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NLRP3 INFLAMMASOME FUNCTION AND PYROPTOTIC CELL DEATH IN HUMAN PLACENTAL HOFBAUER CELLS

Vikki M. Abrahams^{1,*}, Zhonghua Tang¹, Gil Mor², Seth Guller¹

¹Department of Obstetrics, Gynecology & Reproductive Sciences, Yale University, New Haven, CT, USA

²C.S. Mott Center for Human Growth and Development, Department of Obstetrics, Gynecology, Wayne State University, Detroit, Michigan, USA.

Abstract

Alterations in the number and protein/gene expression of Hofbauer cells (HBCs) may play a role in microbial-driven/cytokine-mediated placental inflammation, and in subsequent pregnancy complications such as villitis, histologic chorioamnionitis, and the fetal inflammatory response syndrome. Pyroptosis is an inflammatory form of cell death mediated by the inflammasome, a multi-protein complex which drives the processing and secretion of interleukin 1 beta (IL-1 β). Pyroptosis can be triggered by bacterial lipopolysaccharide (LPS) and adenosine triphosphate (ATP) in non-placental macrophages through activation of the NLRP3 inflammasome. However, the role of inflammasome activation and pyroptosis in HBC pathophysiology remains unclear. HBCs isolated from human term placentas were treated with or without LPS or ATP, alone or in combination. Treatment of HBCs with both LPS and ATP induced the rapid secretion of high levels of IL-1 β and at the same time, cell death associated with nuclear condensation and cellular swelling. HBC treatment with both LPS and ATP induced caspase-1 activation, gasdermin D (GSDMD) cleavage, which mediates pyroptosis, and IL-1 β processing. Caspase-1 activation, GSDMD cleavage, IL-1 β processing, and IL-1 β secretion were all significantly reduced following NLRP3 knockdown; inhibition of caspase-1; and inhibition of P2X7, the receptor that mediates K⁺ efflux. Together, our data indicate that LPS and ATP treatment stimulated NLRP3 inflammasome activation and pyroptosis in HBCs leading to the rapid release of IL-1 β . Since the localization of HBCs confers a unique ability to influence microbial-associated placental and fetal inflammation, these studies suggest a key role for the inflammasome and pyroptosis in mediating HBC driven inflammation.

Keywords

Infection; Inflammasome; Placenta; Pyroptosis; Macrophage

*Corresponding Author: Vikki M Abrahams, PhD. Department of Obstetrics, Gynecology & Reproductive Sciences, Yale School of Medicine, 310 Cedar Street, LSOG 305C, New Haven, CT 06510, USA. vikki.abrahams@yale.edu; Phone: 203-785-2175.

DECLARATION OF COMPETING INTEREST

The authors declare that there are no conflict of interest.

1. INTRODUCTION

Hofbauer cells (HBCs) are large, pleiomorphic, highly vacuolated placental macrophages, thought to be of fetal origin, and located beneath the syncytium and adjacent to fetal capillaries (Castellucci *et al.* 1980), a site critical for the protection of the fetus against microbes migrating from the mother. Recent studies have indicated that alterations in the number and protein/gene expression of HBCs may play a role in microbial-driven/cytokine-mediated inflammation in placenta, and in subsequent pregnancy complications such as villitis, histologic chorioamnionitis (HCA), and the fetal inflammatory response syndrome (FIRS) (Tang *et al.* 2011a, Reyes and Golos 2018).

Based on their surface expression markers, HBCs can be classified as anti-inflammatory M2 macrophages (Joerink *et al.* 2011, Tang *et al.* 2011b), supporting their role in angiogenesis and development (Anteby *et al.* 2005, Ingman *et al.* 2010). However, while expressing M2 markers, HBCs can also produce robust inflammatory cytokine responses towards bacterial and viral triggers (Young *et al.* 2015, Hendrix *et al.* 2020, Schliefssteiner *et al.* 2020), indicating that HBCs actively sense and respond to infections (Tang 2011 103). Yet, little is known about the mechanisms involved.

One important inflammatory factor that is associated with pregnancy complications including preterm labor, chorioamnionitis, and FIRS is interleukin 1 beta (IL-1 β) (Menon *et al.* 2010, Nadeau-Vallee *et al.* 2016, Xiong and Wintermark 2020). Experimental models have demonstrated a role for placental-derived IL-1 β in mediating placental inflammation and injury, and poor fetal/neonatal outcomes (Girard *et al.* 2010, Adams Waldorf *et al.* 2011, Leitner *et al.* 2014, Bergeron *et al.* 2016, Brien *et al.* 2017, Presicce *et al.* 2018). Furthermore, macrophages may play a contributing role (Girard *et al.* 2010). Indeed, we previously reported that HBCs infected with a live herpes virus (MHV-68) secreted IL-1 β , which in turn induced a pro-neutrophilic response in endothelial cells (Hendrix *et al.* 2020).

Since IL-1 β is such a potent inflammatory cytokine that if not properly controlled causes tissue injury, its production and secretion is regulated by a two-step process culminating in activation of the inflammasome, a multi-protein complex which, classically though caspase-1, drives the processing and secretion of mature IL-1 β (Franchi *et al.* 2009). The first step is a priming signal whereby microbial activation of a pattern recognition receptor, such as a Toll-like receptor (TLR), upregulates expression of pro-IL-1 β and the Nod-like receptor (NLR) that will form the inflammasome. The second step is a triggering signal whereby activation of the NLR leads to inflammasome assembly and activation (Franchi *et al.* 2009). In non-placental macrophages, the NLPR3 inflammasome has been well described. Bacterial lipopolysaccharide (LPS) can prime macrophages to increase NLRP3 and pro-IL-1 β expression, while adenosine triphosphate (ATP) activates the NLRP3 inflammasome (Mariathasan *et al.* 2006, Bauernfeind *et al.* 2009, Netea *et al.* 2009). In parallel, caspase-1 activation of gasdermin D (GSDMD) leads to pyroptosis, a form of rapid cell death that contributes to IL-1 β release (He *et al.* 2015, Shi *et al.* 2015). Since the mechanism by which IL-1 β is produced by HBC remains unclear, the aim of this study was to explore the regulation of NLRP3 inflammasome function and pyroptotic cell death in HBCs pathophysiology.

2. MATERIALS AND METHODS

2.1. Cell Isolation

Placentas obtained from uncomplicated cesarean delivery at term (n=6) were used to isolate HBCs cells and were processed within 1 h of delivery. Isolation of HBCs from placenta was carried out as we have described (Tang *et al.* 2011b, Young *et al.* 2015). Briefly, villous tissue was sequentially digested with trypsin/deoxyribonuclease I and collagenase A/deoxyribonuclease I, single cells were pelleted, resuspended, and then loaded onto a discontinuous Percoll gradient (35/30/25/20%). Cells from 20/25% to 30/35% interfaces were combined and were immunopurified by negative selection using anti-epidermal growth factor receptor and anti-CD10 antibodies conjugated to magnetic beads. Cells were plated, and after 1 hr, floating and weakly adherent cells were removed and discarded. This procedure yielded HBCs with purity of at least 98% based on flow cytometry and morphological criteria (Tang *et al.* 2011b, Young *et al.* 2015). Cells were plated in DMEM/F12 (cat# D2906, Sigma, St. Louis, MO) supplemented 10% FBS (cat# 100–500, Gemini BioProducts, West Sacramento, CA) and 1% Pen-Strep, and studies were carried out in serum-free medium consisting of DMEM/F12 with 50µg/ml ascorbic acid and ITS+ Premix (cat# 354352, Corning, Corning, NY), a universal culture supplement which yields a final concentration of insulin of 6.25mg/ml; transferrin, 6.25mg/ml; selenous acid, 6.25ng/ml; bovine serum albumin, 1.25mg/ml; and linoleic acid, 5.35µg/ml as we have previously described (Tang *et al.* 2013, Young *et al.* 2015).

2.2. Time Course Study

Isolated HBCs were treated with 1ng/ml LPS for 4 hr, after which ATP was added to a final concentration of 5mM. After 20 min to 120 min, media were collected, and cells were lysed in RIPA cell lysis buffer (50mM Tris-HCl, pH 8.0 containing 150mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 5% glycerol supplemented with a complete protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail, cat# 11836170001 and 4906845001, respectively, from Roche Diagnostics, Mannheim, Germany) for Western blotting.

2.3. Cell death assay

HBCs were treated with 1ng/ml LPS for 4 hr, and then with 2 mM ATP for 40 min, after which cells were stained with H33342 (cat# B2261, Sigma, St. Louis, MO) and SYTOX® Orange (cat# S11368, Invitrogen, Carlsbad, CA) for 1 min. Photographic images were captured using a IX71 fluorescent microscope (Olympus, Shinjuku, Tokyo, Japan).

2.4. Inhibitor assays

HBCs pretreated with either caspase-1 inhibitors Z-WEHD-FMK (10µM; cat# FMK002, R&D Systems, Minneapolis, MN) and VX765 (10µM; cat# 28825, Cayman Chemical, Ann Arbor, Michigan), or P2X7 receptor antagonist, KN-62 (5µM, cat# 1277, TOCRIS, Minneapolis, MN) for 30 min, were then incubated with 1ng/ml LPS for 4 hr, and followed by treatment with 5mM ATP for 40 min. Media were collected and cells were lysed in RIPA buffer.

2.5. siRNA studies

HBCs were transfected with 50nM of each ON-TARGETplus Human NLRP3 siRNA SMARTpool (cat# L-017367-00, Horizon Discovery/Dharmacon, Cambridge, MA) and ON-TARGETplus non-targeting control pool (cat# D-001810-10, Dharmacon, for 24 hr in DMEM/F12 complete culture media using TransIT-X2® transfection reagent (cat# MIR 6000, Mirus Bio, Madison, WI). Cells were then treated with 1 ng/ml LPS in SFM for 4 hr followed by 5 mM ATP for 40 min. Culture media were collected and cells were lysed in RIPA buffer.

2.6. ELISA

Levels of secreted IL-1 β in culture media was measured by ELISA using a Human IL-1 β /IL-1F2 DuoSet ELISA kit (R&D Systems).

2.7. Western Blotting

Protein was extracted from HBCs using RIPA cell lysis buffer and levels of total cellular protein were quantitated using DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Electrophoretic separation of proteins was carried out on a Tris-glycine 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane. After transfer, the membrane was incubated for 1 hr at room temperature in Odyssey (LI-COR, Lincoln, NE) blocking buffer (TBS) and then overnight at 4°C with the following primary antibodies: rabbit anti-NLRP3 (1:1,000; cat# 13158, Cell Signaling Technology, Danvers, MA), mouse anti- GSDMD (1:500; cat# sc-81868, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-caspase-1 (1:1,000; cat# 3866, cell signaling technology), rabbit anti IL1 α (1:1,000; cat# sc-7884, Santa Cruz Biotechnology), mouse anti-IL1 β (1:1,000; cat# MAB201, R&D Systems, Minneapolis, MN) and mouse anti-Human HSP90 (1:5,000; cat# 610418, BD Bioscience, San Jose, CA). All primary antibodies were prepared in TBS containing 0.1% Tween with 50% Odyssey blocking buffer (TBS). IRDye 800cw conjugated donkey anti-mouse and anti-rabbit (cat# 926-32212 and 926-32213, LI-COR), and Alexa Fluor 680 conjugated donkey anti-mouse and anti-rabbit (cat# A10038 and A10043, Invitrogen, Carlsbad, CA) secondary antibodies were all used at a 1:50,000 dilution. Target and housekeeping proteins were detected simultaneously on the same membrane. The membrane was washed in TBS containing 0.1% Tween, and secondary antibodies were added for 1 hr at room temperature. After washing, the red signal from the 680-nm fluorophore and the green one from the 800-nm fluorophore were visualized and quantitated with an Odyssey infrared imager (LI-COR).

2.8. Statistical Analysis

Statistical analysis was carried out using SigmaStat version 4 (Systat Software, San Jose, CA). One-way analysis of variance (ANOVA) was used to compare results that were normally distributed and are presented as a mean $\pm$ standard error of the mean (SEM). Kruskal-Wallis ANOVA was carried out for data that were not normally distributed and are presented as a median with quartiles. Student-Newman-Keuls analysis was carried out for pairwise comparisons. A $p < 0.05$ was considered significant.

3. RESULTS

3.1. Treatment of HBCs with combination LPS and ATP induces rapid IL-1 β secretion

HBCs were treated with LPS for 4 hr followed by 5 mM ATP for an additional 20 min before culture supernatants were collected and measured for levels of secreted IL-1 β by ELISA from 20–120 min. As shown in Figure 1, levels of IL-1 β secreted by HBCs treated with combination LPS and ATP were significantly greater at all time points compared to the control (Ctrl) (20,000- to 100,000-fold), and compared to ATP alone (2,000- to 10,000-fold). Combination LPS and ATP induced a 3- to 7.5-fold increase in IL-1 β secreted by HBCs when compared to cells treated with LPS alone with statistical differences noted at all time points studied, with the exception of at 20 min (Figure 1). These results indicate that treatment of HBCs with both LPS and ATP induce the rapid secretion of high levels of IL-1 β by HBCs ranging from 1,750 pg/ml at 20 min to 88,200 pg/ml at 120 min.

3.2. LPS and ATP treatment of HBCs promotes pyroptotic-like cell death

Pyroptosis is a cell death pathway characterized by DNA fragmentation, nuclear condensation and cellular swelling prior to lysis and subsequent IL-1 β secretion (Jorgensen and Miao 2015). Morphological assessment was initially used to assess whether the combined treatment of LPS and ATP promoted pyroptosis in HBCs. As shown in Figure 2A, treatment of HBCs with both LPS and ATP induced pronounced nuclear condensation and cellular swelling (indicated by arrows in the right panel). In addition, compared to LPS or ATP alone, the majority of HBCs treated with LPS+ATP stained positive for the dye SYTOX orange, the uptake of which is blocked in live cells, indicative of significant HBC cell death (Figure 2B). Together, this indicated that combination LPS and ATP treatment of HBCs promoted pyroptotic-like cell death.

3.3. Modulation of the NLRP3 inflammasome and GSDMD in HBCs by LPS and ATP treatment

Pyroptosis of non-placental macrophages has been shown to be mediated by activation of the NLRP3 inflammasome, with subsequent activation of caspase-1 and the processing of pro-IL-1 β into active-IL-1 β , and in parallel, caspase-1-mediated activation of GSDMD (He *et al.* 2016, Evavold *et al.* 2018). The formation of active N-terminal domain GSDMD pores on lipid membranes following its activation is viewed as the effector process of pyroptosis and a hallmark feature that can lead to the release of mature IL-1 β (He *et al.* 2016). To demonstrate the induction of pyroptosis in HBCs following treatment with LPS and ATP, Western blot was performed for these key components of the pyroptotic pathway. As shown in Supplemental Figure 1 and Figure 3A, under control conditions, HBC expressed NLRP3 and the pro-forms of GSDMD and caspase-1, but not the pro-form of IL-1 β . Similarly, under control conditions HBC lacked expression of the active forms of GSDMD, caspase-1 and IL-1 β . Treatment of HBCs with LPS alone, or in combination with ATP, increased the expression of NLRP3 and pro-IL-1 β , while treatment with ATP alone had no effect on their expression (Supplemental Figure 1 and Figure 3A). Similarly, only combination LPS and ATP triggered HBC expression of the active forms of GSDMD, caspase-1, and IL-1 β (Supplemental Figure 1 and Figure 3A). This indicated that LPS provided the priming signal 1 for HBC NLRP3 and pro-IL-1 β expression, while ATP provided the triggering signal 2 for

subsequent HBC inflammasome assembly and activation (Mariathasan *et al.* 2006, Bauernfeind *et al.* 2009, He *et al.* 2016). VX765, a caspase 1 inhibitor, significantly reduced the induction of the active forms of GSDMD; caspase-1; and IL-1 β in HBCs following LPS and ATP treatment by 67.1%; 65.5%; and 91.9%, respectively. Similarly, WEHD, another caspase-1 inhibitor, significantly suppressed the activation of caspase-1 by 65.4% and processing of active IL-1 β by 81.3%. The 32.9% reduction in active GSDMD by WEHD did not reach statistical significance (Figure 3A). KN-62, a P2X7 receptor antagonist which blocks K⁺ efflux (He *et al.* 2016), significantly reduced the induction of the active forms of GSDMD; caspase-1; and IL-1 β in HBCs following LPS and ATP treatment by 91.7%; 99.1%; and 93.8%, respectively. Of note, the inhibitors, VX765, WEHD, and KN-62 did not significantly affect the levels of NLRP3, pro-GSDMD, pro-caspase-1, or pro-IL-1 β in cells treated with LPS and ATP. WEHD, VX765, and KN-62 significantly inhibited HBC IL-1 β secretion following treatment with LPS and ATP by 92.7%, 95.9%, and 95.5%, respectively (Figure. 3B), reflecting the patterns of protein activation seen by Western blotting (Figure 3A).

3.4. The NLRP3 inflammasome mediates pyroptotic IL-1 β release by HBCs treated with LPS and ATP

Studies using siRNA were then used to directly examine the role of the NLRP3 inflammasome in the activation of caspase-1; the cleavage of GSDMD; and the processing and release of IL-1 β . As shown in Figure 4A, under combination LPS and ATP conditions, NLRP3 siRNA significantly knocked down HBC expression levels of NLRP3 by 71.5% when compared to the scrambled control. Knockdown of NLRP3 significantly inhibited the activation of GSDMD; caspase-1; and IL-1 β in HBCs treated with LPS and ATP by 66.5%; 67.4%; and 41.9%, respectively. Levels of pro-caspase-1 and pro-IL-1 β were not significantly affected by NLRP3 siRNA, while pro-GSDMD levels were significantly increased by 11.2% (Figure 4A). Knockdown of NLRP3 also significantly inhibited the release of IL-1 β by 84.2% in HBCs treated with LPS and ATP when compared to the scrambled control (Figure 4B). These results indicate that NLRP3 regulates the activation of GSDMD and caspase-1, as well as the processing and secretion of mature IL-1 β in HBCs treated with LPS and ATP.

4. DISCUSSION

Although clinical and experimental models have demonstrated a role for placental inflammation in poor pregnancy and fetal/neonatal outcomes, in particular, in the setting of an infection, our understanding of the cell types and mechanisms involved are still incomplete. IL-1 β , whose production is mediated by the inflammasome, promotes a number of immune responses including the recruitment of innate immune cells to sites of infection and modulation of the adaptive immune response. However, if not adequately controlled, its production can also lead to tissue injury. Indeed, IL-1 β represents a major driver of placental inflammation and injury (Girard *et al.* 2010, Adams Waldorf *et al.* 2011, Leitner *et al.* 2014, Bergeron *et al.* 2016, Brien *et al.* 2017, Presicce *et al.* 2018); and placental-derived IL-1 β mediates fetal brain injury (Girard *et al.* 2010, Leitner *et al.* 2014). A number of studies have demonstrated that the placental trophoblast and the chorioamnion exhibit inflammasome

activity in response to infectious and non-infectious triggers; as do fetal membranes from women with labor, and this is further elevated with preterm labor, infection, and chorioamnionitis [reviewed in (Gomez-Lopez *et al.* 2019)]. Moreover, blocking the NLRP3 inflammasome in a mouse model reduced LPS-induced preterm birth (Faro *et al.* 2019). Together these studies highlight the importance of inflammasome function in both normal placental physiology and pathophysiology. However, little is known about this pathway in placental HBCs. Herein, we report that bacterial LPS provides the priming signal for the NLRP3 inflammasome in human HBCs, while ATP provides the activating signal leading to pyroptosis and the secretion of mature IL-1 β .

Studies in non-placental macrophages have established the ability of LPS to prime the NLRP3 inflammasome, while ATP triggers NLRP3 activation and subsequent inflammasome assembly and IL-1 β secretion (Mariathasan *et al.* 2006, Bauernfeind *et al.* 2009, Netea *et al.* 2009). We previously demonstrated that human HBCs have the ability to secrete high levels of IL-1 β (~1,000 – 3,000pg/ml) in response to a viral infection (Hendrix *et al.* 2020). In this current study we found that while LPS or ATP alone were not sufficient to trigger significant human HBC IL-1 β secretion, in combination there was an even more potent release of mature IL-1 β (~20,000 – 40,000pg/ml). Furthermore, LPS and ATP triggered IL-1 β secretion was released much quicker (20 – 120min) than after viral infection (24 hr) (Hendrix *et al.* 2020), suggesting that the mechanisms involved may be distinct. In a study using isolated CD14⁺ placental macrophages the saturated lipid and metabolic stressor palmitic acid also induced IL-1 β secretion but only after 4 – 24 hr, and this was associated with apoptotic cell death, again suggesting a distinct mechanism (Rogers *et al.* 2020).

To assess the role of the NLRP3 inflammasome in HBC responses to LPS and ATP, we examined the expression of NLRP3 and pro-IL-1 β as well as the activation of caspase-1, and the processing and secretion of IL-1 β . In keeping with previous studies in non-placental macrophages, we found that the priming signal LPS increased HBC expression of NLRP3 and pro-IL-1 β , while combination LPS and ATP induced caspase-1 activation, and IL-1 β processing. Caspase-1 activation, IL-1 β cleavage and IL-1 β secretion were all reduced by NLRP3 knockdown; inhibition of caspase-1; and blocking of P2X7, the receptor that mediates K⁺ efflux - an important contributor to inflammasome function (He *et al.* 2016). Together, this demonstrates that in human HBCs activation of the NLRP3 inflammasome mediates IL-1 β secretion in response to LPS and ATP. In another study, CD14⁺ placental macrophages treated with palmitic acid exhibited NLRP3 inflammasome assembly, and caspase-1 activation and this was associated with IL-1 β secretion (Rogers *et al.* 2020).

Since combination LPS and ATP triggered a rapid and very robust IL-1 β response by human HBCs, we investigated whether this was associated with NLRP3 inflammasome-mediated pyroptosis. Pyroptosis is a rapid form of cell death that contributes to IL-1 β release by forming pores on the inner lipid membrane (He *et al.* 2015, Shi *et al.* 2015, He *et al.* 2016). These pores are formed by the active N-terminal domain of GSDMD which is cleaved by caspase-1 in parallel to caspase-1-mediated IL-1 β processing (He *et al.* 2016). Thus, both IL-1 β and GSDMD cleavage can be triggered upon NLRP3 inflammasome activation. In our studies we found that following treatment with LPS and ATP, the majority

of HBCs exhibited cell death as evidenced by SYTOX Orange uptake, and this was associated with pronounced nuclear condensation and cellular swelling, in keeping with the morphological profile of pyroptosis (Jorgensen and Miao 2015). Moreover, following treatment with LPS and ATP, HBCs exhibited GSDMD cleavage and this was blocked by NLRP3 knockdown; by inhibition of caspase-1; and by inhibition of P₂X₇. Together, this indicates that NLRP3 inflammasome activation in HBCs leads to pyroptotic cell death and this facilitates IL-1 β secretion. While the vast majority of HBCs treated with LPS and ATP exhibited cell death, some remained intact, evidenced by SYTOX orange exclusion. A recent study reported that mature IL-1 β release by both live intact and pyroptotic cells can be mediated by GSDMD activation and subsequent pore formation, even in the absence of cell lysis (Evavold *et al.* 2018). Therefore, it is possible that in our system both intact and pyroptotic HBCs could have contributed to the secretion of IL-1 β , however, the large amount of cell death and very high levels of IL-1 β suggests that the dominant mechanism was through pyroptotic cell death.

In summary, our data indicate that LPS and ATP treatment stimulated NLRP3 inflammasome activation and pyroptosis in HBCs leading to the rapid release of IL-1 β . The localization of HBCs confers a unique ability to influence microbial-associated placental and fetal inflammation. Therefore, these studies suggest a key role for the inflammasome and pyroptosis in mediating HBC driven inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- LPS and ATP induce rapid secretion of high levels of IL-1 β by Hofbauer cells (HBC)
- HBC IL-1 β triggered by LPS and ATP is mediated by the NLRP3 inflammasome
- The NLRP3 inflammasome also triggers HBC pyroptotic cell death

Figure 1. Combination LPS and ATP induce a rapid secretion of IL-1 β by HBCs.

HBCs were treated with media alone (Ctrl), LPS alone, ATP alone, or combination LPS and ATP (LPS+ATP) for 20–120 min. Levels of secreted IL-1 β , quantitated in four independent experiments, were determined by ELISA. Results are presented as Mean $\pm$ SEM. * p <0.05: LPS+ATP vs LPS, ATP, and Ctrl; + p <0.05 LPS+ATP vs ATP, and Ctrl.

Figure 2. LPS and ATP treatment of HBCs promotes pyroptosis.

HBCs were treated with or without LPS or ATP, alone or in combination. After 40 min cells were stained with H33342 (Blue) and SYTOX® Orange (Pink). (A) Left hand panels show bright field (black and white) images of HBCs at 40x magnification; right hand panel shows the enlarged bright field image of HBCs treated with LPS+ATP. (B) Fluorescent microscopy of HBCs at 10x magnification. Results are representative of five independent experiments.

Figure 3. Modulation of the NLRP3 inflammasome and GSDMD in HBCs by LPS and ATP treatment.

HBCs were treated with media alone (Ctrl) or with combination LPS and ATP (LPS+ATP) either alone or with inhibitors to caspase-1 (WEHD or Vx-765) or P2X7 (KN-62). (A) After 40 min HBC protein was analyzed by Western blot ($n=4-5$) for expression of NLRP3; pro- and active-GSDMD; pro- and active-caspase-1; and pro- and active-IL-1 β . Blots are from a representative experiment. Charts show protein expression levels as determined by densitometry after normalization to HSP90. (B) After 40 min HBC supernatants were analyzed by ELISA for IL-1 β secretion ($n=5$). Data are either presented as Mean $\pm$ SEM for bar charts or are presented as medians and percentiles; the lines inside the box indicate the median, the ends of the box describe the lower and upper quartiles, and the whiskers define the smallest and largest values. * $p < 0.05$ relative to LPS+ATP.

Figure 4. The NLRP3 inflammasome mediates pyroptotic IL-1 β secretion by HBCs treated with LPS and ATP.

HBCs were treated with media alone (Ctrl), with LPS alone or with combination LPS and ATP (LPS+ATP) either alone or scrambled or NLRP3 siRNA. (A) After 40 min HBC protein was analyzed by Western blot for expression of NLRP3; pro- and active-GSDMD; pro- and active-caspase-1; and pro- and active-IL-1 β . Blots are from a representative experiment. Charts show protein expression levels as determined by densitometry after normalization to HSP90 (n=5). (B) After 40 min HBC supernatants were analyzed by ELISA for IL-1 β secretion (n=5). Data are either presented as Mean $\pm$ SEM for bar charts or are presented as medians and percentiles; the lines inside the box indicate the median, the ends of the box describe the lower and upper quartiles, and the whiskers define the smallest and largest values. * p <0.05 relative to scrambled siRNA control unless otherwise specified.