Role of Golgi apparatus in the NLRP3 inflammasome activation and proinflammatory cytokine production

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Directed by Professor Je-Wook Yu

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홍수정 배상

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ABBREVIATION LIST

AIM2	Absent in melanoma 2
ARF	Adenosine diphosphate ribosylation factor
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
BFA	Brefeldin A
BIG1	Brefeldin A-inhibited guanine nucleotide exchange factor 1
BMDMs	Bone marrow-derived macrophages
BSA	Bovine serum albumin
COPI	Coat protein 1
DAPI	4’,6-diamidino-2-phenylindole
Drp1	Dynamin-related protein 1
DSS	Dextran sodium sulfate
EDTA	Ethylenediaminetetraacetic acid
EGA	4-bromobenzealdehde N-(2,6-dimethylphenyl) semicarbazon
FBS	Fetal bovine serum
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescence protein
GSDMD	Gasdermin D

IgG	Immunoglobulin G
IL	Interleukin
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAM	Mitochondria–associated endoplasmic reticulum membranes
MAVS	Mitochondrial antiviral-signaling protein
MFI	Mean fluorescent intensity
MSU	Monosodium urate
NBD	Nucleotide binding domain
NLRs	Nucleotide-binding oligomerization domain-like receptors
NLRP3	Nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3
P. aeruginosa	*Pseudomonas aeruginosa*
PBMCs	Peripheral blood mononuclear cells
PMA	Phorbol-12-myristate-13-acetate
PYD	Pyrin domain
ROS	Reactive oxygen species
TGN	*trans*-Golgi network
TLR	Toll-like receptor
TNF	Tumor necrosis factor

ABSTRACT

Role of Golgi apparatus in the NLRP3 inflammasome activation and proinflammatory cytokine production

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(Directed by Professor Je-Wook Yu)

Nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome is a multi-protein complex, comprised of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and cysteine protease pro-caspase-1. NLRP3 inflammasome is activated by a variety of stimulations such as microbial infection and endogenous metabolites. The activation of NLRP3 inflammasome causes the production of active caspase-1, which then induces the maturation and secretion of interleukin-1β (IL-1β), a major proinflammatory cytokine. Recent studies suggest that excessive NLRP3 inflammasome activation is related with progression of some metabolic and degenerative disease. Therefore, the regulation step of NLRP3 inflammasome signaling can be a prominent target for developing therapeutic strategies of NLRP3 inflammasome-related diseases. However,

the detailed molecular mechanisms of NLRP3 inflammasome activation and of IL-1β secretion remain still unclear. In the present study, I presents evidences that the Golgi apparatus-mediated intracellular vesicle trafficking is crucial for the activation of NLRP3 inflammasome. NLRP3 inflammasome activation was significantly inhibited by brefeldin A (BFA), which blocks endoplasmic reticulum (ER)-Golgi vesicle trafficking, in primary bone marrow-derived macrophages (BMDMs). Moreover, the reduced expression of BFA-inhibited guanine nucleotide exchange factor (GEF) 1 (BIG1), a target molecule of BFA, by shRNA-mediated knockdown clearly attenuated the activation of NLRP3 inflammasome in BMDMs. Moreover, prevention of Golgi-mediated vesicle trafficking through BFA treatment and BIG1-knockdown impaired LPS-promoted proinflammatory cytokine production. These results indicate that Golgi plays a significant role in the priming and activation step of NLRP3 inflammasome pathway. Collectively, our data suggest a potential implication of Golgi apparatus-mediated vesicle trafficking in NLRP3 inflammasome regulation.

Key words: inflammasome, NLRP3, Golgi apparatus, vesicle trafficking, Brefeldin A, BIG1

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Ⅰ. INTRODUCTION

The Inflammasome is protein complex consisting of nucleotide-binding oligomerization domain-like receptors (NLRs) or absent in melanoma 2 (AIM2), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) as adaptor protein and cysteine protease pro-caspase-1. Once the inflammasome is activated, inactive pro-caspase-1 is cleaved to active caspase-1 which proteolytically cleaves the proinflammatory cytokine interleukin-1β (IL-1β)[1,2]. Usually, activation of inflammasome has protective role against harmful stimuli. However, excessive inflammasome activation can cause chronic or auto inflammatory disease such as atherosclerosis, rheumatoid arthritis and crohn's disease[3-8]. So far, four inflammasomes involving NLR family pyrin domain-containing 1 (NLRP1), NLRP3, NLR family caspase recruitment domain (CARD) domain-containing 4 (NLRC4) and AIM2 have been described[1,9,10]. Among these inflammasomes, NLRP3 inflammasome has been under intense investigation.

Generally, NLRP3 inflammasome activation is known to require two signals[11]. Signal 1, such as toll-like receptor 4 (TLR4) signal, leads to the up regulation of NLRP3 and pro-IL-1β protein expression through the NF-κB activation[12]. Also, recent studies suggest that signal 1 can be involved in post-translational modification of NLRP3 and ASC to ready for inflammasome activation[13-16]. Additionally, signal 2, induced by various stimulations that include microbial toxins, crystalline molecules and endogenous danger signals[17-20], is required for NLRP3 inflammasome assembly and activation. Until now, several events have been proposed to explain the mechanism of NLRP3 inflammasome activation including cytosolic potassium efflux, production of reactive oxygen spices (ROS), mitochondrial damage and lysosomal damage[17,21-23]. However, it is unclear how these variety events are related to NLRP3 inflammasome activation.

Even in this circumstance, mitochondria have been strongly suggested as key organelle of NLRP3 inflammasome activation. Mitochondria-derived molecules (ROS and DNA) and protein located in mitochondrial membrane (MAVS and cardiolipin), even protein involved in mitochondrial dynamics (mitofusin2 and Drp1) influence the activation of NLRP3 inflammasome[24-29]. Moreover, in the recent studies, mitochondria–associated endoplasmic reticulum (ER) membranes (MAM) has been proposed that activation site of NLRP3 inflammasome[30]. This membrane is formed by result of mitochondrial movement to ER membrane. MEM is crucial for correct communication between the mitochondria and ER, including the selective transmission of Ca^{2+} signals from the ER to mitochondria[31,32]. Mitochondrial Ca^{2+} overload leads to mitochondrial dysfunction. In 2013, Takuma Misawa and colleagues identify that mitochondria move to ER through microtubule dependent manner in response to NLRP3 inflammasome activators[30]. They suggest that mitochondrial movement to ER is necessary event for the activation of NLRP3 inflammasome.

Endoplasmic reticulum is a continuous membrane system and serves major role in the synthesis, folding, modification and transport of protein and lipid. Especially, almost of secretory proteins, such as IL-6 and tumor necrosis factor α (TNFα), are expressed in ER lumen because their mRNA has leader sequence which leads to ribosome in the rough ER[33]. After protein expression, these proteins are further transported to the Golgi and the extracellular space through vesicle trafficking[34]. This protein transport is called classical protein secretory pathway. However, although IL-1β is secretory protein, IL-1β mRNA lacks a leader sequence. Based on this fact, researchers presume that IL-1β is released through non-classical protein secretory pathway independent on ER and Golgi. As expected, in 1990, Anna Rubartelli and colleagues verify that secretion of IL-1β does not though the classical protein secretory pathway in human monocytes using brefeldin A (BFA), classical secretory pathway inhibitor via blocking the vesicle formation from Golgi memebrane[35]. Thus, recent publications suggest several mechanisms involved in IL-1β secretion including the exosome pathway, microvesicle pathway, autophagy pathway, secretory lysosome pathway, caspase-1-dependent pyroptosis and necrosis pathway[36-41]. However, these mechanisms cannot explain exactly the IL-1β secretory pathway.

Almost of secretory cytokines are secreted immediately without additional process after protein synthesis. Unlike other secretory cytokines, IL-1β requires additional process for secretion. IL-1β is expressed as inactive pro-IL-1β which is non-functional and non-secretory protein. For activation and secretion of the IL-1β, active-caspase-1 cleaves pro-IL-1β to active-IL-1β[42]. Moreover, according to recent study, active-caspase-1 also cleaves and activates gasdermin D (GSDMD) that generates the membrane pore which induces cell death and leads the secretion of IL-1β. Further, active-caspase-1 is also detected in extracellular space with active-IL-1β. Based on these investigates, it is obvious that IL-1β secretion is tightly related

with caspase-1 activation.

In this report, I tried to identify the relation between IL-1β secretory pathway and caspase-1 activation. First, I confirm that IL-1β is secreted via non-classical protein secretory pathway using BFA treatment. However, surprisingly, in macrophages, NLRP3-dependent IL-1β secretion is inhibited by BFA treatment. On the basis of this experiment, I hypothesize that Golgi apparatus, target organelle of BFA, is a novel organelle involved in IL-1β secretory pathway and caspase-1 activation.

Ⅱ. MATERIAL & METHODS

1. Reagents and antibodies

ATP, brefeldin A (BFA), MCC950, glibenclamide, LPS, nigericin sodium salt, phorbol-12-myristate-13-acetate (PMA) and Poly(dA;dT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ac-YVAD-CMK was purchased from BACHEM. Alum crystal was purchased from InvivoGen (San Diego, CA, USA). Golgi-Plug was purchased from BD Biosciences (San Jose, CA, USA). EGA was purchased from Calbiochem (Sandhausen, Germany). Pitstop® 2 (Pitstop2) was purchased from Abcam (Cambridge, MA, USA).

Mouse monoclonal anti-caspase-1 (p20) antibody and mouse monoclonal anti-NLRP3/NALP3 antibody were purchased from Adipogen (San Diego, CA, USA). Rabbit polyclonal anti-ASC antibody, rabbit polyclonal anti-TOM20 antibody and mouse monoclonal anti-α-tubulin antibody were purchased from Santa cruz (Santa Cruz, CA, USA). Goat polyclonal anti-IL-1β/IL-1F2 antibody was purchased from R&D systems (Minneapolis, MN, USA). Rabbit polyclonal anti-BIG1 antibody was purchased from Abcam. Rabbit monoclonal anti-IL-6 antibody was purchased from Cell Signaling Technology. Mouse monoclonal anti-GM130 antibody was purchased from BD Biosciences. Peroxidase affinipure donkey anti-rabbit IgG (H+L) antibody, peroxidase affinipure donkey anti- mouse IgG (H+L) antibody and Peroxidase affinipure donkey anti-goat IgG (H+L) antibody were purchased from Jackson Immuno Research (West Grove, PA, USA).

2. Mice

C57BL/6 mice were purchased from OrientBio (Gyeonggi-do, Korea). Nlrp3 knockout (-/-) mice was purchased from Jackson Laboratories (Stock Number 017971, Bar Harbor, ME, USA). Nlrp3 knockout mice were crossed with C57BL/6 mice to produce Nlrp3 hetero (+/-) mice. Wild type mice and Nlrp3 knockout mice used in this report were generated from Nlrp3 hetero mice. All mice care and procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Animal Care Committee of Yonsei University College of Medicine. All mice were maintained in specific pathogen-free conditions.

3. Cell cultures

Primary bone marrow-derived macrophages (BMDMs) were prepared from the femurs and tibias of C57BL/6 mice. Bone marrow progenitor cells were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Corning, NY, USA) ,that is supplemented with 10% fetal bovine serum (FBS, Corning), 100 U/mL penicillin and streptomycin (Gibco, Gran Island, NY, USA), containing 8% L929-conditional media for three days. Then culture medium with non-adherent cells was replaced with fresh complete DMEM containing 8% L929-conditional media. On day 2 after media change, adherent cells were detached by 0.25% trypsin-EDTA and plated in 6 or 12 well plate with complete DMEM containing 5% L929-conditional media. To isolation of peritoneal macrophages, 2 ml of 3% thioglycollate solution was injected into peritoneal cavity of wild type or Nlrp3 knockout mice. 4 days after the injection, cells were harvested by intraperitoneal lavage with ice-cold phosphate-buffered saline (PBS). Then, harvested cells were plated in 6 or 12 well plate with complete DMEM. Immortalized macrophages

including NLRP3-green fluorescence protein (GFP)-expressing macrophages and NLRP3-overexpressing macrophages were kindly provided by Dr. E.S. Alnemri (Thomas Jefferson University, Philadelphia, PA, USA). These cells were grown in complete DMEM containing 5% L929-conditional media and plated 6 or 12 well plates with complete DMEM containing 5% L929 conditional media. $CD14^+$ primary human monocytes isolated from the human peripheral blood were kindly provided by Dr. Won-woo Lee (Seoul National University Hospital Biomedical Research Institute, Seoul, Korea). THP1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning) supplemented with 10% FBS, 100 U/mL penicillin and streptomycin, 2 mM glutamine (Gibco), 10 mM HEPES (Gibco), 1 mM sodium pyruvate (Gibco) and 50 μM 2-mercaptoethanol (Gibco). 0.5 μM PMA was added to THP1 cells for 1-2 hrs to differentiate the monocytes into macrophage-like cells. Differentiated THP1 cells were sub-cultured in 6 well or 12 well plates.

4. Generation of BIG1-knockdowned macrophages

To achieve stable knockdown of target gene in macrophages, three packing plasmid (pLP1, pLP2 and VSVG, kindly gifted from Dr. HP, Kim, Yonsei University, Seoul, Korea) and pLKO.1-puro expressing mouse *Arfgef* (gene that encode BIG1)-targeting shRNAs or non-targeting shRNAs were transfected into 293FT cells. Supernatants containing the shRNA-expressing lentivirus were collected after 72 hrs transfection. Then, macrophages infected with collected supernatants for another 48 hrs. Target shRNA-positive cells were selected by 4 μg/mL puromycin treatment.

5. Measurement of cytokine production

Total RNAs were isolated with TRIzol reagent (Invitrogen) and reverse transcription was done with PrimeScript™RT Master Mix (TaKaRa Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The cDNA fragments were amplified by reverse transcription PCR using AccPower HotStart PCR Premix (Bioneer, Daejeon, Korea) and Veriti 96well Thermal Cycler instrument (Applied Biosystems). For quantitative PCR, cDNA fragments were amplified with SYBR Premix Ex Taq™ II (TaKaRa) and detected by a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The expression of target mRNA was normalized to Rn18s mRNA expression using the $2^{-\Delta\Delta Ct}$ method. Specific primer pairs (Table 1.) All primers were purchased from Genothech (Daejeon, Korea). Secreted mouse IL-1β and IL-6 (Biolegend) as well as human IL-1β and IL-6 (R&D Systems) were measured in cell-free supernatants by enzyme-linked immunosorbent assay (ELISA).

6. Immunoblotting analysis

After treatments, cell supernatants and cell lysates were collected for immunoblotting analysis. Soluble cell lysates were prepared using 20 mM HEPES (pH 7.5) buffer containing 0.5% NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, protease inhibitors and phosphatase inhibitors. Proteins in the supernatants were precipitated by methanol-chloroform extraction. Proteins in cell lysates and supernatants were separated on SDS-PAGE gel and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with 2% skim milk in PBS with 0.1% Tween 20 for 30 mins at room temperature and incubated with the appropriate primary antibodies. Membranes were then incubated with HRP-conjugated secondary antibody and proteins were visualized using Pierce

ECL Western Blotting Substrate (32106, Thermo Fisher Scientific, Waltham, MA, USA).

7. Stimulation for inflammasome activation

To activate the NLRP3 inflammasome in macrophages, BMDMs or macrophages were primed with 0.25 μg/mL LPS for 3 hrs followed by 2 mM ATP for 20 or 40 mins, 5 μM nigericin for 20 or 40 mins or 500 μg/mL alum for 6 hrs. In monocytes, such as PMA-differentiated THP1 cells or primary human monocyte cells, NLRP3 inflammasome was activated by treatment of 0.5 μg/mL LPS for 6 hrs or 5 μM nigericin for 1 hr 30 mins. For activation of NLRC4 inflammasome, *Pseudomonas aeruginosa* (PAO1, kindly gifted Dr. SS, Yoon, Yonsei University, Seoul, Korea) was grown overnight at 37℃ with aeration, and then seeded in Luria-Beritani (LB) medium by 1/50 dilution and grown for additional 2 hrs. Bacteria were collected by centrifugation. The macrophages were infected at multiplicity of infection (MOI) of 3 for 3 hrs. AIM2 inflammasome was activated by poly(dA;dT) transfection using Lipofectamine 2000 (Invitrogen, Gran Island, NY, USA). Prolonged LPS-induced IL-1β secretion is induced by treatment of 0.5 μg/mL ultra-pure LPS (UP-LPS, Invivogen) for 3, 12 or 24 hrs in BMDMs or peritoneal macrophages (PMs).

8. Measurement of caspase-1 activation by flow cytometry

For flow cytometry-based monitoring of caspase-1 activation, treated cells were washed in fluorescence-activated cell sorting (FACS) buffer (PBS with 5% FBS) and stained with YVAD-FLICA (FAM-YVAD-FMK, caspase-1 fluorescein) reagent (Immunochemistry, Bloomington, MN, USA) for 1 hrs. Next, stained cells

were collected by centrifugation and resuspended with FACS buffer for analysis using flow cytometry (FACS Verse, Beckman coulter, CA, USA).

9. Assessment of inflammasome assembly

To determine formation of NLRP3 specks, NLRP3-GFP-expressing macrophages were treated with NLRP3 inflammasome activators. The treated cells were washed with PBS and fixed in 4% paraformaldehyde for 30 mins at room temperature, followed by counterstaining by ProLong Gold reagent with DAPI. NLRP3 specks were visualized, counted and imaged using a confocal microscope (LSM700; Zeiss, jena, Germany). For assessment of ASC oligomerization, treated cells with inflammasome activators were lysed with 20 mM HEPES (pH 7.5) buffer containing 0.5% NP-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1,mM EGTA, protease inhibitors and phosphatase inhibitors. Cell lysates were centrifuged at 12,000 rpm for 10 mins at 4℃, and supernatants and pellet were separated. The pellets were resuspended in 500 μL of PBS. 0.75 mM disuccinimydyl suberate (DSS, Pierce, Gran Island, NY, USA) was added to the resuspended pellets, which were incubated at room temperature for 30 mins at dark condition, and centrifuged at 12,000 rpm for 10 min at 4℃. Supernatants and pellet samples were analyzed by immunoblotting. Measurement of interaction between NLRP3 and ASC was performed by proximity ligation assay (PLA) using Duolink *In situ* Red Starter kit mouse/rabbit (Sigma, St. Louis, MO, USA). For calculation of the percentage of cells positive for proximity ligation, at least five fields were observed per sample.

10. Determination of mitochondrial damage and mitochondrial reactive oxygen species (ROS) production

To assess mitochondrial damage, cells were double stained with MitoTracker Deep Red and MitoTracker Green (all from Invitrogen). Treated cells were detached using 0.25% trypsin-EDTA and incubated with FACS buffer containing 25 nM MitoTracker Deep Red and 50 nM MitoTracker Green for 10 mins at room temperature. To determine reactive oxygen species generated from mitochondria, cells were stained with MitoSOX (Invitrogen) according to the manufacture's protocol. Treated cells were detached and incubated with FACS buffer containing 2.5 μM MitoSOX for 10 mins at room temperature. Stained cells were measured by flow cytometry (FACS Verse, Beckman coulter) and analyzed with FlowJo analytical software (TreeStar, Ashland, OR, USA).

11. Immunofluorescence analysis

To perform the immunofluorescence analysis, cells were plated on coverslip in 12 well plates. These cells were fixed in 4% paraformaldehyde for 30 mins and then wash with PBS. Fixed cells were permeabilized with 0.2% triton X-100 for 15 mins and then wash with PBS. After washing with PBS, cells were blocked with 4% bovine serum albumin (BSA, Affymetrix, Santa Clara, CA, USA) for 1 hrs and then incubated with primary antibodies (in 4% BSA) for 1 hrs. Next, cells were washed with PBS and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 4% BSA for 30 mins and washed with PBS. The stained cells on coverslip were mounted using ProLong Gold reagent with DAPI. Images were acquired using confocal microscope (LSM700, Carl Zeiss, Oberkochen, Germany).

12. Statistical analysis

Data are presented as average values ± standard error of the mean (SEM) from multiple individual experiments. The data were considered significant when p values ≤ 0.05 calculated using Student's t-test.

Table 1. Primer sequence for PCR

Primer sequence	
Target	Sequence
Mouse IL-6	Forward: 5'-AGT TGC CTT CTT GGG ACT GA-3'
	Reverse: 5'-TCC ACG ATT TCC CAG AGA AC-3'
Mouse IL-1β	Forward: 5'-GCC CAT CCT CTG TGA CTC AT-3'
	Reverse: 5'-AGG CCA CAG GTA TTT TGT CG-3'
Mouse Rn18s	Forward: 5'-CGC GGT TCT ATT TTG TTG GT-3'
	Reverse: 5'-AGT CGG CAT CGT TTA TGG TC-3'
Mouse *Arfgef1*	Forward: 5'-GCT GCA TCA GAC CAA GAT GA-3'
	Reverse: 5'-GAA AGC CTG CGG TCT ATC AG-3'
Human IL-6	Forward: 5'-TAC CCC CAG GAG AAG ATT CC-3'
	Reverse: 5'-TTT TCT GCC AGT GCC TCT TT-3'
Human IL-1β	Forward: 5'-GGG CCT CAA GGA AAA GAA TC-3'
	Reverse: 5'-TTC TGC TTG AGA GGT GCT GA-3'
Human Rn18s	Forward: 5'-AAA CGG CTA CCA CAT CCA AG-3'
	Reverse: 5'-CCT CCA ATG GAT CCT CGT TA-3'

Ⅲ. RESULT

1. Brefeldin A inhibits IL-1β secretion in macrophages

IL-1β is the leaderless protein and considered to be released through non-classical protein secretory pathway. Previous studies reported that secretion of IL-1β does not follow the endoplasmic reticulum (ER)-Golgi secretory pathway[35]. To confirm the IL-1β secretory pathway, I employed brefeldin A (BFA) that is known as classical protein secretion inhibitor. In accordance with previous reports, BFA did not inhibit the IL-1β secretion in $CD14^+$ monocytes isolated from peripheral blood mononuclear cells (PBMCs), whereas IL-6 secretion, which is known to follow the classical protein secretory pathway, was inhibited by BFA treatment in response to LPS stimulation (Fig 1A). However, unexpectedly, not only IL-6, but also IL-1β secretion were significantly inhibited by BFA treatment in response to LPS plus ATP, the most well-known NLRP3 inflammasome activators, in primary bone marrow-derived macrophages (BMDMs) (Fig 1B). I confirmed this result using GolgiPlug (commercially available BFA-based classical protein secretion inhibitor) treatment. GolgiPlug also inhibited NLRP3 inflammasome activator-induced IL-1β secretion in BMDMs (Fig 1C). These results indicate that BFA inhibits NLRP3-dependent IL-1β secretion in macrophages.

To investigate the effect of BFA on IL-1β secretory pathway in macrophage-like cells, human monocytic THP1 cells were tested. THP1 cells were differentiated into macrophage-like cells by phorbol-12-myristate-13acetate (PMA) treatment. In PMA-differentiated THP1 cells, BFA did not inhibit LPS or nigericin-induced IL-1β secretion (Fig 2). This result indicated that BFA does not inhibit IL-1β secretion induced by LPS or nigericin stimulation in monocyte-derived macrophage-like cells.

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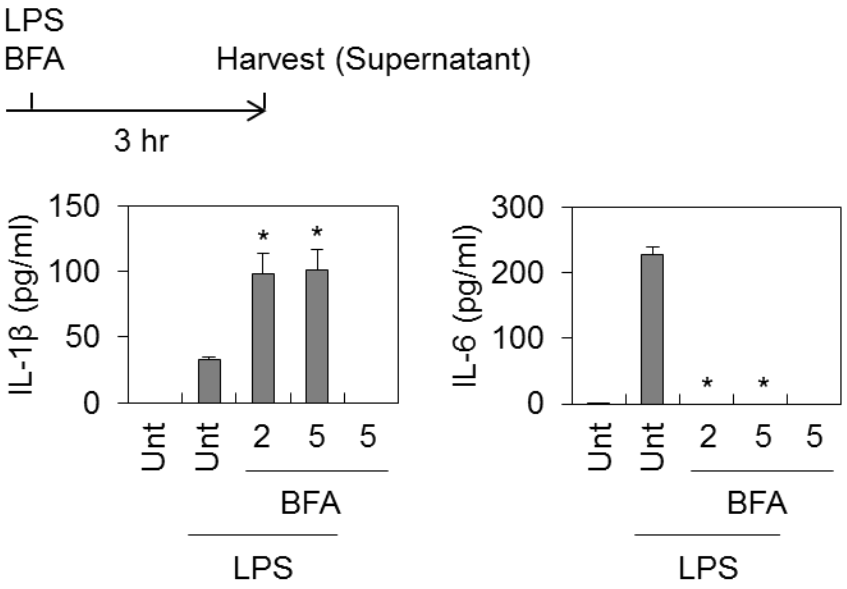

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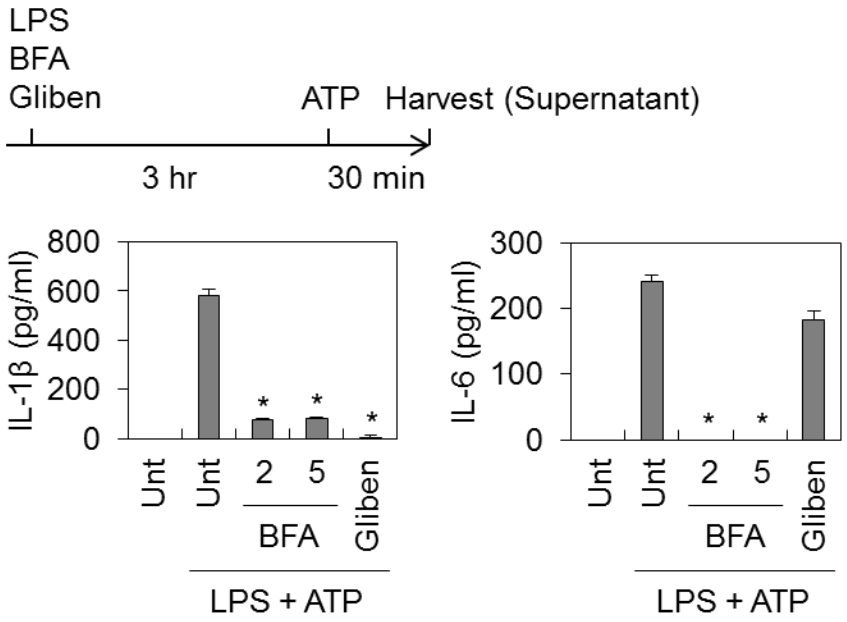

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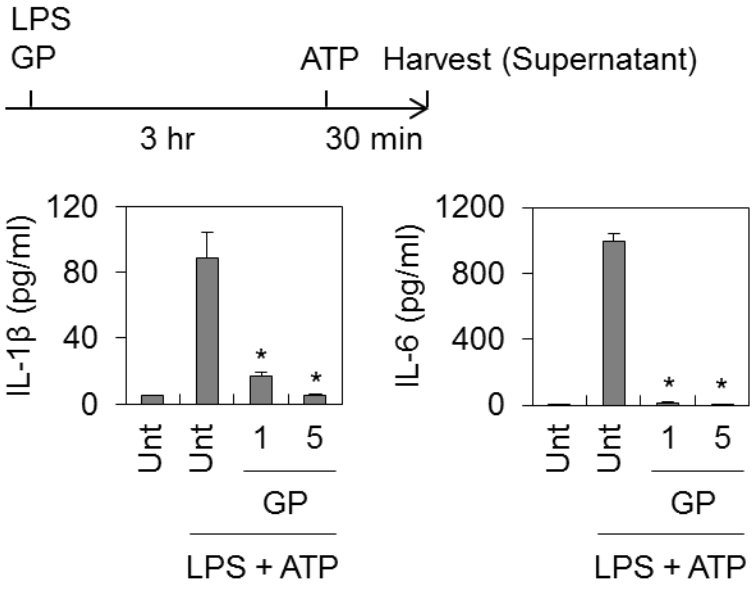

Figure 1. Brefeldin A inhibits IL-1β release in macrophages. (A) Quantification of IL-1β and IL-6 in culture supernatants from primary human $CD14^+$ monocytes from peripheral blood pretreated with LPS (0.5 μg/mL) in the presence or absence of BFA (2 or 5 μg/mL) for 3 hours (hrs). (B) Quantification of IL-1β and IL-6 in culture supernatants from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL or 5 μg/mL) or glibenclamide (50 μM) for 3 hrs, followed with ATP (2 mM) for 30 minutes (mins). (C) Quantification of IL-1β and IL-6 in culture supernatants from primary bone marrow-derived macrophages (BMDMs) pretreated with LPS (0.25 μg/mL) in the presence or absence of GolgiPlug (1 or 5 μL/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor), GP: GolgiPlug (BFA-based classical protein secretion inhibitor), Gliben: glibenclamide (potassium channel inhibitor).

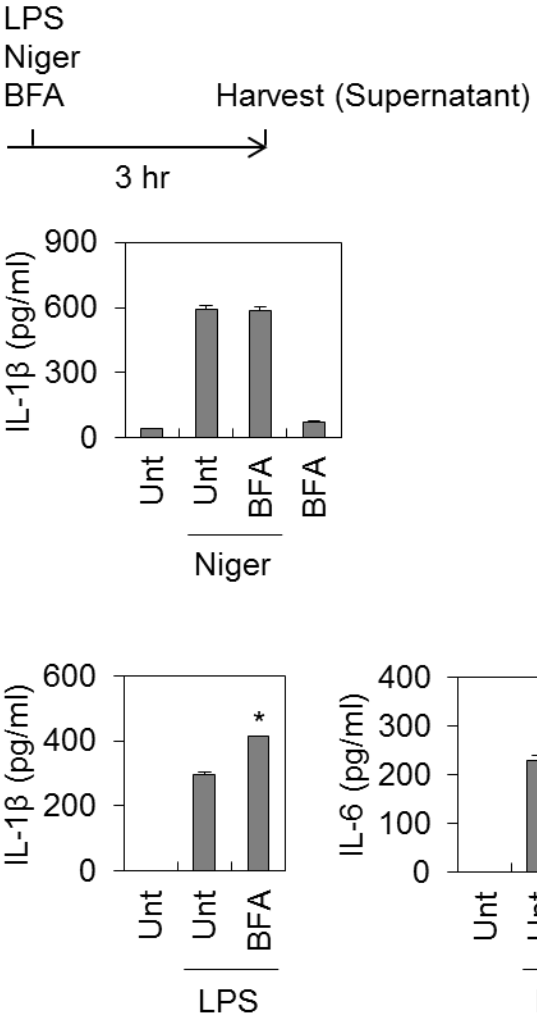

Figure 2. Brefeldin A does not inhibit IL-1β release in humnan monocytic THP1 cells. Quantification of IL-1β and IL-6 in culture supernatants from PMA-differentiated THP1 cells pretreated with nigericin (5 μM) in the presence or absence of BFA (2 μg/mL) for 45 min (upper), or LPS (0.5 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs (lower). The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), Niger: nigericin (bacterial toxin), BFA: brefeldin A (classical protein secretion inhibitor).

2. Brefeldin A inhibits NLRP3-dependent caspase-1 activation in macrophages

Because activated caspase-1 is prominent factor of IL-1β secretion, I wondered whether BFA regulates caspase-1 activation. To examine the possible effect of BFA on caspase-1 activation, first I checked the caspase-1 activation in macrophages. BMDMs were primed with LPS or LPS plus BFA and then stimulated the cell with ATP, nigericin or alum crystals to activate NLRP3 inflammasome. Caspase-1 activation in response to NLRP3 inflammasome activators was diminished by BFA treatment (Fig 3A). However as expected, same as IL-1β secretion, BFA did not inhibit caspase-1 activation in PMA-differentiated THP1 cells (Fig 3B).

Next, to further confirm caspase-1 inhibitory effect of BFA in macrophages, I performed the fluorescence-activated cell sorting (FACS) analysis. FAM-YVAD-FMK (FLICA) stains intracellular active caspase-1. FLICA-stained BMDMs were increased in response to NLRP3 inflammasome activators. However, BFA reduced FLICA-stained BMDMs (Fig 4). These results indicated that BFA inhibits NLRP3 inflammasome activator-induced caspase-1 activation in macrophage.

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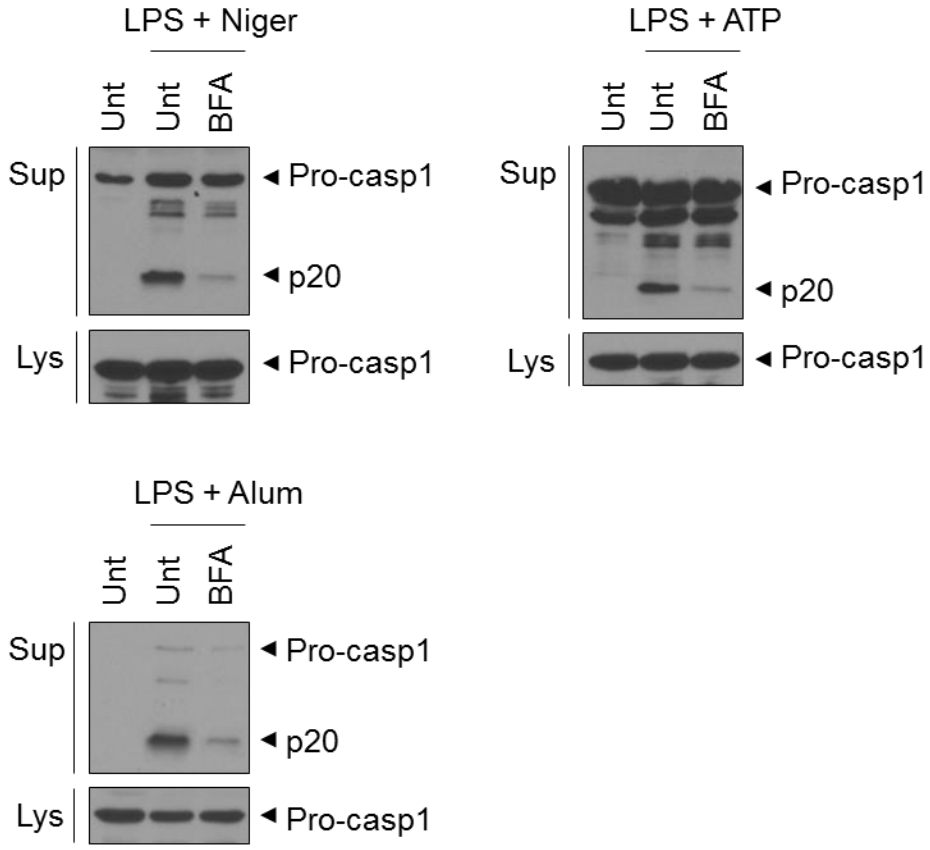


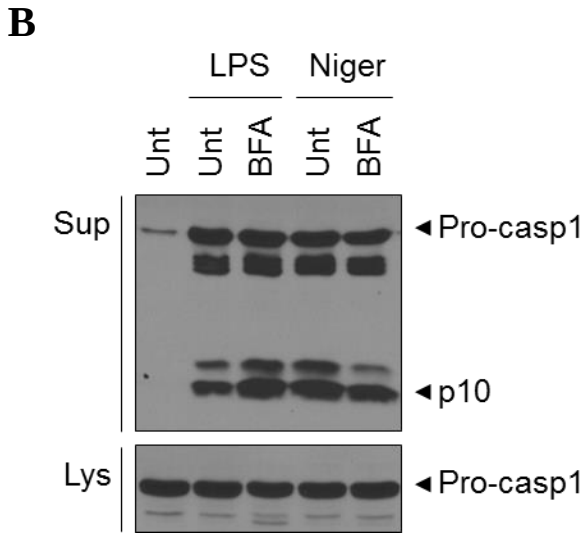
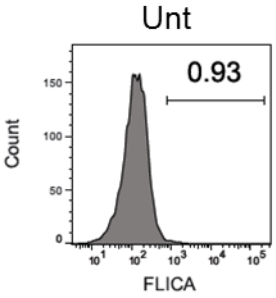
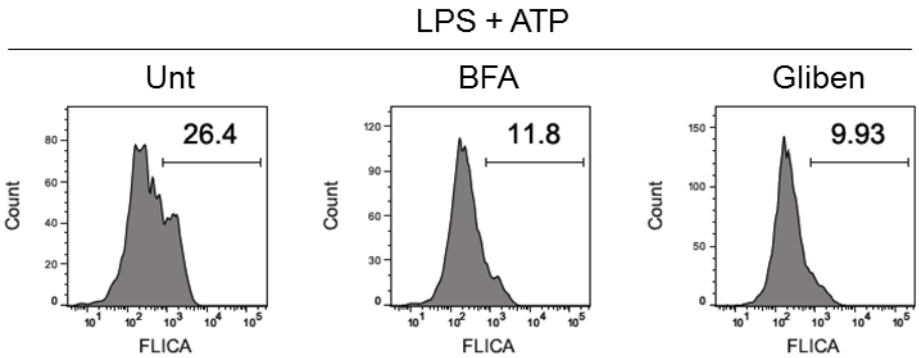


Figure 3. Brefeldin A inhibits NLRP3-dependent caspase-1 activation in macrophages. (A) Immunoblotting of culture supernatants and lysates from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs, followed with nigericin (5 μM) for 40 mins or ATP (2 mM) for 30 mins or alum (500 μg/ml) for 6 hrs. (B) Immunoblotting of culture supernatants and lysates from PMA-differentiated THP1 cells pretreated with nigericin (5 μM) for 40 mins or LPS (0.5 μg/mL) for 6 hrs in the presence or absence of BFA (2 μg/mL). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor), Niger: nigericin (bacterial toxin), Alum: alum crystal, Sup: supernatant, Lys: lysate, Pro-casp1: pro-caspase-1, p10/p20: active-caspase-1.





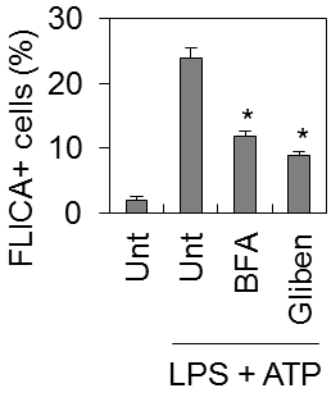


Figure 4. Brefeldin A reduces the number of active caspase-1-containing cells in response to NLRP3 inflammasome activators. FLICA analysis of BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) or glibenclamide (50μM) for 3 hrs, followed with ATP (2 mM) for 30 mins. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor), Gliben: glibenclamide (potassium channel inhibitor). FLICA: caspase-1 fluorescein (indicator of active caspase-1).

3. Brefeldin A does not inhibit NLRP3-independent caspase-1 activation

To date, four inflammasomes have been discovered. Unlike NLRP3 inflammasome, double-strand DNA-induced AIM2 inflammasome and pseudomonas aeruginosa infection-induced NLRC4 inflammasome activation did not inhibited by BFA (Fig 5A, B). Unlike macrophages, inflammasome is activated in response to only LPS treatment in monocytes. To identify that single signal pathway leads to different response to BFA, macrophages were treated with only ultra-pure LPS. In short-term treatment, IL-1β is not secreted in response to only ultra-pure LPS. However, in long-term treatment, a small amount of IL-1β is secreted in BMDMs (Fig 6A). Also in peritoneal macrophages, IL-1β is secreted in response to only long-term ultra-pure LPS treatment (Fig 6B). However, I founded that LPS-single stimulation in macrophages leads to NLRP3-independent IL-1β secretion unlike monocytes (Fig 6B). Moreover, LPS-single stimulation-induced and NLRP3-independent IL-1β secretion did not inhibited by BFA treatment (Fig 6C). These results imply that BFA does not inhibit NLRP3-independent caspasae-1 activation in macrophages.

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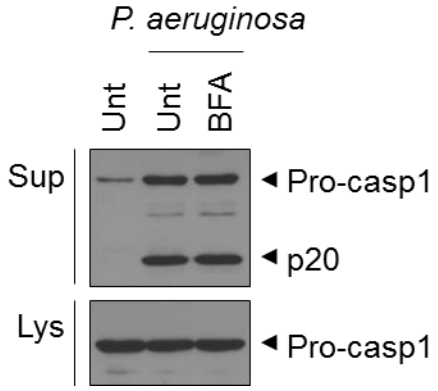

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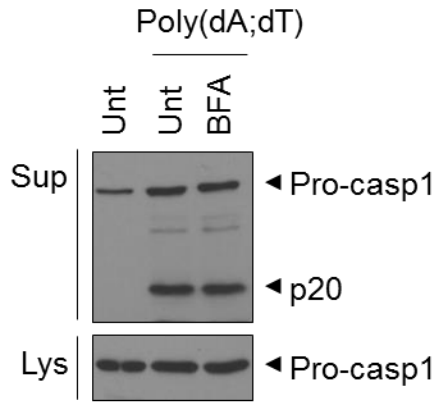

Figure 5. Brefeldin A does not inhibit NLRC4 and AIM2-dependent caspase-1 activation. (A) Immunoblotting of cell culture supernatants and lysates from BMDMs infected with *Pseudomonas aeruginosa* (3 MOI), NLRC4 inflammasome activator, for 3 hrs in the presence or absence of (2 μg/mL) BFA. (B) Immunoblotting of cell culture supernatants and lysates from BMDMs transfected with Poly (dA;dT) (1 μg/mL), AIM2 inflammasome activator, for 6 hrs in the presence or absence of (2 μg/mL) BFA. Unt: untreated, BFA: brefeldin A (classical protein secretion inhibitor), *P. aeruginosa*: *Pseudomonas aeruginosa*, Poly(dA;dT): double-stranded DNA, Sup: supernatant, Lys: lysate, Pro-casp1: pro-caspase-1, p20: active-caspase-1.

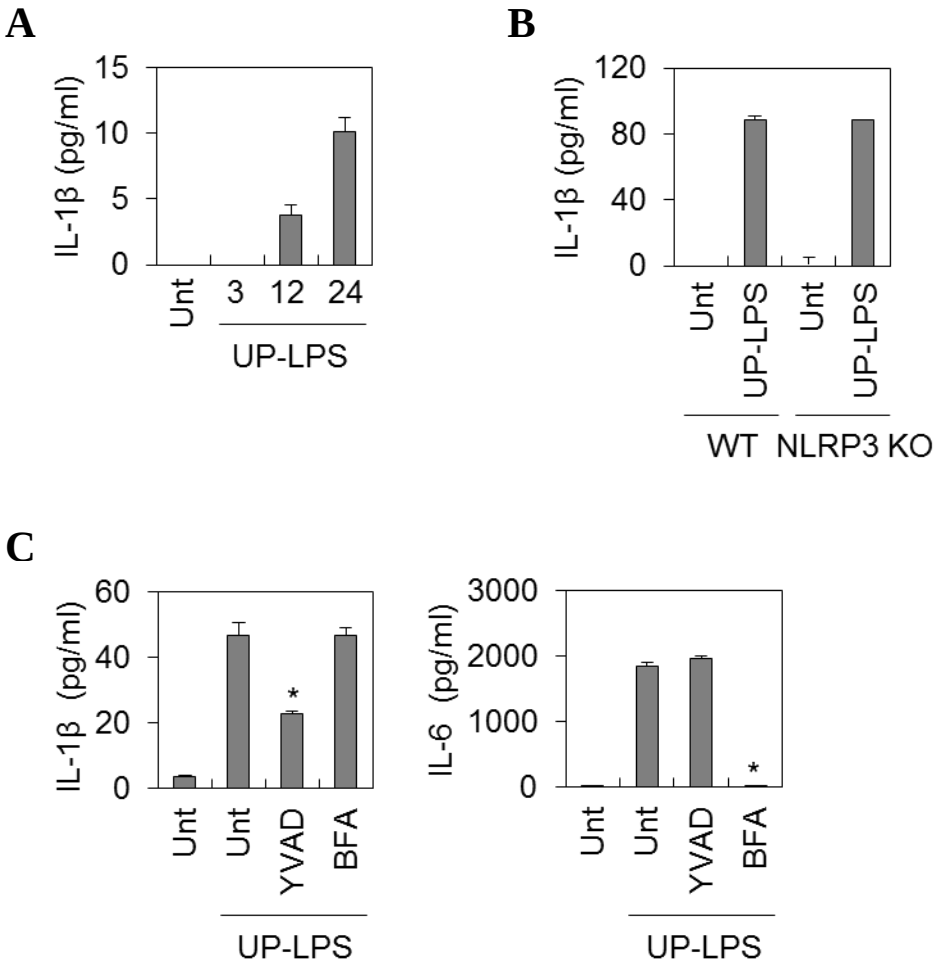

Figure 6. Brefeldin A does not inhibit prolonged LPS-induced IL-1β secretion in macrophages. (A) Quantification of IL-1β in culture supernatants from BMDMs pretreated with ultra-pure LPS (0.5 μg/mL) for 3, 12 or 24 hrs. (B) Quantification of IL-1β in culture supernatants from peritoneal macrophages isolated from wild type or NLRP3 knockout mice pretreated with ultra-pure LPS (0.5 μg/mL) for 12 hrs. **.** (C) Quantification of IL-1β and IL-6 in culture supernatants from peritoneal macrophages isolated from NLRP3 knockout mice pretreated with ultra-pure LPS (0.5 μg/mL) in the presence or absence of BFA (2 μg/mL) for 12 hrs. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, UP-LPS: ultra-pure lipopolysaccharide (TLR4 agonist), YVAD: Ac-YVAD-cmk (caspase-1 inhibitor), BFA: brefeldin A (classical protein secretion inhibitor), WT: wild-type, NLRP3 KO: NLRP3 knockout.

4. Brefeldin A does not directly inhibit IL-1β secretion

Active caspase-1 cleaves pro-IL-1β to IL-1β. This process is required for IL-1β secretion. Therefore, the role of BFA in the IL-1β secretion after caspase-1 activation was examined. To exclude the inhibitory effect of BFA on activated caspase-1, BFA was treated after caspasae-1 is activated with LPS plus ATP or nigericin. Also, NLRP3 inflammasome activators-contained medium was changed to fresh medium before BFA treatment to stop the sustained caspase-1 activation by NLRP3 inflammasome stimulators. In this condition, BFA did not inhibit caspase-1 activation and IL-1β secretion even in BMDMs (Fig 7A, B). However IL-6 secretion was inhibited by BFA treatment (Fig 7B). Taken together, these results indicated that BFA does not directly inhibit IL-1β secretory pathway in macrophages.

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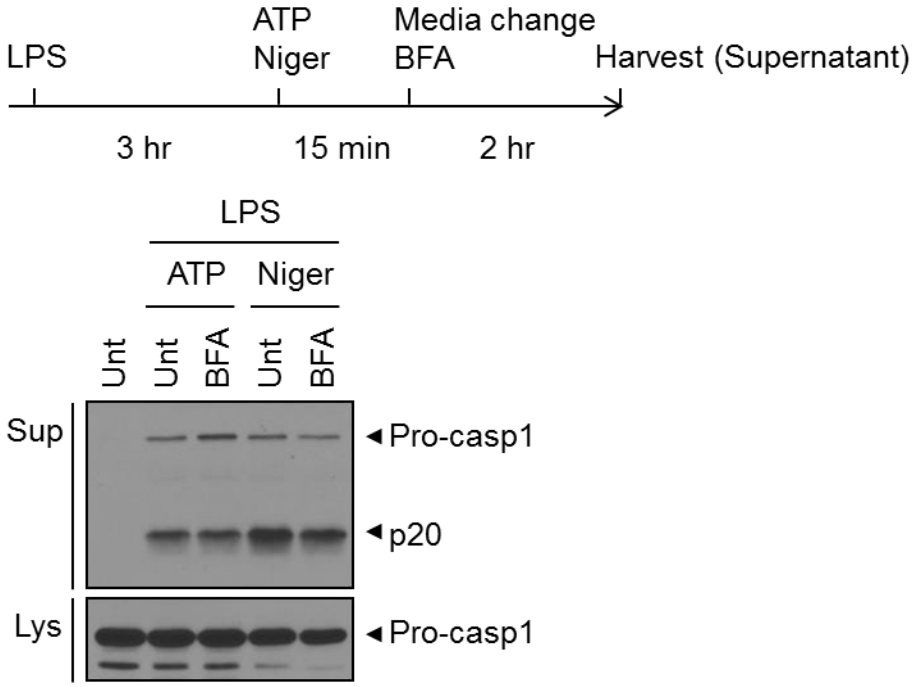

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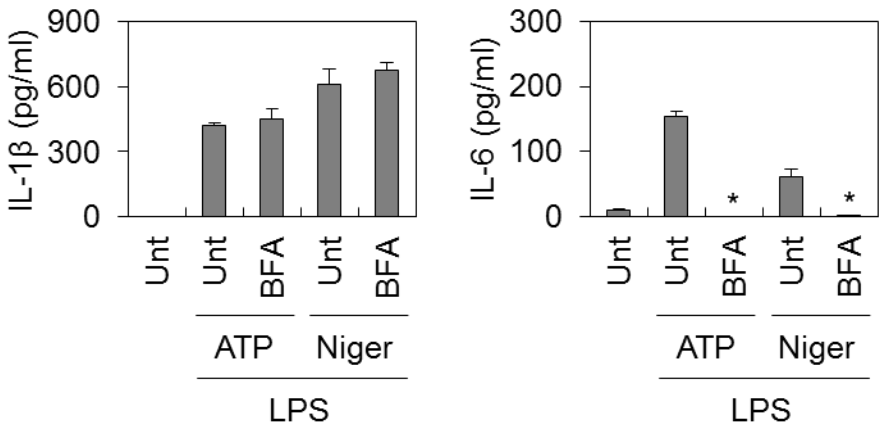

Figure 7. Brefeldin A does not inhibit IL-1β secretion after caspase-1 activation in macrophages. (A) Immunoblotting of culture supernatants and lysates from BMDMs. BMDMs were pretreated with LPS (0.25 μg/mL) for 3 hrs, followed with ATP (2 mM) or nigericin (5 μM) for 15 mins. Then media were changed to fresh media. After changing the media, BMDMs were treated with or without BFA (2 μg/mL) for 2 hrs. (B) Quantification of IL-1β and IL-6 in culture supernatants from BMDMs pretreated as in A. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), Niger: nigericin (bacterial toxin), BFA: brefeldin A (classical protein secretion inhibitor), Sup: supernatant, Lys: lysate, Pro-casp1: pro-caspase-1, p20: active-caspase-1.

5. Brefeldin A inhibits the assembly of NLRP3 inflammasome

As previous studies reported, inflammasomes are multiprotein complex and they are assembled by their activators[43,44]. These events are significant for caspase-1 activation. To explore the effect of BFA on NLRP3 inflammasome assembly, the formation of NLRP3 speck-like structure that refers to NLRP3 self oligomerization was observed in NLRP3-GFP-expressing macrophages. The formation of NLRP3 speck-like structure was inhibited by BFA treatment (Fig 8A). Moreover, I checked ASC self oligomerization triggered by NLRP3 inflammasome activators. LPS plus ATP, a NLRP3 inflammasome activator, induced robust ASC oligomerization in BMDMs determined by crosslinking experiment. However, this ASC oligomerization was abrogated by BFA (Fig 8B). Furthermore, to examine the interaction between NLRP3 and ASC, I employed proximal ligation assay (PLA). I found that BFA also inhibited their interaction (Fig 8C). These observations indicated that BFA negatively regulates the assembly of NLRP3 inflammasome.

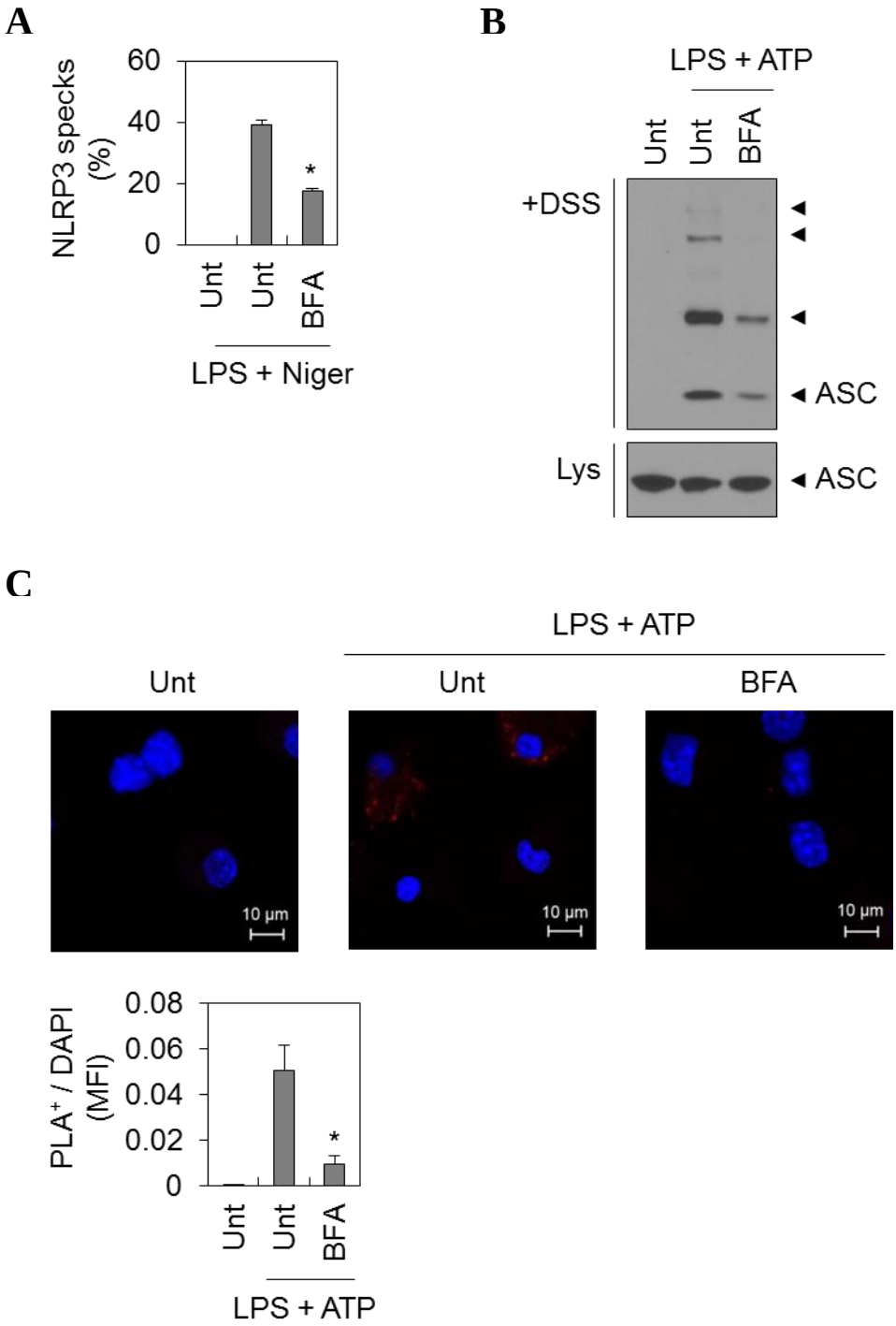

Figure 8. Brefeldin A inhibits the assembly of NLRP3 inflammasome. (A) Confocal microscope imaging (data not shown) of NLRP3-GFP-expressing macrophages pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs, followed with nigericin (5 μM) for 40 mins. Quantification of NLRP3 speck-like structure was performed by counting cells in 3 individual filed. (B) Immunoblotting of culture lysates and cross-liking pellet from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. (C) Confocal microscope imaging of PLA (ASC-NLRP3)-stained BMDMs treated as in B. PLA signals (red) were quantified by measurement the mean fluorescent intensity (MFI) of PLA per MFI of DAPI (blue). The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), Niger: nigericin (bacterial toxin), BFA: brefeldin A (classical protein secretion inhibitor), +DSS: dextran sodium sulfate-induced lysate pellet, Lys: lysate, PLA: proximal ligation assay.

6. Brefeldin A inhibits mitochondrial damage but not mitochondrial movement triggered by LPS plus ATP stimulation.

Mitochondria are major source of cellular reactive oxygen species (ROS). The electron transport chain in the mitochondrial inner membrane is involved in the production of energy. In this place, oxygen acts as a final electron acceptor. When the electron transport chain breaks down, ROS can be accumulated to toxic levels within the cells[45]. Several studies suggest the possibility that ATP or monosodium urate (MSU) crystal-induced ROS production activates NLRP3 inflammasomes[23]. Because damaged mitochondria can produce numerous ROS, I observed mitochondrial damage. By using live cell co-staining with Mito Tracker Green (staining mitochondria independent on mitochondrial membrane potential) and Mito Tracker Deep Red (staining mitochondria dependent on mitochondrial membrane potential), I found that mitochondrial damage was enhanced in NLRP3 inflammasome activated BMDMs. However, BFA reduced mitochondrial damage (Fig 9A). Next, to investigate the ROS production in mitochondria, I analyzed mitochondrial ROS level in BMDMs. MitoSOX, the mitochondrial ROS specific indicator,-stained cells are increased in response to NLRP3 inflammasome activators in BMDMs. However, BFA inhibited production of mitochondrial ROS (Fig 9B).

Additionally, recent studies suggest that mitochondria move to ER in microtubule-dependent ways and this mitochondrial movement mediates NLRP3 inflammasome assembly[30]. To observe the mitochondrial movement in response to NLRP3 inflammasome activators, I performed immunofluorescence assay in BMDMs. Mitochondria were stained with anti-tom20 protein antibody and nucleus was stained with 4’,6-diamidino-2-phenylindole (DAPI) mount solution. Cytoplasm was determined by staining with anti-tubulin antibody. In this experiment, mitochondria move to peri-nuclear space in response to NLRP3 inflammasome

activators in BMDM and this mitochondrial movement was induced by signal 2 (ATP) not signal 1 (LPS) (Fig 10A). Moreover, BMDMs isolated from NLRP3 knockout mice also appeared same mitochondrial movement as wild-type BMDMs (Fig 10A). This result implies that NLRP3 does not necessary for mitochondrial trafficking in response to NLRP3 inflammasome signal 2 activators. Next, I investigated whether inhibition of vesicle transport between ER and Golgi by BFA treatment has a significant effect on mitochondrial movement. But BFA with NLRP3 inflammasome activators also transports mitochondria to peri-nuclear area in BMDMs (Fig 10B). Collectively, BFA abrogates the mitochondrial damage and ROS generation that involved in NLRP3 inflammasome activation but not mitochondrial transport to peri-nuclear region.

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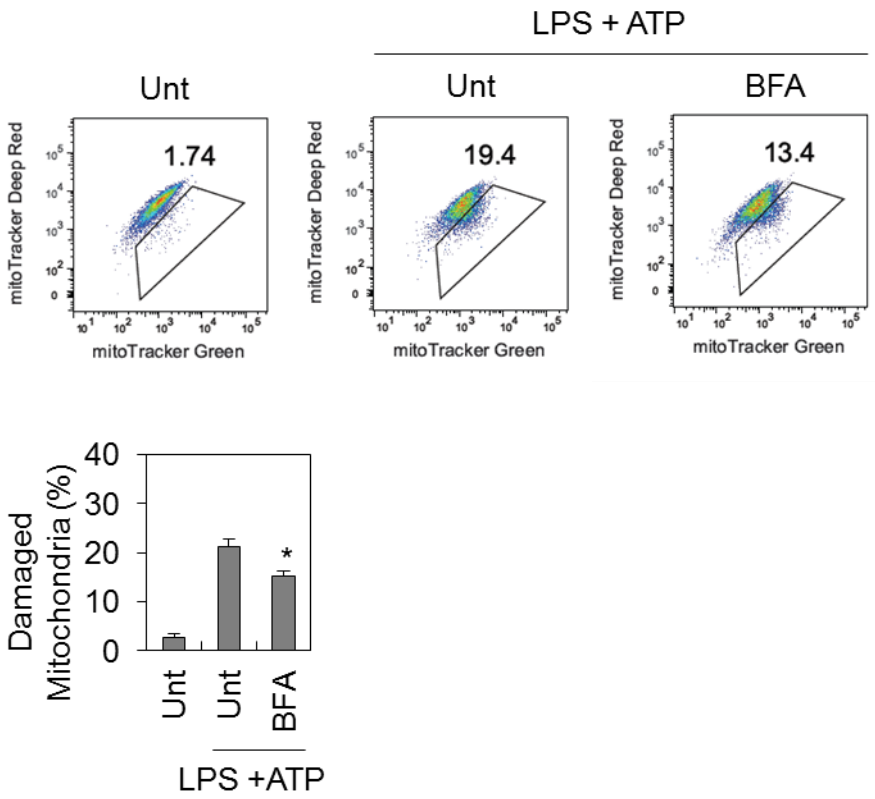

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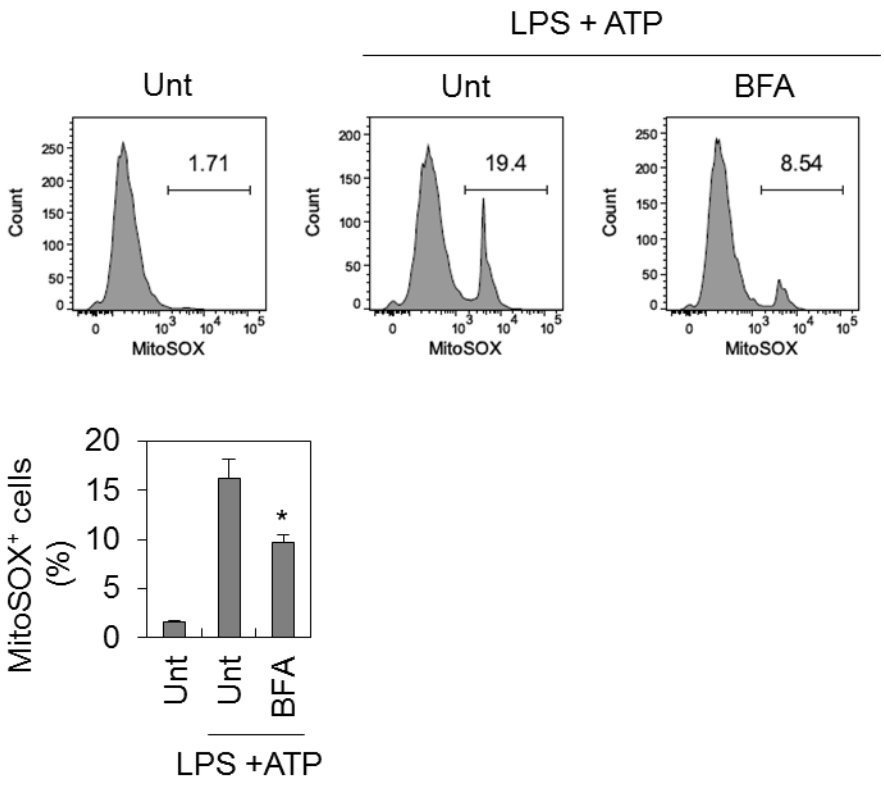

Figure 9. Brefeldin A reduces mitochondrial damage and the production of mitochondrial reactive oxygen species (ROS) in response to NLRP3 inflammasome activators. (A) FACS analysis of MitoTracker Deep Red (mitochondrial membrane potential sensitive mitochondria probe) and MitoTracker Green (mitochondria membrane potential insensitive mitochondrial probe)-stained BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. (B) FACS analysis of MitoSOX (mitochondrial ROS indicator)-stained BMDMs pretreated as in A. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor).

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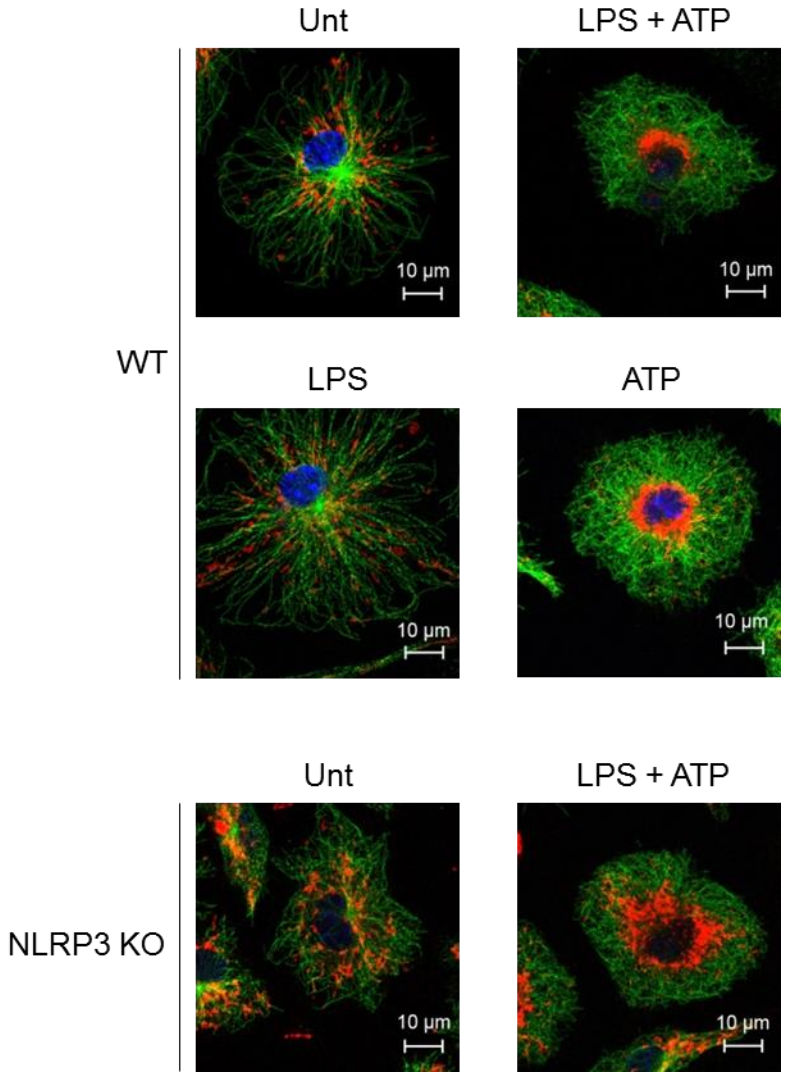

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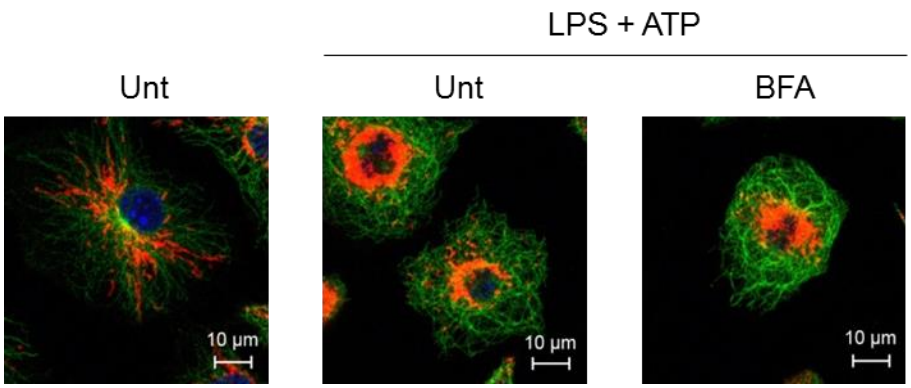

Figure 10. NLRP3 inflammasome activators induce mitochondrial movement to peri-nuclear space independent on brefeldin A. (A) Confocal microscope imaging of tom20 (red), α-tubulin (green) and nuclei (blue) stained BMDMs isolated from wild type or NLRP3 knockout (KO) mice. BMDMs were pretreated with LPS (0.25 μg/mL) 3 hrs, followed with ATP (2 mM) for 30 mins. (B) Confocal microscope imaging of tom20 (red), α-tubulin (green) and nuclei (blue) stained BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor), WT: wild-type, NLRP3 KO: NLRP3 knockout.

7. Knockdown of brefeldin A-inhibited guanine nucleotide exchange factor 1 (BIG1) abolishes the activation of NLRP3 inflammasome

BFA directly inhibit guanine nucleotide exchange factor (GEF). This inhibitory effect on GEF leads to ADP ribosylation factor (ARF) activation[46]. Active-ARF recruits coat protein (COP) to Golgi membrane for vesicle production. Until now, various Golgi-related GEFs have been discovered. Among these GEFs, Brefeldin A inhibited GEF 1 (BIG1) is representative BFA-sensitive GEF. To identify the potent role of BIG1 in inflammasome activation, macrophages were infected with letivirus carrying scramble or *Arfgef1* (gene name of BIG1)-target shRNA and selected by puromycin. *Arfgef1* (#1) shRNA-stably expressing macrophages exhibit reduced expression of *Arfgef1* mRNA (Fig 11A). *Arfgef1*-knockdowned macrophages (BIG1-konckdowned macrophages) elicit abrogated IL-1β secretion (Fig 11B). Not only IL-1β secretion, also caspase-1 activation is reduced in BIG1-konckdowned macrophages (Fig 12A) in response to NLRP3 inflammasome activators. Moreover BIG1-konckdowned macrophages fail to oligomerization of ASC in response to NLRP3 inflammasome activators (Fig 12B). These results indicated that BIG1, a target molecule of BFA, has important role for NLRP3 inflammasome activation.

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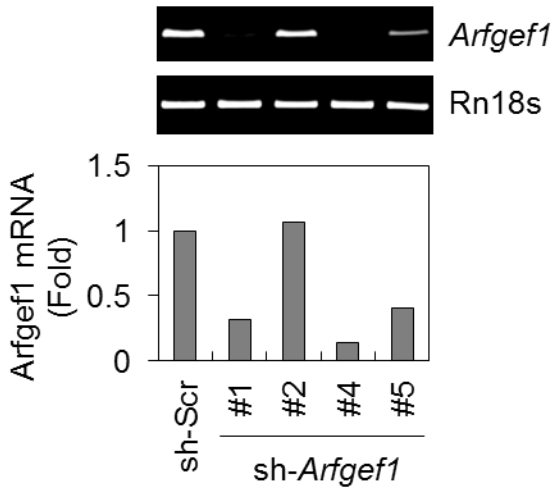

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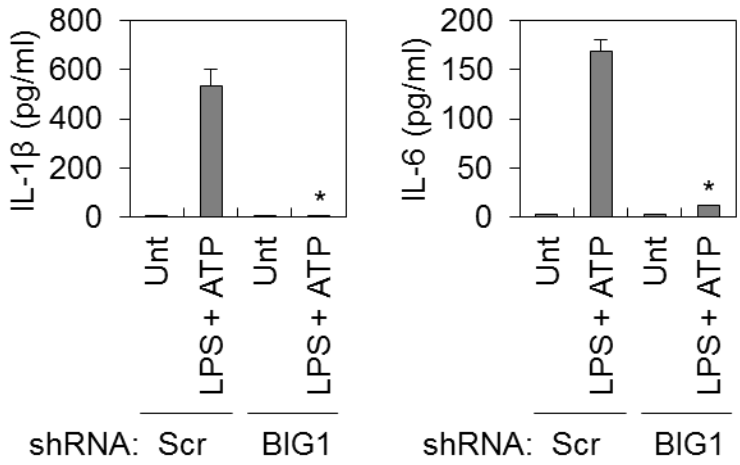
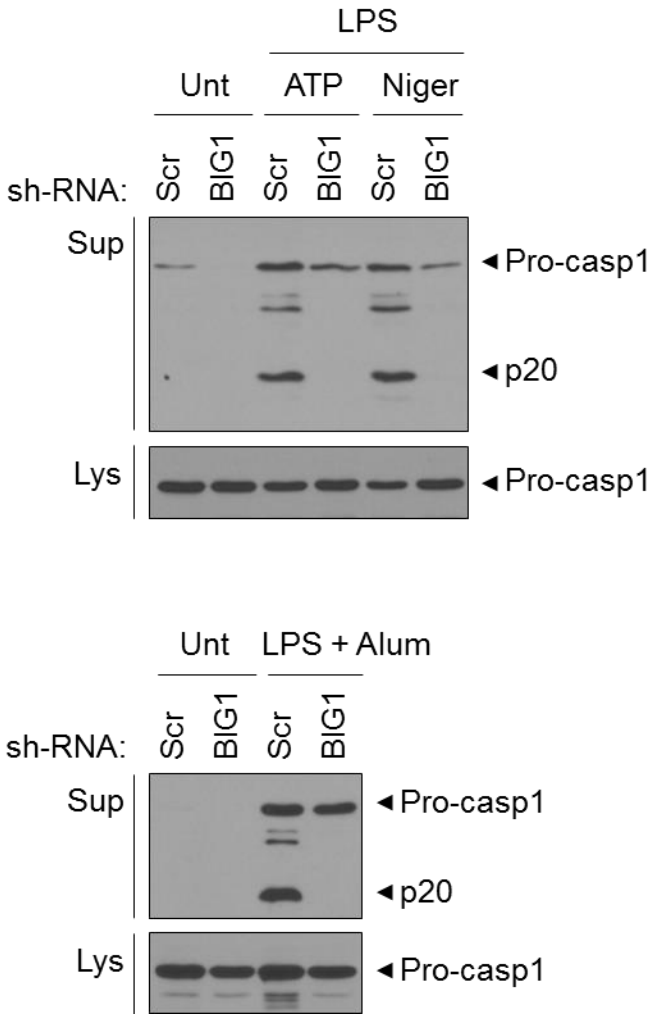


Figure 11. BIG1-knockdowned macrophages exhibit reduced IL-1β secretion. (A) Quantitative PCR and PCR analysis of scramble or BIG1-konckdowned macrophages. (B) Quantification of IL-1β and IL-6 in culture supernatants from sh-scramble or BIG1-konckdowned macrophages pretreated with LPS (0.25 μg/mL) for 3 hrs followed with ATP (2 mM) for 30 mins. All mRNA expression level is relative to Rn18s mRNA. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). *Arfgef1*: BIG1 encoded gene. Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), sh-Scr: scrambled short hairpin RNA, sh-*Arfgef1*: short hairpin RNA for *Arfgef1*, *Arfgef1*: BIG1 encoded gene, Rn18s: 18S ribosomal RNA.

A



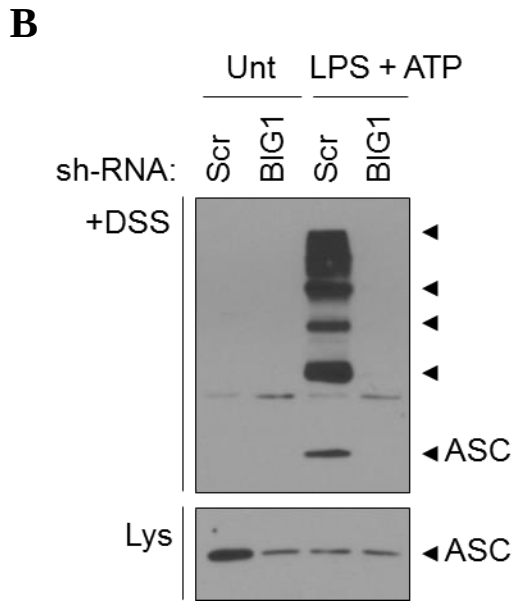


Figure 12. BIG1-knockdowned macrophages exhibit reduced NLRP3 inflammasome activation. (A) Immunoblotting of supernatants and lysates from scramble or BIG1-konckdowned macrophages pretreated with LPS (0.25 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins or nigericin (5 μM) for 40 mins or alum (500 μg/mL) for 6 hrs. (B) Immunoblotting of culture lysates and cross-linked pellet from scramble or BIG1-konckdowned macrophages pretreated with LPS (0.25 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), Niger: nigericin (bacterial toxin), Alum: alum crystal, sh-RNA: short hairpin RNA, Scr: scramble, *Arfgef1*: BIG1 encoded gene, Sup: supernatant, Lys: lysate, +DSS: dextran sodium sulfate-induced lysate pellet, Pro-casp1: pro-caspase-1, p20: active-caspase-1.

8. Brefeldin A and BIG1-knockdown impair TLR4-triggered cytokine production

Generally, activation of NLRP3 inflammasome requires two independent signals in BMDMs. Most of signal 1 stimuli, such as LPS, activate TLRs and induce TLR-dependent mRNA expression of proinflammatory cytokines. To verify the effect of BFA treatment or BIG1-knockdown on TLR-dependent mRNA expression of proinflammatory cytokine, I examined the mRNA expression of IL-6 and IL-1β in response to LPS. IL-6 and IL-1β mRNA expression were decreased in BFA-treated BMDMs (Fig 13A) and BIG1-knockdown macrophages (Fig 13B). These results suggest that BFA and BIG1-knockdown system inhibit TLR4-induced cytokine production.

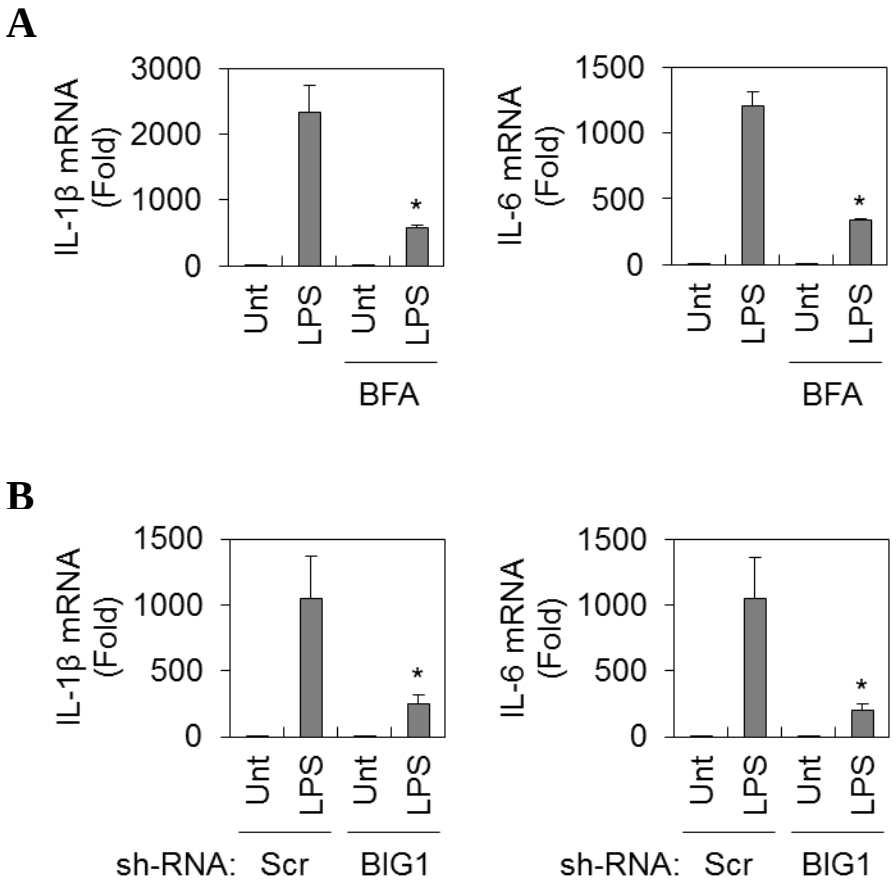


Figure 13. Brefeldin A and BIG1-knockdown inhibit TLR4-triggered IL-6 and IL-1β mRNA expression. (A) Quantitative PCR analysis of BMDMs pretreated with LPS (0.5 μg/mL) in the presence or absence of BFA (2 μg/mL) for 3 hrs. (B) Quantitative PCR of scramble or BIG1-konckdowned macrophages pretreated with LPS (0.5 μg/mL) for 3 hrs. All mRNA expression level is relative to Rn18s mRNA. The values are expressed as means ±SEM. *, $P < 0.05$ (*t* test). Unt: untreated, LPS: lipopolysaccharide (TLR4 agonist), BFA: brefeldin A (classical protein secretion inhibitor), sh-RNA: short hairpin RNA, Scr: scramble, *Arfgef1*: BIG1 encoded gene.

9. Brefeldin A does not inhibit signal 2 stimuli-induced caspase-1 activation

Next I wondered that BFA could control signal 2 response, I employed NLRP3-overxpressing macrophages. One of the roles of signal 1 is NLRP3 production. In normal condition, NLRP3 is barely expressed for restriction of NLRP3 inflammasome activation. In NLRP3-overexpressing macrophages, NLRP3 inflammasome is activated in response to only signal 2 activators (Fig 14). Thus I used NLRP3-overexpressing macrophages to confirm the effect of BFA on signal 2 activators. However, in this condition, BFA does not inhibit NLRP3 inflammasome activation (Fig 14). These results indicated that BFA does not inhibit signal 2 activator-induced response.

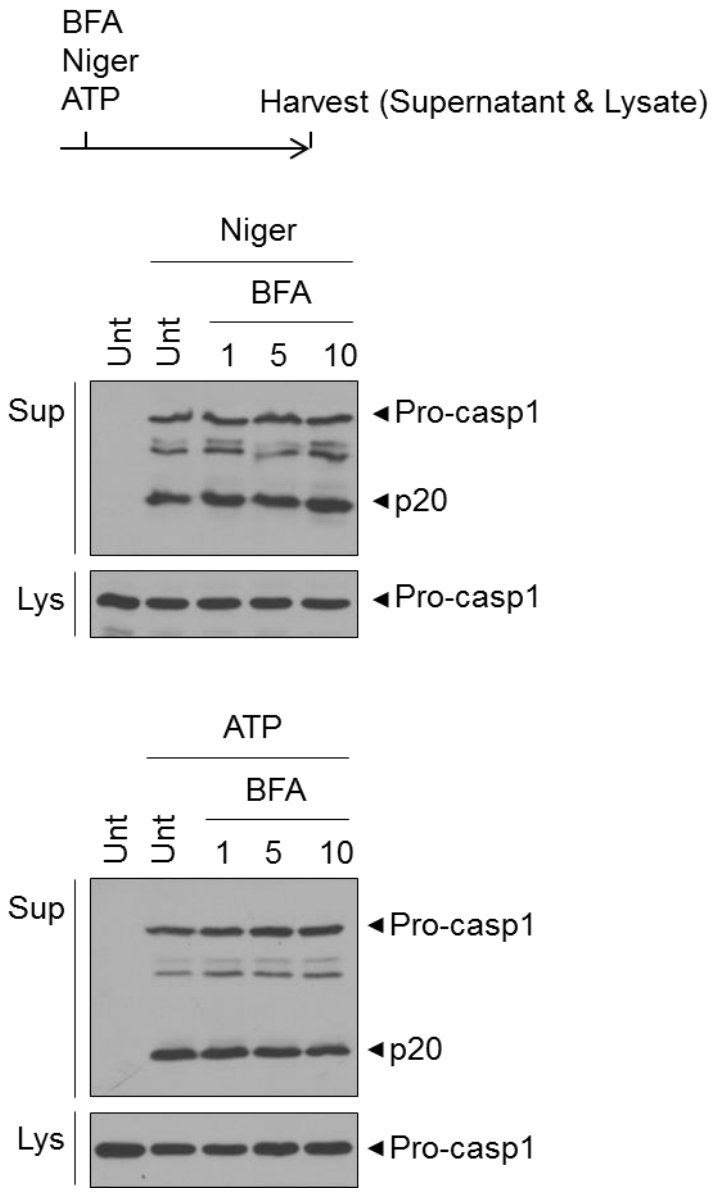
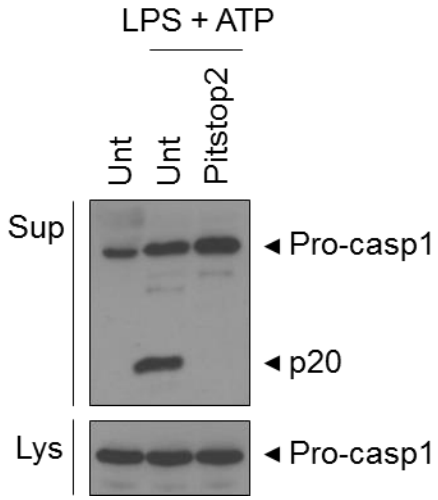


Figure 14. Brefeldin A does not inhibit signal 2 stimuli-induced caspase-1 activation in NLRP3-overexpressing macrophages. Immunoblotting of culture supernatants and lysates from NLRP3-overexpressing macrophages pretreated with nigericin (5 μM) or ATP (2 mM) in the presence or absence of BFA (2 μg/mL) for 2 hrs or 3 hrs, respectively. Unt: untreated, Niger: nigericin (bacterial toxin), BFA: brefeldin A (classical protein secretion inhibitor), Sup: supernatant, Lys: lysate, Pro-casp1: pro-caspase-1, p20: active-caspase-1.

10. Clathrin inhibits the activation of NLRP3 inflammasome

Vesicle production from Golgi apparatus occurs in “cis” and “trans” Golgi membrane. COPI is involved in vesicle formation from “cis” Golgi membrane and this vesicle is transported to ER[47]. Otherwise, clathrin is major component of vesicle formation complex from “trans” Golgi and plasma membrane[48]. To identify the relation between vesicle formation from Golgi in “trans” side and NLRP3 inflammasome activation, BMDMs were treated with Pitstop2 which is clathrin inhibitor. Pitstop2 inhibits NLRP3 inflammasome activation (Fig 15A) and ASC oligomerization (Fig 15B). Further, I used 4-bromobenzaldehyde N-(2, 6-dimethylphenyl) semicarbazone (EGA) which is late endosome trafficking inhibitor to identify the effect of endosomal trafficking on NLRP3 inflammasome activation. However, endosomal trafficking does not inhibit NLRP3 inflammasome activation (Fig 15C). According to these studies, vesicle formation from Golgi is important to NLRP3 inflammasome activation and their assembly. Collectively, I demonstrated that Golgi apparatus has important role for NLRP3 inflammasome activation (Fig 16).

A



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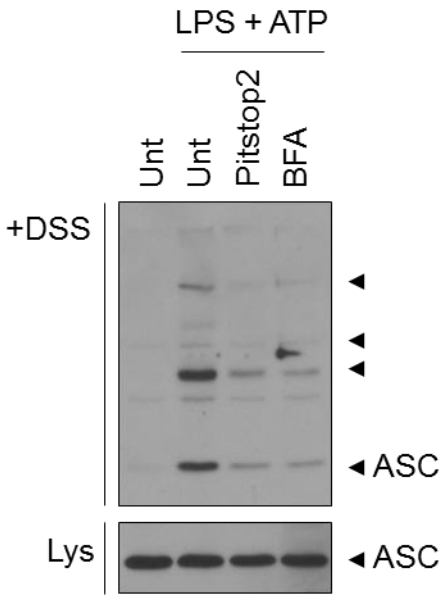

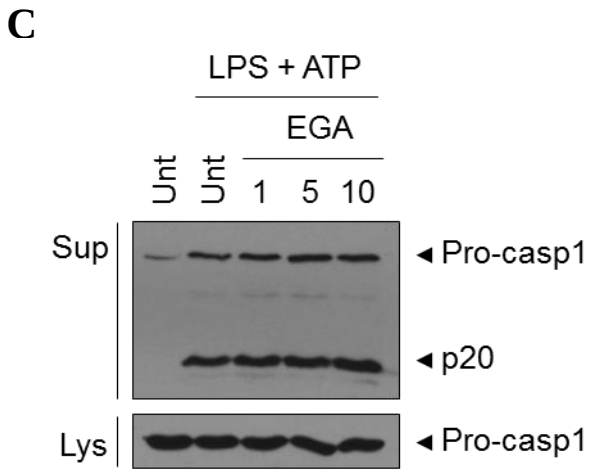

Figure 15. Pitsop2 inhibits the activation of NLRP3 inflammasome. (A) Immunoblotting of supernatants and lysates from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of pitstop2 (10 μM) for 3 hrs, followed with ATP (2 mM) for 30 mins. (B) Immunoblotting of culture lysates and cross-liking pellet from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of pitstop2 (10 μM) or BFA (2 μg/mL) for 3 hrs, followed with ATP (2 mM) for 30 mins. (C) Immunoblotting of supernatants and lysates from BMDMs pretreated with LPS (0.25 μg/mL) in the presence or absence of EGA (1, 5 or 20 μM) for 3 hrs, followed with ATP (2 mM) for 30 mins. Unt: untreated, BFA: brefeldin A (classical protein secretion inhibitor), Pitstop2: Pitstop® 2 (clathrin inhibitor), EGA: 4-bromobenzealdehde N-(2,6-dimethylphenyl) semicarbazon (late endosome trafficking inhibitor), +DSS: dextran sodium sulfate-induced lysate pellet, Sup: supernatant, Lys: lysate, Pro-casp1: pro-caspase-1, p20: active-caspase-1.

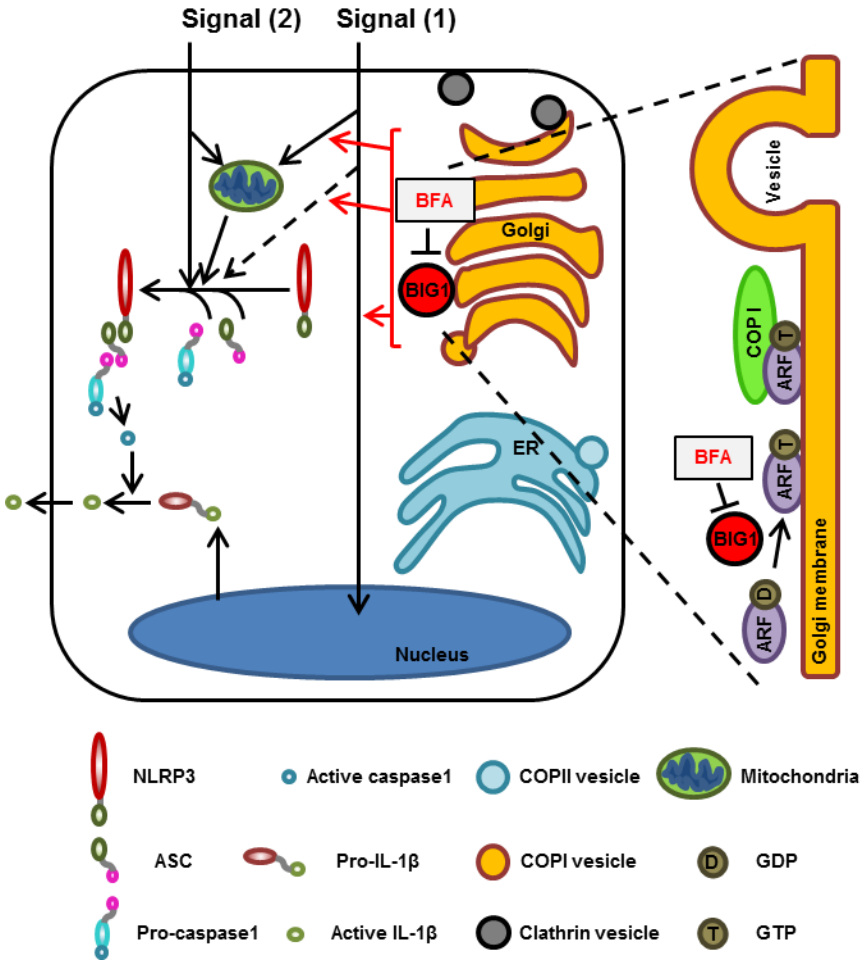

Figure 16. Proposed role of the Golgi apparatus in NLRP3 inflammasome activation in macrophages. Brefeldin A (BFA) that induces Golgi structure inhibits NLRP3 inflammasome assembly through signal 1 response inhibition in macrophages. This response also induced by BFA-inhibited guanine nucleotide exchange factor 1 (BIG1)-knockdowned macrophages.

Ⅳ. DISCUSSION

Golgi apparatus consists of dozens of cisternae, which is flattened and tacked pouches. Golgi membrane divides three primary compartments, known generally as “cis”, “medial” and “trans”. The Golgi apparatus receives proteins and lipids from the rough endoplasmic reticulum (ER). And then proteins and lipids are modified, sorted and packed into vesicles for delivery to targeted destinations. Up to date, several researchers investigate various organelles connected with NLRP3 inflammasome activation, such as mitochondria, ER and lysosome[23,49-51]. However involvement of Golgi apparatus in NLRP3 inflammasome activation remains unclear.

In this study, I try to explain the inflammasome activation pathway and IL-1β secretory pathway using brefeldin A (BFA). BFA is a lactone antibiotic isolated from the fungal organisms such as *Eupenicillium brefeldianum*. BFA inhibits the transport of proteins from ER to Golgi indirectly by interfering interaction of coat protein, conserved coat protein complex I (COPI), with Golgi membrane. Because BFA prevents the protein secretory system, it allows measurement of secretory cytokine with fluorescence-activated cell sorting (FACS) analysis. Up to now, BFA has been used to inhibitor of classical protein secretory pathway. IL-1β has been thought that it is secreted through non-classical protein secretory pathway. I also showed that IL-1β secretory pathway is ER-Golgi-independent pathway using BFA in monocytes. However, in macrophages, BFA inhibits NLRP3 inflammasome activation. These opposing responses probably arise from characteristics of each cell. The monocytes and macrophages are mononuclear phagocytes that have critical and distinct roles in tissue homeostasis and immunity. Monocytes can differentiate in to macrophages in response to appropriate stimulus and it changes transcriptional profiling[52]. The cell type-dependent effect of BFA on caspase-1 activation requires further experiments.

Mitochondrial dysfunction and release of mitochondrial-derived materials are important events of NLRP3 inflammasome activation. In this reports, mitochondrial damage and production of mitochondrial reactive oxygen species (ROS) are diminished by BFA. It is crucial evidence of relation between mitochondria and Golgi apparatus. I considered that Golgi apparatus plays a role in NLRP3 inflammasome activation after mitochondria move to peri-nuclear region because this mitochondrial movement is not disturbed by BFA. Previous studies show that mitochondria migrate to ER and form mitochondria-associated ER membranes (MEMs) in response to NLRP3 inflammasome activators[30]. The MEMs are inferred to platform of NLRP3 inflammasome assembly. Since Golgi is near the ER, there is possibility that NLRP3 inflammasome assembly can be influenced by Golgi.

In mammalian and yeast cells, the major target of BFA is Sec7 guanine nucleotide exchange factors (GEFs), especially brefeldin A –inhibited guanine nucleotide-exchange protein 1 (BIG1) and BIG2[53]. Sec7 GEFs are required for activation of ADP ribosylation factors (ARFs) through the exchange of GDP to GTP. Activated ARFs have been shown that regulate the binding of vesicle coat complex including COPI on Golgi, Clathrin-AP1 on the *trans*-Golgi network (TGN) and COPII on ER[47,48]. These membrane-bounded proteins produce vesicle from their located organelle. BIG1 is COPI-related GEF. Therefore, BIG1 regulates production of vesicle from Golgi membrane. Because BIG1 is a target of BFA, BFA prevents vesicle formation from Golgi membrane. To inhibit specific target of BFA, I used knockdown system of *Arfgef1*, gene encoded BIG1. In this experiment, BIG1-knockdowned macrophages completely fail to activate the NLRP3 inflammasome. Knockdown of BIG1 probably evokes malfunction of Golgi and it means that Golgi has important role for NLRP3 inflammasome activation.

I tried to find out the mechanism of inhibitory effect of BFA and role of

BIG1 on NLRP3 inflammasome activation. BFA-treated or BIG1-knockdowned macrophages exhibit reduced signal 1 (LPS)-induced proinflammatory cytokine mRNA expression. Furthermore, to investigate the effect of BFA treatment on signal 2, NLRP3-overexpressing macrophages were tested. In NLRP3-overexpressing macrophages, NLRP3 inflammasome is activated in response to only signal 2 activators. However, BFA does not inhibit the NLRP3 inflammasome activation in response to only signal 2 activators in NLRP3-overexpressing macrophages. Moreover, signal 2-induced mitochondrial movement does not inhibited by BFA treatment. Together these experiments, I draw a conclusion that BFA inhibits signal 1 response not signal 2 response. Further experiments are required to explain the effect of BFA on signal 1 response.

Not only BFA, Pitstop2 (clathrin inhibitor) also inhibits the activation of NLRP3 inflammasome. Clathrin is responsible for vesicle formation from trans-Golgi membrane and plasma membrane[48]. However, because not only vesicle formation from Golgi, vesicle formation from plasma membrane is also dependent on clathrin, vesicle formation from plasma membrane might influence NLRP3 inflammasome activation. Further studies are needed to explain how clathrin can contribute to NLRP3 inflammasome activation.

Collectively, Golgi apparatus is related with NLRP3 inflammasome activation through regulation of signal 1 response and mitochondrial dysfunction. Based on these results, I demonstrate that Golgi apparatus are associated with NLRP3 inflammasome activation and proinflammatory cytokine production.

Ⅴ. CONCLUSION

This study proposes further evidence for role of the Golgi apparatus on NLRP3 inflammasome activation. I show that BFA-treated macrophages exhibit diminished NLRP3 inflammasome assembly and activation but not AIM2 and NLRC4 inflammasome. Further, BFA inhibits mitochondrial damage and ROS generation in response to NLRP3 inflammasome activators but not mitochondrial trafficking. Moreover, BIG1, BFA-sensitive GEF, is a critical molecule that necessary for NLRP3 inflammasome activation. Also BFA-treated or BIG1-knockdowned macrophages exhibit reduced inflammatory cytokine mRNA expression in response to TLR4 stimulation. These findings provide a new insight of Golgi apparatus function on regulation of NLRP3 inflammasome activation and proinflammatory cytokine production.

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ABSTRACT (IN KOREAN)

Golgi apparatus의 NLRP3 inflammasome 활성화 및 염증성 사이토카인 생성 조절 기전 규명

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홍수정

NLRP3 인플라마좀 (inflammasome) 은 NLRP3, ASC, pro-caspase-1으로 이루어진 단백질 복합체이다. NLRP3 인플라마좀은 미생물 감염 및 내부에서 생성된 대사 물질 등 다양한 자극 의해 활성화 된다고 알려져 있다. NLRP3 인플라마좀 활성화는 pro-caspase-1을 활성화를 유도하고, 이는 대표적 염증성 사이토카인 (cytokine) 인 인터류킨 (Interleukin)-1β (IL-1β) 를 활성화 형태로 성숙시키고 분비시켜 염증의 초기 반응을 유도한다. 과도한 NLRP3 inflammasome의 활성화는 최근 일부 대사성, 퇴행성 질환의 병인기전에 연계되어 있음이 보고되고 있다. 이러한 이유로 NLRP3 인플라마좀 경로의 조절 과정은 매우 중요한데 아직 활성화 과정과 IL-1β의 분비과정은 명확하게 알려져 있지 않다. 본 연구에서는 골지체 (Golgi apparatus)를 중심으로 하는 세포 내 소수포 (vesicle)의 이동 과정이 NLRP3 인플라마좀 활성화에 중요한 역할을 하고 있음을 보여주려고 한다. brefeldin A (BFA)는 Golgi apparatus에서 소수포 형성의 억제를 통해 소포체 (endoplasmic reticulum)와 Golgi apparatus 사이의 소수포 이동을 저해시킨다고 알려져 있다. 본 논문에서는 BFA를 사용한 대식 세포에서의 실

험을 통해 NLRP3 인플라마좀 활성화를 억제하고 있음을 확인하였다. 이와 마찬가지로 BFA의 표적인 BFA-inhibited guanine nucleotide exchange factor (GEF) 1 (BIG1)의 발현을 저해 시킨 대식 세포도 앞선 BFA를 처리한 세포와 동일하게 NLRP3 인플라마좀 활성이 억제되어 있음을 확인하였다. BFA와 BIG1 발현 저해를 통한 골지체에서 생성되는 소수포 이동의 억제는 lipopolysaccharide (LPS) 에 의한 사이토카인 발현을 저해시키는 것으로 보아 signal 1으로 유도되는 준비 (priming) 단계의 저해를 통해 NLRP3 인플라마좀 활성을 조절하는 것으로 생각되었다. 본 연구결과를 통해 BIG1을 중심으로하는 골지체에서의 소수포 전달 기능이 NLRP3 인플라마좀 활성화를 조절할 수 있다는 가능성을 제시하였다.

핵심되는 말: 인플라마좀, NLRP3, 골지체, 소수포 이동, Breflecin A, BIG1

PUBLICATION LIST

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