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Original Article

Different expressions of TLRs and related factors in peripheral blood of preterm infants

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Abstract: Objective: TLR insufficiency increases newborn's susceptibility to infectious disease. Methods: The peripheral blood of four premature births has been collected weekly from the 28th gestational week (GW) until maturity at 36th GW. Microarray assays were used to derive dynamic follow-up data of TLR1-10 and other TLR signaling pathway associated factor changes. Results: The follow-up results showed that the transcription level of TLR1 increased at the 36th, TLR 3 decreased at the 33rd and TLR7 increased at the 34th GW significantly, whereas NFkB and its activator TBK1 were highest transcribed in the 28th and 32nd GW. Low TLR4 transcription in addition to late MD-2 maturation (33rd GW) indicated a lack of defense mechanisms against bacterial infections in preterm births particular in the first weeks after birth. Late transcriptional enhancements of TLR1 and MYD88 (35th week) as well as β 2 microglobulin (35th GW) also indicated a weak immune system in the early maturation stages. Conclusion: The transcription levels of TLR1, 3, 7 and the signaling pathway associated cofactors were different transcribed during the 28th and 36th GWs of the premature newborns. In the early stage after preterm birth, beside peak transcriptions of NFkB and TBK1, the immune system is not fully developed and maturation takes place mainly between the 33th and 35th GW.

Keywords: TLR, preterm birth, immune system, NFkB, TLR4

Introduction

Premature birth accounts for 11% of all live-born infants in the world [1]. Relative to all newborns, premature are more susceptible to disease since their innate immunity and acquired immunity not have been physically well developed. Severe infections can even lead to a mortality rate up to 35% [1]. Nupponen et al. suggested that innate immune signaling pathways responding to inflammatory stimuli are strongly functional in leucocytes of preterm neonates, which was attributed to high NFkB phosphorylation rates, but in both preterm and full-term newborns, responses needed against intracellular pathogens, and regulatory functions showed immaturities, possibly contributing to worse control of infections [2]. On the other hand it has been postulated, that the level of neutrophil and hyaline leukocytes in premature births are relatively low and function less compared to term infants, resulting in the significantly higher clinical infection rate of premature than that of term infants [1]. Toll-like receptors (TLRs) function as sensors of the innate immunity, monitoring and identifying various challenging agents, and become the organism's first barrier resisting pathogenic microorganisms. Previous studies have shown that under infection, prematures' TLR changes are notably lower than that of term infants (Sadeghi et al., 2007), which leads to less leukocyte activation and secretion of pro-inflammatory cytokines as well as less up-regulation of various factors; the acquired immune system facilitates TLRs to adjust the functions of T-lymphocytes (Roach et al., 2013), but it is still not clear about the specific change of TLR1-10 and which factors among the cytokine on TLR signaling pathway have played the decisive role in the process of cultivating a premature into a term infant. TLR is a transmembrane glycoprotein family and a pathogen surface recognition receptor [3]. TLRs are widely distributed in various immune effector cells, such as macrophages, dendritic cells (DC), natural killer cells and neutrophil granulocytes [4-6]. The TLR family comprises 10 proteins (TLR1-TLR10) in humans [7]. TLR2 acts as

Table 1. Basic characteristics of the infants

	P1	P2	P3	P4
Sex	Male	Male	Female	Female
Maternal age (year)	27	32	24	34
GA (week)	$28^{+2\ days}$	$28^{+6\ days}$	$30^{+1\ day}$	$29^{+2\ days}$
BW (gram)	1226	1330	1450	1390
Apgar score (1, 5, 10)	8, 9, 9	8, 8, 9	8, 9, 9	9, 9, 9

a heterodimer in concert with TLR1 or TLR6 and mediates responses to lipoproteins, lipoteichoic acids, peptidoglycan and zymosan. TLR3 is involved in the recognition of dsRNA from viruses and the synthetic dsRNA analogue poly (I:C) [8]. TLR5 recognizes bacterial flagellin [9], while TLR7 and TLR8 mediate responses to viral ssRNA and imidazoquinolines such as imiquimod (R-837) and resiquimod (R-848) [10]. TLR9 responds to bacterial and viral DNA containing unmethylated CpG motifs [11]. A set of endosomal TLRs comprising TLR3, TLR7, TLR8 and TLR9 recognizes nucleic acids derived from viruses as well as endogenous nucleic acids in context of pathogenic events. Activation of these receptor leads to production of inflammatory cytokines as well as type I interferons to cope with viral infection. After TLRs and ligands combine, transmembrane signal activates nuclear factor KB (NF-KB) through the intracellular domain of TLRs and up regulate the expression of tumor necrosis factor (TNF) and other cytokines in order to enhance the organism's inflammatory reaction and achieve the purpose of killing the causative organisms [12].

In this study, peripheral blood of newborns was collected and then analyzed by gene chip assays to detect the expression changes of the TLR family and their signaling pathway factors in the gestational immune system development process during different gestational ages of preterm newborns, which were in non-infected states.

Subjects and methods

Subjects

The study was approved by the ethical committee of the School of Medicine, Fudan University and the participating parents gave their written informed consents. We analyzed four neonatal hospitalized newborns with gestational age between 28 to 30 weeks. There were no major differences of birth weight, maternal ages and apgar scores among the preterm births (**Table 1**). The mothers of the preterm newborns were medicated with prenatal hormones and antibiotics before delivery and there were no infection indications between birth and discharge, which was monitored by blood routine examinations, C-reactive protein (CRP) test and urine culture. All four included preterm births had no intracranial hemorrhage, chronic lung disease or other premature complications and were discharged with weights of 2 kg at the gestational age of 34-35 weeks.

Methods

0.5 ml peripheral blood was collected from the preterm newborns immediately after admission and then once every week.

RNA isolation, amplification and labeling: Total RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA). Total RNA was amplified and labeled (cRNA) using the Ambion MessageAmpTM II-Biotin enhanced kit (Invitrogen, USA). Both total RNA and cRNA concentration and quality were determined using NanoDrop-1000 (Thermo scientific, USA) and an Agilent 2100 Bio-analyzer (Agilent Technologies, USA) respectively.

Microarray hybridization and scanning: 20 mg fragmented, biotin-labeled cRNA (n = 5) was hybridized to Gene Chip Human U133 plus 2.0 microarray platforms (Affymetrix, USA) for 18 h at 45°C and were scanned by a Gene Chip 3000 scanner (Affymetrix, Santa Clara, USA).

Microarray data analysis: Raw data (n = 4) were uploaded onto GeneSifter (Geospiza, USA), log transformed and normalized using GC-RMA. Then, gene expression changes were defined as a fold change of ≥ 1.5. KEGG (Kyoto Encyclopedia of Genes and Genomes) gene pathways (significance = z score of $2 < Z < -2$) and were used to functionally categorize genes before further analyses of specific gene changes. Individual gene changes were also examined based on previous work measuring TLRs and cytokines detected in peripheral blood collected from the preterm newborns.

Quantitative real-time PCR (qRT-PCR): The TLR family and associated signaling pathway factors were first analyzed by using a microarray

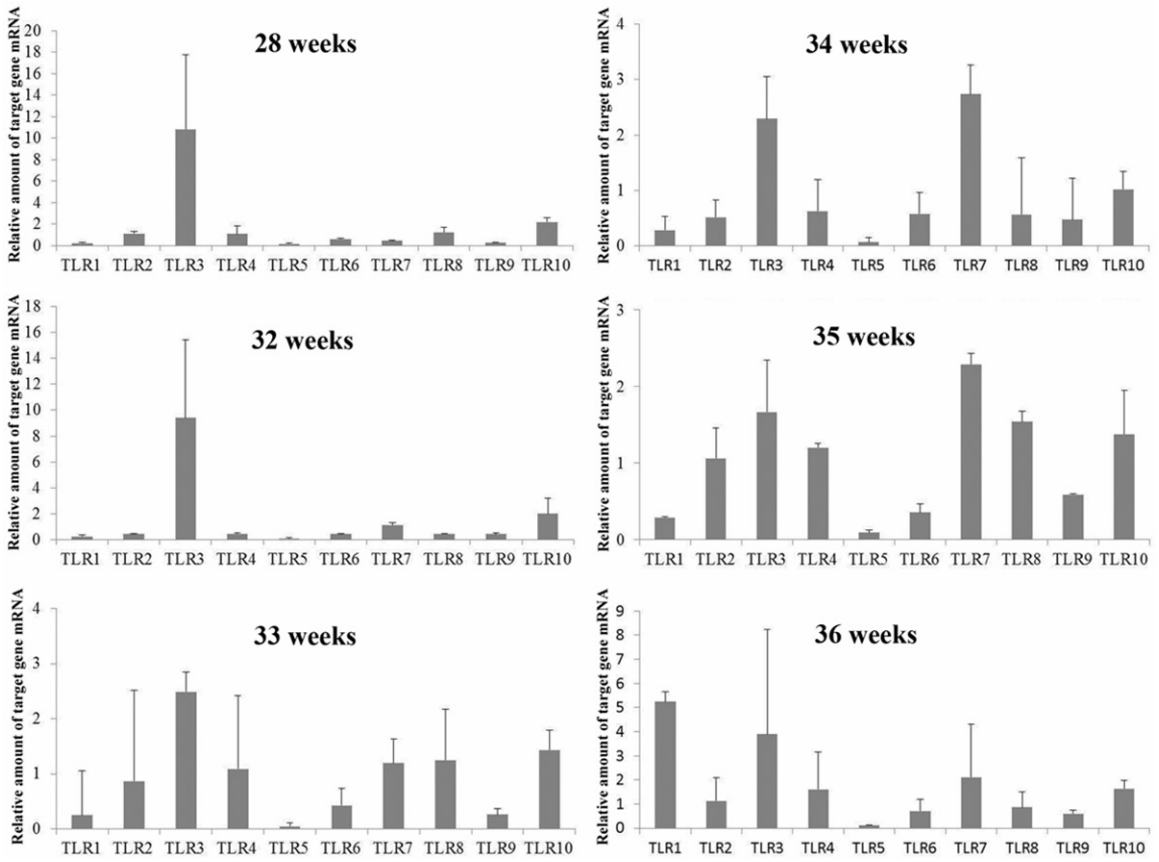

Figure 1. The transcription of TLR 1-10 from the 28th to the 36th gestational week.

assay. Individual gene expression was measured by a comparative CT (ΔΔCT) real time PCR using an ABI step one plus instrument with Quantifast SYBR green kit (Qiagen, USA). Beta-actin (B-ACT) expression was used as an internal control. Before using the ΔΔCT method for quantitation, a validation experiment was performed for primer efficiency investigation. Real time PCR was performed under the following conditions; 40 cycles during 5 minutes at 95°C for hot star taq polymerase activation, 10 seconds at 95°C for denaturation and 30 seconds at 60°C for annealing and extension.

Statistical analyses: All data are presented as mean ± sd. Independent sample t-test, paired sample t-test, ANOVA and linear correlation analyses were performed by using SPSS for Windows (Version 12.0. Chicago, SPSS Inc.)

Results

Information of the four preterm births are listed in **Table 1**. There were two cases of 28, one case of 29 and one case of 30 GWs at birth.

The transcriptions of TLR1-10 changed between 28 weeks and 36 weeks. At 28 weeks the m-RNA level of TLR3 was about 6-12 times of all other TLRs, while at 36 weeks the proportion of TLR3 m-RNA was about 2-4 times of other TLRs except TLR1 (**Figure 1**).

We found that TLR1, TLR3 and TLR7 transcription changed significantly. TLR1 transcription started dramatically to increase from the 36th GW and TLR3 began to decrease at the 28th GW, while TLR7 transcription significantly increased after the 33rd GW (**Figures 2**, **3**).

NFkB was highest expressed in GW 32, which was accompanied by high TBK1 expression at week 28 and 32 and indicated an NFkB activation peak at this period. At the same time the transcription rates of the kinases TAK1 and IRAK4, the interferon transcription factor IRF3, the cytokine IL-4, the differentiation factors CD14, CD80 and CD86 as well as the adapter molecule TIRAP peaked, indicating a boost of immune system component development dur-

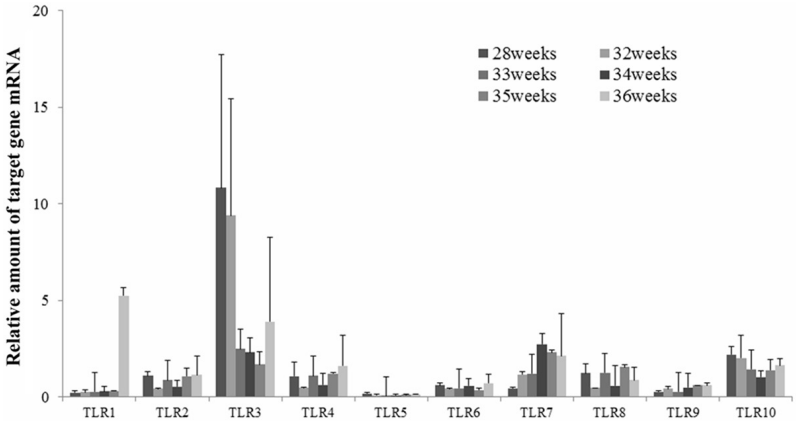

Figure 2. The expression of TLR1-10 from the 28th to 36th gestational weeks.

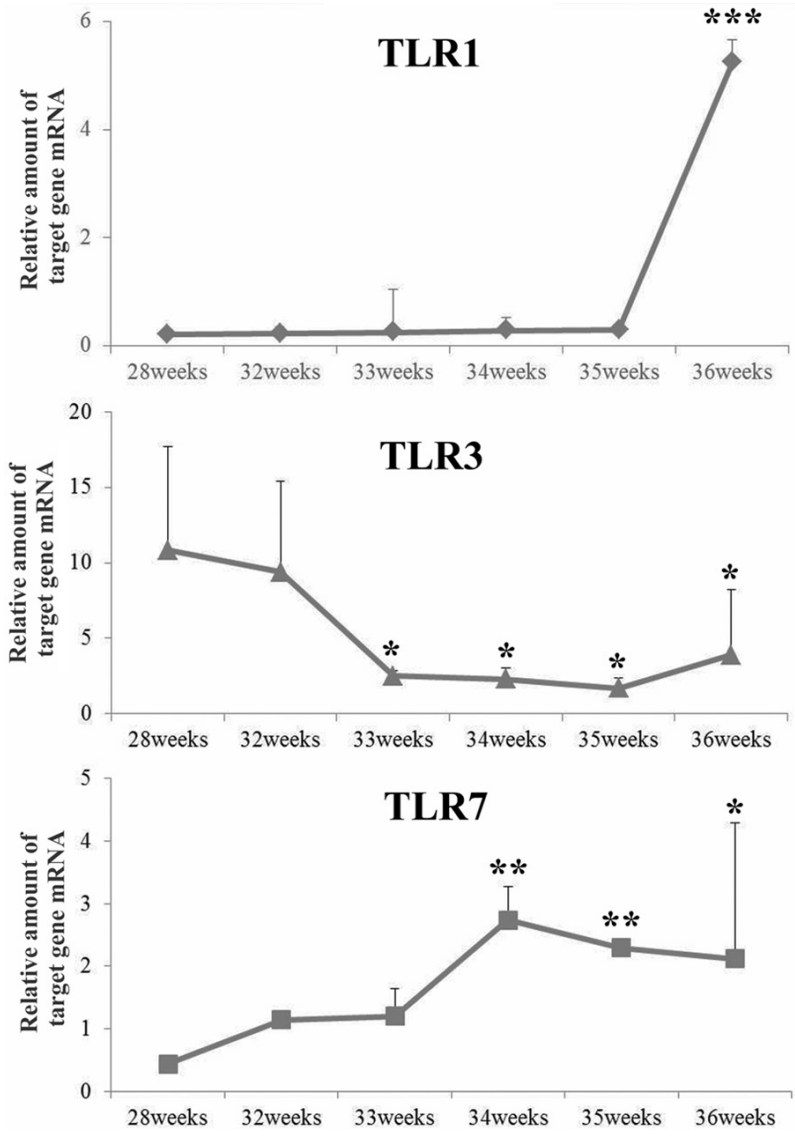

Figure 3. Transcriptions of TLR1, TLR3 and TLR7 at different gestational ages.

ing the 28th to 32nd GW. Thereafter in the 33rd to 34th GW the TLR4 specific surface LPS recognition protein MD2, the adapter molecule TRIF, the signaling transducer TRAF6, interferon (IFN) as well as the kinase IRAK1 transcription peaked, the later one accompanied with a second NFkB transcription enhancement at week 34. In the final phase of maturation at the 35th GW, only MYD88 and β 2 microglobulin showed highest transcription rates, whereas in the 36th GW all determined factors were transcribed to lower degrees than their peak levels during the development (**Table 2**).

Discussion

In our study, we analyzed TLR transcription pattern of premature births from the 28th to the 34th GW. Dasari. et.al noted no expression differences of the TLR family members in mononuclear cells and T-lymphocytes between full-term child and adult peripheral blood [13]. Therefor the TLR system must be developed during the last GWs and might be an important factor for the weaker immune functions in preterm newborns. Our results showed this trend, since in the 28th and 32nd GW, beside TLR3, all other TLRs were only marginal transcribed. Impairment of TLR3 and TLR7 were suggested to be the reason for preterm births [14] and our data revealed that TLR3 was high but TLR7 low transcribed at the 28th GW and the low TLR7 m-RNA level might have been a causative factor for the preterm birth

Table 2. The expression of immune system related factors at different gestational stages

		28 weeks	32 weeks	33 weeks	34 weeks	35 weeks	36 weeks
NF-KB	NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA.	1.28±0.03	2.37±0.08	1.06±0.15	1.66±0.23	1.51±0.06	1.24±0.57
TBK1	TANK-binding kinase 1 (TBK1) is similar to IKB kinases and can mediate NFKB activation via inactivation of the inhibited by I-kappa-B (IKB) proteins thereby allowing activation and nuclear translocation of the NFKB complex	2.20±0.42	2.00±0.65	1.43±0.54	0.85±0.25	0.98±0.22	0.89±0.10
Adapter Molecules							
MD-2	The Lymphocyte antigen 96 (MD-2) is associated with toll-like receptor 4 on the cell surface and confers responsiveness to lipopolysaccharides (LPS)	1.11±1.26	0.81±0.79	1.25±0.84	1.20±0.15	0.70±0.25	1.09±0.85
MYD88	Myeloid differentiation primary response gene 88 (MYD88) is a universal adapter protein used by almost all TLRs (except TLR 3) to activate the transcription factor NF-κB. TIRAP is necessary to recruit Myd 88 to TLR 2 and TLR 4, and MyD88 then signals through IRAK.	0.31±0.48	0.51±0.10	0.32±0.02	0.62±0.79	0.83±0.33	0.73±0.18
TRIF	TIR-domain-containing adapter-inducing interferon-β (TRIF) is an adapter in responding to activation of TLRs.	0.63±0.56	0.64±0.38	0.63±0.01	1.44±0.14	0.45±0.17	0.34±0.57
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) is an adapter molecule associated with the TLR4 signaling pathway of the immune system.	0.73±0.46	2.85±2.43	0.54±0.18	0.87±0.34	0.62±0.01	1.37±0.64
Kinases							
IRAK1	The interleukin-1 receptor-associated kinase 1 is partially responsible for IL1-induced up-regulation of the transcription factor NF-kappa B.	0.45±0.35	0.60±0.24	0.48±0.08	0.86±0.06	0.83±0.05	0.79±0.23
IRAK4	IRAK-4 (interleukin-1 receptor-associated kinase 4), in the IRAK family, is a protein kinase involved in signaling innate immune responses from Toll-like receptors. It also supports signaling from T-cell receptors.	0.94±0.26	1.61±0.33	0.81±0.24	0.97±0.23	0.87±20	0.88±0.14
TAK1	Transforming growth factor beta activated kinase-1 (TAK1) is a member of the serine/threonine protein kinase family. In response to IL-1, this protein forms a kinase complex including TRAF6, MAP3K7P1/TAB1 and MAP3K7P2/TAB2, which is required for the activation of nuclear factor kappa B.	2.15±0.98	1.15±0.60	1.69±1.34	0.74±0.11	0.90±0.29	0.73±0.53
TAB1	TGF-beta activated kinase 1/MAP3K7 binding protein 1 (TAB1) is a regulator of the MAP kinase MAP3K7/TAK1 and activates TAK1 kinase	0.42±0.19	0.99±0.09	0.52±0.13	1.12±0.19	0.76±0.01	0.93±0.84
Signal transducers							
TRAF6	The TNF receptor associated factor (TRAF) 6 protein mediates signal transduction from members of the TNF receptor superfamily and from the Toll/IL-1 family. Signals from receptors such as CD40, TNFSF11/TRANCE/RANKL and IL-1	0.68±0.20	0.49±0.05	1.16±1.21	1.04±0.40	0.70±0.05	0.64±0.21
IRF3	The interferon regulatory transcription factor 3 (IRF3) plays an important role in the innate immune system's response to viral infections.	1.97±0.39	2.15±1.17	1.55±0.36	1.20±0.40	0.97±0.57	1.15±0.23
Differentiation molecules							
CD14	Cluster of differentiation (CD14) is a component of the innate immune system and is anchored to the membrane by a glycosylphosphatidylinositol tail (mCD14) or exists as a soluble form (sCD14)	2.45±0.82	1.76±0.15	1.98±0.79	0.74±0.55	0.53±0.10	0.70±0.15
CD80	Cluster of Differentiation 80 (also CD80 and B7-1) is expressed on activated B cells and monocytes that provides a co-stimulatory signal necessary for T cell activation and survival	2.20±0.39	2.00±1.17	1.43±0.36	2.11±1.53	1.38±0.57	0.52±0.32
CD86	Cluster of Differentiation 86 (also known as CD86 and B7-2) is expressed on antigen-presenting cells that provide co-stimulatory signals necessary for T cell activation and survival.	0.37±0.35	1.65±1.11	1.43±0.15	1.29±0.61	0.91±0.01	0.65±0.44
B2M	β 2 microglobulin also known as B2M is a component of major histocompatibility complex (MHC) class I molecules.	1.06±0.39	1.00±0.08	0.80±0.45	1.33±0.02	1.38±0.07	1.19±0.56
Cell signaling molecules							
IFN	Interferons (IFNs) are released by host cells in response to pathogens	3.19±0.71	3.36±1.93	5.42±1.93	3.92±1.92	2.42±1.64	3.25±0.79
IL4	Interleukin 4 is a cytokine that stimulates the proliferation of activated B-cells	0.21±0.09	1.63±0.15	0.36±0.21	0.41±0.41	0.37±0.09	0.48±0.63

time. TLR3 has been shown to be highly expressed in the immature but lower in the mature brain and was suggested to mediate preconditioning against ischemic injury [15]. Our results showed a similar TLR3 transcription pattern, with high transcription rates at 28th-32nd GWs, which decreased then until maturity (**Figure 3**). m-RNA levels of TLR4 which is the major LPS activated TLR were low and MD-2 peaked late in the 33rd GW, indicating a lack of defense mechanisms against bacterial infections of preterm births particular in the first weeks after birth, which is in accordance with previous literature proposing an asynchronous developmental hierarchy of anti-microbial response maturation in neonates born early in gestation [16].

TLR signaling is divided in the MyD88-dependent and TRIF-dependent pathway and four adapter molecules are involved in the signaling. These proteins are MyD88, Tirap, Trif, and toll-like receptor 4 adaptor protein (Tram) [17-19]. The MyD88-dependent response occurs on dimerization of the TLR receptor and is utilized by every TLR except TLR3. Its primary effect is activation of NFκB and Mitogen-activated protein kinase. Ligand binding and conformational change leads to receptor recruitment of the adaptor protein MyD88, a member of the TIR family. MyD88 then recruits IRAK 4, IRAK1 and IRAK2. IRAK kinases then phosphorylate and activate TRAF6, which in turn polyubiquinates the TAK1, as well as itself in order to facilitate binding to IKKβ. On binding, TAK1 phosphorylates IKKβ, which then phosphorylates IκB causing its degradation and allowing NFκB to diffuse into the cell nucleus and activate transcription and consequent induction of downstream factors. Both TLR3 and TLR4 utilize the TRIF-dependent pathway, which is triggered by dsRNA and LPS, respectively. For TLR3, dsRNA leads to activation of the receptor and recruitment of the adaptor TRIF. TRIF activates the kinases TBK1 and RIP1, which creates a branch in the signaling pathway. The TRIF/TBK1 signaling complex phosphorylates IRF3 allowing its translocation into the nucleus and production of Interferon type I. Meanwhile, activation of RIP1 causes the polyubiquitination and activation of TAK1 and NFκB transcription in the same way as the MyD88-dependent pathway [20]. The data shown in **Table 2** indicated that immune system-related factor expressions occur mainly in the 32th to 35th GW of preterm births. Among these factors, TAK1 is essential for the activation of NF-kB and TAB1, and the increased activity of TIRAP in the 32nd and in the 36th GW might improve the preterm newborns' innate immunity and resistance to environmental microbial challenge, whereas the late transcriptional enhancements of TLR1 and MYD88 as well as β 2 microglobulin might be immune system compromising in the early maturation stages.

A drawback of our study was that we analyzed blood of only four preterm births and we cannot conclude, that our data reflect the pattern in normal term infants during this period of development. In addition premature births might already be immune system compromised [14, 21]. Furthermore, environmental factors might have influenced the immune system maturation, which is otherwise protected by intrauterine mechanisms.

Conclusion

In the early stage of maturation, the immune system is not mature, but high transcription levels of TLR1, MyD88 and B2M appeared in the later stage of maturation, suggesting that these factors might contribute to an enhanced defense during maturing of preterm births.

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Disclosure of conflict of interest

None.

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