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Placental and intra-amniotic inflammation are associated with altered fetal immune responses at birth

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ABSTRACT

Introduction: High-grade placental inflammation is associated with preterm birth and poor neonatal outcomes. Recent reports suggest that low-grade placental inflammation is common in uncomplicated pregnancies. The relationship between placental inflammation and innate immune anti-microbial responses is unknown. In this study we sought to identify any association between placental inflammation and fetal immune responses.

Methods: Cord blood samples collected from late preterm and full-term Caesarean section deliveries (n = 44) were exposed to various immune challenges (resiquimod, LPS, PGN, poly (I:C), cGAMP, and 5′ppp-dsRNA) and production of inflammatory mediators (G-CSF, IFN-γ, IL-1β, IL-6, IL-8, IL-10, and TNF-α) was measured by multiplex assay. Hospital histology reports were used to assess the extent of inflammation in the placenta.

Results: Almost half (47.7%) of placentae examined here showed histological evidence of inflammation. Resiquimod, LPS, and PGN elicited strong inflammatory responses in neonatal cord blood, while poly (I:C), cGAMP, and 5′ppp-dsRNA elicited weaker responses. Fetuses with evidence of chorioamnionitis and fetal inflammatory reaction in their placentae had significantly increased immune responses to cGAMP and 5′ppp-dsRNA (ligands for STING and RIG-I, respectively) and significantly decreased immune responses to poly (I:C) (a TLR3 agonist). Interestingly, STING, RIG-I, and TLR3 are all involved in viral response pathways, suggesting that fetuses exposed to chorioamnionitis or fetal inflammatory reaction might respond differently to viruses postnatally.

Conclusion: Our data suggest that low-level placental inflammation is associated with altered innate cytokine responses at birth.

1. Introduction

Placental inflammation is typically associated with infection, preterm birth, and poor neonatal outcomes [1]. Placental inflammation can be broadly categorised according to the site and extent of histopathological inflammation [2–7]. Placental inflammation includes deciduitis (DEC), subchorionitis, chorionitis, chorioamnionitis (CAM), chorionic vasculitis, umbilical vasculitis, funisitis, and villitis (including villitis of unknown aetiology; VUE). Different inflammatory lesions within the placenta are associated with different pathologies, aetiologies, and clinical outcomes. The three major chronic inflammatory lesions of the placenta are CAM, VUE, and DEC [8]. CAM, most commonly diagnosed histologically after delivery (hCAM), is a significant cause of preterm birth, and is characterised by maternal neutrophil and $CD8^+$ T-cell infiltration of the placental membranes [3]. Bacterial and fungal infections of the amniotic cavity are frequently identified in cases of hCAM, although a significant proportion are apparently sterile. VUE is characterised by infiltration of maternal lymphocytes into the chorionic villi, and can be associated with destruction of the villous architecture by macrophages [7]. VUE is less common in placentae delivered preterm. DEC is characterised by the presence of lymphocytes or plasma cells in the decidua basalis. Both infectious and sterile immune mechanisms have been implicated in this condition.

While placental inflammation has been extensively studied in the context of fetal and pregnancy pathologies, such as preterm birth and intra-uterine growth restriction, the prevalence and significance of inflammation in placentae from uncomplicated pregnancies has received less attention [9–12]. A recent study by Romero et al. revealed that placental inflammation is common in healthy, full-term pregnancies. These authors examined placentae from 944 uncomplicated full-term pregnancies for histological evidence of inflammation and found that 42.3% of these had acute inflammatory lesions, while 29.9% had

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Table 1
Details of immune stimulation.

Stimulant	Abbreviation	Concentration	Brief description
Unstimulated RPMI 1640 control	US-R	RPMI 1640 media only	Vehicle control used to compare to R848, PLS, PGN, and poly (I:C).
Resiquimod	R848	10 mM in RPMI 1640	TLR7/TLR8 agonist
Lipopolysaccharide	LPS (from *E. coli*)	100 ng/ml in RPMI 1640	TLR4 agonist
Peptidoglycan	PGN (from *S. aureus*)	10 μg/ml in RPMI 1640	TLR2 & NOD1/2 agonist
Polyinosinic–polycytidylic acid	Poly (I:C)	100 μg/ml in RPMI 1640	TLR3 agonist
Unstimulated LyoVec control	US-L	LyoVec transfection reagent only	Vehicle control used to compare to cGAMP and 5′ppp-dsRNA
Cyclic guanosine monophosphate–adenosine monophosphate	cGAMP	20 μg/ml in LyoVec	STING agonist
5′ triphosphate double-stranded RNA	5′ppp-dsRNA	2 ng/ml in LyoVec	RIG-I agonist

chronic inflammatory lesions [13]. Interestingly, most of these cases were mild, with severe inflammation present in only 3.4% of cases. These data suggest that low-level placental inflammation is common in uncomplicated, full-term pregnancies. The consequence of exposure to low-level placental inflammation with respect to innate immune responses is unclear.

The neonatal immune system is biased against Th1 immune responses [14–16], and has a comparatively naïve adaptive immune system [17,18]. However, neonates are able to mount a strong inflammatory innate immune response. Several studies have now shown that activation of Toll-like receptors (TLRs) produce an exaggerated cytokine response in neonatal cord blood compared to adult blood [14,19]. Further, other pattern recognition receptors (PRRs), including nucleotide oligomerization domain (NOD)-like receptors, retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), and stimulator of interferon genes (STING), have been shown produce a heightened cytokine response in neonatal compared to adult blood [20]. Previous studies have shown that *in-utero* events can alter post-natal immune responses [21,22]. Additionally, intrauterine infection and CAM have been correlated with immune-related disorders, including an increased risk of asthma and atopic disease [23–25]. We therefore hypothesised that exposure to placental inflammation would alter innate immune antimicrobial defences.

2. Methods

2.1. Patient recruitment and ethics

Patients giving birth by elective Caesarean section between 34 and 42 weeks gestation (n = 44) at King Edward Memorial Hospital, Subiaco, Western Australia, were invited participate in this study. Cord blood and placental samples were taken at birth with the approval of the Human Research Ethics Committee of the Western Australian Department of Health's Women and Newborns Health Service (2015212 EW). Inclusion criteria were: singleton pregnancies, Caesarean section deliveries, and a gestational age of $\geq 34^{+0}$ weeks. Exclusion criteria were: onset of labor, antibiotic or antimycotic use throughout the pregnancy, antenatal steroid administration, vaginal progesterone administration, fetal genetic abnormalities, and recreational drug abuse. Participants answered a detailed questionnaire regarding their health, diet and lifestyle during this pregnancy. Clinical data was also collected, including gestational age, maternal age, parity, infant sex, indications for Caesarean section (Supplementary Table 1), and previous obstetric history. Maternal and fetal characteristics are summarised in Supplementary Table 2.

2.2. Collection of cord blood

Umbilical cord blood was collected by obstetric surgeons prior to delivery of the placenta. After birth, the cord was clamped and cut. The clamp was removed and cord blood was allowed to drip into a sterile collecting pot, then immediately handed off to the study investigator. Approximately 4 ml of cord blood was then poured into a BD Vacutainer® heparinized blood collection tube and gently mixed by inverting 8–10 times. All cord blood samples were processed within 20 min of collection.

2.3. Immune stimulation

Innate immune phenotyping was performed using a well-established, robust, validated, and quality-controlled protocol from Professor Tobias Kollmann's lab (University of British Columbia, Canada) [14,19,20,26–30]. Cord blood samples were plated out in duplicate in 200 μl aliquots onto custom made immune stimulation plates (plates manufactured by Tobias R Kollmann, University of British Columbia). Each well of each row was coated with one of eight nominated immune stimuli or controls. The stimuli used were selected to cover a broad range of immune pathways, including Gram negative bacterial infections, Gram positive bacterial infections, intracellular bacterial infections, and viral infections (Table 1). We chose four prototypic TLR agonists to stimulate extracellular pathogenic infection: resiquimod (R848), a powerful immune activator, was used as a toll-like receptor (TLR) 7/8 agonist; lipopolysaccharide (LPS), a component of Gram negative bacterial cell walls, was used as a TLR4 agonist; peptidoglycan (PGN), the major outer membrane component of Gram positive bacterial cell walls, was used a TLR2 & nucleotide-binding oligomerization domain (NOD) 1/2 agonist; polyinosinic–polycytidylic acid (Poly I:C) was used as a TLR3 agonist to simulate viral infection. We also selected two agonists of cytoplasmic PRRs (cPRRs) (cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), and 5′ triphosphate double-stranded RNA (5′ppp-dsRNA)) to simulate intracellular pathogenic infection. cGAMP was used as a stimulator of interferon genes (STING) agonist, while 5′ppp-dsRNA was used as a retinoic acid-inducible gene I (RIG-1) agonist. These two ligand were complexed with a transfection agent (LyoVec) to allow them to enter the cell's cytoplasmic compartment to stimulate cPRRs. RPMI 1640 was used as a vehicle control for R848, LPS, PGN, and poly (I:C). LyoVec was used as a vehicle control for cGAMP and 5′ppp-dsRNA.

Samples were incubated in the sealed immune stimulation plates for 24 h at 37 °C and 5% CO_2. After incubation, the plates were centrifuged and the supernatant was transferred to a fresh microplate and frozen at −80 °C until analysis.

2.4. Quantitation of immune markers

All cord blood supernatant samples were diluted 1:2 in Calibrator Diluent RD6-52 prior to analysis. We selected seven markers to broadly cover expected responses to our innate immune stimulation protocol. Human granulocyte-colony stimulating factor (G-CSF), interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 8 (IL-8) and interleukin 10 (IL-10) were measured by multiplex assay (R&D Systems Inc., Minneapolis,

Table 2
Histology results for patients with evidence of placental inflammation. DEC: deciduitis. VUE: villitis of unknown aetiology. hCAM: histological chorioamnionitis. FIR: fetal inflammatory reaction. SGA: placenta small for gestational age. LGA: placenta large for gestational age.

Study ID	DEC	VUE	hCAM	FIRS	Other pathology
12	Focal, chronic				
48	Chronic				
40	Chronic	Chronic, high grade			
45	Chronic	Patchy, high grade, chronic		Early FIR of the fetal vessels in the chorionic plate	Meconium laden macrophages in placental membranes
02		Focal, low grade			
08		Low grade			SGA; meconium laden macrophages in placental membranes
32		Focal, low grade			
38		Focal, low grade			
39		High grade			
47		Multifocal, low grade, chronic villitis			SGA
50		Patchy, high grade, chronic			
13			Acute	Early FIR of the fetal vessels in the chorionic plate; acute cord vasculitis	SGA
23			Acute	Early FIR of the fetal vessels in the chorionic plate; early, acute cord vasculitis	SGA; meconium laden macrophages in placental membranes
26			Early/evolving, acute	Early, acute cord vasculitis	Meconium laden macrophages in placental membranes and chorionic plate
28			Acute	FIR of the fetal vessels in the chorionic plate; early, acute cord vasculitis	
44			Acute	FIR of the fetal vessels in the chorionic plate; early, acute cord vasculitis	
49			Early	FIR of the fetal vessels in the chorionic plate; early cord vasculitis	SGA; meconium laden macrophages in placental membranes
36			Early, acute	Focal FIR of the fetal vessels in the chorionic plate	LGA
41			Early/evolving, acute	FIR of the fetal vessels in the chorionic plate	SGA
43			Early/evolving, acute		
31				Focal, early cord vasculitis	Meconium laden macrophages in placental membranes
21				Focal, acute cord vasculitis	SGA
19				Focal, early cord vasculitis	

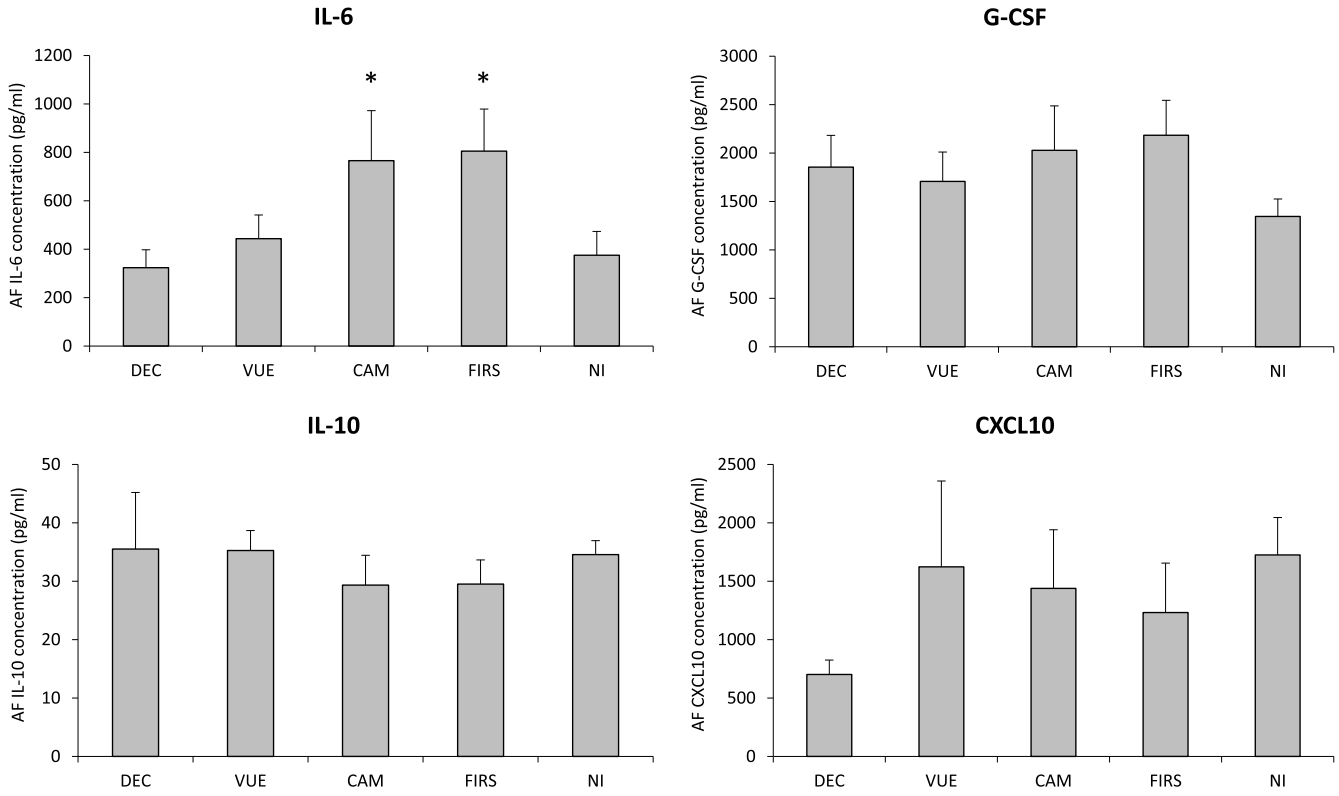

Fig. 1. Amniotic fluid cytokine levels of patients with deciduitis (DEC, n = 3), vasculitis of unknown aetiology (VUE, n = 6), chorioamnionitis (CAM, n = 9), inflammatory reaction of the fetal vessels (FIR, n = 11), or no inflammation (NI, n = 20). Data are mean ± SEM. *$P < 0.05$ compared to the NI group.

MN) on a MAGPIX instrument (Luminex Corp, Austin, TX) as per the manufacturer's instructions. These factors were chosen for investigation as they are well-characterized immune mediators, known to be produced by the placenta and detectable in cord blood, that have been shown to be elevated in neonates with intraamniotic infection/inflammation [31–33]. G-CSF is a hemopoietic growth factor involved in regulation of granulopoiesis, mobilisation of stem cells, and production and activation of neutrophils; it also has neuroprotective properties and ameliorates neuroinflammation in neonates. IL-1β and TNF−α are potent pro-inflammatory cytokines with well-proven roles in driving placental and systemic inflammation in response to pathogen exposure. IFN-γ orchestrates widespread cellular activation, proliferation and apoptosis in response to both bacterial and viral infections via induction of an array of interferon-regulated genes; importantly, it regulates Th1/Th2 balance and macrophage differentiation and activation. IL-6 is a pleiotropic immunomodulatory cytokine produced by multiple tissues in response to infection and tissue injuries; it is a regulator of acute phase responses, haematopoiesis, and immune cell differentiation and activation. IL-8 (CXCL8) is a ubiquitous chemokine responsible for recruitment of neutrophils, basophils and lymphocytes. IL-10 is an immunomodulatory cytokine produced by macrophages, lymphocytes and many other cell types; it inhibits the proliferation and activity of Th1 cells, NK cells, and macrophages and inhibits the production of proinflammatory cytokines and chemokines. IL-10 can also promote some humoral immune responses, such as enhancing class II expression on B cells and inducing immunoglobulin production.

2.5. Placental histology

Placentae and full thickness placental membranes were transported to the hospital's histology department for routine examination by a senior histopathology scientist. The histology reports generated were used to classify inflammatory lesions of the placentae [4]. Standard culture-based screening for *Listeria* spp., *Ureaplasma* spp., and *Mycoplasma* spp. was also performed.

2.6. Amniotic fluid cytokine analysis

Approximately 10 ml of fluid was drawn into a syringe immediately following amniotomy, then transferred to sterile tubes and centrifuged at 40,000 × *g* at 4 °C for 6 min to pellet. Levels of IL-6, IL-10, CXCL10, and G-CSF were measured in amniotic fluid supernatants by multiplex assay (R&D Systems Inc., Minneapolis, MN) on a MAGPIX instrument (Luminex Corp, Austin, TX) as per the manufacturer's instructions. All samples were diluted 1:2 prior to analysis.

2.7. Statistical analysis

Comparisons were made between variables in placentae with VUE, hCAM, FIR, and no inflammation (NI) using the Kruskal-Wallis test. Differences between groups were further analysed using the Mann-Whitney test. Presence of inflammatory reactions in the fetal blood vessels (either cord vasculitis or inflammation of the fetal vessels in the chorionic plate) was classified as FIR. Cytokine concentrations were corrected for dilution (x2) then expressed as a value relative to the appropriate control. SPSS (version 20.0, IBM SPSS) statistical software was used for data analysis. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Low grade placental inflammation is common in full-term pregnancies

Of the 44 patients enrolled in this study 21 (47.7%) showed

histological evidence of placental inflammation. This percentage is similar to that recently reported by Romero et al. for women delivering by elective caesarean section [13]. Of those placentae positive for inflammation, 4 had DEC (of which we were only able to obtain cord blood from 2), 9 had VUE, 9 had hCAM, and 12 had FIR (9 instances of umbilical cord vasculitis, 8 instances of inflammation in the fetal vessels of the chorionic plate). It is interesting to note that cases of VUE and hCAM did not overlap in this study, suggesting separate aetiologies. There was a high level of overlap between placentae with hCAM and those with FIR. In most cases the inflammation was mild, with only four cases of high grade inflammation observed (all cases of VUE). Histology results are described in full in Table 2.

Routine hospital screening for *Ureaplasma* spp., *Mycoplasma* spp., and *Listeria* spp. was negative for all placentae; however, this is not surprising given that the presence of these bacteria in the amniotic cavity is normally associated with early preterm birth [34].

3.2. Histological CAM and FIR are associated with increased amniotic fluid IL-6 levels

Amniotic fluid cytokine levels were within previously-reported ranges in all cases (Fig. 1). Amniotic fluid IL-6 was significantly elevated in pregnancies with hCAM and FIR compared to those with no inflammation ($P = 0.0475$ and $P = 0.0322$, respectively). An amniotic fluid IL-6 level of > 745 pg/mL is diagnostic of microbial invasion of the amniotic cavity and intra-amniotic inflammation [35]. Patients with hCAM in the present study had a mean amniotic fluid IL-6 level of 765.7 pg/mL, while those with FIR had a mean amniotic fluid IL-6 level of 805.4 pg/mL. Similarly, an amniotic fluid G-CSF level of > 2000 pg/mL is diagnostic of CAM [36]. Patients with hCAM in the present study met this criterion, with a mean amniotic fluid G-CSF level of 2027.7 pg/mL. Those with FIR had a mean amniotic fluid G-CSF concentration of 2184.4 pg/mL. There were no significant differences between the groups with respect to concentrations of IL-10 (an immunoregulatory cytokine with anti-inflammatory properties) or CXCL10 (a putative marker of chronic chorioamnionitis arising from maternal fetal allograft rejection [8]).

3.3. Cord blood immune response patterns differ with placental inflammation

With the exception of IL-8, baseline expression of all cord blood cytokines was low (Table 3), as has previously been reported elsewhere [37,38]. All forms of immune stimulation were associated with increased cord blood cytokine levels (Table 4). R848, LPS, and PGN elicited a strong inflammatory response across all cytokines, while poly (I:C), cGAMP, and 5′ppp-dsRNA elicited a weaker inflammatory response. We did not observe any correlation between amniotic fluid cytokine levels and baseline cord blood cytokine levels for the cytokines which were measured in both sample sets (IL-6, IL-10, and G-CSF).

No significant differences were observed between groups after immune stimulation with R848, LPS, or PGN, nor were any significant differences seen in levels of TNF-α relative to controls (data not shown).

Cord blood derived from fetuses exposed to hCAM had significantly lower IL-8, IL-10, IL-1β, and G-CSF responses to poly (I:C) than those with no placental inflammation (IL-8 $P = 0.0179$, IL-10 $P = 0.0262$, IL-1β $P = 0.0268$, G-CSF $P = 0.00135$) (Fig. 2A–D). Similarly, cord blood taken from fetuses with FIR had significantly lower IL-8, IL-10, and G-CSF responses to poly (I:C) than those with no placental inflammation (IL-8 $P = 0.0427$, IL-10 $P = 0.0262$, G-CSF $P = 0.0132$) (Fig. 2A, B, D). Interestingly, the FIR group differed from the CAM group in its response to cGAMP and 5′ppp-dsRNA. Stimulation with cGAMP elicited a significantly higher IL-6, IL-8, and IL-1β response in fetuses with FIR than those not exposed to inflammation (IL-6 $P = 0.00695$, IL-8 $P = 0.0336$, IL-1β $P = 0.0475$) (Fig. 2A, C, F). Stimulation with 5′ppp-dsRNA elicited a significantly higher IL-6 and IL-8 response in fetuses with FIR

Table 3
Baseline levels of cytokines (pg/ml) in cord blood of patients with deciduitis (DEC, n = 2), vasculitis of unknown aetiology (VUE, n = 6), histological chorioamnionitis (hCAM, n = 9), inflammatory reaction of the fetal vessels (FIR, n = 11), or no inflammation (NI, n = 20). Data are median ± IQR.

Cytokine	Concentration in unstimulated RPMI 1640 control (pg/ml)					Concentration in unstimulated LyoVec control (pg/ml)				
	DEC	VUE	hCAM	FIRS	NI	DEC	VUE	hCAM	FIRS	NI
G-CSF	32.0 ± 18.7	42.4 ± 49.5	42.0 ± 174.2	38.1 ± 54.2	32.3 ± 46.6	87.3 ± 70.2	42.4 ± 38.5	48.7 ± 47.7	38.3 ± 30.4	50.7 ± 113.4
IFN-γ	12.8 ± 12.8	8.8 ± 25.9	11.3 ± 14.8	9.1 ± 10.0	11.7 ± 20.5	52.3 ± 40.3	14.7 ± 12.0	19.1 ± 9.2	16.7 ± 13.8	17.4 ± 44.9
IL-1β	45.0 ± 40.0	22.9 ± 30.6	23.5 ± 100.6	20.5 ± 41.5	19.8 ± 57.4	57.2 ± 31.4	30.2 ± 24.4	20.6 ± 177.0	16.8 ± 11.7	36.1 ± 144.8
IL-6	54.4 ± 17.9	90.4 ± 378.9	28.7 ± 75.8	30.2 ± 176.8	24.3 ± 101.7	77.9 ± 15.1	31.1 ± 103.1	21.2 ± 27.5	21.2 ± 19.9	71.8 ± 463.9
IL-8	2797.6 ± 1872.8	1304.5 ± 3453.3	3567.7 ± 12213.2	3525.9 ± 11435.9	1689.2 ± 3672.6	10447.4 ± 9289.8	4470.4 ± 3754.3	1616.2 ± 2069.1	1517.2 ± 2650	4658.9 ± 3640.7
IL-10	4.6 ± 4.6	0.0 ± 2.8	2.1 ± 3.3	1.4 ± 2.9	1.3 ± 4.1	75.8 ± 74.6	3.8 ± 6.3	3.3 ± 3.7	3.0 ± 3.7	4.0 ± 46.9
TNF-α	16.5 ± 14.3	9.1 ± 7.3	8.8 ± 13.8	6.2 ± 12.0	7.2 ± 12.4	16.6 ± 7.0	9.7 ± 4.7	6.1 ± 6.8	7.8 ± 6.8	11.7 ± 16.4

Table 4
Mean fold change in cord blood cytokine levels following 24 h incubation with various immune stimuli.

	R848	LPS	PGN	Poly (I:C)	CGAMP	5′ppp-dsRNA
G-CSF	326.5	598.7	1126.1	17.7	13.8	11.6
IFN-γ	339.6	84.8	77.0	6.5	60.8	18.0
IL-1β	3948.7	2062.7	1157.0	25.1	225.7	163.6
IL-6	847.5	855.1	870.1	172.8	232.2	331.3
IL-8	96.9	92.4	105.6	91.0	5.5	9.7
IL-10	523.4	455.3	139.7	17.2	3.1	12.2
TNF-α	3507.0	610.5	748.0	10.4	178.6	103.8

than those not exposed to inflammation (IL-6 $P = 0.0228$, IL-8 $P = 0.0294$) (Fig. 2A & F).

Immune responses in cord blood taken from fetuses with VUE did not differ from those of fetuses that were not exposed to inflammation. Fetuses whose placentae showed evidence of VUE had lower IL-10 responses to poly (I:C) than those not exposed to inflammation; however, this did not quite reach statistical significance ($P = 0.0505$) (Fig. 2B). Interestingly, fetuses with VUE had significantly higher IFN-γ responses to cGAMP than those that were exposed to CAM ($P = 0.0485$, Fig. 2E).

4. Discussion

We examined innate cytokine response following PRR stimulation of whole cord blood from neonates with and without exposure to placental inflammation. We found that infants exposed to placental inflammation *in-utero* had altered cord blood innate immune responses at birth, and that these responses varied based on the type of inflammatory lesion present. While previous studies have characterised innate immune responses in preterm *vs*. full term infants [30], to our knowledge this is the first study to examine the innate immune responses in cases of low-level placental inflammation.

Placental inflammation is often sub-clinical (diagnosed histologically) and is known to be associated with preterm birth [1]. In this cohort of late preterm and full term deliveries, none of which displayed any clinical indications of intra-amniotic infection/inflammation or risk of preterm birth, a large portion (47.7%) were found to have histological evidence of placental inflammation. This figure is similar to that reported by Romero et al. in a cohort of women delivering by elective Caesarean section [13]. It is possible that many cases of low grade placental inflammation are missed in late preterm and full term births, as placentas from these deliveries are not routinely examined histologically. The significance of such a high rate of "silent" placental inflammation is unclear; however, the data presented in this paper suggest that such inflammation may be associated with altered innate immune responses in the fetus. It is interesting to note that cases of VUE and hCAM did not overlap in this study, suggesting separate aetiologies.

It is unclear whether the inflammation seen in this cohort has a microbiological aetiology or a sterile aetiology. Recent research has suggested that the intra-amniotic space is, in some pregnancies, colonised by bacteria prior to birth [39,40], and that this colonisation may alter fetal immune development [41,42]. Differences in placental inflammation and neonatal immune responses may, therefore, be reflective of differences in prenatal microbial colonisation.

A number of maternal and fetal characteristics could contribute to placental inflammation. Obesity and cigarette smoking are known to cause systemic inflammation [43,44]. In the present study the mean pre-pregnancy BMI of participants was elevated (30.1); however, there were no significant differences in pre-pregnancy BMI values between groups. The incidence of maternal smoking was actually greater in the inflammation negative group than the inflammation positive group (13% vs 5%). Diabetes could also contribute to placental inflammation; however, in the current study the incidence of maternal diabetes was higher in the inflammation-negative group than in the inflammation-positive group (48% *vs*. 33%). Previous studies have demonstrated that placental immune function is partially sex-dependent, and that the placenta responds to maternal inflammation in a sex-specific manner, with heightened responses in male placentae [45]. However, there was no significant difference in the rates of male births between groups in our study.

It is unclear whether differences in immune reactivity observed in this cohort are caused by placental inflammation, or whether both the immune differences and the placental inflammation are symptoms of a common underlying cause, be it sterile or microbial.

Fetuses exposed to hCAM and FIR showed significantly reduced immune responses to stimulation with poly (I:C). Poly (I:C) is a ligand of TLR3 capable of activating downstream NF-κB and interferon regulatory factors 3 and 7 (IRF3/7), stimulating the production of pro-inflammatory cytokines, interferons, and IL-10 [46]. Previous studies have revealed that cord blood natural killer (NK) cells have deficient TLR3 expression associated with an inability to respond to poly (I:C) [47]. Our data indicate that this dampened response to poly (I:C) is exaggerated in fetuses that are exposed to CAM during pregnancy. This is indicative of a greater impairment in NK TLR3 expression, which could result in heightened vulnerability to viral infections postnatally. It is unsurprising that fetuses exposed to hCAM and FIR reacted similarly to poly (I:C), given the overlap in cases of hCAM and FIR, and the likelihood that these conditions share a common aetiology.

Interestingly, the FIR group differed from the hCAM group in its response to the intracellular ligands cGAMP and 5′ppp-dsRNA. Fetuses exposed to FIR showed significantly increased immune responses to stimulation with cGAMP and 5′ppp-dsRNA (ligands of STING and RIG-I, respectively). The same was not true for fetuses exposed to hCAM. Both STING and RIG-I drive downstream type 1 interferon production, NF-κB activation, and antiviral gene transcription to control viral infections [48,49]. There exists much cross-talk and interplay between the STING and RIG-I pathways, so it is not surprising that their stimulation resulted in similar reactions between groups in this study.

In the present study we found that fetuses exposed to hCAM and FIR had dampened cytokine responses to stimulation with poly (I:C), and heightened cytokine responses to stimulation with cGAMP and 5′ppp-dsRNA. Given that TLR3 activates transcription of IRF3, and that STING and RIG-I activate transcription of IRF3 independently of the action of TLR3, this opposing reaction may be a compensatory mechanism. Interestingly, these aberrant reactions were almost exclusive to fetuses exposed to hCAM and FIR and not observed in fetuses exposed to VUE. The significance of such a finding is unclear, but could be related to the differences in aetiology of the placental lesions.

One limitation of this study was the use of whole blood stimulation without matched cell enumeration. We were not able to obtain sufficient sample volume to ascertain cell composition using flow cytometry. Therefore, differences in PRR responses may be due to different levels of innate immune cells in our samples. However, the measurement of immune responses from whole blood provides a global picture of antimicrobial responses. Non-cellular components of neonatal plasma effect TLR-mediated cytokine production [50,51]. Whole blood stimulation is therefore important for demonstrating total immune responsiveness.

5. Conclusion

Exposure to inflammation *in-utero* is associated with altered cytokine responses to PRRs at birth. Different types of inflammatory lesions were associated with distinct fetal innate immune response profiles. Further work is required to determine whether it is the inflammation itself that causes this effect, as opposed to exposure to microbial factors, and/or whether the inflammation and altered immune responses result from changes in the populations of circulating immune cells or their sensitivity and responsivity to challenge.

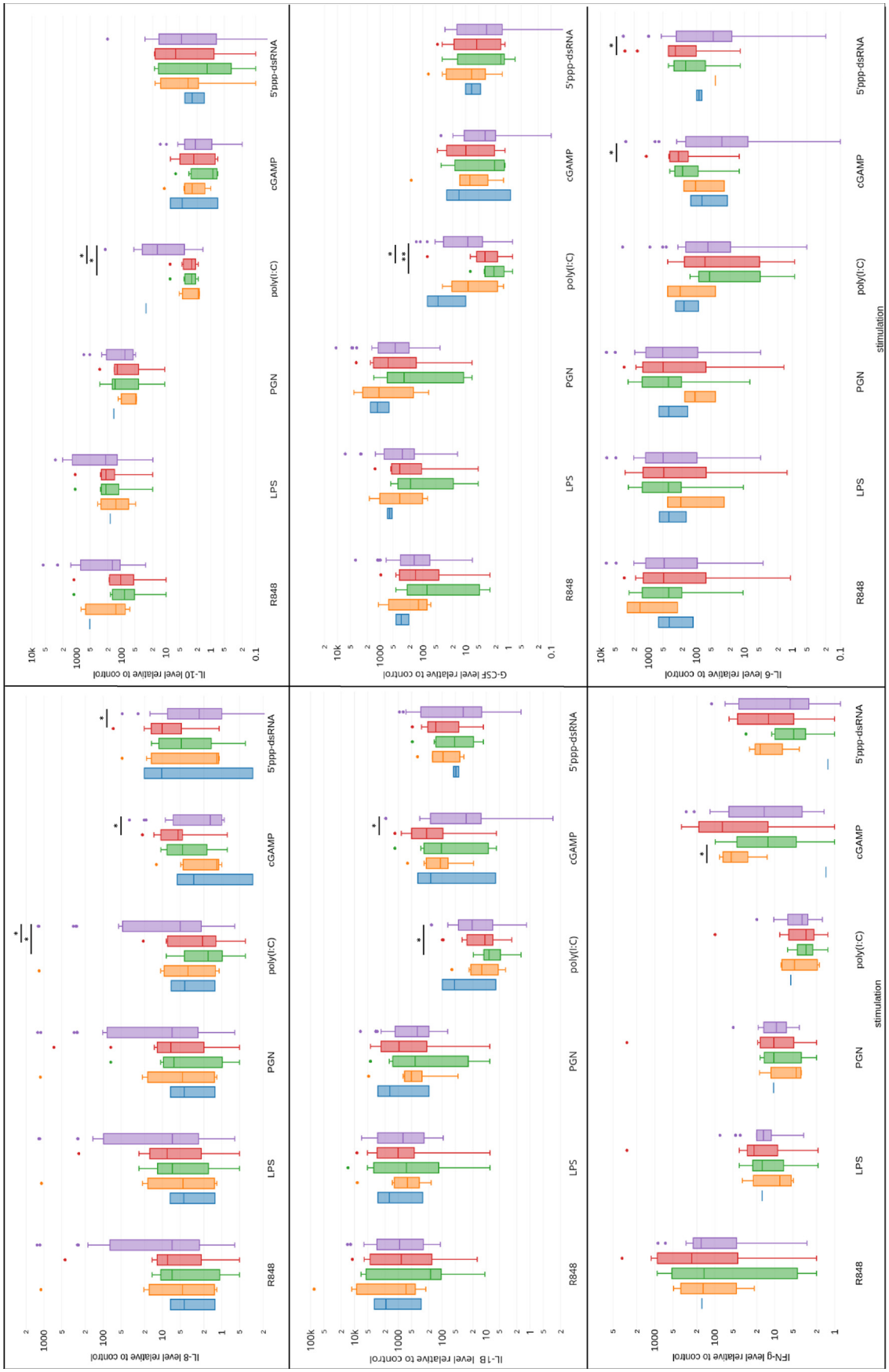

Fig. 2. Levels of IL-8 (panel A), IL-10 (panel B), IL-1β (panel C), G-CSF (panel D), IFN-γ (panel E), and IL-6 (panel F) in fetal cord blood plasma relative to unstimulated controls. Data is stratified according to type of placental inflammation (DEC, VUE, CAM, FIR, NI). $*P < 0.05$, $**P < 0.01$.

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Conflicts of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.08.079.

References

[1] R. Romero, J. Espinoza, L.F. Goncalves, J.P. Kusanovic, L. Friel, S. Hassan, The role of inflammation and infection in preterm birth, Semin. Reprod. Med. 25 (1) (2007) 21–39.
[2] Z. Liu, Z. Tang, J. Li, Y. Yang, Effects of placental inflammation on neonatal outcome in preterm infants, Pediatr. Neonatol. 55 (1) (2014) 35–40.
[3] C.J. Kim, R. Romero, P. Chaemsaithong, N. Chaiyasit, B.H. Yoon, Y.M. Kim, Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance, Am. J. Obstet. Gynecol. 213 (4 Suppl) (2015) S29–S52.
[4] R.W. Redline, Clinically and biologically relevant patterns of placental inflammation, Pediatr. Dev. Pathol. 5 (4) (2002) 326–328.
[5] R.W. Redline, Placental inflammation, Semin. Neonatol. 9 (4) (2004) 265–274.
[6] R.W. Redline, Inflammatory responses in the placenta and umbilical cord, Semin. Fetal Neonatal Med. 11 (5) (2006) 296–301.
[7] R.W. Redline, Villitis of unknown etiology: noninfectious chronic villitis in the placenta, Hum. Pathol. 38 (10) (2007) 1439–1446.
[8] C.J. Kim, R. Romero, P. Chaemsaithong, J.S. Kim, Chronic inflammation of the placenta: definition, classification, pathogenesis, and clinical significance, Am. J. Obstet. Gynecol. 213 (4 Suppl) (2015) S53–S69.
[9] J. Bar, L. Schreiber, A. Golan, M. Kovo, Placental factor in spontaneous term labor in uncomplicated pregnancy, J. Matern. Fetal Neonatal Med. 25 (12) (2012) 2704–2707.
[10] S. Pathak, C.C. Lees, G. Hackett, F. Jessop, N.J. Sebire, Frequency and clinical significance of placental histological lesions in an unselected population at or near term, Virchows Arch. 459 (6) (2011) 565–572.
[11] C.M. Salafia, C. Weigl, L. Silberman, The prevalence and distribution of acute placental inflammation in uncomplicated term pregnancies, Obstet. Gynecol. 73 (3 Pt 1) (1989) 383–389.
[12] I. Hulthen Varli, K. Petersson, M. Kublickas, N. Papadogiannakis, Both acute and chronic placental inflammation are overrepresented in term stillbirths: a case-control study, Infect. Dis. Obstet. Gynecol. 2012 (2012) 293867.
[13] R. Romero, Y.M. Kim, P. Pacora, C.J. Kim, N. Benshalom-Tirosh, S. Jaiman, G. Bhatti, J.S. Kim, F. Qureshi, S.M. Jacques, E.J. Jung, L. Yeo, B. Panaitescu, E. Maymon, S.S. Hassan, C.D. Hsu, O. Erez, The frequency and type of placental histologic lesions in term pregnancies with normal outcome, J. Perinat. Med. 46 (6) (2018) 613–630.
[14] N.P. Corbett, D. Blimkie, K.C. Ho, B. Cai, D.P. Sutherland, A. Kallos, J. Crabtree, A. Rein-Weston, P.M. Lavoie, S.E. Turvey, N.R. Hawkins, S.G. Self, C.B. Wilson, A.M. Hajjar, E.S. Fortuno 3rd, T.R. Kollmann, Ontogeny of Toll-like receptor mediated cytokine responses of human blood mononuclear cells, PLoS One 5 (11) (2010) e15041.
[15] O. Levy, Innate immunity of the newborn: basic mechanisms and clinical correlates, Nat. Rev. Immunol. 7 (5) (2007) 379–390.
[16] H. Zaghouani, C.M. Hoeman, B. Adkins, Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells, Trends Immunol. 30 (12) (2009) 585–591.
[17] B. Adkins, C. Leclerc, S. Marshall-Clarke, Neonatal adaptive immunity comes of age, Nat. Rev. Immunol. 4 (7) (2004) 553–564.
[18] D.J. Dowling, O. Levy, Ontogeny of early life immunity, Trends Immunol. 35 (7) (2014) 299–310.
[19] T.R. Kollmann, J. Crabtree, A. Rein-Weston, D. Blimkie, F. Thommai, X.Y. Wang, P.M. Lavoie, J. Furlong, E.S. Fortuno 3rd, A.M. Hajjar, N.R. Hawkins, S.G. Self, C.B. Wilson, Neonatal innate TLR-mediated responses are distinct from those of adults, J. Immunol. 183 (11) (2009) 7150–7160.
[20] H.K. Bedi, Ontogeny of Cytokine Responses Following Cytoplasmic Pattern Recognition Receptor Stimulation, The University of British Columbia, Vancouver, Canada, 2013.
[21] P.S. Noakes, J. Hale, R. Thomas, C. Lane, S.G. Devadason, S.L. Prescott, Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses, Eur. Respir. J. 28 (4) (2006) 721–729.
[22] P.S. Noakes, P.G. Holt, S.L. Prescott, Maternal smoking in pregnancy alters neonatal cytokine responses, Allergy 58 (10) (2003) 1053–1058.
[23] D. Getahun, D. Strickland, R.S. Zeiger, M.J. Fassett, W. Chen, G.G. Rhoads, S.J. Jacobsen, Effect of chorioamnionitis on early childhood asthma, Arch. Pediatr. Adolesc. Med. 164 (2) (2010) 187–192.
[24] R. Kumar, Y. Yu, R.E. Story, J.A. Pongracic, R. Gupta, C. Pearson, K. Ortiz, H.C. Bauchner, X. Wang, Prematurity, chorioamnionitis, and the development of recurrent wheezing: a prospective birth cohort study, J. Allergy Clin. Immunol. 121 (4) (2008) 878–884 e6.
[25] T. Zhu, L. Zhang, Y. Qu, D. Mu, Meta-analysis of antenatal infection and risk of asthma and eczema, Medicine (Baltim.) 95 (35) (2016) e4671.
[26] D. Blimkie, E.S. Fortuno 3rd, H. Yan, P. Cho, K. Ho, S.E. Turvey, A. Marchant, S. Goriely, T.R. Kollmann, Variables to be controlled in the assessment of blood innate immune responses to Toll-like receptor stimulation, J. Immunol. Methods 366 (1–2) (2011) 89–99.
[27] K. Jansen, D. Blimkie, J. Furlong, A. Hajjar, A. Rein-Weston, J. Crabtree, B. Reikie, C. Wilson, T. Kollmann, Polychromatic flow cytometric high-throughput assay to analyze the innate immune response to Toll-like receptor stimulation, J. Immunol. Methods 336 (2) (2008) 183–192.
[28] B.A. Reikie, R.C. Adams, C.E. Ruck, K. Ho, A. Leligdowicz, S. Pillay, S. Naidoo, E.S. Fortuno 3rd, C. de Beer, W. Preiser, M.F. Cotton, D.P. Speert, M. Esser, T.R. Kollmann, Ontogeny of Toll-like receptor mediated cytokine responses of South African infants throughout the first year of life, PLoS One 7 (9) (2012) e44763.
[29] K.K. Smolen, C.E. Ruck, E.S. Fortuno 3rd, K. Ho, P. Dimitriu, W.W. Mohn, D.P. Speert, P.J. Cooper, M. Esser, T. Goetghebuer, A. Marchant, T.R. Kollmann, Pattern recognition receptor-mediated cytokine response in infants across 4 continents, J. Allergy Clin. Immunol. 133 (3) (2014) 818–826 e4.
[30] E.A. Marchant, B. Kan, A.A. Sharma, A. van Zanten, T.R. Kollmann, R. Brant, P.M. Lavoie, Attenuated innate immune defenses in very premature neonates during the neonatal period, Pediatr. Res. 78 (5) (2015) 492–497.
[31] M.W. Kemp, Preterm birth, intrauterine infection, and fetal inflammation, Front. Immunol. 5 (2014) 574.
[32] K. Mestan, Y. Yu, P. Thorsen, K. Skogstrand, N. Matoba, X. Liu, R. Kumar, D.M. Hougaard, M. Gupta, C. Pearson, K. Ortiz, H. Bauchner, X. Wang, Cord blood biomarkers of the fetal inflammatory response, J. Matern. Fetal Neonatal Med. 22 (5) (2009) 379–387.
[33] R. Romero, P. Chaemsaithong, N. Docheva, S.J. Korzeniewski, A.L. Tarca, G. Bhatti, Z. Xu, J.P. Kusanovic, N. Chaiyasit, Z. Dong, B.H. Yoon, S.S. Hassan, T. Chaiworapongsa, L. Yeo, Y.M. Kim, Clinical chorioamnionitis at term V: umbilical cord plasma cytokine profile in the context of a systemic maternal inflammatory response, J. Perinat. Med. 44 (1) (2016) 53–76.
[34] H.E. Jones, K.A. Harris, M. Azizia, L. Bank, B. Carpenter, J.C. Hartley, N. Klein, D. Peebles, Differing prevalence and diversity of bacterial species in fetal membranes from very preterm and term labor, PLoS One 4 (12) (2009) e8205.
[35] P. Chaemsaithong, R. Romero, S.J. Korzeniewski, A. Martinez-Varea, Z. Dong, B.H. Yoon, S.S. Hassan, T. Chaiworapongsa, L. Yeo, A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes, J. Matern. Fetal Neonatal Med. 29 (3) (2016) 349–359.
[36] I.A. Hoskins, P. Zandieh, F. Schatz, C. Lee, Amniotic fluid granulocyte colony stimulating factor levels: a rapid marker for diagnosing chorioamnionitis, Am. J. Reprod. Immunol. 38 (4) (1997) 286–288.
[37] E.E. Garanina, D. Gatina, E.V. Martynova, A. Rizvanov, S. Khaiboullina, I. Salafutdinov, Cytokine profiling of human umbilical cord plasma and human umbilical cord blood mononuclear cells, Blood 130 (2017).
[38] N. Takahashi, R. Uehara, M. Kobayashi, Y. Yada, Y. Koike, R. Kawamata, J. Odaka, Y. Honma, M.Y. Momoi, Cytokine profiles of seventeen cytokines, growth factors and chemokines in cord blood and its relation to perinatal clinical findings, Cytokine 49 (3) (2010) 331–337.
[39] L.F. Stinson, M.C. Boyce, M.S. Payne, J.A. Keelan, The not-so-sterile womb: evidence that the human fetus is exposed to bacteria prior to birth, Front. Microbiol. 10 (2019) 1124.
[40] L.F. Stinson, M.S. Payne, J.A. Keelan, Planting the seed: origins, composition, and postnatal health significance of the fetal gastrointestinal microbiota, Crit. Rev. Microbiol. (2016) 1–18.
[41] J.L. Kaplan, H.N. Shi, W.A. Walker, The role of microbes in developmental immunologic programming, Pediatr. Res. 69 (6) (2011) 465–472.
[42] J. Romano-Keeler, J.H. Weitkamp, Maternal influences on fetal microbial colonization and immune development, Pediatr. Res. 77 (1–2) (2015) 189–195.
[43] R. Monteiro, I. Azevedo, Chronic inflammation in obesity and the metabolic syndrome, Mediat. Inflamm. 2010 (2010).
[44] M.S. Shiels, H.A. Katki, N.D. Freedman, M.P. Purdue, N. Wentzensen, B. Trabert, C.M. Kitahara, M. Furr, Y. Li, T.J. Kemp, J.J. Goedert, C.M. Chang, E.A. Engels, N.E. Caporaso, L.A. Pinto, A. Hildesheim, A.K. Chaturvedi, Cigarette smoking and variations in systemic immune and inflammation markers, J. Natl. Cancer Inst. 106

(11) (2014).
[45] V.L. Clifton, Review: sex and the human placenta: mediating differential strategies of fetal growth and survival, Placenta 31 (Suppl) (2010) S33–S39.
[46] T. Kawasaki, T. Kawai, Toll-like receptor signaling pathways, Front. Immunol. 5 (2014) 461.
[47] L. Slavica, I. Nordstrom, M.N. Karlsson, H. Valadi, M. Kacerovsky, B. Jacobsson, K. Eriksson, TLR3 impairment in human newborns, J. Leukoc. Biol. 94 (5) (2013) 1003–1011.
[48] G.N. Barber, STING: infection, inflammation and cancer, Nat. Rev. Immunol. 15 (12) (2015) 760–770.
[49] Y.M. Loo, M. Gale Jr., Immune signaling by RIG-I-like receptors, Immunity 34 (5) (2011) 680–692.
[50] M.E. Belderbos, O. Levy, F. Stalpers, J.L. Kimpen, L. Meyaard, L. Bont, Neonatal plasma polarizes TLR4-mediated cytokine responses towards low IL-12p70 and high IL-10 production via distinct factors, PLoS One 7 (3) (2012) e33419.
[51] O. Levy, K.A. Zarember, R.M. Roy, C. Cywes, P.J. Godowski, M.R. Wessels, Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848, J. Immunol. 173 (7) (2004) 4627–4634.