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NADPH oxidase derived reactive oxygen species are involved in human neutrophil IL-1β secretion but not in inflammasome activation

María Laura Gabelloni[1], Florencia Sabbione[1], Carolina Jancic[1,2], Juan Fuxman Bass[1], Irene Keitelman[1], Leonardo Iula[1], Matías Oleastro[3], Jorge R. Geffner[1,2] and Analía S. Trevani[1,2]

[1] Departamento de Inmunología, Instituto de Medicina Experimental (IMEX)-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina
[2] Departamento de Microbiología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina
[3] Hospital Nacional de Pediatría "Juan P. Garrahan", Buenos Aires, Argentina

Neutrophils are essential players in acute inflammatory responses. Upon stimulation, neutrophils activate NADPH oxidase, generating an array of reactive oxygen species (ROS). Interleukin-1 beta (IL-1β) is a major proinflammatory cytokine synthesized as a precursor that has to be proteolytically processed to become biologically active. The role of ROS in IL-1β processing is still controversial and has not been previously studied in neutrophils. We report here that IL-1β processing in human neutrophils is dependent on caspase-1 and on the serine proteases elastase and/or proteinase 3. NADPH oxidase deficient neutrophils activated caspase-1 and did not exhibit differences in NALP3 expression, indicating that ROS are neither required for inflammasome activation nor for its priming, as has been reported for macrophages. Strikingly, ROS exerted opposite effects on the processing and secretion of IL-1β; whereas ROS negatively controlled caspase-1 activity, as reported in mononuclear phagocytes, ROS were found to be necessary for the exportation of mature IL-1β out of the cell, a role never previously described. The complex ROS-mediated regulation of neutrophil IL-1β secretion might constitute a physiological mechanism to control IL-1β-dependent inflammatory processes where neutrophils play a crucial role.

Keywords: Caspase-1 · IL-1β · NADPH-oxidase · Neutrophil · Reactive oxygen species

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Introduction

Neutrophils represent the first line of defense against bacterial and fungal infections. Their pivotal role is highlighted by recurrent infections in individuals suffering from neutrophil functional disorders or neutropenia [1]. Neutrophils kill microbes by the release of destructive molecules such as proteases and highly reactive oxygen species (ROS), and can also produce a variety of proteins, including cytokines, chemotactic molecules, and other mediators that are involved in their effector functions [2]. However, the microbicidal effectiveness of neutrophils is sometimes obscured by the damage suffered by adjacent healthy tissues; this is a price that has to be paid to contain potentially

Correspondence: Dr. Analía S. Trevani
e-mail: analiatrevani@yahoo.com.ar

life-threatening situations [3]. Although infections are the major triggers of neutrophil recruitment, many different sterile stimuli, including mechanical trauma, ischemia, toxins, minerals, crystals, and chemicals also lead to neutrophil accumulation in the tissues. In these situations, neutrophils may contribute to inflict collateral damage on otherwise healthy cells [3].

Interleukin-1 beta (IL-1β) is a key cytokine involved in the development of neutrophilic inflammation induced either by microbial or sterile inflammatory stimuli. It is a potent multifunctional proinflammatory cytokine, whose activity is controlled at the levels of transcription, translation, maturation, and secretion [4]. IL-1β is first synthesized in the cytosol as a biologically inactive pro-form (pro-IL-1β), which requires proteolytic cleavage for its activation and release from cells [5]. The cleavage of pro-IL-1β to mature IL-1β is catalyzed by caspase-1 [6], which is synthesized as a pro-enzyme and is activated within large multiprotein complexes termed inflammasomes [7]. A variety of inflammasomes differing from one another in their Nod-like receptor (NLR) subunit have been described, mainly involved in recognizing microbial products. In contrast, the inflammasome including NLR family pyrin domain containing 3 (NLRP3) has been also implicated in the production of mature IL-1β in response to host-derived stress signals such as extracellular ATP, monosodium urate (MSU), and amyloid-β, as well as crystalline and particulate substances from the environment such as silica, asbestos, and aluminum hydroxide [7]. On the other hand, pro-IL-1β can also be released from cells and cleaved extracellularly by other proteases into mature IL-1β as has been shown for neutrophil proteinase 3 (PR3), elastase, and cathepsin G [8, 9].

Given the structural and chemical divergence between stimuli that lead to NLRP3 inflammasome activation, it has been proposed that these stimuli may elicit common signals recognized by NLRP3, such as: (i) a decrease in intracellular potassium levels, (ii) the presence in cytoplasm of lysosomal proteases due to phagolysosomal membrane disruption induced by phagocytosis of particulate stimuli, and (iii) the generation of intracellular ROS [10]. However, controversial findings have been made regarding the role of ROS in IL-1β maturation and release. Pharmacological inhibition of NADPH oxidase, the enzyme that generates ROS in phagocytes, or siRNA-mediated knockdown of the $p22^{phox}$ subunit of NADPH oxidase have been shown to diminish IL-1β secretion induced by ATP, MSU, asbestos, and silica in THP-1 cells [11]. In contrast, other studies found that inflammasome activation induced by silica, MSU, or ATP was not affected in mouse macrophages deficient in $gp91^{phox}$, another subunit of the NADPH oxidase [12]. Likewise, mononuclear phagocytes from NADPH oxidase deficient patients activated caspase-1 and produced biologically active IL-1β [13]. Intriguingly, ROS were also shown to inhibit caspase-1 activation [12]. Thus, the role of ROS in inflammasome activation and IL-1β secretion is still a matter of debate.

The mechanisms involved in IL-1β processing have been widely studied in some myeloid lineage cells such as monocytes and macrophages, in which different mechanisms were found to regulate IL-1β secretion. It was shown that caspase-1 is constitutively activated in monocytes, thus, stimuli such as LPS that trigger pro-IL-1β synthesis also lead to its release, while macrophages require a second stimulus in order to activate the inflammasome and consequently release IL-1β [14]. In contrast, the mechanisms involved in human neutrophil IL-1β processing and secretion remain to be elucidated. Previous studies have documented that human neutrophils express both NLRP3 and caspase-1 [15–18]. However, as neutrophil proteases were also implicated both in extracellular and intracellular pro-IL-1β processing [5, 19–22], the idea that prevails is that these cells process pro-IL-1β through a protease-dependent and caspase-1-independent mechanism [4].

In the current study, we demonstrate that both serine-protease-dependent and caspase-1-dependent mechanisms contribute to IL-1β processing and secretion in human neutrophils. Our results identify a never before described role of ROS in the release of mature IL-1β, and show that ROS are not required for inflammasome activation and IL-1β processing in neutrophils. Our findings also support that ROS can negatively regulate neutrophil caspase-1 activity.

Results

Caspase-1 and serine proteases are involved in neutrophil pro-IL-1β processing

To investigate the capacity of human neutrophils to produce and release IL-1β, we stimulated highly purified neutrophils (<0.5% of monocyte contamination) either with LPS, a TLR4 agonist that triggers pro-IL-1β synthesis, or with LPS and ATP, a well-known agonist of NLRP3 inflammasome in other cell types. We determined by ELISA that LPS stimulation was sufficient to induce IL-1β release. However, when neutrophils were preincubated with LPS and then stimulated with ATP, higher levels of IL-1β were detected in culture supernatants (Fig. 1A). We detected maximal levels of IL-1β in neutrophil culture supernatants after 5 h of stimulation with LPS+ATP; however, the highest IL-1β release induced by LPS alone was detected after 18 h of culture (Fig. 1B). Thus, taking into account that, as previously described [23, 24], LPS and ATP inhibited neutrophil spontaneous death (Supporting Information Fig. 1A) in the rest of the experiments, we evaluated IL-1β release after an 18-h culture period. Of note, the increased secretion of IL-1β could not be attributable to an augmented cell death or nonspecific membrane permeability because (i) the maximal release of IL-1β detected in neutrophils stimulated with LPS+ATP was determined after 5 h of culture (Fig. 1B), a time point at which apoptosis was as low as 5.3 ± 1.1 ($n = 3$); and (ii) a lower percentage of neutrophils incorporated 7-aminoactinomycin D (7-AAD) stain after 18 h of culture when stimulated with LPS+ATP (Supporting Information Fig. 1A), even though they released higher levels of IL-1β compared with those stimulated only with LPS. The maximal intracellular IL-1β content detected by ELISA in whole cell extracts was observed after 5 h of stimulation (Fig. 1C and data not shown). Thus, the experiments to assess intracellular IL-1β were performed after a 5 h stimulation period. Of note, at 18 h poststimulation with LPS, similar levels of IL-1β were

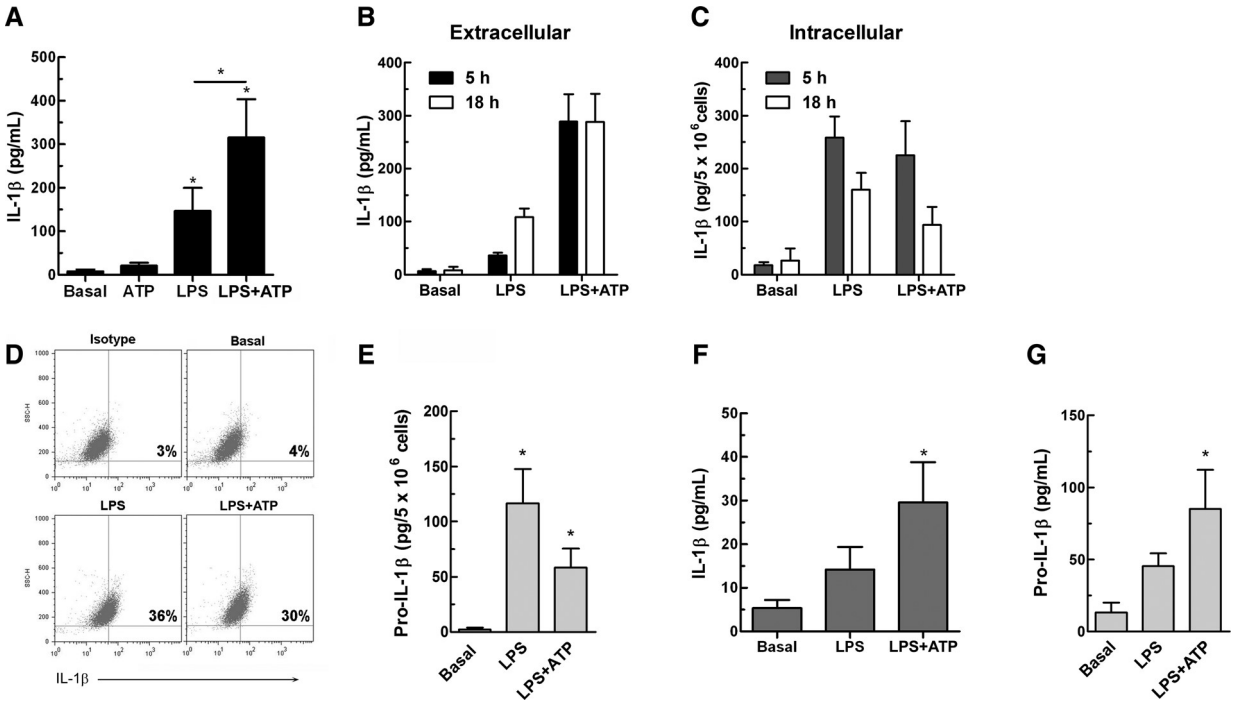
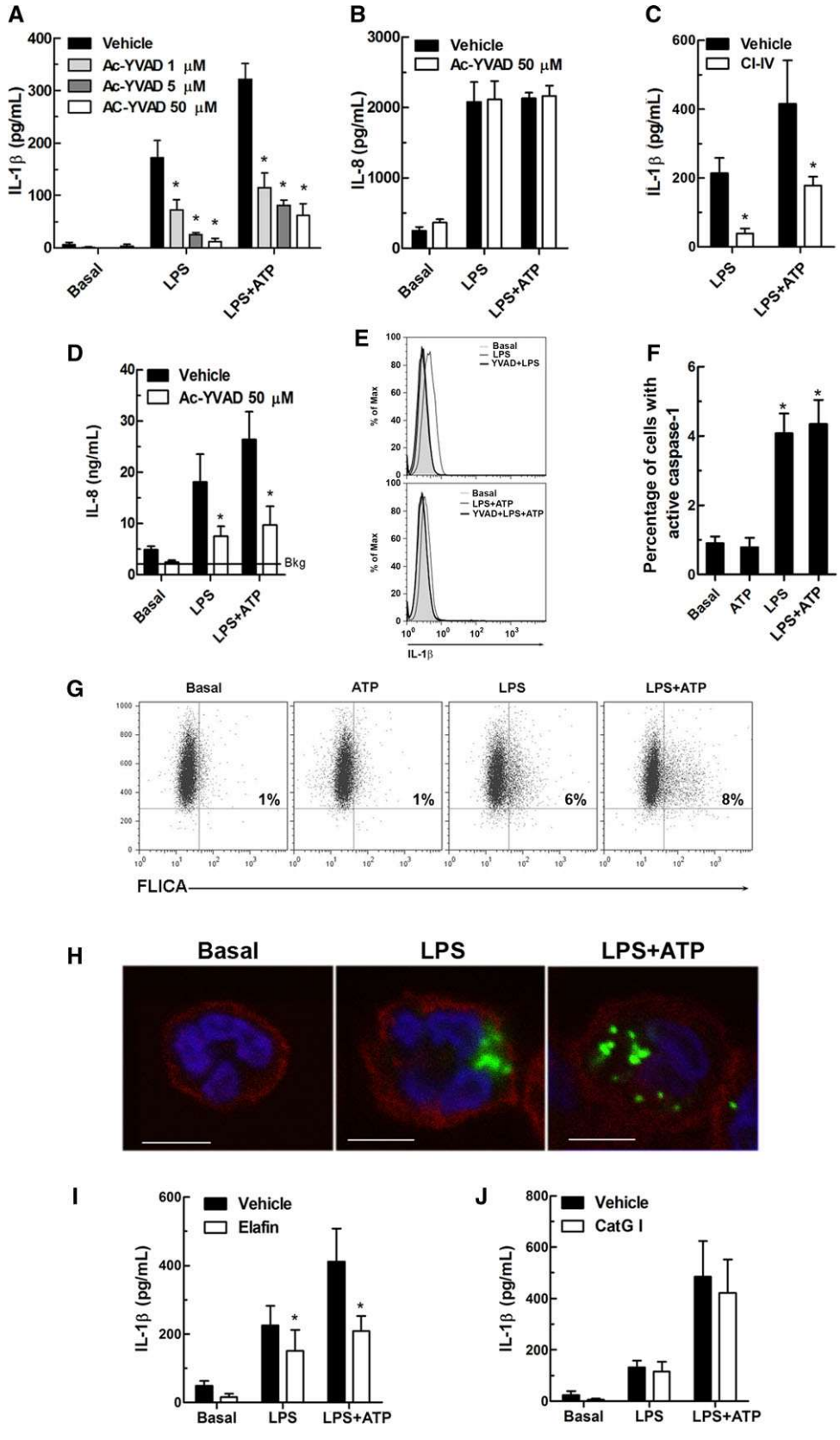


Figure 1. Human neutrophils release IL-1β and pro-IL-1β upon LPS and ATP stimulation. Neutrophils were cultured for 2 h in the presence or absence of LPS (200 ng/mL) and then stimulated or not with ATP (2.5 mM) and cultured for (B) 3 h or (A, B, E, and G) 16 h. The (A, B) IL-1β and (G) pro-IL-1β released into culture supernatants were determined by ELISA. Data are expressed as mean + SEM of samples pooled from 20 (A) or 5 (B, G) donors from ten (A) or three (B, G) independent experiments, each performed in duplicate. (C) IL-1β of whole cell extracts corresponding to cell pellets from (B) was determined by ELISA and shown as mean + SEM of samples pooled from five donors from three independent experiments, each performed in duplicate. (D) Intracellular IL-1β of neutrophils cultured for 2 h in the presence or absence of LPS and then stimulated or not with ATP for 3 h was determined by flow cytometry. (E) Neutrophil intracellular pro-IL-1β was determined by ELISA. Data represent the mean + SEM of samples pooled from six donors from three independent experiments, each performed in duplicate. (F) IL-1β release by neutrophil-differentiated PLB-985 cells stimulated as in (A). Data represent the mean + SEM of four independent experiments, each performed in triplicate. $^*p < 0.05$ versus basal; (A) Friedman test for multiple comparisons with Dunn's posttest and (E, F and G) Wilcoxon matched ranked one-tailed.

found inside and outside cells, while upon LPS+ATP stimulation, approximately threefold higher levels of IL-1β were found in supernatants in relation to those inside the cells. However, intracellular IL-1β in LPS+ATP-stimulated neutrophils decreased after 18 h of culture without a concomitant increase in IL-1β detected in supernatants, suggesting that some intracellular IL-1β degradation took place. By intracellular immunostaining, we confirmed both that neutrophils were indeed the cells responsible for IL-1β synthesis and that LPS stimulation was required for triggering IL-1β synthesis because unstimulated neutrophils did not express significant amounts of this cytokine (Fig. 1D and Supporting Information Fig. 2). This was confirmed by detection of intracellular pro-IL-1β only in stimulated neutrophils (Fig. 1E). Supporting the ability of neutrophils to synthesize IL-1β, we found that the neutrophilic cell line PLB-985 released IL-1β in response to LPS and LPS+ATP (Fig. 1F). Since IL-1β might also be released as a proform (31 kDa), we determined the presence of pro-IL-1β in culture supernatants by a specific ELISA. As shown in Figure 1G, pro-IL-1β was also detected in culture supernatants of stimulated neutrophils.

To elucidate the mechanisms involved in the generation of active IL-1β, neutrophils were pretreated with Ac-YVAD-CMK, a selective and irreversible caspase-1 inhibitor. The IL-1β release induced by either LPS alone or LPS+ATP was concentration-dependently reduced by Ac-YVAD-CMK, evidencing a role for caspase-1 in mature IL-1β generation (Fig. 2A). As expected, the secretion of IL-8, another cytokine synthesized by neutrophils that does not require caspase-1 processing, was not affected by Ac-YVAD-CMK treatment (Fig. 2B). Of mention, the reduced secretion of IL-1β observed in neutrophils treated with Ac-YVAD-CMK was not due to increased cell death as the percentage of neutrophils that incorporated 7-AAD was not significantly modified by this treatment (Supporting Information Fig. 1B). A role for caspase-1 in IL-1β processing was also evidenced by the ability of the caspase-1 inhibitor IV to reduce IL-1β release by LPS- and LPS+ATP-stimulated neutrophils (Fig. 2C) and the fact that Ac-YVAD-CMK was able to reduce the biological activity of IL-1β in culture supernatants of stimulated neutrophils, as measured by a bioassay (Fig. 2D). In agreement with a role for caspase-1 in pro-IL-1β processing, we also found that Ac-YVAD-CMK reduced IL-1β expression inside stimulated neutrophils as determined by flow cytometry (Fig. 2E). Moreover, by employing the reagent for detection of active caspase-1 FLICA, we detected increased levels of active caspase-1 when neutrophils were stimulated with LPS or pretreated with LPS and stimulated with ATP (Fig. 2F and G). Even though we found that both agonists induced caspase-1



activation in a similar percentage of neutrophils, the MFI emitted by cells stimulated with LPS+ATP was higher (MFI = 99 versus 177 for LPS and LPS+ATP, respectively), suggesting that ATP stimulation increased the amount of active caspase-1 in these cells. The presence of active caspase-1 in neutrophils was also detected by confocal laser scanning microscopy (CLSM) (Fig. 2H).

Neutrophils exhibit a high content of serine proteases such as elastase, PR3, and cathepsin G in their granules, which in vitro are able to cleave pro-IL-1β [5, 8]. Thus, we evaluated the effect of specific and permeable irreversible inhibitors of cathepsin G (CatG I) or elastase and PR3 (Elafin) on IL-1β secretion. Elafin significantly reduced IL-1β secretion induced by LPS or LPS+ATP (Fig. 2I), while CatG I did not affect it (Fig. 2J), suggesting that elastase and/or PR3 also play a role in IL-1β processing.

ROS are required for neutrophil IL-1β release

In response to different proinflammatory stimuli, neutrophils activate their NADPH oxidase and deploy a potent oxidative antimicrobial arsenal that includes different ROS, such as superoxide anion, hydrogen peroxide, hypochlorite and cloramines, among others. These species function as potent antimicrobial agents and in some cases also participate as signaling molecules that regulate diverse physiological signaling pathways in neutrophils [25]. As has been previously shown [26], we determined that LPS was able to elicit ROS production by neutrophils (Supporting Information Fig. 3). Because the role of ROS in inflammasome activation and IL-1β release has not been studied in neutrophils, we performed experiments to address this issue. We first analyzed the secretion of IL-1β by neutrophils from X-linked chronic granulomatous disease (X-CGD) patients. X-CGD is an inherited disorder caused by mutations in the gp91^{phox} subunit of the NADPH oxidase that completely impair ROS production [27]. X-CGD neutrophils released very low levels of IL-1β in response to LPS or LPS+ATP when compared with those secreted by healthy neutrophils (Fig. 3A). By contrast, IL-8 secretion was not affected in X-CGD neutrophils (Fig. 3B), ruling out a general impairment in cytokine secretion. In agreement with previous findings [28], X-CGD peripheral blood mononuclear cells (PBMC) did not exhibit defects in IL-1β secretion induced by LPS (Fig. 3C). Together, these findings suggested that in contrast to monocytes, NADPH oxidase generated ROS play a role in neutrophil IL-β release. The requirement of a functional NADPH oxidase for IL-1β release in response to LPS+ATP was also supported by results obtained with the neutrophilic PLB-985 (WT) cell line, the stable PLB-985 cell line with *gp91^{phox}* genetically knockedout (PLB-KO) and with *gp91^{phox}* reexpressed in the same background (PLB-91) [29]. The IL-1β released in response to LPS+ATP by PLB-KO cells was significantly lower than that secreted by PLB-985 and PLB-91 cells (Fig. 3D). Of note, the reduced ability to release IL-1β exhibited by PLB-KO cells was not due to an incapacity to synthesize this cytokine, because IL-1β could be detected by intracellular immunostaining in PLB-KO cells stimulated by LPS or LPS+ATP (Fig. 3E). Noteworthy, the percentage of cells expressing IL-1β in response to these agonists was higher in PLB-KO cells than in PLB-985 cells.

Recent studies in macrophages indicated that ROS inhibitors such as diphenyliodonium block NLRP3 inflammasome activation by inhibiting NLRP3 expression [30]. The authors of that study pointed out that NLRP3 inflammasome activation is critically dependent on priming because NLRP3 is expressed at limiting levels in macrophages. Thus, considering the requirement of ROS for IL-1β secretion we observed in neutrophils, we determined if NLRP3 expression is modified by LPS treatment. As shown in Figure 4A, contrasting with our observations made in monocytes (right), neutrophil NLRP3 expression was not increased by LPS treatment (left), suggesting that ROS produced by NADPH oxidase do not mediate IL-1β secretion by increasing NLRP3 expression. Supporting this conjecture, similar levels of NLRP3 expression were detected in PLB-985 and PLB-KO cells either under resting or stimulated conditions (Fig. 4B); however, when stimulated, PLB-KO cells evidenced a deficiency in IL-1β release (Fig. 3D).

Next, we analyzed if ROS are required to trigger inflammasome activation. To this aim, we evaluated the ability of stimulated PLB-KO cells to activate caspase-1. Noteworthy, we detected higher levels of active caspase-1 by FLICA staining in PLB-KO cells

◀ **Figure 2.** Neutrophil IL-1β release involves caspase-1 and elastase/PR3 processing. Neutrophils were pretreated or not with the caspase-1-specific inhibitor Ac-YVAD-CMK (A, B) or caspase-1 inhibitor IV (25 μM; C), cultured for 2 h in the presence or absence of LPS and then cultured for further 3 (B) or 16 h (A, C) with or without ATP. (A–C) The levels of (A and C) IL-1β and (B) IL-8 released into culture supernatants were determined by ELISA. Data are expressed as mean +SEM of samples pooled from seven (A), three (B), and four (C) donors from four, two, or three independent experiments, respectively, each performed in duplicate. (D) Biological activity of IL-1β in culture supernatants of neutrophils stimulated as in (A) determined by using a MRC-5 fibroblast bioassay, in which the MRC-5 fibroblasts produce IL-8 in response to IL-1. The data represent the mean +SEM of samples pooled from four donors from two independent experiments, each performed in duplicate. Bkg, background level of IL-8 production by MRC-5 fibroblasts. (E) Intracellular IL-1β expression of neutrophils pretreated or not with the caspase-1-specific inhibitor Ac-YVAD-CMK (50 μM), cultured for 2 h in the presence or absence of LPS and then stimulated or not with ATP for 3 h was determined by flow cytometry. (F, G) Neutrophil caspase-1 activation determined by flow cytometry with the fluorescent probe for active caspase-1, FLICA. Neutrophils were cultured with or without LPS for 2 h and then stimulated or not with ATP for 3 h in the presence of FLICA. Data are expressed as mean +SEM of samples pooled from nine donors from five independent experiments, each performed in duplicate. (G) Representative dot plots of one experiment from (F). The percentage of cells with active caspase-1 is indicated. (H) CLSM image of neutrophils stimulated as in (F). Active caspase-1 was detected with FLICA staining (green). The characteristic shape of neutrophil nucleus was visualized with ToPro 3 (blue) and the cell membrane by staining with a phycoerythrin-conjugated anti-CD11b antibody (red). Original magnification x600; Bar, 5 μm. (H) Data shown are from one experiment representative of five performed. (I, J) Elastase and/or PR3 are also involved in pro-IL-1β processing. Neutrophils were pretreated or not with (I) 10 μg/mL Elafin or with (J) 10 μM of the cathepsin G inhibitor I (CatG I) and then cultured for 2 h in the presence or absence of LPS and 16 h with or without ATP. IL-1β released to culture supernatants was determined by ELISA. Data are expressed as mean +SEM of samples pooled from four donors from three independent experiments, each performed in duplicate. *$p < 0.05$; (A) Two-way ANOVA with repeated measures and (C, D, F, and I) Wilcoxon matched ranked one-tailed test.

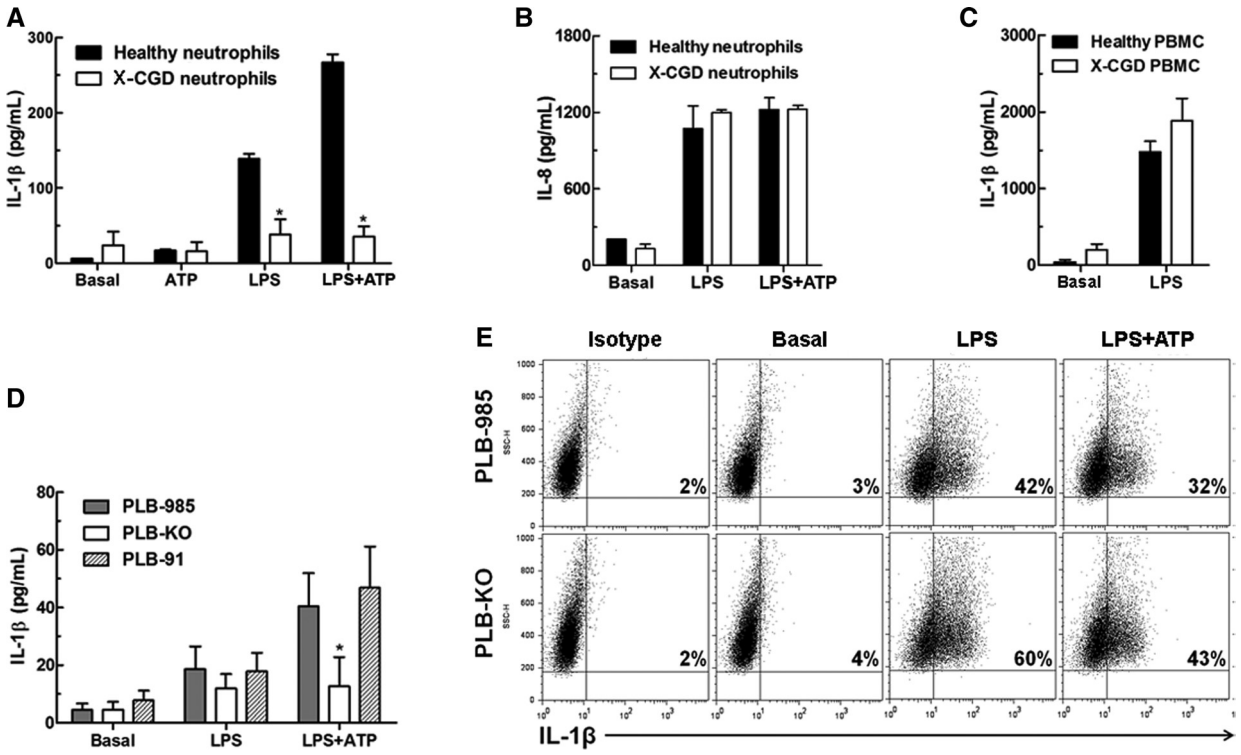


Figure 3. ROS are required for neutrophil IL-1β release. Neutrophils from healthy donors or X-CGD patients were cultured for 2 h in the presence or absence of LPS and then stimulated or not with ATP and cultured for (A) 16 additional hours or (B) 3 additional hours. (A) IL-1β or (B) IL-8 release to culture supernatants was determined by ELISA. (C) IL-1β released by healthy and X-CGD PBMCs cultured in the presence or absence of LPS for 18 h. Data represent the mean +SEM of experiments performed with five (A) and two X-CGD patients (B, C). (D) IL-1β release by neutrophil-differentiated PLB-985, PLB-KO, and PLB-91 cells stimulated as in (A). Data represent the mean +SEM of samples pooled from six independent experiments, each performed in triplicate. (E) Intracellular IL-1β determined by flow cytometry of PLB-985 and PLB-KO cells cultured for 2 h in the presence or absence of LPS and then stimulated or not with ATP for 3 h. A representative experiment of three performed is shown. $^*p < 0.05$; (A, D) Friedman test for multiple comparisons with Dunn's posttest.

(Fig. 5A) than in PLB-985 cells, indicating that cells unable to produce ROS from NADPH oxidase are still able to induce caspase-1 activation. In agreement with these findings, we also observed that stimulated PLB-KO cells, similar to stimulated PLB-985 cells, contained the active IL-1β isoform (Fig. 5B), indicating that the NADPH oxidase deficiency does not affect pro-IL-1β processing. The densitometric quantification of the bands indicated that mature IL-1β levels were higher in stimulated PLB-KO cells than in stimulated PLB-985 cells (Fig. 5C). Taken together, our results suggest that ROS are not necessary to induce inflammasome activation but are required for the release of active IL-1β from neutrophils. Of mention, we were unable to detect pro-IL-1β in PLB cells by Western blot even though it was evidenced in THP-1 cells (Fig. 5B). However, as in neutrophils (Fig. 1E), intracellular pro-IL-1β could be detected in PLB cells by the specific pro-IL-1β ELISA (data not shown).

Since upon stimulation with LPS and LPS+ATP, we found mature IL-1β inside PLB-KO cells (Fig. 3E and 5B), we reasoned that the addition of ROS to these cells after agonist stimulation should induce IL-1β release. To address this issue, X-CGD neutrophils and PLB-KO cells were stimulated with LPS or LPS+ATP for 5 h, and then cell cultures were supplemented with xanthine/xanthine oxidase (X/XO), an enzyme that generates superoxide and uric acid from xanthine. As shown in Fig. 5D and E, the addition of superoxide markedly increased the release of IL-1β by both X-CGD neutrophils and PLB-KO cells stimulated with LPS or LPS+ATP. Together, our results evidenced a not previously described ROS-mediated positive regulation of mature IL-1β secretion.

ROS also negatively regulate neutrophil pro-IL-1β processing

Previous studies in monocytes and macrophages established that caspase-1 activity is negatively regulated by cysteine oxidation [12, 28]. As pro-IL-1β processing takes place before mature IL-1β is released from cells by the ROS-dependent mechanism described above, we reasoned that if neutrophil caspase-1 is also regulated by oxidation, the addition of superoxide before neutrophil stimulation should inhibit pro-IL-1β processing and consequently IL-1β secretion. In accordance with this possibility, we found that when neutrophils from healthy donors (Fig. 6A) and PLB-985 cells (Fig. 6B) were pretreated with X/XO and then stimulated with

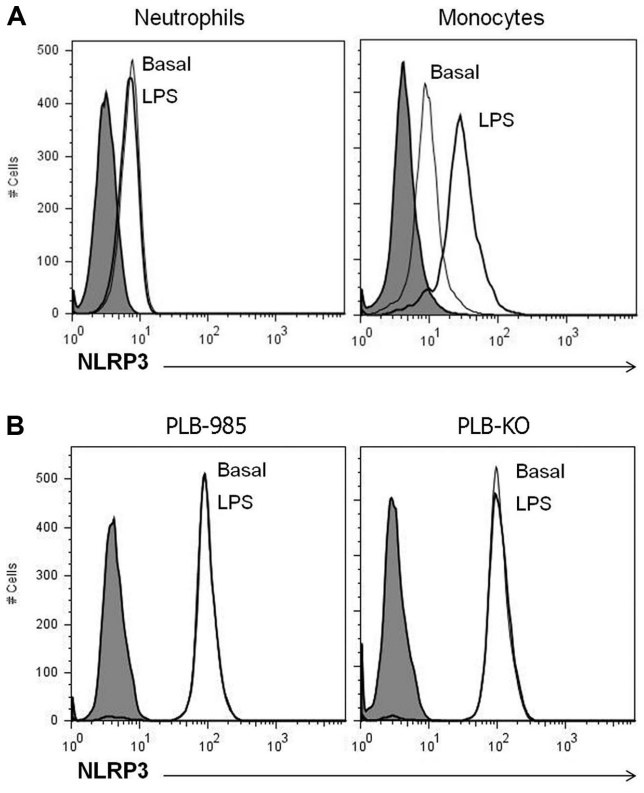


Figure 4. Neutrophil NLRP3 expression is not significantly increased by LPS stimulation. (A) Neutrophils and magnetic-beads purified monocytes or (B) PLB-985 and PLB-KO cells were cultured in the presence or absence of LPS for 3 h and NLRP3 expression was assessed by intracellular staining and flow cytometry. Data shown are from one experiment representative of four experiments performed. Shaded histograms correspond to isotype controls.

LPS and LPS+ATP, IL-1β release was partially inhibited while IL-8 secretion was not significantly modified (Fig. 6C). The reduced IL-1β release observed in X/XO-treated neutrophils was not due to an increase in cell death as the percentage of cells that incorporated 7-AAD did not significantly change upon X/XO treatment (Supporting Information Fig. 1C). Together, these findings are in agreement with the notion that ROS also exert a negative control over IL-1β secretion. To determine if ROS negatively control caspase-1 activity, we evaluated the impact of the presence of X/XO before and during LPS or LPS+ATP stimulation. As shown in Figure 6D, the addition of superoxide reduced the percentage of cells with active caspase-1. Further supporting a negative regulation of ROS over caspase-1 activity, results in Figure 5A also showed a higher percentage of cells with active caspase-1 in PLB-KO cells as related to that exhibited by PLB-985 cells.

Discussion

The ability of neutrophils to generate mature IL-1β has been mostly circumscribed to their capacity to release PR3 and other proteases that could extracellularly process pro-IL-1β [4, 8, 20]. Likewise, there is also evidence indicating that intracellular PR3 and elastase generate active IL-1β [22]. However, previous studies showed that human and mouse neutrophils express NLRP3 and that activity of caspase-1 is induced upon LPS stimulation [15–18, 31]. Moreover, these studies showed the ability of human neutrophils to release IL-1β in a caspase-1-dependent fashion in response to high concentrations of LPS (1 μg/mL) [17]. In this study, we determined that in response to LPS or LPS in combination with ATP, human neutrophils generate active IL-1β not only by means of proteases such as elastase and/or PR3, but also by caspase-1 processing. Moreover, our findings also indicated that a fraction of pro-IL-1β can be found in culture supernatants, suggesting that in vivo, depending on the balance of proteases and antiproteases, it could also be substrate of extracellular proteases, as has been previously suggested [4].

Our studies also suggest that in human neutrophils ROS might play a dual role in IL-1β secretion, being required for the release of mature IL-1β induced by agonists such as LPS and ATP but also playing a negative regulatory role over caspase-1 activation (Fig. 7). The requirement of ROS for IL-1β release is supported both by results obtained with X-CGD human neutrophils (gp91 deficient) and with PLB-KO cells, which released significantly lower amounts of IL-1β upon stimulation as compared to those secreted by healthy neutrophils and PLB-985 cells, respectively, and by the detection of higher levels of intracellular IL-1β in PLB-KO cells as compared to those observed in PLB-985 cells, which suggests that in the absence of ROS, pro-IL-1β is synthesized and processed in response to LPS and ATP but cells are not able to release it.

We also found that ROS are not involved in inflammasome activation, although they are required for the release of mature IL-1β. This conclusion is supported by results obtained with PLB-KO cells and X-CGD neutrophils, that even when deficient in ROS production, they were able to induce caspase-1 activation and to process pro-IL-1β. In fact, in stimulated PLB-KO cells, IL-1β could be found as the mature processed isoform. Furthermore, the addition of superoxide both to X-CGD neutrophils and PLB-KO cells after 5 h of culture in the presence of agonists markedly increased the IL-1β levels found in supernatants, supporting that ROS triggered the release of the accumulated IL-1β (Fig. 7). Previous studies in macrophages showed a reduction in IL-1β secretion when cells were pretreated with the ROS inhibitor N-acetyl-cystein or by knockdown of $p22^{phox}$ upon MSU or asbestos stimulation. The authors concluded that ROS were necessary for inflammasome activation [11, 32]. Contrasting against these findings, other studies showed that monocytes from $p47^{phox}$-deficient patients, which have defective NADPH oxidase activity, exhibited increased caspase-1 activity [28]. Similarly, mononuclear phagocytes from CGD patients with defects in the phagocyte oxidase subunits $p22^{phox}$, $p47^{phox}$, or $gp91^{phox}$ activated caspase-1 in response to danger signals [13], as had been previously shown for gp91-deficient mouse macrophages [12]. On the basis of these results, the authors sustained that ROS not only are dispensable for inflammasome activation but also that exert a negative regulatory role on caspase-1 activity, in accordance to previous studies which had demonstrated that caspase-1 activity can be

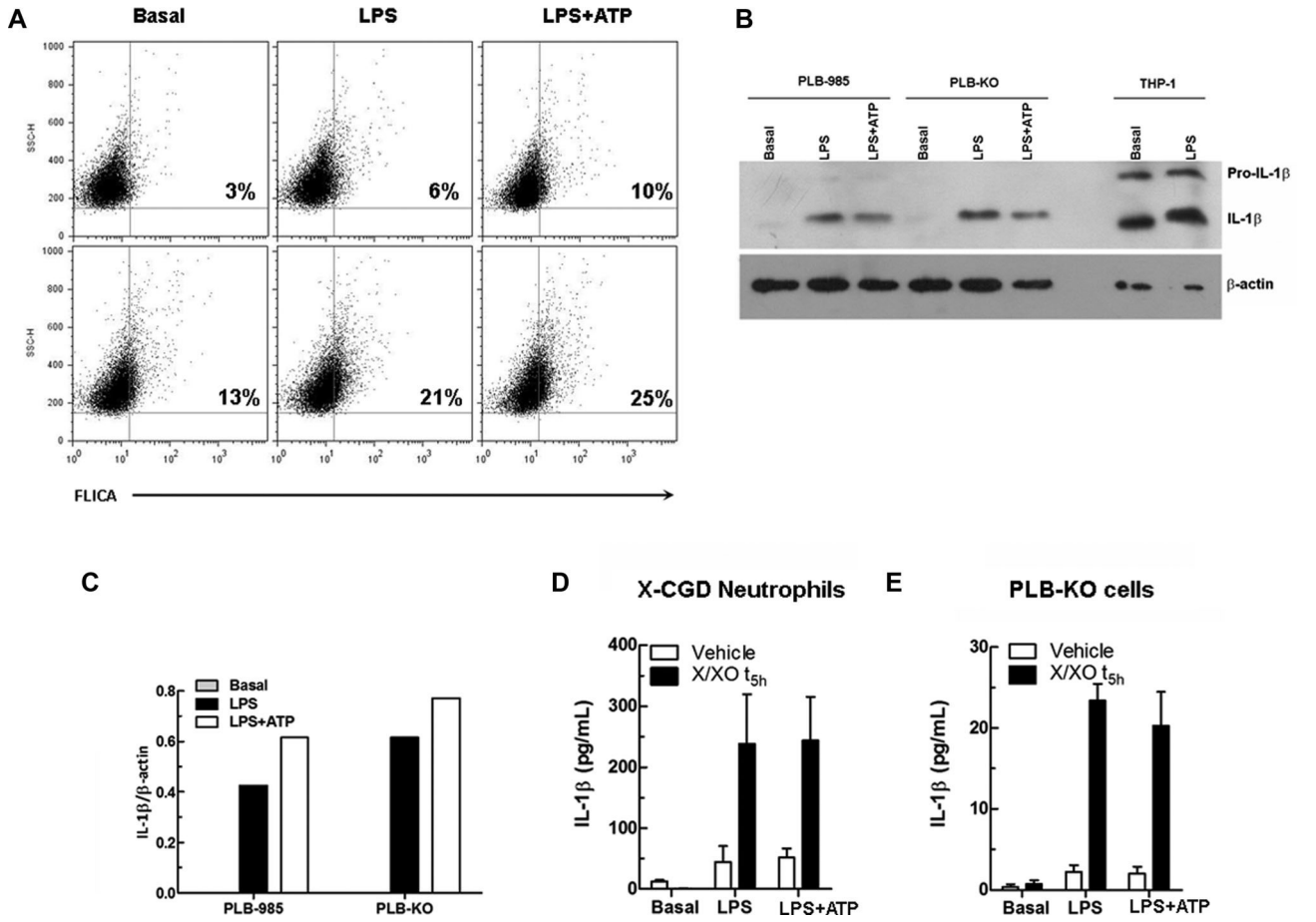


Figure 5. ROS are neither required for caspase-1 activation nor for IL-1β processing but are involved in mature IL-1β secretion. (A) Caspase-1 activation in PLB-985 and PLB-KO cells determined with FLICA by flow cytometry. Cells were cultured for 2 h with or without LPS and then stimulated or not with ATP for 3 h in the presence of FLICA. Results are from one experiment representative of three experiments performed. The percentage of cells with active caspase-1 is indicated. (B) Representative Western blot assay ($n = 4$) of pro-IL-1β and active IL-1β expression in neutrophil-differentiated PLB-985 and PLB-KO cells, and in THP-1 human monocytes differentiated in the presence of 5 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h, employed as controls to show the ability of the antibody to recognize pro-IL-1β. The cell lines were cultured in the presence or absence of LPS for 2 h and then stimulated or not with ATP for 3 h. Then, whole cells extracts were subjected to Western blot. β-actin was used as a loading control. (C) Graphic representation of densitometric analysis of the bands corresponding to mature IL-1β normalized to β-actin in PLB-985 and PLB-KO cells. (D, E) IL-1β secretion by (D) X-CGD neutrophils and (E) neutrophil-differentiated PLB-KO cells can be triggered by the addition of superoxide 5 h after stimulation. Cells were cultured for 2 h in the presence or absence of LPS and then stimulated or not with ATP and cultured for 3 additional h. Then, xanthine (750 μM) and xanthine oxidase (1 mU/mL) were added and cells were cultured for 11 additional hours. Data are expressed as the mean + SEM of samples pooled from (D) two X-CGD patients from two independent experiments each performed in duplicate and (E) four independent experiments performed in triplicate.

regulated by cysteine oxidation [12]. Our findings indicating that PLB-KO cells exhibited a higher percentage of cells with active caspase-1 and that the addition of superoxide to neutrophils by pretreatment with X/XO inhibited caspase-1 activation, also support that in neutrophils, ROS exert a negative regulatory control over caspase-1 activity (Fig. 7). However, in contrast to these studies, which did not find differences in IL-1β secretion in response to LPS or LPS+ATP between NADPH oxidase deficient monocytes and healthy cells [28], or even detected elevated IL-1β secretion in unstimulated CGD monocytes and in response to LPS and MSU [13], we found that X-CGD neutrophils and PLB-KO cells released significantly reduced levels of IL-1β upon stimulation. These contrasting findings might be due to differences in the mechanisms involved in the exportation of IL-1β out of the cell between mononuclear phagocytes and neutrophils, an issue that deserves further investigation.

Previous work in monocytes showed that diphenyliodonium, a NADPH oxidase inhibitor, reduced IL-1β secretion [33, 34]. These studies suggested that ROS generated upon TLR stimulation trigger an antioxidant response that is ultimately required for IL-1β secretion. The authors found that the inhibition of the antioxidant response did not induce intracellular accumulation of mature IL-1β in a cell where IL-1β processing is temporally coupled to secretion, suggesting that the antioxidant response is required for IL-1β processing [34]. We found notable differences in the regulation of IL-1β processing and secretion between neutrophils and those reported for monocytes. In neutrophils, IL-1β processing is not temporarily associated with its secretion because we

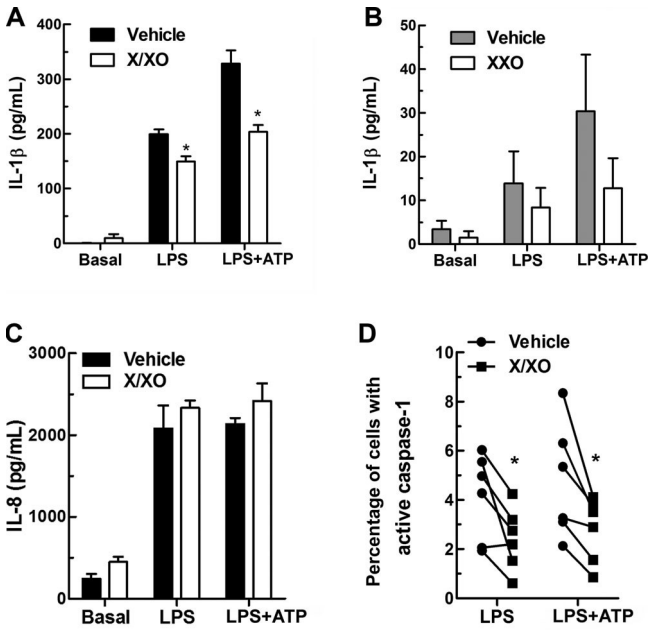


Figure 6. ROS also exert a negative regulation over IL-1β secretion. (A–C) Effect of pretreatment with xanthine/xanthine oxidase on IL-1β and IL-8 release. (A, C) Neutrophils and (B) neutrophil-differentiated PLB-985 cells were preincubated with xanthine (750 μM) and xanthine oxidase (1 mU/mL) for 30 min, then cultured for 2 h in the presence or absence of LPS, and after stimulated or not with ATP and cultured for (A, B) 16 additional hours or (C) 3 additional hours. (A, B) IL-1β and (C) IL-8 release to culture supernatants were evaluated by ELISA. (D) Neutrophils preincubated with X/XO for 30 min were cultured for 2 h with LPS and then stimulated or not with ATP for 3 h in the presence of FLICA. Then, the percentage of cells with active caspase-1 was determined by flow cytometry. Data represent the mean + SEM of samples pooled from (A, B) eight, (C) three, and (D) six donors from two to four independent experiments, respectively, each performed in duplicate; $^{*}p < 0.05$; (A, B) Wilcoxon matched ranked one-tailed test.

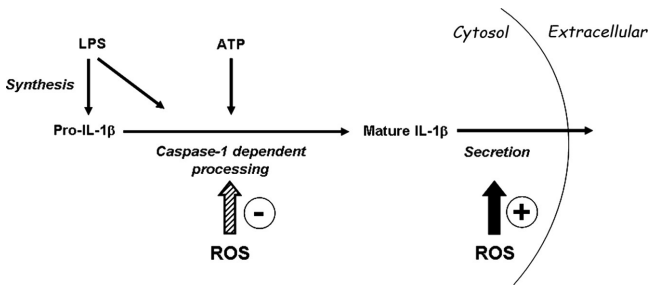


Figure 7. Model for ROS-mediated regulation of neutrophil IL-1β secretion. LPS stimulates pro-IL-1β synthesis. Both LPS and ATP trigger the caspase-1-dependent processing of pro-IL-1β, which is negatively regulated by ROS. Furthermore, ROS are required for IL-1β exportation.

were able to find processed IL-1β inside neutrophils. Besides, we detected mature IL-1β inside stimulated PLB-KO cells, in which this antioxidant response would not take place upon stimulation. Thus, even when a redox response activated upon LPS signaling might be a mechanism involved in controlling neutrophil IL-1β secretion, this response should operate on the exportation of the cytokine out of the neutrophils.

Recent studies by Bauernfeind et al. [30] in mouse macrophages showed that ROS produced by LPS priming are required to induce NALP3 expression and consequently for caspase-1 activation. By contrast, results of our study showed that human neutrophils express NALP3 and this expression is not markedly increased by LPS treatment. Thus, in neutrophils, ROS produced by NADPH oxidase upon LPS stimulation probably have minor impact, if any, in stimulating IL-1β secretion by increasing NALP3 expression. In fact, NALP3 expression levels were similar in PLB-985 and PLB-KO cells and were not modified by LPS stimulation, even though PLB-KO cells evidenced a deficiency in IL-1β release.

Our findings also highlight other differences in the regulation of IL-1β secretion between mononuclear phagocytes and neutrophils. Bauernfeind et al. [30] in macrophages showed that once NLRP3 was expressed, ROS inhibition had no impact on IL-1β release. By contrast, in PLB cells, we observed that ROS deficiency affected neither NLRP3 expression nor inflammasome activation upon stimulation, but did cause a reduction in the ability of PLB-KO cells to secrete IL-1β, evidencing a never before described role of ROS on the release of mature IL-1β.

We found that LPS, a TLR4 agonist, is able per se to induce neutrophil IL-1β secretion. Previous studies indicated that in contrast to macrophages, human blood monocytes are also able to release IL-1β in response to LPS in the absence of a second stimulus [14] because caspase-1 is constitutively active in these cells. Unlike monocytes, we found that neutrophil caspase-1 is not constitutively active, being LPS the stimulus that triggers its activation. Recent studies indicated that human neutrophils constitutively release ATP [35], thus it is possible to speculate that this agonist contributes in an autocrine manner to trigger caspase-1 activation in LPS-primed neutrophils.

Our findings provide evidence that both caspase-1-dependent and -independent mechanisms participate in the production of mature IL-1β in human neutrophils. We found that Elafin, an elastase and PR3 inhibitor, partially reduced human neutrophil IL-1β release induced by LPS and LPS+ATP, suggesting that these serine proteases contribute to pro-IL-1β processing. In recent studies, PR3/elastase double deficient mouse neutrophils did not show differences with WT neutrophils in the amount of IL-1β secreted, whereas caspase-1 or NALP3 KO neutrophils were unable to secrete IL-1β upon stimulation [18]. In contrast, previous studies did not find differences in LPS-induced IL-1β secretion between WT and caspase-$1^{-/-}$ mouse neutrophils [22]. However, in the same work, it was shown that not only a cell-permeable peptide that specifically blocks neutrophil elastase and PR3, but also AC-YVAD-cmk, a caspase-1 inhibitor, blocked IL-1β secretion in vivo. Further investigations performed in vivo in caspase-1-deficient mice indicated that proteases such as PR3 and elastase can compensate caspase-1 absence in the processing of pro-IL-1β [19, 36]. Moreover, studies conducted in a mouse model of acute myocardial infarction indicated that treatment with alpha-1-anti-trypsin, a physiological circulating serine protease inhibitor, reduced caspase-1 activity [37], suggesting that alpha-1-anti-trypsin interferes with the conversion from procaspase to active

caspase-1. Thus, reduction of IL-1β release by Elafin could be due to inhibition of either caspase-1 activation or pro-IL-1β processing by serine proteases. Hence, redundant proteolytic mechanisms probably cooperate in vivo to promote mature IL-1β generation and IL-1β-dependent inflammation. Nevertheless, whatever be the contribution of each mechanism to neutrophil mature IL-1β generation, our results suggest that ROS are required for releasing active IL-1β.

On a per-cell basis, neutrophils produce lower amounts of cytokines than mononuclear leukocytes; however, given that neutrophils consistently outnumber monocytes and macrophages by one to two orders of magnitude in areas of acute inflammation, IL-1β secretion by these cells is likely to play a relevant role in the development of inflammatory responses. Thus, in inflammatory pathologies with massive neutrophil recruitment in which IL-1β secretion by these cells plays a major role, therapeutic interventions including NADPH oxidase inhibitors might turn out useful.

Materials and methods

Human neutrophil isolation

The studies performed in this work have been reviewed and approved by the institutional review board and local ethical committee. Human subjects gave written informed consent.

Blood samples were obtained from healthy donors and X-CGD patients by venipuncture of the forearm vein. Neutrophils were isolated from heparinized human blood by centrifugation on Ficoll-Paque (GE Healthcare, Munich, Germany), dextran sedimentation, and hypotonic lysis, as previously described [38]. Cells were resuspended at 5×10^6/mL in complete medium: RPMI 1640 (HyClone Laboratories, Logan, UT, USA) supplemented with 10% FBS (Gibco Invitrogen, Carlsbad, CA, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL). All neutrophil preparations were stained with an anti-CD14-PE antibody (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed with an FACSCalibur cytometer (Beckton Dickinson, San Jose, CA, USA) after purification to guarantee a monocyte contamination less than 0.5% and that FSC/SSC parameters of the neutrophil population were compatible with those of nonactivated cells. Cells were used immediately after isolation. A representative dot plot of the purified neutrophil preparations is shown in Supporting Information Fig. 4. After treatments, total cell death was determined by evaluating membrane permeability to the fluorescent DNA binding probe 7-AAD. To this aim, cells were incubated with 10 μg/mL of 7-AAD for 15 min at 37°C and immediately analyzed by flow cytometry.

Unless otherwise stated, all the chemicals employed were from Sigma Aldrich (St. Louis, MO, USA).

Neutrophilic cell lines culture and differentiation

PLB-985, *gp91^{phox}* KO-PLB-985 (PLB-KO), and PLB-KO transfected with WT *gp91^{phox}* (PLB-91) were all a kind gift from Dr. Mary Dinauer (Washington University Medical School, St. Louis, MO, USA). The cells were grown in complete medium with 2 mM glutamine and, in the case of PLB-91, with 1 μg/mL puromycin in a humidified incubator at 37°C under an atmosphere of 5% CO_2/95% air [29]. For granulocytic differentiation, logarithmically growing cells at a density of 2.5×10^5 cells/mL were exposed to 0.5% dimethylformamide; differentiation was monitored by acquisition of respiratory-burst activity, as assayed by dihidrorhodamine test. At day 5, cells were washed and resuspended in complete medium at 2×10^6/mL.

Cell stimulation

Neutrophils were treated for 2 h with or without 200 ng/mL LPS from *Escherichia coli* O111:B4. Then, they were stimulated or not with 2.5 mM ATP and cultured for 3 or 16 additional hours. Where indicated, before LPS treatment, cells were pretreated for 30 min with the caspase-1 inhibitor II, AC-YVAD-CMK, the caspase-1 inhibitor IV (both from EMD Biosciences, La Jolla, CA, USA), X/XO (Roche, Basel, Switzerland), Elafin (Anaspec, Fremont, CA, USA), or catepsin G inhibitor I (CatG I, EMD Biosciences). After culture, cell supernatants were recovered and, in some cases, cell pellets were lysed by treatment with Igepal 1% or 0.1% Triton X-100 plus protease inhibitors (PBS pH 7.4, 85 mM EDTA, 2 mM PMSF, 100 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 2 mM AEBSF). Concentrations of IL-1β and pro-IL-1β in culture supernatants and whole cell lysates were determined by ELISA (OptEIA human IL-1β ELISA kit, BD Biosciences or Quantikine Human pro-IL-1β Immunoassay, DLBP00, R&D Systems, Minneapolis, MN, USA). Alternatively, cells were either fixed with 4% paraformaldehyde for intracellular staining or whole cell extracts were prepared with Laemmli buffer with 5% 2-mercaptoethanol for Western blot assays. The biological activity of the IL-1β released to culture supernatants was measured using the previously described MRC-5 fibroblast bioassay [39].

Intracellular immunostainings

Fixed cells were permeabilized with 0.05% saponin and treated with 0.1 M glycine. Cells were incubated with a mouse anti-IL-1β antibody (BD Biosciences) or the corresponding isotype control, and then with a DyLight 488-conjugated goat anti-mouse IgG antibody (Jackson Immunoresearch, West Grove, PA, USA). Alternatively, permeabilized cells were incubated with a rabbit anti-NALP3 antibody (Clone EPR4777, Epitomics, Burlingame, CA, USA), a goat polyclonal anti-NALP3 antibody (#ab4207, Abcam, Cambridge, MA, USA) or the corresponding isotypes controls, and with a DyLight 488-conjugated goat anti-rabbit IgG or Alexa Fluor 488-conjugated donkey anti-goat IgG. Immunofluorescence was determined by flow cytometry and analyzed with FlowJo Software (Tree Star, Ashland, OR, USA). For CLSM detection of intracellular IL-1β, neutrophils were also stained with propidium iodide, cytospun onto slides, and mounted using the antifade reagent

Fluoromont (Sigma-Aldrich). Images were acquired by using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon 60X/1.42 objective. Images were analyzed with Olympus FV10-ASW software.

Determination of caspase-1 activation

Neutrophils were stimulated as described above and FLICA reagent (Immunochemistry, Technologies, Bloomington, MN, USA) was added according to manufacturer's instructions to the corresponding samples, after 2 h of culture, at the moment of the addition of ATP. Cells were incubated for 3 h, washed thoroughly, and analyzed for caspase-1 activation by flow cytometry. For CLSM, neutrophils were seeded on poly-L-lysine-treated Lab-Tek chambered coverglass slides containing a borosilicate glass base (Nunc, Roskilde, Denmark) before stimulation. The cellular membrane of neutrophils was stained with an anti-CD11b-PE antibody (Immunothech-Beckman Coulter, Czech Republic), cells were fixed with 4% paraformaldehyde, and nuclei were dyed with ToPro3 (Molecular Probes-Invitrogen, Carlsbad, CA, USA). Finally, images were acquired with a FluoView FV1000 confocal microscope.

Statistical analysis

Statistical significance was determined using the nonparametric Friedman test for multiple comparisons with Dunn's posttest or Wilcoxon matched ranked test. Statistical significance was defined as $p < 0.05$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **7-AAD**: 7-aminoactinomycin D · **CLSM**: confocal laser scanning microscopy · **MSU**: monosodium urate · **NLR**: Nod-like receptor · **NLRP3**: NLR family pyrin domain containing 3 · **PR3**: proteinase 3 · **X-CGD**: X-linked chronic granulomatous disease · **X/XO**: xanthine/xanthine oxidase

Full correspondence: Dr. Analía Trevani, Departamento de Inmunología, Instituto de Medicina Experimental (IMEX-CONICET), Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina
Fax: +54-11-4803-9475
e-mail: analiatrevani@yahoo.com.ar

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