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TLR2 and TLR4 co-activation utilizes distinct signaling pathways for the production of Th1/Th2/Th17 cytokines in neonatal immune cells

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ABSTRACT

Co-activation of TLR2 and TLR4 by gram negative and gram positive bacterial ligands induces a robust pro-inflammatory response in inflammatory cells. In order to understand the signaling mechanism, we aimed to delineate the signaling molecules involved in TLR2 and TLR4 co-activation in neonatal immune cells for the production of Th1/Th2/Th17 inflammatory cytokines. For this, we pretreated cord blood and peripheral blood mononuclear and human mast cells with specific signaling molecule inhibitors such as BAY117082, PD98059 and LY294002 and then stimulated with LPS and PGN and assayed for cytokines IL-6, IL-12/IL-23p40 (Th1), IL-13 (Th2), IL-23 (Th17) and RANTES secretion. We found that upon co-stimulation the phosphorylation of NFκBp65, ERK1/2 and Akt was found to be higher than when stimulated with individual ligands in CBMCs. Also, when compared to adult cells, neonatal cells were more potent in the activation of ERK and Akt through TLR2 and TLR4 co-activation. In addition, neonatal cells possess similar capacity to activate NFκB as that of adult cells for IL-6 secretion. Furthermore, all three signaling molecules were found to be involved in the production of Th17 cytokines which is detrimental during inflammation induced by infection in neonates whereas NFκB is mainly involved in the induction of pro-inflammatory response and Th2 cytokines production. In conclusion, different signaling molecules were utilized for the production of different cytokines in immune cells.

1. Introduction

Toll-like receptors (TLRs) represents one of the major components of the innate immune system in sensing and responding to various Pathogen Associated Molecular Patterns (PAMPs). Upon engagement with LPS and PGN, TLR4 and/or TLR2 respectively recruit the adaptor proteins myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) [1,2]. In turn, these adaptor molecules activate TLR-mediated downstream signal transduction pathways NF-κB and mitogen-activated protein kinases (MAPKs) including p38, c-Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2), which ultimately leads to the production of various inflammatory mediators including cytokines (TNFα, IL-1β, IL-6, IL-8, and IL-12), macrophage inflammatory proteins (MIP1α and MIP1β), and chemokines (RANTES and MCP-1) [3].

Among the downstream signaling molecules, NF-κB has been reported to play an important role in the regulation of many cytokines and pro-inflammatory mediators essential to the host, and previous studies have reported that ERK1/2 mediates the transcription of various proteases and cytokines in response to various stimuli [4]. The phosphatidylinositol 3-kinase (PI3K)/Akt kinase signaling axis was found to play an important role in cellular processes such as cytoskeletal rearrangement and migration as well as survival and proliferation during bacterial infection [5,6]. One of the most striking differences in cell signaling downstream of TLR2 and TLR4 is the activation of IRF-3 pathway by TLR4 stimulation [7,8].

Furthermore, in response to various stimuli, including most TLR stimuli, human CBMCs tested *in vitro* possess a unique polarization, with impaired production of T_H1-polarizing cytokines including TNF-α, IFN-γ, and IL-12p70 and increased expression of T_H2/T_H17 and anti-inflammatory cytokines such as IL-6, IL-10, IL-17, and IL-23 [9,10]. T_H2-type responses are typically characterized by increases in the levels of IL-4, IL-5, IL-9, IL-13 and IL-21

Abbreviations: IL, interleukin; PBMC, peripheral blood mononuclear cells; CBMC, cord blood mononuclear cells; HMC-1, human mast cells.

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[11]. Also, IL-12 is required for Th1 cell differentiation, whereas IL-23 plays an important role in the survival and multiplication of Th17 cells [12–15].

In addition, the prevalence of polymicrobial infections is found to be around 5–27% among bloodstream infections in adult or pediatric intensive care unit [16–20]. Activation of downstream signaling molecules represents an important step in the activation and progression of the inflammatory host response to co-stimulation of two or more bacterial ligands. Due to the usage of multiple signaling pathways, co-activation of two or more TLR receptors leads to their synergistic actions for the production of various inflammatory mediators that may likely contribute to the development of multiple organ damage [21–25].

Furthermore, the importance of mast cells in the host immune response against bacteria and viruses is increasingly being recognized [56,57]. Human mast cells have been known to express TLR2 and TLR4 receptors on their surface with TLR2 mediated immune response being more elucidated [58–64]. Studies on the inflammatory response to TLR2 and TLR4 report that mast cells release a wide range of inflammatory mediators on stimulation with PGN such as IL-4, IL-5 and IL-13 in TNF-α [65]. This is in contrast to monocytes and macrophages where LPS mediates a higher inflammatory response than PGN [75]. Also, in human cord-blood derived mast cells, stimulation with PGN caused GM-CSF and IL-1β production whereas LPS did not [66,67]. Moreover, TLR4 signaling is known to occur in the cell membrane via the MyD88 dependent pathway whereas internalization of the TLR4 complex occurs in the TRIF-dependent pathway. This internalization of TLR4 does not occur in mast cells [59,68,69]. Although mast cell cytokine production following TLR recognition has been well studied [70–74], much is still not known about TLR signaling and functions during bacterial infection in mast cells.

Previously, we demonstrated that co-stimulation of TLR2 and TLR4 by gram negative and gram positive bacterial ligands induced a robust pro-inflammatory response in neonatal immune cells [26]. To further understand the signaling mechanism, we aimed to delineate the signaling molecules involved in TLR2 and TLR4 co-activation in immune cells for the production of Th1/Th2/Th17 inflammatory cytokines.

2. Materials and methods

2.1. Processing of blood samples

Umbilical cord blood was collected at SRM hospital, Kattankulathur from healthy volunteers without complicated vaginal delivery. It was collected in sample collection tubes containing 38% Sodium citrate that acts as an anti-coagulant. Peripheral blood was collected by vein puncture from age-matched healthy non-pregnant volunteers without any antibiotic use or any medical procedure. Informed consent was obtained from all mothers and healthy volunteers. Blood was used within 4 h of collection for further experiments to prevent cell loss.

2.2. Isolation of mononuclear cells and HMC-1 cell lines

Cord blood and Peripheral blood were processed further by isolating mononuclear cells from them using density gradient centrifugation (Lymphocyte separation Medium LSM (HiSep)). These cells were washed twice in phosphate buffered saline and then resuspended in RPMI 1640 supplemented with 1% BSA and antibiotic solution. Trypan blue staining was used to confirm cell viability.

HMC-1 cells (Provided by Dr. Joseph H Butterfield, Mayo Clinic, USA) were cultured in IMDM supplemented with 10% FBS, glutamine (2 mM), penicillin (100 IU/ml) streptomycin (100 ng/ml) and 5% CO_2 at 37 °C.

2.3. Activation of signaling molecules and Western Blotting

To determine the role of TLR2 and TLR4 in the downstream signal transduction cascade, the mononuclear cells and HMC-1 (1×10^6) were stimulated with LPS *Escherichia coli* (100 ng/ml), PGN *Staphylococcus aureus* (100 ng/ml) and LPS + PGN (100 ng/ml) for 30 min ((p-ERK1/2 and p-Akt antibody) and 1 h (p-NFκB) (Cell Signaling)). The immune cells (1×10^6 cells/ml) were then lysed in RIPA buffer and protein concentration was determined by using Bradford method. Next, the protein samples were separated by 10% SDS-PAGE and electro blotted onto nitrocellulose membrane. The membrane was incubated overnight with primary antibodies (p-ERK1/2, p-Akt, p-NFκBp65) at 4 °C. It was then blocked with 5% skim milk in Wash buffer (PBS-Tween 20) for 1 h at room temperature and then incubated with goat anti-rabbit-IgG-HRP (ERK and Akt) and goat anti-mouse HRP (NFκB) (Santa Cruz) for 2 h. The bands were visualized by SuperSignal West Pico chemiluminescent substrate. Respective blots were stripped and reprobed with anti-NFκBp65, anti-ERK or anti-Akt antibody (Cell Signaling). After washing the membrane in PBS-tween-20 three times, the blot was incubated with goat anti-mouse HRP secondary antibody for (anti-NFκB) and goat anti-rabbit HRP secondary antibody (anti-ERK or anti-Akt) for 2 h and then visualized by ECL method using multi imaging system (cell biosciences).

2.4. Measurements of cytokines using ELISA

Mononuclear cells and HMC-1 cells (1×10^6 cells/ml) were pre-treated with PD98059 (30 μM/ml), LY294002 (20 μM/ml) and Bay 117082 (30 μM/ml) for 1 h and then stimulated with LPS (100 ng/ml), PGN (100 ng/ml) and LPS + PGN (100 ng/ml) for 24 h. The levels of cytokines in the cell culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA): IL-6, IL-12/IL-23p40, IL-13, IL-23, RANTES (R&D systems). The experiments were performed according to manufacturer's instructions.

2.5. Calcium measurements and Degranulation assay

HMC-1 cells containing 2×10^5 cells/well were plated on black flat-bottom 96-well plates and then labeled with FLUO-4 performed according to manufacturer's instructions. The Intracellular Ca^{2+} release measurement was done in a multimode reader. Fluorescence was measured at excitation, 494 nm, emission, 516 nm every 3 s for 600 s and at the 100th second, LPS (100 ng/ml), PGN (100 ng/ml) and LPS + PGN (100 ng/ml) were added to the respective wells.

HMC-1 cells containing 5×10^4 cells/well were seeded into 96-well plates to a volume of 50 μl of SIR buffer containing 0.1% BSA and stimulated with LPS (100 ng/ml), PGN (100 ng/ml) and LPS + PGN (100 ng/ml). Control cells were lysed in 50 μl of 0.1% Triton X-100 to measure the total amount of β-hexosaminidase release. 15 μl of supernatants or cell lysates were incubated with 15 μl of 1 mM *p*-nitro phenyl-*N*-acetyl-β-D-glucosamine for 1 h at 37 °C. 250 μl of 0.1 M Na_2CO_3/0.1 M $NaHCO_3$ buffer was added to stop the reaction. The absorbance was measured at 405 nm.

2.6. Statistical analysis

Statistical analysis of the data was analyzed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California, USA. The difference in estimated parameters between the stimulant and inhibitor were determined by student *t* test.