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Maturation of Toll-like receptor 1–4 responsiveness during early life ☆

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ABSTRACT

Background: Toll-like receptors (TLRs) are part of the highly conserved components of the innate immune system, and have been investigated extensively; however, little is known about TLR function during early postnatal life, a critical period for immune maturation.
Aims: In order to achieve a more complete understanding of the ontogeny of immune system during the first years of life, our study investigated age-matched TLR1–4 responsiveness at several time points up to the age of two years.
Study design: Mononuclear cells were isolated from cord blood (n = 150) and peripheral blood from infants at 6 (n = 68), 12 (n = 75), and 24 (n = 74) months of age, and from 50 adults. Cells were stimulated with Toll-like receptor ligands (TLR1–4) and phytohemagglutinin (PHA). Stimulated cells were assessed for their production of the cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), and for TLR4 gene expression.
Results: Our results suggested that cord response of IL-6 and TNF-α was not affected by allergic background. In addition, neonatal mononuclear cell had enhanced IL-6 production upon TLR1, 2, and 4 stimulations as compared to those of young children and adults. Nevertheless, after 6 months of age, the level remained comparable throughout the first two years of life. While TNF-α response to all TLR stimulations remained fairly similar during early life. This cytokine pattern closely paralleled our findings for TLR4 mRNA expression, and longitudinal cytokine changes within the same individual.
Conclusions: Our findings provided additional information to the understanding of immune development during early life, and offered stronger evidence of neonatal innate immunity being capable of responding adequately to TLR stimulation.

1. Introduction

Early childhood is characterized by increased susceptibility to infection due to relative immaturity of the immune system [1]. Immune maturation continues after birth, and early life events appear to have a critical influence on the ultimate pattern of immune maturation. Toll-like receptors (TLRs) are highly conserved components of the innate immune system, and play critical roles in early innate responses to various invading pathogens. Despite the fact that TLR has been investigated extensively, little is known about the maturation of TLR development during early postnatal life. Ten human TLRs have been identified, among which TLR1, 2 and 4 pathway provides protection against life-threatening bacterial infections during early life, while TLR3 signaling provides for responses against several respiratory viruses, such as respiratory syncytial virus (RSV) [2,3], rhinovirus [4,5], and influenza virus [6,7], which are frequently encountered and pose considerable health threats during infancy.

Although, it is well known that neonates have the highest incidence of infection, but infants might continue to remain at increased risk during early life [8,9]. Furthermore, patients with TLR signaling defect or immune disorder involving the nuclear factor (NF) κβ signaling pathway can have life-threatening infections with poor inflammatory cytokine production starting at a very young age [10–13]. Therefore the development of a clinical assay to evaluate TLR function during early life would be necessary. Although there are several published studies for screening patients with primary immunodeficiency that affect TLR functions [10,14], however, most current information relates only to older children or adults [9,14,15] and data on normal cytokine responses to several TLR stimulations during early childhood are very limited. Furthermore, it has been difficult to assess TLR function in neonates, because despite extensive studies on neonatal innate immunity, conflicts exist as some reports have shown that such susceptibility

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has resulted from impaired cord TNF-α response to LPS when compared to adults' cells [5,16–20], while other studies have drawn opposite conclusions, stating a hyper-responsive state in neonates . Although data for other cytokines, such as IL-6, show consistent observations of increased IL-6 production in neonates [9,21–24], a few reports had different conclusions [25,26]. Variations in experimental methodology may account for some of these different outcomes; however, it is worth noting that most studies that compared neonatal innate immunity to that of adults involved rather small population sizes. This could be an important issue, since cytokine levels analyzed by current methodology could result in large variations with skewed distributions. In addition, neonatal cytokine responses could be influenced by several perinatal factors [27–29], leading to biased results when population number is limited. We intend to re-address this issue by enrolling a considerable population size. Furthermore, we extended our study to investigate the development of TLR1–4 responsiveness during the first two years of life that would be helpful in establishing a basis for the development of a peripheral blood TLR assay that could be useful for screening young children with TLR defects or immune disorders.

2. Materials & methods

2.1. Study population

The Chang Gung ethics committee approved the research, and informed consent was obtained from the adult volunteers (n = 50; age range 25–40 years) or the parents/legal guardians of the neonates and young children. Cord bloods were obtained from uncomplicated full-term deliveries (n = 150). Blood samples were collected from healthy children aged 6 (n = 68), 12 (n = 75), and 24 (n = 74) months. Among these, successive blood collection was performed for 17 children at a 6-month interval for longitudinal results. Neonates were excluded in the presence of major congenital anomaly, gestational age of less than 37 weeks and perinatal infection such as chorioamnionitis, maternal fever, premature rupture of membrane for longer than 18 h, or any other settings suspecting congenital infection. Young children were excluded from the study if they had any acute or chronic infectious disease (including upper respiratory tract infection), took medications, or had any clinically significant disorders known to influence immune status. We recorded patients' histories for vaccination, perinatal history, parental allergy status, household pets, previous infection, breast-feeding, and allergic status. Because our study subjects returned for scheduled vaccination and health checks, the immunization status was similar among individuals in each age group, such that by 6 months, all of them had received a dose of BCG, 2 doses of HBV, and 2 doses of DTPa-Hib-IPV; by 1 year of age, all of them would have had received a dose of BCG, 3 doses of HBV, 3 doses of DTPa-Hib-IPV; and by 2 years of age, they would have an additional dose of DTPa-Hib-IPV, and MMR plus varicella. Blood was always withdrawn right before scheduled vaccination.

2.2. Cell preparation and culture

For neonates, cord blood was collected immediately after delivery. For young infants and toddlers, additional blood was obtained during routine health checks and also partly from an ongoing cohort study (Prediction of Allergies in Taiwanese Children: PATCH cohort) with the parents' agreement. Fresh peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using Ficoll–Hypaque separation (Pharmacia Biotech, Piscataway, NJ). Cell culture medium was RPMI 1640 supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 2 mmol/L glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomycin (complete medium).

2.3. TLR1-2, TLR3, and TLR4 ligand stimulation

TLR ligands used for cell stimulation were obtained from InvivoGen (San Diego, CA). These included: a synthetic bacterial lipoprotein (PAM3csk4) for TLR1–2 at 10 μg/ml; a synthetic analog of double-stranded RNA for TLR3 (poly I:C) at 10 μg/ml; and ultrapure LPS for TLR4 at 20 ng/ml. Culture medium without any added ligand was used to determine any baseline production of each cytokine. As a positive control, cells were treated with the NF-κB activator phytohemagglutinin (Murex Pharmaceuticals, Missouri) at 4 μg/ml in R10-FBS.

To determine TLR responses, 3×10^5 PBMCs in 100 μl of R10-FBS were added to duplicate ligand- or medium-containing wells and incubated for 20 h at 37 °C with 5% CO_2. All assay preparations were done using sterile technique in a laminar flow hood.

2.4. TNF-α and Interleukin-6 measurements

TNF-α and IL-6 levels in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISA; R&D systems, MN) according to the manufacturer's instructions. The detection limits were 15.6 pg/mL for TNF-α and 3.12 pg/mL for IL-6.

2.5. RNA extraction and real-time PCR

Total RNA was extracted from control and stimulated cells with a RNeasy kit (Qiagen, CA) according to the manufacturer's instructions. For a reverse transcriptase reaction, a total of 0.8 μg RNA was reverse transcribed to cDNA using a SuperScript III First-Strand Synthesis System (Invitrogen, CA). Quantitative real-time PCR (Q-RT-PCR) used an assays-on-demand gene expression assay mix specific for TLR4 as the target gene, and 18S rRNA as an internal control. Duplicate CT (cycle threshold) values were analyzed with Microsoft Excel using the comparative CT (ΔΔCT) method as described by the manufacturer (Applied Biosystems, CA). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to the endogenous reference (18S) sample.

3. Statistical analysis

Data analysis was performed with SPSS software. Comparisons between different age groups were made by Student's *t* test and one-way ANOVA, corrected with Bonferroni's. A *p*-value of <0.05 was considered significant.

4. Results

4.1. Influence of perinatal factors and allergic phenotype on TNF-α and IL-6 response to TLR1–2, and 4

Due to the fact that some reports have shown a relationship between maternal allergy and other perinatal elements with cytokine-producing capacity in the cord blood [27–29], we had analyzed whether factors such as parental allergy, house pet, cord IgE level, and maternal smoking have an influence on cord IL-6 and TNF-α response. Although parental allergy and house pet were associated with a decreased IL-6 and TNF-α response to LPS, respectively, the difference was not significant (Table 1). In addition, to determine the influence of allergic status on cytokine expression level in young children, cytokine responses were compared between children with and without allergy. The allergic group is defined as children having total serum IgE level of greater than 100 KU/L with a doctor diagnosed allergic disease (atopic dermatitis, allergic rhinoconjunctivitis or asthma). We did not make comparisons for infants of 6 months of age, because the number of infants having clinical presentation of allergy was too small. The results showed that cytokine production in young children or adults was not affected by