Human newborn polymorphonuclear neutrophils exhibit decreased levels of MyD88 and attenuated p38 phosphorylation in response to lipopolysaccharide

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Abstract

Purpose: Human newborn infants have increased susceptibility to gram-negative bacterial infection. Since lipopolysaccharide (LPS) primes polymorphonuclear neutrophils (PMN) to enhance host defense functions, we investigated its effect on adult and newborn PMN *in vitro*.

Methods: PMN were isolated from blood of healthy adults and umbilical cords of full term newborns using dextran and Ficoll-Paque gradient sedimentation. Gel electrophoresis and Western blotting of membranes were used to probe for Mitogen-Activated Protein (MAP) kinase p38 phosphorylation, Toll-like Receptor-4 (TLR-4) and Myeloid Differentiation Factor 88 (MyD88) on isolated PMN membranes using specific antibodies. LPS induced degranulation was assessed using CD66 expression on PMN measured by flow cytometry.

Results: We show that p38 phosphorylation in newborn PMN is attenuated in response to LPS stimulation even though adult and newborn PMN have similar amounts of p38 protein. The degree of attenuation in newborn PMN is dependent on the osmolarity of the medium. In addition,

LPS-induced degranulation, a process that is p38 dependent, was also absent in newborn PMN. Although the LPS receptor TLR-4 is present at similar levels on newborn and adult PMN, its downstream adaptor protein MyD88 was significantly diminished in newborn PMN compared to adult cells.

Conclusions: Although the mechanism of PMN priming by LPS is not fully understood, our results suggest that MyD88 and p38 phosphorylation are important pathways in the process and contribute to attenuated response of newborn PMN to LPS *in vitro*.

Bacterial sepsis is a leading cause of morbidity and mortality in newborns. This increased risk is attributed to the immaturity of their host defense systems, including both humoral and cellular factors.¹⁻³ Indeed, the risk of bacterial infection in newborns is similar to that seen in adults with abnormal numbers of polymorphonuclear leukocytes (PMN), suggesting possible deficiencies in the functional activity of PMN

of newborns.⁴ When compared with adult cells, newborn PMN exhibit decrease functional activity, including adhesion, bacterial clearance and response to lipopolysaccharide (LPS). For instance, LPS-induced CD14 upregulation and priming of the oxidative burst are diminished in newborn compared with adult PMN.⁵⁻¹⁰ We hypothesize that the decreased functional responses of newborn PMN to LPS may be due to differences in their signaling pathways compared to those of adults.

Although the mechanisms involved in LPS signaling by PMN are not fully understood, it is possible that Toll-like Receptor-4 (TLR-4) and Myeloid Differentiation Factor 88 (MyD88) and Mitogen-Activated Protein (MAP) kinases downstream from these membrane receptors play an important role as with other cells.¹¹⁻¹⁶ For example, we showed that when Extracellular signal Regulated Kinase 1 (ERK1), ERK2 and p38 are inhibited, the effects of LPS on priming of PMN respiratory burst are attenuated.^{17, 18} However, we did not find that LPS-induced activation of ERK1/2 differed between adult and newborn PMN¹⁸, although subtle differences in subcellular distribution and phosphorylation of both ERK1/2 and p38 were noted.¹⁹

Activation of the MAP kinase p38 is associated with several functional responses of PMN, including up-regulation of β_2 integrins, degranulation, adhesion and respiratory burst.^{17, 20-23} There is growing evidence that p38 and MyD88 play important roles in cell activation during infection.^{20, 24} However, the precise role of p38 and its upstream mediator MyD88 and the extent to which they may contribute to deficiencies in human newborn PMN are unknown.

Materials and Methods

The Research Ethics Board of the IWK Health Centre approved the procedures for collecting blood from newborn infant umbilical cords and adult volunteers.

Reagents: Pyrogen-free reagents and plastics were used in all cell preparations. *Escherichia coli* LPS (Serotype 0111:B4) was obtained from List Biological Laboratories Inc. (Campbell, CA). Rabbit IgG anti-phospho-p38 antibody and HRP-conjugated goat

anti-rabbit (IgG) antibody were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-MyD88 and anti-TLR-4 antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Mouse IgG1 anti-human CD66b monoclonal antibody was purchased from Beckman Coulter (Mississauga, ON). Affinity-purified mouse IgG1 isotype control antibody was acquired from eBioscience (San Diego, CA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and IgM secondary antibody was purchased from Bio/Can Scientific (Mississauga, ON). Percoll, ECL Western blotting reagents and nitrocellulose membranes were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC Canada). Red blood cell lysing solution and paraformaldehyde were purchased from Becton Dickinson (Mountain View, CA) and from Cedarlane[®] Laboratories Limited, respectively. Dithiothreitol (DTT), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, sodium bicarbonate solution, and Hanks' balanced salt solution (HBBS) were obtained from GIBCO/BRL (Burlington, ON). E-Toxate kits, 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), formyl-Met-Leu-Phe (fMLP), rabbit anti- β -actin antibody and all other chemicals were purchased from Sigma Chemicals (Oakville, ON). Buffers and plasma were checked for endotoxin by a limulus amoebocyte lysate assay to ensure that LPS was not detectable (limit of detection <12 pg/ml).

PMN isolation and incubation: Blood was collected from peripheral veins of healthy adult volunteers and from umbilical cords of healthy full term newborns delivered by caesarean section as described previously.¹⁸ In some experiments, contaminating RBC (red blood cells) were lysed by briefly resuspending the cells in 3 ml of hypotonic NaCl (0.2%) for 1 min before reconstituting with 7 ml of 1.2% NaCl. Lysis with hypotonic solution was omitted in other experiments to eliminate its effect on altering cell responses. In order to avoid the hypotonic activation of p38, we prolonged the dextran sedimentation to 1 hr and omitted hypotonic lysis to remove RBCs. The small numbers of remaining RBC in the suspension had no effect on the observed PMN activation. In

these experiments, sample equalization was achieved by loading equal numbers of PMN (usually, 0.75×10^6 cells/sample).

Before the beginning of the experiments, PMN were washed and suspended in H-medium supplemented with 1% autologous plasma at a density of $1 \times 10^7/\text{ml}$.¹⁸ The H-medium used was isotonic (127 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 0.8 mM CaCl_2 , 5 mM glucose and 10 mM HEPES, pH 7.4). In some experiments, the H-medium was made slightly hypertonic (320 mOsm) by adjusting the concentration of NaCl (to 146 mM). PMN were incubated in the presence or absence of 5 ng LPS/ml for 30 min at 37°C, unless otherwise indicated.

After incubation, PMN were placed in an ice bath and cold HBSS was added to quench functional activity. PMN were collected by centrifugation ($9,000 \times g$) for 10 seconds and lysed on ice with cold Triton buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 100 μM sodium orthovanadate, 2 mM diisopropylfluorophosphate, 5 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ pepstatin A and 1 mM phenylmethylsulfonylfluoride). PMN lysates were cleared by centrifugation at $12,000 \times g$ for 10 min and stored at -70°C.

Gel electrophoresis and Western blotting: PMN lysates were mixed with the same volume of 2X SDS reducing buffer, separated on 10% SDS-PAGE, and transferred to nitrocellulose membrane. Membranes were incubated with antibodies specific for phospho-p38 (detects dually phosphorylated p38), TLR-4 or MyD88 overnight and the rest of the procedure was performed as previously described.¹⁸ To confirm the equality of protein loading, membranes were stripped and re-probed with antibodies specific for p38 or β -actin.

In all experiments, equal amounts of protein or cell numbers from adult and newborn PMN lysates were loaded, with paired adult and newborn samples loaded on the same gel. Results were calculated as the densitometric ratio of phospho-p38 to its corresponding protein band, or the ratio of LPS-induced vs unstimulated phospho-p38.

Flow cytometry: Leukocyte rich plasma was incubated with 50 ng LPS/ml for 30 min at 37°C. Cells were washed in PBS, followed by IF buffer (1% BSA, 0.2% sodium azide in PBS), and then labeled for 1 hr with either mouse anti-human CD66b monoclonal antibody (IgG1) or IgG1 isotype control. Cells were washed, lysed and fixed as described previously.¹⁷ Samples were analyzed using FCS Express, after gating on the PMN population.

Statistical analysis: The results were analyzed by two-way ANOVA, using Instat Graphpad software. Statistical significance was defined as $P \leq 0.05$.

Results

Cell treatment alters p38 activation: The effects of a hypotonic lysis procedure commonly employed to remove RBC during PMN isolation on p38 activity were examined. Short (about 1 min) hypotonic treatment caused spontaneous activation of p38, as measured by increased phosphorylation of the protein, in both adult and newborn PMN. (Figure 1A). This effect was long lasting, persisting with subsequent incubation in isotonic buffer for at least 1.5 hr. When hypotonically-treated cells were stimulated with LPS (5 ng/ml for 30 min), the phosphorylation of p38 was further increased in adult PMN but not in newborn PMN (Figure 1B). Given these observations, we avoided exposing cells to hypotonic conditions in our subsequent experiments.

When incubated in an isotonic (280 mOsm) H-medium, PMN from both adults and newborns showed increased p38 phosphorylation upon LPS stimulation (Figure 2A). However, the increase in p38 phosphorylation after LPS stimulation in newborn PMN was lower than that of adult cells (Figure 2B).

Attenuated p38 activation in newborn PMN: Phosphorylation of p38 in newborn and adult PMN in response to LPS and fMLP under a higher tonic (320 mOsm) concentration were compared. The concentrations used in vitro are similar to those which may be encountered during shock and sepsis management.^{25,26}

The activation of p38 in adult PMN by LPS (5 ng/ml, 30 min) or fMLP (1 μM , 1 min) was not affected by this increased osmolarity (Figure 3). In contrast,

FIGURE 1. Effects of hypotonic treatment on p38 activation in adult and newborn PMN. (A) PMN were lysed either with (+) or without (-) hypotonic lysis of RBC and lysates were analyzed for p38 phosphorylation by Western blotting. (B) PMN treated with LPS and analyzed as above. Figure is representative of four independent experiments.

A. Western blot:

B. Densitometry of the bands:

FIGURE 2. Activation of p38 by LPS in isotonic condition. Adult and newborn PMN membrane lysates (A) analyzed for p38 phosphorylation and the p38 protein content (B) analyzed by densitometry. Fold increase in phospho-p38 band density (mean $\pm$ SD) in LPS-treated relative to unstimulated cells. **: $P < 0.01$ (Comparison between adults and newborns).

A. Western blot:

B. Densitometry of phospho-p38:

FIGURE 3. Activation of p38 by LPS and fMLP in high-normal tonic condition. PMNs were resuspended in high-normal tonic medium and stimulated with LPS or fMLP lysed without hypotonic treatment. (A) Analyzed for p38 phosphorylation and protein content and (B) by densitometry shown as relative density (mean of percentages $\pm$ SD), calculated by dividing each phosphorylated p38 band by the p38 protein band. **, $P < 0.01$ and #, $P > 0.05$, comparing the unstimulated control for adults and newborns, respectively.

FIGURE 4. Effects of LPS on CD66b surface expression in adult and newborn PMN. Leukocyte-rich plasma from adults and newborns was incubated with or without LPS. Results are shown for mean fluorescence $\pm$ SD (for adult, $n = 5$; for newborn, $n = 3$). **, $P < 0.01$; #, $P > 0.05$.

the modest p38 response to LPS stimulation by newborn PMN was lost under these conditions. The non-responsiveness of p38 in newborn PMN remained even when LPS concentrations were increased up to 200 ng/ml, plasma up to 10%, or incubation times up to 40 min (results not shown). The response of newborn PMN to fMLP was unaffected under these conditions (Figure 3).

Effect of LPS on CD66b expression in adult and newborn PMN: LPS stimulation was carried out with leukocyte-rich plasma, and CD66b surface expression was analyzed using flow cytometry. In adult PMN, surface expression of CD66b was up-regulated by LPS stimulation (Figure 4, $P \leq 0.001$). In contrast, LPS stimulation did not cause a significant change in CD66b surface expression in newborn PMN.

Expression of TLR-4 in adult and newborn PMN: In order to understand why newborn PMN respond differently to LPS, we investigated upstream

A. Western blot: **B. Flow cytometry of adult PMNs:** **C. Flow cytometry of newborn PMNs:**

FIGURE 5. Effects of LPS on TLR-4 expression in adult and newborn PMN. (A) Adult and newborn PMN lysates analyzed for TLR-4. The membrane was stripped and re-blotted for β -actin to assess the equality of loading. (B) Adult or (C) newborn leukocyte-rich plasma was incubated with or without LPS for the times indicated and analyzed for TLR-4 by flow cytometry. Results are shown as relative fluorescence intensity (mean $\pm$ SEM), by dividing the intensities of each experimental group by cells incubated without LPS. *, $P < 0.05$; #, $P > 0.05$ (for adults, $n = 5$; for newborns, $n = 4$).

pathways involved in LPS signaling. First, we assessed the total amount of TLR-4, the major receptor for LPS, in adult and newborn PMN. In both unstimulated and LPS-treated PMN, total TLR-4 detected by Western blotting of cellular lysates was identical in adult and newborn PMN (Figure 5A).

Since the level of surface expression is important for a receptor, we also assessed the surface exposure of TLR-4 in adult and newborn PMN by flow cytometry. We detected no differences between adult and newborn cells, and surface expression of TLR-4 was stable for up to 60 min in unstimulated adult and newborn PMN (Figure 5B and C). However, the response of adult and newborn PMN to LPS was different: addition of LPS to adult PMN for 30 min significantly decreased TLR-4 surface expression (Figure 5B), while no significant change in TLR-4 surface expression in newborn PMN was noted (Figure 5C).

Expression of MyD88 is decreased in newborn PMN: We investigated levels of MyD88 in adult and newborn PMN using Western blotting. As shown in Figure 6, there was a marked difference in protein levels of MyD88 in lysates prepared from newborn compared to adult PMN ($39 \pm 9\%$ of adult PMN MyD88 protein, $p = 0.013$ by paired t -test). This was not linked to a difference in total cellular TLR-4 protein on the same analyzed samples (Figure 6).

Discussion

In this study, we compared the priming of PMN by LPS in human adult and newborn PMN, with special attention to p38-associated signaling pathways. We found that newborn PMN showed decreased p38 phosphorylation in response to LPS. In keeping with this finding, p38-dependent up-regulation of CD66b was attenuated in newborn PMN even though basal CD66b expression on adult and newborn PMN were similar. Adults and newborns were also found to have similar levels of TLR-4, a specific receptor for LPS. However, levels of the intracellular mediator of TLR-4 signaling, MyD88, were decreased in newborn PMN in proportion to the diminished priming response of these cells.

FIGURE 6. Expression of MyD88 in adult and newborn PMN. (A) Paired samples of adult and newborn PMN analyzed for MyD88. (B) The protein bands assessed by densitometry. Results are shown as relative densities (mean $\pm$ SD), calculated by dividing the density of the MyD88 band from each donor with that of the TLR-4 band of the same donor. *, $P = 0.013$.

Over the past decade the mechanism of LPS interaction with phagocytic cells has been more clearly understood.²⁷⁻³⁰ For adult PMNs, monocytes and macrophages, CD14 is the principal cell membrane receptor for the LPS/LPS-binding protein (LBP) complex.²⁸ However, CD14 lacks a cytoplasmic domain to transmit signals intracellularly.³¹ A family of transmembrane receptors with homology to Toll proteins of *Drosophila* are now known to fill this role.³² The Toll-like receptor-4 (TLR-4) imparts ligand-specific recognition of LPS by mammalian cells.²⁷ Through CD14/TLR-4 interactions, LPS induces sev-

eral intracellular responses, including activation of the mitogen-activated protein (MAP) kinase family, particularly extracellular-signal regulated kinases (ERKs) and p38^{17, 18}, that eventually increase superoxide (O_2^-) production in response to additional stimuli. Enhanced O_2^- production by primed PMN may allow the host to resist infection more efficiently since products of O_2^- have potent bactericidal activity. MyD88, a cytosolic adapter protein, is an important mediator of TLR receptor activation, including TLR-4 response to LPS.²⁷⁻³⁰ LPS signaling is altered in animals with inactivated genes for MyD88^{32, 33} and TLR signalling in

general is suppressed by truncation of MyD88.³⁴ Indeed, our findings of diminished LPS responses of newborn PMN are similar to those using cells from MyD88^{-/-} mice or MyD88 truncated cells.¹⁰ In such cells LPS-induced nitric oxide release and degranulation are diminished although responses to other stimuli are preserved.^{30, 35-37}

The increased susceptibility of human newborns to gram-negative bacterial infections is due to a variety of humoral and cellular factors. One of the first cellular responses to gram-negative bacterial infection occurs with priming of PMN by LPS. Our studies shed light on the role of MyD88 and p38 on PMN priming and the basis of the attenuated LPS responses by newborn cells. The findings are consistent with earlier studies showing that adult and newborn PMN have similar levels of p38 protein.¹⁷ However, in the present study we show that phosphorylation of p38 protein in response to LPS is diminished in newborn PMN. This appears to be LPS-specific, since p38 phosphorylation was increased similarly in newborn and adult PMN by other stimuli such as fMLP and osmotic stress. Since p38 signalling is needed for PMN priming, attenuation of its LPS-triggered phosphorylation may affect host defense processes.³⁸

We have previously shown that p38 activation by LPS in adult PMN requires both CD14 and LBP, and that the p38-specific inhibitor SB203580 attenuates the respiratory burst in cells primed by LPS.¹⁷ p38 also plays a role in the regulating PMN degranulation and chemotaxis.^{21,39} Given our findings, we would expect PMN of newborns to have diminished degranulation. Indeed, we observed that the LPS-induced up-regulation of CD66b, a process known to be p38 dependent and associated with degranulation¹¹, was absent in newborn PMN, further suggesting a functional consequence of diminished LPS-induced p38 phosphorylation in these cells. Moreover, the delayed downregulation of surface TLR-4 in newborn PMN in response to LPS may also be attributable to lowered MyD88 level and/or decreased p38 response.

In agreement with our findings, decreased levels of MyD88 in PMN of premature infants have been observed.²⁷ In addition, newborn monocytes have low levels of MyD88, which is associated with diminished

LPS-induced production of TNF- α .^{19, 24} However, no significant attenuation of p38 phosphorylation by LPS was observed in newborn monocytes²⁴ and only subtle age-related differences in subcellular distribution of phosphorylated and total p38 were noted in a more recent study with PMN.¹⁹ The possible role of a MyD88-independent pathway of TLR-4 signaling involving Mal/TIRAP³⁰ in newborn PMN and monocytes should be investigated to explain these differences. The PMN used in our studies were recovered from cord blood of full-term infants born by caesarean section. Future research should be done using PMN of infants born vaginally to assess the effect of labour and delivery on cell function.

In summary, the present investigation has further defined the molecular basis of attenuated degranulation and priming of newborn PMN in response to low levels of LPS. We have identified significant age-specific differences in the LPS activation of a major intracellular MAP kinase, p38, and have shown that newborn PMN have lower concentrations of MyD88, an important mediator of LPS signaling. In this and previous studies¹⁰ we have shown no differences between adult and newborn PMN in the levels of the major LPS membrane receptors (CD14 and TLR-4). Moreover, many of the intracellular kinases implicated in LPS signaling (Erk, p38, Lyn, Hck, Fgr) are present in similar amounts in these cells.^{18, 19} To date, the TLR-4 adaptor protein MyD88 is the only mediator of LPS signaling that has been found to exhibit decreased levels in newborn vs adult PMN; similar age-related differences have also been observed in monocytes.²⁴ Whether differences in MyD88 are solely responsible for other altered LPS responses such as activation of the src-like kinase p53/56lyn¹⁹, p38 phosphorylation, TLR-4 downregulation, and CD66 upregulation is still unknown. Nevertheless, MyD88 and phosphorylation of p38 appear to have an important role in PMN priming with LPS and provide potential therapeutic targets for pediatric infectious disease.

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