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NLRP3 Inflammasome Mediated Production of Th1/Th17 Cytokines in Response to Inflammatory Stimulants in Innate Immune Cells

Doğal Bağışıklık Hücrelerinden Enflamatuvar Uyaranlara Yanıt Olarak NLRP3 İnflamazom aracılı Th1/Th17 Sitokinlerinin Üretimi

Amrithavarshini K[1], Akshaya Keerthi SAIKUMAR[1], Manigandan V[1], Vishnu RAGHURAM[1], Shahana PARVEEN[1], Sugitharini V[1], Rohit SALUJA[2], Elden Berla THANGAM[1]

Abstract

Introduction: Monocytes play a major role in eliciting the immune response against infection. Also mast cells are found to play a critical role in mediating inflammatory immune response not only to allergic reaction but also to infection by inducing the production of inflammatory cytokines such as IL-1β, IL-6, IL-18 and IL-17. This study aims to investigate nod like receptor protein 3 (NLRP3) mediated production of Th1/Th17 cytokines in monocytes and mast cells.

Materials and Methods: Mononuclear cells from peripheral and cord blood were stimulated with lipopolysaccharide (LPS), peptidoglycan (PGN) whereas human mast cell line-1 (HMC-1) cells were stimulated with histamine and LPS to analyse the activation of NLRP3 inflammosome components such as NLRP3, procaspase-1(p45), caspase-1(p10) nuclear factor-κB (NF-κB) and extracellular signal regulated kinase (ERK). The release of cytokines such as IL-1β, IL-6, IL-18 and IL-17 was quantified.

Results: This study shows that stimulating the mononuclear and mast cells with LPS, PGN and LPS, histamine respectively induce the production of IL-1β, IL-6, IL-18 and IL-17 whereas the costimulation with gram positive and gram negative bacterial stimulants such as LPS and PGN showed synergistic response to cytokines whereas HMC-1 cells when stimulated with histamine produces IL-1β, IL-6 and IL-17 but when co-stimulated with LPS level of cytokines were reduced. It is mediated through activation of NLRP3 inflammosome. Also these cells utilized NFκB and ERK pathway.

Conclusion: The activation of NLRP3 inflammosome in innate immune cells leads to the production of IL-1β, IL-6, IL-18 and IL-17 cytokine. The signaling pathways NF-κB and ERK are found to have been involved in the activation of NLRP3 inflammosome complex during inflammatory conditions.

Keywords: NLRP3 Inflammasome, innate immune cells, inflammation

Öz

Giriş: Monositler, infeksiyona karşı gelişen bağışıklık yanıtında çok önemli bir rol oynar. Aynı zamanda, mast hücreleri de sadece allerjik reaksiyonlara değil aynı zamanda IL-1β, IL-6, IL-17 ve IL-18 gibi sitokinlerin üretilmesi ile infeksiyon bağışıklığında da kritik öneme sahiptir. Bu çalışmada düğüm benzeri reseptör proteini 3 (İng; 'nod like receptor protein 3;NLRP3)'e bağlı olarak monosit ve mast hücrelerinden salınan Th1/Th12 sitokinleri araştırılmıştır.

Gereç ve Yöntemler: Periferik kandan ve kordon kanından elde edilen mononükleer hücreler, lipopolisakkarid (LPS), peptidoglikan (PGN) ile uyarılır iken insan mast hücresi hattı-1 (HMC-1) hücreleri histamin ve LPS ile uyarılmış ve bu uyarılmanın NLRP3, prokaspaz-1(p45), kaspaz-1(p10), nükleer faktör κB(NF-κB) ve ERK gibi inflamazon bileşenlerinin salınımına olan etkisi irdelendi. IL-1β, IL-6, IL-17 ve IL-18 gibi sitokinlerin salınımı ölçüldü.

Bulgular: Bu çalışma, mononükleer hücreler ve mast hücrelerinin LPS, PGN, LPS ve uyarılması ile IL-1β, IL-6, IL-17 ve IL-18 uyarılır iken, bu hücrelerin LPS ve PGN gibi gram-negatif ve gram-pozitif bakterilerin uyarıya neden olan molekülleri ile birlikte uyarıldıklarından daha yüksek bir sinerjik uyarım gerçekleşti, HMC-1 hücreleri histamin ile uyarıldığında ise, bu hücreler IL-1β, IL-6 IL-17 üretti, ancak, ortama LPS eklendiğinde bu sitokinlerin üretimi azaldı. Bu azalma, NLRP3 inflamazomunun uyarılmasına bağlı idi. Bu üretim NFκB ve ERK yolağına bağlı idi.

Sonuç: oğal bağışıklık hücrelerinin NLRP3 inflamozomu ile bu hücrelerden IL-1β, IL-6, IL-17 ve IL-18'in salınımına yol açar. Bu NLRP inflamazom kompleksinin yangısal durumlarda uyarılmasından NF-κB ve ERK moleküllerinin yer aldığı yolak sorumludur.

Anahtar Kelimeler: NLRP3, inflamazom, doğal bağışıklık hücreleri, yangı

[1] Dept of Biotechnology, School of Bio-engineering, SRM Institute of Science and Technology, Kattankulathur, Chennai-603203, India

[2] Department of Biochemistry, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh-462024, India

Correspondence:
E. Berla THANGAM
Department of Biotechnology,
School of Bioengineering,
SRM Institute of Science and Technology ,
Kattankulathur – 603203,
Tamil Nadu, India.
E-mail: berlathagam.e@ktr.srmuniv.ac.in
Phone: +91 9444681340

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Introduction

Innate immune response against bacterial pathogens are mainly regulated by various cytokines and chemokines.[1] Accumulating evidence has documented the role of IL-18 and IL-1β during infections with a variety of pathogens.[1] Almost invariably, both cytokines were found to have a protective function.[1] Maturation of these cytokines are executed by multiprotein complex called nod like receptor protein 3 (NLRP3) inflammasome.[2] NLRP3 inflammasome consists of the sensor NLRP3, adaptor apoptosis associated speck like protein containing CARD (ASC) and effector procaspase-1.[3] The inflammasome complex assembles during tissue damage and infection.[4] Especially, it is activated during infection of macrophages with various bacterial, viral, and fungal pathogens and is required for host immune defense against these pathogens.[5,6,7] Expression of NLRP3 is induced by priming with microbial components such as TLR ligands or endogenous molecules such as tumor necrosis factor or IL-β, through the activation of NF-κB.[5] Next, it is activated by potassium efflux, lysosomal destabilization or mitochondrial dysfunction producing functional IL-1β and IL-18.[8] After the activation of the NLRP3 inflammasome complex, caspase-1 proteolytically cleaves the inactive forms of the cytokines IL-1β and IL-18 into their mature forms.[9] The pro forms of IL-1β and IL-18 are produced by the TLR mediated NF-κB and ERK signaling. Previous reports have shown that monocyte derived macrophages respond to the bacterial ligands LPS and peptidoglycan (PGN) and produce the pro-inflammatory cytokines IL-1β and IL-18 through NF-κB activation.[10]

It is also known that exogenous ATP and monosodium urate crystals further activate the NLRP3 inflammasome pathway through purigenic receptors and help in the secretion of IL-1β cytokine in human macrophages.[11] The complement C5a and NLRP3 inflammasome have a bidirectional crosstalk and the complement protein interactions are critical for enhancing the IL-1β secretion during inflammatory conditions.[12] Several studies show the activation of NLRP3 inflammasome in peripheral blood mononuclear cells against bacterial stimulants. Not much research have been carried out on the neonatal immune response involving NLRP3 inflammasome. Many aspects of the specific signaling events that lead to ligand recognition, inflammasome assembly, and finally to IL-1β/IL-18 release and cell death remain uncharacterized in neonatal immune cells. The monocytes play a major role in eliciting the immune response against infection and express a broad range of functions like immune response regulation, wound healing and repair etc.[13]

Mast cells are important effector cells of the innate immune system present at the strategic locations of the body, exposed to external environment such as skin, respiratory, gastro-intestinal tract and provide immune response against invading pathogens.[14] They have been extensively studied for their allergic inflammatory response.[15] Previous reports on mast cell lines have showed the production of IL-1β or IL-18 in response to IgE stimulation.[14] Moreover, Mast Cells contribute to inflammation via the release of IL-1β in an NLRP3, ASC, and caspase-1 dependent manner in skin inflammation, arthritis and multiple sclerosis.[14,16] However, reports on HMC-1 mediated NLRP3 activation has not been reported during bacterial infections.

Therefore, in this study we have studied the activation of NLRP3 inflammasome in mononuclear cells of peripheral blood and cord blood and HMC-1 cells, thus comparing the activation in adult and neonatal innate immune cells and mast cells during the allergic and inflammatory conditions. We have also focused on the involvement of NF-κB and ERK signaling pathways in the activation of NLRP3 inflammasome. This study may help us to understand the mechanism of action of NLRP3 inflammasome pathway in different cells and thereby devise potential therapeutic strategies in future.

Materials and Methods

Chemical and reagents

Penicillin-Streptomycin, IMDM and FBS were purchased from Gibco Invitrogen, Carlsbad, CA. Cryopyrin, Caspase-1, caspase-1 p10, Total NF-κBp65, phospho NF-κBp65, phospho ERK1/2, Goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP conjugates, β-actin antibodies were purchased from Santa Cruz Biotechnology, Dallas, TX. Total ERK 1/2 antibody was purchased from Cell Signaling, Burlington, NC. LPS and PGN were purchased from Invivogen, SanDiego. Histamine, and other basic chemicals were from Sigma, St. Louis, MO. ELISA Kits were purchased from R & D Systems, Minneapolis, MN. Clarity western ECL substrate was purchased from Bio-rad, USA.

Blood sample processing

Cord blood was collected from healthy volunteers without complicated vaginal delivery from SRM hospital, Kattankulathur. Blood was collected in tubes containing 38% sodium citrate as anti-coagulant and processed within 4 h. Peripheral blood was collected from healthy volunteers by venipuncture without any type of antibiotic use or any medical intervention. Informed consent was obtained from all mothers and healthy volunteers.

Isolation of mononuclear cells

PBMCs and CBMCs were isolated by density gradient centrifugation using lymphocyte separation Medium. Isolated cells were washed twice in phosphate buffered saline and resuspended in RPMI 1640 supplemented with 1% BSA and antibiotic solution. Cell viability was confirmed by trypan blue staining.

Culture of HMC-1 cell line

HMC-1 cells were kindly provided by Dr. Joseph H Butterfield, Mayo Clinic (Rochester, MN), and cultured in IMDM supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ ml) and streptomycin (100 ng/ml).

Activation of immune cells with respective ligands

The mononuclear cells were plated at a density of 10^6/ml and stimulated with 100 ng/ml each of LPS from E. coli and PGN from S. aureus. The HMC-1 cells were plated at a density of 10^6/ml and stimulated with 100 ng/ml of LPS from E. coli and 10μM of histamine. The supernatants were collected and stored in -20°C until further analysis.

Determination of various inflammatory cytokines and chemokines by ELISA

The levels of IL-6, IL-1β, IL-17 and IL-18 in the culture supernatants were measured by enzyme-linked immunosorbent assay. Experiments were performed according to manufacturer's instructions.

Western blotting

Mononuclear cells and mast cells (1×10^6 cells/ml) were lysed in RIPA buffer. Protein concentration was quantified by Bradford method and was separated by SDS–PAGE. Western Blotting was performed for with Rabbit anti cryopyrin antibody, rabbit anti procaspase-1 antibody, rabbit anti caspase-1 p10 antibody, total and phospho NF-κBp65 and ERK1/2 and probed with goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP secondary antibodies. Immunoreactive bands were visualized by ECL using multi imaging system (Cell Biosciences, USA).

Statistical analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data are expressed as mean ± SD. All the parameters were analyzed at 95% confidence intervals and p values of <0.05 was considered to be statistically significant. Statistical analysis of the data was performed using GraphPad Prism version 5.00, San Diego, CA.

Results

Production of IL-1 Family of cytokines in immune cells is mediated by NLRP3 inflammasome in response to different stimulants

NLRP3 inflammasome activation proteolytically cleaves the inactive cytokines to form active cytokine and release extracellularly. The amounts of IL-1β were higher in PBMCs than in CBMCs whereas IL-6 amounts were more in CBMC than in PBMCs (Figure 1). There was no significant difference in the levels of IL-18 between PBMCs and CBMCs (Figure 1). Furthermore, PBMCs and CBMCs co-stimulated with LPS and PGN showed a synergistic effect in the levels of Th1 response (Figure 1) IL-1β in PBMC and IL-6 in CBMC. No synergistic effects was found in the case of IL-18 in both cells and HMC-1 (Figure 1). Pro-inflammatory cytokines were upregulated in histamine treated HMC-1 cells and downregulated by LPS (Figure 1).

IL-1β, IL-6 and IL-18 production was measured using ELISA after 24 hr of stimulation with LPS (100 ng/ml), PGN (100 ng/ml) and LPS+PGN (100 ng/ml) in PBMCs and CBMCs. HMC-1 cells were stimulated for 24 hr with histamine (10 μM) , LPS (100 ng/ml) and histamine(10 μM) + LPS (100 ng/ml).

Mononuclear cells when stimulated with bacterial ligands LPS, PGN and co-stimulation with LPS and PGN produced higher amount of IL-17 in PBMCs than in CBMCs (Figure 2). Furthermore, PBMCs co-stimulated with LPS and PGN showed a synergistic effect in the level of IL-17. Production of IL-17 was upregulated in HMC-1 in response to histamine whereas when co-simulated with histamine and LPS the level of IL-17 was reduced compared to histamine (Figure 2).

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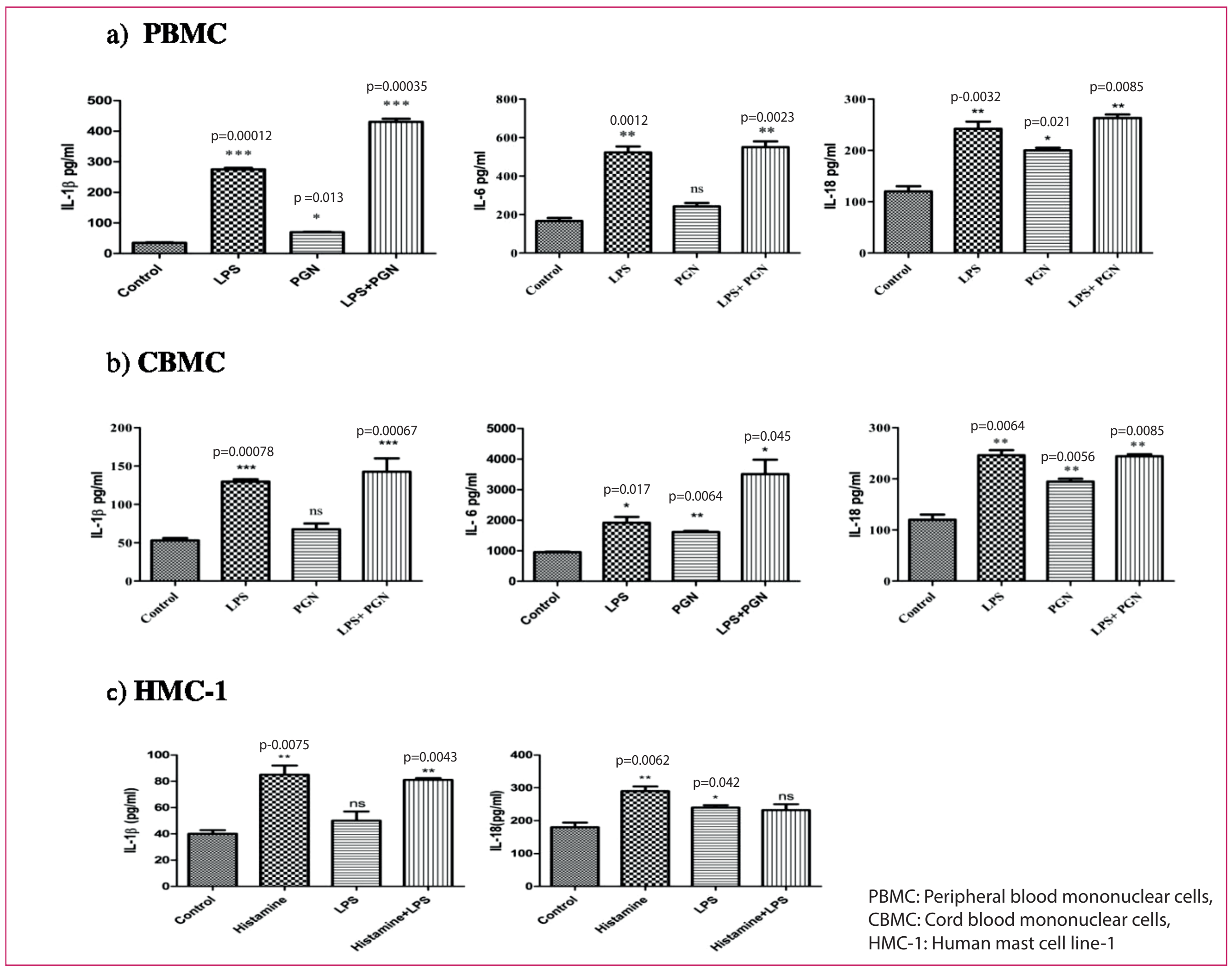

Figure 1. Measurement of IL-1β, IL-6 and IL-18 levels upon co-stimulation with LPS and PGN in **a)** PBMCs, **b)** CBMCs, and histamine and LPS in **c)** HMC-1. IL-1β, IL-6 and IL-18 production was measured using ELISA after 24 hr of stimulation with LPS (100 ng/ml), PGN (100 ng/ml) and LPS+PGN (100 ng/ml) in PBMCs and CBMCs. HMC-1 cells were stimulated for 24 hr with histamine (10.M) , LPS (100 ng/ml) and histamine (10.M) + LPS (100 ng/ml). Data expressed as mean ± SD of three experiments done in duplicates. ns: not significant.

Activation of NLPR3 inflammosome signaling components with various stimulants in immune cells

We stimulated mononuclear cells with LPS and PGN and determined the protein expression of NLRP3, procaspase-1(p45) and caspase-1 (p10) by western blot (Figure 3). From the blot, it is observed that the NLRP3 and caspase-1 protein expression is upregulated and procaspase-1 (p45) expression is down-regulated indicating its conversion to active caspase-1 (Figure 3). We also stimulated HMC-1 cells with histamine (10 μM) and LPS (100 ng/ml) and the expression of NLRP3, procaspase-1 and caspase-1 were determined. Expression of NLRP3 and caspase-1 was found to be up-regulated in histamine treated HMC-1 cells and down-regulated in LPS and co-stimulation with histamine and LPS (Figure 3).

Involvement of ERK and NF-κB signaling pathways in NLRP3 Inflammasome activation

We stimulated PBMC with LPS and PGN (100 ng/ml) and HMC-1 cells with histamine (10 μM) and LPS (100 ng/ml) for 30 mins for ERK and 2hrs for NF-κB and their expression were determined (Figure 4). It was observed that the expression of ERK and NF-κB were increased in LPS and co-stimulation of LPS+PGN in PBMCs and where as in HMC-1 cells the expression was prominent when stimulated with histamine (Figure 4). During co-stimulation with histamine and LPS, the expression of ERK and NF-κB is down-regulated (Figure 4).

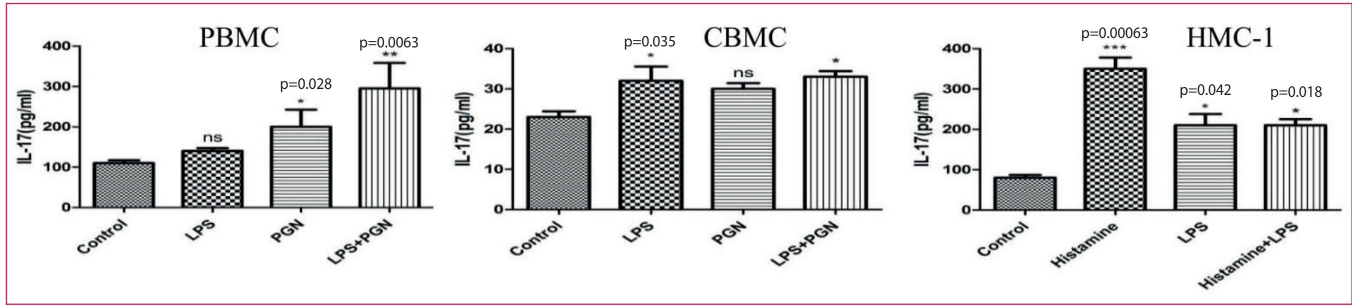

Figure 2. Analysis of IL-17 production upon co-stimulation with LPS and PGN in mononuclear cells and histamine and LPS in HMC-1 cells. IL-17 production using ELISA after 24 hr of stimulation with LPS (100 ng/ml), PGN (100 ng/ml) and LPS + PGN (100 ng/ml) in mononuclear cells and stimulation with histamine (10.M), LPS (100 ng/ml) and histamine (10.M) + LPS (100 ng/ml) in HMC-1 cells. Data expressed as mean ± SD of three experiments done in duplicates. ns: not significant.

a) **PBMC**

Caspase-1 (≅10kDa)
Pro-caspase-1 (≅45kDa)
NLRP3 (≅109kDa)
β-actin (≅42kDa)
Control LPS PGN LPS+PGN

% NLRP3 activation
p=0.0056
**
ns
p=0.0093
**
Control LPS PGN LPS+PGN

Relative caspase-1/ procaspase-1
p=0.0061
**
p=0.043
*
p=0.0067
**
Control LPS PGN LPS+PGN

b) **CBMC**

Caspase-1 (≅10kDa)
Pro-caspase-1 (≅45kDa)
NLRP3 (≅109kDa)
β-actin (≅42kDa)
Control LPS PGN LPS+PGN

% NLRP3 activation
0.017
*
0.043
*
p=0.0085
**
Control LPS PGN LPS+PGN

Relative caspase-1/ procaspase-1
p=0.0056
**
p=0.031
*
p=0.0047
**
Control LPS PGN LPS+PGN

c) **HMC-1**

Caspase-1 (≅10kDa)
Pro-caspase-1 (≅45kDa)
NLRP3 (≅109kDa)
β-actin (≅42kDa)
Control His LPS His +LPS

% NLRP3 activation
p=0.00096
***
ns
0.037
*
Control Histamine LPS Histamine+LPS

Relative caspase-1/ procaspase-1
0.023
*
ns
p=0.031
ns
Control Histamine LPS Histamine+LPS

Figure 3. Analysis of inflammosome signaling components in **a)** PBMCs **b)** CBMCs and **c)** HMC-1 by Immunoblotting. Mononuclear cells (1x106) were stimulated with LPS (100ng/ml), PGN (100 ng/ml) and LPS+PGN (100 ng/ml) and HMC-1 cells (1x106) were stimulated with histamine (10.M), LPS (100 ng/ml) and histamine (10.M) + LPS (100 ng/ml) cells for 24 hours and lysed. Cell lysates and supernatant were separated on SDS-PAGE and blots were probed with anti cryopyrin, anti procaspase-1, anti caspase-1 and anti-β-actin. Immunoreactive bands were visualized by ECL substrate. Bar graph represents a summary of the expression of NLRP3 and relative caspase-1/procaspase-1 expression. Data expressed as mean ± SD of three experiments. ns: not significant.

Discussion

Previously we reported the signaling molecules that are involved in the production of inflammatory cytokines through TLR2 and TLR4 activation in neonates.[17] Activation of the inflammasome leads to the activationof caspase1, followed by its self-cleavage from the inactive

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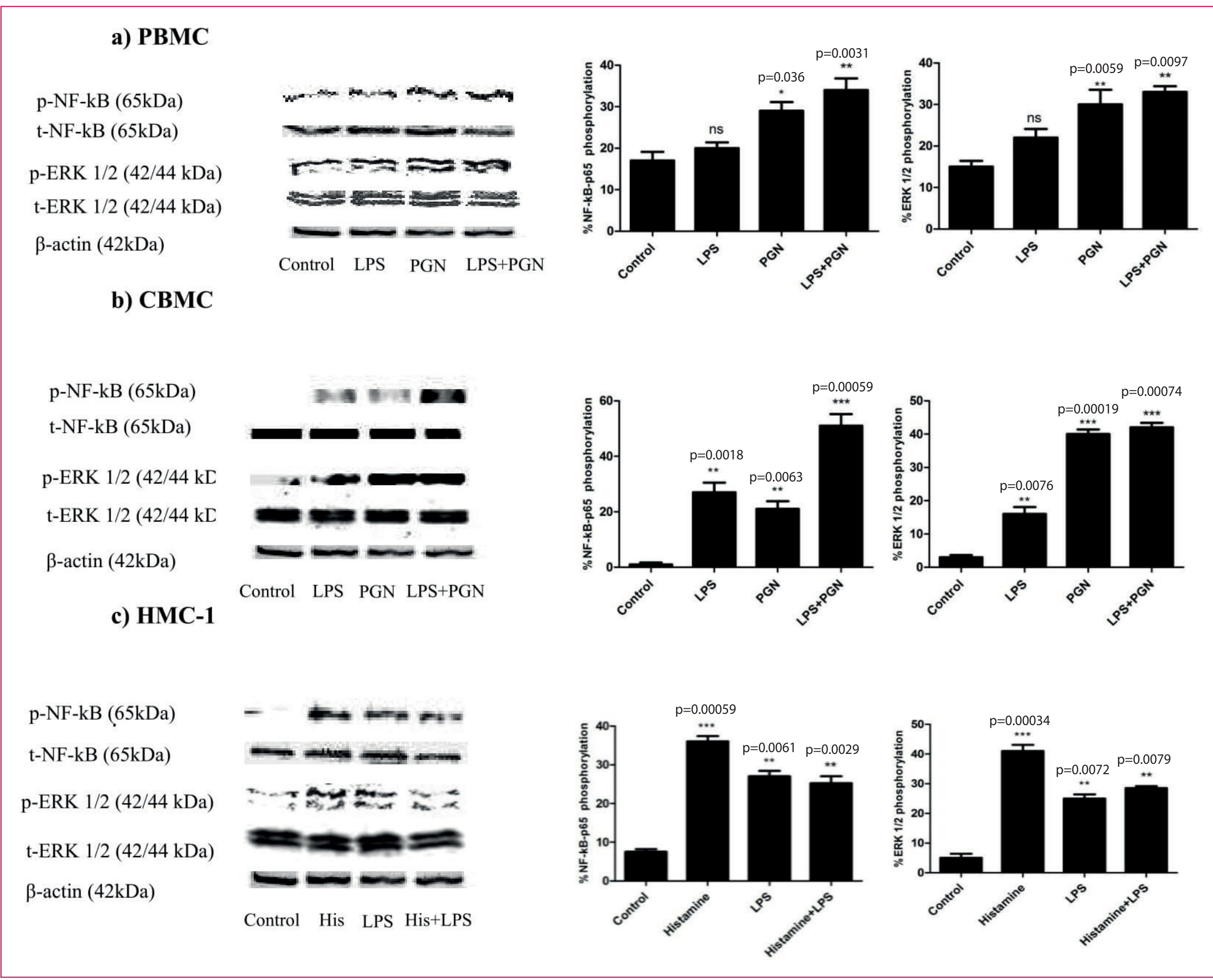

Figure 4. Activation of NFκB and ERK upon co-stimulation with LPS and PGN in **a)** PBMC, **b)** CBMC and histamine and LPS on **c)** HMC-1 by Immunoblotting. Mononuclear cells were stimulated with LPS (100ng/ml), PGN (100 ng/ml) and LPS+PGN (100 ng/ml) and HMC-1 cells were stimulated with histamine (10.M), LPS (100 ng/ml) and histamine (10.M) + LPS (100 ng/ml) cells for 2 hours and . hour respectively. Cell lysates were separated on SDS-PAGE and blots were probed with anti-phospho-NFκB p65, anti-phospho ERK1/2 and anti-β-actin. Immunoreactive bands were visualized by ECL substrate. Bands were quantified by densitometry and expressed as a percentage. Data expressed as mean ± SD of three experiments. ns: not significant.

precursor procaspase-1 to the active form.[18] Though the TLR/MyD88/IRAK4 pathway is defective in neonates, neonatal immune cells are capable of producing an immune response similar to that of adult immune cells.[13,19] Triggering of TLRs on monocytes by microbial products activates common mediator NF-κB leading to cytokine production. We investigated the NLRP3 and caspase activation that plays an important role in IL-1β and IL-18 production in neonates. Consistent with current reports, we found a significant increase in IL-1β and IL-18 production upon LPS stimulation in both peripheral and cord blood mononuclear cells. It also has been reported that the amount of IL-1β produced in PBMCs is more when compared to CBMCs, which could be attributed to the distinct difference in ability to produce TLR mediated inflammatory cytokines between neonates and adults.[20,21] Another finding demonstrated that although IL-1β was found to be less in production, it quickly reaches adult level at about 2 weeks of age. This may be due to the high production of placental derived prostaglandins that play an important role in suppressing IL-1β responses during birth.[22]

The cytokine IL-1β produced as a result of Th1 response induces the differentiation of Th-17 response which produces the pro-inflammatory cytokine IL-17.[23] We

found a significant increase in IL-17 production in both CBMCs and PBMCs upon LPS and PGN stimulation. Similar to previous reports neonatal cord blood was found to demonstrate that TLR-induced cytokine responses were significantly different from adult TLR responses. One study suggests that a continuous cross-talk between innate and adaptive immunity as such may contribute to the development and maintenance of chronic inflammation in pathogenic conditions.[24]

It has reported previously that human mast cell activation by histamine leads to the production of various inflammatory cytokines.[25] Pro-inflammatory cytokines derived from mast cells play an important role in the acute and late phase allergic reactions.[13,26] Histamine induced a significant production of IL-1β, IL-18 and IL-17 in HMC-1 cells compared to control. During co-stimulation, the endotoxin LPS lowers the allergic reaction. Studies have shown that exposure to endotoxin suppresses the allergic reaction.[27,28] However, it was also reported that the amount of LPS and TLR4 signals encountered can determine whether Th1 or Th2 types of inflammatory responses will be generated. Therefore, LPS can either lead to susceptibility of allergic asthma or exacerbate the severity of allergy or protect from allergic conditions, which is possibly dictated by the timing and level of exposure to the endotoxin.

Monocytes have constitutively activated caspase-1 as they require only a single stimulus for the activation and secretion of IL-1β.[29] Consistent with this report, we found a decrease in the level of expression of inactive caspase1 subunit p45 in LPS, PGN and co-stimulated CMBCs and PBMCs, indicating the cleavage and activation of caspase1. We also found an increased amount of caspase1 protein in PBMCs as compared to CBMCs. One can only speculate that this difference is due to an immaturity in the development of the NLRP3 inflammasome pathway. Studies suggests that preterm labor associated with infection is also associated with a high concentration of caspase-1, suggesting that infection may induce caspase-1 production and activation of the inflammosome.[30] The level of expression of inactive caspase-1 subunit p45 in histamine stimulated HMC-1 cells were decreased in HMC-1 cells, whereas LPS and co-stimulation of histamine and LPS showed increased levels of inactive caspase-1.

Previous reports suggest that NLRP3 is activated by both Gram positive and Gram negative bacterial ligands. Also, LPS/ATP was utilized to induce the activation of the NLRP3 inflammasome in RAW264.7 cells.[31] Consistent with these reports we found that NLRP3 increases on stimulation with LPS, PGN and LPS and PGN and increases gradually with the highest concentration in co-stimulated cells. It was reported that the ability of CBMCs to secrete IL-1β is dependent on a high CD14-expressing monocyte subset producing pro-1L-1β but characterized by impaired release of IL-1β attributed to low expression of NLRP3;P2X7 and pro- caspase-1 expression when compared to adult levels due to gestational age-dependent immaturity of signal transduction pathways.[32,33] In accordance with previous report we also found low expression of NLRP3, caspase-1 and IL-1β production in neonatal cells when compared to adult cells.

In conclusion, the inflammasome has emerged as a central signaling complex driving the maturation of pro-inflammatory cytokines and regulating pyroptotic cell death. In addition to being important for pathogen recognition, the high incidence of hereditary and acquired diseases, in which inflammasome activity is deregulated, highlights the importance of this signaling platform for human health. We found a distinct difference in activation of the NLRP3 inflammasome pathway between neonatal and adult mononuclear cells and mast cells, complemented by a difference in IL-1β production. Further studies are required to determine the potential of this pathway being a target for inhibition of IL-1β induced inflammatory diseases which could also provide new targets for the development of therapeutic strategies. This may contribute to the development of interventions to control bacterial infection and allergy and also provide protective innate immunity to the host.

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Author Contributions: Study design/planning: BT, RS; Data collection/entry: AK, AK, MV, VR, SP; Data Analysis/statistics: AK, AK, MV, VR, SP, SV; Data Interpretation: BT; Manuscript preparation: AK, AK, MV, VR, SP, SV; Literature search/analysis: AK, AK, MV, VR, SP, SV.

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