

# Impaired trophoblast Toll-Like Receptor 3 signaling pathway involves in hepatitis B vaccine non- or hypo-response of infants born to HBsAg-positive mothers

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## Research article

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# Abstract

**Background:** Babies born to hepatitis B surface antigen (HBsAg) positive mothers bear a high risk of being non- or hypo-responsive to hepatitis B (HB) vaccine with unilluminated mechanisms. Placental immunity is closely related to the development of baby immune system, however, the roles of the placental immunity in the insufficient response of these babies are unclear. This study was aimed to investigate the role of placental trophoblast Toll-Like Receptor 3 (TLR3) signaling pathway in HB vaccine non- or hypo-response of these special babies.

**Methods:** A total of 399 pairs of HBsAg-positive mothers and their neonates were recruited to perform a nested case-control study. The maternal and children's HBV DNA and the HBV serological markers were detected by Fluorescence Quantitative Polymerase Chain Reaction (FQ-PCR) and Electrochemiluminescence Immunoassay (ECLIA). The trophoblast TLR3 signaling pathway proteins and infant cytokines IL-6, IL-12, TNF- $\alpha$ , IFN- $\alpha$  and IFN- $\gamma$  were tested by immunohistochemistry (IHC) and Enzyme-Linked Immunosorbent Assay (ELISA).

**Results:** The expression of TLR3 and NF- $\kappa$ B, a TLR3 downstream protein, were significantly decreased in the non- or hypo-responders ( $Z = -3.00$  and  $-2.46$ ,  $P < 0.01$  and  $= 0.01$ ). Furthermore, the trophoblast TLR3 expression negatively correlated with maternal HBV DNA ( $r = -0.29$ ,  $P = 0.003$ ), HBeAg ( $r = -0.28$ ,  $P = 0.01$ ) and HBV DNA+HBeAg ( $r = -0.24$ ,  $P = 0.02$ ). Besides, NF- $\kappa$ B positively correlated with infant IL-6 ( $r = 0.24$ ,  $P = 0.026$ ). By comprehensive analysis of maternal, placental and infant information, a Bayesian network model showed that the trophoblast TLR3 signaling pathway contacted with the non- or hypo-responsiveness.

**conclusions:** Maternal HBV infection affected the trophoblast TLR3 signaling pathway protein expression, and consequently the impaired TLR3 signaling pathway involved in the HB vaccine non- or hypo-responsiveness mainly by influencing infant IL-6.

## Background

The hepatitis B virus (HBV) is the second most important human carcinogen, and chronic HBV infection causes great problems globally [1]. According to the World Health Organization (WHO), there are approximately 257 million people living with chronic HBV infection and about 887,000 HBV-related deaths worldwide each year [2]. Children under the age of 1 are at the greatest risk of HBV infection, because nearly 90% of infant infection would remain chronic [3] and 15–25% of infected infants would die from HBV related liver disease [4]. At present, HBV infection can be safely and effectively prevented by available vaccines [1]. However, according to the epidemiological data, 5–10% of population exhibit poor immune responses to the vaccine which may lead to subsequent prophylactic failure [5]. What's worse, infants of hepatitis B surface antigen (HBsAg) positive mothers experience a higher rate of non- or hypo-response, which is around 10–40% [6–8]. Consequently, improving the immune response rate of this

population is a key to control the HBV infection. Identifying the related causes of the poor immune response to the vaccine is therefore crucial to achieving this aim.

The special intrauterine environment of HBsAg-positive mothers may be one of the causes influencing the baby's immune system and eventually lead to a poor response to the hepatitis B (HB) vaccine. Based on current researches on the HB vaccine response of infants born to HBsAg-positive mothers, maternal HBV infection status and baby's body immunity both participate in HB vaccine non- or hypo-response [9–11]. Placenta, an important organ that connects mother and child, is therefore likely to act as the intermediary through which maternal exposure to HBV can affect the baby response to hepatitis B vaccine. However, studies that examine this hypothesis are very rare.

Trophoblast cells are the characteristic cell of placenta, locating at the outermost layer of the placental villi and contacting with the maternal blood supply directly. Therefore, trophoblast cells play a vital role in protecting fetal growth and affecting the long-term health of the offspring [12, 13]. Beside macrophages, dendritic cells, lymphocyte, etc., current studies have demonstrated that trophoblast cells also are a component of the innate immune system and perform important immune functions [14]. The innate immune system is essential in producing protective antibodies after HB vaccination [15, 16]. The Toll-Like Receptors (TLRs) are important components of Pattern Recognition Receptors (PRRs) [17–19]. Study found that TLRs not only take part in innate immune but also trigger the adaptive immune response following vaccination [20]. It is notable that the trophoblast cells TLRs play very important role in immune responses against HBV [21]. 10 types of the TLRs have been found in humans including TLR1-10 in which TLR3 highest express in trophoblast cells [22]. TLR3 can specifically recognize HBV-related double-stranded RNA (dsRNA) and involves in anti-HBV activity [23, 24]. However, the role of the trophoblast cells TLR3 in the immune response to HB vaccine is significantly understudied.

TLR3 is activated by a MyD88-independent pathway and signals through TRIF, the only adaptor protein of TLR3 [25]. By combining with TRIF, TLR3 signals inducing the activation of NF- $\kappa$ B and IRF3 [26, 27]. In our present study, we focused on the TLR3, TRIF, NF- $\kappa$ B and IRF3 within trophoblast cells to understand how the TLR3 signaling pathway relates to non- or hypo-responsiveness to the hepatitis B vaccine in babies who were born to HBsAg-positive mothers. Additionally, the information about mother and newborn HBV infection status as well as infant cytokines was collected to investigate the interplay among maternal HBV infection, the trophoblast cells TLR3 signaling pathway, and baby's response to hepatitis B vaccinations. By analyzing the comprehensive data, we hope this study will provide new insights into the mechanism of non- or hypo-response in infants borne of HBsAg-positive mothers.

## Methods

### Subjects and specimens

A total of 399 pairs of HBsAg-positive mothers and their children were recruited from the Third People's Hospital of Taiyuan City, Shanxi province, China during June 2011 to July 2013. All mothers had no

history of infection with Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV) or other viruses.

This study was approved by the Ethics Committee of Shanxi Medical University and all mothers and legal guardians of children signed the informed consent forms for the participation of this study.

After delivery, face-to-face interviews were conducted to collect the general demographic information of the mothers and their children by well-trained interviewers with standardized questionnaires. Before delivery, peripheral blood samples were collected from the median cubital vein from all mothers. Placentas were collected within 30 minutes after delivery. Then tissue samples were fixed in 4% paraformaldehyde, dehydrated by gradient alcohol, transparentized by xylene and embedded in paraffin. We collected two femoral venous blood samples from the children: 1) within 24 hours of birth and prior to the administration of the HB vaccine and hepatitis B immunoglobulin (HBIG), 2) after 12 months following completion of the HB vaccination regimen. All blood samples were pretreated and stored at -80 °C until the next use in successive experiments.

## **Determination of HBV serological markers**

HBsAg, anti-HBs, hepatitis B e antigen (HBeAg), anti-HBe and anti-HBc of the mothers and their babies were measured by Electrochemiluminescence Immunoassay (ECLIA) (Roche Diagnostics GmbH, Germany) according to the manufacturers' instructions. Children who had completed the HB vaccination regimen with anti-HBs titers < 100mIU/ml were defined as non- or hypo-response, those with anti-HBs levels  $\geq$  100mIU/ml were defined as high-response [28].

## **Quantification of serum HBV-DNA**

The Fluorescence Quantitative Polymerase Chain Reaction (FQ-PCR) kit was used to detect the serum HBV DNA loads. The process and result judgment were carried out following the instruction manual (DAAN Gene Co. Ltd., Sun Yat-sen University, Guangdong, China).

## **Detection of TLR3, TRIF, NF- $\kappa$ B and IRF3 in placental trophoblast cells**

For immunostaining, the placental tissue was sectioned into 4  $\mu$ m thick samples and deparaffinized. Thereafter, the slides were subjected to a heat-induced epitope retrieval step for 2 minutes before incubation with primary antibodies. The TLR3 (ab62556), TRIF (ab13810) and NF- $\kappa$ B (ab86299) were rabbit polyclonal antibodies obtained from Abcam (USA) and adopted a diluted concentration of 1:400, 1:400 and 1:1000 respectively. IRF3 (D9J5Q), a mouse monoclonal antibody, was purchased from Cell Signaling (USA) and adopted 1:400 diluted concentration. We used EnVision Plus to detect these antibodies, developing them in 1:1000 DAB for 5 minutes then counterstaining them in hematoxylin. Appropriate positive and negative control cases were stained in parallel. The results were scored by the integral of staining intensity (1, weak; 2, moderate; and 3, strong) and positive cells percentage (0, negative; 1, < 25%; 2, 25–50%; 3, > 50%), and graded into the following categories: 0(0), 1(1–2), 2(3–4), and 3(> 4).

# Measurement of cytokines

Cytokines relating to TLR3 signaling pathway, including IL-6, IL12, TNF- $\alpha$ , IFN- $\alpha$  and IFN- $\gamma$ , were assessed by Enzyme-Linked Immunosorbent Assay (ELISA) kits (eBioscience, USA) according to the manufacturer's instructions.

## Statistical analysis

Database creation was performed using Epidata 3.1. Statistical analysis was performed using SAS version 9.4 software package. Descriptive data was presented in mean  $\pm$  standard deviation (SD), median with interquartile ranges (IQR) or absolute numbers (n) with percentages based on the data type and distribution. Statistical significance was tested by t-test or non-parametric test for continuous data and by Chi-square test for categorical data. Correlation coefficients were determined using the Spearman rank correlation method. All statistical tests were two-tailed, with a P value  $< 0.05$  considered indicative of statistically significant results. The Bayesian network utilized the R3.3.4 and Netica software for analyzing the influencing factors of hepatitis B vaccine non- or hypo-responsiveness and the relationship among these factors.

## Results

### The general information of the HBsAg-positive mothers and the children

The standard passive-active immunoprophylaxis was administered to all 399 neonates of HBsAg-positive mother, and the follow-up was performed until the age of one. Of these, 295 infants eventually completed the follow-ups, with 241 high responsive individuals and 54 non- or hypo-responsive individuals. The rate of non- or hypo-response was 18.31%. A total of 50 non- or hypo-responders with complete placenta and blood samples, proceeded for further analysis as the case group. 50 high- responders were randomly selected as the control group. The average age of the 100 mothers was  $27.71 \pm 4.33$  years and the average gestational time was  $39.09 \pm 1.13$  weeks. On average, the 100 babies were weighted  $3385 \pm 395.33$  g and measured  $50.05 \pm 1.43$  cm in length at birth. At the age of one, the average weight and height were  $11.20 \pm 3.39$  kg and  $76.84 \pm 4.12$  cm, respectively. Table 1 (placed at the end of the document text file) shows the descriptive information of the 100 HBsAg-positive mothers and children. Except for neonatal serum HBsAg status, there were no significant differences between cases and controls. Serum HBsAg-positive neonates had a significantly increased rate of non- or hypo-responsiveness.

Table 1  
Descriptive Information of the 100 HBsAg-positive Mothers and Their Children

Characteristics	Non- or Hypo-Responders (n = 50)	High- Responders (n = 50)	t/ $\chi^2$	P Value
Mothers				
Age (year)	28.16 ± 4.74	27.24 ± 3.85	-0.99	0.32 <sup>a</sup>
Gestational Week	39.24 ± 1.14	38.94 ± 1.11	1.11	0.27 <sup>a</sup>
Educational Level			0.18	0.67 <sup>b</sup>
<High School	18(36.00)	16(32.00)		
≥High School	32(64.00)	34(68.00)		
Mode of Delivery			1.48	0.22 <sup>b</sup>
Vaginal Delivery	24(48.00)	18(36.00)		
Caesarean Section	26(52.00)	32(64.00)		
Serum HBeAg			0.65	0.42 <sup>b</sup>
Positive	20(40.00)	24(48.00)		
Negative	30(60.00)	26(52.00)		
Serum HBV DNA			3.35	0.07 <sup>b</sup>
Positive	16(32.00)	25(50.00)		
Negative	34(68.00)	25(50.00)		
Serum HBV DNA(copies /ml)			0.75	0.69 <sup>b</sup>
< 10 <sup>3</sup>	32(64.00)	31(62.00)		
10 <sup>3</sup> ~ 10 <sup>6</sup>	6(12.00)	4(8.00)		
> 10 <sup>6</sup>	12(24.00)	15(30.00)		
Children				
Neonatal Length(cm)	50.08 ± 1.42	50.02 ± 1.46	-0.50	0.62 <sup>a</sup>
Neonatal Weight(g)	3404.00 ± 410.30	3366.00 ± 383.00	-0.63	0.53 <sup>a</sup>

<sup>a</sup>:t-test, described by  $\bar{x} \pm SD$  ; <sup>b</sup>:chi-square test, described by n (%)

Characteristics	Non- or Hypo-Responders (n = 50)	High- Responders (n = 50)	t/ $\chi^2$	P Value
Gender			0.36	0.55 <sup>b</sup>
Male	28(56.00)	25(50.00)		
Female	22(54.00)	25(50.00)		
Neonatal Serum HBsAg			4.33	0.04 <sup>b</sup>
Positive	10(20.00)	3(6.00)		
Negative	40(80.00)	47(94.00)		
Neonatal Serum HBeAg			2.10	0.15 <sup>b</sup>
Positive	15(30.00)	22(44.00)		
Negative	35(70.00)	28(56.00)		
Infant Height (cm)	77.18 ± 4.43	76.48 ± 3.79	-0.84	0.40 <sup>a</sup>
Infant Weight (kg)	11.55 ± 4.54	10.84 ± 1.48	-1.03	0.30 <sup>a</sup>
<sup>a</sup> :t-test, described by $\bar{x} \pm SD$ ; <sup>b</sup> :chi-square test, described by n (%)				

## The placental trophoblast cells expression of TLR3, TRIF, NF- $\kappa$ B and IRF3 in the non- or hypo-responders and the high-responders

To evaluate trophoblast TLR3 signaling pathway protein expression, placental tissue sections were detected by immunohistochemistry that possesses the advantage of expression localization. TLR3, TRIF, NF- $\kappa$ B and IRF3 were primarily expressed in trophoblastic cells, as shown in Fig. 1, they were also present in decidual cells, villous mesenchymal cells and villous capillary endothelial cells. These proteins were stained in the cytoplasm except for NF- $\kappa$ B. As a transcriptional activator, the activation of NF- $\kappa$ B must be phosphorylated and translocate into the nucleus. Therefore, NF- $\kappa$ B localized in the nucleus.

We analyzed the expression levels of TLR3, TRIF, NF- $\kappa$ B and IRF3 in trophoblastic cells to evaluate the TLR3 signaling pathway in non- or hypo-response. The expression of TLR3 and NF- $\kappa$ B were significantly decreased in the non- or hypo-responsive group as compared to the high-responsive group. The expression of TRIF and IRF3 were lower in the non- or hypo-response group than in the high-response group, though the difference was not statistically significant. The T value of TLR3, TRIF, NF- $\kappa$ B and IRF3 was 2150.00, 2394.00, 2252.00 and 2302.00 in the non- or hypo-response group and was 2900.00, 2656.00, 2798.00 and 2748.00 in the high-response group. Table 2 and Fig. 2 showed the expression of

TLR3, TRIF, NF-κB and IRF3 in the non- or hypo-response group and the high-response group. Further, we found that TLR3 expression positively correlated with the expression of TRIF ( $r = 0.26$ ,  $P = 0.01$ ) and NF-κB ( $r = 0.30$ ,  $P = 0.003$ ).

Table 2

The expression integral category of trophoblast cell TLR3, TRIF, NF-κB and IRF3 proteins in the non- or hypo-responders and the high-responders

Protein Expression Integral Category	Non- or Hypo- Responders (n = 50)	High- Responders (n = 50)	Z Value	P Value
TLR3, n (%)			-3.00	< 0.01
0	0(0.00)	0(0.00)		
1	3(6.00)	6(12.00)		
2	32(64.00)	14(28.00)		
3	15(30.00)	30(60.00)		
TRIF, n (%)			-1.12	0.26
0	0(0.00)	1(2.00)		
1	13(26.00)	6(12.00)		
2	25(50.00)	25(50.00)		
3	12(24.00)	18(36.00)		
NF-κB, n (%)			-2.46	0.01
0	4(8.00)	1(2.00)		
1	35(70.00)	26(52.00)		
2	5(10.00)	8(16.00)		
3	6(12.00)	15(30.00)		
IRF3, n (%)			-1.81	0.07
0	5(10.00)	4(8.00)		
1	25(50.00)	17(34.00)		
2	11(22.00)	11(22.00)		
3	9(18.00)	18(36.00)		

## The relationship between the TLR3 signal pathway and the HBV infection status of HBsAg-positive mother

To find potential reasons for the TLR signal pathway protein changes, we analyzed the relationship between these proteins and the mother HBV infection status. The TLR3 was negatively correlated with quantitatively measured maternal HBV DNA ( $r = -0.29$ ,  $P = 0.003$ ), maternal HBeAg status ( $r = -0.28$ ,  $P = 0.01$ ) and maternal HBV DNA + HBeAg + status ( $r = -0.24$ ,  $P = 0.02$ ). However, maternal HBV DNA and HBeAg status were not correlated with TRIF, NF- $\kappa$ B or IRF3. Furthermore, in the situations where mothers were positive for either HBeAg or both HBeAg and HBV DNA, the expression of TLR3 was significantly decreased when compared with mothers who were positive for neither condition (shown in Table 3). Additionally, we did not observe that the mode of delivery was correlated with the TLR3 signal pathway.

Table 3

The information of TLR3 expression and maternal HBV DNA and HBeAg

Characteristics	n	TLR3 (T)	Z	P Value
Maternal HBV DNA and HBeAg			-2.66	< 0.01
Double Positive	34	1418.50		
Not Double Positive	66	3631.50		
Maternal HBeAg			-2.74	0.01
Positive	44	1854.50		
Negative	56	3195.50		
Maternal HBV DNA			-1.31	0.19
Positive	41	1911.00		
Negative	59	3039.00		

## The expression of TLR3 signal pathway proteins and the HBV infection status of new babies

There were 37 cases of serum HBeAg positive new babies and 63 cases of serum HBeAg negative new babies investigated in this study. The expression of TLR3 was significantly decreased in the HBeAg positive babies when compared with the HBeAg negative ones ( $P < 0.001$ ). However, the expressions of TRIF, NF- $\kappa$ B or IRF3 were not significantly different between the two serum HBeAg statuses of the newborns. What's more, whether new babies were serum HBsAg (or HBV DNA) positive or negative, there were no significant differences be found in expressions of TLR3, TRIF, NF- $\kappa$ B or IRF3.

## The relationship between the TLR3 signal pathway and the infant cytokines

When babies were one year old and had completed the hepatitis B vaccination regimen, their cytokines levels were observed. Although there was no significant difference between the two groups, nearly all cytokines, except for IL-12, exhibited a decrease in the non- or hypo-response group (shown in Fig. 3).

Furthermore, we found that NF- $\kappa$ B was positively correlated with infant IL-6 ( $r = 0.24$ ,  $P = 0.026$ ), IL-12 ( $r = 0.23$ ,  $P = 0.035$ ) and TNF- $\alpha$  ( $r = 0.23$ ,  $P = 0.03$ ), while TLR3, TRIF, or IRF3 were not correlated with the infant cytokines.

## The Bayesian network of the relation between placental trophoblast cell TLR3 signal pathway and hepatitis B vaccine non- or hypo-responsiveness

In order to have a comprehensive evaluation of the role of TLR3 signal pathway in the non- or hypo-response to HB vaccine, factors that were potentially associated to non- or hypo-response were put into a Bayesian model. Continuous data was categorized as above or below median point in control group. As shown in Fig. 4, the Bayesian network model revealed that NF- $\kappa$ B, infant IL-6 and IRF3 were directly contacted to the non- or hypo-response. According to the conditional distribution, the probability of the non- or hypo-response was much higher when all the three markers were below the median (63.27%) than when the three were above the median (10.00%). TLR3 via NF- $\kappa$ B was indirectly contacted to the non- or hypo-response. Furthermore, maternal HBeAg was directly contacted to TLR3 which again supported our previous speculation that maternal HBV infection affected trophoblast cells TLR3 signal pathway. Although NF- $\kappa$ B was correlated with infant IL-6, IL-12 and TNF- $\alpha$ , the Bayesian network model found that the contact between NF- $\kappa$ B and IL-6 was direct but the contact between NF- $\kappa$ B and IL-12 and TNF- $\alpha$  was indirect.

## Discussion

The purpose of the present study was to investigate the relationship between trophoblast TLR3 signaling pathway and the HB vaccine response of the infants who were born to HBsAg-positive mothers. Considering the high morbidity and mortality of the HBV infection sequelae, it is imperative to clarify the possible cause and mechanism of non- or hypo-response in this population who is suffer from the high risk of HBV infection and considerably inadequate response rate to the HB vaccine. Our results showed that the TLR3 signaling pathway proteins, TLR3 and NF- $\kappa$ B, were significantly decreased in the non- or hypo-responders. Moreover, by comprehensively analyzing information from HBsAg-positive mothers, trophoblast cells TLR3 signaling pathway protein expression and the babies, we found that HBsAg-positive mother's HBV infection status impaired trophoblast cells TLR3 signaling pathway and the impaired TLR3 pathway eventually affected infant immune response to the HB vaccine. The findings suggested that, as the intermediary, trophoblast cells TLR3 signaling pathway may involve in the vaccine non- or hypo-response.

It has been demonstrated that HBV can affect TLR3 signaling [29]. In the case of long-term HBV infection, intrahepatic TLR3 expression was lower and TLR3 elevation was slower compared with normal controls [30]. Besides, the mRNA of TLR3 was significantly down-regulated in peripheral blood mononuclear cell of patients with chronic HBV infection [31]. Consistent with these researches, our study found that

trophoblast cells TLR3 expression was significantly decrease in the non- or hypo-response. The placental function can be modified by the maternal environment [32, 33]. By analyzing the maternal HBV infection, we found that the HBsAg-positive mothers with both HBV DNA- and HBeAg-positive had a significant reduction of TLR3 expression. Furtherly, the Bayesian network model indicated that the maternal HBeAg status was directly contacted to TLR3. These findings indicated that the affected TLR3 involved in the non- or hypo-response and the reduced TLR3 expression may attribute to the HBsAg-positive mother's HBV infection status.

The impairment of trophoblast cells' TLR3 signaling pathway was multifaceted in the non- or hypo-responsiveness to the HB vaccine. In this study, the expressions of the TLR3 downstream protein NF- $\kappa$ B were significantly decreased among inadequate responders. This phenomenon may be related to the diminished expression of TLR3, because TLR3 expression directly positively correlated to NF- $\kappa$ B expression in our investigation. However, it has also recently been proven that the HBV polymerase (Pol) inhibited the activation of NF- $\kappa$ B by suppressing the phosphorylation of I $\kappa$ B kinase, blocking degradation of I $\kappa$ B $\alpha$  and by restraining translocation of NF- $\kappa$ B to the nucleus [34]. In Bayesian network model, the direct contact between NF- $\kappa$ B and non- or hypo-response suggested a complicated underlying mechanism of decreased trophoblast cell NF- $\kappa$ B expression, which requires further investigation.

In our study, the expressions of TRIF and IRF3 were lowered in the inadequate response cases, even though the difference was not statistically significant. The Bayesian model indicates that IRF3 directly contacted to the non- or hypo-response condition. It has been proven that the HBX protein encoded by the HBV genome reduces the TRIF protein expression in human hepatoma cell lines and liver tissue samples by affecting protein ubiquitination [35, 36]. In addition, the HBX protein could suppress IRF3 activation by inhibiting IRF3 phosphorylation, dimerization and nuclear translocation [35]. However, the increased expression of HBX is mainly in HBV-related hepatocellular carcinoma [37]. Therefore, it is possible that HBX expression might be low in HBsAg-positive mothers, which results in insignificant decreases in TRIF and IRF3. However, better understanding of this mechanism needs to be supported with additional confirmations.

As an intermediary, placental transfers maternal pathogenic microbiota exposure to baby and affects the development of the offspring's immune system [38]. In our study, significantly decreased trophoblast cells TLR3 expression was observed in HBeAg positive neonate. The result indicated that the suppressed expression of trophoblast cells TLR3 signaling pathway proteins might relate to neonate HBV infection status. Besides, the trophoblast cells NF- $\kappa$ B expression was positively correlated with infant IL-6, IL-12 and TNF- $\alpha$ , that is, the decrease NF- $\kappa$ B affected these cytokines expression. Studies have shown that decreased levels of cytokines such as IL-6, TNF and IL-12 are involved in inadequate response to the HB vaccine, and even lead to the vaccination failure [11, 39–41]. Not only that, the Bayesian model presented in this study, suggested that trophoblast cells TLR3 signaling pathway influenced the offspring immune response profoundly, and IL-6 might be a crucial cytokine because IL-6 levels were not only contacted to the non- or hypo-response status but also to IL-12, TNF- $\alpha$ , IFN- $\alpha$  and IFN- $\gamma$ . All these indicated that by affecting baby cytokines, mainly IL-6, the impaired trophoblast cells TLR3 signaling pathway involved in

the HB vaccine non- or hypo-responsiveness. Although TLR signaling pathway can be triggered via vaccination [20], how the activation of TLR3 signaling pathway followed HB vaccine need to be illuminated furtherly.

In summary, the findings presented in this study indicated that impaired TLR3 signaling pathway of trophoblast cells involved in the HB vaccine non- or hypo-response. The vaccines containing TLR ligands as adjuvants can enhance innate immunity and activate adaptive immunity [42]. Therefore, TLR3 ligands should be considered as an alternative adjuvant of HB vaccine. Our study was limited by its sample size, and by the ethical constraints of performing invasive diagnostics and sampling techniques on the newborn, which resulted in a small neonatal blood sample volume and an inability to perform more extensive analysis on the neonatal blood. Therefore, further studies are needed to focus on inadequate immune response resulting from inhibition of the TLR3 pathway and other TLR pathways in trophoblast as well as in other cell types of the placenta.

## Conclusions

Impaired TLR3 signaling pathway of trophoblast cells involved in the HB vaccine non- or hypo-response of infants born to HBsAg-positive mothers.

## Abbreviations

dsRNA: double-stranded RNA; ECLIA: electrochemiluminescence immunoassay; ELISA: enzyme-linked immunosorbent assay; FQ-PCR: fluorescence quantitative polymerase chain reaction; HB: hepatitis B; HBeAg: hepatitis B e antigen; HBIG: hepatitis B immunoglobulin; HBsAg: hepatitis B surface antigen; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; IQR: interquartile ranges; PRRs: Pattern Recognition Receptors; SD: standard deviation; TLR3: Toll-Like Receptor 3; TLRs: Toll-Like Receptors; WHO: World Health Organization

## Declarations

### Ethics approval and consent to participate

This study was approved by the Shanxi Medical University ethics committee. All study participants and legal guardians of children provided written, informed consent for the participating in the study, including blood and tissue sample collection. Their right to keep information confidential was guaranteed. The study was carried out in accordance with the principles of the Declaration of Helsinki.

### Consent for publication

Not applicable

### Availability of data and materials

The datasets used in this study are available from the corresponding author on reasonable request.

## Competing interests

All authors declare that they have no competing interests.

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## Authors' Contributions

Conception and design of the study: Lina Wu and Suping Wang. Specimen collection: Ruijun Zhang, Shuying Feng and Bo Wang. Laboratory detections: Lina Wu, Ruijun Zhang, Huili Wan and Junli Li. Data acquisition and statistical analysis: Lina Wu, and Tian Yao. Manuscript preparation: Lina Wu, and Linzhu Yi. Critical review of manuscript: Suping Wang and Yongliang Feng.

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## Figures



### Figure 1

TLR3, TRIF, NF- $\kappa$ B and IRF3 expressed in the placenta. A-D. The expressions of TLR3, TRIF, NF- $\kappa$ B and IRF3 in high-responders. E-H. The expressions of TLR3, TRIF, NF- $\kappa$ B and IRF3 in non- or hypo-responders. The expression of TLR3 and NF- $\kappa$ B was significantly decreased in the non- or hypo-responders. (En Vision original magnification  $\times$  200).

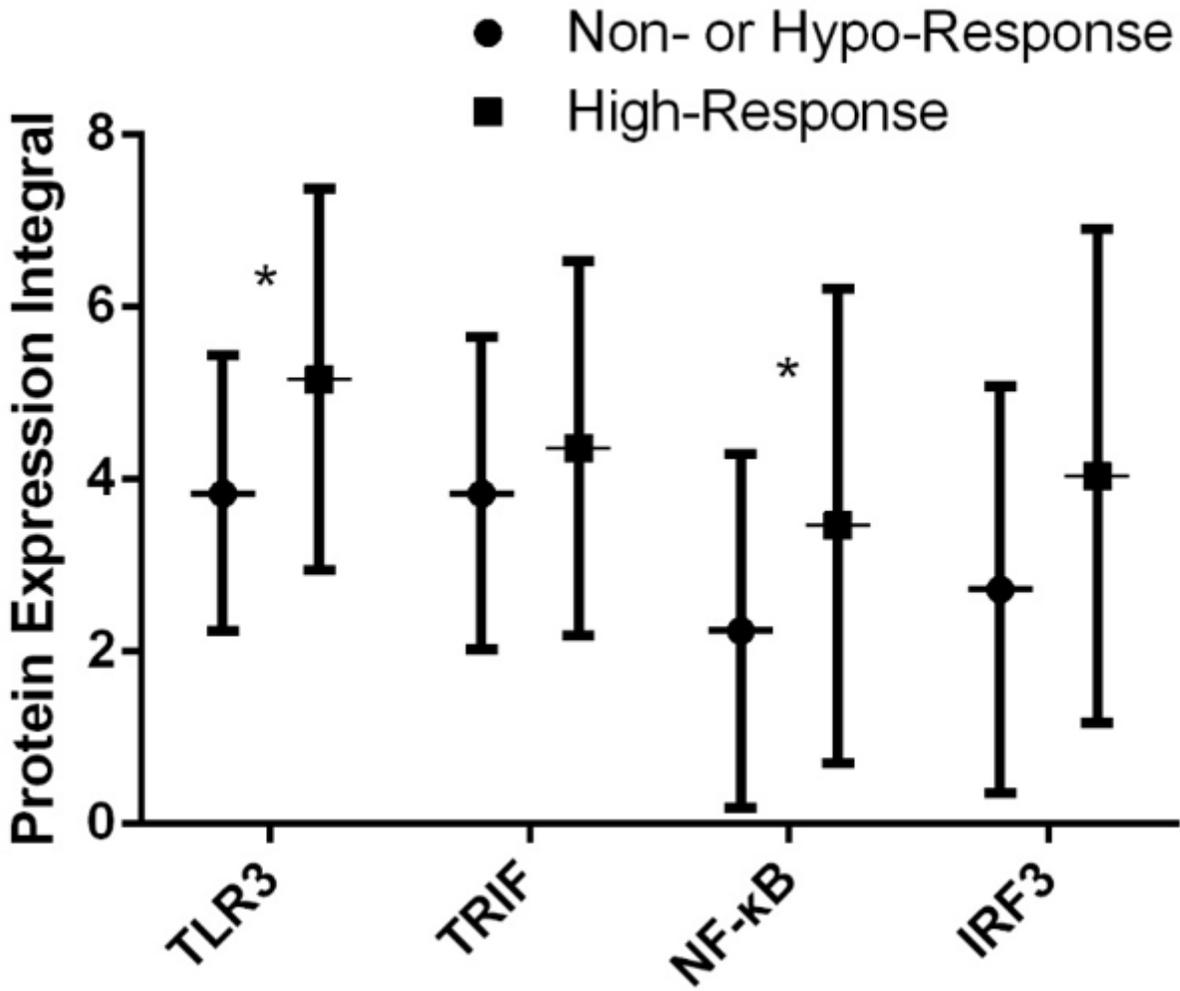


Figure 2

The integral of TLR3, TRIF, NF-κB and IRF3 expression in on- or hypo-response and high-response (\*, P<0.05).

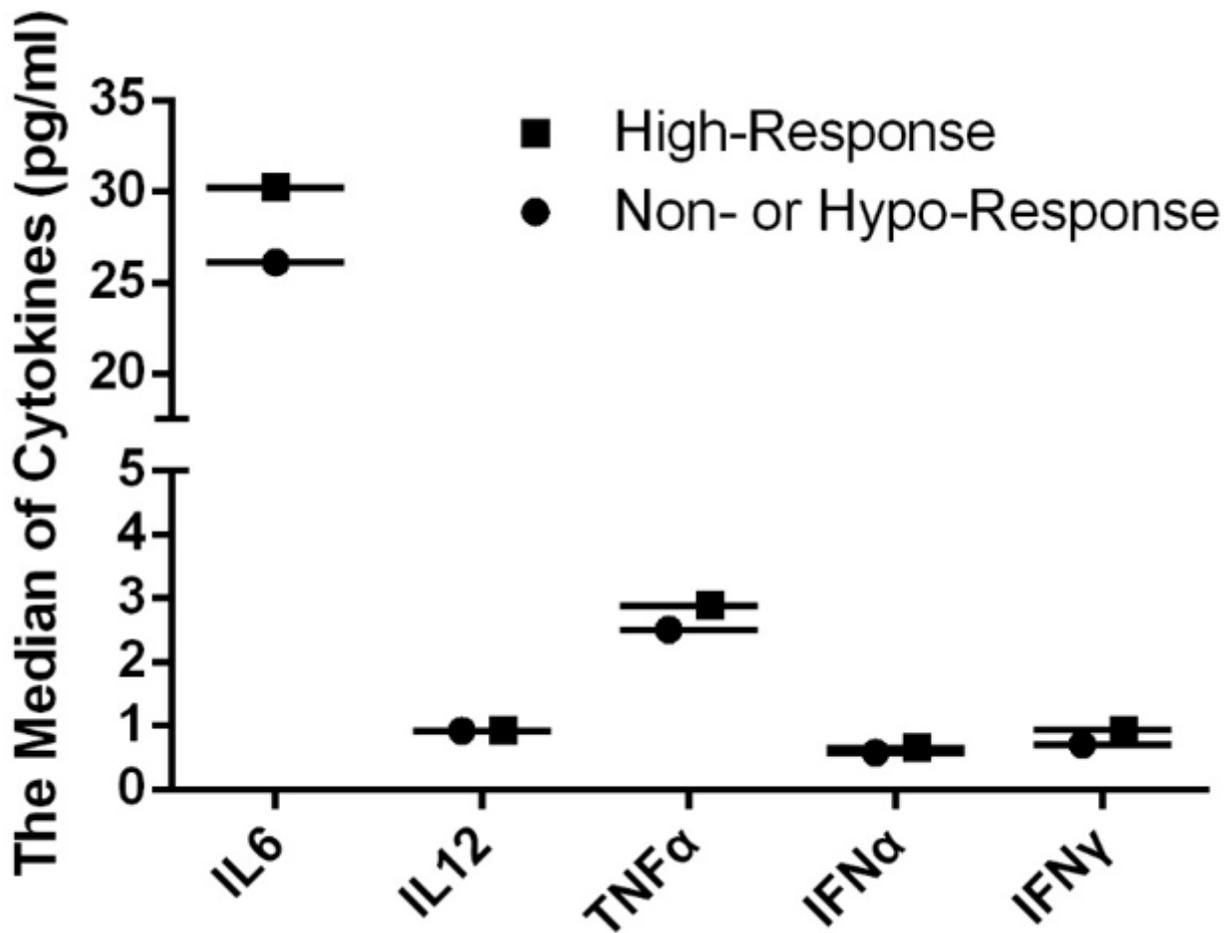
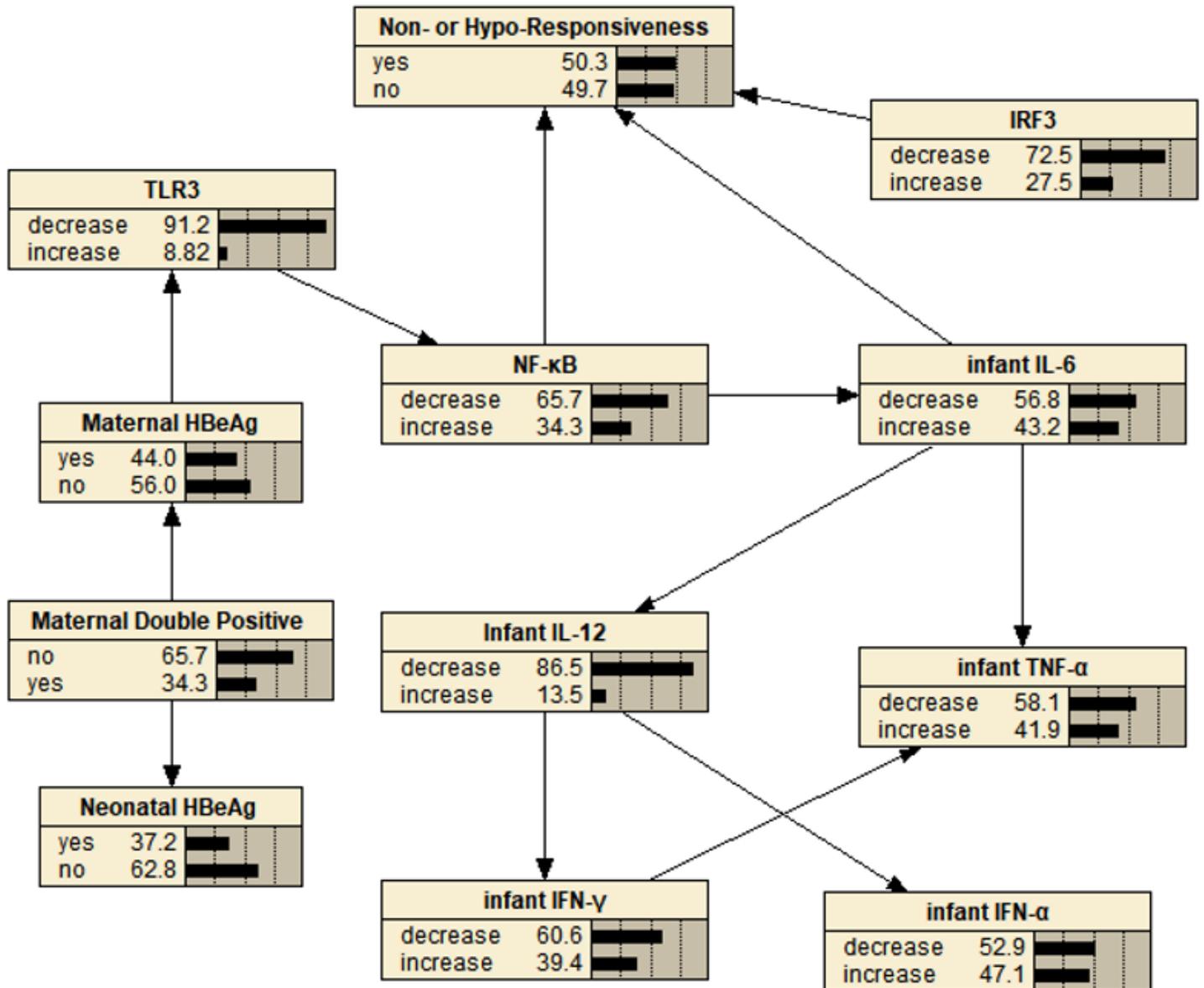


Figure 3

The level of infant cytokines in non- or hypo-response and high-response. Nearly all cytokines, except IL-12, showed a decrease, though the differences were not significant between the two groups.



**Figure 4**

The Bayesian network of placental trophoblast cell TLR3 signal pathway in hepatitis B vaccine non- or hypo-responsiveness. Maternal double positive means maternal HBV DNA and HBeAg were positive at the same time.