신호와 활성화 이후의 차이에 대한 연구는 아직 미흡하다. 본 연구는 ATP 에 노출된 호중구는 대식세포와는 다르게 파이롭토시스를 유발하지 않고 NLRP3 인플라마좀을 활성화할 수 있다는 사실을 발견하였다. 더 나아가, LPS 에 의해 생성되는 염증성 사이토카인이 대식세포에서만 ATP 에 의해 감소되는 것을 확인하였다. NLRP3 인플라마좀 활성 이후, 호중구는 파이롭토시스가 일어나지 않았으나 여전히 다른 세포사멸을 유발하는 특성은 존재하였다. 비슷한 관점으로 호중구는 NLRP3 인플라마좀 활성 이후 다른

세포 기능을 유지하였다. 결과적으로 세포자멸이 일어난 호중구와는 달리 NLRP3 인플라마좀이 활성화된 호중구는 염증 종료에 중요한 efferocytosis 를 유도하지 못하였다. 종합적으로 위의 결과들은 염증 환경의 위험인자인 ATP에 노출된 호중구는 대식세포와 달리 NLRP3 인플라마좀을 활성 시킬 수 있으며 파이롭토시스가 발생하지 않기에, 지속적인 염증을 통해 보다 과한 면역반응 유발할 수 있다는 것을 제시한다.

핵심되는 말: 손상 연관 분자패턴, 고혈당 최종 당화물, 인플라마좀, 대식세포, 호중구

PUBLICATION LIST

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