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**Caspase-1 activation and mature interleukin-1β release are uncoupled events in monocytes**

Galliher-Beckley A *et al.* Uncoupled processing and release of IL-1β

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**Abstract**

**AIM:** To investigate whether caspase-1 activation/intracellular processing of pro-interleukin-1β (proIL-1β) and extracellular release of mature IL-1β from activated monocytes are separable events.

**METHODS:** All experiments were performed on **f**resh or overnight cultured human peripheral blood monocytes (PBMCs) that were isolated from healthy donors. PBMCs were activated by lipopolysaccharide (LPS) stimulation before being treated with Adenosine triphosphate (ATP, 1 mmol/L), human α-defensin-5 (HD-5, 50 μg/mL), and/or nigericin (Nig, 30 μmol/L). For each experiment, the culture supernatants were collected separately from the cells. Cell lysates and supernatants were both subject to immunoprecipitation with anti-IL-1β antibodies followed by western blot analysis with anti-caspase-1 and anti-IL-1β antibodies.

**RESULTS:** We found that proIL-1β was processed to mature IL-1β in LPS-activated fresh and overnight cultured human monocytes in response to ATP stimulation. In the presence of HD-5, this release of IL-1β, but not the processing of proIL-1β to IL-1β, was completely inhibited. Similarly, in the presence of HD-5, the release of IL-1β, but not the processing of IL-1β, was significantly inhibited from LPS-activated monocytes stimulated with Nig. Finally, we treated LPS-activated monocytes with ATP and Nig and collected the supernatants. We found that both ATP and Nig stimulation could activate and release cleaved caspase-1 from the monocytes. Interestingly, and contrary to IL-1β processing and release, caspase-1 cleavage and release was not blocked by HD-5. All images are representative of three independent experiments.

**CONCLUSION:** These data suggest that caspase-1 activation/processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

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**Key words:** Caspase-1; Human defensin; Monocytes; Interleukin-1β processing and release; Inflammasome

**Core tip:** Activated macrophages release large amounts of interleukin-1β (IL-1β) and macrophages deficient in caspase-1 expression have undetectable IL-1β secretion. This suggests that IL-1β release and caspase-1 activation are closely related events. We found that α-defensin 5 (HD-5) inhibited the release of IL-1β, but not the processing of proIL-1β to IL-1β in lipopolysaccharide-activated monocytes stimulated with ATP or Nig. Different from IL-1β processing and release, the activation and release of caspase-1 from stimulated monocytes was not blocked by HD-5. These data suggest that caspase-1 activation/processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

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**INTRODUCTION**

Interleukin-1beta (IL-1β) is an important acute response factor of host defense against microbial infections and a key mediator of inflammation in multiple organs[[1](#_ENREF_1),[2](#_ENREF_2)]. Viral and bacterial pathogens trigger inflammasome formation and subsequent caspase-1 activation and IL-1β maturation[[3](#_ENREF_3)]. IL-1β is synthesized as a biologically inactive 31 kDa proIL-1β polypeptide and must be post-translationally processed by caspase-1 to generate the mature 17 kDa pro-inflammatory cytokine IL-1β[[4](#_ENREF_4)]. Overproduction of IL-1β is associated with multiple autoimmune diseases and septic shock and animals deficient in IL-1β are highly susceptible to microbial infections[[5](#_ENREF_5),[6](#_ENREF_6)].

Activated monocytes and macrophages rapidly release large amounts of mature IL-1β and inflammasome components including caspase-1[[7](#_ENREF_7)]; macrophages deficient in caspase-1 expression have impaired processing of pro-IL-1β and undetectable IL-1β secretion[[8](#_ENREF_8)]. This suggests that IL-1β release and caspase-1 activation are closely related events. Because most known inhibitors of IL-1β production block caspase-1 activation, previous studies are not able to determine whether caspase-1 activation/proIL-1β processing and IL-1β release are separate or linked processes. We have previously demonstrated that human defensin peptide human α-defensin-5 (HD-5) can block the release of IL-1β, but not tumor necrosis factor-α (TNF-α), from lipopolysaccharide (LPS)-activated human monocytes stimulated with Adenosine triphosphate (ATP)[[9](#_ENREF_9)]. Here, using HD-5 as a molecular tool, we explored whether IL-1β release is an indivisible process from caspase-1 activation/proIL-1β processing in human monocytes.

**MATERIALS AND METHODS**

***Human HD-5***

Synthetic human defensin HD-5 was prepared by t-Boc solid-phase synthesis as described previously[[9](#_ENREF_9)]. All peptides were folded and purified to homogeneity by reversed-phase-HPLC and their molecular weights verified by electrospray ionization mass spectrometry.

***PBMC Isolation***

Peripheral blood monocytes (PBMC) from healthy adult donors were prepared as described previously [[9](#_ENREF_9)]. Briefly, blood was diluted with RPMI 1640, overlaid on lymphocyte separation medium (Mediatech), and centrifuged at 400 ×g for 35 min. The mononuclear cell layer was washed with PBS and centrifuged at 250 ×g for 10 min. The cells were then resuspended in 25 mL of PBS plus citrate solution and overlaid on Percoll (GE Healthcare) prediluted 9/1 with 1.5 mol/L NaCl. After a 35-min centrifugation at 400 ×g, the PBMC were washed with PBS again. Cells were counted using trypan blue exclusion and cells were resuspended in monocyte medium [RPMI 1640, 5% FBS, 20 mmol/L HEPES (pH 7.3), 1% streptomycin/penicillin] and incubated at 37°C for 2 h to allow for adherence, after which medium supernatants were discarded. Attached cells were rinsed twice with monocyte medium and used immediately or incubated in monocyte medium overnight at 37°C in a 5% CO2 environment.

### *IL-1β posttranslational processing and release assay*

Fresh or overnight cultured PBMCs were treated with 20 ng/mL LPS for 2 h at 37°C. In some experiments, the media was removed then replaced with RPMI (without Met, Cys, or Glu), + 1% FBS + 25 mmol/L HEPES pH 7.4 + 300 mg/L Glutamine + 83 μCi/mL 35S-Met/Cys and incubated at 37°C for 1 h. Cells were then rinsed and media was replaced with RPMI (+Glu). Adenosine triphosphate (ATP, 1 mmol/L), human α-defensin-5 (HD-5, 50 μg/mL), and/or nigericin (Nig, 30 μmol/L) was added to culture media and incubated at 37°C for 1.5 h. The supernatants were collected, 1% triton X-100 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States) added, then spun down. Cells were lysed in 500 μL lysis buffer (25 nmol/L HEPES pH 7.4 + 150 mmol/L NaCl + 0.1% Triton X-100 + Protease Inhibitor cocktail) then spun down. Cell lysates and supernatants were subject to IL-1β immunoprecipitation and western blot analysis of caspase-1 as described previously[[9](#_ENREF_9)]. Results shown are a representative from three independent experiments.

***Statistical analysis***

All experiments were repeated 3 times to ensure reproducibility. Image J software (NIH) was used to quantitate protein bands. Proteins bands between two different treatment groups were considered statistically significant when *P* < 0.05, by *t*-test analysis.

**RESULTS**

***HD-5 blocks the release of IL-1β from both freshly isolated and overnight cultured monocytes***

Our previous studies have shown that HD-5 can block the release of proIL-1β and mature IL-1β from ATP stimulated human monocytes that were cultured overnight prior to LPS exposure[[9](#_ENREF_9)]. Since culturing monocytes *in vitro* can lead to further differentiation into macrophage-like cells[[10](#_ENREF_10),[11](#_ENREF_11)], we determined whether the subcultured monocytes behaved similarly to freshly isolated cells. As shown in Figure 1 (upper panel) the extracellular release of mature IL-1β from both freshly isolated and overnight cultured monocytes was blocked by HD-5. Interestingly, we also observed that freshly isolated monocytes had enhanced intracellular processing of pro-IL-1β to IL-1β (Figure 1, lower panel, lanes 1-2 *vs* 3-4) suggesting that increased time in culture can decrease the ability of monocytes to process IL-1β. Most importantly, it was found that the intracellular processing of proIL-1β to mature IL-1β was not inhibited by HD-5 as evidenced by the presence of intracellular mature IL-1β in cell lysates from monocytes treated with HD-5 (Figure 1, lower panel).

***HD-5 treatment significantly blocks IL-1β release from monocytes stimulated with Nigericin***

To determine whether the ability of HD-5 to block IL-1β release in overnight-cultured, LPS-activated monocytes was specific to ATP stimulation, we treated LPS-activated monocytes with ATP or nigericin (Nig). Similar to ATP, nigericin is a microbial toxin that acts as an inflammasome inducer, leading to caspase-1 maturation and IL-1β processing and release[[12](#_ENREF_12),[13](#_ENREF_13)]. We found that, similar to its effect on ATP-mediated IL-1β release, HD-5 treatment was also able to significantly block IL-1β release from monocytes stimulated with Nig (Figure 2, upper panel). Furthermore, HD-5 did not block Nig-induced intracellular processing of proIL-1β to mature IL-1β as indicated by the presence of mature IL-1β in the cell lysate (Figure 2, lower panel).

***ATP and Nigericin-mediated caspase-1 activation and extracellular release from LPS-activated monocytes is not affected by HD-5***

Caspase-1 activation has been implicated in both the processing and release of IL-1β[[14](#_ENREF_14),[15](#_ENREF_15)]. Because our current studies have shown that HD-5 selectively blocked the release but not the processing of proIL-1â to mature IL-1â, it is important to know whether HD-5 has any effect on caspase-1 activation and release. As shown in Figure 3, ATP/Nig-mediated caspase-1 activation and extracellular release from LPS-activated monocytes were not affected by HD-5. This observation is consistent with the finding that HD-5 did not block the intracellular processing of proIL-1β to IL-1β (Figures 1 and 2).

**DISCUSSION**

Current research suggests that once caspase-1 becomes active it leads to both the processing and release of IL-1β[[16-18](#_ENREF_16)]. Although the processing and release of IL-1β are rapid and probably concurrent events, it has been suggested previously that the cleavage of proIL-1β and release of mature IL-1β are likely independent of each other[[19](#_ENREF_19)]. However, this speculation remains hypothetical because, due to the technical limitations, the presence of mature IL-1β inside LPS-primed, ATP-stimulated monocytes have not been documented prior to this report. Here, under certain conditions we have clearly shown that IL-1β release but not its processing from proIL-1β by caspase-1 in ATP/Nig-stimulated monocytes can be blocked by HD-5. To our knowledge, this report provides the first direct evidence that the processing of proIL-1β to mature IL-1β and extracellular release of mature IL-1β are two divisible events in human monocytes.

Our results also indicate that the majority of proIL-1β is processed intracellularly by activated caspase-1 in freshly isolated monocytes (Figure 1A). This observation is consistent with the report that human blood monocytes release processed IL-1β after a one-time stimulation with toll-like receptor 4 ligands due to the resulting constitutively activated caspase-1[[16](#_ENREF_16)]. Furthermore, because the release of IL-1â, but not the externalization of caspase-1 was affected by HD-5, our studies suggest that IL-1β seems to be released directly to the extracellular environment without the involvement of caspase-1 inflammasome.

The mechanisms by which proIL-1β and mature IL-1β are released from cytokine producing cells have been an intriguing and unsolved question of IL-1β biology for decades. Known activators of caspase-1 and IL-1β release include ATP, adjuvants, and various microbial molecules[[20](#_ENREF_20)]. Using different experimental systems, studies on ATP-induced IL-1β maturation have posited four different and conflicting models of IL-1β secretion, including secretory lysosome exocytosis[[21](#_ENREF_21),[22](#_ENREF_22)], microvesicle shedding[[23-25](#_ENREF_23)], direct transport across the plasma membrane[[26](#_ENREF_26)], and exocytosis of exosome-containing multivesicular bodies[[7](#_ENREF_7)]. The multiple models of IL-1β secretion reflect the confusion in this area. It is true that although ATP-induced caspase-1 activation is followed by the processing of proIL-1β to IL-1β in the cytosol and secretion of IL-1β, other protein release mechanisms, such as externalization of caspase-1, secretory lysosomes and microvesicle shedding, do occur. However, externalization of caspase-1 and other inflammasome components, secretory lysosomes and microvesicle shedding may be cell-type specific processes after ATP stimulation and may not be involved in IL-1β secretion from monocytes. Here, we provide direct evidence that IL-1β secretion is a separable process from inflammasome activation/caspase-1 externalization and IL-1β processing in human monocytes. Inflammasome activation and caspase-1 activity are required but not sufficient for the release of IL-1β.

Taken together, our studies clearly demonstrated that intracellular processing of pro-IL-1β to mature IL-1β and the externalization of mature IL-1β are divisible events in human monocytes. Although the molecular mechanisms by which HD-5 blocks the release of mature IL-1β has yet to be revealed in future studies, a better understanding of IL-1β processing and release may lead to the discovery of novel molecular targets for IL-1β blockade and the development of new therapeutic approaches to treat life-threatening microbial infections and inflammatory diseases.

**COMMENTS**

***Background***

Interleukin-1beta (IL-1β) is secreted by monocytes and macrophages and is an important acute response factor of host defense against microbial infections. IL-1β is synthesized as a biologically inactive 31 kDa proIL-1β polypeptide and must be post-translationally processed by caspase-1 to generate the mature 17 kDa IL-1β that is released into the extracellular space.

***Research Frontiers***

Macrophages deficient in caspase-1 expression have undetectable IL-1β secretion. This suggests that IL-1β release and caspase-1 activation are closely related events. Because most known inhibitors of IL-1β production block caspase-1 activation, previous studies are not able to determine whether caspase-1 activation/proIL-1β processing and IL-1β release are separate or linked processes. This report shows that processing of pro-IL-1β by caspase-1 and the release of mature IL-1β from human monocytes are distinct and separable events.

***Innovations and breakthroughs***

Recent reports suggest IL-1β secretion involves the formation of the inflammasome, leading to the cleavage and activation of caspase-1, which in turn proteolytically processes pro-IL-1β. Biologically active IL-1β is subsequently secreted by the cell. Here authors propose that IL-1β secretion involves a more complex regulatory mechanism.

***Applications***

Standard therapy for patients with autoimmune diseases or lymphomas involves blocking IL-1β activity. By knowing how IL-1β is processed and released, this study may lead to the development of novel therapeutics that can block IL-1β release and prevent or enhance treatment for these debilitating pro-inflammatory disorders.

***Terminology***

The inflammasome is a multi-protein complex responsible for the activation of caspase-1, an enzyme that cleaves and activates downsteam targets such as IL-1β. IL-1β is a pro-inflammatory cytokine secreted by immune cells to aid in the defense of microbial infection. Human α-defensin 5 (HD-5) is an anti-microbial peptide that normally functions by binding to the microbial cell membrane to form a lethal pore.

***Peer review***

The authors examined the ability of HD-5 to block the release, but not the activation, of IL-1β from ATP and nigericin stimulation human monocytes. It revealed that while HD-5 can block IL-1β release, caspase-1 activation is not affected. The results are interesting and may represent a new molecular mechanism in IL-1β secretion.

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**Figure 1** **Human α-defensin 5 blocks the release from but not the processing of** **interleukin-1β in fresh and overnight cultured human monocytes.** Freshly isolated or overnight cultured human monocytes were primed with lipopolysaccharide (LPS) (20 ng/mL) for 2 h before being labeled with 35*S*-methionine/cysteine for 1 h and then washed and treated with Adenosine triphosphate (ATP, 1 mmol/L) and/or human α-defensin 5 (HD-5) (50 μg/mL) for another 1.5 h. Media and cell-associated fractions were harvested separately. Interleukin-1β (IL-1β) was recovered from each by immunoprecipitation with anti-hIL-1β antibodies (1:1000) that recognize both the pro-IL-1β (31 kDa) and mature IL-1β (17 kDa) proteins. The resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Images are representative from three independent experiments.

**Figure 2** **Human α-defensin 5 blocks interleukin-1βrelease from monocytes** **stimulated with Adenosine triphosphate or** **nigericin**. Overnight-cultured, lipopolysaccharide (LPS)-activated, 35*S*-Met/cys-labeled human monocytes were treated with ATP (1 mmol/L) or nigericin (Nig) (30 μmol/L) in the presence or absence of human α-defensin 5 (HD-5) (50 μg/mL) for 1.5 h. Media and cell-associated fractions were harvested separately. Interleukin-1β (IL-1β) was recovered from each by immunoprecipitation and resulting immunoprecipitates were analyzed by SDS-PAGE and autoradiography. Images are representative from three independent experiments.

**Figure 3 Adenosine triphosphate / nigericin-induced caspase-1 activation and externalization in human monocytes are not blocked by human α-defensin 5.** Lipopolysaccharide (LPS)-activated monocytes were stimulated with Adenosine triphosphate (ATP, 1 mmol/L) or nigericin (Nig) (30 μmol/L) in the presence or absence of human α-defensin 5 (HD-5) (50 μg/mL) for 1.5 h. Media were harvested, precipitated, and subjected to SDS-PAGE and western blot analysis with anti-caspase-1 p10 antibodies (1:1000) that recognize both the pro-caspase-1 (45 kDa) and cleaved caspase-1 p10 subunit proteins.