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NLRP3 inflammasome activation and interleukin-1 β release in macrophages require calcium but are independent of calcium-activated NADPH oxidases

Balázs Rada^{1,2}, Jonathan J. Park¹, Payel Sil², Miklós Geiszt³, and Thomas L. Leto^{1,4}

Balázs Rada: radab@uga.edu; Jonathan J. Park: jon.j.park@gmail.com; Payel Sil: payel@uga.edu; Miklós Geiszt: geiszt.miklos@med.semmelweis-univ.hu

¹Laboratory of Host Defenses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Drive, Rockville, MD 20852, U.S.A

²Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, 501 DW Brooks Drive, Athens, GA, 30602 U.S.A

³Department of Physiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary and “Lendület” Peroxidase Enzyme Research Group of the Semmelweis University and the Hungarian Academy of Sciences, Budapest, Hungary

Abstract

Objective and design—We studied the involvement of calcium and calcium-activated NADPH oxidases in NLRP3 inflammasome activation and IL-1 β release to better understand inflammasome signaling in macrophages.

Material or subjects—Human volunteer blood donors were recruited to isolate monocytes to differentiate them into macrophages. Wild-type or DUOX1-deficient C57/B6 mice were used to prepare bone marrow-derived macrophages.

Treatment—Murine or human macrophages were treated *in vitro* with NLRP3 inflammasome agonists (ATP, silica crystals) or calcium agonists (thapsigargin, ionomycin) in calcium-containing or calcium-free medium.

Methods—Intracellular calcium changes were followed by measuring FURA2-based fluorescence. Gene expression changes were measured by quantitative real-time PCR. Protein expression was assessed by western blotting. Enzymatic activity was measured by fluorescence caspase-1 activity assay. IL-1 β release was determined by ELISA. ELISA data were analyzed by ANOVA and Tukey’s post-hoc test.

Results—Our data show that calcium is essential for IL-1 β release in human macrophages. Increases in cytosolic calcium alone lead to IL-1 β secretion. Calcium removal blocks caspase-1 activation. Human macrophages express Duox1, a calcium-regulated NADPH oxidase that

Correspondence: Thomas L. Leto, Ph.D., Laboratory of Host Defenses, NIAID, NIH, USA., 12441 Parklawn Drive, Rockville 20852 MD USA., tlet@niaid.nih.gov, Phone: 301 402-5120. Fax: 301 480-1731.

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produces reactive oxygen species. However, Duox1-deficient murine macrophages show normal IL-1 β release.

Conclusions—Human macrophage inflammasome activation and IL-1 β secretion requires calcium but does not involve NADPH oxidases.

Keywords

Calcium; Duox; macrophage; inflammasome; NADPH oxidase

1. Introduction

Interleukin-1 β (IL-1 β) is a major pro-inflammatory cytokine secreted by several cell types but its main sources are monocytes and macrophages.[1] IL-1 β is produced as an inactive form, pro-IL-1 β , (35 kDa molecular mass) that needs to be cleaved to form the bioactive 17 kDa form.[2] Proteolytic cleavage of IL-1 β is carried out by caspase 1.[2, 3] Formation of active caspase 1 requires proteolysis of procaspase 1 by an intracellular multimeric protein complex called the inflammasome.[4, 2, 5]

Inflammasome activation and consequent IL-1 β release can be triggered by diverse innate immune stimuli that are danger- or pathogen-associated molecular patterns including silica crystals and extracellular ATP.[2, 6, 7] Several different inflammasomes have been discovered but the best-described one is the NLRP3 inflammasome that mediates effects of both silica and ATP.[2]

Details of NLRP3 inflammasome regulation are largely unclear but ion fluxes and cell stress play a role in the process.[2] Earlier observations implicated a role for potassium efflux.[8, 9] Recent publications show that Ca²⁺ ions are essential in NLRP3 inflammasome activation.[1, 10, 11] The role of Ca²⁺ in NLRP3 inflammasome activation has been characterized in murine bone marrow-derived macrophages (BMDM) and human monocytes but this has not been described in human macrophages.

Reactive oxygen species (ROS) are also involved in NLRP3 inflammasome activation.[12, 13] The two main ROS sources in cells are mitochondria and NADPH oxidases.[14, 15] Mitochondrial-derived ROS have been already linked to the NLRP3 inflammasome.[16] The Nox/Duox family of NADPH oxidases consists of seven proteins (Nox1-5 and Duox1-2) that produce superoxide or hydrogen peroxide as their primary enzymatic products.[17, 18] Earlier observations suggested involvement of the phagocytic Nox2-based NADPH oxidase (also expressed in monocytes and macrophages) during inflammasome activation.[12] Later studies, however, disproved this theory since blood cells isolated from patients deficient in NADPH oxidase subunits (Nox2, p22^{phox}) showed normal, or even enhanced, inflammasome activation.[10, 19, 20] Because p22^{phox} is an essential subunit of not only the Nox2-based NADPH oxidase but also Nox1, Nox3 and Nox4, inflammasome activation does not require any of these oxidases (Nox1-4) either.[20] The remaining three NADPH oxidases (Nox5, Duox1 and Duox2) are all activated directly by increases in cytosolic Ca²⁺ levels [21–23]. It is unknown whether Nox5, Duox1 or Duox2 are expressed in monocytes or macrophages and could contribute to NLRP3 inflammasome activation.

In this article, we show that IL-1 β secretion and NLRP3 inflammasome activation in human monocyte-derived macrophages (hMDMs) requires Ca²⁺. ATP- and silica-stimulated IL-1 β secretion is abolished by chelating intra- and extracellular Ca²⁺. Cytosolic Ca²⁺-enhancing agonists induce IL-1 β release. Among the three Ca²⁺-activated NADPH oxidases, only Duox1 expression could be detected in hMDMs. Duox1 is, however, not required for NLRP3 inflammasome regulation since Duox1-deficient murine BMDMs stimulated by silica or ATP had normal IL-1 β release.

2. Materials and Methods

2.1. Purification of human monocytes and macrophage differentiation

Human subjects provided written informed consent for participation under the guidelines of NIH and UGA IRB-approved protocols.[24, 25] Whole blood was drawn at the Transfusion Medicine Branch of the National Institutes of Health or the University of Georgia Health Center. 40 mL blood was anticoagulated by ACD (anticoagulant citrate dextrose) or heparin. Red blood cells were removed by Dextran sedimentation. White blood cells were layered on 5-step Percoll (Sigma) gradient: 85%-80%-75%-70%-65% and centrifuged (800 xg, 30 min, 13° C). PBMCs containing monocytes (PBS/65% Percoll interface) were separated, washed, suspended in RPMI-1640 medium (Life Technologies, Grand Island, NY) and added to 6-well plates (BD Primaria, Swedesboro, NJ). After 30 min, adherent cells were washed four times with HBSS. Cells were differentiated into macrophages by incubating them in RPMI-1640 medium supplemented with 10% pooled human heat-inactivated serum and 50 ng/ml human macrophage colony stimulating factor (hM-CSF, R&D Systems, Minneapolis, MN) for 5–8 days. When mentioned, MDMs were primed with 1 μ g/ml *Salmonella typhimurium* LPS (Sigma Aldrich, St. Louis, MO) for 30 min.

2.2. Collection of human serum

Blood (10 ml) was also collected from healthy volunteers to separate serum. After coagulation, sera were collected, centrifuged (10000g, 5min), filtered through 0.22 μ m size sterile filter and stored at –80 °C for future use. Serum samples from at least 5 different donors were thawed, pooled and heat-inactivated (56 °C, 30 min). 10% heat-inactivated pooled human serum was used on MDM cultures.

2.3. Differentiation of murine BMDMs

Duox1-deficient mice (purchased from Lexicon Pharmaceuticals, Inc., The Woodlands, TX, USA) were previously characterized.[26] These mice were shown to contain a gene-trapping cassette between DUOX1 exons 20 and 21, resulting in the failure to produce detectable Duox1 protein in uroepithelial cells. Mice were maintained in a specific pathogen-free facility at the NIAID according to IUCAC animal protocols approved at the NIH. Bone marrow cell suspensions were isolated from tibias and femurs of 8- to 12-week-old C57Bl/6 mice by flushing bone marrows with RPMI1640 medium (+10% FCS, +1% Pen/Strep). Cell clumps were dislodged by pipetting, and debris was removed by passaging through a 70- μ m cell strainer (Fisher Scientific, Pittsburg, PA, USA). Cells were washed twice with medium and seeded on 10 cm tissue culture dishes (for Ca²⁺ measurements) or on 24-well plates (for ELISA) (Corning Costar, Tewksbury, MA). Cells were grown in complete RPMI-1640

medium supplemented with 10 ng/mL recombinant murine M-CSF (R&D Systems, Minneapolis, MN) and cultured for 5–7 days in a humidified incubator at 37°C and 5% CO₂.

2.4. Manipulations of intra- and extracellular calcium

To scavenge extracellular Ca²⁺, human and murine macrophages were incubated in HBSS supplemented with 1 mM MgCl₂, 10 mM HEPES, 5 mM glucose and 100 μM EGTA, pH 7.4. Corresponding Ca²⁺-containing solutions contained 1 mM CaCl₂ and no EGTA (pH 7.4). To chelate intracellular Ca²⁺, human MDMs were incubated with 50 μM BAPTA-AM for 15 min at 37°C in HBSS and washed twice in PBS afterwards to remove excess dye.

2.5. Calcium measurements

Murine macrophages were primed with 1 μg/ml *Salmonella typhimurium* LPS (Sigma Aldrich, St. Louis, MO) for 1 hr. After two washes, cells were incubated in HBSS containing 2 μM FURA2-AM (Life Technologies, Grand Island, NY) for 30 min in the dark. Cells were washed, suspended in HBSS and were added to black 96-well plates (Corning Costar, Tewksbury, MA). After 10-minute incubation, cells were stimulated by different doses of ATP (0–3 mM) (Sigma Aldrich, St. Louis, MO) and changes in 510nm fluorescence emission while exciting at two wavelengths (340nm_{ex}/510nm_{em}, 390nm_{ex}/510nm_{em}) were followed for 60 min with shaking. The ratio of 340 nm/510nm and 390 nm/510 nm values were calculated and presented to reflect kinetic changes in cytosolic Ca²⁺ levels.

2.6. RNA isolation and quantitative real-time RT-PCR

RNA was isolated by Trizol/chloroform extraction and isopropanol precipitation from differentiated hMDMs. cDNA synthesis was carried out (Thermoscript cDNA synthesis kit, Life Technologies, Grand Island, NY). Human *DUOX1*, *DUOX2*, *NOX5*, *NLRP3* and *P2RX7* gene expression was measured by reverse transcription/quantitative real-time PCR using SYBR Green (Invitrogen) and the following primers:

Human *DUOX1* (F: 5'-CACCTCCTGGAGACCTTTTTC -3', R: 5'-GGCCTGGTTGATGTCCAG -3', 60 bp product), human *DUOX2* (F: 5'-GATGGTGACCGCTACTGGTT -3', R: 5'-GCCACCACTCCAGAGAGAAG -3', 323 bp product), human *NOX5* (F: 5'-CAAGAATGAAGCCGCAGAC -3', R: 5'-CCTGCAATGGTCTTAAACTGC -3', 95 bp product), human *NLRP3* (F: 5'-CTTCTCTGATGAGGCCCAAG -3', R: 5'-GCAGCAAAGTGGAAAGGAAG -3', 200 bp product), human *P2RX7* (F: 5'-GGGAACCAGAAGACCTGTGA -3', R: 5'-AGTTTTTCGGCACTGTTCAAGA -3', 94 bp product) and β-actin (F: 5'-CCAACCGCGAGAAGATGA -3', R: 5'-CCAGAGGCGTACAGGGATAG -3', 97 bp product). Changes in fluorescence were followed in 7500 Real-time PCR system (Applied Biosystems, Grand Island, NY). Reaction parameters were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The relative abundance of mRNAs was obtained using the comparative cycle threshold method and was normalized to beta-actin as the endogenous control.

2.7. Measurement of human and murine IL-1 β release by ELISA

Human MDMs or mouse BMDMs cultured on 24-well plates were primed with *Salmonella typhimurium* LPS (1 μ g/ml, Sigma Aldrich, St. Louis, MO) for 60 min. Cells were washed twice with Ca²⁺-free HBSS before addition of inflammasome stimuli (ATP, 0–4 mM or 100 μ g/ml silica crystals (Sigma Aldrich, St. Louis, MO)). At indicated time points, supernatants were collected, centrifuged (500g, 10 min) to remove cell debris, crystals etc. Supernatants were either frozen or immediately used for further analysis. Human and murine IL-1 β release in macrophage supernatants was determined by commercial ELISA kits (DuoSet, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cytokine release was calibrated using standard curves.

2.8. Western Blotting

HEK-293 cells or human MDMs were washed three times with cold Ca²⁺- and magnesium-free PBS and lysed by NP-40 lysis buffer (Boston Biosciences, Ashland, MA) containing 150 μ M PMSF (Fluka Biochemika-Sigma Aldrich, St. Louis, MO) and 1% protease inhibitor cocktail (dissolved in DMSO, Sigma Aldrich, St. Louis, MO). Lysates were centrifuged and protein concentrations in supernatants were determined using the BCA assay (Pierce, Rockford, IL). Equal amounts of protein were added to SDS sample buffer containing 2-mercaptoethanol (without boiling), and electrophoresed on SDS-polyacrylamide gels (8%; Tris-Glycine Gel, Life Technologies, Grand Island, NY). Gels were blotted on nitrocellulose membrane (Invitrogen) using the TE Series Transphor Electrophoresis Unit (Hoefer, Holliston, MA) wet blotting system. Blots were blocked overnight in TTBS (TBS-buffer containing 5% milk powder and 0.05% Tween-20). Blots were incubated with primary antibodies (RT, 1hr, TTBS), washed three times with TTBS and then probed with secondary HRP-linked antibodies (RT, 1hr, TTBS, GE Healthcare, Piscataway, NJ). After repeated washes, blots were developed by chemiluminescence using the Lumigen DS detection kit (GE Healthcare, Piscataway, NJ). The primary antibodies used were: anti-Duox1 (Clone T-14, 1:500, rabbit, Santa Cruz, Dallas, TX), anti-Hsp90 (1:1000, mouse, mouse, Santa Cruz, Dallas, TX), anti-caspase-1 p20 (rabbit, 1:500, Cell signaling, Danvers, MA), anti-actin (rabbit, 1:2000, Sigma Aldrich, St. Louis, MO), HA (mouse, 1:100, Covance, Princeton, NJ).

2.9. Caspase-1 enzymatic activity

Differentiated human MDMs were primed with 1 μ g/ml *Salmonella typhimurium* LPS (Sigma Aldrich, St. Louis, MO, USA) for 30 min. To scavenge intracellular calcium, MDMs were treated with 50 μ M BAPTA-AM (Dojindo laboratory, Rockville, Maryland USA) in a PBS with 5 mM glucose for 10 min. Equal volume of DMSO (Sigma Aldrich, St. Louis, MO, USA), solvent for BAPTA-AM, was used as a control. MDMs were then stimulated with 3mM ATP in HBSS containing 2mM glucose with or without 1mM Ca²⁺ for 10 min. Ca²⁺-free HBSS contained 1 mM MgCl₂ (Sigma Aldrich, St. Louis, MO, USA) and 100 μ M EGTA (Sigma Aldrich St. Louis, MO, USA) was used to chelate any remaining Ca²⁺. MDMs were then scraped off to measure caspase-1 enzymatic activity using caspase-1 fluorometric assay kit (Enzo Life Sciences, Farmingdale, NY, USA). Following the manufacturer's instruction, cold lysis buffer was added to macrophages. Equal volume of

the reaction buffer containing 10 mM DDT was then added to the lysis buffer. Finally, 50 μ M of caspase-1 YFAD-AFC substrate was used to detect fluorescence at excitation and emission wavelengths of 450 nm and 505 nm, respectively. Results were reported as relative fluorescence units (RFU) and kinetics of the caspase-1 signal development was obtained using Varioskan™ Flash Multimode Reader (Thermo-scientific, USA) for 1.5 hours at 37 °C. Final endpoint fluorescent values were compared between 2 groups treated, one treated with media containing Ca^{2+} and once treated without Ca^{2+} .

2.10. Statistical analysis

Data are represented as mean $\pm$ S.E.M. or mean $\pm$ S.D. Significance was calculated with ANOVA and Tukey's post-hoc test and was marked as * when $p < 0.05$, ** when $p < 0.01$ and *** when $p < 0.001$.

3. Results

3.1. Calcium is critical for IL-1 β secretion of human macrophages stimulated by ATP or silica

Recently, a critical role of Ca^{2+} in NLRP3 inflammasome activation has been shown.[1] IL-1 β release from murine BMDMs and human monocytes is inhibited by intra- or extracellular chelation of Ca^{2+} . [1, 11] Since the role of Ca^{2+} in IL-1 β secretion by human macrophages has not been demonstrated so far and differences exist between human monocytes and macrophages in terms of regulation of IL-1 β expression and inflammasome activation, we differentiated monocytes isolated from peripheral human blood into macrophages by treatment with 50 ng/ml human M-CSF and measured IL-1 β release by ELISA.[27, 2] We stimulated inflammasome activation by ATP, a well-described stimulus to induce IL-1 β release in monocytes/macrophages.[28] Extracellular ATP enhanced IL-1 β secretion in a concentration-dependent manner (Fig. 1A). Chelation of extracellular Ca^{2+} by ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) or intracellular Ca^{2+} by 1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA) decreased ATP- and silica-stimulated cytokine release (Fig. 1B). Combined effects of EGTA and BAPTA resulted in an 80.2 $\pm$ 8.0% (mean $\pm$ S.E.M., n=6) inhibition of silica- and 70.4 $\pm$ 7.4% inhibition (mean $\pm$ S.E.M., n=5) of ATP-activated IL-1 β release (Fig. 2B). We observed similar robust inhibition of IL-1 β secretion of human MDMs by using two additional known inflammasome activators, antimycin-A and calcium pyrophosphate dehydrate crystals (data not shown). Thus, Ca^{2+} plays an essential role in IL-1 β release by human macrophages.

3.2. Calcium-inducing agonists trigger IL-1 β release

To investigate whether the elevation in intracellular Ca^{2+} alone is sufficient to induce IL-1 β secretion in human MDMs we used pharmacological manipulation to disrupt Ca^{2+} homeostasis. Thapsigargin (TG) is a widely used inhibitor of the sarco-/endoplasmic reticulum Ca^{2+} ATPase.[3] TG first empties intracellular calcium stores that activates the store-operated calcium entry pathway leading to subsequent calcium influx from the extracellular space.[3] TG-treatment of lipopolysaccharide (LPS)-primed human MDMs alone induced release of IL-1 β (Fig. 2A). Ionomycin (iono), a calcium ionophore, enhances

intracellular Ca^{2+} concentrations abruptly by releasing Ca^{2+} from both, extra- and intracellular stores.[6] Addition of ionomycin to human MDMs increased IL-1 β secretion (Fig. 2B). ATP-induced IL-1 β release did not differ in the presence or absence of ionomycin suggesting that ATP also releases Ca^{2+} from stores both inside and outside of the cell (Fig. 2B). Our data show that increases in cytosolic Ca^{2+} alone are enough to induce IL-1 β secretion by human macrophages.

3.3. Macrophage inflammasome activation requires Ca^{2+}

Next, we asked whether Ca^{2+} is required for IL-1 β release either upstream or downstream of NLRP3 inflammasome activation. We stimulated human MDMs in the presence or absence of Ca^{2+} (BAPTA+EGTA) with 3 mM ATP, a well-characterized NLRP3 agonist, and measured the true indicator of inflammasome activation, procaspase-1 cleavage. Once assembled, the direct consequence of NLRP3 inflammasome activation is proteolytic activation of procaspase-1 into the bioactive, 20kD-size caspase-1.[20] While in presence of calcium the 20 kD caspase-1 cleavage product appeared upon stimulation by ATP, in absence of Ca^{2+} , this band was missing (Fig. 3A). In addition, we measured caspase-1 enzymatic activity in human MDMs stimulated with 3mM ATP in the presence or absence of calcium (BAPTA+EGTA), and we experienced an inhibition in caspase-1 enzymatic activity upon calcium removal (Fig. 3B). These results show that Ca^{2+} is required for NLRP3 inflammasome activation in human macrophages (Fig. 3).

3.4. Duox1 is expressed in human macrophages

ROS were suggested to play a role in NLRP3 inflammasome regulation.[12] Mitochondria have been identified as the source of ROS but certain NADPH oxidases have been excluded. [16, 19, 20, 10] Studies on phagocytic NADPH oxidase-deficient monocytes showed that four members of the Nox/Duox family of NADPH oxidases (Nox1-4) that are dependent on $\text{p}22^{\text{phox}}$ are not involved in inflammasome activation.[10, 19, 20] There are, however, three other members of this family which could be ROS sources required for inflammasome activation in macrophages: Nox5, Duox1 and Duox2.[17] Whether these oxidases are required for inflammasome activation is unknown. Nox5, Duox1 and Duox2 are all directly activated by increases in intracellular Ca^{2+} levels through their intracellular Ca^{2+} -binding EF-hand motifs.[22, 21, 23] Innate immune functions of Duox1 have already been previously proposed but it is unknown if these enzymes are expressed in human macrophages.[29, 17, 30] Therefore, we measured gene expression levels of *NOX5*, *DUOX1* and *DUOX2* in human MDMs by real-time PCR. Nox5 mRNA was not detected at all, Duox2 mRNA was only detected in one of the four donors whereas Duox1 mRNA was consistently detected (Fig. 4A). *DUOX1* expression was significantly lower than that of *NLPR3* or *P2RX7* (Fig. 4A). Probing human MDM cell lysates of two donors with a commercial Duox1 antibody, we detected a clear band at about 180 kDa molecular mass in both donors (Fig. 4B). Thus, we identified a new cell type in humans expressing Duox1: macrophages.

To test the isoform specificity of the Duox antibody, we used cell lysates prepared from our previously characterized HEK-293 cell model lines stably expressing bicistronic pcDNA5.1TM/FRT constructs of human Duox1+DuoxA1 α or human Duox2+DuoxA2.[23]

We used cell lines containing HA-tagged constructs to reliably determine the amounts of Duox by Western blotting.[10] The parental cell line (HEK-293) did not show detectable HA or Duox (Fig. 4C). The intensity of the HA-band was stronger in Duox2/DuoxA2-expressing cells compared to that of the Duox1/DuoxA1S sample, indicating higher protein/cell expression for Duox2 than Duox1 (Fig. 4C). The used Duox1 antibody detected Duox1 but not Duox2 (Fig. 4C), confirming that human MDMs express the Ca^{2+} -activated NADPH oxidase Duox1.

3.5. Duox1 plays no role in NLRP3 inflammasome activation

To address the question whether Duox1 is required for inflammasome activation and IL-1 β release, first we tested whether murine BMDMs produce cytosolic Ca^{2+} responses to ATP. Cytosolic Ca^{2+} levels did not rise above baseline unless cells were stimulated with 1 mM or higher doses of ATP (Fig. 5A). 1mM ATP triggered a short, temporary Ca^{2+} transient whereas 3mM ATP, a concentration triggering maximal inflammasome activation resulted in a robust, long-lasting Ca^{2+} signal (Fig. 5A). We next exposed wild-type and Duox1-deficient murine BMDMs to ATP or silica and measured IL-1 β release.[26] Duox1-deficiency does not impair the ability of murine BMDMs to secrete IL-1 β (Fig. 5B). Thus, we conclude that Duox1 does not play a role in inflammasome activation in macrophages.

Discussion

Several diverse stimuli (cytokines, crystals, danger signals, microbial products) converge on the NLRP3 inflammasome in monocytes/macrophages and activate production and secretion of bioactive IL-1 β . [31–34] The links between extracellular stimuli and NLRP3 activation are still largely unknown. Decreased intracellular potassium concentrations have been implicated in inflammasome activation by ATP, crystals, toxins and bacteria.[35, 36] The proposed role of potassium was based on experiments showing that caspase 1 activation and IL-1 β secretion is blocked in the presence of high extracellular potassium levels that inhibit potassium efflux from cells.[36, 35] More recently, another ion, Ca^{2+} has been shown to be essential for NLRP3 inflammasome activation in murine BMDMs and human monocytes.[1, 11] Our data (Fig. 1–3) show that Ca^{2+} has a pivotal role in IL-1 β secretion and NLRP3 inflammasome activation in human MDMs as well. We experienced a difference in the magnitudes of the cytosolic Ca^{2+} increase between two Ca^{2+} -releasing agonists, ionomycin and thapsigargin (Fig. 3.). Ionomycin triggers an almost immediate, very large Ca^{2+} signal whereas thapsigargin requires time to deplete the intracellular Ca^{2+} stores to induce the store-operated Ca^{2+} influx.[37] We think these differences in their kinetics and modes of action are the reason for the different Ca^{2+} responses (Fig. 2.). Cytosolic Ca^{2+} has already been implicated in IL-1 β secretion from macrophages in an earlier study but recent data link it to inflammasome activation.[38, 1] Several of the known NLRP3 inflammasome agonists induce cytosolic Ca^{2+} signals in monocytes/macrophages: ATP, MSU crystals, nigericin.[1, 38] Another inflammasome agonist, antimycin-A induces Ca^{2+} signal in osteoblasts.[39] Increase in extracellular potassium levels depolarizes the plasma membrane and blocks Ca^{2+} entry in phagocytes.[1, 40] This and the fact that potassium -unlike Ca^{2+} - has no known direct role in cell signaling suggest that Ca^{2+} rather than potassium affects inflammasome activation directly. Most of the inflammasome agonists lead to plasma membrane damage or

pore formation (crystals, toxins, ionophores, ATP) that always results in potassium efflux and Ca^{2+} influx at the same time according to their electrochemical gradients.[34] Thus, an increasing number of studies including ours show that Ca^{2+} is an essential element of NLRP3 inflammasome activation.

ROS were proposed to be the convergence point in inflammasome signaling.[12, 41] ATP and crystals stimulate ROS production in macrophages.[42–46] The phagocytic NADPH oxidase was first thought to be the ROS source promoting inflammasome activation, but studies on gp91^{phox}- and p22^{phox}-deficient human monocytes showed that inflammasome activation is NADPH oxidase-independent.[10, 19, 20] P22^{phox} is also a partner of three other members of the NADPH oxidase enzyme family, Nox1, 3 and 4, so inflammasome activation does not require these proteins and has been declared to be NADPH oxidase-independent.[47, 29] There are, however, three other members of this family (Nox5, Duox1, Duox2) that are all directly activated by cytosolic Ca^{2+} and have never been studied before regarding their potential participation in IL-1 β metabolism in human macrophages.[21–23] We could detect only *DUOX1* gene expression and Duox1 protein in human MDMs (Fig. 4). Using Duox1-deficient murine BMDMs we show, however, that IL-1 β secretion is not dependent on Duox1 (Fig. 5.). Our data indicate that Ca^{2+} -activated NADPH oxidases are not ROS sources required for inflammasome activation and therefore none of the seven NADPH oxidases are involved in IL-1 β processing or release. The contribution of ROS to NLRP3 activation is still controversial. Recent data suggest mitochondria to be the major source.[16] Another study suggests that ROS are only required for inflammasome priming but not activation.[48] Several studies proposed that primary monocytes, macrophages and their model cell lines have different overall redox states and this will ultimately determine the efficiency of inflammasome activation.[27, 2, 49]

The function of Duox1 in macrophages could be participation in ATP-induced inflammatory responses since Duox1 is known to be activated in airway epithelial cells in response to extracellular ATP and mediates wound inflammatory responses in *Drosophila* embryos.[50–52] Considering its low expression, Duox1 could have a role in different signaling pathways similar to its suggested roles in airway epithelial cells and lymphocytes.[53, 54] The detected low Duox1 expression makes it unlikely that Duox1-derived ROS have any role in macrophage bactericidal activities. The main NADPH oxidase in macrophages is the NOX2-based NADPH oxidase that has the potential to produce enough ROS to kill microbes. NOX2, however, has already been clearly shown to be dispensable for inflammasome activation.[19] Aside from these speculations, the function of Duox1 in macrophages remains an open question.

Abbreviations

BMDM	bone marrow-derived macrophage
Duox	dual oxidase
DuoxA	Duox Activator
MDM	monocyte-derived macrophage

Nox5 NADPH oxidase 5
ROS reactive oxygen species

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Figure 1. IL-1 β secretion by human macrophages stimulated with ATP or silica requires calcium
 A) LPS-primed human MDMs were stimulated with different concentrations of ATP and release of human IL-1 β was measured by ELISA (mean $\pm$ S.E.M., n=3). B) LPS-primed human MDMs were treated (or not) with 50 μ M BAPTA-AM and stimulated with 3 mM ATP or 100 μ g/ml silica crystals in the presence (1 mM Ca²⁺) or absence (No Ca²⁺ and 100 μ M EGTA) of extracellular Ca²⁺. Release of IL-1 β was measured by ELISA. Mean $\pm$ S.E.M. n=5. **, p<0.01.

Figure 2. Increased cytosolic calcium levels trigger IL-1 β release by human macrophages

A) Human MDMs with or without LPS-priming (1 μ g/ml, 1hr) were stimulated with thapsigargin (TG, 0.1 μ M or 1 μ M) and release of human IL-1 β was measured at the indicated time points (24, 36 or 48 hrs post-treatment). Average $\pm$ S.D. of two independent donors. B) LPS-primed human MDMs were stimulated with 1 μ M ionomycin and/or 3 mM ATP and extracellular IL-1 β release was measured by ELISA. Mean $\pm$ S.E.M., n=4. *, p<0.05.

Figure 3. Human macrophage NLRP3 inflammasome activation requires calcium

LPS-primed human MDMs were stimulated with 3 mM ATP in the absence (“-Ca”: treatment with 50 μ M BAPTA-AM, absence of extracellular Ca^{2+} , 100 μ M EGTA) or presence (“+Ca”: no BAPTA-AM treatment, 1 mM extracellular Ca^{2+}) of calcium. A) After 15 min, cell lysates were prepared and cleavage of 45 kD procaspase-1 into active 20kD capsase1 p20 was detected by western blotting. Hsp90 was used as loading control. Another donor gave similar data. B) After 10 min intracellular caspase-1 activity was measured (mean $\pm$ S.E.M., n=5). RFU, relative fluorescence unit.

Figure 4. Human macrophages express Duox1

A) Gene expression levels of human *DUOX1*, *DUOX2*, *NOX5*, *NLRP3* and *P2RX7* were determined in LPS-primed human MDMs by real-time quantitative PCR using gene specific primers and SYBR Green. Mean $\pm$ S.E.M. of gene expression levels relative to actin are shown (n=4). B) Immunoblots of LPS-primed human MDMs or Duox1+DuoxA1 α -expressing HEK-293 cells were probed with anti-Duox1 (T-14) antibody. Macrophages from two different donors were tested side by side. C) Cell lysates were prepared from HEK-293 cell lines stably expressing bicistronic pcDNA5.1TM/FRT constructs of human

Duox1+DuoxA1 α (D1+DA1S) or human Duox2+DuoxA2 (D2+DA2) or from the parental line. D1 and D2 are HA-tagged, whereas DA1S and DA2 are tagged with V5. Different amounts of proteins (1, 3 and 10 μ g) were loaded on western blots and probed with three different antibodies: Duox1 (Santa Cruz T-14 clone), HA-tag and actin. The anti-Duox1 and anti-HA antibodies were used on the same blot, the blots were stripped and reprobed. One representative gel, n=3.

Figure 5. Macrophage IL-1 β secretion is Duox1-independent

A) LPS-primed murine BMDMs were loaded with Ca^{2+} -sensitive dye FURA 2-AM and stimulated with different doses of extracellular ATP. Changes in cytosolic Ca^{2+} over 60 min time were monitored as a ratio of 510nm fluorescence emission based on excitation at two different wavelengths (340/510 nm and 390/510 nm) using a Fluoroskan microplate fluorimeter. One representative recording of three similar ones isolated from different mice.

B) Duox1-deficient BMDMs produce normal amounts of IL-1 β . Duox1-deficient and wild-type mouse BMDMs were primed with LPS and stimulated with 3 mM ATP (30 min) or 100 $\mu\text{g/ml}$ silica crystals (6 hrs). Secretion of murine IL-1 β in culture supernatants was determined by ELISA (mean $\pm$ S.E.M., n=4).