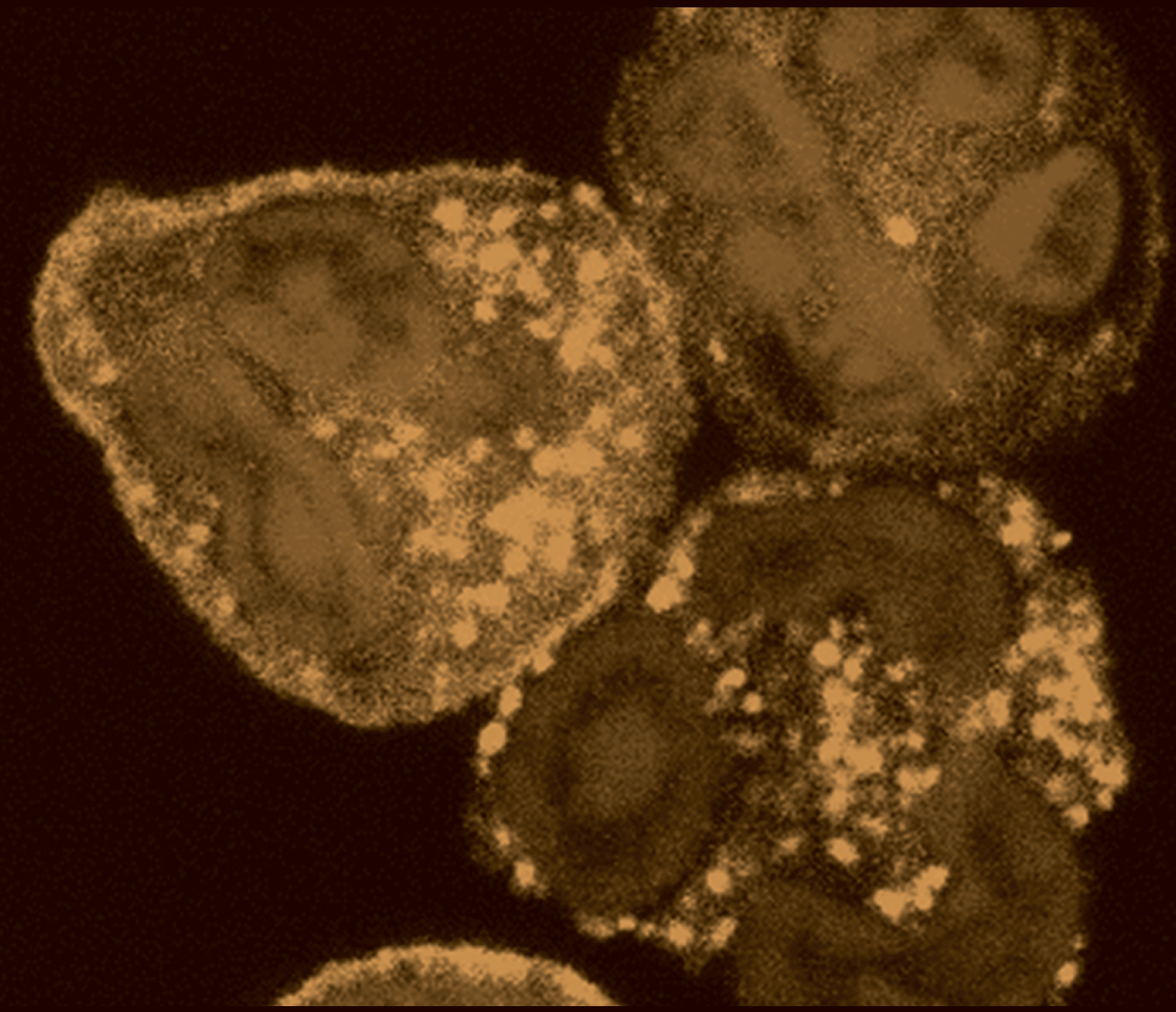
Mediators of Inflammation

Cytokines in Placental Physiology and Disease

Guest Editors: Dariusz Szukiewicz, Felipe Vadillo-Ortega, Morgan R. Peltier, and Noboru Uchide



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Editorial Board

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Editorial

Cytokines in Placental Physiology and Disease

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The placenta forms connection between growing fetus and uterine wall and is a unique, temporary, highly specialized organ required for the development of the embryo and fetus. In humans, as early as 3-4 days after fertilization trophoblastic cells can be identified on the outer layer of cells within the blastocyst. These precursor cells differentiate into all the other placental cell types that build the future, highly specialized organ. After approximately 12-13 weeks (end of the first trimester of gestation) development of the maternal blood supply to the placenta is completed. The most important functions and roles of the human placenta include metabolic (synthesis of glycogen, cholesterol, and fatty acids), transport and exchange within maternal-placental-fetal interface (gases, nutrients, and waste products), endocrine (synthesis and secretion of the pregnancy supporting hormones), immunological (immuno-suppressive function that protects the fetal "allograft" from T-cell-mediated immune aggression as well as—to some degree—protective role against infectious agents).

Many research papers elucidated that physiological phenomena related to normal growth, development, and reproduction of living organisms are govern by complex cytokine interactions. It is becoming increasingly apparent that cytokines (CKs) not exclusively are synthesized within the "immune cells," but are the product of a whole host of cell types, including nonimmune and/or structural cells such as epithelial cells, cytotrophoblast cells, and fibroblasts. Almost all CKs are pleiotropic effectors showing multiple biological activities. Interestingly, the human placenta produces a variety of CKs and expresses virtually all known CKs. Both successful placentation with trophoblast invasion and normal placental development accompanied by vascular and angiogenesis require precise balance between all members of the local (utero-placento fetal) cytokine network.

Increased number of the innovative research approaches devoted to the better understanding of the connections between CKs, their receptors, and placental malfunction have recently been undertaken. On this background, the data about the role of cytokines in placental physiology and disease are still accumulating. The studies are focusing on the involvement of CKs in the processes of implantation, invasion of the trophoblast into the spiral uterine arteries, placental angiogenesis, response to inflammatory and immunologic factors in the uteroplacental interface, and induction of term/preterm labour.

In this special issue of Mediators of Inflammation, I am pleased to present to the reader several articles written by experts in the field. All of them share the same subject mater, CKs in the human uteroplacental compartment.

Modifications in the production of CKs by human placenta and fetal membranes are observed in response to infection-caused inflammation. However, precise changes in the placental cytokine profile during the course of an infection are hard to predict because of the heterogeneity of infectious agents. Extensive studies in this field, particularly related to the influenza virus infection, were performed by N. Uchide et al.

Recently, the results of significant studies on heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence have been published. The role of the so-called epigenetic factors may be greater than previously thought. Moreover, in contrast to genetically governed inheritance, epigenetic heritability is potentially reversible, which may facilitate implementation of new therapeutic strategies. The pathomechanism of preterm labour involves a shift in the balance between pro- and anti-inflammatory CKs synthesized by gestation tissues. Modulation of the placental

cytokine production by epigenetic mechanisms has been proved. The original research paper dealing with the mentioned issues in the context of inhibitors of DNA methylation and a histone deacetylation is presented by M. D. Mitchell et al. It may be of high relevance, considering that immaturity remains a leading cause of neonatal morbidity and mortality worldwide.

A specific form of immunosuppression is required for a successful pregnancy, while protection of the fetus, which resembles a semiallogenic graft, should not be compromising for the mother. Depending on their pattern of cytokine synthesis, subpopulations Th1 and Th2 of T helper cells are distinguished. A shift from Th1 response towards Th2 profile was reported and proposed as the important mechanism of induction of maternal tolerance and suppression. An interesting review paper on the relationship between preterm labour and an aberrant Th1:Th2 profile is presented by L. Sykes et al. Elsewhere in this special issue, L. Sykes et al. published original paper on the influence of prostaglandin J_2 ($15dPGJ_2$) on the production of proinflammatory cytokines by T helper cells in pregnancy. Authors suggest potential therapeutic benefit of $15dPGJ_2$ in inflammation-induced preterm labour.

Increased concentration of chemokine interleukin-8 (IL-8) has been demonstrated in the uterus during term and preterm labour. Another contribution to the knowledge of cytokine-dependent mechanisms that trigger contractile activity of the uterus was given by S. Khanjani et al. This work focuses on the roles of transcription factors (NF-κB, AP-1, CEBP) in interleukin-1β (IL-1β) regulation of the IL-8 gene in myometrial cells cultured in vitro.

The pathogenesis of preeclampsia or pregnancy-induced hypertension remains largely unknown. It has been hypothesized that placental ischemia due to failed invasion of the trophoblastic cells in uterine spiral arteries may be an initiating factor. Different distribution of mast cells in the placenta and corresponding changes in histamine concentration may be involved in the defective placental vascularization seen in preeclampsia as reported by G. Szewczyk et al.

Acknowledgment

I would like to thank all contributors and reviewers, especially the Guest Editors of this special issue Morgan R. Peltier, Noboru Uchide, and Felipe Vadillo-Ortega for their excellent work, commitment, and support.

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Review Article

Possible Roles of Proinflammatory and Chemoattractive Cytokines Produced by Human Fetal Membrane Cells in the Pathology of Adverse Pregnancy Outcomes Associated with Influenza Virus Infection

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Pregnant women are at an increased risk of influenza-associated adverse outcomes, such as premature delivery, based on data from the latest pandemic with a novel influenza A (H1N1) virus in 2009-2010. It has been suggested that the transplacental transmission of influenza viruses is rarely detected in humans. A series of our study has demonstrated that influenza virus infection induced apoptosis in primary cultured human fetal membrane chorion cells, from which a factor with monocyte differentiation-inducing (MDI) activity was secreted. Proinflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-β, were identified as a member of the MDI factor. Influenza virus infection induced the mRNA expression of not only the proinflammatory cytokines but also chemoattractive cytokines, such as monocyte chemoattractant protein (MCP)-1, regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1β, IL-8, growth-regulated oncogene (GRO)-α, GRO-β, epithelial cell-derived neutrophil-activating protein (ENA)-78, and interferon inducible protein (IP)-10 in cultured chorion cells. These cytokines are postulated to associate with human parturition. This paper, therefore, reviews (1) lessons from pandemic H1N1 2009 in pregnancy, (2) production of proinflammatory and chemoattractive cytokines by human fetal membranes and their functions in gestational tissues, and (3) possible roles of cytokines produced by human fetal membranes in the pathology of adverse pregnancy outcomes associated with influenza virus infection.

1. Introduction

The human fetal membranes play a critical role as defensive barriers against infectious agents in order to maintain normal pregnancy. They produce a wide variety of cytokines that can initiate and regulate inflammatory responses. The proinflammatory and chemoattractive cytokines produced by the fetal membranes have been postulated to play a central role in the physiology of normal parturition and the pathology of premature delivery associated with intrauterine infections.

Based on the data from pandemic H1N1 2009, it has been clearly demonstrated that pregnant women are at an increased risk of influenza-associated adverse outcomes, such as premature delivery. It has been suggested that the transplacental transmission of human influenza A viruses, such as A(H1N1)pdm09 and (H3N2), human influenza B virus, and highly pathogenic avian influenza A virus (H5N1), is uncommon but rarely detected in humans. However, the etiology of adverse pregnancy outcomes associated with influenza virus infection has remained unclear. In order to understand the etiology, we have been investigating direct effects of influenza virus infection on human fetal membrane tissues.

A series of our study has demonstrated that influenza virus infection induced apoptosis in primary cultured human fetal membrane chorion cells, from which a factor with monocyte differentiation-inducing (MDI) activity

was secreted. Influenza virus infection induced the gene expression of various proinflammatory and chemoattractive cytokines in cultured human fetal membrane cells.

This paper reviews (1) lessons from pandemic H1N1 2009 in pregnancy, (2) production of proinflammatory and chemoattractive cytokines by human fetal membranes and their functions in gestational tissues, and (3) possible roles of cytokines produced by human fetal membranes in the pathology of adverse pregnancy outcomes associated with influenza virus infection.

2. Lessons from Pandemic H1N1 2009 in Pregnancy

We have experienced the pandemic with a novel influenza A (H1N1) 2009 virus in 2009-2010 [1], then we are now facing to a postpandemic situation [2]. Some countries, such as India and New Zealand, are still in significant levels of infection with the pandemic strain of influenza A (H1N1) 2009 virus [2], a strain of which is now termed as A(H1N1)pdm09 virus by the World Health Organization [3]. Before the emergence of A(H1N1)pdm09 virus, much of what we knew about influenza in pregnancy was based on indirect evidence, such as studies that used acute respiratory hospitalizations during influenza season as a proxy for influenza illness [4–6] and observations from a series of pandemics cases of pregnant women with uncertain representativeness [7–11]. Indeed, lessons learned from pandemic H1N1 2009 expand our knowledge with better information for treatment of pregnant women infected with influenza viruses and the adverse effect of influenza virus infection on pregnant women and neonates [12]. It is evidently reconfirmed that pregnant women are at an increased risk for influenza-associated adverse outcomes, based on data from the pandemic H1N1 2009 and recent seasonal influenza [13].

2.1. Adverse Pregnancy Outcomes Associated with A(H1N1)pdm09 Virus Infection

2.1.1. Increased Risk of Hospitalization, Intensive Care Unit Admission, Death, and Premature Delivery. Many studies demonstrated that pregnant women, when compared to nonpregnant women of similar age or when compared to the general population, have an increased risk of hospitalization, intensive care unit (ICU) admission, death, and severe outcomes due to pandemic H1N1 2009 (Table 1) [14–28]. Five studies demonstrated that neuraminidase (NA) inhibitors (i.e., oseltamivir (1) and zanamivir (2)) administrated within 48 hour of symptom onset conferred the decreased risk of developing severe disease (Figure 1) [17, 19, 29–31]. New NA inhibitors (i.e., peramivir (3) and laninamivir (4)) are now available for treatment of influenza (Figure 1). It is not established whether peramivir and laninamivir are safe for pregnant woman and her baby or not.

Preterm delivery, whether spontaneous or iatrogenic, was commonly reported, particularly with severe maternal illness (Table 2) [15, 17, 19, 21, 29–42]. In 3 of 6 series reporting >50 pregnancy outcomes, preterm birth rates approximated or exceeded 30% [15, 30, 31]. Yates et al. found that when pregnant women infected with A(H1N1)pdm09 virus were compared to uninfected pregnant women, they had increased odds of preterm birth (odds ratio, 5.5; 95% confidence interval, 3.5–8.3) and of very preterm birth at <32 weeks (odds ratio, 4.3; 95% confidence interval, 2.1–8.9) [31]. Creanga et al. found that preterm deliveries more frequently (3.3-fold, $P < 0.001$) occurred in pregnant women who admitted in ICU than those who did not [43]. Cesarean delivery was also commonly reported among mothers with pandemic H1N1 2009 and performed in 90% of fatal cases due to maternal refractory gas exchange abnormalities and in 37% of nonfatal cases [44].

No fatal case of pregnant woman infected with A(H1N1)pdm09 virus was observed in Japan. Nakai et al. reported data including 181 pregnant women hospitalized for A(H1N1)pdm09 virus infection from 2,082 clinical facilities in Japan (Table 3) [39]. Pregnant women who required hospitalization were more likely to give birth prematurely (relative risk, 2.5; 95% confidence interval, 1.7–3.6) than those in the general population. Pregnant women developing pneumonitis were more likely to give birth prematurely than those in the absence of pneumonitis ($P < 0.05$).

2.1.2. Neonatal Infection with A(H1N1)pdm09 Virus. Many neonates required neonatal ICU (NICU) admission and extended hospital stays, and these were largely for preterm birth rather than neonatal influenza [15, 29]. Fourteen papers reported that all specimens were negative for A(H1N1)pdm09 virus among tested 81 neonates, 7 maternal sera, 32 placentas, and 7 amniotic fluids in totally [19, 21, 29, 34, 41, 45–53]. In contrast, among tested 20 neonates in the Australia and New Zealand, 2 neonates were positive for A(H1N1)pdm09 virus infection [15]. In another study, 1 of 6 neonates died from A(H1N1)pdm09 virus infection [36].

As the risk of transmission of influenza virus from mother to fetus is unknown, the neonate should be considered potentially infected if delivery occurs during the 2 days before through to 7 days after illness onset in the mother [54]. Intrauterine infection of the fetus is potentially possible from maternal influenza viremia [55]. Since influenza has rarely been detected in vaginal secretions, it is most likely that the neonate will be infected postnatally through the respiratory route [54]. Consequently, the neonate should be considered potentially infected irrespective of delivery route. Gérardin et al. observed that a neonate delivered by cesarean section was positive for A(H1N1)pdm09 virus but asymptomatic [19], in the case postnatal transmission should be suspected.

2.1.3. Transplacental Transmission of Influenza Viruses. The transplacental transmission of influenza viruses is uncommon but rarely detected in humans. It has been raised a possibility of vertical transmission in 4 neonates delivered by cesarean section that have not been exposed to their mothers with infection [56–59]. Although two publications have demonstrated that viremia with A(H1N1)pdm09 virus occurred in 16 severe cases [60, 61], Gérardin et al. failed

Table 1: Relative risk of hospitalization, intensive care unit admission, death, or any severe outcome in pregnant women due to 2009 H1N1 influenza.

Paper	No. of hospitalized pregnant women with laboratory-confirmed A(H1N1)pdm09 virus infection	Risk of hospitalization	Risk of ICU admission	Risk of death	Risk of severe outcome
ANZIC [15]	64[†]		RR, 7.4[a]		
Campbell et al. [16]	170		RR, 0.7 (0.4–1.2)[a]	RR, 1.1 (0.3–4.1)[a]	RR, 0.7 (0.4–1.3)
Creanga et al. [17]	62	RR, 7.2[a]			RR, 4.3[a]
Fuhrman et al. [18]	18			aOR, 0.3 (0.04–3.0)	aOR, 0.5 (0.2–0.8)
Gérardin et al. [19]	141	RR, 1.1 (0.5–2.0)	RR, 0.4 (0.0–2.6)[a]		
Hanslik et al. [20]	59		RR, 5.2 (4.0–6.9)	RR, 1.4 (0.3–4.2)	
Jamieson et al. [21]	34	RR, 4.3 (2.3–7.8)[a]			
Kelly et al. [22]	273	RR, 5.2 (4.6–5.8)[b]	RR, 6.5 (4.8–8.8)[b]	RR, 1.4 (0.4–4.5)[b]	
Koegelenberg et al. [23]	6			OR, 1.13 (0.14–8.88)	
New South Wales public health network [24]	16		RR, 5.8[a]	RR, 10.2[a]	
Oliveira et al. [25]	525			RR, 1.07 (0.82–1.41)[a]	
Yang et al. [26]	17			OR, 0.8 (0.2–3.5)	OR, 0.4 (0.2–3.4)
Zarychanski et al. [27]	22		OR, 3.64 (0.86–15.4)[a,c]		

Abbreviations used: ANZIC: ANZIC Influenza Investigators and Australasian Maternity Outcomes Surveillance System; aOR: adjusted odds ratio; ICU: intensive care unit; OR: odds ratio; RR: relative risk. Superscript: [a]compared to nonpregnant women of reproductive age; [b]compared to general population; [c]this number reports increased odds that pregnant women would require ICU admission over that they would require only outpatient treatment. Symbol: [†]40 pregnant women, 22 postpartum women, and 2 miscarried women. Ranges of 95% confidence interval are shown in parentheses. This table is reproduced from Mosby et al. [14] with minor modifications.

to detect viral RNA of A(H1N1)pdm09 virus in 17 sera obtained from pregnant women infected with the virus.

Many other studies suggest that human influenza A (H3N2) virus, human influenza B virus, and highly pathogenic avian influenza A (H5N1) virus can cross the placenta in humans [62–72]. Viremia with influenza viruses has been reported but appears to be rare with human influenza A viruses (H2N2, H3N2) [73]. In 2007, Parkins et al. have documented that a pregnant woman at 32 weeks' gestation was hospitalized for pneumonia caused by seasonal influenza and subjected to emergency cesarean section at 3 days after admission; RNA for influenza A (subtype H1) virus was detected in serum sample collected at hospital admission [55]. Therefore, it is believed that transplacental transmission of influenza virus may occur through the bloodstream.

Most recently, Lieberman et al. published their findings from an investigation of placenta associated with a 20-week intrauterine fetal demise that occurred after exposure to seasonal influenza A (H1N1) virus early during the pregnancy at 2–6 weeks of gestation [74]. Light microscopy revealed that histiocytes were abundant in the maternal space (chronic intervillositis) and were noted within the fetal chorionic villi. Electron microscopy revealed that histiocytes identified from the maternal intervillous space and fetal chorionic villi demonstrated characteristics of viral production and that several well-formed viral capsids were noted within the cytoplasm, each containing regularly spaced projections along the surface of the virion corresponding to the hemagglutinin (HA) and NA spikes. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirmed the presence of M1 capsid genes of influenza A virus. Immunohistochemical analysis using influenza A (H1N1) virus-specific antibody revealed that viral antigen was detected in the histiocytes of the intervillous space (maternal) and fetal intravillous histiocytes in the placenta. Viral antigen was detected on the surface of epithelial cells and histiocytes in the fetal respiratory and gastrointestinal tracts. The fetus was XY karyotype. Y-specific immunofluorescent staining revealed that only the intravillous (fetal) histiocytes were stained positively, confirming that chronic inflammatory responses were associated with maternal and fetal macrophages.

2.2. Questions about the Etiology of Adverse Pregnancy Outcomes Associated with Influenza. Lessons learned from pandemic H1N1 2009 pose questions about potential, yet unknown, pathophysiological associations among maternal influenza, transplacental transmission of the virus, and adverse pregnancy outcomes. Multiple factors may influence the transplacental passage of influenza virus and its subsequent effects on the fetus. As proposed by various

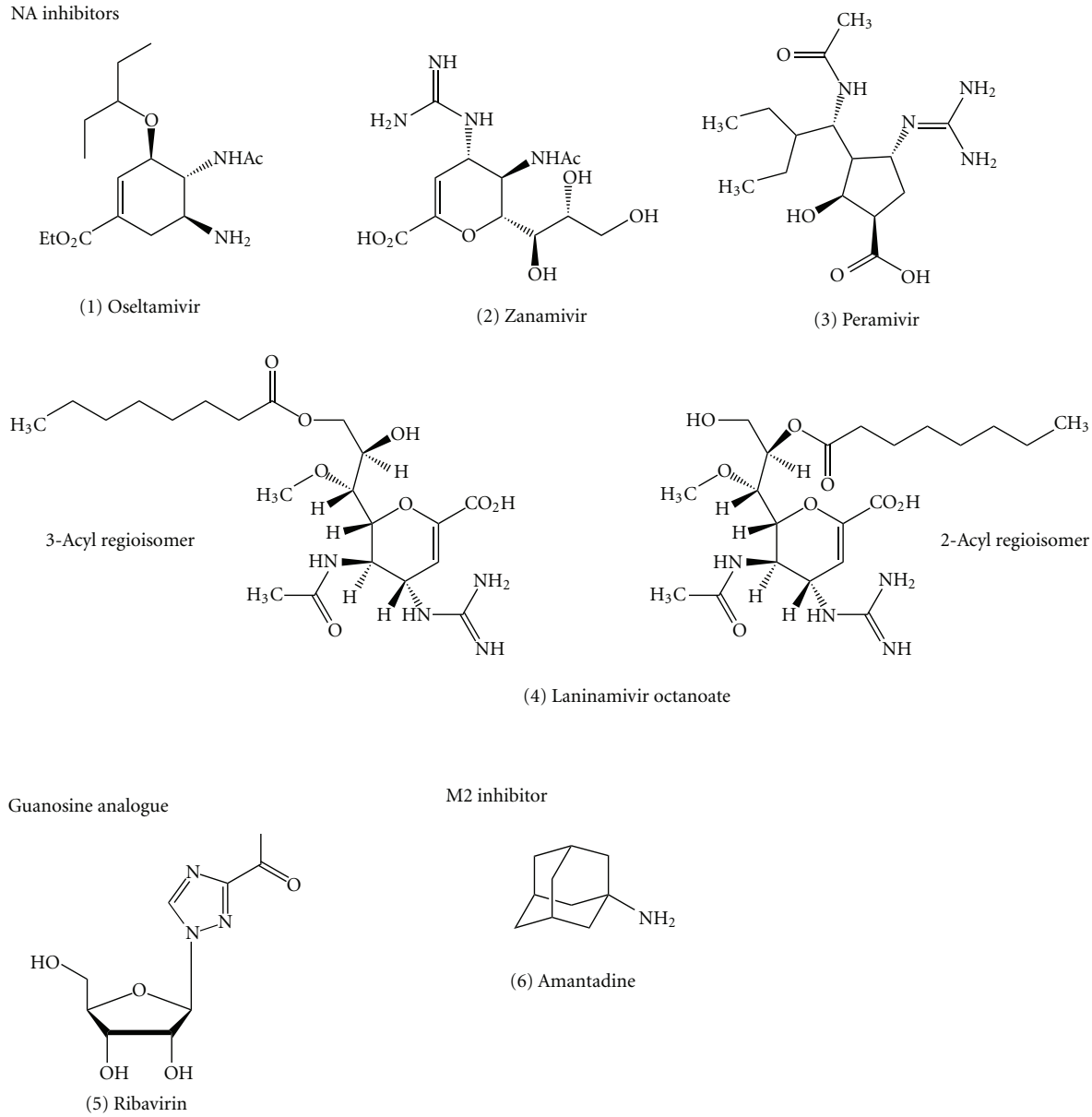

Figure 1: List of anti-influenza drugs currently available.

authors, key factors among these may be the virulence of the strain of influenza virus [68], the timing of exposure [74], maternal viremia [55], maternal immune response, and the use of antiviral agents [75]. Importantly, as Rasmussen et al. noted, the different degrees of influenza illness severity in the mother and the difficulty in separating the effects of the infection itself from those of the medications used to treat the infection will make research and interpretation of research findings difficult [75]. Fridman et al. documented that chorioamnionitis was suspected in 1 of 2 pregnant women infected with A(H1N1)pdm09 virus at 37 weeks' gestation, but they did not clarify the infectious agents for chorioamnionitis [48]. Yet, clinical research efforts are greatly needed.

The clinical evidence of transplacental transmission of influenza virus in humans is incomplete yet. Now, a pregnant mouse model established recently is available to understand the adverse effect of influenza virus infection on pregnancy through transplacental transmission of the virus. The intranasal infection with highly pathogenic avian influenza A (H5N1) virus in pregnant mice has been shown to cause vertical transmission of the virus and preterm delivery [76]. Furthermore, our *in vitro* study using primary cultured human fetal membrane cells may provide a good model to understand the etiology of adverse pregnancy outcomes associated with intrauterine influenza virus infection. It is useful to elucidate the direct effect of influenza virus infection on cultured human fetal membrane cells and will provide presumable molecular and cell biological mechanisms of adverse pregnancy outcomes associated with influenza virus infection. On the basis of results obtained from the *in vitro* study, target molecules to diagnose and treat

Table 2: Number of deliveries, gestational age, mode of delivery, and neonatal outcome in pregnancies affected by 2009 H1N1 influenza.

Papers	No. of deliveries	Proportion of preterm deliveries	Proportion of cesarean deliveries	Fetal/neonatal survival
United States of America				
CDC, 2010 [32]	9	5/9 (56%)	4/9 (44%)	1 stillbirth, 1 neonatal death
Creanga et al., 2010 [17]	22 while ill 22 after recovery	6/44 (14%) While ill: 3/22 (2 in women with severe disease) After recovery: 3/22	While ill: 11/22 (50%) After recovery: 7/22 (32%)	2 neonatal deaths
Jamieson et al., 2009 [21]	6‡	6/6 (100%)	6/6 (100%; 5 in cases with maternal death)	1 PROM
Louie et al., 2010 [29]	35	3/35 (9%) second trimester (25–28 weeks) 32/35 (91%) third trimester (>28 weeks)	At least 9, including 4 in ICU	11 NICU admissions
Miller et al., 2010 [33]	7	6/7 (86%)	4/7 (57%)	2 spontaneous abortion
Siston et al., 2010 [30]	169	51/169 (30%)	109/188 (58%)	
Australia				
ANZIC, 2010 [15]	59	22/60† (36.7%) 11 (32–36 weeks) 11 (20–31 weeks)	While ill: 13/14 (93%) in ICU After recovery: 6/23 (26%)	4 stillbirths, 3 infant deaths 32 NICU admissions
Hewagama et al., 2010 [34]	15	6/15 (40.0%)		2 stillbirths, 1 neonatal death due to prematurity
France (La Reunion)				
Gérardin et al., 2010 [19]	115	17/115 (14.8%) 3 very preterm (<33 weeks) 14 late preterm (33–36 weeks)	21/114 (18%)	13 PTL concomitant to flu, 1 PROM concomitant to flu, no adverse neonatal outcome
United Kingdom				
Yates et al., 2010 [31]	152	45/152 (30%) (OR, 5.5; CI 3.7–8.3)		6 stillbirths
Pierce et al., 2011 [37]	256	59/251 (23.7%) (aOR, 4.0; CI, 2.7–5.9; P = 0.046)	100/250 (40.0%) (aOR, 2.3; CI, 1.7–3.2)	5 loss of pregnancy before 24 weeks, 7 stillbirths (aOR, 4.2; CI, 1.4–12.4; P = 0.001), 3 neonatal death (aOR, 5.6; CI, 0.5–64.2) 10 perinatal death (aOR, 5.7; CI, 2.2–15.1), 94 low birth weight (aOR, 3.2; CI, 2.1–4.9)
Singapore				
Lim et al., 2010 [35]	42*	13/42 (31%)	6/42 (14%)	
Israel				
Honarvar et al., 2010 [36]	6	1/6 (17%)	5/6 (83%)	1 neonatal death due to H1N1 infection¶
India				
Pramanick et al., 2011 [38]	13	5/13 (38%)	11/13 (85%)	1 abortion

Table 2: Continued.

Papers	No. of deliveries	Proportion of preterm deliveries	Proportion of cesarean deliveries	Fetal/neonatal survival
Japan				
Nakai et al., 2011 [39]	181	5/178 (3%) (22–31 weeks) 21/178 (12%) (32–36 weeks)		3 abortions
Brazil				
Figueiró-Filho et al., 2011 [40]	31	9/31 (29%)	19/31 (61%)	3 neonatal death
Turkey				
Özyer et al., 2011 [41]	10	4/11 (36%)		
Canada				
Oluyomi-Obi et al., 2010 [42]	6	1/6 (17%; ≤14 weeks) 1/6 (17%; 15–28 weeks) 4/6 (66%; ≥29 weeks)	3/6 (50%)	1 PPROM, 1 PTL, 1 stillbirth, 3 NICU admission, 1 neonatal death

Abbreviations used: ANZIC: ANZIC Influenza Investigators and Australasian Maternity Outcomes Surveillance System; aOR: adjusted odds ratio; CDC: Centers for Disease Control and Prevention; CI: 95% confidence interval; ICU: intensive care unit; NICU: neonatal ICU; OR: odds ratio; PROM: preterm rupture of membranes; PTL: preterm labor. Superscript: [a]Number of deliveries of fetuses of potentially viable gestational age (definition varied by study). Symbol: *excluded 5 spontaneous and iatrogenic abortions; †includes one set of twins; ‡all of 6 were fatal cases; §127 with PCR-confirmed 2009 H1N1 influenza; ¶confirmed by RT-PCR. This table is reproduced from Mosby et al. [14] with minor modifications, and the other reports from India, Japan, Brazil, Turkey, and Canada are added in the original table.

Table 3: Risk of preterm birth and abortion among 181 women who needed hospitalization for A(H1N1)pdm09 virus infection.

Characteristics	Japan[a]	Study	Pneumonitis	
			Absent	Present
No. of pregnant women	1,091,156	181	164	17
abortion at <22 weeks	NA	3/181 (1.7%)	2/164 (1.2%)	1/17 (5.9%)
Preterm birth				
22–31 weeks	7,876/1,091,156 (0.7%)	5/178 (3.8%)†	5/162 (3.1%)†	0/16 (0.0%)
32–36 weeks	54,932/1,091,156 (5.0%)	21/178 (11.8%)†	16/162 (9.8%)†	5/16 (29.4%)†‡
Term birth	1,028,348/1,091,156 (94.2%)	152/178 (85.4%)†	141/162 (86.0%)†	11/16 (68.8%)†

NA, national statistics concerning spontaneous abortion at <22 weeks of gestation is not available.
†$P < 0.01$ versus Japan (national statistics); ‡$P < 0.05$ versus women group without pneumonitis.
[a]National data of Japan in 2008 were presented as a comparison group.
This table is reproduced from Nakai et al. [39] with minor modifications.

adverse pregnancy outcomes associated with influenza virus infection will be conferred.

3. Production of Proinflammatory and Chemoattractive Cytokines by Human Fetal Membranes and Their Functions in Gestational Tissues

3.1. Structures and Constituent Cells of Human Fetal Membranes. Human fetal membranes surrounding the amniotic cavity are composed of the fetus-derived amnion and chorion tissue layers consisting of several types of cells and extracellular matrix fibres (Figure 2) [77–79]. The amniotic epithelium is a single cell layer of apparently simple non-ciliated cuboidal cells resting on a basement membrane. The compact layer is acellular and comprised of a markedly dense network of fibres. The fibroblast layer is composed of bundles of fibres with embedded fusiform and stellate-shaped cells. Both the compact and fibroblast layers formed the connective tissue of the amnion. Between the amnion and the chorion is the sponge layer which is comprised of a fine, loose, wavy, fibrillar network. The chorion is composed of three layers: the reticular, chorionic basement membrane, and trophoblast. The reticular layer is composed of a network of fibres in which fusiform and stellate-shaped cells are embedded. The connective tissues of the amnion and chorion include two layers containing mesenchymal cells, the fibroblast layer of the amnion and the reticular layer of the chorion. The exact cellular composition of the mesenchymal connective tissues of amnion and chorion has been controversial. The mesenchymal cells exhibit plasticity among fibroblast/myofibroblast cells and macrophages [80]. The chorionic basement membrane underlies the trophoblast

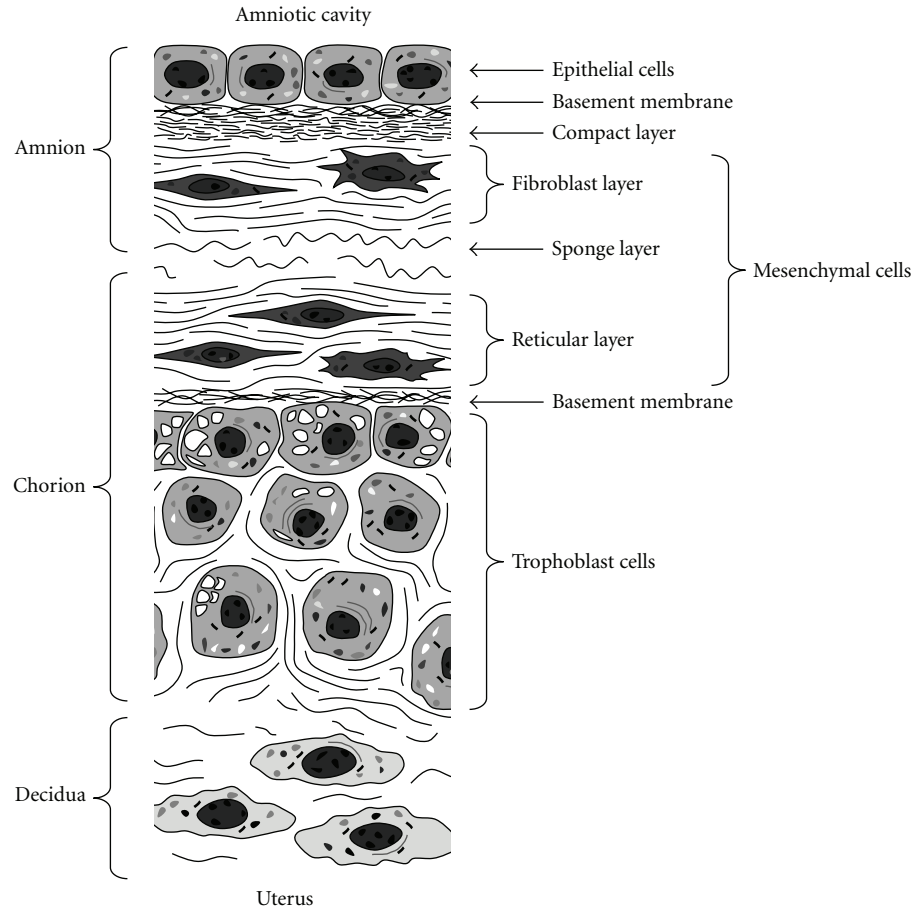

Figure 2: Diagrammatic representation of the human fetal membranes. The amnion and chorion are attached together by the sponge layer. The amnion is formed of a single-layered amniotic epithelial cell and compact and fibroblast layers. The chorion is formed of multilayered trophoblast cells and reticular layer. The fibroblast layer of the amnion and the reticular layer of the chorion contain mesenchymal cells. The mesenchymal cells exhibit plasticity among fibroblast/myofibroblast cells and macrophages.

layer which is composed of round to polygonal trophoblast cells. The basal cells near to the basement membrane (termed basal trophoblast) are tightly adherent to each other with narrow intercellular spaces. Near to the decidua, the trophoblast cells (termed superficial trophoblast) become separated by the wider intracellular spaces. Degenerated villi (areas of connective tissue) are occasionally detected in the trophoblast layer. The decidua is formed of few layers of elongated cells that exhibited less dense eosinophilic cytoplasm and more regular nuclei than the trophoblasts.

3.2. Pathology of Preterm Rupture of Fetal Membranes: Focusing on Apoptosis and Matrix Metalloproteinases. The human fetal membranes form boundaries between the fetus and the external world in order to provide a sterile environment to the fetus and play a critical role as defensive barriers against infectious agents in order to maintain normal pregnancy [79, 81]. The membranes normally rupture during labor. Preterm labor is defined in the presence of painful regular uterine contractions and cervical changes occurred between 22 and 37 weeks of amenorrhea [19]. Premature rupture of the fetal membranes (PROM) is defined as the rupture of the amniotic membranes with release of the amniotic fluid more than 1 hour prior to the onset of labor. PROM may be subdivided into term PROM (i.e., PROM after 37 weeks of gestation) and preterm PROM (PPROM, i.e., PROM prior to 37 weeks of gestation). PPROM occurs in approximately 3% of pregnancies and is responsible for a third of all preterm deliveries, which greatly contributes to infant morbidity and mortality [79]. The etiology of PPROM is multifactorial including infection, behavioral factors (smoking, substance abuse, nutritional status, and coitus), obstetric complications (multiple gestation, polyhydramnios, cervical operations, gestational bleeding, and antenatal trauma), and possibly environmental changes (barometric pressure) [82].

In the past two decades, many investigators have been investigating the molecular and cell biology of rupture of human fetal membranes. A growing body of evidence suggests that the biochemical processes, including constituent cell degradation through apoptotic pathway and collagenous fibril degradation by matrix metalloproteinases (MMPs), appear to be related to the pathology of PROM [79, 82–85]. It is likely that proinflammatory cytokines may play a central role in the etiology of infection-derived PPROM because they activate initiator and effector caspases and

MMPs in the fetal membranes in response to infection [86]. Accumulating evidence suggests that reactive oxygen species (ROS) are also implicated in the rupture of human fetal membranes by altering certain biochemical events, including apoptosis induction, degradation of the connective tissues, and promoting inflammatory responses [87].

3.2.1. Apoptosis Induction

(a) Apoptosis Induction under Physiological and Pathological Conditions. In the chorion trophoblast cell layer of fetal membranes at full term, a large amount of degraded cells have been observed [77, 88]. In our knowledge, Parmley has disclosed for the first time that apoptotic cells were detected in the chorionic trophoblast cell layer of human fetal membrane tissues [89]. The study using 121 specimens obtained from clinically heterogeneous patients has suggested that the trophoblast cell layer of the chorion laeve showed widespread apoptotic cells and loss of the trophoblast cell layer as term approached, and that the chorion trophoblast cells was prematurely destroyed by infiltrating maternal leukocytes in cases of chorioamnionitis [89]. The number of apoptotic cells in the chorionic trophoblast and decidual cell layers in the 37–42 week group of uncomplicated cases at term was greater than that in the 23–30 week group of complicated cases with preeclampsia and diabetes at preterm [90]. The apoptotic bodies were quite abundant in the chorionic trophoblast cell layer of fetal membranes located over the cervix [91]. Recent studies have revealed that the number of apoptotic cells was much higher in the chorion of fetal membranes with histological chorioamnionitis at term than those without chorioamnionitis [92] and that the chorion of fetal membranes from patients with premature rupture of membranes had significantly more apoptotic cells than those without chorioamnionitis [93]. In addition, apoptosis was detected in also amnion epithelial cells at term, which was associated with onset of labor [94]. It is likely that apoptotic cell death in broad area of the fetal membranes is responsible for the rupture of fetal membranes both at term under physiological conditions and at preterm with chorioamnionitis.

(b) Induction of Apoptosis by ROS. Apoptosis of chorion trophoblast cells in the amniochorion tissues observed at the end of pregnancy was progressed by the *in vitro* incubation, which was suppressed by the addition of glucocorticoids, antioxidative reagents (pyrrolidine dithiocarbamate (PDTC) (1) in Figure 4, *N*-acetyl-L-cysteine (1) in Figure 3, nordihydroguaiaretic acid (NDGA) (2) in Figure 4, 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E), general and selective cyclooxygenase (COX)-2 inhibitors (indomethacin and nimesulide, resp.), and inducible nitric oxide synthase (iNOS) inhibitor (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine) to the medium [95–97]. The expression levels of COX-2 and iNOS mRNAs as well as proteins were increased in the isolated chorion tissues during the *in vitro* incubation [95], resulting in the production of ROS, such as superoxide and nitric oxide (NO). Furthermore, apoptosis was induced in cultured chorion, but not amnion, cells by the treatment with a NO donor reagent, sodium nitroprusside [95]. It has been known that peroxynitrite, a strong oxidant, is formed when superoxide and NO are produced at near equimolar ratios [98]. Moreover, Trolox inhibits peroxynitrite-mediated apoptosis in rat thymocytes [99]. These results suggest that the induction of apoptosis in the chorion trophoblast cells is mediated through peroxynitrite resulting from the induction of COX-2 and iNOS gene expression. It has been known that bacterial toxin lipopolysaccharide (LPS) induces COX-2 and iNOS gene expression simultaneously in macrophages [100]. On the basis of these results, it is possible that macrophages are a major source for COX-2 and iNOS enzymes in isolated amniochorion tissues.

Our previous study has identified the contribution of enzymes capable of producing superoxide and NO (i.e., COX-2 and iNOS, resp.) to the apoptosis induction in the chorion cells as described above [97]. Furthermore, we examined the role of enzymes capable of eliminating ROS (e.g., glutathione peroxidase and catalase) in the apoptosis induction of the cultured chorion cells [101], since the apoptosis induction by oxidative stress is a result of imbalance between production and elimination of ROS. The treatment of cultured chorion and amnion cells with mercaptosuccinic acid (glutathione peroxidase inhibitor) and 3-amino-1,2,4-triazole (catalase inhibitor) resulted in an inhibition of glutathione peroxidase and catalase activities, respectively. The incubation with glutathione peroxidase inhibitor alone induced apoptosis in the cultured chorion cells, the levels of which were enhanced by the addition of catalase inhibitor, while catalase inhibitor alone hardly induced apoptosis. However, none of these reagents induced apoptosis in the cultured amnion cells. Therefore, we have concluded that glutathione peroxidase played a more critical role than catalase in the control of the apoptosis induction of the chorion cells, suggesting that the threshold levels of stress tolerance in the chorion cells are much lower than those in the amnion cells [101].

As described above, an intracellular oxidative stress may play a critical role in the process of apoptosis induction in chorion trophoblast cells [97, 101]. Hence, in order to further elucidate the direct contribution of iNOS gene expression to the apoptosis induction in these cells, we examined the effect of iNOS gene transfection into the cultured chorion and amnion cells on apoptosis induction [102]. A significant increase in the levels of iNOS protein expression and nitrite accumulation in both chorion and amnion cells was observed after the iNOS gene transfection. The induction of apoptosis was observed in an approximately 70% of chorion cells transfected with iNOS gene. Transfection of the iNOS gene into the cultured chorion cells resulted in the activation of p38 mitogen-activated protein (MAP) kinase and downregulation of heme oxygenase-1 protein expression, whereas no such events were observed in the transfected amnion cells. These results suggest that apoptosis induced in the chorion trophoblast cells by the iNOS gene expression is closely linked to a physiological consequence, such as the rupture of fetal membranes.

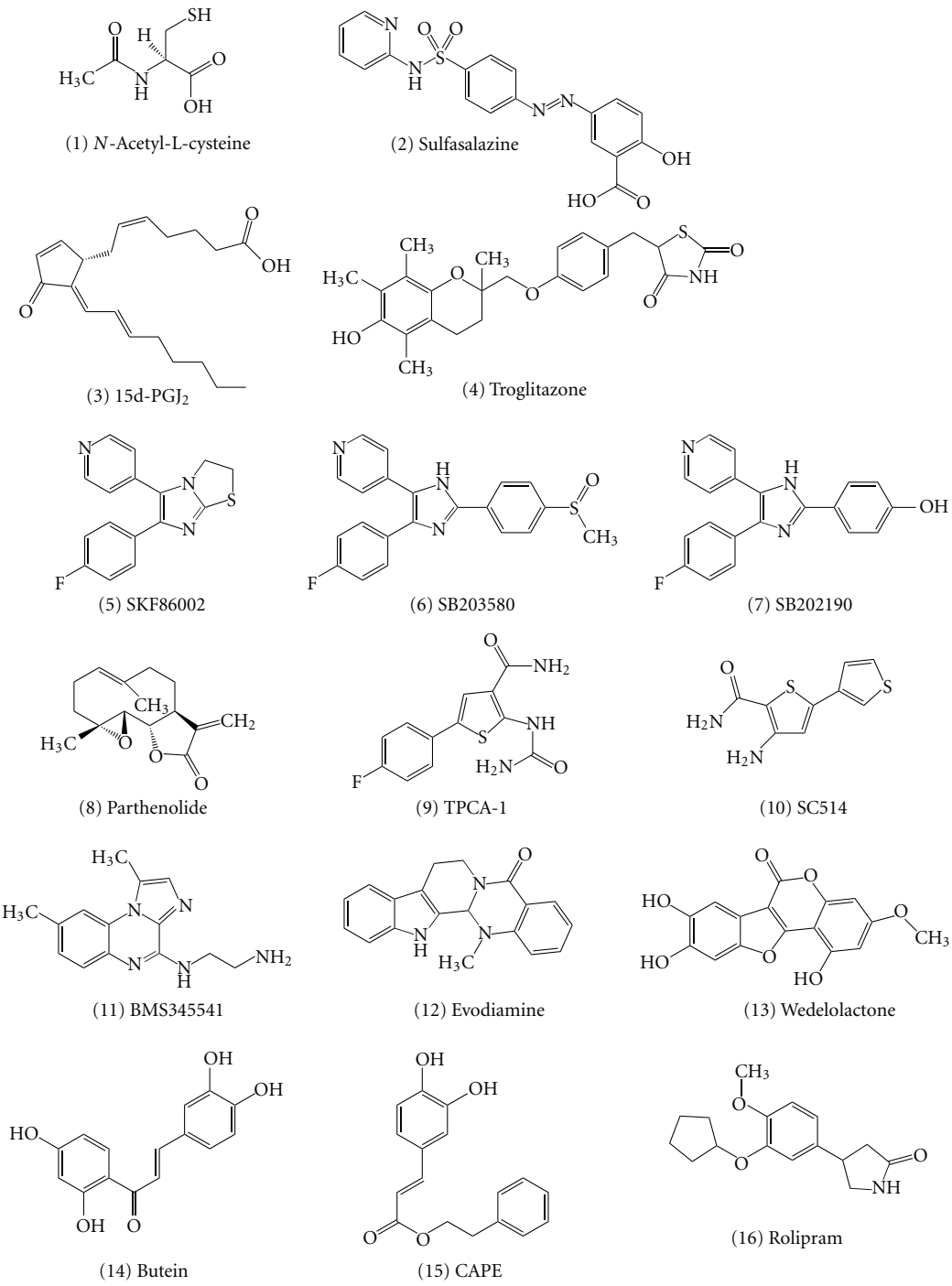

Figure 3: Pharmacological agents with inhibitory effect on the production of proinflammatory cytokines by human fetal membrane cells.

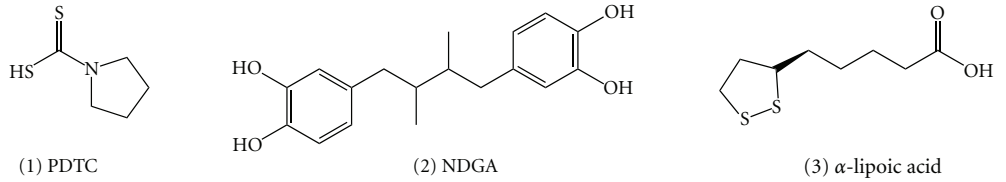

Figure 4: Pharmacological agents with inhibitory effect on the induction of apoptosis in human fetal membrane cells. Abbreviations used: NDGA, nordihydroguaiaretic acid; PDTC, pyrrolidine dithiocarbamate.

3.2.2. Extracellular Matrix Homeostasis of Human Fetal Membranes

(a) Gene Expression of MMPs and Tissue Inhibitor of Metalloproteinases. Extracellular matrix homeostasis is a key process in the maintenance of the tensile strength of the amniochorion [85]. This tensile strength guarantees the role of the membranes as a physical and functional boundary for the fetus during human pregnancy. Although the expression of MMP-9 was barely detectable in isolated fetal membrane tissues before the onset of labor at activity and protein levels, it was enhanced after the onset of labor [103]. Both MMP-9 mRNA and protein were coexpressed in the amnion epithelial cells, the fibroblasts/macrophages in chorioamniotic mesenchymal layers, and the chorion trophoblast cells of fetal membranes after labor [103]. MMP-3 and tissue inhibitor of metalloproteinase-1 (TIMP-1) proteins were expressed in the amnion epithelial cells, the fibroblasts/macrophages, and the chorion trophoblast cells of fetal membranes obtained at term prior to labor [104]. These results suggest that the increased expression of MMP-9 may result in degradation of the extracellular matrix of the fetal membranes and facilitate their rupture and moreover that MMP-3, MMP-9, and TIMP-1 may influence the extracellular matrix homeostasis of the amniochorion.

(b) Regulation of MMP-9 Activity by ROS. The gene expression of MMP-9 is partly controlled by a transcription factor, nuclear factor (NF)-κB [105]. Superoxide anion increased the activity of MMP-9, but not MMP-2, in culture supernatants of isolated amniochorion tissues, which was inhibited by the presence of either superoxide dismutase or *N*-acetyl-L-cysteine [106]. The treatment with *N*-acetyl-L-cysteine inhibited the activation of NF-κB and subsequent the induction of MMP-9 activity in culture supernatants of isolated amnion and choriodecidua tissues after the stimulation with LPS [107]. Thus, these results suggest that ROS may regulate the activity of MMP-9 through the activation of NF-κB in the human fetal membranes.

3.3. Proinflammatory Cytokines

3.3.1. A Fundamental Role of Proinflammatory Cytokines in the Pathogenesis of Preterm Delivery. Elevated levels of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, can be found in the amniotic fluid of patients with preterm labor and intra-amniotic infection [108–110]. Both IL-1α and IL-1β induced preterm delivery in mice, as demonstrated by the prevention of preterm delivery with the pretreatment with IL-1 receptor antagonist [111]. Significantly higher numbers of tryptase-positive mast cells, $CD8^+$ T cells and TNF-α-positive cells were observed in the decidua tissues obtained from women with spontaneous abortion during the first trimester, which was closely related to higher stress scores estimated by questionnaires [112]. The results suggest that stress-triggered abortion in humans is linked to immunological imbalances. In mice, stress-triggered abortion was prevented by neutralizing TNF-α and IL-1 with soluble receptors [113]. Thus, proinflammatory cytokines, such as IL-1β and TNF-α, have been postulated to play a fundamental role in the pathogenesis of preterm delivery during intrauterine infection and receiving intensive stresses [111–113].

3.3.2. Production of Proinflammatory Cytokines by Human Fetal Membranes. Production of many cytokines has been shown to change during normal labor and parturition. These alterations may play a significant role in the processes that culminate in the successful delivery. Concentrations of inflammatory cytokines in amniotic fluid, for example, increase at term in normal pregnancies. Proinflammatory cytokines may play a regulatory role in parturition by stimulating the local production of uterotonic prostaglandin (PG) E_2 [114] and MMPs and by inducing apoptosis in gestational tissue cells. As listed in Table 4, isolated amniochorion tissues have shown to produce a wide variety of cytokines classified into proinflammatory, lymphocyte-derived, macrophage-derived, anti-inflammatory, antiviral, and chemoattractive cytokines constitutively or in response to diverse stimuli with physical stretching, PGE_2, IL-1β, TNF-α, and bacterial products (LPS), and infections with bacteria (*Escherichia coli* [115], *Streptococcus agalactiae* [116], and *Ureaplasma urealyticum* [117]) and viruses (influenza virus and New-Castle disease virus [118]) [119–127]. Proinflammatory and chemoattractive cytokines produced by fetal membranes have been postulated to play a central role in the physiology of normal parturition and the pathology of premature delivery associated with intrauterine infections [119, 120, 123, 125, 128–130].

Explants of fetal membranes obtained at term were mounted and incubated in a Transwell device, which allowed testing the amnion and the choriodecidua compartments independently. *Escherichia coli* was added to either the amniotic, the choriodecidual regions, or both [115]. The stimulation with *Escherichia coli* regardless of side of the membranes enhanced the secretion of IL-1β, IL-6, IL-8, and IL-10 from the choriodecidual compartment. The stimulation of both sides with *Escherichia coli* enhanced the secretion of TNF-α from both choriodecidual and amniotic compartments. When the amnion was stimulated directly, the secretion of IL-1β and IL-8 from the amniotic compartment increased. The study demonstrated that selective stimulation of fetal membranes with *Escherichia coli* resulted in a differential production of IL1β, IL-6, TNF-α, IL-8, and IL-10. In contrast, the stimulation with *Streptococcus agalactiae* enhanced the secretion of both IL-1β and TNF-α from isolated choriodecidua tissues but that of only TNF-α from isolated amnion tissues [116]. Therefore, on the basis of these results, it has been hypothesized that the choriodecidua may play a primary role during an ascending intrauterine infection, being the main barrier to progression of the infection into the amniotic cavity.

3.3.3. Implication of Toll-Like Receptors in Cytokine Production by Fetal Membrane Cells. The Toll-like receptor (TLR)-2 and TLR-4 recognize microbial products that are associated with

Table 4: Diverse cytokines produced by human fetal membranes.

Proinflammatory cytokines		IL-1α/β, IL-6, TNF-α
Lymphocyte-derived cytokines		IL-2
Macrophage-derived cytokines		IL-15
Anti-inflammatory cytokines		IL-4, IL-10, IL-1RA, TGF-β1
Anti-viral cytokines		IFNs-$\alpha/\beta/\gamma$
Chemoattractive cytokines	CC chemokines	MCP-1/2/3/4, RANTES, MIP-1α/β
	CXC chemokines	IL-8, IP-10, ENA-78, MIF
Hematopoietic growth factor		M-CSF

Abbreviations used: ENA: epithelial cell-derived neutrophil-activating protein; IFN: interferon; IL: interleukin; IL-1RA: interleukin 1 receptor antagonist; IP: interferon inducible protein; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; MIF: macrophage migration inhibitory factor; M-CSF: macrophage colony-stimulating factor; RANTES: regulated on activation, normal T cell expressed and secreted; TGF: transforming growth factor; TNF: tumor necrosis factor.

gram-positive and gram-negative bacteria, respectively. TLR-4 is crucial in mediating the response to LPS. Ligation of TLRs leads to the activation of NF-κB, a transcription factor that is involved in the expression of many chemokines (e.g., IL-8), proinflammatory cytokines (e.g., IL-1β and TNF-α), and antimicrobial peptide (defensins) [131].

Spontaneous labor at term and preterm delivery with histologic chorioamnionitis, regardless of the membrane status (intact or ruptured), is associated with an increased expression of TLR-2 and TLR-4 in the fetal membranes [132]. TLR-2 and TLR-4 proteins were strongly expressed in the amnion epithelial cells and acute inflammatory cells, macrophages, and neutrophils, but weakly in the decidua cells; the expressions of TLR-2 and TLR-4 proteins were increased during spontaneous labor at term and at the lesions with chorioamnionitis [132]. The chorion expressed significantly higher levels of TLR-4 protein than the amnion, which decreased significantly with the progression of gestation [133]. These results suggest that TLR-2 and TLR-4 expressed in the fetal membranes may regulate intrauterine inflammatory response during pregnancy and that TLR-4 may be involved in preterm delivery.

The activation of TLR-5 and TLR-6/2 with respective specific agonists stimulated the secretion of IL-6 and IL-8 from cultured amnion epithelial cells, concomitantly with the activation of NF-κB signaling pathway, and MMP-9 induction [134]. In contrast, the activation of TLR-4 with a specific agonist reduced amniotic epithelial cell viability and induced cell apoptosis evidenced by an elevated Bax/Bcl-2 ratio and cleavage of caspase-3 [134]. These results suggest that TLRs in the amnion epithelial cells may regulate the production of cytokines and MMP-9 through NF-κB activation and the induction of apoptosis.

3.3.4. Functions of IL-1α/β, IL-6, and TNF-α in Gestational Tissues. IL-1α increases the production of MMP-1 in cultured chorion cells [135]. IL-6 stimulates the production of uterotonic PGE_2 in cultured amnion and decidual cells [136]. IL-6 and TNF-α induce the secretion of MMP-2 and MMP-9 from cultured amnion epithelial cells [137]. TNF-α stimulates the production of MMP-1/3 and PGE_2 in cultured chorion cells [138] and the production of PGE_2 in cultured amnion fibroblast and epithelial cells [139]. On the other hand, TNF-α induces apoptosis in cultured myometrial cells [140]. TNF-α alone induces apoptosis in cultured placental trophoblast cells, the activity of which is enhanced by the presence of interferon (IFN)-γ [141]. Furthermore, a recent study has demonstrated that IL-1β and TNF-α induces the differentiation of myofibroblast cells to macrophages [80].

Apoptosis was induced in isolated amniochorion tissues when incubated with proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α [142, 143]. IL-1β and TNF-α increase the protein levels of MMP-9 in isolated human amniochorion tissues, resulting in actual physical weakening of fetal membranes [144]. The intra-amniotic injection of IL-1β and TNF-α induces histologic chorioamnionitis characterized by extensive neutrophil infiltration and patchy necrosis in the chorion layer and preterm labor in rhesus monkeys, while the intra-amniotic injection of IL-6 induces histologic chorioamnionitis characterized by macrophage infiltration and patchy necrosis but not preterm labor [145].

3.3.5. Inhibition of Cytokine Production by Physiological and Pharmacological Agents. Several inhibitors for cytokine production are listed in Table 5. Physiological agents, such as IL-10 and activin A, inhibit the production of proinflammatory cytokines in fetal membranes. IL-10 showed downregulation of mRNA expression and protein production of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, in isolated human amniochorion tissues stimulated with LPS [146–149], suggesting that biosynthesis of proinflammatory cytokines in the fetal membranes can be controlled by IL-10 as an anti-inflammatory cytokine during infectious processes. Activin A inhibited IL-6, IL-8, and TNF-α production by isolated amniochorion tissues [150].

Diverse types of pharmacological agents inhibit the production of proinflammatory cytokines in human fetal membranes in response to LPS and to infection with influenza virus (Table 5 and Figure 3). Antioxidant (*N*-acetyl-L-cysteine (1)), anti-inflammatory compound (sulfasalazine (2)), and peroxisome proliferator-activated receptor (PPAR)-γ ligands (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) (3) and troglitazone (4)) inhibited the production of IL-1β, -6, -8, and TNF-α and the activation of NF-κB in LPS-stimulated fetal membrane tissues (Figure 3) [120–123]. Thus, ROS may promote inflammatory response through the production of

Table 5: Physiological and pharmacological inhibitors for cytokine production in human fetal membranes.

Properties	Inhibitors	References
Antioxidant	*N*-Acetyl-L-cysteine	[123]
Anti-inflammatory cytokines	IL-10	[115–118]
	Activin A	[119]
Anti-inflammatory compounds	Sulfasalazine	[122]
p38 MAK inhibitors	SKF86002	[120]
	SB203580 and SB202190	[125]
NF-κB inhibitors		
Selective and competitive IKKβ inhibitor	SC-514	[126]
Selective allosteric IKKβ inhibitor	BMS 345541	[126]
IKK inhibitor	Evodiamine	[126]
Nonselective IKK inhibitor	Wedelolactone	[126]
Partially selective IKK inhibitor	Butein	[126]
Inhibitor of NF-κB translocation	CAPE	[126]
Inhibitor of the IKK complex	Parthenolide	[126]
Selective IKKβ inhibitor	TPCA-1	[126]
Phosphodiesterase 4 inhibitor	Rolipram	[127]
PPAR-γ ligand	15d-PGJ_2	[121]
	Troglitazone	[121]

proinflammatory cytokines by NF-κB activation in human fetal membranes.

SKF86002 ((5) in Figure 3), an inhibitor for p38 MAP kinase, blocked the secretion of IL-1β protein from isolated amniochorion tissues after stimulation with LPS but not the induction of its mRNA expression [151, 152], indicating that SKF86002 blocked the secretion of IL-1β protein at a post-transcriptional level via the inhibition of MAP kinase activity. We have demonstrated that p38 MAP kinase inhibitors, such as SB203580 (6) and SB202190 (7), blocked TNF-α protein secretion from cultured chorion cells after influenza virus infection but not TNF-α mRNA accumulation and viral gene replication and transcription in the cells (Figure 3) [153]. These studies suggest that influenza virus infection uses common p38 MAP kinase pathway in the production of proinflammatory cytokines at a post-transcriptional level.

A recent study demonstrated that two inhibitors of κB kinase (IKK), such as parthenolide (8) and TPCA-1 (9), strongly inhibited the secretion of IL-6 and TNF-α and the nuclear translocation of NF-κB in cultured chorion cells stimulated with LPS [154]. The other NF-κB inhibitors, such as SC514 (10), BMS345541 (11), evodiamine (12), wedelolactone (13), butein (14), and caffeic acid phenylmethyl ester (CAPE) (15), also inhibited the cytokine production and NF-κB nuclear translocation (Figure 3) [154]. This study expands the possibility of potential use of IKK inhibitors for the treatment of inflammation in human fetal membranes.

Cyclic nucleotide phosphodiesterases (PDEs) are the enzymes catalyzing the hydrolysis and inactivation of the second messengers, cAMP and cGMP. Eleven PDE families are described to date, and selective inhibitors of some PDEs families are currently used in clinic for treating cardiovascular disorders, erectile dysfunction, and pulmonary hypertension. Isoforms of the PDE4 family are involved in smooth muscle contraction and inflammation. PDE4 selective inhibitors are currently in clinical trials for the treatment of diseases related to inflammatory disorders. LPS induces the production of TNF-α and the nuclear translocation and activation of NF-κB in cultured human fetal membrane chorion cells, which were blocked by the treatment with rolipram (16), an inhibitor of PDE4 (Figure 3) [155]. These results suggest that the PDE4 family interacts with the LPS signaling pathway during the inflammatory response of human fetal membrane chorion cells. PDE4-selective inhibitors may represent a new therapeutic approach in the management of inflammation-induced preterm delivery.

On the basis of data using pharmacological agents, intracellular intermediates for the production of proinflammatory cytokines are revealed. These studies suggest that in cellular oxidation process, PPAR-γ, p38 MAP kinase, NF-κB, and PDE4 regulate the induction of gene expression of proinflammatory cytokines in the fetal membranes during infection. In particular, the functions of NF-κB in human fetal membranes have been investigated extensively, many evidence of which are accumulating. Recently, Lappas and his colleagues review that NF-κB plays a pivotal role in the cellular signaling for apoptosis induction and MMP and PG productions as well as proinflammatory cytokine production in human fetal membranes under inflammatory milieu [156].

3.3.6. Inhibition of Cytokine-Induced Weakening of Fetal Membranes by Antioxidant α-Lipoic Acid. An antioxidant, α-lipoic acid ((3) in Figure 4), inhibits the TNF-α-induced physical wakening of isolated amniochorion tissues [157]. Cultured amnion epithelial and mesenchymal cells were incubated with either TNF-α or IL-1β in the presence or absence of α-lipoic acid pretreatment. The pretreatment with α-lipoic acid inhibited the induction of MMP-9 activity and its protein release by either TNF-α or IL-1β in cultured amnion epithelial cells, as well as PGE_2 production in both cultured amnion epithelial and mesenchymal cells.

Kumar and his colleagues have demonstrated that thrombin also induced the physical weakening of isolated amnion membrane tissues, which were separate from choriodecidua tissues, accompanying with the increases in poly(ADP-ribose) polymerase cleavage and reciprocal increases and decreases, respectively, in MMP-9 and tissue inhibitor of metalloproteinases-3 protein [158]. Although TNF-α and IL-1β weakened isolated full-thickness amniochorion tissues, neither TNF-α nor IL-1β weakened isolated amnion membrane tissues. However, culture supernatants of choriodecidua tissues incubated with either TNF-α or IL-1β weakened isolated amnion membrane tissues. α-Lipoic acid blocked the weakening of full-thickness amniochorion tissues by thrombin. These results suggested that thrombin weakens amnion membrane directly, whereas TNF-α and IL-1β weaken amnion membrane indirectly by causing the release of soluble intermediates from the choriodecidua. Kumar and his colleagues review their comprehensive study [121].

3.4. Chemoattractive Cytokines. Chemoattractive cytokines (i.e., chemokines) cause leukocytes to migrate through postcapillary venule endothelium into a discrete organs or the lymphatic circulation. Chemokines can be divided into mainly two groups based on the arrangement of cysteine residues within the receptor-binding domain. For cysteine-X-cysteine (CXC) chemokines, two cysteines are separated by a single amino acid. Cysteine-cysteine (CC) chemokines have two adjacent cysteines. CC chemokines principally attract and activate monocytes as well as lymphocytes, whereas CXC chemokines mainly attract and activate neutrophils.

Using cDNA arrays, the profiles of chemokine gene expression in human fetal membrane tissues associated with chorioamnionitis have been investigated [159]. The expression levels of mRNAs for CC chemokines, such as monocyte chemoattractant protein (MCP)-1, regulated on activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β, and CXC chemokines, such as IL-8, growth-related oncogene (GRO)-α, GRO-β, epithelial cell-derived neutrophil-activating protein (ENA)-78, and interferon-inducible protein (IP)-10, were increased in fetal membranes ruptured prematurely in patients with chorioamnionitis as compared to those without chorioamnionitis. Simultaneously, the expression of CD14 mRNA, a marker of monocyte/macrophage, was increased. Isolated amniochorion tissues obtained after labor exhibit much greater chemoattractive activity for monocytes than those for T and B lymphocytes, natural killer (NK) cells, and polymorphonuclear leukocytes, the activity of which is associating with the presence of IL-8, MCP-1, IP-10, and MIP-1α [124]. These results suggest that neutrophils and monocytes/macrophages are recruited from maternal decidua tissue to amniochorion tissue by chemoattractive cytokines described above, which may be involved in the physiology of labor and the pathology of PROM associated with infections [124, 160].

3.4.1. CC Chemokines

(a) MCP-1. MCP-1 is a chemokine capable of recruiting monocytes/macrophages into sites of inflammation as well as stimulating the respiratory burst required for macrophage activation [161]. Concentrations of MCP-1 in the amniotic fluid were elevated in women in preterm labor with intra-amniotic infection, those without intra-amniotic infection who delivered preterm, and those who displayed histological chorioamnionitis [162]. The levels of MCP-1 mRNA expression were increased in the fetal membrane tissues in term laboring patients as well as the myometrial tissues, and the production of MCP-1 protein in the myometrial tissues was increased in during term labor [163]. These results suggest that MCP-1 may play a role in both term labor and preterm labor regardless of the presence of intra-amniotic infection.

(b) RANTES. RANTES, a potent and versatile chemokine, is capable of attracting monocytes, lymphocytes, basophils, and eosinophils. This cytokine has been implicated in the regulation of the inflammatory response and in the recruitment of macrophages to the implantation site in early pregnancy. Concentrations of RANTES in the amniotic fluid decrease with advancing gestational age. Labor at term was associated with an increase in concentrations of RANTES. Women with preterm labor who delivered preterm in the absence of microbial invasion had a higher concentration of RANTES in amniotic fluid than those who delivered at term. Microbial invasion of the amniotic cavity was associated with a significant increase in concentration of RANTES in amniotic fluid in both preterm and term labor. These results support a role for RANTES in the mechanisms of human parturition and in the regulation of the host response to intrauterine infection [164].

Secretion of MCP-1, IL-8, and RANTES from isolated amnion, chorion, and decidua tissues has been investigated [126]. Considerable amounts of MCP-1 and RANTES were released from the isolated chorion, and decidua tissues, but the amounts of MCP-1 released from the isolated amnion tissues were much lower. In contrast, higher concentrations of IL-8 were released from the isolated amnion, chorion, and decidua tissues. The study suggests that fetal membranes may be one of sources of tissues capable of producing chemokines.

(c) MIP-1α/β. MIP-1α is undetectable in most amniotic fluid samples from patients in the mid trimester of pregnancy and at term not in labor. Microbial invasion of the amniotic cavity is associated with increased concentrations of amniotic fluid MIP-1α protein in both term and preterm gestations. MIP-1α concentrations correlate with IL-8 levels and white blood cell count in amniotic fluid. These results suggest that MIP-1α may play a role in the mechanisms responsible for the recruitment of leukocytes into the amniotic cavity during the course of intrauterine infection [165].

The production of IL-8 and MIP-1α proteins in cultured chorion cells was increased by the infection with group B streptococci or the stimulation with IL-1β, suggesting that

chorion cells may produce specific types of chemokines to attract different types of inflammatory cells and thus may participate in the pathophysiology of infection-mediated preterm labor by directing specific inflammatory responses [166]. The production of both IL-6 protein and PGE_2 in cultured chorion cells increased the stimulation with MIP-1α [167]. Although the production of PGE_2 in cultured amnion cells was increased by the stimulation with MIP-1α, that of IL-6 protein was not [167]. Conversely, the production of IL-6 protein, not PGE_2, in cultured decidual cells was increased by the stimulation with MIP-1α [167]. These results suggest that amnion, chorion, and decidual cells differentially respond to MIP-1α with regard to PGE_2 and IL-6 production and that MIP-1α may play a role in both initiation and propagation of the inflammatory response associated with intrauterine infection.

Advanced glycation end products (AGEs) are known to accumulate in patients with diabetes, autoimmune diseases, or that smoke, on human trophoblasts. The receptor for AGEs was localized in trophoblasts of human chorionic villi obtained at the first trimester (6–10 weeks gestation). AGEs stimulated the secretion of both MIP-1α and MIP-1β from isolated human trophoblasts and induced apoptosis in the cells, the effect of which was inhibited by the treatment with pharmacological agents, such as aminoguanidine (nitric oxide synthase inhibitor) and nafamostat mesilate (a suppressor of transcription factor NF-κB activation) [168]. These results suggested that AGE-mediated changes in trophoblasts may lead to impairment of implantation and placentation.

When the migration of cells was assayed using chemotaxis chambers *in vitro*, the migration of human trophoblast cell line ACIM-88 was stimulated by MIP-1β. Culture supernatants of cultured human endometrial epithelial cells also stimulated trophoblast migration, the activity of which was suppressed with neutralizing antibody against MIP-1β [169].

3.4.2. CXC Chemokines

(a) IL-8. An increased level of IL-8, a potent neutrophil chemoattractant, has been demonstrated in the amniotic fluid with chorioamnionitis [170], suggesting that IL-8 is part of the host response to microbial invasion of the amniotic cavity. The biosynthesis of IL-8 in human amnion and chorion cells and its regulation by other inflammatory cytokines has been investigated. Both cultured amnion and chorion cells were found to produce IL-8 in response to IL-1β and TNF-α [171, 172].

(b) ENA-78. Intra-amniotic secretion and abundance of ENA-78, a potent chemoattractant and activator of neutrophils, have been studied in the context of term and preterm parturition [127]. Immunohistochemical analysis revealed that ENA-78 protein was localized predominantly in the chorion trophoblast and amnion epithelial cells in the fetal membranes at term and preterm. The concentrations of ENA-78 protein in membrane tissue homogenates were significantly elevated with term labor in the amnion and with preterm labor in amnion and choriodecidua. In extract of amnion tissue homogenate, the levels of ENA-78 protein were positively correlated with the extent of leukocyte infiltration. In amniotic fluids, ENA-78 levels from pregnancies with preterm labor without intra-amniotic infection were significantly lower than those from pregnancies with preterm deliveries with infection; levels in samples derived from term pregnancies were similar before and after labor. The treatment with IL-1β, TNF-α, and LPS stimulated the production of ENA-78 by cultured amnion cells. These results suggest that ENA-78, derived from the fetal membranes, increased in the amniotic cavity in response to intrauterine infection. Therefore, it is possible that IL-8 and ENA-78 play a role in the mechanism of infection-driven preterm birth and rupture of membranes secondary to leukocyte recruitment and activation.

(c) GRO-α/β. Cultured human amnion mesenchymal cells produced GRO-α, IL-6, IL-8, MCP-1, and macrophage migration inhibitory factor (MIF) [173]. Surfactant protein-A suppressed the secretion of GRO-β and ENA-78 from isolated amnion tissues [174].

(d) IP-10. The expression of IP-10 gene is induced by different factors (IL-1, TNF-α, IFN-α, and IFN-γ) in many cell types. It also has chemoattractive properties over Th1-lymphocytes, eosinophils, monocytes, and dendritic cells. IP-10 shows pleiotropic biological activity including the migration and stimulation of T-cell adhesion to endothelial cells, the modulation of adhesion molecules, the inhibition of tumor growth *in vivo*, and inhibition of angiogenesis. Recently, it has been demonstrated that human endometrial stromal cells secrete IP-10 [175] and that IP-10 elicits the migration of human trophoblast JEG3 cells [176].

IP-10 as well as other cytokines, such as granulocyte colony-stimulating factor, IFN-γ, IL-1 receptor antagonist, IL-4, IL-6, IL-7, IL-8, MCP-1, MIP-1β, and platelet-derived growth factor, are detectable in all samples of amniotic fluids obtained from 100 singleton pregnant women undergoing elective amniocentesis at 14–16 weeks gestation for karyotype analysis due to older maternal age (range 30–47 years, median 37 years) [177]. In contrast, granulocyte macrophage colony-stimulating factor, IL-10, IL-12, IL-15, IL-17, and TNF-α were detectable in <50% of amniotic fluids. The concentrations of IL-1 receptor antagonist, IP-10, and MCP-1 were significantly higher than maternal serum levels in matched pairs.

4. Possible Roles of Proinflammatory and Chemoattractive Cytokines Produced by Human Fetal Membranes in the Pathology of Adverse Pregnancy Outcomes Associated with Influenza Virus Infection

The etiology of adverse pregnancy outcomes, such as premature delivery, associated with pandemic H1N1 2009 has been remained unclear. Interestingly, we have found

that influenza virus infection induced apoptotic cell death in primary cultured human fetal membrane chorion cells, from which a factor with MDI activity was secreted. It should be noted that these phenomena are not observed in primary cultured human fetal membrane amnion cells. Proinflammatory cytokines, such as IL-6, TNF-α, and IFN-β, were identified as a member of the MDI factor. Influenza virus infection induced the gene expression of not only the proinflammatory cytokines but also chemoattractive cytokines in cultured human fetal membrane cells. The expression profiles were different among cell types. Hence, we present knowledge obtained from our comprehensive study and discuss the implication of apoptosis induction and macrophage activation in human fetal membranes responding to influenza virus infection with a possible etiology of premature delivery.

4.1. Induction of Apoptosis in Cultured Chorion, but Not Amnion, Cells by Influenza Virus Infection

4.1.1. Cytocidal and Persistent Infections. Cultured chorion and amnion cells were infected with influenza A/PR/8/34 (H1N1) virus. Both in chorion cells and in amnion cells viral HA gene was replicated and transcribed [178], and viral nucleoprotein (NP) was *de novo* synthesized depending on the density of virus particles inoculated [179]. Virus yields in culture supernatants increased after influenza virus infection [180]. Significant cytopathic effects (CPEs), such as cell rounding and detachment, were observed in only cultured chorion cells after the virus infection [180]. Moreover, lactate dehydrogenase (LDH) activity in the culture supernatants of chorion cells was elevated by the virus infection [178, 180]. These results indicated that the intracellular LDH leaked into the extracellular medium as a result of an enhanced permeability of cellular membrane. In contrast, these phenomena were not observed in cultured amnion cells after the virus infection [178, 180]. Consequently, these results suggest that the infection of influenza A/PR/8/34 (H1N1) virus to chorion cells was cytocidal accompanied with virus proliferation and cell lysis, whereas the infection of amnion cells with the cytopathogenic strain of influenza virus resulted in persistent state accompanied with virus proliferation but without cell lysis.

4.1.2. Cellular Degradation through Apoptotic Pathway. DNA fragmentation into oligonucleosomes was detected in the cultured chorion cells after influenza virus infection by agarose gel electrophoresis [180]. As evidenced in our previous study, the virus infection promoted the apoptotic cellular degradation in organ-cultured human amniochorion tissues [179]. The extent of DNA fragmentation was further determined at a single cell level by the terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein isothiocyanate (FITC) nick-end labeling (TUNEL) method [181]. Numerous numbers of fragmented nuclei with FITC-labeled DNA were detected in the cultured chorion cells after the inoculation with active influenza viruses, but not heat-inactivated viruses [182]. No nucleus labeled with FITC was observed in the cultured amnion cells after the virus infection. These DNA laddering and TUNEL-positive reactions are biochemical characteristic changes for apoptotic cellular degradation [181, 183]. Therefore, these results indicated that influenza virus infection induced apoptotic degradation in cultured chorion cells but not in cultured amnion cells, yet viruses were proliferated in both cells.

4.1.3. Virus Replication as Requirement for Apoptosis Induction. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) ((5) in Figure 1) is a guanosine analogue that inhibits influenza virus ribonucleoprotein synthesis through reducing the size of the cellular guanosine 5′-triphosphate pool and by directly affecting viral replicative enzymes, RNA-dependent RNA polymerase [184]. The treatment of chorion cells with ribavirin inhibited the virus gene replication and transcription throughout the infection, resulting in the inhibition of both virus particle production and apoptosis induction [180, 185]. Furthermore, PDTC (1) and NDGA (2) also inhibited both apoptosis induction and virus proliferation in the chorion cells (Figure 4) [186–188]. The inhibition of influenza virus replication by treating infected host cells with various antiviral drugs, such as zanamivir of NA inhibitor, amantadine of viral membrane protein (M2) inhibitor ((6) in Figure 1), and ammonium chloride, also resulted in the inhibition of virus-induced apoptosis [189]. In addition, UV-inactivated influenza virus induced little or no apoptosis [190]. Therefore, it is most likely that influenza virus replication is prerequisite to the induction of apoptosis by the virus infection in chorion cells.

4.1.4. Execution of Apoptosis by Caspase-3 Activation. Our study has demonstrated that influenza virus infection induced the cleavage of procaspase-3 protein into an active form in cultured chorion, but not amnion, cells [178]. A general caspase inhibitor, *N*-*t*-Boc-Asp(OMe)-fluoromethyl ketone (Boc-D-fmk), inhibited the cleavage of procaspase-3 protein and the induction of DNA fragmentation in the virus-infected chorion cells. In contrast, the treatment with Boc-D-fmk did not interfere in virus infection (i.e., viral NP expression) and virus particle release in the chorion cells [178]. Therefore, these results suggest that Boc-D-fmk inhibits apoptotic cellular degradation in the virus-infected chorion cells through inhibiting the process of caspase-3 cleavage irrespective of virus proliferation.

4.1.5. Implication of ER Stress in Apoptosis Induction. In influenza virus-infected amnion cells, procaspase-3 protein cleavage and DNA fragmentation were not observed, yet the virus proliferation was observed [178]. Apoptosis is a tightly regulated cellular process involving several checkpoints before irreversible cellular degradation begins. The process consists of initiation, commitment, and degradation phase [191, 192]. It is possible that a failure of procaspase-3 protein cleavage in amnion cells is implicated in the mechanism of persistent infection without the commitment to apoptosis induction.

Maruoka and coworkers demonstrated that the expression levels of immunoglobulin heavy-chain binding protein (BiP) mRNA, one of major molecular chaperons in the lumen of endoplasmic reticulum (ER), increased in human bronchial epithelial cells by the virus infection, indicating that ER stresses occur in the virus-infected cells [193].

Our previous study has demonstrated that the levels of BiP protein expression increased in the chorion, but not amnion, cells at 48 hr after the virus infection [194]. The cleaved form of caspase-3 protein (19 kDa) was detected in the virus-infected chorion cells in the absence of Boc-D-fmk but not in the presence of Boc-D-fmk. The treatment with Boc-D-fmk did not alter the extent of accumulated BiP protein in the virus-infected chorion cells. Conceivably, these data raise the hypothesis that the ER stress accompanied by the BiP accumulation occurs prior to caspase-3 activation, which relates to the apoptosis induction by the virus infection in chorion cells. Further study to elucidate the hypothesis is needed.

4.2. Secretion of MDI Factor from Chorion Cells Infected with Influenza Virus

4.2.1. Concept of MDI Factor as Grow-Eater Signal Presented by Apoptotic Cells. Apoptosis, programmed cell death, is involved not only in the physiological processes of development and tissue homeostasis but also in the pathological processes of a number of human diseases including influenza virus infection [195, 196]. Apoptotic cell death occurs sporadically during the development and tissue homeostasis [183]. Resident macrophages present in noninflamed normal tissues in limited numbers and undertake to scavenge scattering apoptotic cells as well as nonprofessional phagocytes such as fibroblasts [183, 197]. In contrast, apoptotic cell death induced by viral pathogens occurs focally and extensively in order to destruct infected cells [198, 199]. It has been observed that a plenty of professional phagocytes (i.e., macrophages and neutrophils) are recruited into the site of infection with influenza virus in order to scavenge a large number of apoptotic cells resulting from the virus infection [200]. The phagocytosis of apoptotic cells resulting from influenza virus infection by macrophages has been considered to play a critical role in the construction of host defense mechanisms against the virus infection. The process results in the presentation of viral antigens to T lymphocytes [201], the abortion of virus growth [202], the prevention of virus dissemination [203], the elimination of viral pathogens from the body [204], and the reduction of virulence [200].

Apoptotic cells present unique signals, such as "eat-me" markers on cell surface to be recognized and engulfed by phagocytes and soluble "come-get-me" signals to attract phagocytes to the site where apoptosis occurs [205]. The exposure of phosphatidylserine on cell surface is one of the most common and best-characterized "eat-me" signals. Influenza virus-infected cells are phagocytosed by macrophages anchored with phosphatidylserine that appears on the surface of infected cells during the process of apoptosis [206–208]. Moreover, influenza virus-infected cells secrete various chemoattractive cytokines (e.g., MIP-1α, MIP-1β, MCP-1, and RANTES) that can recruit macrophages as professional phagocytes into the site of infection before apoptotic cell degradation fulfills [209]. The secretion of chemoattractive cytokines from host cells may associate with the soluble "come-get-me" signal presented by apoptotic cells as well as the release of lysophosphatidylcholine from apoptotic cells [205]. Thus, apoptotic cells as a result of influenza virus infection present "eat-me" and "come-get-me" signals as well as apoptotic cells induced by other stimuli.

Physiological program of monocyte differentiation to macrophage normally proceeds under the control of several cytokines in a coordinate manner. For example, IL-6 induces the differentiation of human monocytic leukemia cell lines including THP-1 cells to macrophages capable of producing superoxide, the activity of which is synergistically enhanced in combination with either IL-1, TNF-α, or IFN-γ [210, 211]. Interestingly, we have found that influenza virus infection induces apoptosis and the gene expression of a set of proinflammatory cytokines, such as IL-6, TNF-α, and IFN-β, in cultured human fetal membrane chorion cells [179, 180, 212]. The treatment with heated culture supernatants of influenza virus-infected chorion cells induces the differentiation of human peripheral blood monocytes as well as human monoblastic THP-1 and histiocytic U937, but not promyelocytic HL-60, leukemia cell lines to well-matured macrophages capable of adhering, phagocytosing, and producing superoxide anion [182, 213]. The adhered THP-1 cells phagocytose corpses of chorion cells resulting from apoptosis induced by the virus infection [194, 214, 215]. It should be noted that these phenomena are not observed in cultured human fetal membrane amnion cells where apoptosis is not induced by influenza virus infection [179, 180, 182, 212, 213]. These results have suggested that influenza virus-infected chorion cells undergoing apoptosis secrete heat-stable soluble factors with MDI activity in order to scavenge corpses of themselves by matured mononuclear phagocytes (i.e., macrophages), not polymorphonuclear phagocytes [182]. Therefore, cultured human fetal membrane chorion cells undergoing apoptosis after influenza virus infection present a unique signal, which is apparently different from "eat-me" or "come-get-me" signal, to differentiate monocytes to matured macrophages. Consequently, we herein define for the first time the unique signal presented by apoptotic cells as "grow-eater" signal that increases the number of macrophages in order to phagocytose and eliminate apoptotic cells.

4.2.2. Molecular and Cell Biological Characteristics of MDI Factor

(a) Induction of Capability of Adhering and Phagocytosing Latex Particles. We developed a new simple multiwell plate-based assay for evaluating MDI activity using human monoblastic leukemia THP-1 cells [185]. It is based on an enhanced adherence activity of macrophage after the induction of monocyte differentiation. This method is highly

sensitive and easy to perform, especially in case of analyzing a large number of samples. In order to elucidate whether or not influenza virus-infected cells undergoing apoptosis secrete soluble factors with MDI activity, we examined the effect of culture supernatants of chorion and amnion cells on monocyte differentiation by the method [182, 213]. Culture supernatants of the virus-infected cells were heated at 56°C for 30 min in order to inactivate virus prior to use. THP-1 cells became adherent to plastic plates by the incubation with heated culture supernatants of influenza virus-infected chorion cells (IV-C-sup), the extent of which was much higher than that with culture supernatants of mock-infected chorion cells (Mock-C-sup). Interestingly, THP-1 cells did not acquire the adherence activity when the cells were incubated with culture supernatants of mock and influenza virus-infected amnion cells (Mock-A-sup and IV-A-sup, resp.). The Giemsa staining showed that nontreated THP-1 cells were round, the nucleocytoplasmic ratio was >1, and the cytoplasm was highly basophilic with a few vacuoles. In contrast, THP-1 cells adhered to coverslips after the incubation with IV-C-sup were irregularly shaped, the nucleocytoplasmic ratio decreased to <1, and the cytoplasm was weakly basophilic with many vacuoles. Furthermore, adhered THP-1 cells phagocytosed many fluorescent latex particles. These results demonstrate that THP-1 cells are morphologically and functionally differentiated to macrophages by the incubation with heat-stable soluble factors in IV-C-sup. Therefore, we have suggested that influenza virus-infected chorion cells undergoing apoptosis secrete heat-stable MDI factor [213].

(b) Induction of Capability of Producing Superoxide. The cellular biological characteristics of MDI factor were further analyzed by the nitroblue tetrazolium (NBT) reduction test for measuring the ability of superoxide production [182]. When human peripheral blood monocytes as well as monoblastic THP-1 and histiocytic U937, but not promyelocytic HL-60, leukemia cells were treated with IV-C-sup, these cells acquired the ability of NBT reduction, which was much higher than that of Mock-C-sup [182]. The induced NBT reduction was inhibited by the addition of superoxide dismutase and diphenyleneiodonium chloride, an inhibitor for reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, indicating that NBT was reduced by superoxide resulting from the activation of NADPH oxidase [182]. In contrast, the treatments with Mock-A-sup and IV-A-sup had no effect on the NBT reduction ability in THP-1 cells. Therefore, these results suggest that the MDI factor induces the differentiation of human peripheral blood monocytes and cells in monocytic lineage to well-matured macrophages capable of producing superoxide through NADPH oxidase enzyme complex.

(c) Induction of mRNA Expression for Class A Scavenger Receptor and gp91^{phox}. Our unpublished data showed that a large proportion (76%) of THP-1 cells acquired both adherence and superoxide production abilities after the incubation with IV-C-sup, but a small proportion (24%) acquired only superoxide production ability. It is well known that class A scavenger receptor (SR-A) on the cell surface of macrophages is one of molecules responsible for cell-adhering and recognizing apoptotic cells [215], and the treatment with TPA dramatically induces the expression of SR-A mRNA in THP-1 cells [216]. Additionally, membrane-integrated protein gp91^{phox}, existing as a heterodimer with p22^{phox}, functions as the catalytic core of the phagocyte NADPH oxidase [217], and the treatment with IFN-γ or TPA induces the expression of gp91^{phox}, not p22^{phox}, mRNA in THP-1 cells [218]. We investigated the effect of MDI factor on SR-A [213], gp91^{phox}, and p22^{phox} mRNA expression in adhered and suspended THP-1 cells discriminately (our unpublished data). The levels of SR-A mRNA expression were increased in only adhered, not suspended, THP-1 cells after the incubation with IV-C-sup, while the levels of gp91^{phox} mRNA expression were increased in both adhered and suspended THP-1 cells. However, the levels of p22^{phox} mRNA expression were not changed. The acquisition of capabilities of adhering and superoxide production was coincidence with the induction of SR-A and gp91^{phox} mRNA expression, respectively. These results, therefore, suggest that the MDI factor induces the expression of SR-A and gp91^{phox} genes, resulting in the differentiation of monocytes to well-matured macrophages capable of adhering, phagocytosing, and producing superoxide by NADPH oxidase.

(d) Phagocytosis of Apoptotic Cells by Macrophages Matured with MDI Factor. We investigated phagocytosis of chorion cells undergoing apoptosis after influenza virus infection by macrophages matured with the MDI factor [214]. Since chorion cells were detached from culture flasks due to apoptosis resulting from the virus infection [180], the chorion cells undergoing apoptosis were collected for the analysis. Adherent THP-1 cells were obtained by the treatment with IV-C-sup and then incubated with the chorion cells undergoing apoptosis in the presence or absence of IV-C-sup for phagocytosis assay. As incubated in the presence of IV-C-sup, viral NP-positive particles were detected within adherent THP-1 cells by immunohistochemical analysis, but not in the absence of IV-C-sup. These results suggest that adhered macrophages phagocytose the chorion cells undergoing apoptosis after the virus infection and that chorion cells secrete heat-stable soluble factors to facilitate phagocytotic reaction by macrophages [214].

4.2.3. IL-6, TNF-α, and IFN-β as a Member of MDI Factor

(a) Common Responses among Certain Types of Host Cells. Apoptosis induction has been defined as the elimination of dying cells without inducing an inflammatory response [183]. However, this conventional definition may not be fit in a certain situation, such as pathogen invasion that induces an inflammatory response, resulting in the activation of an immune response [219]. Influenza virus infection commonly induces apoptosis and the secretion of proinflammatory and monocyte chemoattractive cytokines in certain types of cells, such as monocytes/macrophages [209, 220], bronchial

Table 6: Cytokines induced by influenza A virus infection in host cells undergoing apoptosis.

Cell types	Cytokines	References
Chorion cells	Proinflammatory cytokines: IL-6, TNF-α Antiviral cytokines: IFN-β, IFN-γ CC chemokines: MCP-1, RANTES, MIP-1β CXC chemokines: IL-8, GRO-α, GRO-β, ENA-78, IP-10	[94, 151, 182]
Monocytes or macrophages	Proinflammatory cytokines: IL-1, IL-6, TNF-α, IFN-α/β CC chemokines: MCP-1, RANTES, MIP-1α, MIP-1β	[179, 190]
Bronchial epithelial cells	Proinflammatory cytokine: IL-6 CC chemokine: RANTES CXC chemokine: IL-8	[191, 192]

Abbreviations used: ENA: epithelial cell-derived neutrophil-activating protein; GRO: growth-related oncogene; IFN: interferon; IL; interleukin; IP: interferon-inducible protein; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; RANTES: regulated on activation, normal T cell expressed and secreted; TGF: transforming growth factor; TNF: tumor necrosis factor.

epithelial cells [221, 222], and fetal membrane chorion cells [120, 179, 212], as listed in Table 6.

(b) Gene Expression of a Set of Proinflammatory Cytokines. Influenza virus infection induced the mRNA expression of a set of proinflammatory cytokine genes, such as IL-1β, IL-6, TNF-α, IFN-β, IFN-γ, and GM-CSF, in cultured chorion cells; no such induction was observed in cultured amnion cells [212]. In contrast, in cultured amnion cells, the mRNA expression of TNF-α and IFN-β was induced in cultured amnion cells, although that of IL-1β, IL-6, IFN-γ, and GM-CSF was not [212]. Hence, the contribution of IL-1β, IL-6, TNF-α, IFN-β, and IFN-γ to MDI activity in IV-C-sup was investigated. Immature form of IL-1β (proIL-1β) protein was accumulated within cultured chorion cells in response to influenza virus infection, although IL-1β protein was not secreted from the cells [179]. Considerable amounts of bioactive IL-6, TNF-α [179], and IFN-β proteins [212] and a trace amount of IFN-γ protein [212] were secreted from the virus-infected chorion cells prior to undergoing apoptosis. The secretion of TNF-α protein from the amnion cells was not changed after the virus infection, and the TNF-α protein produced by the amnion cells had no biological activity [209]. The induction of both adhesion and NBT reduction abilities was well correlated with the increase of IL-6 protein concentrations in IV-C-sup [179].

(c) Neutralization with Antibodies and Reconstitution of MDI Activity with Recombinant Cytokines in Part. It is known that IL-6 receptor α-chain (gp80) binds to IL-6 [223], whereas IL-6 receptor β-chain (gp130) itself does not bind to IL-6 but associates with the α-chain/IL-6 complex and is responsible for signal transduction [224]. Our study has demonstrated that the addition of respective antibodies against IL-6 and its receptor subunits, gp80 and gp130, inhibited the induction of adhesion and NBT reduction abilities by IV-C-sup [182]. Our unpublished data demonstrated that the combination of these antibodies suppressed >60% of NBT reduction activity induced by IV-C-sup. Moreover, the addition of either anti-TNF-α antibody or anti-IFN-β antibody also inhibited. Although the addition of antibody against IFN-γ inhibited the induction of NBT reduction ability by recombinant human (rh) IFN-γ, it did not inhibit the inducible effect of IV-C-sup on NBT reduction. In addition, both superoxide production and adhesion abilities were partly reconstituted with recombinant cytokines (e.g., rhIL-6, rhTNF-α, and rhIFN-β). It has been reported that IL-6, TNF-α, and IFN-β molecules are heat-stable at 56°C for 30 min, but IFN-γ molecule is labile [179, 225, 226]. On the basis of these results, our studies suggest that MDI activity is predominantly influenced by IL-6 molecule in culture supernatants and partly by TNF-α and IFN-β, but not IFN-γ, molecules.

(d) Existence of MDI Factor in Isolated Fetal Membrane Tissues. MDI activity was also detected in the supernatants of homogenate of amniochorion tissues obtained from pregnant women at term by elective cesarean section (our unpublished data). Monocytes/macrophages are normally present in the decidua tissue in large numbers but limited numbers in the amniochorion tissue of normal pregnancy [227–232]. The MDI activity present in steady states may contribute to maintain the occurrence of macrophages in normal amniochorion tissues at term.

(e) Secretion of MDI Factor from Isolated Fetal Membrane Tissues in Organ Cultures. Influenza virus infection promoted apoptotic cellular degradation in isolated amniochorion tissues in organ cultures and stimulated the secretion of MDI activity and IL-6 and TNF-α proteins from the tissues [179, 182]. Intra-amniotic infusion of IL-6 results in the infiltration of macrophages in the chorion trophoblast cell layer of rhesus monkeys [145], the expression of TNF-α converting enzyme (TACE), which is essential for the release of TNF-α, increases in the fetal membranes with chorioamnionitis as compared to those from normal pregnancies, and in parallel there is an increased infiltration of monocytes/macrophages within the choriodecidua tissues [233]. The chorion is the fetal-derived tissue that interfaces directly with the maternal decidua. That is, fetal chorion cells are a good location for cell communication with maternal monocytes/macrophages in decidua via cytokine secretion. It is possible that chorion cells contribute to the production of MDI factor containing IL-6 and TNF-α by amniochorion tissues in response to influenza

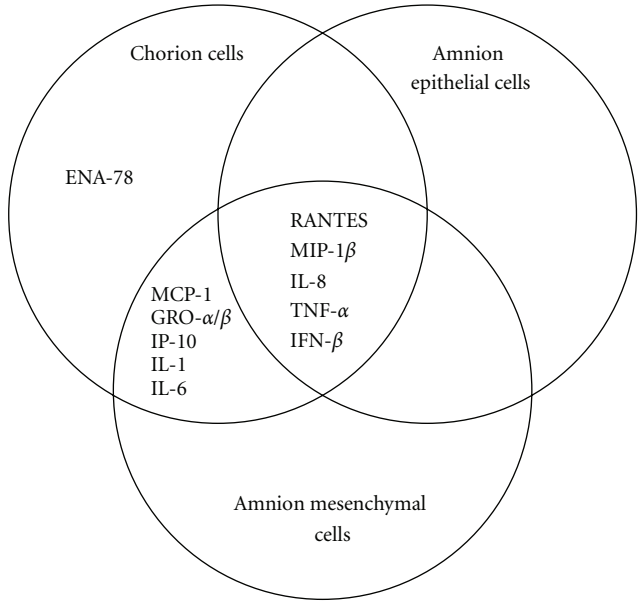

Figure 5: Differential mRNA expression of chemoattractive cytokines and MDI factor among cultured chorion, amnion mesenchymal, and amnion epithelial cells in response to influenza virus infection. In cultured chorion cells, the levels of mRNA expression of all of 7 chemokines tested (i.e., ENA-78, MCP-1, GRO-α/β, IP-10, RANTES, MIP-1β, and IL-8) were increased by influenza virus infection. In cultured amnion mesenchymal cells, the levels of mRNA expression of 6 chemokines except for ENA-78 (i.e., MCP-1, GRO-α/β, IP-10, RNATES, MIP-1β, and IL-8) were increased. In cultured amnion epithelial cells, the levels of mRNA expression of 3 chemokines (RANTES, MIP-1β, and IL-8) were increased. Influenza virus infection induced the expression of IL-1β and IL-6 mRNAs in chorion and amnion mesenchymal cells but not in amnion epithelial cells. The expression of TNF-α and IFN-β mRNAs was induced in three types of cells by influenza virus infection. Abbreviations used: ENA-78, epithelial cell-derived neutrophil-activating protein 78; GRO-α/β, growth-related oncogene α/β; IL, interleukin; IP-10, interferon inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MDI, monocyte differentiation-inducing; MIP-1β, macrophage inflammatory protein 1β; RANTES, regulated on activation, normal T cell expressed and secreted.

virus infection and play a pivotal role in the pathogenesis of adverse pregnancy outcomes associated with the virus infection [119, 214, 234]. Therefore, our results raise a possibility that chorion cell-derived MDI factor induces the differentiation of maternal monocytes in the decidua tissue to well-matured macrophages during intrauterine influenza virus infection.

(f) Intermediate Molecules for Gene Expression of MDI Factor. The transcription of IL-6, TNF-α, and IFN-β genes is activated by NF-κB, ROS-sensitive transcription factor [235, 236]. The expression of influenza virus proteins, such as HA, M2 and NP, activates the NF-κB-dependent transcription activity as demonstrated by luciferase gene assay. The transcription was inhibited by the addition of some antioxidants, such as dithiothreitol [237, 238]. Conceivably, it is possible that the transcription of IL-6, TNF-α, and IFN-β genes is activated in chorion cells in response to oxidative stress after the synthesis of the virus macromolecules.

PDTC and ribavirin are shown to inhibit the replication and transcription of influenza virus gene [185–187]. Both reagents also inhibited the induction of IL-6 and TNF-α mRNA expression in chorion cells after the virus infection and the secretion of IL-6 and TNF-α proteins from the cells [153, our unpublished data]. These results suggest that the synthesis of viral macromolecules is prerequisite for the induction of the expression of proinflammatory cytokine genes, such as IL-6 and TNF-α, in chorion cells after the virus infection. Since NDGA is shown to inhibit the virus proliferation in chorion cells [188], it is predicted that NDGA can also inhibit the induction of proinflammatory cytokine gene expression as well as PDTC and ribavirin.

4.3. Differential Gene Expression of Chemoattractive Cytokines among Types of Human Fetal Membrane Cells Responding to Influenza Virus Infection. In order to understand the involvement of chemoattractive cytokines (chemokines) in pathology of adverse pregnancy outcomes associated with influenza virus infection, we have examined the effect of influenza virus infection on chemokine gene expression in addition to proinflammatory cytokines in cultured amnion epithelial cells, amnion mesenchymal cells, and chorion cells [120]. Cultured amnion epithelial cells, amnion mesenchymal cells, and chorion cells were infected with influenza virus. Significant morphological changes, such as cell rounding and detachment, were observed in the cultured amnion mesenchymal and chorion, but not amnion epithelial, cells. The profiles of mRNA expression induced by influenza virus infection among three types of cells are shown in Figure 5. In cultured chorion cells, the levels of mRNA expression of all of 7 chemokines tested (i.e., ENA-78, MCP-1, GRO-α/β, IP-10, RANTES, MIP-1β, and IL-8) were increased by influenza virus infection. In cultured amnion mesenchymal cells, the levels of mRNA expression

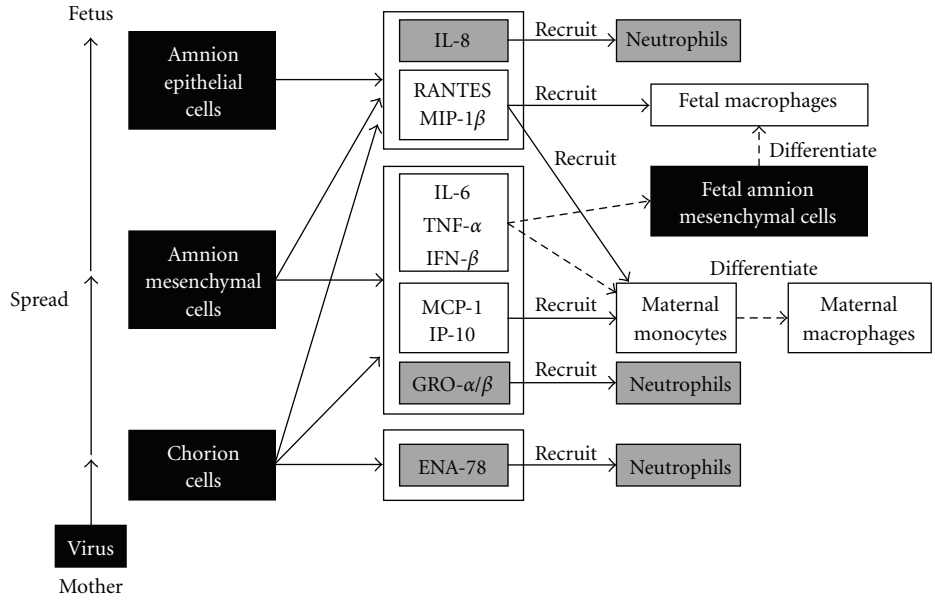

Figure 6: Multistage roles of chemoattractive cytokines and MDI factor produced by chorion, amnion mesenchymal, and amnion epithelial cells in the recruitment and differentiation of phagocytes. When influenza viruses spread from mother to the chorion cell layer at the first stratum, ENA-78, GRO-α/β, and IL-8 may be firstly produced to recruit a first unit of neutrophils by only chorion cells that are located on the maternal-fetal interface. The chorion cells may produce MCP-1, IP-10, RANTES, and MIP-1β to recruit maternal circulating monocyte, IL-6, TNF-α, and IFN-β to differentiate the recruited maternal monocytes, and the staying fetal amnion mesenchymal cells to macrophages. When the viruses got over the chorion cell layer and spread to the amnion mesenchymal cell layer at the second stratum, the amnion mesenchymal cells may produce GRO-α/β and IL-8 to recruit a second unit of neutrophils as reinforcements, MCP-1, IP-10, RANTES, and MIP-1β to recruit maternal circulating monocytes, and IL-6, TNF-α and IFN-β to differentiate the recruited maternal monocytes and the staying fetal amnion mesenchymal cells to macrophages as well as the chorion cells. If the virus spread to the amnion epithelium as a final cellular barrier, the amnion epithelial cells may produce IL-8 to recruit a third unit of neutrophils and RANTES and MIP-1β to recruit fetus-derived macrophages differentiated from the staying amnion mesenchymal cells. Abbreviations used: ENA-78, epithelial cell-derived neutrophil-activating protein 78; GRO-α/β, growth-related oncogene α/β; IL, interleukin; IP-10, interferon inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MDI, monocyte differentiation-inducing; MIP-1β, macrophage inflammatory protein 1β; RANTES, regulated on activation, normal T cell expressed and secreted.

of 6 chemokines except for ENA-78 (i.e., MCP-1, GRO-α/β, IP-10, RANTES, MIP-1β, and IL-8) were increased. In cultured amnion epithelial cells, the levels of mRNA expression of 3 chemokines (RANTES, MIP-1β, and IL-8) were increased. The profiles of mRNA expression for proinflammatory (IL-1β, IL-6, and TNF-α) and antiviral cytokines (IFN-β) in cultured amnion epithelial cells and chorion cells were consistent with our previous observations. These results suggest that the combinations of chemoattractive, proinflammatory, and antiviral cytokines induced by influenza virus infection are different among types of cultured cells. The differential combination of cytokines may produce varying results of recruiting neutrophils and monocytes, and the differentiation of maternal circulating monocytes, and fetal staying amnion mesenchymal cells to macrophages.

During the acute inflammatory response, huge numbers of neutrophils are mobilized and recruited to the tissues, where they survive for only a short time before undergoing apoptosis. Apoptotic neutrophils retain plasma membrane integrity so that release of harmful cellular contents is limited. Apoptotic neutrophils are recognized and ingested by macrophages, which are thought to be important steps in preventing the release of toxic granules and chemotactic factors into the extracellular fluid. Clearance of apoptotic cells by macrophages plays a significant role in the resolution of inflammation [239]. Notably, as reported by Lieberman et al., placentitis with seasonal influenza A (H1N1) virus was characterized by chronic inflammatory responses associated with maternal and fetal macrophages [74].

On the basis of our results and Lieberman's observations [74], it is possible that chemoattractive and proinflammatory cytokines induced by influenza virus infection among chorion, amnion epithelial, and amnion mesenchymal cells play a multistage role in the recruitment of maternal circulating neutrophils and monocytes and the differentiation of maternal circulating monocytes and fetal staying amnion mesenchymal cells to macrophages within each stratum of multilayered fetal membranes depending on the spread of virus infection from mother to the fetus (Figure 6). When influenza viruses spread from mother to the chorion cell layer at the first stratum, ENA-78, GRO-α/β, and IL-8 may be firstly produced to recruit a first unit of neutrophils by only chorion cells that are located on the maternal-fetal interface. The chorion cells may produce MCP-1, IP-10, RANTES, and MIP-1β to recruit maternal circulating monocyte and IL-6, TNF-α, and IFN-β to differentiate the recruited maternal monocytes and the staying fetal amnion mesenchymal cells [80] to macrophages as scavengers for huge numbers of

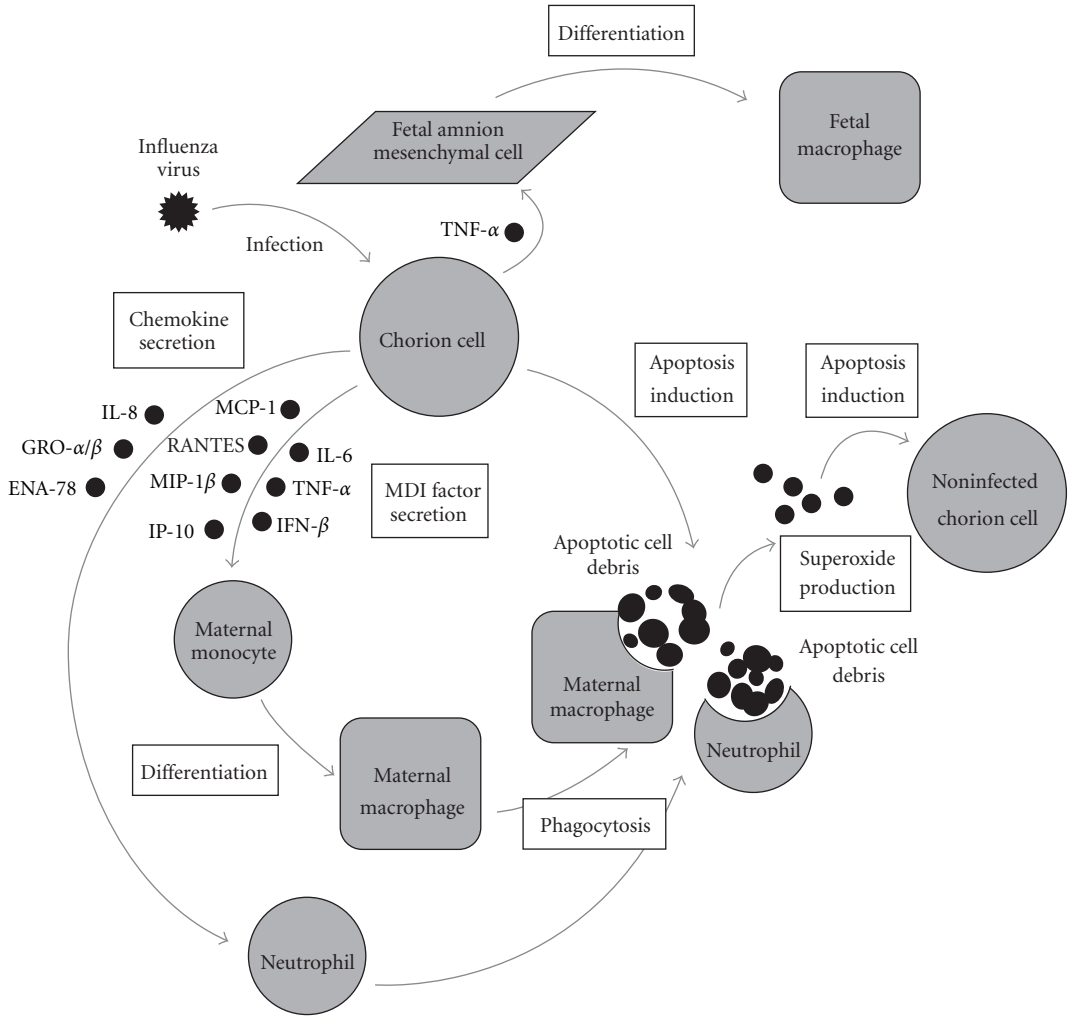

Figure 7: Hypothetical tissue injury model with special emphases of the interaction between human fetal membrane chorion cells and two types of phagocytes. Influenza virus infection induces apoptosis and the gene expression of the MDI factor (i.e., IL-6, TNF-α and IFN-β), monocyte-attractive chemokines (MCP-1, RANTES, MIP-1β and IP-10) and neutrophil-attractive chemokines (IL-8, GRO-α/β and ENA-78) in human fetal membrane chorion cells. The monocyte- and neutrophil-attractive chemokines recruit maternal monocytes and neutrophils circulating in the bloodstream into the infected region, respectively. The MDI factor (i.e., IL-6, TNF-α and IFN-β) differentiates the recruited maternal monocytes and the staying fetal amnion mesenchymal cells to mature macrophages. The mature macrophages and neutrophils phagocytose the apoptotic cell debris of chorion cells resulting from apoptosis. Subsequent to phagocytosis, an abrupt increase in superoxide production by macrophages and neutrophils, known as the oxidative burst, occurs, which is catalyzed by NADPH oxidase enzyme complex. Superoxide produced by phagocytes engulfing chorion cells undergoing apoptosis may injure tissues through inducing apoptosis in noninfected cells *in vivo* situation, resulting in the formation of necrotic foci. Abbreviations used: ENA-78, epithelial cell-derived neutrophil-activating protein 78; GRO-α/β, growth-related oncogene α/β; IL, interleukin; IP-10, interferon inducible protein 10; MCP-1, monocyte chemoattractant protein 1; MDI, monocyte differentiation-inducing; MIP-1β, macrophage inflammatory protein 1β; NADPH, nicotinamide adenine dinucleotide phosphate; RANTES, regulated on activation, normal T cell expressed and secreted.

corpses of neutrophils and massive number of corpses of chorion cell themselves resulting from apoptosis induced by the virus infection. If the viruses were not eliminated with the acute inflammation, a next stage will start. When the viruses got over the chorion cell layer and spread to the amnion mesenchymal cell layer at the second stratum, the amnion mesenchymal cells may produce GRO-α/β and IL-8 to recruit a second unit of neutrophils as reinforcements, MCP-1, IP-10, RANTES, and MIP-1β to recruit maternal circulating monocytes, and IL-6, TNF-α, and IFN-β to differentiate the recruited maternal monocytes and the staying fetal amnion mesenchymal cells to macrophages as well as the chorion cells. If the virus spread to the amnion epithelium as a final cellular barrier, the amnion epithelial cells may produce IL-8 to recruit a third unit of neutrophils and RANTES and MIP-1β to recruit fetus-derived macrophages differentiated from the staying amnion mesenchymal cells.

5. Conclusions

The data from pandemic H1N1 2009 in pregnancy clearly demonstrate that pregnant women are at an increased risk of adverse pregnancy outcomes, such as premature delivery. It has been suggested that transplacental transmission of

human influenza A viruses, such as A(H1N1)pdm09 and (H3N2), human influenza B virus, and highly pathogenic avian influenza A (H5N1) virus, is uncommon but rarely detected in humans.

Apoptosis induction and MMPs production are postulated to weaken the fetal membranes, resulting in the rupture of fetal membranes. Proinflammatory cytokines produced by fetal membranes regulate both apoptosis induction and MMPs production in the tissues. It is likely that neutrophils and monocytes/macrophages are recruited from maternal decidua tissue to amniochorion tissue by chemoattractive cytokines derived from the amniochorion tissue. It has been suggested that the chemoattractive cytokines are involved in both the physiology of labor and the pathology of PROM associate with infections.

A hypothetical tissue injury model with special emphases on the interaction between human fetal membrane chorion cells and phagocytes during intrauterine influenza virus infection is illustrated in Figure 7. Influenza virus infection induces apoptosis and the gene expression of the MDI factor (i.e., IL-6, TNF-α, and IFN-β), monocyte-attractive chemokines (MCP-1, RANTES, MIP-1β, and IP-10) and neutrophil-attractive chemokines (IL-8, GRO-α/β, and ENA-78) in human fetal membrane chorion cells. The monocyte- and neutrophil-attractive chemokines recruit maternal monocytes and neutrophils circulating in the bloodstream into the infected region, respectively. The MDI factor (i.e., IL-6, TNF-α, and IFN-β) differentiates the recruited maternal monocytes and the staying fetal amnion mesenchymal cells to mature macrophages. The mature macrophages and neutrophils phagocytose the apoptotic cell debris of chorion cells resulting from apoptosis. Subsequent to phagocytosis, an abrupt increase in superoxide production by macrophages and neutrophils, known as the oxidative burst, occurs, which is catalyzed by NADPH oxidase enzyme complex [240]. The production of superoxide by phagocytes is necessary for remodeling tissues damaged by infectious agents [241]. However, an excessive production of superoxide by NADPH oxidase in phagocytes is known to implicate in the lethal or toxic effect of influenza virus infection [242–244]. Conceivably, superoxide produced by phagocytes engulfing chorion cells undergoing apoptosis resulting from influenza virus infection may injure tissues through inducing apoptosis in noninfected cells *in vivo* situation, resulting in the formation of necrotic foci. Consequently, the MDI factor and chemokines derived from influenza virus-infected chorion cells undergoing apoptosis play a possible pathological role in adverse pregnancy outcomes associated with the virus infection through the recruitment, maturation, and activation of maternal and fetal phagocytes [119, 194, 214, 215, 235, 245]. It is possible that the MDI factor is implicated in the pathology of chronic inflammatory responses associated with maternal and fetal macrophages in placenta infected with influenza virus [74].

Since PDTC and NDGA exhibit not only antiviral activity but also superoxide-scavenging activity, they are potential candidates for drugs of choice for anti-influenza treatment as multifunctional agents with antiviral and antioxidant activities [246–249]. Physiological agents (IL-10 and activin A) and pharmacological agents, such as antioxidants (*N*-acetyl-L-cysteine and α-lipoic acid), PPAR-γ ligand (15d-PGJ_2 and troglitazone), p38 MAP kinase inhibitors (SKF86002, SB203580, and SB202190), NF-κB inhibitors (SC-514, BMS 345541, evodiamine, wedelolactone, butein, CAPE, parthenolide, and TPCA-1), and PDE4 inhibitor (rolipram), may be potential therapeutic drugs to prevent PROM associated with infections because they inhibit the production of the MDI factor (IL-6 and TNF-α) by human fetal membranes. The combination of anti-influenza drugs with these agents may provide a new strategy for the prevention of adverse pregnancy outcomes associated with influenza virus infection.

Abbreviations

15d − PGJ_2:	15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2
A(H1N1)pdm09:	The pandemic strain of influenza A (H1N1) 2009 virus
ADP:	Adenosine diphosphate
AGE:	Advanced glycation end products
BiP:	Immunoglobulin heavy-chain binding protein
Boc-D-fmk:	*N*-*t*-Boc-Asp(OMe)-fluoromethyl ketone
cAMP:	Cyclic adenosine monophosphate
CAPE:	Caffeic acid phenylmethyl ester
CC:	Cysteine-cysteine
CD:	Cluster of differentiation
cDNA:	Complementary DNA
cGMP:	Cyclic guanosine monophosphate
COX:	Cyclooxygenase
CPE:	Cytopathic effect
CXC:	Cysteine-X-cysteine
DNA:	Deoxyribonucleic acid
dUTP:	Deoxyuridine triphosphate
ENA:	Epithelial cell-derived neutrophil-activating protein
ER:	Endoplasmic reticulum
FITC:	Fluorescein isothiocyanate
gp130:	Glycoprotein with a molecular mass of 130 kDa
gp80:	Glycoprotein with a molecular mass of 80 kDa
$gp91^{phox}$:	91 kDa glycoprotein component of phagocyte NADPH oxidase
GRO:	Growth-related oncogene
HA:	Hemagglutinin
ICU:	Intensive care unit
IFN:	Interferon
IKK:	Inhibitor of κB kinase
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
IP:	Interferon inducible protein
IV-A-sup:	Culture supernatants of influenza Virus-infected amnion cells
IV-C-sup:	Culture supernatants of influenza Virus-infected chorion cells

LDH: Lactate dehydrogenase
LPS: Lipopolysaccharide
M1: Membrane protein 1
M2: Membrane protein 2
MAP: Mitogen-activated protein
MCP: Monocyte chemoattractant protein
M-CSF: Macrophage colony-stimulating factor
MDI: Monocyte differentiation-inducing
MIF: Macrophage migration inhibitory factor
MIP: Macrophage inflammatory protein
MMP: Matrix metalloproteinase
Mock-A-sup: Culture supernatants of mock-infected amnion cells
Mock-C-sup: Culture supernatants of mock-infected chorion cells
mRNA: Messenger RNA
NA: Neuraminidase
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
NBT: Nitroblue tetrazolium
NDGA: Nordihydroguaiaretic acid
NF: Nuclear factor
NICU: Neonatal intensive care unit
NK: Natural killer
NO: Nitric oxide
NP: Nucleoprotein
$p22^{phox}$: 22 kDa protein component of phagocyte NADPH oxidase
PDE: Cyclic nucleotide phosphodiesterase
PDTC: Pyrrolidine dithiocarbamate
PG: Prostaglandin
PPAR: Peroxisome proliferator-activated receptor
PPROM: Preterm premature rupture of the membranes
PROM: Premature rupture of the membranes
RANTES: Regulated on activation, normal T cell expressed and secreted
rh: Recombinant human
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RT-PCR: Reverse transcriptase-polymerase chain reaction
SR-A: Class A scavenger receptor
TACE: TNF-α converting enzyme
TGF: Transforming growth factor
TIMP: Tissue inhibitor of metalloproteinase
TLR: Toll-like receptor
TNF: Tumor necrosis factor
TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein isothiocyanate nick-end labeling
UV: Ultraviolet.

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Research Article

Mast Cells and Histamine: Do They Influence Placental Vascular Network and Development in Preeclampsia?

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The physiological course of pregnancy is closely related to adequate development of the placenta. Shallow invasion of trophoblast as well as decreased development of the placental vascular network are both common features of preeclampsia. To better understand the proangiogenic features of mast cells, in this study we aim to identify the potential relationship between the distribution of mast cells within the placenta and vascular network development. *Material and Methods*. Placentas from preeclampsia-complicated pregnancies ($n = 11$) and from physiological pregnancies ($n = 11$) were acquired after cesarean section. The concentration of histamine was measured, and immunohistochemical staining for mast cell tryptase was performed. Morphometric analysis was then performed. *Results*. We noticed significant differences between the examined groups. Notably, in the preeclampsia group compared to the control group, we observed a higher mean histamine concentration, higher mast cell density (MCD), lower mean mast cell (MMCA) and lower vascular/extravascular (V/EVT) index. In physiological pregnancies, a positive correlation was observed between the histamine concentration and V/VEVT index as well as MCD and the V/VEVT index. In contrast, a negative correlation was observed between MMCA and the V/EVT index in physiological pregnancies. *Conclusions*. Based on the data from our study, we suggest that a differential distribution of mast cells and corresponding changes in the concentration of histamine are involved in the defective placental vascularization seen in preeclamptic placentas.

1. Introduction

Angiogenesis is a crucial process for the growth and development of new tissues. We can observe angiogenesis in neoplasms, during tissue repair after injury and in the placenta. Proper placental angiogenesis is necessary for the normal course of pregnancy and labor [1]. The pathogenesis of preeclampsia is still unclear, but it is known that shallow spiral artery invasion may contribute to preeclampsia development. Shallow spiral artery invasion results in poor placental perfusion and may lead to hypoxic stress in the fetus. Immaturity of extravillous trophoblastic cells has been identified as a cause of diminished spiral artery invasion [2]. The placental vascular network is defectively developed as well. In some preeclampsia-complicated pregnancies, the placenta and associated placental vascular network are diminished. Mast cells are found in the placenta in every stage of placenta development. Their potential role, apart from immunological properties, can be associated with proangiogenic activity. Mast-cell-derived mediators of known angiogenetic potential include vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), histamine, tumor necrosis factor alpha (TNF-α), interleukin-8, and basic fibroblast growth factor [3]. The activation and degranulation of mast cells in the place of angiogenesis stimulate vessel sprouting and sustain mast cell attraction and activation [4]. Data from the literature and our own experience suggest that mast cells may be involved in the pathogenesis of

Table 1: The characteristics of patients included into the study.

	PE group $n = 11$		Control group $n = 11$	
	Median	Range	Median	Range
Mother's age	30	27–42	30	23–37
Weeks of gestation	37,5	35–40	39	37–40
Birth weight (g)	2485	1650–3650	3400	2630–3810
1st minute Apgar's score	9,5	3–10	10	9-10

preeclampsia-complicated pregnancies [5, 6]. In this study, we examined the relationship between mast cells (number and morphological features), histamine concentration, and microvascular density in placentas obtained after delivery from normal and preeclampsia-complicated pregnancies.

2. Material and Methods

The characteristics of the patients are detailed in Table 1. Placental samples were obtained in a standardized manner after the dissection of fetal membranes. Three samples were excised from the maternal side of the placenta and two were excised from the fetal side. Macroscopically changed areas, large vessels, and fibrous tissues were avoided. Samples were taken immediately after cesarean sections in each group: preeclamptic women (PE, $n = 11$) and healthy women (control group, $n = 11$). In the PE group, cesarean sections were performed due to severe preeclampsia. In the control group, cesareans were performed due to severe myopia and breech presentation of the fetus. None of the patients included in the study had contractile activity [7]. The study was reviewed and accepted by the local ethic committee.

3. Immunocytochemical Stainings

The tissue fragments were fixed in formaldehyde solution, dehydrogenized with 96% alcohol, acetone, and xylene and then paraffinized. Next, they were cut in microscopic slides and deparaffinized, and the intrinsic peroxidase activity was blocked with hydrogenium superoxide. The samples were then washed with PBS and incubated with normal human serum for 20 minutes. Excess antibody was removed, and the slides were incubated with mouse anti-tryptase antibody (Novocastra, 1 : 3000), followed by secondary anti-mouse biotinylated antibody and Novostain Super ABC Reagent (Novocastra). Both incubations lasted for 30 minutes. The slides were washed with PBS and exposed to 3,3′-diaminobenzidine (Immunotech) for 3 minutes as an electron donor and hydrogen peroxide as a substrate, resulting in a brown reaction product. The cells were then counterstained with Mayer's hematoxylin (Sigma) for 1 minute. Finally, the slides were mounted with DPX (Sigma). As a negative control, the slides were incubated with PBS instead of the primary antibody.

4. Histamine Concentration Assay

A fluorimetric method was applied as previously described [8]. The determination of histamine was based on a precolumn derivatization with o-phthaldialdehyde using reversed-phase high-performance liquid chromatography in perchloric acid extracts. A fluorescence detection system was used, with the excitation set at 360 nm and the emission read at 455 nm. The intra- and inter-assay coefficients of variation were 8.5% and 10.0%.

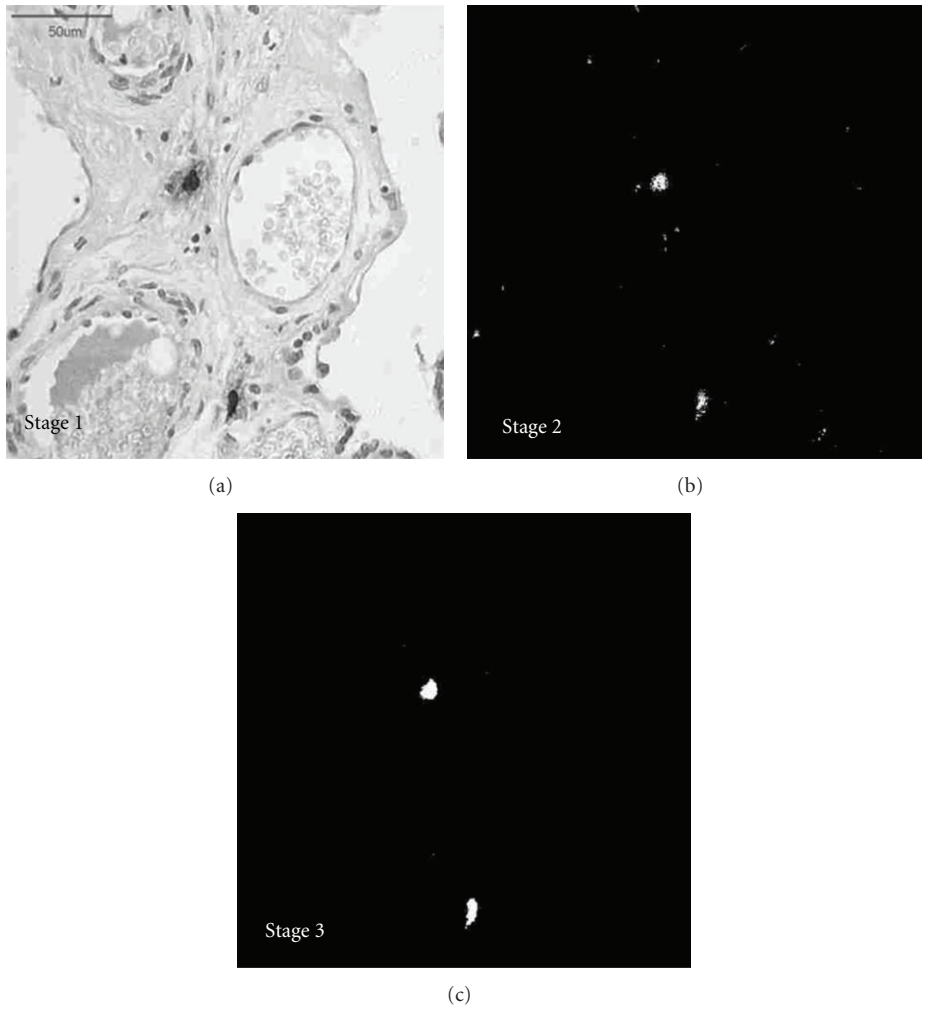
5. Morphometric Analysis

Morphometric analysis was carried out with the computer image analysis system Leica Quantimet 500C+ (Leica Cambridge Ltd. Cambridge, UK). The system consisted of an IBM Pentium computer operating at 120 MHz equipped with an ARK Logic 2000MT graphic card and graphic processor. The computer was connected to a CCD video camera JVC TK-1280E and Leica DMLB light microscope. Sections of placentas were imaged using a 20 : 1 objective and a 10 : 1,20 ocular. The optical image was focused by a video camera, and an analogue video signal was generated. An analogue to digital converter (ADC) produced a digitized video with distinct color level values in HSI system. The images were processed, and mast cells and placental vessels were clearly identified [9] (Figure 1).

Two independent researchers were responsible for image acquisition and analysis. All measurements were recorded in a blinded fashion. Neither researcher had previous knowledge of the clinical data. For each case, 50 random visual fields were analyzed. After system calibration, the area of a single analyzed image (visual field) was defined as approximately 0,14 mm^2. The following parameters were analyzed: mast cell density (MCD), defined as number of mast cells per mm^2 of placental tissue; mean mast cell area (MMCA), the mean area of mast cells cross-sections; shape of mast cells, defined as the ratio of long to short axis of a cell (with perfectly round cells defined as having 1.00 index); vascular/extravascular tissue index (V/EVT index), the ratio of vessel cross-section area to remaining placental tissue. Technical error caused by uniaxial sections of vessels was eliminated by accepting the lowest value of Ferret's diameter as the diameter for a single lumen. Vessels between 10 and 70 μm in diameter were included for analysis.

6. Statistical Analysis

Statistical analysis was performed with Statistica 8.0 (StatSoft, Poland). Groups were compared with Student's t-test. In each group analysis, correlation was measured between the histamine concentration, V/EVT index, and



(a) (b)

(c)

Figure 1: Process of mast cells identification with morphometric software. Stage 1: initial picture obtained from microscope and saved in HSI colour system. Stage 2: binary image after detection of mast cells in distinct hue values. Stage 3: final reduction of noises and smoothing of detected fields.

morphometric parameters of the mast cells. Differences were deemed statistically significant if $P < 0{,}05$.

7. Results

Specific differences were observed in several examined parameters between the PE and control groups. The mean histamine concentration (ng of histamine per 1g of tissue) was significantly higher in the PE group compared to the control group (245,6 ± SD 19,8 versus 175,1 ± SD 15,1; $P = 0{,}002$). MCD (in cells/mm^2) was also significantly higher in the PE group compared to the control group (7,67 ± SD 3,56 versus 2,89 ± SD 1,34; $P = 0{,}004$). In contrast, the MMCA was significantly lower in the PE group in comparison to the control group (62,25 μm^2 ± SD 18,91 versus 101,98 μm^2 ± SD 57,91; $P = 0{,}0428$). We also observed some differences in cell shape. Mast cells in the control group were longer than mast cells in the PE group (shape index 1,88 ± SD 0,8 versus 1,52 ± SD 0,39; $P = 0{,}051$; refer to Table 2).

Morphometric assessment of placental circulature was performed and revealed a decrease in the V/EVT index in the PE group compared to the control group (0,15 ± SD 0,04 versus 0,23 ± SD 0,074; $P = 0{,}005$; refer to Table 2).

The analysis revealed a positive correlation between the histamine concentration and the V/VEVT index as well as between MCD and the V/VEVT index. A negative correlation existed between the MMCA and V/EVT index in the control group, while the PE group showed no significant correlation between these parameters. Specific values of correlation for these parameters are provided in Table 3.

8. Discussion

Angiogenesis is the process of vessel growth from preexisting vessels, a process that requires stimulation by proangiogenic factors. Important stimulants of placental angiogenesis include VEGFs and placental growth factor, which act through the VEGF receptor family. VEGF production is stimulated by histamine acting through the H_2 receptor [10]. Mast cells are pointed to as a potential source of potent proangiogenic factors during angiogenesis, including histamine, VEGF, bFGF, TGF-beta, TNF-alpha, and IL-8.

Table 2: Morphometric parameters analyzed in the study compared with Student's *t*-test. V/EVT index: vascular/extravascular tissue index, MCD: mast cell density, and MMCA: mean mast cell area. The statistically significant results are in bold.

	PE group		Control group		
	mean	SD	mean	SD	*P*
V/EVT index	**0,15**	**±0,04**	**0,23**	**±0,074**	**0,005**
MCD (n/mm^2)	**7,67**	**±3,56**	**2,89**	**±1,34**	**0,0004**
MMCA (μm^2)	**62,25**	**±18,91**	**101,98**	**±57,91**	**0,0428**
Shape index	1,52	±0,39	1,88	±0,8	0,051

Table 3: The indexes of correlation between histamine concentration and morphometric parameters of mast cells and V/EVT index in each group. V/EVT index: vascular/extravascular tissue index, MCD: mast cell density, MMCA: mean mast cell area. The statistically significant results are in bold.

	PE group $n = 11$	Control group $n = 11$
	V/EVT index	V/EVT index
Histamine concentration (ng/1 g of tissue)	0,48	**0,74**
MCD (n/mm^2)	0,03	**0,82**
MMCA (μm^2)	0,22	**0,67**
Shape index	0,51	0,42

Additionally, mast cells are a source of extracellular matrix-degrading proteinases [4].

In vitro models of angiogenesis observed in hypoxic conditions provide us with information on increased angiogenesis, which occurs mainly through increases in VEGF synthesis [11]. Histamine proangiogenetic action is provided through H_1- and H_2-receptor-mediated VEGF synthesis. Mast cell degranulation leads to a local increase in histamine concentration and therefore an increase in VEGF synthesis. Mast cells, however, synthesize and secrete VEGF apart from histamine. The final effect is vigorous formation of new vessels in place of mast cell degranulation [12, 13].

Decreases in mast cell density in connection with decreased histamine concentration correlated with lower V/EVT index values; nevertheless, this correlation was observed only in the control group. Decreased mast cell area may indicate changes in mast cell activation, perhaps as an effect of degranulation. Hypoxia, which is dominant during placenta formation, is a potent stimulator for mast cell activation and new vessel formation. The most important pathway through which hypoxia stimulates angiogenesis is the activation of hypoxia inducible factor-1α (HIF-1α) transcription and further synthesis of VEGF. It is also observed that the synthesis of histamine within mast cells and their degranulation is increased after stimulation with HIF-1α that is achieved through histidine decarboxylase (HDC, EC:4.1.1.22) [14].

Preeclampsia is a specific state of pregnancy associated with hypertension and proteinuria. Shallow trophoblast invasion of maternal spiral arteries results in an increase in systemic blood pressure. The leading hypothesis for preeclampsia pathogenesis suggests it may arise in order to maintain placental perfusion pressure at a satisfactory level [15]. The vascular bed of the placenta is diminished as a whole, with reduced branching and malformations observed; blood vessels are characterized by decreased number, lumen diameter, and total lumen area [16]. Data from our study support this previous finding, as the V/EVT index was decreased in the PE group compared to the control group. The reduced proportion of vascular area may reflect diminished placental angiogenesis in the first trimester of pregnancy. The decreased vascular network development is a result of a multifactorial pathogenetic course as well as inherited conditions.

The differences in mast cell organization observed between the PE and control groups suggest that mast cells take part in the process of vessel development. Because mast cells are observed to gather close to blood vessels just before the process of angiogenesis begins (this is particularly characteristic for neoplasm growth [17]), we expect an expanded vascular network in preeclamptic placentas. In our study, we observed an increase in mast cell density and an increase in histamine concentration but a low V/EVT ratio. We conclude that in PE, susceptibility to histamine and/or other mast cell proangiogenic compounds may be decreased. In PE placentas, the mast cells had a different shape and smaller area in comparison to the control group. The data suggest that we observed mast cells after an intensive degranulation, as we also found an increased concentration of histamine [18]. Increased mast cell density and histamine concentration can be a compensation effect for incorrect vascular network development. On the other hand, we cannot exclude impairments in histamine receptor configuration. Functional predominance of intracellular histamine receptor (H_{IC}) over H_1 and H_2 receptors may be a causative factor in the observed decreased angiogenesis [19].

The reason for the decreased V/EVT index in preeclamptic placentas may be associated not only with decreased angiogenesis but also with fibroblast proliferation and fibrosis in the extravascular area. In the examined material, the V/EVT index was assessed in placentas obtained during the

third trimester. A remodeling of extravascular tissue during the pregnancy should also be taken into consideration. Mast cells are sources of matrix-degrading enzymes including collagenases and gelatinases [4]. Prolonged stimulation of mast cells with hypoxia leads to an increase in collagenolytic activity and an accumulation of low molecular collagen fragments, thus providing a stimulatory factor to fibroblasts and smooth muscle cell proliferation [20]. A dominance of activated fibroblasts may lead to a decrease in the V/EVT index.

We conclude that mast cells are strongly involved in the pathogenesis of preeclampsia, as their concentration and activity are changed in preeclamptic placentas in comparison to physiological placentas. Low vascularization in preeclamptic placentas despite higher histamine concentration and accumulation of mast cells suggests that mast cells fail in their proangiogenic potential, concurrently increasing extravascular activity.

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Review Article

The Th1:Th2 Dichotomy of Pregnancy and Preterm Labour

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Pregnancy is a unique immunological state in which a balance of immune tolerance and suppression is needed to protect the fetus without compromising the mother. It has long been established that a bias from the T helper 1 cytokine profile towards the T helper 2 profile contributes towards successful pregnancy maintenance. The majority of publications that report on aberrant Th1:Th2 balance focus on early pregnancy loss and preeclampsia. Over the last few decades, there has been an increased awareness of the role of infection and inflammation in preterm labour, and the search for new biomarkers to predict preterm labour continues. In this paper, we explore the evidence for an aberrant Th1:Th2 profile associated with preterm labour. We also consider the potential for its use in screening women at high risk of preterm labour and for prophylactic therapeutic measures for the prevention of preterm labour and associated neonatal adverse outcomes.

1. Introduction

Preterm labour occurs in some 10% of pregnancies [1]. In many developed countries, the rates are rising. Birth before 37 weeks of gestation is thought to account for up to 70% of neonatal deaths, and the extremely high neonatal intensive care costs required to support those who do survive make preterm birth both a social and economic burden. It is now widely acknowledged that the aetiology of preterm labour is multifactorial, and, as such, the underlying cause of preterm labour is often unknown. There is a strong association between preterm labour and infection and inflammation, and research in this field has dramatically increased over the last few decades [2]. However, we still have made little significant progress in the prevention of preterm labour. Evidence of the detrimental direct impact of maternal infection/inflammation on neonatal outcome is emerging, yet we do not fully understand if anti-inflammatory therapeutic agents would provide benefit or harm to the neonate born under conditions of infection/inflammation-induced preterm labour.

The immunology of pregnancy is complex, in that the mother must tolerate the "foreign" fetus, and thus requires a degree of immunosuppression whilst on the other hand needs to maintain immune function to fight off infection. One mechanism which is involved in successful pregnancy maintenance is the proposed switch from the T helper 1 (Th1) cytokine profile to the T helper 2 (Th2) profile. This paper explores the evidence for an imbalance in the Th1:Th2 profile in women at risk of and who are in established preterm labour.

2. The Immunology of Pregnancy

The fetus can be described as a semiallogeneic graft, being a product of two histoincompatible individuals [3, 4]. This poses a challenge to the mother, to both tolerate and accommodate the fetus, which will express paternal antigens, and maintain an ability to reject in case of overwhelming infection [5]. This challenge is undertaken in part by the immune system. The immune system has two main defence systems: the innate and the adaptive. The innate immune response is a nonspecific reaction towards foreign antigens, whereas the adaptive response forms a very specific reaction towards antigens [6]. Although different immune components are involved in these systems, much overlap and

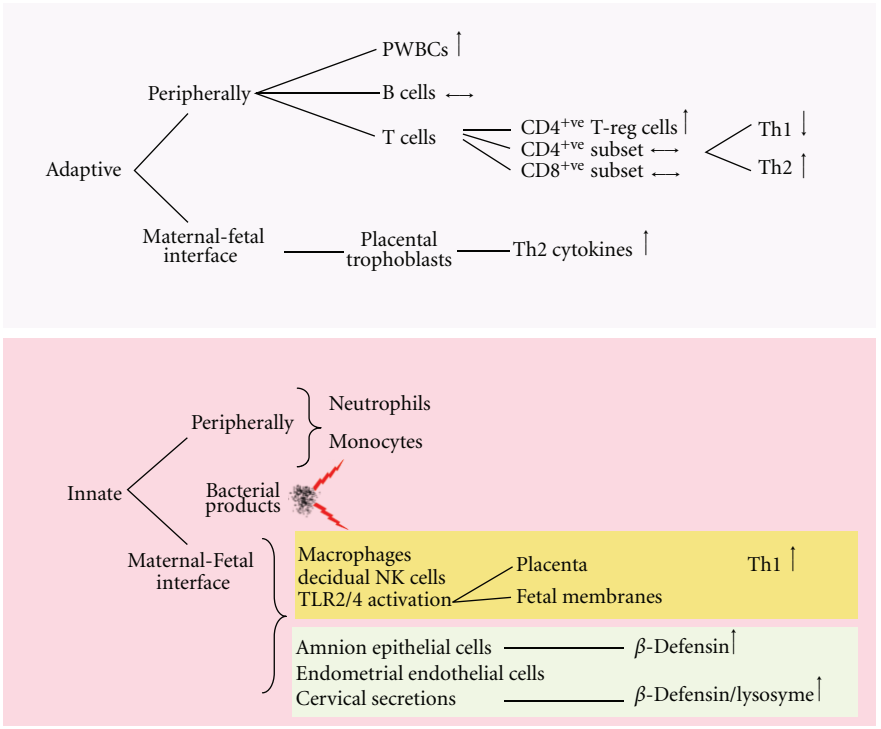

Figure 1: Summary of the adaptive and innate immune system in pregnancy. Mediators of the adaptive and innate immune system work in parallel to facilitate a balance between immune tolerance of the fetus whilst maintaining the ability to mount a response against invading pathogens. PWBC: peripheral white blood cells.

cross-talk exist between the two. Figure 1 summarises the key elements of these systems during pregnancy.

The immune cells that make up the adaptive immune response include B and T lymphocytes. Activation by antigen presenting cells and cytokines leads to cytokine release by T cells in a cell-mediated response, or antibody release by B cells in a humoral response [7]. Although Medawar originally hypothesised that pregnancy represents a time of immune suppression [8], a more complex picture has recently emerged where a change in the ratio and function—rather than a complete suppression—of the maternal leukocytes occurs during pregnancy. For example, there is an increase in the total peripheral white cell count from the early stages of pregnancy with no change in the CD4 and CD8 counts [9]. Within the CD4 positive population, an increase in T regulatory cells is seen in pregnancy [10]. The function of the T cells adapts in pregnancy to favour the T helper 2 cytokine profile, which is more pronounced at the maternal fetal interface [11]. Nonimmune cells, for example, placental trophoblasts also contribute to the Th2 cytokine predominance in pregnancy [12].

The innate immune system provides a less specific response nevertheless is critical for the prevention of microbial invasion. Cellular components include neutrophils, monocytes, and macrophages, which protect against pathogens by phagocytosis. The Toll-like receptors (TLRs) TLR2 and TLR4 are pattern recognition receptors stimulated by Gram-positive and Gram-negative bacteria, respectively [1]. TLRs are expressed on nonimmune cells in the placenta and fetal membranes, which mediate part of the innate immune system at the maternal fetal interface [13]. TLR2 and 4 mutations are associated with an increased risk of preterm birth [14, 15]. During pregnancy, there is tight regulation and considerable cross-talk between the adaptive and the innate adaptive immune system that is responsible for preventing or activating rejection of the conceptus.

3. Th1:Th2 Cytokines

T helper 1 and 2 cell subsets originate from undifferentiated Th0 cells under the influence of interferon-gamma (IFN-γ) and interleukin-4 (IL-4), respectively. Pregnancy hormones such as progesterone [16], leukaemic inhibitory factor [17], estradiol [18], and prostaglandin D_2 (PGD_2) [19] promote the T helper 2 cell profile and are likely to be in part responsible for the Th2 bias associated with pregnancy.

Type 1 $CD4^+$ T cells (Th1) produce an array of inflammatory cytokines including IFN-γ [20], IL-2 [21], and Tumor necrosis factor-alpha (TNF-α) [22] and are the major effectors of phagocyte-mediated host defence, protective against intracellular pathogens [21, 23, 24]. Type 2 $CD4^+$ T cells (Th2) produce IL-4, IL-5, IL-13, IL-10 [20], and IL-6. Whilst IL-4 and IL-10 are considered to be anti-inflammatory cytokines [25], IL-6 has proinflammatory properties [26]. Although IL-10 and IL-6 are frequently referred to as Th2 cytokines [27–32], they are both produced by other cell types including Th1 cells, macrophages, and B cells for IL-10 [33, 34], and macrophages, fibroblasts, and B

cells for IL-6 [35]. The T helper 2 cytokines are commonly associated with strong antibody responses [36], for example, IL-4 stimulates IgE and IgG_1 antibody production [37]. However, the Th2 cytokines also serve other functions, for example; IL-5 promotes the growth and differentiation of eosinophils, whereas IL-13 and IL-10 inhibit the activity of macrophages [37]. T helper 2 cell responses are also associated with protection against parasites, since IL-4 mediates IgE production, and IL-5 mediates an eosinophilia, both of which are hallmarks of parasitic infection [38]. It is important to note that, although the Th1 and Th2 responses can be seen as discrete responses, there is considerable cross-talk and overlap between the functions of the T helper cells. For example, the Th1 cytokines can promote the production of complement-fixing antibodies involved in antibody-dependent cell cytotoxicity [39], and thus the dichotomy described may be an oversimplified representation of the complex immune system. Transcriptional regulation of the predominant Th2 cytokine IL-4 is by STAT-6, c-maf, GATA-3, and NFAT [40], whereas Th1 cell cytokine production is transcriptionally regulated by T-bet and STAT-4 [11].

4. Th1:Th2 and Pregnancy Maintenance

The hypothesis of Th2 predominance and downregulation of the Th1 response originated from Wegmann and colleagues [41] and was reinforced by evidence from both murine studies and the clinical course of Th2 and Th1 based conditions in pregnancy. IL-2, IFN-γ, and TNF-α induce miscarriage in mice, which can be reversed by inhibitors of the Th1 cytokines or by administering the anti-inflammatory Th2 cytokine IL-10 [42, 43]. Autoimmune conditions where Th1 is involved in the pathophysiology generally improve in pregnancy (e.g., rheumatoid arthritis [44]), whereas the Th2 autoimmune spectrum tends to worsen (e.g., systemic lupus erythematosus [45]). With a Th2:Th1 bias, the diminished cell-mediated immunity may be responsible for the increased susceptibility in pregnancy of conditions caused by intracellular pathogenesis (e.g., influenza, leprosy, and *Listeria monocytogenes* [46]).

4.1. Peripheral Blood. Several techniques are available to establish the function of Th1 and Th2 cells in pregnancy; enzyme-linked immunosorbent assay (ELISA) can be used to measure maternal serum interleukins; peripheral T cells can be isolated and stimulated with a mitogen such as phorbol myristate acetate (PMA) or phytohaemagglutinin (PHA) to measure the cytokine production either by ELISA or flow cytometry during pregnancy compared with nonpregnant controls.

Marzi and colleagues isolated PBMCs, stimulated them with PHA, and measured interleukin secretion by ELISA showing a reduction in IFN-γ and IL-2 and an increase in IL-4 and IL-10 in pregnancy compared with nonpregnant controls [47]. In support of this study, Reinhard et al. stimulated cells with PMA and demonstrated by flow cytometry a reduction in intracellular IFN-γ and IL-2, and an increase in intracellular IL-4 production in pregnancy compared with nonpregnant controls [48]. *In vivo* confirmation of this bias has since been demonstrated by polymerase chain reaction (PCR) reflecting decreasing messenger ribonucleic acid (mRNA) of IFN-γ through pregnancy and a concurrent increase in IL-4 mRNA which peaks in the 7th month compared with nonpregnant controls [49]. However, not all studies support the Th1 to Th2 bias. Shimaoka et al. reported a reduction in PMA-stimulated IL-4 during pregnancy [50], while Matthiesen and colleagues presented data suggesting an increase in both IL-4 and IFN-γ secreting cells in pregnancy compared with nonpregnant controls [51, 52]. Such discrepancies may be due to characterisation of cytokine profiles in either isolated cell populations or whole blood, the latter arguably being a more biologically relevant system.

4.2. Maternal Fetal Interface and Nonimmune Cells. While much research has been dedicated toward circulating cytokines in pregnancy, local cytokine production at the maternal interface may be of greater significance than measurements obtained in the peripheral blood [23]. IL-4, IL-10, and macrophage colony-stimulating factor (m-CSF) production by T cells at the maternal fetal interface is associated with successful pregnancy [23]. Trophoblast, decidua, and amnion all contribute to the Th2 cytokine environment by production of IL-13 [53], IL-10 [54], IL-4 and IL-6 [55, 56]. Coculture of trophoblasts and T cells results in an increase in the transcription factors GATA-3 and STAT-6 (which regulate Th2 cytokine production), and a reduction in the Th1 transcription factor STAT-4 and subsequently decreased production of IFN-γ and TNF-α [57]. The placenta also synthesises PGD_2, which may act as a chemoattractant of Th2 cells to the maternal fetal interface via the classic Th2 receptor CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) [28]. $CRTH2^+$ cells are reduced at the maternal fetal interface of women suffering from recurrent loss compared with women undergoing elective termination [58].

Local production of IL-4 and IL-10 inhibits the function of both Th1 cells and macrophages, which serves to prevent fetal allograft rejection [59]. Other anti-inflammatory effects of these interleukins result in inhibition of the Th1 cytokine TNF-α [60], and TNF-α-induced cyclo-oxygenase-2 (COX-2), and/or PGE_2 synthesis in amnion-derived wish cells. Similar effects are observed in decidual and placental cells *in vitro* [61–64], which is thought to inhibit the onset of labour. Consistent with such a role, decidual CD4 positive cells from women undergoing unexplained recurrent pregnancy loss typically exhibit reduced IL-4 and IL-10 production [65].

5. Th1:Th2 Cytokines in Labour

5.1. Peripheral Blood. The Th2 cytokine predominance which exists during pregnancy has been shown to return to nonpregnant Th1:Th2 ratios by 4 weeks postpartum [66]. Labour is often seen as a proinflammatory state marking the end of the pregnancy, and thus it is plausible that labour is associated with a reversal in the bias back towards Th1 rather than Th2. Rather, Kuwajima and colleagues

have shown that the Th2:Th1 ratio remained constant in favour of Th2 through pregnancy and labour, with a reversal back to nonpregnant parameters at 7 days postpartum [67]. However, this finding is somewhat contrary to an earlier report indicating that serum IL-4 levels measured by ELISA in women through pregnancy and at different stages of labour were reduced in the later part of labour and by day 1 postpartum in both normotensive and preeclamptic women [68]. In this study, the Th1 proinflammatory cytokine TNF-α peaked in early labour consistent with labour being a proinflammatory state. Consistent with this, an increase in IFN-γ and IL-1β in women in active labour has also been reported [69].

5.2. Maternal Fetal Interface and Nonimmune Cells. There is substantial evidence that the Th1 cytokines play a role in the initiation of labour at term [22]. The importance of local rather than peripheral production of the cytokines is highlighted by their direct input into the biochemical pathways involved in parturition. Fetal membranes [70, 71] and myometrium [72] produce IL-1β at term, a potent inducer of NF-κB [73]. This transcription factor regulates the expression of numerous labour-associated genes including COX-2, the oxytocin receptor, IL-8, and matrix metalloproteinase-9 (MMP-9) [74]. TNF-α and IL-1β are both increased in amnion, amniotic fluid, and decidua at term [75] and can induce PGE_2 production in amniocytes and decidual cells *in vitro* [76, 77]. Despite the proinflammatory nature of the Th1 cytokines they are required for successful pregnancy contributing to the physiology of term labour.

6. Th1:Th2 Cytokines in Infection

Activation of the Th1 cytokines occurs as a specific response to infection caused by intracellular bacteria, parasites, and viruses [78]. The necessary proinflammatory type 1 response elicited by infection, along with the action of the activated T cells, drives local and systemic cytokine production that, if left unchecked, can be harmful to the host [78]. In some situations, the Th1 response is balanced by the production of Th2 cytokines, particularly IL-4 and IL-10 [79–82]. In the early stages of infection, IL-12 is produced by macrophages and dendritic cells [83, 84], which lead to polarisation from Th0 to Th1 type cells [24]. IFN-γ enhances Th1 development by upregulating the IL-12 receptor and inhibiting the growth of Th2 cells [85]. IFN-γ also primes macrophages to begin phagocytosis and to stimulate the release of interleukin-1 [86].

While the Th1 cytokine response may be suppressed by both the maternal and fetal immune system during pregnancy [87], it still maintains the capacity to mount a defensive response in the context of infection. For example, cord blood mononuclear cells cultured with lipopolysaccharide (LPS) *in vitro* show an increased production of IFN-γ concurrent with reduced IL-4 secretion [88]. Similarly, neonates exposed to intrauterine infection have an increased percentage of IFN-γ-producing cells, with some neonates also showing an increase in IL-4-producing cells [89]. In response to LPS amnion, chorion, deciduas, and placenta also release proinflammatory cytokines [64, 90, 91].

7. Th1:Th2 Cytokines in Preterm Labour

Approximately, 30% of preterm births are associated with infection [92], with a higher rate of 80–85% in early preterm birth (<28 weeks) [93]. Immune and nonimmune cells contribute to a cytokine-rich environment in the presence of infection and inflammation. Proinflammatory cytokines such as TNF-α and IL-1β ultimately result in the production of prostaglandins and MMPs [86], via NF-κB. This triggers a cascade of prolabour events including uterine contractility and fetal membrane rupture, and if this cascade is activated early in pregnancy, preterm labour can ensue.

7.1. Peripheral Blood. As discussed above, the peripheral response may not be as potent as the local Th1:Th2 response and may instead reflect a more significant inflammatory response at the fetal placental compartment. A large case control study of 101,042 Danish women showed that an elevated mid pregnancy IFN-γ plasma level was associated with moderate and late spontaneous preterm delivery, whereas no increased risk was seen with elevated TNF-α or IL-2 [94]. However, a study comparing women in active preterm labour and no labour looked at mitogen-stimulated production of IFN-γ and the Th2 cytokines IL-4, IL-10, and IL-13 and showed no difference in median cytokine production in the supernatant *in vitro* [86]. The differing results between these studies could be explained by the fact that the *in vitro* cells lack the presence of other cells of the immune system and thus lack the ability to reflect the complexity of the immune system as a whole. This same study did however show a higher IL-12 and lower IL-4 in cervical secretions of women in preterm labour, reflecting the localised Th1:Th2 dichotomy. Bahar and colleagues did not demonstrate any difference in serum TNF-α or IFN-γ in women with preterm labour compared to term labour or matched controls not in labour [95]. However, those women in the preterm labour group received indomethacin, an anti-inflammatory COX-2 inhibitor, which could have dampened a typical proinflammatory response. Serum taken from women with preterm prelabour rupture of membranes (pPROM) compared to women who delivered at term exhibit a higher concentration of IFN-γ. Levels of IL-4 and IL-5 were undetectable in both groups [96]. In a study of 30 women in preterm labour, mitogen- and antigen-stimulated PBMCs showed a higher production of the proinflammatory cytokines IFN-γ and IL-2, along with an altered Th1:Th2 ratio favouring a Th1 response compared with controls who delivered at term [97]. Taken together, these results suggest that, rather than a decrease in the Th2 response, preterm labour most likely represents an activation of the Th1 response. Thus, future development of therapeutic targets would likely be more effective if directed towards the modulation of the Th1 cytokines.

The Th1:Th2 dichotomy likely represents an oversimplification of the complexity of the cross-talk between the

Th1 and Th2 cytokines. The ratio of Th1:Th2 is likely to be of more physiological importance than the actual concentrations produced. In support of such a notion, women in threatened preterm labour with high serum levels of IL-12 (which induces a Th1 cytokine response) and no change in serum IL-18 (which can induce both Th1 and Th2 response) do not show significant associations with preterm labour. However, women with high IL-12 levels and low IL-18 and thus a high IL-12 : IL-18 ratio increasing the Th1 predominance are associated with a twofold risk of preterm labour when presenting with threatened preterm labour [98].

7.2. Maternal Fetal Interface and Nonimmune Cells. The inflammatory response at the maternal fetal interface more likely reflects the true importance of the Th1:Th2 dichotomy and the aberrant profile in preterm labour. A recent meta-analysis concluded that proinflammatory cytokines at the maternal fetal interface play a role in the events leading to spontaneous preterm labour, while systemic inflammation does not appear to be present in asymptomatic women early on in pregnancy who then go on to deliver preterm [99]. This is consistent with a more local intrauterine inflammatory response syndrome, where no organisms are identified. Understanding the pathophysiology at the maternal interface is essential for developing new therapies for the prevention of inflammation-induced preterm labour, although using such local changes for the prediction is challenging because of lack of access to the maternal fetal interface.

Placentas from women with pPROM and preterm delivery have higher Th1, inducing cytokines [100], and placentas from women following preterm delivery compared with term delivery show a bias towards the Th1 profile with significantly higher levels of IFN-γ and IL-2 as well as the Th1-inducing cytokine IL-12 [100]. Moreover, term placentas exhibit comparatively higher levels of the Th2 cytokines, IL-4, and IL-10, compared with the preterm placentas.

TNF-α is increased in choriodecidual tissues [71] and amniotic fluid [101] in preterm labour. TNF-α is known to stimulate PG production through the TNF receptor 2, leading to uterine contractions likely via activation of NF-κB, but is also likely to contribute to MMP-9 production leading to PROM via activation of its receptor TNF Receptor 1 (TNFR1) [102]. Interestingly, samples of myometrium collected women in preterm labour and samples collected preterm before labour express comparable mRNA levels of TNF-α. However, mRNA levels of the receptors, TNF R1 A and B, are increased in preterm labour and term labour compared with nonlabour controls [103] suggesting a receptor-mediated increase in sensitivity to TNF-α.

Although placental, amnion, and choriodecidual cells secrete proinflammatory cytokines, cytokine levels in tissues from preterm deliveries (with and without intrauterine infection) correlate with the extent of leukocyte infiltration in fetal membranes [75]. In the presence of infection, the primary cellular source of cytokine production in fetal membranes is likely to be infiltrating leukocytes rather than amniocytes or choriodecidual cells. [75].

7.3. Polymorphisms of the Th1 and Th2 Cytokines. Studying genetic polymorphisms of the Th1 and Th2 cytokines could provide a novel screening method for determining women at high risk of preterm labour. Polymorphisms giving rise to functional alterations can also provide information on the importance of the interleukins in preterm labour. There has yet to be any promising genetic polymorphisms identified in the Th1:Th2 cytokines for the prediction of preterm labour, the work conducted warrants consideration (see Table 1).

8. Non-Th1:Th2 Interleukins

8.1. IL-8. Interleukin 8 is a chemokine produced by many immune cells but primarily macrophages and monocytes [116]. Its production is stimulated by LPS, TNF, and IL-1 [117] and, in the context of pregnancy, is thought to attract leukocytes to the gestational tissues and the cervix at the onset of term and preterm labour. IL-8 mRNA expression has been reported to be increased more than 50-fold in preterm labour and more than 1000-fold in preterm labour with evidence of chorioamnionitis in amnion and choriodecidua [118]. A number of studies have also identified increases of IL-8 in the myometrium and cervix with the onset of labour [119, 120]. Placental IL-8 is also higher in preterm deliveries compared with term deliveries [71].

8.2. IL-6. Although IL-6 is produced by Th2 cells, it is a proinflammatory cytokine and a major mediator of host response to inflammation and infection [121]. IL-6 levels are moderately increased in placenta, significantly increased in amnion and choriodecidua in women with preterm delivery compared with term delivery [71]. IL-6 appears to be among the most sensitive and specific indicators of infection-associated preterm labour [122, 123]. The presence of an increase in IL-6 in amniotic fluid and cervicovaginal fluid is an independent risk factor for preterm labour and neonatal morbidity [124] including cerebral palsy [125] and bronchopulmonary dysplasia [126].

9. Therapeutic Modulation of Th1 and Th2 Profile

Various therapeutic strategies have been proposed to prevent preterm labour, with the primary objectives of (1) delaying delivery to increase gestation at delivery and (2) to improve neonatal condition at birth [127]. Currently, many of the strategies adopted for the prevention of preterm labour involve targeting the proposed pathways and events that result in uterine contractions and cervical shortening and dilation rather than targeting immune activation. As described here, an aberrant proinflammatory profile exists in both term and preterm labour, which is associated with neonatal morbidity. The limitation of tocolytics is the inability to counteract the exposure of the fetus to proinflammatory cytokines, which lead to the fetal inflammatory response syndrome. This may in fact worsen neonatal outcome by prolonging the exposure of the fetus to a hostile

Table 1: Cytokine polymorphism associations with preterm labour (PTL).

Gene	Polymorphism	Th1/Th2	Function	Reference
IFN-γ	+874A>T	Th1	Classic Th1 cytokine. Proinflammatory. No clear association between IFN-γ polymorphisms and PTL	[104, 105]
TNF-α	−308G>A	Th1	Regulatory role in PG synthesis elevated at maternal fetal interface controversial link between PTL and TNF-α polymorphisms	Refute association [104, 106, 107] Support association [108, 109]
IL-4	−590	Th2	Classic Th2 cytokine. The IL-4 590 C/C genotype is associated with preterm birth but unclear. IL-4-590 SNP has been associated with both low and high IL-4 expression. Link also exists between IL-4 promoter polymorphisms and preterm birth in multiple pregnancies; however, polymorphism actually associated with increased IL-4	[110, 111]
IL-10	−1082G>A −819C>T −592C>A	Th2	Anti-inflammatory Th2 cytokine inhibits production of cytokines, chemokines, and prostaglandins in LPS stimulated amnion, choriodecidual, and placental explants [112–114]. However, no clear association between IL-10 polymorphisms and PTL or adverse neonatal outcome	[104–106, 115].

environment. There is mounting evidence that periventricular leukomalacia and cerebral palsy are associated with fetal exposure to intra-amniotic inflammation and the development of fetal inflammatory response syndrome [128]. Thus, a strategy for targeting immune activation through the modulation of the Th1:Th2 bias may be beneficial for both the prevention of preterm labour as well as the reduction of neurological insult to the fetus.

9.1. Progesterone. There have been several studies indicating a positive response to progesterone treatment for the prevention of preterm labour in specific patient populations [129–131]. The strongest evidence for improvement in neonatal outcomes comes from the most recent multicentre randomised controlled trial which showed a 45% reduction in preterm labour (<33 weeks) and a 60% reduction in respiratory distress syndrome at <33 weeks using 90 mg of vaginal progesterone in women with a short cervix of 10–20 mm [132]. The mechanism by which progesterone contributes to pregnancy maintenance has traditionally been attributed to maintenance of uterine quiescence by increasing cyclic AMP (cAMP) and a reduction in intracellular calcium thus reducing contractility [133]. Moreover, progesterone appears to inhibit the phosphorylation of myosin, a critical step in the activation of the myometrial contractile machinery required for labour onset [134, 135].

Progesterone also has immunomodulatory effects on the Th1:Th2 bias. Progesterone is able to suppress Th1 differentiation and enhance Th2 differentiation in peripheral blood mononuclear cells *in vitro* [136]. A more potent and orally bioavailable progestogen, dydrogesterone (6-dehydro-9β,10α-progesterone) upregulates IL-4 and downregulates IFN-γ in PHA-stimulated PBMCs more significantly than progesterone *in vitro* [137]. There is also *in vivo* evidence of an anti-inflammatory effect of prolonged administration of vaginal progesterone. In a study of pregnant women receiving either progesterone or placebo from 24 to 34 weeks, peripheral blood leukocytes were collected before and after treatment [138]. mRNAs of the proinflammatory cytokines IL-1β and IL-8 were reduced with progesterone treatment, whereas the anti-inflammatory IL-10 was increased. A multicentre placebo controlled trial (OPPTIMUM, https://www.opptimum.org.uk/: ISRCTN 14568373) powered on neonatal outcome will provide us with evidence of any potential beneficial effect of vaginal progesterone on neonates born preterm.

9.2. NF-κB Inhibitors. Inhibition of NF-κB activation is another attractive strategy to prevent preterm labour as NF-κB activation is central to the activation of labour-associated genes in labour [139]. NF-κB activation also leads to a proinflammatory response in various cytokines including IFN-γ [140], IL-1β [74], TNF-α, and IL-8 [141]. *Ex vivo* studies with the anti-inflammatory sulfasalazine suppress LPS-induced IL-6 and TNF-α production in fetal membranes via inhibition of translocation of p65 to the nucleus [142]. The reported clinical safety profile of sulfasalazine has been variable [143–145], however, if used in pregnancy is often supplemented with folate. The anti-inflammatory characteristics of the cyclopentenone PG, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$) appears to be derived from its ability to inhibit NF-κB activation in human amnion and myometrial cell culture [146]. We have also shown that 15dPGJ$_2$ inhibits activation of NF-κB in human peripheral blood mononuclear cells and reduces the percentage of cells producing the proinflammatory cytokines, IFN-γ and TNF-α, [147]. Work conducted in our laboratory has also shown that 15dPGJ$_2$ is able to delay labour and provide

neuroprotection by reducing pup mortality from 75% to 5% in a murine model of inflammation induced preterm labour [148].

10. Conclusion

There has been extensive interest in the Th1:Th2 dichotomy for the maintenance of successful pregnancy. A trend towards the Th2 cytokine profile and a suppression of the Th1 cytokine profile appears to exist both in the peripheral blood but more significantly at the maternal fetal interface. Activation of the proinflammatory Th1 profile—rather than suppression of the Th2 profile—is apparent in preterm labour and thus should be considered as the logical target for immunomodulating therapies for the prevention of preterm labour and improving neonatal outcome.

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Research Article

Changes in the Th1 : Th2 Cytokine Bias in Pregnancy and the Effects of the Anti-Inflammatory Cyclopentenone Prostaglandin 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2

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Pregnancy is a complex immunological state in which a bias towards T helper 2 (Th2) protects the fetus. Evidence suggests that proinflammatory cytokines increase the risk of poor neonatal outcome, independently of the direct effect of preterm labour. The anti-inflammatory prostaglandin 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15dPGJ$_2$) inhibits nuclear factor Kappa B (NF-κB) in amniocytes and myocytes *in vitro* and is a ligand for the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptor. Here we examine the Th1:Th2 cytokine bias in pregnancy and whether 15dPGJ$_2$ could be used to inhibit the production of the proinflammatory cytokines through inhibition of NF-κB while simultaneously promoting Th2 interleukin 4 (IL-4) synthesis via CRTH2 in T helper cells. Peripheral blood mononuclear cells (PBMCs) from women at 28 weeks, term pre-labour, term labour as well as non-pregnant female controls were cultured with 15dPGJ$_2$ or vehicle control and stimulated with phorbol myristyl acetate (PMA)/ionomycin. The percentage of CD4$^+$ cells producing interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) in response to PMA/ionomycin was significantly reduced in pregnancy. 15dPGJ$_2$ reduced IFN-γ and TNF-α production in stimulated T helper cells, but did not alter IL-4 production in CRTH2^{+ve} cells. 15dPGJ$_2$ also reduced phospho-p65 in stimulated PBMCs. In summary, 15dPGJ$_2$ suppresses the Th1 response of PBMCs during pregnancy and active labour whilst maintaining the Th2 response suggesting a therapeutic benefit in reducing neonatal morbidity in inflammation-induced PTL.

1. Introduction

Preterm labour <34 weeks occurs in about 4% of pregnancies [1]. In 80–85% of cases of spontaneous preterm labour (PTL) <28 weeks, there is evidence of intrauterine infection [2]. Despite increased awareness of the association between inflammation and preterm labour [3], there have been no major advances in the prevention of preterm labour which have been shown to improve long-term neonatal outcomes. During normal term labour, the uterus and cervix become infiltrated with leukocytes and undergo changes in response to local secretion of proinflammatory cytokines. A similar pattern of biochemical events occur during PTL; however, the triggers for this premature activation are still not fully understood. Regardless, the presence of increased proinflammatory cytokines during pregnancy is clearly associated with a poor prognosis for babies born preterm [4].

The immune system can generally be divided into the innate and adaptive immune system. The former is a nonspecific system providing immediate defence against pathogens, while the latter is more targeted, characterised by T and B lymphocytes. Although cross-talk between these lymphocytes exist, B cells and their antibodies mainly give rise to humoral immunity, whereas T cells primarily provide cell mediated immunity [5]. T helper cells (CD4$^+$) form a subset of T cells and can be further subdivided into T helper 1 cells (Th1) and T helper 2 cells (Th2) depending on their pattern of cytokine production. Th1 cells secrete

pro inflammatory cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), whereas the Th2 cells secrete anti-inflammatory cytokines such as interleukin 4 (IL-4), IL-10, and IL-13 [6]. A mutually exclusive interaction exists between the Th1 interleukin, IFN-γ, and the Th2 interleukin, IL-4. IL-4 is the dominant factor for promoting growth and differentiation from the Th0 to the Th2 subtype, and directly inhibits the development of the Th1 cells [7]. IFN-γ indirectly promotes Th1 differentiation by upregulating the IL-12 receptor whilst inhibiting the growth of Th2 cells [8, 9].

Wegmann and colleagues first developed the concept that during pregnancy there is a shift from a T helper 1 (Th1) response to a T helper 2 (Th2) bias during pregnancy that functionally induces maternal tolerance and suppression [10]. Consistent with this notion, administration of the Th1 interleukins IFN-γ [11] and IL-2 [12] leads to fetal loss and preterm labour in the mouse. Similarly, CBA $\times$ DBA/2 mice that have placentas deficient in IL-4 and IL-10 are prone to fetal resorption. Treatment of these mice via intraperitoneal injection of IL-10 protects the fetuses from resorption [13]. Several human studies have shown a Th2 bias in the ratio of circulating T helper cytokine profile in normal pregnancy; and an increase in the Th1 ratio in cases of recurrent miscarriage [14] and in preeclampsia [15]. Peripheral blood lymphocytes taken from women in the first trimester show an increased production of IL-4 and IL-10 and less IFN-γ compared to nonpregnant controls *in vitro* [16]. There is *in vivo* evidence in pregnant women of increased expression of IL-4 messenger ribonucleic acid (mRNA) and lower expression of the IFN-γ mRNA [17].

Not all studies support the requirement of the shift from Th1 to Th2 for successful pregnancy outcome [18, 19]. Despite showing a suppression of IFN-γ and an increase in IL-10 during pregnancy compared to nonpregnant controls, Bates et al. showed no difference in IFN-γ, IL-10, or IL-4 secretion in women who subsequently miscarried compared with those who went on to complete their pregnancy [18]. However, contrary to this study, women with recurrent miscarriage have been shown to have increased IFN-γ and TNF-α levels compared with women that go on to have successful pregnancies [20]. While the mechanism regulating the Th1 : Th2 ratio is yet to be fully elucidated the importance of maternal immune tolerance during pregnancy is unquestionable. Several pregnancy-related proteins are known to promote Th2 bias such as leukemia inhibitory factor [21], progesterone, progesterone-induced blocking factor [22], and estradiol [23]. Prostaglandin D_2 (PGD_2) promotes IL-4, IL-13, IL-5, and IL-10 production in T helper 2 cells *in vitro* via the second PGD_2 receptor; chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) [24]. PGD_2 is produced by the placenta and may play a role in the chemoattraction of Th2 cells via the CRTH2 receptor to the maternal fetal interface to produce a localised Th2 bias [25].

15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 ($15dPGJ_2$), an endogenous prostaglandin (PG), is a product of PGD_2 via a series of dehydration reactions [26]. As well as being a ligand for Peroxisome proliferator-activated receptor gamma (PPARγ), it is also an agonist of the CRTH2 receptor [27]. We have previously demonstrated that this anti-inflammatory PG inhibits nuclear factor Kappa B (NF-κB) in amniocytes and myocytes *in vitro* [28] and delays infection induced preterm labour and increases pup survival in the mouse via NF-κB inhibition [29]. Moreover, recent work has shown that the first trimester of pregnancy is associated with the suppression of available T cell NF-κB and reduced levels of IFN-γ compared to nonpregnant controls [30, 31]. Collectively these data suggest that the Th1 : Th2 ratio is modulated through the regulatory interplay of both Th1 suppression and Th2 promotion.

In this study, we examined the expression of the dominant Th1 interleukins IFN-γ and TNF-α, and the Th2 interleukin IL-4 in T helper cells obtained from nonpregnant women, women in the early and late third trimester, and during labour. We examined the effect of the CRTH2 agonist, $15dPGJ_2$, on these interleukins and on NF-κB activation at different gestational time points to determine its potential role in suppressing the Th1 response via NF-κB whilst simultaneously promoting the Th2 response via CRTH2.

2. Materials and Methods

2.1. Subjects. Pregnant women at 28 weeks and term (prelabour and in labour) and nonpregnant women of child bearing age were included in the study in accordance with ethical approval from the South East London Ethical Committee Ref. 10/H0805/54. Exclusion criteria included women with Asthma and HIV, women not of childbearing age, and women with a fever. After obtaining informed consent, 5 mL of peripheral venous blood was collected in sodium citrate tubes, and processed within 30 mins of collection. Unless otherwise stated, a minimum of 4 subjects were included for each experimental sample group.

2.2. Isolation of Peripheral Blood Mononuclear Cells (PBMCs). Blood was diluted 1 : 1 with phosphate-buffered saline (PBS) and carefully layered onto Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) before centrifuging at 400 $\times$g for 40 mins at room temperature. After centrifugation, the halo containing PBMCs was carefully transferred into a clean centrifuge tube and washed twice with 7 mL of PBS. After centrifugation (400 $\times$g for 10 mins), the cell pellet was resuspended in either PBS or RPMI 1640 (Invitrogen Life Technologies, Grand Island, NY, USA) culture medium.

2.3. CRTH2 Expression Studies. The PBMC pellet was resuspended PBS, and cells were counted with a Neubauer haemocytometer and then resuspended in staining buffer (1% fetal calf serum; 0.09% sodium azide in PBS) to obtain roughly 3.5×10^6 cells per sample. Preparations were incubated in the dark for 1 h at 37°C with 20 μL of CRTH2-phycoerythrin (CRTH2-PE) (Beckman Coulter, High Wycombe, UK) and 3 μL of CD4-Allophycocyanin (CD4-APC) (BD Pharminogen, Oxford, UK) or the relevant isotype controls; Rat Immunoglobulin (Ig)G_{2a}-PE (Beckman Coulter) and Mouse $IgG_{1\kappa}$-APC (BD Pharminogen). After incubation, the PBMC suspension was washed twice in

1 mL of PBS and then resuspended in PBS for analysis. Flow cytometry settings were as follows: forward scatter *E*0 voltage, 1.00 Amp gain Lin, and side scatter of 329 voltage, 1.00 Amp gain Lin. A total of 50,000 cells were counted and gating was on lymphocytes based on the forward and side scatter.

2.4. Intracellular Cytokine Studies (IL-4, IFN-γ, and TNF-α). For intracellular cytokine studies, cells were resuspended in RPMI 1640 (Invitrogen) media supplemented with 10% fetal calf serum, 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA) before being plated in 24-well plates and incubated for 18 h in 5% CO_2/humidified air at 37°C. Following this, 10 μg of brefeldin A (Sigma) was added to each well to immobilize the interleukins in the golgi apparatus. Cells were pretreated with 32 μM of 15dPGJ$_2$ (Cayman Chemicals, Ann Arbor, MI, USA) or dimethyl sulfoxide (DMSO) (Sigma) vehicle control for 2 h in the case of IFN-γ and TNF-α experiments or 1 h for IL-4. Following this 50 ng/mL of phorbol myristate acetate (PMA) and 0.5 μg/mL of ionomycin were added for 4 h in the case of IFN-γ and TNF-α experiments or 5 h for IL-4. Prior to intracellular staining, cell surface staining with either CRTH2 and CD4, or CD4 antibodies alone, was performed as described in Section 2.3. Cells were then fixed with 2% paraformaldehyde (PFA) and incubated at 37°C for 15 mins in the dark. Cells were washed and resuspended in 0.5% saponin and incubated for 30 mins on ice in the dark to permeabilize the cells. After incubation, cells were washed and resuspended in 0.5% saponin for intracellular staining with the relevant antibody; IL-4 PE/Cy7 (BioLegend, San Diego, CA, USA), IFN-γ-(fluorescein isothiocyanate (FITC) or TNF-α-FITC (BD Biosciences, Franklin Lakes, NJ, USA). Appropriate isotype controls were used; PE/Cy7 Rat IgG$_{1\kappa}$ (Biolegend), or FITC Mouse IgG$_{1\kappa}$ (BD Biosciences, Oxford, UK). Cells were incubated in the dark for 1 h at room temperature for IL-4 staining or 20 mins on ice for IFN-γ and TNF-α staining. Finally, cell suspensions were washed with 0.5% saponin and resuspended in PBS for flow cytometry analysis as follows: Forward scatter *E*0 voltage; 1.00 Amp gain Lin; side scatter of 329 Voltage; 1.00 Amp gain Lin.

2.5. Flow Cytometry. Flow cytometry of lymphocytes was carried out using FACSCalibur flow cytometer (BD Biosciences) equipped with FACSCalibur software for analysis. The lymphocyte population was gated on the scatter plot as determined by the characteristic forward scatter (FS) and side scatter (SC) which indicates the cell size and shape. The analysis was of CD4$^+$, CRTH2$^+$, and CRTH2$^+$/CD4$^+$ in the CRTH2 expression studies and IL-4$^+$, IL-4$^+$/CRTH2$^+$, TNF-α^+/CD4$^+$, and IFN-γ^+/CD4$^+$ for the intracellular cytokines. The expression levels of CRTH2, CD4, and intracellular cytokines were evaluated by the percentage of cells expressing the protein of interest or by mean fluorescence intensity (MFI).

2.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting. Following culture and treatments, cells were collected using a 1 mL pipette and incubated on ice in whole cell lysis buffer (Cell Signalling, Beverly, MA, USA) with 5 μL/mL of protease inhibitor (Sigma) lysis buffer for 5 mins and centrifuged for 20 mins at 13,000 rpm at 4°C. The supernatant was stored at −80°C until use. Prior to SDS-PAGE, protein concentrations were determined using the BIORAD quantification assay measuring absorbance at 655 nm. Approximately 15 μg of extracted protein per sample was resolved by SDS-PAGE and subsequently transferred onto polyvinyl difluoride (PVDF) membranes (GE Healthcare) 100 V constant at 4°C. Following transfer, the membrane was then blocked in 5% (wt/vol) milk in tris-buffered saline (TBS) supplemented with 0.1% Tween 20 (TBST) for 1 h. The membrane was then probed with phospho-p65 (Ser 536) (Cell signalling) primary antibody (1 : 1000 in tris-buffered saline) overnight at 4°C followed by the appropriate secondary antibody (1 : 2000 in 1% milk/TBS) for 1 h at room temperature. Chemiluminescence detection was then carried out with ECL Plus (GE Healthcare), and the membranes developed using a high-performance chemiluminescence film (GE Healthcare). Blots were scanned, and densitometry was performed with ImageJ (v1.44p).

2.7. Statistical Analysis. Experimental sample groups consisted of at least 4 biological replicates unless otherwise stated. Statistical analysis was performed with Graph-Pad Prism (v4.0; GraphPad Software, San Diego, CA, USA). Paired Student's *t*-test or repeated measures ANOVA was conducted where appropriate. Samples with $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Gestational Effect on Th1 and Th2 Cytokines. A change in the production of the Th1 and Th2 cytokines in pregnancy has previously been described. In this study, we employed flow cytometry to examine the effect of stimulation by the mitogen PMA/ionomycin on cytokine production at different gestational stages of pregnancy and during labour (see Figure 1). Th1 cytokine profiles of CD4 positive cells were assessed for intracellular IFN-γ and TNF-α (Figures 1(a)–1(d)) and compared to levels of nonpregnant controls. The percentage of peripheral T cells producing IFN-γ in response to stimulation reduced in pregnancy from 10.7% in nonpregnant women to 6.7% at 28 weeks, 5.1% at term ($P < 0.05$), and 5.6% at term in labour ($P < 0.05$). Similarly, the proportion of TNF-α producing cells was reduced, although not significantly, from 20.6% in nonpregnant women to 14.5% at 28 weeks, 15.8% at term and 13.3% at term in labour. Overall levels of Th1 cytokine production (expressed as mean fluorescence intensity), in the CD4$^+$/IFN-γ^+ or CD4$^+$/TNF-α^+ cells remained consistent throughout gestation and unchanged compared to nonpregnant controls. The Th2 cytokine, IL-4, was similarly assessed in CRTH2 positive cells (Figures 1(e) and 1(f)). While PMA/ionomycin stimulation did not increase the percentage of IL-4 expressing cells, the mean fluorescence intensity of

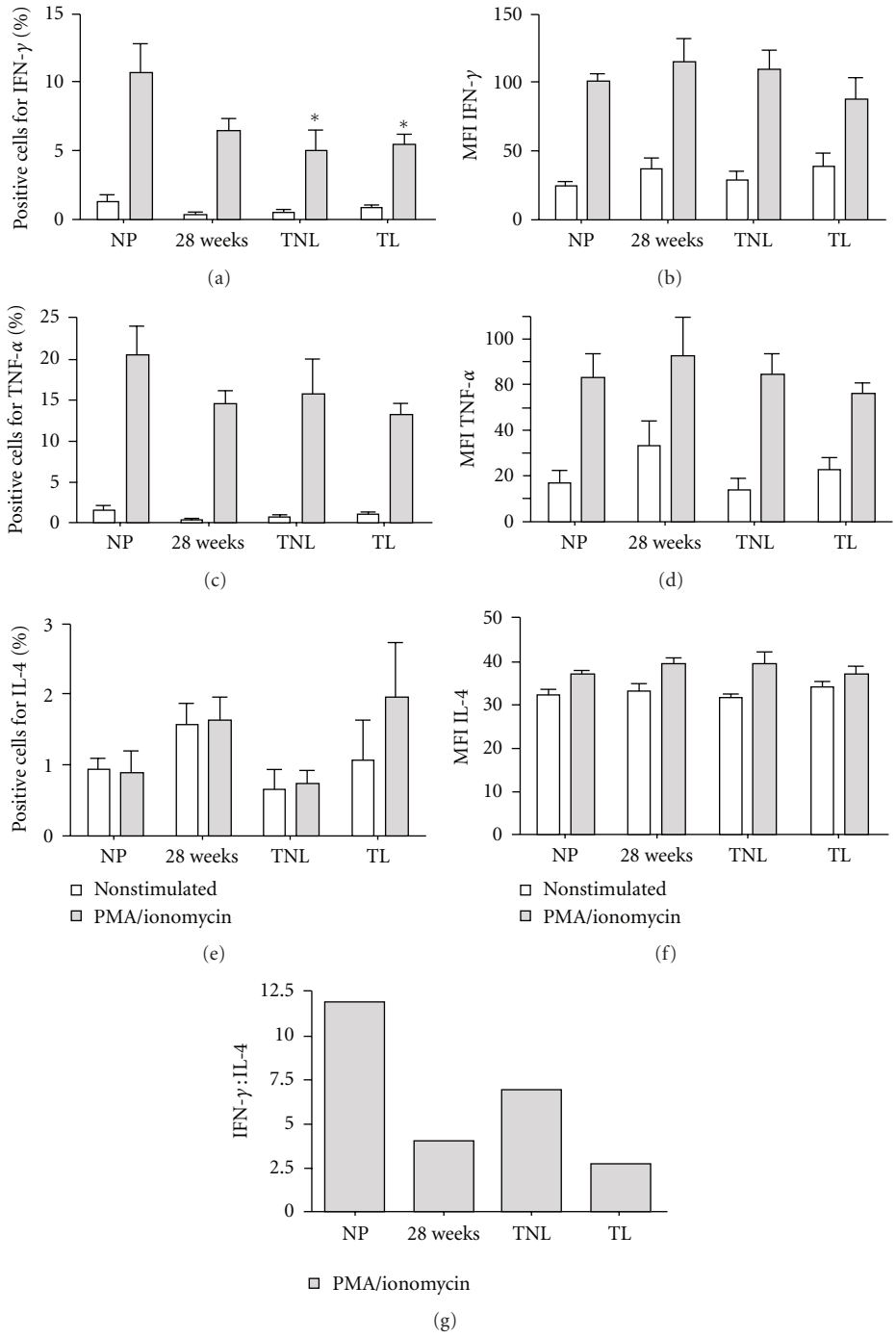

Figure 1: Th1 and Th2 cytokine production in peripheral T cells from nonpregnant and pregnant women. Peripheral blood mononuclear cells were isolated and stimulated with PMA/ionomycin. The percentage of $CD4^+/IFN\text{-}\gamma^+$, $CD4^+/TNF\text{-}\alpha^+$, and $CRTH2^+/IL\text{-}4$ positive cells were detected by flow cytometry in samples derived from nonpregnant (NP), 28-week pregnant, term no labour (TNL), and term labour (TL) samples (a), (c), and (e). A reduction in the percentage of peripheral CD4 positive cells secreting the Th1 cytokines IFN-γ and TNF-α was observed, whereas the percentage of cells secreting the Th2 cytokine, IL-4, remained consistent throughout gestation. Mean fluorescence intensity (MFI) was also assessed as a measure of total cytokine production (b), (d), and (f). PMA/ionomycin stimulation induced both Th1 and Th2 cytokine production in all sample groups compared to NL controls. No differences in gestation-dependent responses were detected. (g) a ratio of IFN-γ : IL-4 revealed a decrease in the Th1 : Th2 ratio during pregnancy, which was increased prior to the onset of labour. For statistical analysis ANOVA with Dunnett's multiple comparison test with NP as a control was used; $^{**}P < 0.01$ and $^{*}P < 0.05$.

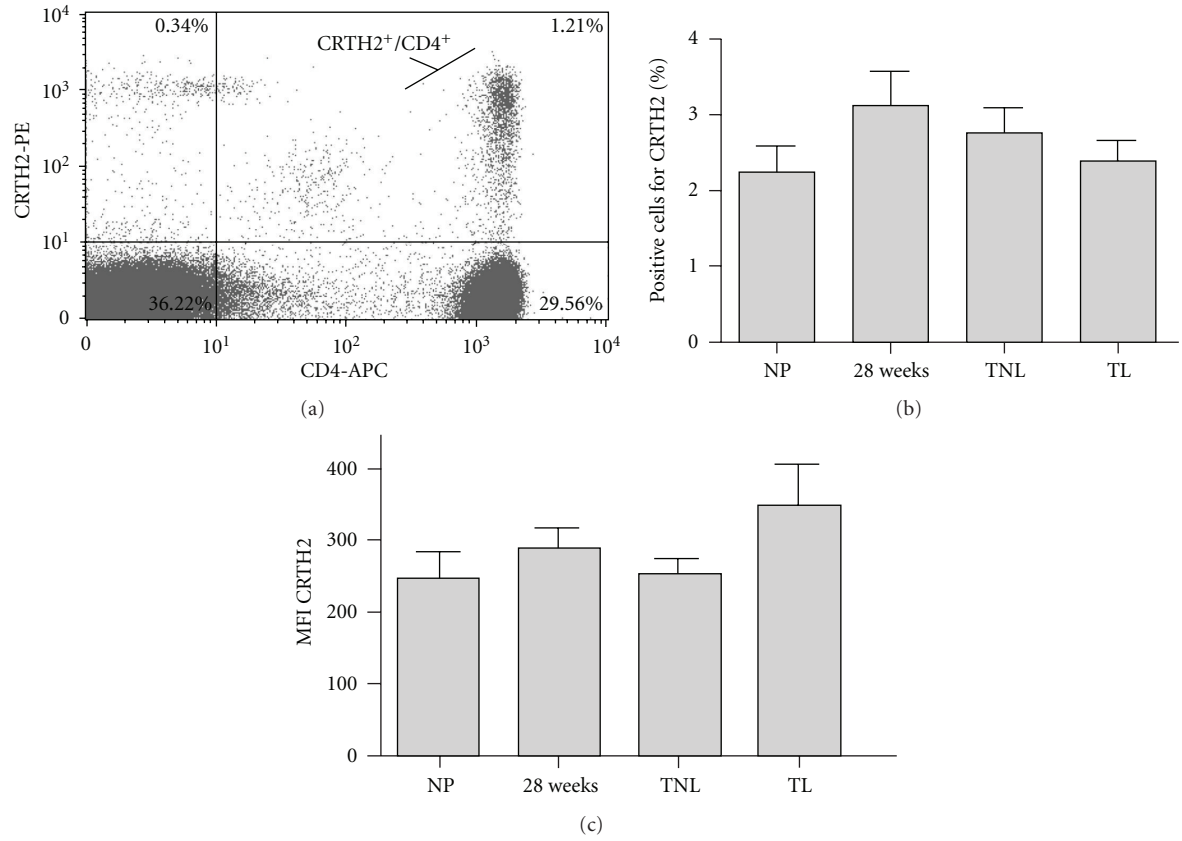

Figure 2: CRTH2 expression in lymphocytes derived from nonpregnant and pregnant subjects. Lymphocytes were gated according to forward scatter and side scatter and T helper cells were identified using CD4 as a cell surface marker ($n = 8$ for each group). A representative cytogram from a woman of 28-week gestation is presented with the right upper quadrant showing $CRTH2^+/CD4^+$ lymphocytes (a). The percentage of CRTH2 positive T helper cells (presented as a percentage of positive CD4 positive cells) increased slightly in the third trimester, although statistical significance was not reached (b). No change in mean fluorescence intensity was detected in either the nonpregnant or pregnant samples (c). For statistical analysis ANOVA with Dunnett's multiple comparison test with NP as a control was used.

IL-4 was significantly increased in samples collected from women at 28 weeks (39.3, $P < 0.01$) and at term (39.4, $P < 0.05$) compared to levels of nonpregnant controls (37.1). Levels of IL-4 in term labouring samples were consistent with nonlabouring samples (37.1). The ratio of the IFN-γ : IL-4 producing cells reduces during pregnancy, due to the suppression of the Th1 rather than the promotion of the Th2 cytokine production (Figure 1(g)).

3.2. CRTH2 Expression. To determine whether the Th2 response in pregnancy is reflected by an increase in the percentage of CRTH2 positive cells, we used CD4 as a marker of T helper cells and calculated the percentage these cells that express CRTH2 (Figure 2(a)). The percentage of CRTH2 positive cells in the CD4 positive population was observed to increase in the second trimester of pregnancy from 2.24% in nonpregnant women to 3.12% at 28 weeks before reducing to 2.75% at term and 2.4% in term labour, although statistical significance was not reached (Figure 2(b)). There was no significant increase in the mean fluorescence intensity of CRTH2 in T helper cells between nonpregnant and pregnant subjects (Figure 2(c)), although a slight increase in CRTH2 production was noted in term labouring samples ($P = 0.15$).

3.3. The Effect of 15dPGJ$_2$ on the Th1 and Th2 Cytokines. 15dPGJ$_2$ is an anti-inflammatory PG and a ligand for CRTH2. We therefore explored the effect of 15dPGJ$_2$ on the Th1 cytokines, IFN-γ and TNF-α. To do this, cells were preincubated with 32 μM of 15dPGJ$_2$ or vehicle control prior to the addition of PMA/ionomycin before intracellular cytokines were detected by flow cytometry (Figures 3(a) and 3(b)). 15dPGJ$_2$ reduced the production of PMA/ionomycin stimulated IFN-γ production (measured as mean fluorescence intensity) in T helper cells from 101.9 to 53 ($P < 0.01$) in nonpregnant controls, 115.7 to 52.4 ($P < 0.01$) in 28 week samples and from 110 to 60.7 in term ($P < 0.01$) samples. There was a nonsignificant decrease observed in term labouring samples also (88.3 to 66.3, Figure 3(c)). Similarly, a significant decrease in PMA/ionomycin-induced TNF-α production following treatment with 15dPGJ$_2$ was detected in all pregnant samples assessed (Figure 3(d)).

The percentage of T helper cells producing IFN-γ was also reduced from 10.7% to 5.13% in nonpregnant women

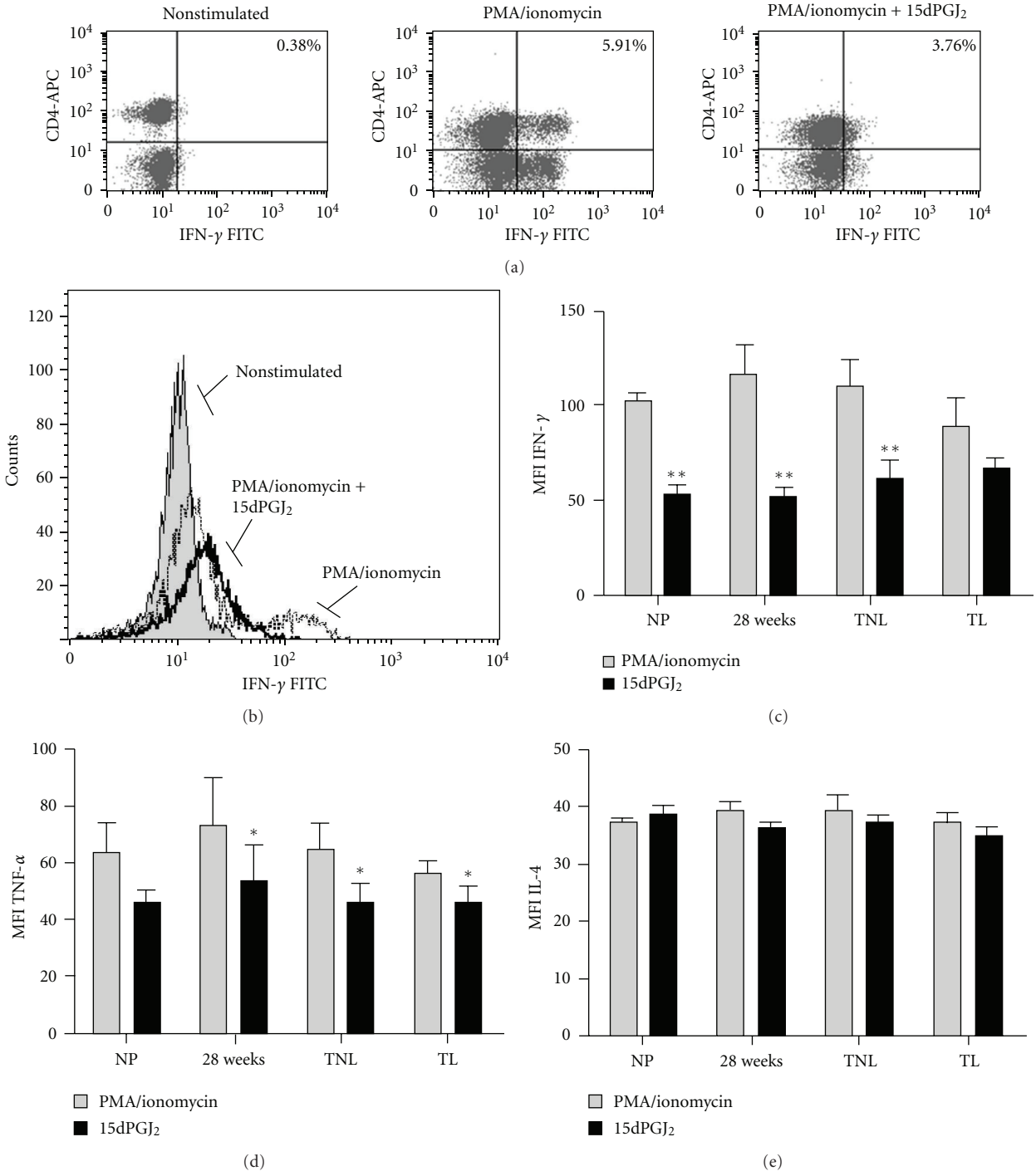

Figure 3: Effect of 15dPGJ2 on Th1 and Th2 cytokines. Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM of 15dPGJ$_2$ and stimulated with PMA/ionomycin before being gated on the lymphocyte population according to forward and side scatter. A representative 28-week patient sample is presented with CD4$^+$/IFN-γ^+ cells shown in the right upper quadrant (a). A representative histogram reveals a clear shift to the right upon stimulation indicative of increased IFN-γ producing cells (b). This effect was attenuated with 15dPGJ$_2$ pre-treatment. PMA/ionomycin induced IFN-γ and TNF-α production was decreased with 15dPGJ$_2$ treatment (c) and (d); however, levels of IL-4 remained unchanged. No change in IL-4 producing cells was detected (e). For statistical analysis a Student's t-test was used to compare means between paired treated and nontreated samples; $^{**}P < 0.01$ and $^{*}P < 0.05$.

($P < 0.01$), 6.6% to 3.4% at 28 weeks ($P < 0.01$), 5.1% to 2.5% at term ($P < 0.05$), and 5.6% to 2.9% ($P < 0.01$). A reduction in the percentage of T cells producing TNF-α was also seen with $15dPGJ_2$ from 20.6% to 16.7% in nonpregnant women, 14.5% to 9.0% at 28 weeks ($P < 0.05$), 15.8% to 12.9% at term ($P < 0.01$), and 13.2% to 8.93% at term in labour ($P = 0.05$) (data not shown).

Considering its anti-inflammatory properties, it was hypothesized that $15dPGJ_2$ would induce an increase in IL-4 production via its action on the CRTH2 receptor. Contrary to what was anticipated, $15dPGJ_2$ had no effect on the production of IL-4 (Figure 3(e)). Consistent with this, the percentage of CRTH2+/IL-4+ lymphocytes remained constant following $15dPGJ_2$ treatment except for samples collected at 28 weeks gestation (1.65% to 1.16%, $P < 0.05$; data not shown).

3.4. $15dPGJ_2$ Inhibits PMA/Ionomycin-Stimulated NF-κB Activation in Peripheral Blood Mononuclear Cells. Peripheral blood mononuclear cells were incubated with vehicle control or 32 μM $15dPGJ_2$ and stimulated with PMA/ionomycin for 10 min before being extracted and assessed for levels of phosphorylated p65, the transcriptionally active subunit of NF-κB. PMA/ionomycin treatment stimulated NF-κB phosphorylation in all samples and at all gestational time points (Figure 4). Pretreatment of cells with $15dPGJ_2$ significantly reduced levels of p-p65 in samples collected from women at 28-week gestation. A clear trend of decreased p-p65 following $15dPGJ_2$ was observed in the remaining samples yet they did not return to basal levels of p-p65.

4. Discussion

The immunological paradox of pregnancy relies on a careful balance of both immune tolerance and immune suppression. Work by Medawar, and later Wegmann and colleagues, has led to the proposal that this balance is determined by a functional immune suppression via a shift from a Th1 response to a Th2 bias during pregnancy [10, 20, 32]. Although not all human and murine studies support the importance or the simplification of the Th1 : Th2 paradigm [18, 19, 33], further understanding of this hypothesis to aid the development of new therapeutic strategies is still required. In this study, we have explored the Th1 cytokines IFN-γ and TNF-α, and the Th2 cytokine IL-4 throughout pregnancy, and examined the potential of the cyclopentenone PG CRTH2 agonist, $15dPGJ_2$, to act as a therapeutic agent to modulate the Th1 : Th2 response.

Consistent with the notion of suppressed Th1 cytokine response throughout gestation [16, 17, 34, 35], our results revealed a reduction in the percentage of IFN-γ and TNF-α-secreting T helper cells during pregnancy in response to PMA/ionomycin stimulation (Figures 1(a) and 1(c)). However, mean production of IFN-γ and TNF-α remained constant within the $CD4^+$/IFN-γ^+ and $CD4^+$/TNF-α^+ cell populations (Figures 1(b) and 1(d)). This indicates that while fewer cells maintain the capacity to initiate a Th1 response to inflammatory stimuli with advancing gestation, a compensatory mechanism facilitates increased IFN-γ and TNF-α production. These results suggest that Th1 response during gestation is likely to be regulated at both an individual cellular level as well as at the level of the cell population.

As normal human labour reflects a proinflammatory state, we anticipated that the percentage of IFN-γ and TNF-α producing T helper cells would increase with the onset of labour. However, no difference between term nonlabouring and term labouring samples was detected. This may be due to the proinflammatory state of labour being a localized response in the uterus and fetal membranes, rather than an extending to peripheral inflammation. This would be consistent with the localised increase in the DNA binding activity of NF-κB in amnion cells postlabour compared to prelabour [36] and in myometrium during labour compared to pre-labour [37] as well as the reported increase in labour of IFN-γ in amnion, choriodecidua, and placenta [38], and TNF-α in the amniotic fluid, myometrium, and cervix [39, 40] rather than in the peripheral blood.

A number of studies have shown that mitogen stimulation can induce IL-4 production in PBMCs of the second and third trimesters of pregnancy *in vitro* [16, 35, 41]. Intrinsic IL-4 production by PBMCs also shows an increase in pregnancy compared with nonpregnant controls [17, 42]. We therefore hypothesized that PMA/ionomycin stimulation of PMBCs would drive IL-4 production during pregnancy, and that this phenomenon would subside at term concurrent with the activation of inflammatory pathways involved in normal labour pathways. To examine any evidence of a shift towards the Th2 profile in peripheral blood during pregnancy, PBMCs were gated for CRTH2 (a marker for Th2 cells) and the level of IL-4 assessed (Figures 1(e) and 1(f)). The percentage of $CRTH2^+$/$IL\text{-}4^+$ secreting cells, as well as mean production levels of IL-4 remained consistent throughout pregnancy and advancing gestation (Figures 1(f) and 1(e)) although a slight increase in the percentage of IL-4 secreting cells was observed in both nonstimulated and stimulated 28-week samples. Between 0.4–6.5% of peripheral blood $CD4^+$ cells of nonpregnant women express CRTH2 and secrete IL-4, but not IFN-γ, in response to PMA and ionomycin and are thus typical of Th2 cells [43]. Consistent with this, we found that between 2–4% of $CD4^+$ PMBCs express CRTH2 (Figure 2(a)). This percentage was maintained throughout gestation with a minor, statistically nonsignificant, increase observed at 28 weeks (Figure 2(b)). Tsuda et al. have previously reported an increase in CRTH2 expression in $CD4^+$ cells between nonpregnant (2.08%) and 7-week gestation (3.43%, $P = 0.02$) [44], but it is possible that this effect is diminished with advancing gestation. Mean levels of CRTH2 expressed in $CD4^+$ cells were also unchanged through gestation and labour (Figure 2(c)). Taken together with the fact that we were unable to detect any difference in IL-4 secretion levels in these cells upon mitogen stimulation, our results collectively suggest that the Th2 response in PMBCs is maximised during pregnancy and cannot be further stimulated. This would imply that any beneficial effects of therapeutic agents intended to push the Th2 : Th1 ratio towards Th2 would need to do so through inhibition of Th1, rather than upregulation of Th2. Although

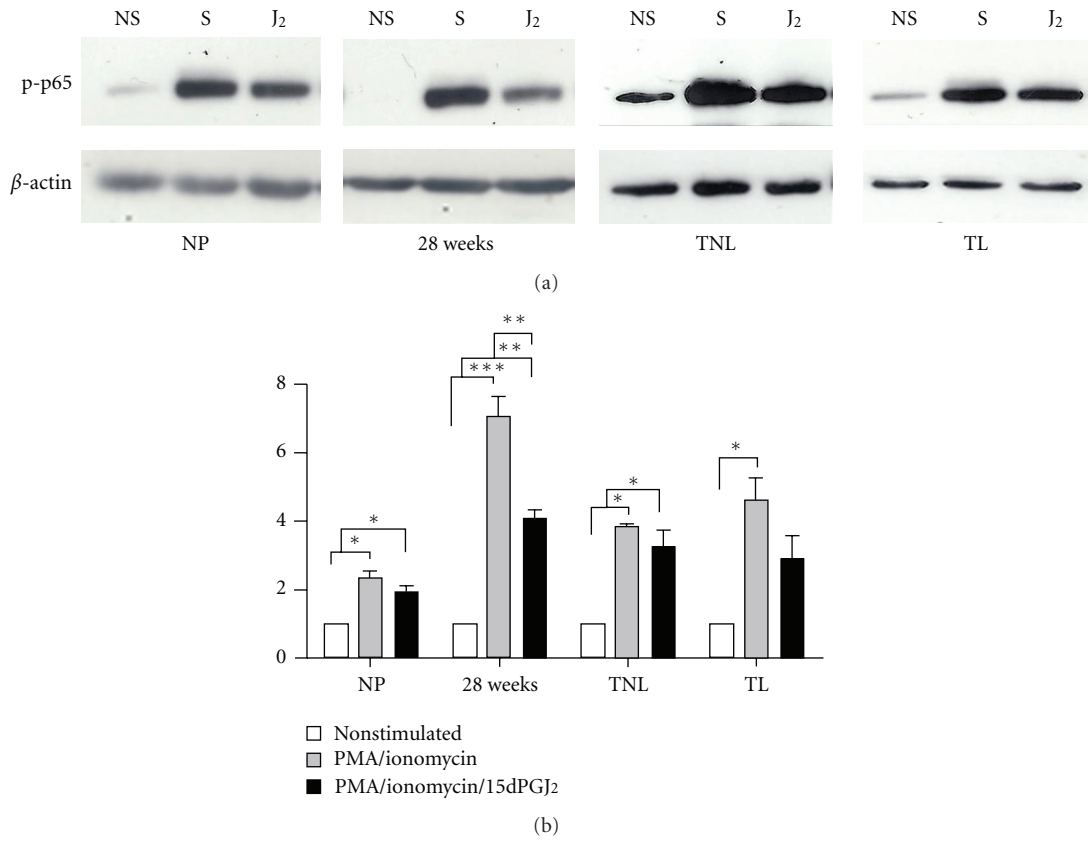

Figure 4: The effect of 15dPGJ2 on NF-κB p65 phosphorylation in peripheral blood mononuclear cells. PBMCs were treated as previously described before being extracted and levels of phosphorylated p65 (p-p65) examined using immunoblotting. Representative immunoblots are shown for each gestational time point (a). Immunoblots were reprobed for β-actin as an internal loading control. Densitometric analysis of the immunoblots was conducted revealing a significant increase in p-p65 levels upon stimulation with PMA/ionomycin in all samples (b). A significant decrease in PMA/ionomycin stimulated p-p65 was observed following $15dPGJ_2$ treatment in 28-week samples. The capacity of $15dPGJ_2$ to inhibit PMA/ionomycin-induced p-p65 was lost in those samples collected at term. NS: nonstimulated, S: PMA/ionomycin stimulated, and J_2: $15dPGJ_2$ pretreatment. Effect of treatment was examined for statistical significance at each gestational timepoint using ANOVA with Bonferroni's multiple comparison test; $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$.

the percentage of CRTH2 expressing $CD4^+$ cells is low in the peripheral blood, we are unable to rule out any modest yet biologically significant changes in IL-4 production from these cells at a tissue-specific level.

To further investigate the potential of Th2 promotion versus Th1 suppression, we examined the response of PBMCs to $15dPGJ_2$, an endogenous product of Prostaglandin D_2 (PGD_2) (and potential therapeutic agent) that has been shown to promote Th2 bias through IL-4 production in T helper 2 cells *in vitro* via CRTH2 [24]. PGD_2 is readily converted to $15dPGJ_2$ by a series of dehydration reactions in the presence of albumin and is found in the amniotic fluid [45], placenta [46], and urine [47]. $15dPGJ_2$ pretreatment significantly inhibited the PMA-stimulated production of both Th1 cytokines: IFN-γ and TNF-α, in nonpregnant and all pregnant samples (Figures 3(a)–3(d)). However, no effect on the production of the Th2 cytokine, IL-4, could be detected in response to $15dPGJ_2$ treatment. This may be due to an overriding effect of a non-CRTH2-mediated mechanism such as the inhibition of other signalling proteins involved the transcriptional regulation of IL-4 expression. Evidence for this has been previously reported in a PMA/Ionomycin-activated T-cell line, where $15dPGJ_2$ inhibited IL-4 production in a dose-dependent manner through the downregulation of nuclear factor of activated T-cells (NF-AT) activation [48].

The anti-inflammatory properties of $15dPGJ_2$ are thought to be at least partly via the inhibition of the transcription factor NF-κB [49]. NF-κB is known to play a key regulatory role in controlling the Th1 immune response by modulating the expression of Th1 cytokines [31]. NF-κB activity in PBMCs during pregnancy is reduced compared to nonpregnant controls [50], and it has therefore been suggested that this is responsible for suppressed Th1 response in pregnancy [30]. To determine whether the observed inhibitory effects of $15dPGJ_2$ on Th1 cytokine production may be via NF-κB, treated cells were extracted, and levels of the transcriptionally active NF-κB subunit p-p65 were examined by immunoblotting. Our results indicate that $15dPGJ_2$ significantly inhibits p-p65 in PMA/ionomycin stimulated T helper at 28 weeks of gestation. A nonsignificant reduction was also observed in nonpregnant controls and

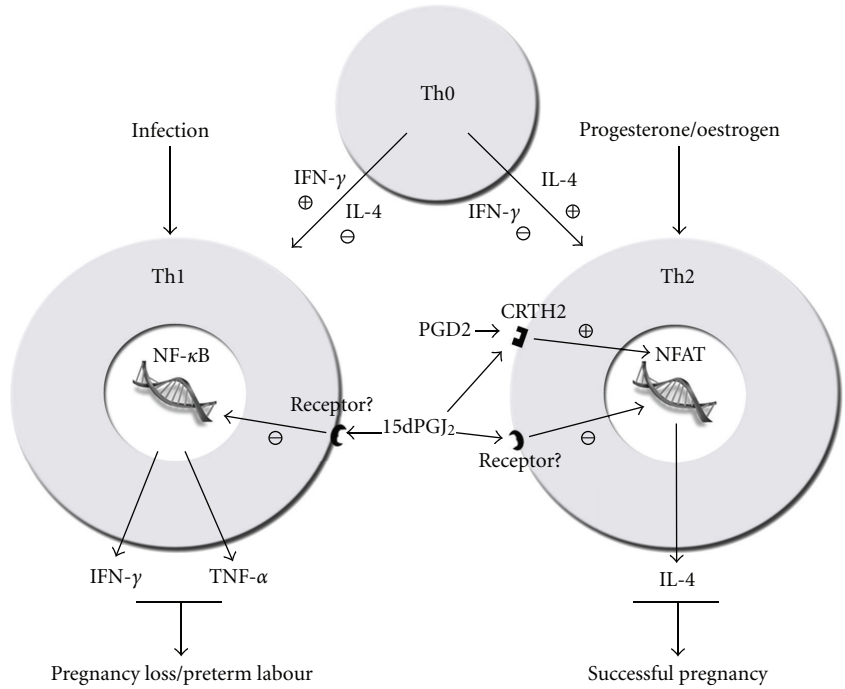

Figure 5: Schematic proposal of the Th1 and Th2 balance and the role of CRTH2 and NF-κB. T-helper cell precursors are directed toward committed immunophenotype by the typical Th1 and Th2 interleukins, IFN-γ and IL-4, respectively. Infection or propregnancy hormones such as progesterone can further modulate the Th1/Th2 bias. Based upon our findings and those of others, we propose that $15dPGJ_2$ maintains a Th2 bias principally through the suppression Th1 interleukins through the inhibition of NF-κB.

samples acquired at term. This data provides evidence that the Th1 inhibitory properties of $15dPGJ_2$ are likely regulated through NF-κB.

$15dPGJ_2$ is a cyclopentenone PG which possesses an α,β-unsaturated carbonyl group in the cyclopentenone ring [51]. This group can form Michael adducts with I Kappa B kinase (IKK) [52], and the p65 [53] and p50 subunits leading to modification of these proteins, thus inhibition of NF-κB activity [51]. We have shown that in amnion and myometrial cells, inhibition of NF-κB by $15dPGJ_2$ is not via PPAR-γ [28]. However, $15dPGJ_2$-mediated suppression of Th1 interleukin expression in lymphocytes via PPAR-γ receptor inhibition or activator protein 1 (AP-1) has not been ruled out in this study.

While $15dPGJ_2$ appears to have no effect on the peripheral Th2 response, the demonstrated inhibition of the Th1 response represents a collective shift toward a Th2 bias, or in other words, an increase in the Th2:Th1 ratio. This has important ramifications for the future design of therapeutic strategies to prevent preterm labour and pregnancy loss (Figure 5). The percentage of CRTH2 positive decidual lymphocytes is far higher, accounting for 10.5% of T lymphocytes and 18% of CD4+ lymphocytes [44]. Exploring the effect of $15dPGJ_2$ on purified decidual T cells may give rise both Th1 suppression and Th2 cytokine promotion.

Immunomodulating therapies such as progesterone (for women at high risk of preterm labour) and intravenous Immunoglobulins (for women with recurrent miscarriage) are currently being used in a clinical context. Progesterone suppresses IFN-γ production in peripheral blood mononuclear blood cells cultured with trophoblasts [54] and dydrogesterone suppresses both IFN-γ and TNF-α whilst increasing IL-4 in phytohaemagglutinin (PHA) stimulated PBMCs leading to a substantial Th1 to Th2 shift [55]. Immunoglobulins are capable of altering the Th1 : Th2 balance leading to a reduction in the $CD4^+IFN\text{-}\gamma^+/CD4^+IL\text{-}4^+$ lymphocyte ratio [56]. The endogenous protein, progesterone-induced blocking factor (PIBF), is capable of increasing the production of IL-4 and IL-10 in PBMC, but has no effect on the Th1 cytokines IFN-γ and TNF-α [57].

$15dPGJ_2$ may show more promising immunomodulating effects for the prevention of preterm labour exploiting its ability to both suppress the Th1 cytokines with the added benefit of inhibiting NF-κB activity. Preterm labour, particularly in the presence of chorioamnionitis, is associated with a shift towards the Th1 cytokine response [58]. PBMCs of women in preterm labour when stimulated with PMA produce significantly higher IFN-γ compared to women who go on to deliver at term [59]. The presence of IFN-γ in cervicovaginal fluid in the late second and early third trimesters has also been shown to be a risk factor for preterm labour in asymptomatic women [60]. Similarly, TNF-α is thought to be key cytokine involved in the initial trigger of biochemical events leading to infection-mediated preterm birth, for example, by causing prostaglandin E_2 (PGE_2) synthesis by intrauterine tissues which drive pathways leading to uterine contractility and cervical ripening [61–63]. While global changes in the PBMCs likely reflect important systemic inflammatory events, their sub-components (e.g., T cells, B cells, and monocytes) may play functionally independent

roles at the maternal fetal interface. Future work using an *in vivo* model examining effects of $15dPGJ_2$ and CRTH2 agonists on Th1 and Th2 interleukins at local sites (e.g., decidua and myometrium) would allow us to extrapolate how immune cells influence nonimmune cell function.

Prophylactic administration of $15dPGJ_2$ to women at high risk of preterm birth may lead to the inability of infection to activate NF-κB and thus preventing transcription of labour associated genes, and the inability to alter the Th1 : Th2 bias to favour Th1, thus preventing the pro inflammatory detrimental environment of the fetus. Consideration for the route of administration should be supported by further studies of the potential local effect of $15dPGJ_2$ on interleukin production from the cervix, myometrium, and fetal membranes. The anti-inflammatory effect on NF-κB and the Th1 interleukins may not be replicated in the cervix, for example, which would serve as the least invasive route of administration of $15dPGJ_2$ in the form of a pessary. Absorption at the local level is also unlikely to provide systemic levels high enough to effect peripheral cytokine production, likewise, the effect of peripheral administration on the local maternal fetal interface is not guaranteed to result in a physiological effect. Nevertheless, this study shows promising potential for the future application of $15dPGJ_2$ as an immunomodulating therapy for infection-mediated preterm labour, and thus further studies examining its effect on gestational tissues should be pursued.

5. Conclusion

This study presents evidence that the Th1 : Th2 balance is changed in pregnancy through Th1 suppression rather than Th2 promotion. We have also demonstrated the potential anti-inflammatory effects of $15dPGJ_2$ through the suppression of NF-κB and the Th1 proinflammatory cytokines IFN-γ and TNF-α. These properties may be of therapeutic benefit for the prevention of infection-mediated preterm labour and the reduction of inflammation induced neonatal morbidity, where an aberrant profile favouring the Th1 response is seen. Thus, future work in this area may be best directed toward designing therapeutic strategies aimed to manipulate the Th1 response during pregnancy as a rational strategy for the prevention of preterm labour and pregnancy loss/miscarriage.

Conflict of Interests

The authors report that no conflict of interests exist.

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Research Article

NFκB and AP-1 Drive Human Myometrial IL8 Expression

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The uterine expression of the chemokine IL8 increases dramatically with the onset of labour both at term and preterm. The IL8 promoter contains binding sites for the transcription factors nuclear factor-kappa B (NFκB), activator protein-1 (AP-1), and CCAAT/enhancer-binding protein (CEBP). In this study we investigated the roles of these transcription factors in IL1B regulation of the IL8 gene in human myometrium. Using chromatin immune precipitation (ChIP) assay, we showed that each of NFκB, CEBP, and AP-1 binds to the IL8 promoter upon IL1B stimulation. To examine the relative importance of each site in IL8 gene expression, site-directed mutagenesis of each of these sites was performed. We found that the NFκB site was essential for basal and IL1B-stimulated gene expression. Mutation of the AP-1 site reduced both basal and IL1B-stimulated expression but to a lesser extent. Mutation of the CEBP site had no effect upon basal expression but eliminated the IL1B response. Small interfering RNA (siRNA) silencing of NFκB abolished the IL8 response to IL1B significantly; siRNA against AP-1 reduced it to a lesser extent whilst knockdown of CEBP enhanced the response. Our data confirms a central and essential role for NFκB in regulation of IL8 in human myometrium.

1. Introduction

The onset of labour is associated with changes in gene expression in the myometrium consistent with activation of inflammatory mediators and leukocyte chemotaxis [1, 2]. The uterine expression of the chemokine IL8 increases dramatically with the onset of term and preterm labour [3, 4] and is thought to promote cervical remodelling [5, 6] and myometrial contractility by promoting neutrophil infiltration and activation [4, 7]. Myometrial IL8 expression is increased by stretch and the inflammatory cytokines in a MAPK- and NFκB-dependent manner [8, 9]. Consistent with these observations, IL8 expression has been shown to be critically dependent on a region in its promoter, spanning nucleotides −1 to −133, which contains binding sites for the transcription factors AP-1, CEBP, and NFκB [10–13]. The binding sites are in close proximity to each other and to the coding region of the gene, forming a transcriptional enhanceosome (Figure 1(a)). Many researchers have investigated the role and interaction of these transcription factors in regulating the IL8 gene and have shown different mechanisms of regulation depending on the cell type [13–21]. Studies in reproductive tissues show that NFκB is important in IL1B-driven IL8 expression in amnion and myometrium [14], AP-1 is involved in the stretch-induced expression of IL8, OXTR, and COX2 in human myometrial and amnion cells [9, 22, 23], and CEBP has been shown to bind to two different regions of the IL8 promoter and interact with NFκB to regulate the IL8 expression [24].

The marked inflammatory infiltration of the myometrium and cervix associated with human labour is driven by increased expression of IL8 and other chemokines. The transcription factors, NFκB, CEBP, and AP-1, have all been implicated in IL8 expression in various tissues/cell types, but which actually regulate myometrial IL8 expression remains unclear. In this study, we have used IL1B stimulation to mimic labour in order to define the relative importance of NFκB, CEBP, and AP-1 in myometrial IL8 expression.

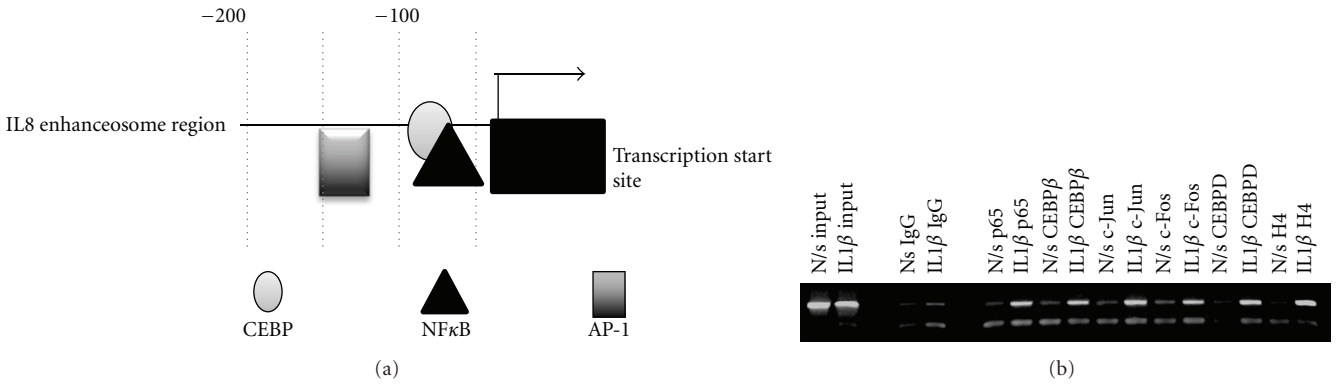

Figure 1: (a) Schematic of the IL8 promoter enhanceosome region. (b) ChIP analysis demonstrates the *in vivo* binding of NFκBp65, CEBPβ and d, c-Jun, c-Fos, and H4 to the transcriptional enhanceosome of the IL8 promoter. ChIP assay using antibodies against NFκBp65 and CEBPβ and -d, c-Jun, and c-Fos was performed in nonstimulated (N/s) and IL1B stimulated conditions. The immunoprecipitates were subjected to PCR analysis using primer pairs spanning the IL8 promoter transcriptional enhanceosome. The first two lanes are "input" lanes where no immunoprecipitation was performed prior to PCR. The second two lanes contained IgG antibody for immunoprecipitation (negative controls).

Table 1: Primers used in site-directed mutagenesis.

Construct	Forward primer sequence	Reverse primer sequence
IL8 mAP-1	5′-GATCTGAAGTGTGATATCTCAGGTTTGCCCTGA-GGG-3′	5′-CCCTCAGGGCAAACCTGAGATATCACACTTCAG-ATC-3′
IL8 mκB	5′-GGGCCATCAGTTGCAAATCGTTAACTTTCCTCT-GAC-3′	5′-GTCAGAGGAAAGTTAACGATTTGCAACTGATGG-CCC-3′
IL8 mCEBP	5′-GGGCCATCAGCTACGAGTCGTGGAATTTCCTCT-GAC-3′	5′-GTCAGAGGAAATTCCACGACTCGTAGCTGATGG-CCC-3′

2. Materials and Methods

2.1. Myometrial Cell Culture. Myometrial cells were extracted from biopsies taken at the time of elective caesarean section at term with the Ethics Committee approval and patient consent. None of the patients was in labour or had received uterotonics or tocolytics. Tissue was minced and digested for 45 min in DMEM with 1 mg/mL collagenase type IA and IX (Sigma-Aldrich Corp., St. Louis, MO, USA). Cells were centrifuged at 400 ×g for 10 min and grown in DMEM with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin (37°C and 5% CO_2). Cells were serum starved for 16 h before treatment with 1 ng/mL IL1B (R&D Systems, Inc., Minneapolis, MN, USA). Cells were used at passage three for all experiments.

2.2. Western Blotting. To obtain whole cell lysates, cells were lysed for 20 min on ice in radioimmunoprecipitation assay buffer (1% NP-40, 1% Triton, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris, PH 8.0, and 2 mM NaF). Protein concentrations were determined using detergent-compatible protein assay reagents (Bio-Rad Laboratories). Protein samples (50 μg) were denatured by boiling for 5 min and run on a 10% SDS-PAGE for 60 min at 140 V, followed by a transfer to a Hybond ECL nitrocellulose membrane (Amersham Biosciensces). The membrane was blocked in 5% milk protein solution over night or for 1 hour, washed and hybridized with the primary antibody for 1 hour at room temperature in a fresh blocking buffer (1 × PBS, 1% milk protein and 0.1% Tween-20) containing antibodies for RELA and CEBPβ (Santa Cruz Biochemicals, SC-8008 and SC-7962, resp.) or beta actin (Abcam, Ab6276). This process was repeated with the secondary antibody. The antibodies were used at 1 : 1000 dilution. Immunoreactivity was then visualised using a chemiluminescent substrate for HRP (ECL plus; Amersham Biosciensces).

2.3. Site-Directed Mutagenesis. The promoter region of the IL8 gene (−135/+46 bp) was amplified by polymerase chain reaction, and the fragment was ligated into the luciferase reporter plasmid pGL2-Basic (Promega, Southampton, UK) to give the wild-type construct. The mutation of individual base pairs in plasmid DNA was achieved using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene, UK) according to manufacturer's instructions. The mutagenic oligonucleotide primers were designed individually and are shown in Table 1.

2.4. Transient Transfection. Myocytes were grown in 24-well plates to 80% confluence. Transient transfections were performed using FuGene 6 transfection reagent (Roche, Indianapolis, IN, USA). Cytomegalovirus-*Renilla* vector (1/10th of reporter) was used to control for transfection efficiency and cell number. Luciferase reporter vectors containing either the wild-type IL8 promoter or the mNFκB (mutation of NFκB) or the mCEBP (mutation of CEBP) or the mAP-1

(mutation of AP-1) were transfected at 0.2 μg /well. Cells were cultured for a total of 48 h (including 24 h of IL1B stimulation for half the experiment) followed by harvesting and analysis with a dual firefly/*Renilla* luciferase assay (Luclite, Packard Bell, and Coelenterazine CN Biosciences). Transfections were performed in triplicates. Luciferase/*Renilla* activity was measured 48 hours after transfection.

2.5. Chromatin Immunoprecipitation (ChIP). DNA-protein complexes were crosslinked in situ with 1% formaldehyde. The cells were lysed, and chromatin was sheared into 200 to 1000 bp fragments by sonication. Antibodies recognizing the C-terminal of RELA, CEBPβ (Santa Cruz Biochemicals, SC-8008 and SC-7962 resp.), and acetyl-histone H4 (Upstate cell signalling solutions, 06-866) were used (1 : 1000 dilution) for immunoprecipitation, and the chromatin fragments containing the crosslinked protein were purified by immunoabsorption and elution from protein A/G beads. The crosslinks were reversed, and the DNA was purified using a QIAquick nucleotide removal kit (QIAGEN Inc., Valencia, CA, USA). The DNA region crosslinked to the protein was determined by PCR analysis. PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems Ltd.). Pre-PCR cycle was 10 min at 95°C followed by 35 cycles of 95°C for 1 min, 56–60°C for 1 min, and 72°C for 1 min followed by final extension at 72°C for 10 min. The primers are shown in Table 2.

2.6. siRNA Transfection. ON-TARGETplus SMART pool human NFκBp65, CEBPβ, CEBPδ, c-Fos, and c-Jun siRNAs (Dharmacon) were used. SiGLO (Dharmacon) was used as a positive control, giving a high transfection efficiency of approximately 90%, and ON-TARGETplus nontargeting pool (Dharmacon) was used as a negative control. The siRNAs were transfected using DharmaFECT 2 (Dharmacon) transfection reagent at a final concentration of 100 nM according to manufacturer's instructions. Briefly, cells were grown to 80% confluence. Two separate tubes were used for siRNA preparation. Tube one contained the siRNA diluted in phenol-red-free DMEM, and tube two contained the transfection reagent diluted in phenol-red-free DMEM. Each tube was incubated at room temperature for 5 min before mixing the contents of the two tubes. The mixture was incubated at room temperature for an additional 20 min. Prewarmed DMEM containing 10% FBS and 2 mM L-glutamine, but no antibiotics were then added to the mixture. The culture media was removed from the cells, and the cells were fed with the siRNA mixture. The media was changed to DMEM containing 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin 24 hours after the transfection. The siRNA transfections were performed at different time courses (12, 24, 48, and 72 h and 5, 6, and 7 days). The changes were of the same pattern at all time points, but maximal response was observed at 5 days. The cell viability was monitored closely before harvesting the cells for appropriate analysis.

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). At the end of incubation assays, 1 mL of medium was collected and immediately frozen at −80°C for analysis by IL8 ELISA kit (Biosource, KHC0081). The IL8 ELISA had a sensitivity of 5 pg/mL; the inter- and intra assay variations were 7.8% and 5.3%, respectively. ELISA data were normalised to cellular protein. Protein concentrations were determined using detergent-compatible protein assay reagents (Bio-Rad Laboratories).

Table 2: IL8 ChIP primer covering bps −177 to +22.

Forward primer sequence	Reverse primer sequence
5′-GAAAACTTTCGT-CATACTCCG-3′	3′-GAAAGTTTGTGC-CTTATGGAG-5′

2.8. Statistical Analysis. Data are expressed as mean ± SEM. Statistical analysis was performed by ANOVA with the level of significance defined as $P < 0.05$.

3. Results

3.1. IL1B Stimulates Binding of NFκB, CEBP, and AP-1 to the IL8 Promoter. ChIP studies showed that IL1B induces the binding of NFκBp65, CEBP and AP-1 to the endogenous IL8 promoter. IL1B stimulated the recruitment of NFκBp65, CEBPβ, and -δ, cFos, and cJun to their respective *cis*-elements in the transcriptional enhanceosome at 6 hrs (Figure 1(b)). This was associated with concomitant acetylation of H4 at that region of the promoter.

3.2. Site-Directed Mutagenesis Reveals an Essential Role for NFκB in the Regulation of IL8 in Human Myometrium. A sequence extending from −135 to +46 which contains the transcription enhanceosome was used as a wild-type control. Site-directed mutagenesis was performed to make three further constructs with mutations for each of the NFκB, CEBP, or AP-1 sites individually (Figure 2). Basal luciferase activity following transient transfection of the wild-type construct was significantly increased by 2-fold upon IL1B stimulation. In primary human myometrial cells, mutation of the NFκB site reduced the unstimulated reporter activity to 10% of that of the wild type and obliterated the response to IL1B. Mutation of the CEBP site had no effect on basal promoter activity but did abolish the response to IL1B. Mutation of the AP-1 site decreased basal IL8 promoter activity although a response to IL1B stimulation was retained (Figure 2).

3.3. Small Interfering RNA (siRNA) Knockdown of NFκB, CEBP, or AP-1 Demonstrates a Central Role for NFκB in the Expression of IL8. Knockdown mediated by siRNA against NFκBp65, CEBPβ, c-Jun, and c-Fos was performed with protein expression studies showing highly-specific and better than 95% gene silencing (Figure 3(a)). The effect of siRNA knockdowns against NFκBp65, CEBPβ, c-Jun, and c-Fos was measured on IL8 protein production and its response to IL1B stimulation (for 24 h) using ELISA.

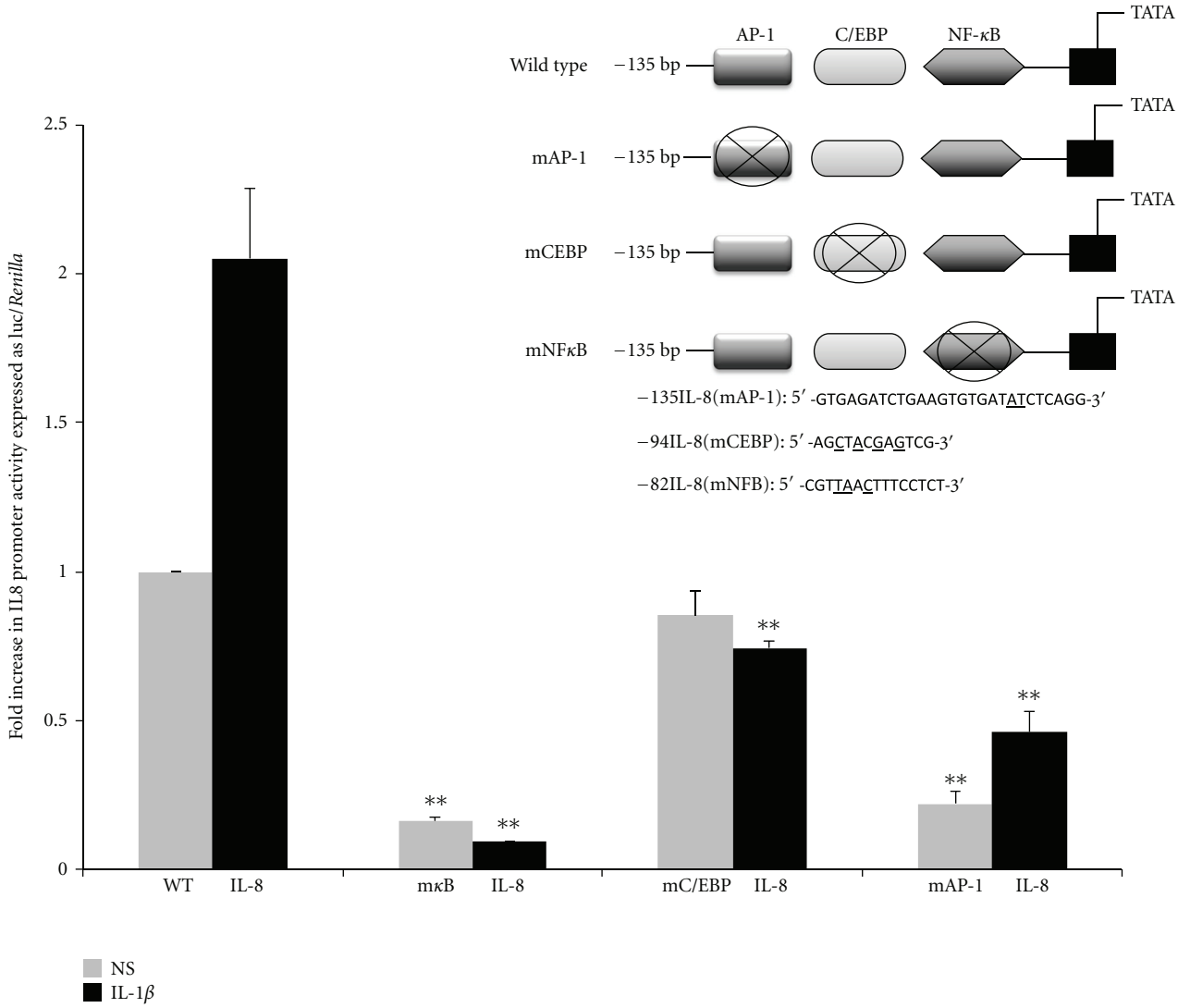

Figure 2: Representation of the IL8 promoter region and the mutations used in this study, as indicated by the underlined nucleotides. mAP-1: mutation of AP-1. mNFκB: mutation of NFκB and mCEBP: mutation of CEBP binding site. Functional effect of site-directed mutagenesis of transcription factor binding sites in the IL8 promoter in primary human myometrial cells. Data are presented as mean ± SEM ($n = 4$). An Anova test was performed with a Dunnett's multiple comparison post-test. **indicates a significant difference of P less than 0.01 compared to WT promoter.

Silencing of each individual transcription factor had no effect on basal IL8 protein release into the culture medium, measured by ELISA. Knock-down NFκBp65 abolished the response to IL1B significantly. Knockdown of CEBPβ increased the response to IL1B by 1.5-fold whilst c-Jun and c-Fos knockdown reduced the response by half (Figure 3(b)). Since CEBPδ may also bind to the IL8 promoter (see Figure 1), we repeated the experiment using siRNA knockdown of CEBPδ which also increased the response to IL1B by 1.5-fold as with knockdown of CEBPβ (Figure 3(c)).

4. Discussion

NFκB, CEBP, and AP-1 are important transcription factor families that are involved in immune and inflammatory functions as well as in cell growth and differentiation. NFκB, CEBP, and AP-1 are each expressed in human myometrium, and their expression is increased in response to the proinflammatory cytokine, IL1B. Labour-associated genes such as PGHS-2, OTR, IL8, IL6, and TNF-α have been shown to be regulated by either one or a combination of theses transcription factors [14, 16, 20, 21, 24–29].

Targeting activators of inflammation has been proposed as a strategy for prevention or delay of preterm birth and to improve outcome in preterm neonates. Condon et al. have demonstrated a central role for NFκB in the onset of term labour in the mouse, and that inhibition of NFκB can delay parturition [30]. Drugs that act through inhibition of NFκB are available. For example, sulfasalazine is a synthetic anti-inflammatory drug comprising 5-aminosalicylic acid (5-ASA), linked to sulfapyridine, which is routinely used for the

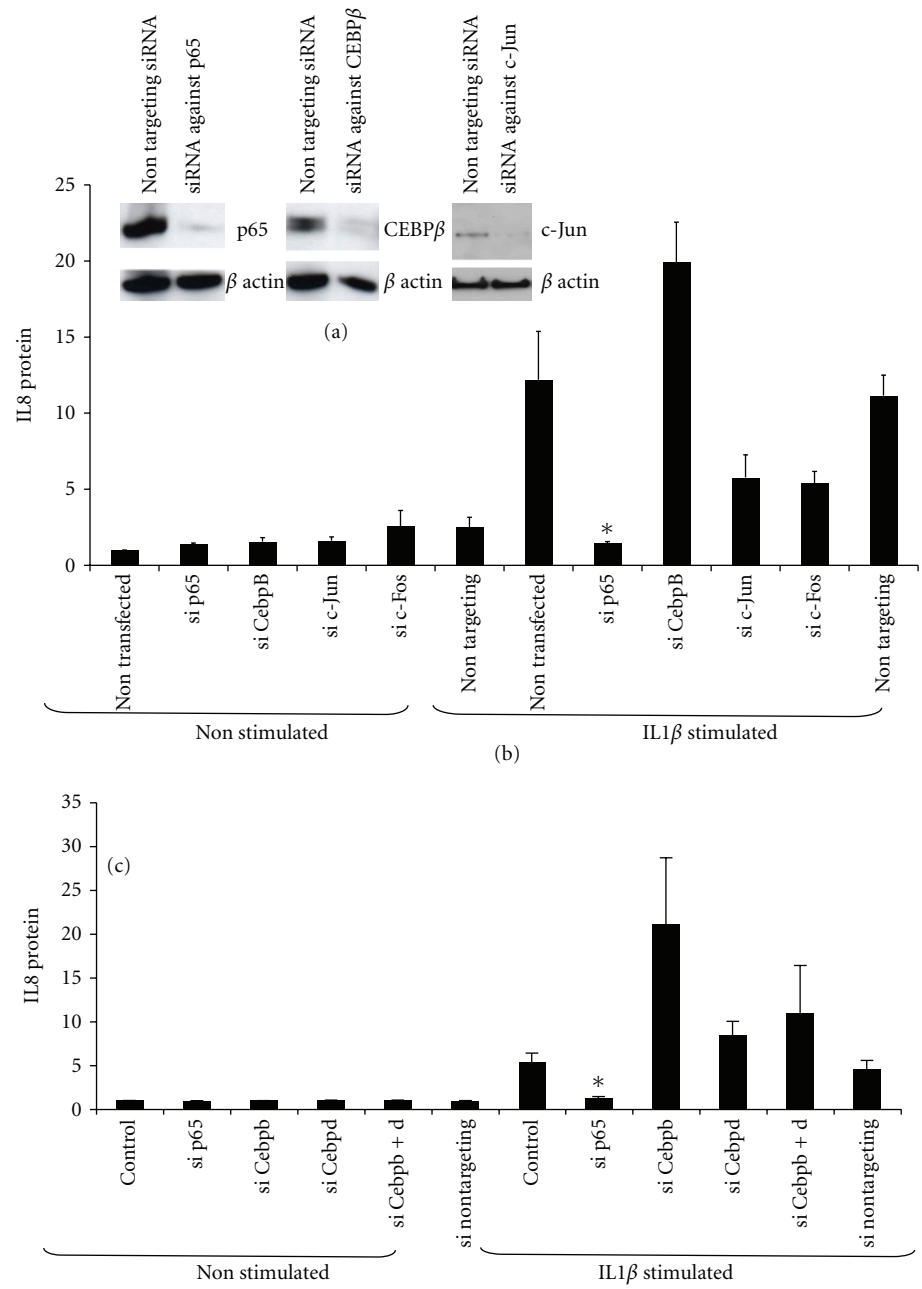

Figure 3: (a) siRNA knockdown of p65 (a), CEBPβ (b), and c-Jun (c). (b) The effects of siRNA against NFκBp65, CEBPβ, c-Jun, and c-Fos on IL8 protein production in a nonstimulated and IL1B-stimulated state. Data is normalised to the nontargeting control. Data are presented as mean ± SEM ($n = 4$). An ANOVA was performed with a Dunnett's multiple comparison post-test. *Indicates a significant difference of P less than 0.05. (c) The effects of siRNA against NFκBp65, CEBPβ, and CEBPD on IL8 protein production in a nonstimulated and IL1B-stimulated state. Data is normalised to the non-targeting control. Data are presented as mean ± SEM ($n = 4$). An ANOVA was performed with a Dunnett's multiple comparison post-test. *Indicates a significant difference of P less than 0.05.

treatment of inflammatory bowel disease and rheumatoid arthritis. This has been shown to inhibit labour-associated gene expression in cell culture models [31, 32], although there are currently no animal or clinical studies. In a mouse model of inflammation-induced preterm labour the cyclopentenone prostaglandin J2, which acts via inhibition of NFκB, both delays preterm birth and improves pup survival [33]. Glucocorticoids such as dexamethasone or prednisolone have well-established anti-inflammatory and immunosuppressive activities through their inhibitory effects on AP-1 and NFκB pathways [34] although there is no evidence that these drugs will delay or prevent preterm birth. Progesterone, which, when used clinically, can reduce the risk of preterm labour, has been shown to repress IL1B-induced expression of NFκB regulated genes in human amnion and myometrium [35, 36].

In this study, we examined the role of NFκB, CEBP, and AP-1 in regulation of IL8 expression in human myometrium

and used IL1B to mimic the effects of inflammation seen with human labour. We used ChIP to show that IL1B induces the binding of all three transcription factors to the IL8 promoter. This is consistent with the observations of Soloff et al. that IL1B increases both NFκBp65 and CEBPβ binding to the endogenous IL8 promoter in myometrial cells [24]. Studies in other cell types have shown different patterns of binding. For example in a study in human conjunctiva epithelial cells ChIP showed no binding of NFκB, CEBP, or AP-1 alone, but when immunoprecipitation was performed for both NFκB and CEBP simultaneously, ChIP showed binding of both to the IL8 promoter [37]. Conversely treatment of human airway smooth muscle cells with tryptase, which mimics inflammation, induced the binding of all three transcription factors to the promoter [38].

Since we found that, in human myometrial cells all three transcription factors bound to the IL8 promoter, we considered two approaches to identify the relative importance of each transcription factor in IL8 expression. First we used site-directed mutagenesis to alter the binding sites for AP1, CEBP, and NFκB. We found that mutating both the NFκB and AP-1 sites markedly reduced both basal and stimulated IL8 promoter activity. However, mutating the CEBP site did not change the basal promoter activity but did completely inhibit the IL1B-induced increase in promoter activity. These data suggest that the NFκB and AP-1 binding sites are essential for both basal and IL1B-stimulated IL8 expression. In contrast, it appeared that CEBP binding sites are not important for basal promoter activity but are essential for the IL1B-induced increase in promoter activity. Using a similar approach in human amnion and cervical epithelial cells, Elliott et al. found similar results for the NFκB binding site but found no effect of mutating the CEBP or AP-1 binding sites on the IL8 promoter activity [14]. However, site-directed mutagenesis of DNA binding sites gives limited information about the role and importance of transcription factors, particularly where, as in the IL8 promoter, those sites are close to each other or overlap. Therefore in an alternative approach we used the more novel technique of siRNA against AP-1, CEBP, and NFκB to knock down the expression of their endogenous proteins. The data generated by siRNA knockdown is probably the most reliable in terms of the role of individual transcription factors since it is highly specific. In addition, the readout is activity of the endogenous promoter, not a transfected construct. In contrast to the finding from the site-directed mutation studies, basal IL8 levels were not reduced by knockdown of any of the transcription factors. This may be because although we achieved 90% knockdown, this was not complete. However, knockdown of p65 did completely inhibit the IL1B-stimulated increase in IL8 levels. The effects of c-Jun and c-Fos knockdown were less marked but still inhibitory. CEBP knockdown did not inhibit the IL1B-induced increase in IL8 expression and actually tended to increase it. In some cell types CEBP has been shown to inhibit the action of NFκB in regulation of IL8. Our knock-out data suggests that this phenomenon may apply in myometrium. Certainly our data does not point to a central positive role for CEBP in driving IL8 expression.

Our data show that, in human myometrial cells, NFκB is essential for IL8 expression; AP-1 plays a less important but still stimulatory role, while the role of CEBP is less clear. The site-directed mutagenesis studies suggested a positive role in IL8 expression; however, the NFκB and CEBP binding sites are close together, and it remains possible that mutation of the CEBP site may have interfered with NFκB binding or function. The probably more reliable data from siRNA would suggest that pharmacological inhibition of CEBP might enhance IL8 expression and may therefore not be beneficial. The dominant role played by NFκB in IL8 expression confirms that it is an attractive therapeutic target for the prevention of preterm labour.

5. Conclusion

These data show that NFκB is essential for IL8 myometrial expression, AP-1 plays a less important but still stimulatory role, while the role of CEBP is less clear. These results provide further support for the notion that NFκB represents an attractive therapeutic target for the prevention of preterm labour.

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Research Article

Epigenetic Regulation of Cytokine Production in Human Amnion and Villous Placenta

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The mechanisms of human preterm labour appear inextricably linked to cytokine biosynthesis by gestational tissues. In turn, cytokine production by gestational tissues has been shown to be regulated by epigenetic mechanisms. In this paper, we demonstrate that cytokine production in gestational tissues is regulated epigenetically in a tissue-specific manner. Furthermore, we show that treatment with a histone deacetylation inhibitor can partially abrogate LPS-stimulated TNFα production in villous placenta but not amnion. LPS treatment significantly ($P < 0.05$) increased the production of IL-1β (~10–34-fold), TNFα (~23–>100-fold) and IL10 (~6–10-fold) after 24 h of treatment in villous explants, as expected. There were no significant LPS effects on IL1Ra production. AZA treatment did not have any significant effect on any cytokines' production tested either alone or in combination with LPS. Interestingly, however, the stimulatory effects of LPS on TNFα production were partially mitigated ($P < 0.05$) by TSA treatment in villous explants. We suggest caution in the consideration of histone deacetylation inhibitors in pregnancy due to the different responses in gestational tissues.

1. Introduction

Preterm birth is the leading cause of neonatal death worldwide with one million deaths directly resulting from premature birth. More children under the age of 5 years die due to preterm birth than to AIDS, malaria, or tuberculosis [1]. The rate of preterm birth in Australia is gradually increasing [2]. Recently, the importance of epigenetics as a regulator of gene expression has become apparent and, potentially, epigenetic differences are driving gene expression leading to preterm birth.

Epigenetics is the process by which interactions between genes and environment lead to stable changes in phenotype [3]. Epigenetic changes (e.g., DNA methylation, covalent histone modifications, etc.) are by definition heritable, meaning that effects brought about by the environment at critical periods of development (such as gestation) can have long-term detrimental consequences [4]. Indeed, certain epigenetic changes are passed between generations which might explain some of the familial and intergenerational effects observed for preterm birth [5]. Importantly and in contrast to genetic inheritance, epigenetic heritability is potentially reversible [6]. An epigenomics approach, thus, opens new avenues for therapeutic interventions.

Epigenetic information is conveyed via a synergistic interaction between mitotically heritable patterns of DNA methylation and histone-mediated modifications to the chromatin structure [7]. In mammals, DNA methylation primarily involves the addition of methyl groups to cytosine residues present in a CpG dinucleotide. Hypermethylation of promoter regions of genes is typically associated with transcriptional repression whereas hypomethylation is usually permissive for gene activity. Histone modifications consist of a plethora of enzymatic modifications including acetylation, methylation, ubiquitination, and phosphorylation of different amino acids in the N terminal tails of histone proteins, the combination of which is thought to determine the local conformational state of the chromatin. Currently, over 60 different histone modifications have been identified although actual numbers are likely to be significantly higher

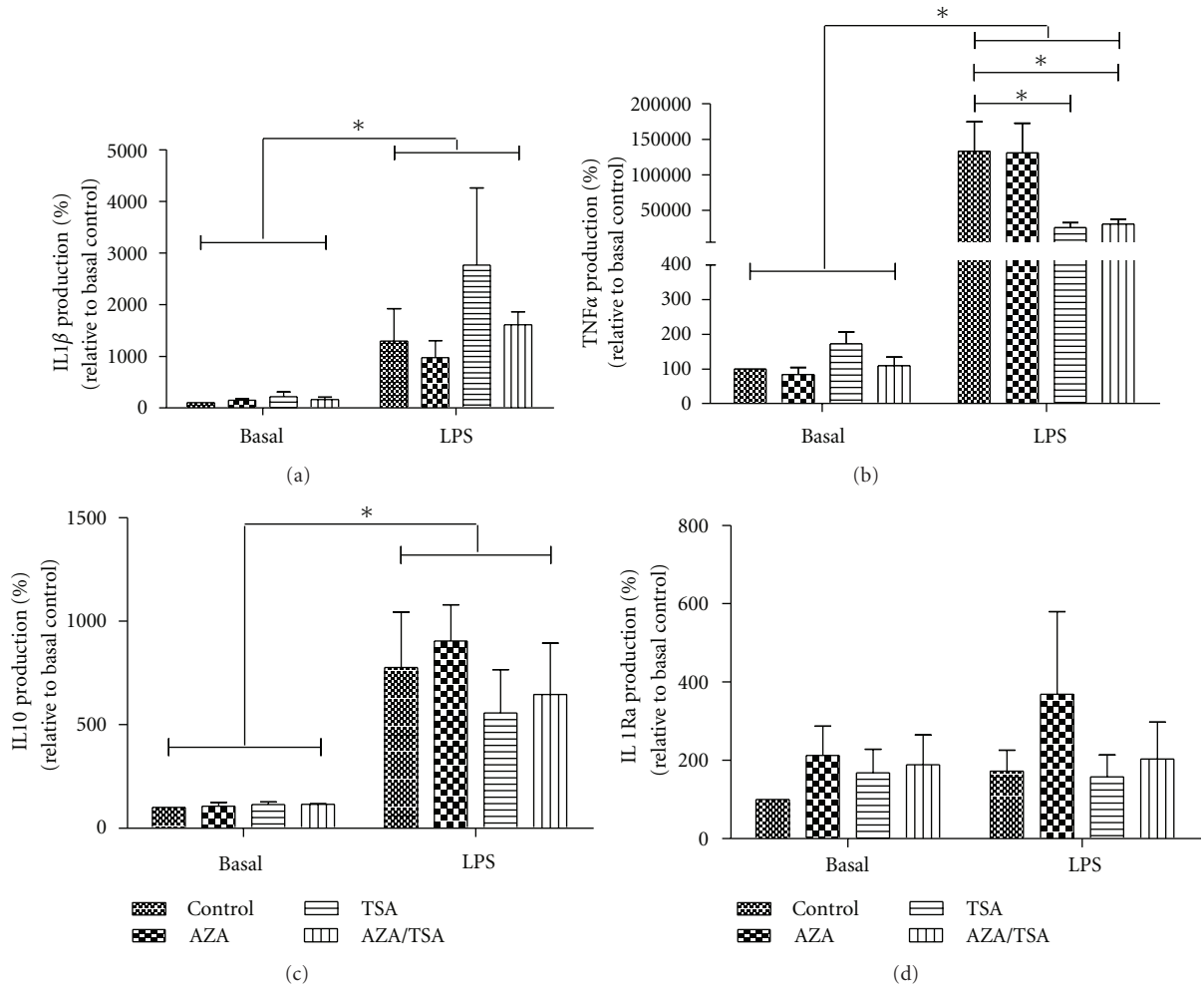

Figure 1: Effect of AZA and TSA on cytokine production by villous explants in the presence or absence of LPS. The levels of cytokines were measured by ELISA. The y-axis shows the rate of mean IL1β (a), TNFα (b), IL10 (c), and IL1Ra (d) production (as % basal control values), normalized against the wet tissue weight, and the x-axis shows the different treatment groups. Error bars represent 1 S.E.M. $^*P < 0.05$, $n = 3$.

[8]. Histone modifications are generally considered a less stable epigenetic mark than DNA methylation. Recent work has clearly demonstrated that epigenetic marks determine time- (temporal) and tissue-specific (spatial) aspects of gene expression throughout development. In contrast, others might carry an epigenetic signature that is less sensitive to the normal stimuli of labour, therefore, leading to dysfunctional labour or postdates delivery. The environment presumably plays a crucial role in bringing about these epigenetic changes that are critical for the safe passage of gestation and later outcomes. An increasing number of diverse factors are now known to epigenetically regulate genes, including age [9], inflammation [10], gender [11], genotype [12], stress [13], nutrition [14], metabolism [15], drugs [16], and infection [17], thus heightening the relevance of the study of the epigenetics of preterm birth.

Both classes of epigenetic modification (e.g., DNA methylation and covalent histone modifications) can be reversed through treatment with a chemical inhibitor: 2′-deoxy-5-azacytidine (AZA) that inhibits DNA methylation leading to global hypomethylation of DNA, while treatment with an inhibitor of histone deacetylation such as trichostatin A (TSA) leads to increased levels of histone acetylation. In both cases, intervention favours the transcriptionally active epigenetic state.

We demonstrated in an earlier study [16] that the combination treatment of human choriodecidual explants with AZA and TSA leads to a massive increase in IL-1β production in response to LPS. In the present study, we extend those findings to amnion and villous placenta.

2. Materials and Methods

2.1. Reagents. Lipopolysaccharide (LPS), 5-aza-2′-deoxycytidine (AZA), and trichostatin A (TSA) were purchased from Sigma Chemical (St. Louis, MO). Culture media and fetal calf serum (FBS) were from Life Technologies (Carlsbad, CA, USA). Human IL1β, TNFα, IL10, and IL1Ra ELISA kits were purchased from BD Bio Sciences.

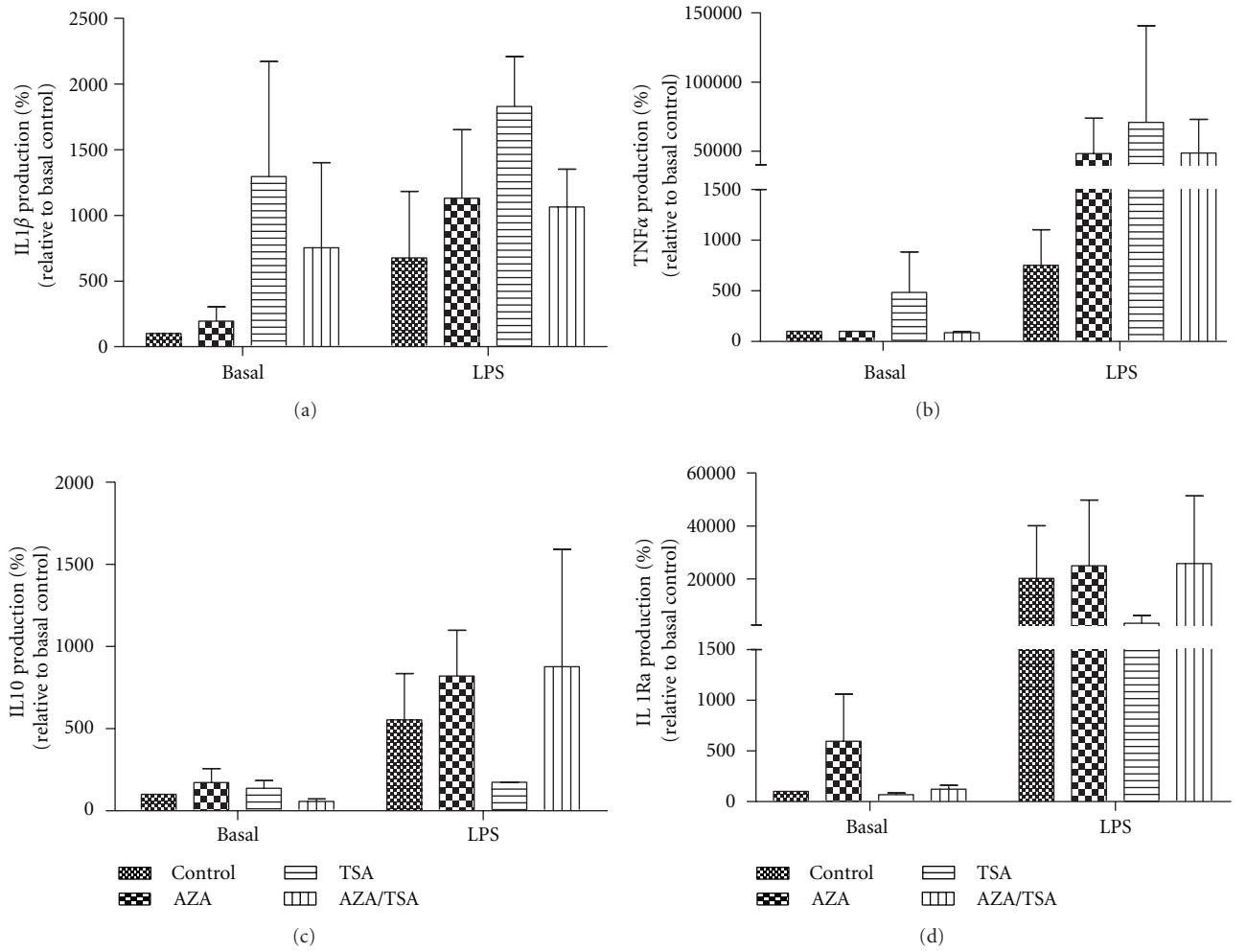

Figure 2: Effect of AZA and TSA on cytokine production by amnion explants in the presence or absence of LPS. The levels of cytokines were measured by ELISA. The y-axis shows the rate of mean IL1 β (a), TNFα (b), IL10 (c), and IL1Ra (d) production (as % basal control values), normalized against the wet tissue weight, and the x-axis shows the different treatment groups. Error bars represent 1 S.E.M.

2.2. Explant Cultures. Placental tissues were collected from women undergoing elective caesarean section at term, with the approval of the NorthernX Regional Ethics Committee. Three individual placentae from singleton pregnancies of healthy nonsmoking mothers were used in the study. In triplicate, villous and amnion tissues explants were cultured in 12-well plates (three pieces of tissue to a well) in DMEM/F12 media supplemented with glutamax, 10% FBS, and 1% Antibiotic-antimycotic and incubated at 37°C in humid 5% CO_2/95% air for 24 h (modified from [18, 19]).

Explant treatments included the use of 5-aza-2′-deoxycytidine (AZA), which inhibits DNA methylation, and/or Trichostatin A (TSA) which inhibits histone deacetylation. Doses and times were chosen to be consistent with published literature [18, 20]. 24 hours following placental dissection, the media were replaced with serum-free media (DMEM/F12 with glutamax and 0.1% Bovine gamma globulin and 1% Antibiotic-antimycotic medium). Explants were treated with either 200 nM AZA alone, TSA (300 nM) alone, 5 μM AZA with TSA (300 nM), or the DMSO carrier and cultured for 48 h, with media refreshed once during this time. This represented a 48 h preincubation ± AZA (the action of AZA is passive and requires cell division for action). The treatment period commenced 72 h postdissection and consisted of the addition of 300 nM TSA or DMSO carrier in fresh media, in the presence or absence of LPS (5 μg/mL), to the explants for a duration of 24 h. The media were collected following the treatment and the wet weight of the tissue in each well determined so that cytokine production rates could be normalized. Measurements of pro- (IL1β, TNFα) and anti-inflammatory (IL10, IL1Ra) cytokines were conducted on cultured media using ELISAs according to manufacturers' specifications.

2.3. Statistical Analysis. Production rates of cytokines were calculated as picograms per gram wet tissue weight per 24 h and are presented as % basal control values (means ± SE, $n = 3$). Statistical significance was determined by ANOVA, and $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of AZA and TSA on Cytokine Production by Villous Explants in the Presence or Absence of LPS. LPS treatment significantly ($P < 0.05$) increased the production of IL-1β (~10–34-fold), TNFα (~23–>100-fold) and IL10 (~6–10-fold) after 24 h of treatment in villous explants, as expected. There were no significant LPS effects on IL1Ra production. AZA treatment did not have any significant effect on any cytokines' production tested either alone or in combination with LPS. Interestingly, however, the stimulatory effects of LPS on TNFα production were partially mitigated ($P < 0.05$) by TSA treatment in villous explants.

3.2. Effect of AZA and TSA on Cytokine Production by Amnion explants in the Presence or Absence of LPS. Neither LPS nor the TSA or AZA had any significant effect on the cytokine production in amnion explants after 24 h of treatment.

4. Discussion

Conceivably, epigenetic modifications (e.g., DNA methylation and covalent histone modifications) might render individuals more or less susceptible to either term or preterm birth by modulating the expression of genes that are effectors of the parturition pathway, for example, the contraction-associated proteins, COX-2, oxytocin, and prostaglandin receptors in myometrium [20]. Thus, some individuals may have a sensitive epigenetic phenotype that leads to an increased susceptibility to preterm labour. Global DNA methylation is increased in preterm preeclamptic placentae [21]. In this study, in conjunction with our previous work [18] we have shown that epigenetic regulation of cytokine production is tissue specific in gestational tissues. Caution of course should be exercised in the comparison of noncontemporaneous data. In choriodecidua AZA/TSA treatment resulted in a massive increase in IL-1β production in response to LPS whereas in amnion and villous placenta this did not occur although a small positive trend in mean production was noted (Figures 1 and 2). Our finding of TSA mitigation of LPS-stimulated TNFα production is most fascinating. This raises the question of TSA-like moieties being developed to attenuate inflammatory process in the human placenta. The disadvantage of such an approach is, of course, the enhancement of IL-1β production in choriodecidua by such an agent [18]. The tissue-specific nature of these regulatory mechanisms means that we must be cautious in our approach to such matters.

Amnion clearly does not respond to the inflammatory challenge of LPS although it remains a significant source of cytokine production and once activated can respond vigorously to modifications of the cytokine environment [22–25].

Villous placenta seems crudely to be more active in pro-inflammatory cytokine biosynthesis than anti-inflammatory cytokine biosynthesis. This is clearly dangerous for the fetus and may play a role in the fetal inflammatory syndrome [26], although clearly direct evidence must be obtained before conclusions are drawn.

5. Conclusions

Cytokine production in gestational tissues is regulated epigenetically in a tissue-specific manner. Furthermore, treatment with a histone deacetylation inhibitor can partially abrogate LPS-stimulated TNFα production in villous placenta but not amnion.

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