Original Article

Role of Zc3h12a in enhanced IL-6 production by newborn mononuclear cells in response to lipopolysaccharide

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Zc3h12a;
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Background: The uncontrolled inflammatory response following infection is closely related to the morbidity and mortality of neonates. Interleukin 6 (IL-6) plays an important role in the pathogenesis and prognosis of this process. To better elucidate the secretion of IL-6 following infection in neonates, we investigated the IL-6 level and mechanism of IL-6/TLR4 signaling pathways.

Methods: We compared the IL-6, procalcitonin (PCT), and CRP levels between septic neonates and toddlers. In vitro cord blood samples from healthy term neonates and peripheral venous blood from healthy adult volunteers were collected. Protein expression was analyzed by Western blotting, mRNA expression by real-time PCR and membrane molecule expression by flow cytometry.

Results: The IL-6 concentrations were significantly higher in the neonate group than in the toddler group (p < 0.05). In the toddler group, the IL-6 concentrations were correlated positively with both PCT and CRP (PCT: r = 0.451, p = 0.001; CRP: r = 0.243, p = 0.023). In vitro, the secretion of IL-6 increased with the rising concentrations of LPS; at 1000 ng/ml
1. Introduction

Neonatal sepsis remains one of the leading causes of morbidity and mortality in both term and preterm infants. The incidence of sepsis in the first month of life ranges from 1 to as high as 10 cases per 1000 live births.1 In China, the incidence of neonatal sepsis in different regions varies considerably, and the accurate incidence of neonatal sepsis is not known. According to the limited and unpublished clinical statistical data of few developed areas in China, the incidence of neonatal sepsis is 3–6.5/1000 live births.2 Sepsis in neonates is distinct in clinical symptoms and prognosis compared with that in older children or adults. The clinical course of neonatal sepsis may suddenly progress toward shock, disseminated intravascular coagulation (DIC), and death within a few hours from the onset of disease. Additional causes of death are long-term complications—e.g., chronic lung disease, neurocognitive disabilities together with neurological sequelae, and growth retardation.

It has become evident, however, that many infants die despite the sterilization of blood cultures with antimicrobial agents. It is now appreciated that the physiologic derangements that occur during sepsis are secondary to the host response induced by pathogenic microorganisms.3 During overwhelming sepsis, the host produces proinflammatory cytokines that initiate a cascade of events, resulting in tissue injury at distant sites and generalized multiorgan system failure.4,5 The balance between proinflammatory and anti-inflammatory cytokines may ultimately determine the outcome of sepsis in newborn infants.

Among many cytokines, Interleukin 6 (IL-6) expression is significantly increased in neonates and is higher than that in adults after infection compared with other cytokines such as TNF-α and IL-1.6,7 Evidence has suggested that IL-6 in the fetus and neonates play a critical role in the pathophysiology of severe neonate diseases, including sepsis, bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC).8,9 IL-6 was even recommended as a biomarker for BPD and NEC recently. More importantly, IL-6 has been identified as a key mediator of prenatal inflammation and cerebral palsy.10 Clinical studies have suggested that preterm neonates born to mothers with elevated IL-6 levels in the amniotic fluid are at increased risk for the subsequent development of periventricular leukomalacia (PVL) and cerebral palsy.11

To understand the secretion of IL-6 after the immune response of neonates, we investigated the level of IL-6 both in vivo and in vitro after stimulation. Furthermore, we investigated the mechanism of abnormally increased IL-6 in the inflammatory response of neonates by detecting the expression of key molecules of TLR4/MyD88 signaling pathways. We report here that enhanced LPS-induced IL-6 protein secretion by monocytes from neonates is associated with diminished Zc3h12a expression.
2.4. Flow cytometric analysis

Surface staining was performed in 100 μl of PBS with 3% (v/v) PBS and antibody for the monocyte surface marker anti-CD14 at room temperature for 30 min. Subsequently, the intracellular cytokines were stained using intracellular staining kits from BD Biosciences. The cells were incubated with anti-IL-6 for 40 min at room temperature. All the samples were analyzed using an EPICS XL flow cytometer and System II, Version 1.0 software.

2.5. Chemiluminescence assay

The supernatants were collected after LPS stimulation and were centrifuged at 3000 g for 10 min at 4 °C to remove insoluble materials. The IL-6 levels were determined by the immunochemiluminescent analyzer “Immulite 2000” (Siemens). The assays were performed according to the manufacturer’s instructions. The results were expressed as the concentration of IL-6 (ng/L).

2.6. RNA isolation, complementary DNA (cDNA) synthesis, and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from 4 × 10^6 cells using the RNeasy Mini Kit (Qiagen) and was transcribed to cDNA using the First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer’s instructions. Standard real-time PCR was carried out using a TaqMan 7900 (Applied Biosystems) and the DNA intercalating dye SYBR Green. We used the following primer sequences:

- Zc3h12a Up-TCAGGGGcGATAcAAcTTGcA
- Down-AGCCTTcTcCTCATGGTGTTGAAGAC
- GAPDH Up-cGGAGtCAGGctTGGcCTACGAg
- Down-tcAGGGGCcATAAcAcTTGcA

2.7. Western blot assays

Adherent monocytes from adults and newborns were prepared and incubated in the absence or presence of LPS (1 μg/ml) as described above for 20 min at 37 °C with 5% CO₂. After the incubation, the medium was aspirated, and the cells were washed twice with ice-cold PBS containing 1 mM diisopropyl fluorophosphate. The PBS was then replaced with 0.5 ml of M2 buffer (20 mM Tris [pH 7.0], 0.5% NP-40, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 2 mM dithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 5 g of leupeptin/ml, 5 g/ml pepstatin A, 10 g of aprotinin/ml, 0.1 mM Na₃VO₄). After incubation for 30 min at 4 °C, the lysates were transferred to microcentrifuge tubes and were clarified by centrifugation at 12,000 g for 10 min at 4 °C to remove insoluble materials. The protein concentrations in the lysates were determined using the Bio-Rad Bradford protein assay. Twenty micrograms of protein from each sample was analyzed by Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransfer, with antibodies specific for MyD88, NF-κB, and IκB (Cell Signaling Technology; diluted 1:1000), and β-actin (Santa Cruz Biotechnology; diluted 1:3000). The binding of the antibodies was detected with HRP-conjugated goat anti-rabbit or mouse IgG and ECL Western blotting reagents, followed by exposure to Kodak X-ray films. The band density was quantified by densitometry analysis using NIH ImageJ software.

2.8. Statistical analysis

The data are presented as means ± the standard deviation (SD). For comparison between the adult and newborn samples, a nonpaired t test was used; Pearson’s correlation was used to analyze the relationships among the variables of interest, and a p value of 0.05 was considered significant. Statistical determinations were performed using SPSS17.0.

3. Results

3.1. Demographics and clinical characteristics

Twenty-one neonates and 21 toddlers were finally enrolled. The blood samples were collected when they were admitted to the hospital. The mean age of the neonate group was 11.76 ± 10.68 days and that of the toddler group was 4.90 ± 1.33 years. In the toddler group, complications occurred in 8 patients (4 had atelectasis, 2 had pulmonary abscess, 2 had pleural effusion). All the patients from these two groups were cured and discharged from the hospital. The mean hospital length of stay was 13.24 ± 4.36 days in the neonatal group and 10.86 ± 3.72 days in the toddler group (Table 1).

3.2. Comparison of IL-6, CRP and PCT in neonates and toddlers

The IL-6 concentrations were significantly higher in the neonate group than in the toddler group (p < 0.05, Table 1). By contrast, the CRP concentrations of the neonate group were significantly lower than those of the toddler group (p < 0.01, Table 1). There was no difference between the PCT concentrations of the two groups. Additionally, in the toddler group, the IL-6 concentrations were correlated positively with both PCT and CRP (r = 0.451, p = 0.001 and r = 0.243, p = 0.023, respectively). However, such a relationship was not seen in the neonate group (p > 0.05) (Fig. 1). There was no relationship between the hospital length of stay and serum IL-6 level in either the neonate or toddler group (p = 0.146 and p = 0.095, respectively). The IL-6 level in toddlers with complications was significant higher than that in patients without complications (30.74 ± 5.32 vs. 12.41 ± 6.53; p = 0.001).
3.3. IL-6 secretion by adult and neonatal monocytes

To better understand IL-6 regulation in neonates, we used LPS, the major component of the outer membrane of Gram-negative bacteria, to stimulate adult or cord-adherent monocytes by different concentrations (10 ng/ml, 100 ng/ml, 1000 ng/ml). We detected both intracellular IL-6 secretion and IL-6 concentration in supernatant with or without LPS stimulation. As shown in Fig. 2A, the IL-6 MFI increased with the rising concentration of LPS; at 1000 ng/ml LPS, the MFI of IL-6 following LPS stimulation in newborns was significantly higher than that in adults (Fig. 2 A,C,D). The trend of the IL-6 concentration after LPS stimulation in the supernatant was consistent with the intracellular MFI of IL-6, although it was not statistically significant between the neonates and adults (Fig. 2B).

3.4. Responses of the LPS-TLR4/NF-κB signal pathway in monocytes from adults and neonates to LPS stimulation

To understand the increased IL-6 expression in neonates at a molecular level, we further examined the key molecule involved in the IL-6 signaling pathway of monocytes from adults and neonates following LPS stimulation. Because membrane-bound CD14 and TLR-4 are required for effective LPS-induced IL-6 secretion by monocytes, we characterized CD14 and TLR-4 expression on monocytes from adults and neonates before or after LPS stimulation. As shown in Fig. 3A, adult and neonate monocytes expressed similar levels of TLR-4 and CD14 before and after LPS stimulation, suggesting that TLR-4, by itself, was unlikely to be responsible for the enhanced neonatal IL-6 production.

The inducible IL-6 gene expression by monocytes is regulated by IκB/NF-κB nuclear transcription factors. Because the LPS-induced secretion of IL-6 from neonatal monocytes was remarkably increased compared with that from adult monocytes, we assessed the signaling molecule activation in adult and neonatal monocytes after LPS stimulation. The activation of the NF-κB signaling pathway is presented as the phosphorylation and degradation of its cytoplasmic inhibitor, IκB. As shown in Fig. 3E and F, IκB degradation was similar for both adult and neonatal monocytes.

MyD88, a cytosolic adapter protein, has been reported to be an essential mediator in LPS receptor signaling. Thus, we investigated whether there was any difference in the expression of MyD88 in adult versus neonatal monocytes by Western blotting. As shown in Fig. 3, the expression of MyD88 protein in newborns was remarkably decreased compared with that in adult monocytes (p < 0.01 as determined by paired t test).

3.5. Expression of Zc3h12a in monocytes from adults and newborns

Zc3h12a is a lipopolysaccharide-inducible gene and has a CCHC-type zinc-finger domain. A study showed that Zc3h12a is a nuclease involved in the destabilization of IL-6 mRNA via the stem loop structure within the 3′ UTR of these genes. Thus, we investigated the expression of Zc3h12a in adult versus neonatal monocytes after stimulation with 1 μg/ml LPS using real-time PCR. As shown in Fig. 4, the mRNA levels of Zc3h12a in neonates were significantly lower than those in adults (t = −5.615, p = 0.000).

4. Discussion

Neonatal sepsis is a common and significant health care burden disease characterized by many non-specific clinical symptoms such as fever or hypothermia, respiratory distress, feeding difficulties, and unexplained jaundice, but it rapidly progresses, resulting in sequelae or even death. Although antibiotics therapy has achieved great efforts, sepsis remains the main cause of mortality for neonates. Elder children and adults are usually able to restrict bacterial infections, whereas neonates often develop a systemic inflammatory response syndrome (SIRS) with detrimental effects. Growing evidence has suggested that this process is mainly mediated by inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-18. Due to the immature immune system, most of the inflammatory cytokines are

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**Table 1 Demographic data and serum IL-6, CRP and PCT concentrations in the neonate and toddler groups.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Neonates (N = 21)</th>
<th>Toddlers (N = 21)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male ratio</td>
<td>12/9</td>
<td>10/11</td>
<td>0.537</td>
</tr>
<tr>
<td>Mean age at diagnosis</td>
<td>11.76 ± 10.68</td>
<td>4.90 ± 1.33</td>
<td>/</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>39.20 ± 1.57</td>
<td>≥37b</td>
<td>/</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>3404.52 ± 713.25</td>
<td>NDc</td>
<td>/</td>
</tr>
<tr>
<td>Delivery mode (V/C)d</td>
<td>13/8</td>
<td>14/7</td>
<td>0.747</td>
</tr>
<tr>
<td>Length of stay (days)</td>
<td>13.24 ± 4.36</td>
<td>10.86 ± 3.72</td>
<td>0.064</td>
</tr>
<tr>
<td>Mortality</td>
<td>0%</td>
<td>0%</td>
<td>/</td>
</tr>
<tr>
<td>Serum IL-6 (pg/ml)</td>
<td>36.77 ± 30.95</td>
<td>19.39 ± 13.72</td>
<td>0.026</td>
</tr>
<tr>
<td>Serum CRP (mg/ml)</td>
<td>8.39 ± 13.59</td>
<td>34.14 ± 32.76</td>
<td>0.003</td>
</tr>
<tr>
<td>Serum PCT (ng/ml)</td>
<td>0.87 ± 0.40</td>
<td>0.63 ± 0.82</td>
<td>0.56</td>
</tr>
</tbody>
</table>

a The unit of mean age at diagnosis for neonates is days and for toddlers is years.

b The gestational age of toddlers is not in detail; all the toddlers were born term.

c ND: not determined.

d V/C: Vaginal delivery vs caesarean.
markedly attenuated in neonates. However, it is reported in some studies that the IL-6 level of neonates was enhanced after infection. IL-6 is a multifunctional cytokine involved in regulating the immune response, hematopoiesis, acute phase response and inflammation. After infection, the IL-6 level raises quickly, peaks at 24–48 h and is closely related to the severity of disease, antibiotic effect and prognosis.15,16 In addition, IL-6 has been reported in other inflammatory diseases of newborns such as pulmonary hypoplasia and necrotizing enterocolitis.17–19

To better characterize the secretion of IL-6 in neonates, we investigated the IL-6 level both in vivo and vitro. We found that the serum IL-6 of neonates with sepsis was significantly higher than that in toddlers with sepsis. Additionally, in vitro, after LPS stimulation, IL-6 secreted by the monocytes of neonates was also significantly higher than that of adults. These results were consistent with the results of previous studies that IL-6 secretion is enhanced in neonates.20–22 At low concentrations of LPS, LBP facilitates the binding of LPS to CD14 and greatly enhances IL-6 secretion. Thus, some studies indicate that one possible explanation for the phenomenon is that LBP or some other constituents of plasma may be more active in neonates than in adult plasma. However, LBP is unlikely to be responsible because studies have shown previously that concentrations in newborn and adult plasma are similar. Additionally, in our study, we also evaluated the relationship among IL-6, PCT and CRP. We found that the serum IL-6

Figure 1  Correlation between the serum IL-6 level and PCT or CRP of the two groups. (A): Correlation between IL-6 and PCT of neonates (p > 0.05); (B): Correlation between IL-6 and CRP of neonates (p > 0.05); (C): Correlation between IL-6 and PCT of toddlers (r = 0.451, p = 0.001); (D): Correlation between IL-6 and CRP of toddlers (r = 0.243, p = 0.023).
concentrations were correlated positively with PCT and CRP in toddlers, whereas the serum IL-6 concentrations did not correlate with either PCT or CRP in neonates. This correlation in toddlers showed that increased IL-6 was consistent with other inflammatory markers. However, in neonates, the increased IL-6 did not match other inflammatory markers. It has been reported that the increase in the IL-6 level in the peripheral blood of neonates is closely related to the occurrence of BPD, and evidence has also shown that there is abundant IL-6 in the bronchoalveolar lavage fluid of children with BPD. In addition, IL-6 overexpression in the alveoli appear with a similar pathogenesis of BPD in animal experiments. Recently, IL-6 was considered an important diagnostic biomarker for BPD.

Increased IL-6 not only affects neonatal lung development but, more seriously, also plays a key role in neonatal brain injury. In some animal models, IL-6 can affect the integrity of the blood–brain barrier, thus causing brain damage. Preterm infants with an immature blood–brain barrier, when destroyed by cytokines, can lead to extremely serious consequences for intracranial hemorrhage. Additionally, it was reported that the level of inflammatory cytokines is very high in premature infants with brain injury. A postmortem study found that IL-6-positive cells in the central nervous system of periventricular leukomalacia (PVL) neonates were significantly higher than those in neonates without PVL. Increased umbilical cord blood IL-6 levels were also related to PVL and cerebral palsy of neonates. Thus, we considered that IL-6 may be harmful to the target organs.

Therefore, we speculate that aberrantly increased IL-6 levels in neonates after infection may result in certain organ damage. Superfluous IL-6 may be one of the reasons that neonates are more vulnerable to complications after infection.

Why is IL-6 expression in neonates higher than that in adults while that of other cytokines of the same signaling pathways are not? To better understand this question, in neonates, we examined the signaling pathway of IL-6 secretion. We compared the expression of key molecules TLR4, MyD88, NF-κB, IκB, and Zc3h12a in the monocytes of neonatal cord blood and adult peripheral blood following stimulation with LPS.

The expression of TLR4 in both neonatal and adult monocytes was significantly increased following LPS stimulation, but there was no significant difference between the two groups before or after stimulation. Studies have shown that the expression of TLR4 in premature infants was significantly lower than that in term neonates and adults, while the expression of TLR4 in term neonates was not significantly different from that in adults. However, further study showed that the functional TLR4 of neonates was significantly lower than that of adults. Thus, the ability of the response to LPS in neonates is also decreased. Therefore,
there may be no significant difference in the number of TLR4 molecules after birth, but the function is still immature.

Our study further showed that the expression of MyD88 in neonates was significantly lower than that in adults. It was reported that diminished TNF-α secretion is correlated directly with levels of MyD88 in newborn cells.27 In addition, mice with an inactivated MyD88 gene lack LPS-induced cytokine production and NO2 release but preserve NF-κB and JNK activation.13,28 Because of its central place in signaling pathways, MyD88 may become a “bottleneck”, affecting TLR-triggered cytokines in general.

These findings suggested that IL-6 secretion after LPS stimulation is controlled primarily by posttranscriptional mechanisms. We detected the negative feedback molecule Zc3h12a in the signaling pathway and found that the expression of this specific RNA enzyme in newborns was significantly lower than that in adults. Zc3h12a is a nuclease involved in the destabilization of IL-6 mRNA via the stem loop structure within the 3 UTR of these genes.29 Zc3h12a does not affect TNF-α and other cytokine mRNAs. In addition, in vitro experiments showed that Zc3h12a could bind to its own mRNA and regulate its degradation.30 The existence of this negative feedback pathway makes the regulation ability of Zc3h12a more precise. Thus, we believed that the lack of neonatal Zc3h12a expression may be the cause of abnormally increased IL-6 after infection in neonates.

In summary, our study showed that neonates displayed enhanced IL-6 production after infection. The low expression of MyD88 was consistent with that in the literature and may be one of the reasons for the reduction in the expression of most cytokines in the neonatal period. Our study, for the first time, reported a significant decrease in the expression of Zc3h12a in neonates and may be one of

Figure 4  Zc3h12a mRNA expression of neonatal and adult monocytes. The relative mRNA expression of Zc3h12a in monocytes from adults and neonates after LPS stimulation was assessed by real-time PCR. The data are representative of six independent experiments. **P < 0.01 vs the newborn group.
the important reasons for the abnormal increase in IL-6 after neonate infection. The immaturity of the neonatal immune system is due to not only the lack of immune cells or immune factors but also the ability to regulate the homeostasis of the immune system.

Conflict of interest
None.

References