

# ***In vitro* screening of LPS-induced miRNAs in leukocytes derived from cord blood and their possible roles in regulating TLR signals**

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**BACKGROUND:** microRNAs (miRNAs) are involved in a wide variety of biological processes and play roles in the regulation of Toll-like receptor (TLR) signals. We hypothesized that lipopolysaccharide (LPS)-induced miRNAs in the leukocytes from cord blood (CB) play an important role in newborn innate immunity, specifically in the regulation of the TLR signaling pathway.

**METHODS:** The expression profiles of LPS-induced miRNAs and TLR-related genes were studied by microarray and PCR arrays, respectively. A bioinformatics analysis was used to identify the potential biological processes and targets involved in the TLR signals affected by these miRNAs.

**RESULTS:** A total of 85 miRNAs and 41 TLR-related genes were differentially expressed. The bioinformatics analysis showed that the potential target genes of these miRNAs were involved in regulation of cellular biosynthetic process, regulation of gene expression, regulation of macromolecule biosynthetic process, etc. Thirteen potential miRNA–mRNA interaction sites were found within the cDNA sequences of 11 differentially expressed TLR signaling pathway genes.

**CONCLUSION:** We identified a global miRNA expression signature occurring during LPS-induced acute inflammation in leukocytes derived from CB. The target genes were mainly involved in several biological processes, and these miRNAs may play important roles in the regulation of TLR signals. However, the precise mechanisms require further validation.

The innate immune response mediated by immune cells involves the initial recognition of conserved pathogen-associated molecular patterns by members of the Toll-like receptor (TLR) family. Lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, is the principal pathogen-associated molecular pattern of TLR4 (1). The immune system detects and responds to LPS via TLR4 and activates various transcription factors, leading to the strong production of proinflammatory cytokines (2). Sepsis, which leads to severe adverse outcomes such as organ dysfunction and death, can develop when the host defensive response

to an infection is magnified (3). In human cord blood (CB) mononuclear cells, this LPS-triggered activation of the proinflammatory response results in many dysregulated genes that have been previously characterized by genome-wide expression profiling methods (4).

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression either by the cleavage of mRNA targets or by the inhibition of protein translation. miRNAs act as key regulators in a wide variety of biological processes, including proliferation, differentiation, cell fate determination, apoptosis, signal transduction, and organ development, and abnormal miRNAs expression is a common feature of inflammatory diseases (5). Furthermore, the differential expression of miRNAs may help to distinguish between disease states (6). miRNAs have been identified as important regulators of immune responses (7) and as fine-tuners of TLRs (8). Multiple molecules involved in the TLR pathway are thought to be targeted by miRNAs, such as the expression of the TLRs themselves, TLR signal molecules, TLR-induced transcription factors, regulators of the TLR signaling pathway, and even the final functional cytokines involved in TLR signaling (9).

Although miRNAs (miR-18a and miR-155) have been proven to show significant expression changes in CB-derived CD14<sup>+</sup> cells in response to LPS (10) and the involvement of miR-146 in neonatal TLR4 signaling has been discussed to some extent (11), the global changes in the miRNA expression of leukocytes derived from CB during LPS activation and their biological and molecular mechanisms are still unclear. In this study, we will explore the LPS-induced miRNA expression profile in the cord leukocytes and speculate about the role of these miRNAs in newborn innate immunity, focusing specifically on TLR signaling pathway regulation.

## RESULTS

### LPS-Induced miRNA Expression Profiling

To identify the miRNAs involved in the regulation of the LPS-induced immune response, we used a commercial microarray chip that can examine the global expression level for each *Homo sapiens* miRNA annotated in miRBase 14.0. In

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leukocytes derived from newborn CB ( $n = 3$ ), we identified 85 miRNAs that were differentially expressed between the experimental and control groups, with 37 miRNAs being upregulated and 48 being downregulated (Table 1).

**Microarray-Based GO Analysis**

To assess the functions of these differentially expressed miRNAs, we performed computational predictions of the potential targets for these miRNAs via two algorithms (TargetScan and miRDB) and focused on those targets that were predicted by both. Gene Ontology (GO) analysis was applied to these putative target genes using DAVID (Database for Annotation, Visualization and Integrated Discovery). Interestingly, the most enriched GO terms for these target genes of the up- or downregulated miRNAs were extremely consistent. They were regulation of cellular biosynthetic process, regulation of gene expression, regulation of macromolecule biosynthetic process, regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, regulation of transcription, etc. (Figure 1a,b).

**LPS-Induced TLR-Related Genes Expression**

To determine the involvement of the TLRs in LPS treatment, 84 TLR-related genes were profiled by PCR array after an LPS challenge. LPS stimulation modulated the expression of 41 of the 84 genes examined; of those 41 genes, 28 were upregulated and 13 were downregulated compared with their expression levels in unstimulated cells ( $n = 3$ ; fold change > 2; Table 2). These differentially expressed genes in the TLR signaling pathway can be classified into several groups. (i) TLRs: *TLR2* and *TRL7* were upregulated, whereas *TLR1*, *TLR5*, *TLR6*, *TLR8*, and *TLR9* were downregulated. (ii) TLR signaling. **MyD88-dependent:** *MyD88* and *IRAK2* were upregulated. **Toll/IL-1 receptor domain-containing adaptor inducing IFN- $\beta$ -dependent:** *TBK1* was upregulated. **Negative regulation:** *SIGIRR* was downregulated. (iii) Downstream pathways and target genes. **Nuclear factor (NF)- $\kappa$ B pathway:** *IKKBK*, *REL*, *UBE2N*, *NFKB1*, *NFKBIA*, and *CHUK* were upregulated, whereas *BTK*, *UBE2V1*, and *NFRKB* were downregulated. **c-Jun N-terminal kinase/p38 pathway:** *FOS*, *MAP3K7IP1*, and *MAPK8IP3* were downregulated. **Janus kinase/signal transducer and activator of transcription pathway:** *CCL2*, *CSF2*, and *interleukin (IL)-6* were upregulated. **IRF pathway:** *CXCL10*, *IFNA1*, and *IFNB1* were upregulated. **Cytokine-mediated signaling pathway:** *CSF3*, *IL-1A*, *IL-1B*, and *TNF* were upregulated. (iv) Regulation of adaptive immunity: *CD80* and *IL-10* were upregulated, whereas *CD86* was downregulated. (v) Adaptors and TLR-interacting proteins: *CLEC4E* and *TICAM1* were upregulated. (vi) Inflammatory mediators: *LTA*, *IL-8*, and *PTGS2* were upregulated.

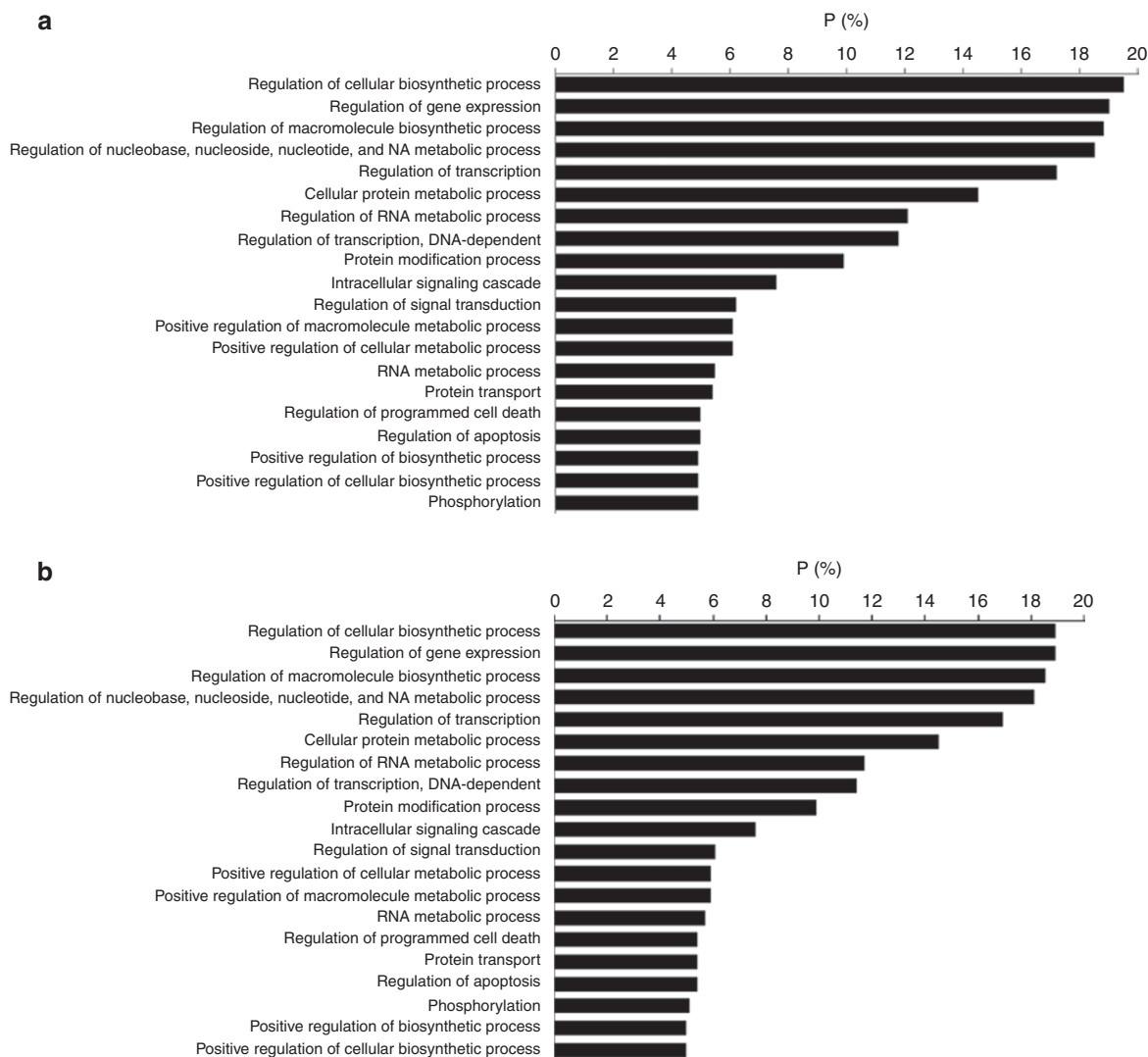
**Predicted Target Genes of LPS-Responsive miRNAs in the TLR Signaling Pathway**

To gain insight into the function of the LPS-responsive miRNAs in the TLR signaling pathway, we used TargetScan and miRDB to predict the potential targets in the TLR signaling pathway for these miRNAs and focused on miRNA-mRNA

**Table 1.** The list of differentially induced miRNAs in leukocytes derived from infant cord blood stimulated with LPS for 2 h

miRNA name (upregulated)	Fold change	miRNA name (downregulated)	Fold change
hsa-miR-130a	2.1	hsa-let-7i	-2.8
hsa-miR-146b-5p	3.2	hsa-miR-107	-2.3
hsa-miR-141	36.6	hsa-miR-16	-2.2
hsa-miR-142-3p	3.0	hsa-miR-182	-48.8
hsa-miR-193a-3p	2.8	hsa-miR-183	-7.8
hsa-miR-203	16.1	hsa-miR-194	-3.3
hsa-miR-214	10.4	hsa-miR-215	-2.2
hsa-miR-377	13.3	hsa-miR-29b	-2.9
hsa-miR-200c	8.7	hsa-miR-29c	-3.3
hsa-miR-491-3p	3.1	hsa-miR-96	-23.9
hsa-miRPlus-A1015	2.5	hsa-miR-627	-3.7
hsa-miR-199a-5p	3.5	hsa-miR-550a-3p	-3.1
hsa-miR-125b	28.7	hsa-miR-92b	-3.5
hsa-miR-129-5p	3.0	hsa-let-7b	-8.8
hsa-miR-933	3.0	hsa-let-7f	-3.9
hsa-miR-326	3.6	hsa-miR-106b-3p	-2.4
hsa-miR-34b	2.0	hsa-miR-500	-2.7
hsa-miR-720	2.8	hsa-miR-186	-2.3
hsa-miR-181a	3.2	hsa-miR-190	-4.9
hsa-miRPlus-E1104	3.1	hsa-miR-574-3p	-2.0
hsa-miRPlus-E1088	2.1	hsa-miR-486-5p	-2.1
hsa-miR-1246	4.1	hsa-miR-126-5p	-4.6
hsa-miRPlus-E1117	2.4	hsa-miR-576-5p	-5.1
hsa-miRPlus-F1195	2.0	hsa-miR-362-5p	-4.4
hsa-miR-205	1407.1	hsa-miR-582-5p	-6.6
hsa-miR-136	103.8	hsa-miR-338-3p	-2.6
hsa-miR-142-5p	2.8	hsa-miR-20a-3p	-2.5
hsa-let-7c	2.5	hsa-miR-25	-3.1
hsa-miR-210	4.1	hsa-miR-943	-3.1
hsa-miR-33a	3.6	hsa-miR-339-5p	-2.3
hsa-miR-494	2.8	hsa-miR-197	-2.0
hsa-miR-27b	2.4	hsa-miR-652	-2.6
hsa-miR-33b	4.2	hsa-miR-451	-3.2
hsa-miR-200b	125.0	hsa-miR-16-2-3p	-9.8
hsa-miR-1260	3.6	hsa-miRPlus-E1285	-2.2
hsa-miR-3202	10.5	hsa-miRPlus-F1181	-2.1
hsa-miR-1973	7.8	hsa-miRPlus-E1245	-71.9
		hsa-miR-1827	-2.3
		hsa-miR-1297	-5.5
		hsa-miR-29a-5p	-2.5
		hsa-let-7i-3p	-4.2
		hsa-miR-484	-2.4
		hsa-miR-374a	-2.6
		hsa-miR-20b	-2.7
		hsa-let-7d	-3.3
		hsa-let-7a	-2.7
		hsa-miR-502-3p	-3.3
		hsa-miR-103a-2-5p	-3.9

LPS, lipopolysaccharide; miRNA, microRNA.



**Figure 1.** Gene Ontology (GO) category for putative target genes. (a) and (b) show the significant GOs of differential genes targeted by the upregulated and downregulated miRNAs, respectively. P is the percentage of predicted target genes, and larger P values indicate higher levels of enrichment. NA, nucleic acid.

pairs that were coexpressed in leukocytes. A total of 13 potential miRNA–mRNA interaction sites for 10 differentially expressed miRNAs were found within the cDNA sequences of 11 differentially expressed TLR signaling pathway genes (Table 3).

#### Quantitative Real-Time PCR Verification of miRNA Array Results

To confirm the results of the miRNA array, we selected six miRNAs from Table 3 for further validation in another set of samples from different subjects ( $n = 3$ ). The quantitative real-time (RT)-PCR results showed that the miRNAs were up- or downregulated in a manner consistent with the miRNA array results (Figure 2). MiR-129-5p and miR-1260 were upregulated in the LPS-stimulated group, whereas miR-183, miR-186, miR-339-5p, and miR-374a were downregulated.

#### DISCUSSION

In the present study, we analyzed the miRNA expression profiles in the leukocytes derived from newborn CB after 2 h of

LPS stimulation. We found 85 miRNAs, with 37 being upregulated and 48 being downregulated after LPS stimulation. These findings indicated that the response to LPS might induce rapid and specific *in vitro* changes to the miRNA expression profile of leukocytes derived from CB and that these miRNAs might play important roles in the neonatal immune response. However, some miRNAs, including miR-150, miR-342, let-7g, and miR-143, which have been shown to be differentially expressed in LPS-stimulated leukocytes derived from adult venous blood (12), were not changed in the LPS-stimulated leukocytes derived from CB; in addition, miR-146b, which was downregulated in adults, was upregulated in the CB, indicating that miRNAs might respond differently in LPS-stimulated leukocytes in newborns than in adults and play contrastingly different roles between them.

Among the differentially expressed miRNAs, miR-205, miR-136, miR-200b, miR-141, and miR-182 were highly deregulated. Most of these miRNAs have been shown to be involved in both normal development and cancer. The miR-200 family

**Table 2.** LPS-induced gene expression in TLR signaling pathway

Gene symbol	Gene description	Gene bank	Fold change
TLRs			
<i>TLR2</i>	Toll-like receptor 2	NM_003264	2.3
<i>TLR7</i>	Toll-like receptor 7	NM_016562	3.4
<i>TLR1</i>	Toll-like receptor 1	NM_003263	-2.8
<i>TLR5</i>	Toll-like receptor 5	NM_003268	-3.7
<i>TLR6</i>	Toll-like receptor 6	NM_006068	-2.5
<i>TLR8</i>	Toll-like receptor 8	NM_138636	-2.7
<i>TLR9</i>	Toll-like receptor 9	NM_017442	-2.4
TLR signaling			
MyD88-dependent			
<i>MyD88</i>	Myeloid differentiation primary response gene (88)	NM_002468	2.3
<i>IRAK2</i>	Interleukin-1 receptor-associated kinase 2	NM_001570	30.3
TRIF-dependent			
<i>TBK1</i>	TANK-binding kinase 1	NM_013254	2.3
Negative Regulation			
<i>SIGIRR</i>	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	NM_021805	-2.0
Downstream pathways and target genes			
NF- $\kappa$ B pathway			
<i>IKKB</i>	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase $\beta$	NM_001556	2.4
<i>REL</i>	V-rel reticuloendotheliosis viral oncogene homolog (avian)	NM_002908	2.2
<i>UBE2N</i>	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	NM_003348	2.4
<i>NFKB1</i>	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NM_003998	8.8
<i>NFKBIA</i>	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, $\alpha$	NM_020529	9.3
<i>CHUK</i>	Conserved helix-loop-helix ubiquitous kinase	NM_001278	2.3
<i>BTK</i>	Bruton agammaglobulinemia tyrosine kinase	NM_000061	-2.4
<i>UBE2V1</i>	Ubiquitin-conjugating enzyme E2 variant 1	NM_021988	-3.8
<i>NFRKB</i>	Nuclear factor related to kappaB binding protein	NM_006165	-3.3
JNK/p38 pathway			
<i>FOS</i>	V-fos FBJ murine osteosarcoma viral oncogene homolog	NM_005252	-3.3
<i>MAP3K7IP1</i>	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	NM_006116	-2.1
<i>MAPK8IP3</i>	Mitogen-activated protein kinase 8 interacting protein 3	NM_015133	-2.1
JAK/STAT pathway			
<i>CCL2</i>	Chemokine (C-C motif) ligand 2	NM_002982	55.0
<i>CSF2</i>	Colony stimulating factor 2 (granulocyte-macrophage)	NM_000758	3.7
<i>IL-6</i>	Interleukin 6 (interferon $\beta$ 2)	NM_000600	272.7
IRF pathway			
<i>CXCL10</i>	Chemokine (C-X-C motif) ligand 10	NM_001565	129.8
<i>IFNA1</i>	Interferon $\alpha$ 1	NM_024013	2.6
<i>IFNB1</i>	Interferon $\beta$ 1, fibroblast	NM_002176	17.6
Cytokine-mediated signaling pathway			
<i>CSF3</i>	Colony stimulating factor 3 (granulocyte)	NM_000759	13.8
<i>IL-1A</i>	Interleukin 1 $\alpha$	NM_000575	486.4
<i>IL-1B</i>	Interleukin 1 $\beta$	NM_000576	118.3
<i>TNF</i>	Tumor necrosis factor (TNF superfamily, member 2)	NM_000594	32.0

Table 2. Continued on next page.

**Table 2.** (Continued)

Gene symbol	Gene description	Gene bank	Fold change
Regulation of adaptive immunity			
<i>CD80</i>	CD80 molecule	NM_005191	2.0
<i>IL-10</i>	Interleukin 10	NM_000572	3.5
<i>CD86</i>	CD86 molecule	NM_006889	-2.5
Adaptors and TLR-interacting proteins			
<i>CLEC4E</i>	C-type lectin domain family 4, member E	NM_014358	5.8
<i>TICAM1</i>	Toll-like receptor adaptor molecule 1	NM_182919	7.0
Inflammatory mediators			
<i>LTA</i>	Lymphotoxin $\alpha$ (TNF superfamily, member 1)	NM_000595	4.3
<i>IL-8</i>	Interleukin 8	NM_000584	20.9
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	NM_000963	3.1

JAK/STAT, Janus kinase/signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; NF, nuclear factor; TLR, Toll-like receptor; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN- $\beta$ .

(miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 are frequently silenced in advanced cancer. A study on mammary gland stem or progenitor cells showed that miR-205 overexpression led to an expansion of the progenitor-cell population, decreased cell size, and increased cellular proliferation (13). However, there is no report on the effect of the miR-200 family on immune cells. In our study, the higher expression levels of miR-200b, miR-141, and miR-205 may be related to the increased number of stem cells in the CB. As an important immune regulator, miR-182 has been demonstrated to have a central role in the physiological regulation of the IL-2-driven helper T-cell-mediated immune response (14). Therefore, the downregulation of miR-182 might have special significance for LPS-induced immune response. However, although these miRNAs were obviously deregulated in LPS-stimulated leukocytes derived from CB, their immune functions in newborn inflammation must be carefully interpreted.

To gain significant insight into the function of these LPS-induced differentially expressed miRNAs, their target genes were predicted, and GO analysis was applied to these predicted target genes. The GO categories showed that the biological processes regulated by the differentially expressed miRNAs mainly included regulation of cellular biosynthetic process, regulation of gene expression, regulation of macromolecule biosynthetic process, regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process, regulation of transcription, etc. for both the up- and downregulated miRNAs. This finding indicated that these miRNAs might act together on the same biological processes to control the robust proinflammatory response in the acute inflammatory state induced by LPS stimulation. Additionally, the regulation of gene expression and transcription by target genes could be an indirect mechanism to help the miRNAs strengthen this control.

In contrast with the study that used cRNA microarray technology to identify differentially expressed genes involved in the LPS-stimulated response of mononuclear cells from CB on a "global" level (4) and the study that detected certain factors

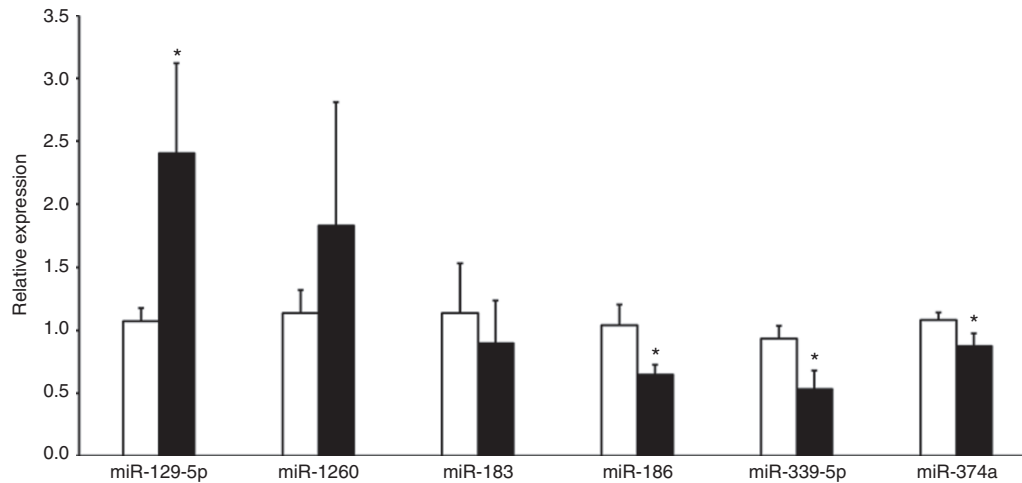
**Table 3.** Target genes of LPS-responsive miRNAs in TLR signaling pathway

miRNA	Gene symbol
Upregulated	
hsa-miR-129-5p	<i>CD86</i>
hsa-miR-494	<i>TLR6</i>
hsa-miR-1260	<i>CD86</i>
Downregulated	
hsa-miR-16	<i>CD80</i>
hsa-miR-183	<i>NFKB1, PTGS2</i>
hsa-miR-186	<i>UBE2N</i>
hsa-miR-339-5p	<i>CSF3, IRAK2</i>
hsa-miR-374a	<i>CCL2, PTGS2</i>
hsa-miR-500a	<i>IL-8</i>
hsa-miR-1827	<i>LTA</i>

LPS, lipopolysaccharide; miRNA, microRNA; TLR, Toll-like receptor.

related to the LPS-induced TLR pathway response in the monocytes derived from CB (11), the aim of this exploratory study was to determine which genes in the LPS-stimulated leukocytes from CB that are associated with TLRs and their signaling elements were differentially expressed on the whole. In our study, 41 of the 84 genes examined were found to be differentially expressed in TLR signaling pathways between leukocytes from CB with or without LPS stimulation. Via the MyD88-dependent pathway, which induces NF- $\kappa$ B-dependent proinflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ), and the MyD88-independent pathway, which induces Toll/IL-1 receptor domain-containing adaptor inducing IFN- $\beta$ -dependent interferon-inducible cytokines (15,16), LPS-stimulated leukocytes from CB showed a predominant initial increase in the production of inflammatory cytokines.

The components of TLR signaling can be directly targeted by miRNAs. For example, the mRNA encoding MyD88, which links members of the TLR family to the downstream



**Figure 2.** Selected microRNA (miRNA) array results were confirmed by real-time PCR in another set of samples from different subjects ( $n = 3$ ). The expression of six miRNAs in lipopolysaccharide-stimulated group (black bar) was compared with that of the control group (white bar). The relative expression values are the normalized mean  $\pm$  SD. \* $P < 0.05$  by the  $t$ -test.

activation of NF- $\kappa$ B and mitogen-activated protein kinases (17), could be directly repressed by miR-200b and miR-200c (18). Proinflammatory IL-6 and *TNF* are direct targets of Let-7a (19) and miR-16 (20), respectively. We think that the inhibition of the interaction between the LPS-induced miRNAs and their targets and/or insufficient expression of these miRNAs that target TLR signaling might contribute to the robust proinflammatory response in the acute inflammatory state induced by LPS stimulation. Further studies will be needed to evaluate these issues.

miRNA expression can be directly regulated by TLR signals. For example, miR-181a was found to be concurrently upregulated with IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in a mouse acute inflammatory state via a TLR4-dependent pathway (21). MiR-107, which was downregulated in response to LPS in multiple cell types (22), and let-7i, which was decreased in LPS-stimulated cholangiocytes (23), were found to be affected in a MyD88- and NF- $\kappa$ B-dependent manner. Similar coexpression patterns between these miRNAs and TLR signaling compartments in our study might support those results. The interaction between miRNAs and the TLR signaling pathway reveals a feedback loop that controls TLR signaling activation (24).

In LPS-stimulated THP-1 cells, upregulated miR-146 has been proposed to play a role in the control of TLR and cytokine signaling through a negative feedback regulation loop (25). The upregulation of miR-146 after LPS stimulation was also observed in monocytes derived from adult peripheral veins and from newborn CB (11). After LPS engagement, monocytes upregulate miR-146b which modulates the TLR4 signaling pathway by direct targeting of multiple elements, including TLR4, MyD88, IRAK-1, and TRAF6, via an IL-10-mediated STAT3-dependent loop (26). In addition, activation of the MAP kinase/EGR pathway by proinflammatory cytokines regulates the transcription of the miR-146, which can in turn repress the NF- $\kappa$ B, AP-1, and MAPK/EGR pathways. Thus, a negative feedback loop is formed to control the proinflammatory signaling in endothelial cells that may impact the

pathogenesis of vascular inflammatory diseases such as sepsis (27). In our study, the expression of miR-146b was upregulated in CB leukocytes after LPS stimulation, indicating that miR-146b might have an important role in controlling LPS-stimulated neonatal early phases of inflammation by engaging in negative feedback loops.

The prediction analyses of the miRNA-mRNA pairs that are coexpressed in leukocytes, which may offer intriguing new perspectives on the interaction between miRNA regulation and TLR signals, identified 11 different candidate genes in the TLR signaling pathway for degradation control by miRNAs. *PTGS2*—a candidate target of the LPS-decreased miR-183 and miR-374a—was upregulated in leukocytes during LPS-induced inflammation. *PTGS2* is considered a proinflammatory mediator during the early phases of inflammation and a chief target for the treatment of inflammatory diseases (28). Hence, the decrease in miR-183 and miR-374a could play a role in supporting the upregulation of *PTGS2*, which may be partially responsible for the upregulation of some cytokines in our model.

This preliminary study has potential limitations. First, an *in vitro* study of whole blood may not reflect the patterns of response *in vivo*. Second, our study did not conduct functional studies in cells to validate the predicted mRNA targets. Third, the whole blood stimulation analysis limits the conclusions on cell-specific mechanisms involving interactions between miRNA regulation and TLR signaling. Further studies are necessary to determine whether these differentially expressed miRNAs regulate each of these predicted target genes and how these miRNAs influence the development of neonatal sepsis.

In conclusion, we identified differentially expressed miRNAs in the LPS-induced acute inflammation of leukocytes derived from CB and performed a functional bioinformatic analysis that demonstrated that the target genes regulated by these miRNAs were mainly involved in several biological processes. In addition, these miRNAs may play important roles in the regulation of TLR signals. Further investigation is needed to validate the involvement of these miRNAs in neonatal sepsis.

## METHODS

## Blood Sampling

CB samples were obtained by sterile puncture of the umbilical cords of six healthy, full-term infants delivered at the Obstetrics Department of the International Peace Maternity and Child Health Hospital of China Welfare Institute, Shanghai after obtaining informed consent from the parent(s). The newborns exhibited no signs of bacterial infection during a follow-up of 1 wk. Blood was collected in lithium heparin collection tubes and processed within 2 h after collection. The study was performed in accordance with the policies of and approved by the Ethics Committee of Children's Hospital of Fudan University, Shanghai, China.

## Whole Blood Cell Culture and LPS Stimulation

The blood samples were diluted 1:1 with RPMI 1640 culture medium supplemented with penicillin (100 IU/ml) and streptomycin (0.1 mg/ml) and immediately transferred as 6-ml aliquots into two pyrogen-free 15-ml Corning tubes. The diluted whole neonatal CB with (experimental group) or without (control group) 1 µg/ml *Escherichia coli* LPS (Sigma-Aldrich, St. Louis, MO) was incubated at 37 °C in a humidified chamber with 5% CO<sub>2</sub> for 2 h.

## Leukocyte Isolation and Total RNA Extraction

A 6-ml aliquot of whole blood culture was mixed with 1.2 ml of 6% dextran (Pharmacia, Uppsala, Sweden) solution in saline. After sedimentation for 30 min at 37 °C, the leukocyte-enriched supernatant was collected and washed twice with saline before the RNA isolation. Total RNA was isolated from leukocytes using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNA contamination was removed from the RNA preparations using DNase I (Qiagen, Hilden, Germany) followed by an RNA cleanup kit (RNeasy MinElute Cleanup Kit, Qiagen). The total RNA concentration and purity were determined by the NanoDrop ND-1000 (Thermo, Wilmington, DE). Electrophoresis (1% agarose gel containing ethidium bromide) was used to confirm the total RNA integrity. Only RNA samples passing the purity (OD<sub>260</sub>/OD<sub>280</sub>: 1.8–2.1) and integrity (28S:18S bands in a ratio of ~2:1 with a clear and trail-free manner and no nonspecific bands) controls were used for further miRNA microarray and reverse transcription PCR analyses.

## miRNA Array

Equal amounts of RNA from three different samples were pooled for each experimental condition and then labeled using the miRCURY Hy3/Hy5 Power labeling kit (Exiqon, Vedbaek, Denmark). The labeled RNA was hybridized on the miRCURY LNA Array (v.14.0) (Exiqon). Scanning was performed with the Axon GenePix 4000B microarray scanner (Axon, Foster, CA). The GenePix pro V6.0 software (Axon) was used to read the raw intensity of the image. The ratio of green to red signals was calculated after background subtraction and normalization using the global Lowess (Locally Weighted Scatter plot Smoothing) regression algorithm (MIDAS, TIGR Microarray Data Analysis System software; J. Craig Venter Institute, Rockville, MD and La Jolla, CA). The threshold value used to screen the up- and downregulated miRNAs was a fold change of >2.0.

## Quantitative RT-PCR

Quantitative RT-PCR was used to confirm the miRNA array results. Briefly, 10 ng of total RNA was reverse-transcribed using the TaqMan miRNA reverse transcription kit with the primers for miR-129-5p, miR-1260, miR-183, miR-186, miR-339-5p, miR-374a, and U6 small nuclear RNA (Applied Biosystems, Foster, CA). Then, the TaqMan probes for these miRNAs were used in the following real-time PCRs. The relative expression of each miRNA was determined in reference to an internal U6 small nuclear RNA control. In the statistical analysis, we presented the results as the mean ± SD and assessed the differences between the groups with the independent *t*-test using SPSS software 16.0 (SPSS, Chicago, IL).

Quantitative RT-PCR arrays were used to detect the expression of the TLR pathway genes. A total of 1.5 µg of RNA was used for cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen). Real-time PCR was performed using the 384-well RT<sup>2</sup> Profiler PCR Array (SuperArray Bioscience, Frederick, MD). A total of 84 genes involved in the human TLR pathway (full details are provided in

Supplementary Table S1) were analyzed for each sample. Two genes (*B2M* and *RPL13A*) were used as internal controls, and the average of their cycle threshold (Ct) values was used to normalize the gene expression ( $2^{-\Delta Ct}$ ) and determine the fold change between groups ( $2^{-\Delta\Delta Ct}$ ). Gene expression was considered up- or downregulated when the fold change was greater than 2.

## Target Gene Prediction and Functional Analysis

To predict the target genes of differentially expressed miRNAs, two online search algorithms, TargetScan Release 6.2 database (Whitehead Institute for Biomedical Research, Cambridge, MA) and miRDB Version 4.0 database (Department of Radiation Oncology, Washington University School of Medicine, St. Louis, MO) (29,30), were used for target prediction. The genes that were identified by both the algorithms were considered to be the target genes. To further understand the functions of the predicted target genes, we used the ontology classification of genes based on gene annotation and summary information available through DAVID Bioinformatics Resources 6.7 database (Laboratory of Immunopathogenesis and Bioinformatics, SAIC-Frederick, Frederick, MD) (31,32) to perform a preliminary analysis of the forecasted target genes.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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