Single- and double-stranded viral RNA generate distinct cytokine and antiviral responses in human fetal membranes

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Abstract: There has been growing interest in the role of viral infections and their association with adverse pregnancy outcomes. However, little is known about the impact viral infections have on the fetal membranes (FM). Toll-like receptors (TLR) are thought to play a role in infection-associated inflammation at the maternal–fetal interface. Therefore, the objective of this study was to characterize the cytokine profile and antiviral response in human FMs exposed to viral dsRNA, which activates TLR3, and viral ssRNA, which activates TLR8; and to determine the mechanisms involved. The viral dsRNA analog, Poly(I:C), induced up-regulated secretion of MIP-1α, MIP-1β, RANTES and TNF-α, and down-regulated interleukin (IL)-2 and VEGF secretion. In contrast, viral ssRNA induced a broader panel of cytokines in the FMs by up-regulating the secretion of IL-1β, IL-2, IL-6, G-CSF, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α and GRO-α. Using inhibitory peptides against TLR adapter proteins, FM secretion of MIP-1β and RANTES in response to Poly(I:C) was MyD88 dependent; MIP-1α secretion was dependent on MyD88 and TRIF; and TNF-α production was independent of MyD88 and TRIF. Viral ssRNA-induced FM secretion of IL-1β, IL-2, IL-6, G-CSF, MIP-1α, RANTES and GRO-α was dependent on MyD88 and TRIF; MIP-1β was dependent upon TRIF, but not MyD88; and TNF-α and MCP-1 secretion was dependent on neither. Poly(I:C), but not ssRNA, induced an FM antiviral response by up-regulating the expression of IFNβ, myxovirus-resistance A, 2′,5′-oligoadenylate synthetase and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G. These findings demonstrate that human FMs respond to two viral signatures by generating distinct inflammatory cytokine/chemokine profiles and antiviral responses through different mechanisms.

Keywords: antiviral factors / cytokines / fetal membranes / Toll-like receptors / viral infections

Introduction

Bacterial infection and inflammation of the placental fetal membranes (FM) play a major role in chorioamnionitis, preterm premature rupture of membranes (PPROM) and preterm birth (ACOG Practice Bulletin No. 80, 2007; Goldenberg et al., 2000; Lamont, 2003; Caughey et al., 2008; Goldenberg et al., 2008). The FMs are likely the first tissues colonized by an ascending bacterial infection (Herve et al., 2008). Although most normal term deliveries have evidence of bacteria in the chorionamnion, it is the association with inflammation that correlates with pathology and prematurity (Romero et al., 2007). An intrauterine infection gaining access to the FMs is thought to trigger prematurity by activating innate immune responses (Romero et al., 2007). Indeed, clinical and experimental studies have correlated bacteria/bacterial components and inflammation at the maternal–fetal interface with preterm birth (Elovitz and Mrinalini, 2004; Elovitz et al., 2006; Romero et al., 2006; Pettker et al., 2007; Koga et al., 2009; Pirianov et al., 2009; Burd et al., 2010; Cardenas et al., 2011). However, in spite of this association, no single bacterium has been attributed to preterm birth, and antibiotic interventions have proved unsuccessful (Ganu et al., 2012).

More recently there has been growing interest in the role of viral infections and adverse pregnancy outcomes. The presence of a number of viral infections in the amniotic fluid or gestational tissues have been reported to be linked to increased risk for chorioamnionitis and spontaneous preterm birth, such as adenovirus (Srinivas et al., 2006; Tsekoura et al., 2010); Epstein–Barr virus, cytomegalovirus (CMV), herpes virus (Gibson et al., 2008, 2011), human papillomavirus (Gomez et al., 2008), coxsackie virus, group B type 1 (Strong and Young, 1995), the enterovirus herpangia (Chen et al., 2010) and hepatitis virus (Elefsiniotis et al., 2010; Connell et al., 2011). Infection with adenovirus has also been associated with second trimester pregnancy loss (Srinivas et al., 2006). Similarly, infection with Parvovirus B19 or herpes virus early in pregnancy has been linked to second trimester miscarriage or very preterm birth (Johansson et al., 2008). In addition, women with H1N1 are more...
likely to have adverse pregnancy outcomes such as spontaneous miscarriages and preterm birth (Creanga et al., 2010; Investigators and Australasian Maternity Outcomes Surveillance, 2010; Siston et al., 2010), and the rate of preterm birth correlates with maternal disease severity (Michaen et al., 2012).

While FMs are permissive to viral infections, including influenza virus (Uchide et al., 2002a, b, 2006, 2009, 2012), CMV (Figueroa et al., 1978; Kumazaki et al., 2002; Matsunaga et al., 2013), and herpes virus (Rokos et al., 1998), and viral infections can trigger an inflammatory cytokine response in the chorioamnion (Uchide et al., 2002b, 2006, 2012), little is known about the mechanisms involved. One way in which infection-associated inflammation at the maternal–fetal interface arises is through activation of the innate immune Toll-like receptors (TLRs) (Abrahams, 2008). We have previously shown that normal human FMs at term constitutively express TLRs 1–10, as well as the two major TLR adapter proteins, MyD88 and TRIF (Hoang et al., 2014). Moreover, in response to bacterial agonists, TLR2, TLR4, TLR5 and TLR9 mediate distinct FM cytokine profiles (Hoang et al., 2014). Thus, we hypothesized that this might also be the case for TLR3, which senses viral dsRNA and TLR8, which detects viral ssRNA (Kumar et al., 2011). Therefore, the objective of this study was to characterize the cytokine profile and antiviral response in human FMs exposed to the viral signatures, dsRNA and ssRNA, and to determine the mechanisms involved.

Materials and Methods

Fetal membrane collection, preparation and stimulation

FM (n = 16) were collected from uncomplicated normal term pregnancies (39–41 weeks) delivered by elective repeat Cesarean section, without signs of labor, infection or PPROM. No patients received prostaglandins or any other induction agent prior to Cesarean section. Sample collection was approved by Yale University’s Human Investigation Committee. After washing the FMs with sterile PBS supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Grand Island, NY, USA), adherent blood clots were removed and sections where both the chorion and amnion were intact were cut using a 6-mm biopsy punch. The FM explants were then placed in 0.4–m cell culture inserts (BD Falcon, Franklin Lakes, NJ, USA) with 500 μl of the Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and these were placed in a 24-well plate containing 500 μl of the DMEM media for 24 h, as previously described (Luo et al., 2010; Hoang et al., 2014). The next day the media was removed and replaced with serum-free OptiMeM media (Gibco). FM explants were then treated with: no treatment (NT); the TLR3 agonist and synthetic analog of viral dsRNA, Poly(I:C), at 20 μg/ml (Invivogen, San Diego, CA, USA); or the TLR8 agonist, viral ssRNA at 5 μg/ml (Invivogen). To determine the involvement of caspase-1, FM explants were pretreated for 1 h with the specific caspase-1 inhibitor, Z-WEHD-FMK at 1 μM (R&D Systems, Minneapolis, MN, USA). To determine the involvement of MyD88 or TRIF, FM explants were pretreated for 1 h with either a MyD88 inhibitor peptide (Pepin-MYD; 10 μM); a TRIF inhibitor peptide (Pepin-TRIF; 10 μM) or a control peptide (Pepin-Control; 10 μM) (Invivogen). The optimal Poly(I:C) and viral ssRNA concentrations were determined in preliminary experiments (data not shown) and the time point determined from previous studies (Hoang et al., 2014). After 24 h of treatment, cell-free culture supernatants were collected and the explants snap frozen. Supernatants and tissues were then stored at −80°C until further analysis was performed.

Cytokine analysis

FM supernatants were analyzed for the following cytokines/chemokines using multiplex analysis (BioRad): IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, G-CSF (CSF3), GM-CSF (CSF2), IFNγ, MCP-1 (CCL2), MIP-1α, MIP-1β, RANTES (CCL5), TNF-α, VEGF and GRO-α as previously described (Luo et al., 2010; Hoang et al., 2014). FM supernatants were also analyzed for IL-1β by ELISA (R&D Systems).

Quantitative real-time RT–PCR

FM explant biopsies were homogenized and total RNA extracted as previously described (Abrahams et al., 2013; Krikun et al., 2013; Hoang et al., 2014). Quantitative real-time PCR was performed using the KAPA SYBR Fast qPCR kit (Kapa Biosystems, Woburn, MA, USA), and PCR amplification performed on the BioRad CFX Connect Real-time System (BioRad, Hercules, CA, USA). Detection of human IFNβ, 2′, 5′-oligoadenylate synthetase (OAS), myxovirus-resistance A (MxA) and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G) and secretory leukocyte protease inhibitor (SLPI) was performed using primer sequences as previously described (Krikun et al., 2013). Data were normalized to the housekeeping gene, GAPDH, analyzed using the ΔΔCT method and presented as fold change (FC) in the expression of gene of interest relative to the NT control, as previously described (Abrahams et al., 2013; Krikun et al., 2013).

Statistical analysis

Experiments were performed at least three times and data presented as mean ± SEM. Prism from Graphpad Software, Inc. (La Jolla, CA, USA) was used to calculate significance (P < 0.05). Statistical analysis was performed using either the paired t-test, or for multiple comparisons, one-way ANOVA.

Results

Viral signatures induce distinct FM cytokine profiles

In a previous study we established that human FMs from normal term pregnancies constitutively express the viral sensors, TLR3 and TLR8, as well as the TLR adapter proteins MyD88 and TRIF (Hoang et al., 2014). Therefore, in this study, FMs were treated with the TLR3 agonist, Poly(I:C), and the TLR8 agonist, viral ssRNA (Kumar et al., 2011). After treatment with Poly(I:C), FMs secreted significantly higher levels of TNF-α (2.1-fold), MIP-1α (8-fold), MIP-1β (4.8-fold), and RANTES (16.4-fold) compared with the NT control. In parallel, FM secretion of IL-2, and VEGF was significantly dampened by 4-fold and 4.1-fold, respectively, after exposure to Poly(I:C) (Fig. 1). FM secretion of IL-1β, IL-6, IL-8, G-CSF, MCP-1, GM-CSF and IFNγ were not significantly changed after Poly(I:C) treatment (Fig. 1). After treatment with viral ssRNA, FMs secreted significantly higher levels of IL-1β (13.8-fold), IL-2 (1.3-fold), IL-6 (4.1-fold), TNF-α (4.2-fold), MCP-1 (1.9-fold), G-CSF (3.8-fold), MIP-1α (4.8-fold), MIP-1β (3.3-fold), RANTES (4.3-fold) and GRO-α (6.6-fold) (Fig. 1). FM secretion of IL-8, GM-CSF, IFNγ and VEGF were not significantly changed after ssRNA treatment (Fig. 1). Levels of IL-4, IL-10, IL-12 and IL-17 were below the assay’s detection limit.
Role of MyD88 and TRIF in viral dsRNA- and ssRNA-induced FM cytokine secretion

Having established that human FMs respond to the viral TLR agonists, Poly(I:C) and ssRNA by secreting cytokines and chemokines, we next sought to determine the role of the adapter proteins MyD88 and TRIF. Typically, TLR3 utilizes the adapter protein TRIF (Yamamoto et al., 2002; Oshiumi et al., 2003), while TLR8 signals through MyD88 (Han et al., 2012; Heil et al., 2004). As shown in Fig. 2A, the Poly(I:C)-induced up-regulation of FM MIP-1α, MIP-1β, and RANTES in the presence of the control peptide was significantly reduced by the presence of the MyD88 inhibitor. The TRIF inhibitor also significantly reduced Poly(I:C)-induced secretion of MIP-1α, but not MIP-1β or RANTES (Fig. 2A). Neither the MyD88 nor the TRIF inhibitor had any significant effect on the FMs Poly(I:C)-induced TNF-α response (Fig. 2A). As shown in Fig. 2B, the ssRNA-induced up-regulation of IL-1β, IL-2, IL-6, G-CSF, MIP-1α, RANTES and GRO-α secretion by the FMs was significantly reduced by both the MyD88 inhibitor and the TRIF inhibitor, when compared with the control peptide. Viral ssRNA-induced up-regulation of MIP-1β secretion by the FMs was significantly inhibited by the presence of the TRIF inhibitor, but not the MyD88 inhibitor (Fig. 2B). ssRNA-induced TNF-α and MCP-1 secretion was not significantly altered by either inhibitor (Fig. 2B).

FM IL-1β secretion in response to viral ssRNA is caspase-1 dependent

Since viral ssRNA, but not Poly(I:C), induced the FMs to produce IL-1β, we further examined the mechanism involved. We previously demonstrated that human FMs express the inflammasome components Nalp1, Nalp3, ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 (Hoang et al., 2014), which mediate the processing of intracellular pro-IL-1β into its active, secreted form (Agostini et al., 2004; Martinon and Tschopp, 2004). In the presence of a caspase-1 inhibitor, IL-1β secretion induced by viral ssRNA was significantly reduced by 54.7% (Fig. 3).

Poly(I:C), but not viral ssRNA, induces an FM antiviral response

TLR3 and TLR8 activation can induce a type I interferon (IFN) response (Arpaia and Barton, 2011), and the subsequent production of antiviral factors, such as IFN-inducible OAS, MxA and APOBEC3G (Samuel, 2001; Turelli et al., 2004; Abrahams et al., 2006; Bonvin et al., 2006; Krikun et al., 2013) and SLPI (Schafer et al., 2005; Abrahams et al., 2006; Krikun et al., 2013). As shown in Fig. 4, Poly(I:C), but not ssRNA, significantly induced FMs to express elevated levels of IFNβ.
MxA, OAS and APOBEC3G mRNA. In contrast, neither Poly(I:C) nor ssRNA induced a significant increase in SLPI mRNA levels (Fig. 4).

**Discussion**

Infection-associated pregnancy complications such as chorioamnionitis, PPROM and preterm birth have been strongly associated with bacterial infection and inflammation of the FMs (ACOG Practice Bulletin No. 80, 2007; Goldenberg et al., 2000; Lamont, 2003; Caughey et al., 2008; Goldenberg et al., 2008). Although, much less is known about the role of viral infections, there is growing evidence to suggest an association with pregnancy mortality and morbidity and gestational tissues including the FMs may be a target (Figueroa et al., 1978; Rokos et al., 1998; Kumazaki et al., 2002; Uchide et al., 2002a, b, 2006, 2009, 2012; Matsunaga et al., 2013). The functional role of innate immune pattern recognition receptors, such as the TLRs and Nod proteins, in response to bacterial components in the FMs has been described (Kim et al., 2004; Adams et al., 2007; Leroy et al., 2007; Abrahams et al., 2013; Lappas, 2013, 2014; Hoang et al., 2014), however, little is known about this tissue’s response to viral components or the mechanisms involved. In this current study we have demonstrated that FMs exposed to the TLR3 agonist, viral dsRNA, and the TLR8 agonist, viral ssRNA, generate distinct cytokine/chemokine and antiviral profiles. Furthermore, we have demonstrated a differential role for the TLR adapter proteins, MyD88 and TRIF, in

**Figure 2** FM cytokine responses to Poly(I:C) and viral ssRNA are regulated by MyD88 and TRIF. FM explants were treated with NT, or either (A) Poly(I:C) or (B) viral ssRNA all in the presence of either a control peptide (Control), a MyD88 inhibitor peptide (MyD88) or a TRIF inhibitor peptide (TRIF) (10 μM). Bar charts show cytokine secretion as FC relative to the NT controls (*P < 0.05 relative to the control; n = 5).

**Figure 3** Viral ssRNA induces FM IL-1β secretion in a caspase-1 dependent manner. FM explants (n = 10) were treated with NT or ssRNA in the presence of media or a caspase-1 inhibitor after which supernatants were measured for IL-1β (*P < 0.05, **P < 0.001 relative to NT unless otherwise indicated).
mediating FM cytokine/chemokine production in response to these two viral signatures. We have also demonstrated a role for the inflammasome component, caspase-1, in mediating FM IL-1β production following exposure to viral ssRNA.

When we compared the cytokine profile generated by human FMs after exposure to the viral TLR3 and TLR8 agonists, Poly(I:C) and viral ssRNA, respectively, these two viral signatures induced quite different cytokine/chemokine responses. Poly(I:C) up-regulated the secretion of the pro-inflammatory cytokine, TNF-α, and the inflammatory chemokines MIP-1α, MIP-1β, RANTES, while down-regulating the constitutive of IL-2 and VEGF. In contrast viral ssRNA triggered a much broader response by increasing the tissue’s section of the pro-inflammatory cytokines, IL-1β, TNF-α, IL-6 and IL-2; the inflammatory chemokines, MCP-1, MIP-1α, MIP-1β, RANTES and GRO-α and the growth factor, G-CSF. So while both viral components triggered distinct profiles, both responses were a combination of pro-inflammatory cytokines and chemokines.

The production of the chemokines, MCP-1, MIP-1α, MIP-1β, RANTES and GRO-α by FMs exposed to viral RNA, suggests that in immune cell recruitment. Although histologic chorioamnionitis is characterized by a neutrophil infiltrate, and the classic chemokine for this, IL-8, is often elevated in these cases (Menon et al., 2010), in our studies FMs treated with Poly(I:C) or ssRNA did not produce elevated levels of IL-8. Interestingly, basal IL-8 levels produced by the FMs were much higher than the other chemokines detected. Neutrophil chemotaxis can, however, be regulated by a number of other chemokines. In mice, for example, it has been shown that neutrophils express two major chemokine receptors, CCR1 and CXCR2 (Chou et al., 2010; McDonald and Kubes, 2010). CCR1 can be ligated by MIP-1α, MIP-1β and RANTES, while CXCR2 can be activated by GRO-α (Chou et al., 2010; McDonald and Kubes, 2010). Thus, it seems that after TLR3 and TLR8 stimulation, human FMs are producing mostly CCR1 ligands and may recruit neutrophils preferentially through this receptor.

The production of the pro-inflammatory cytokine, TNF-α, by the FMs in response to TLR3 and TLR8 activation, and IL-1β and IL-6 in response to viral ssRNA also suggests a role for the FMs in contributing to preterm birth. Levels of IL-1β, IL-6 and TNF-α are elevated in the amniotic fluids of patients with preterm birth and intra-amniotic infection (Romero et al., 1989, 1990, 1992a, b) and IL-1β plays a role in promoting preterm labor (Sadovsky et al., 2006; Christiaens et al., 2008; Kemp et al., 2010). These findings are also in keeping with previous reports that infection of human chorion cells with influenza virus, which is an ssRNA virus, induces the production of the pro-inflammatory cytokines, IL-1β, IL-6 and TNFα (Uchide et al., 2002b, 2006).

In order to achieve IL-1β secretion, intracellular pro-IL-1β must be processed into its active, secreted form (Agostini et al., 2004; Martinon and Tschopp, 2004), and this is often mediated by caspase-1 (Neta et al., 2010) that associates with the other inflammasome components, Nalp1 or Nalp3 and ASC (Agostini et al., 2004; Martinon and Tschopp, 2007). We have previously reported that normal human FMs at term express all these components and in response to the bacterial TLR agonists, lipopolysaccharide (LPS), peptidoglycan and flagellin induce IL-1β processing and secretion via caspase-1 (Hoang et al., 2014). A recent study by Lappas, also showed LPS-induced IL-1β by FMs to be mediated by caspase-1 (Lappas, 2014). In this current study we demonstrated that human FMs exposed to viral ssRNA secrete IL-1β in a caspase-1 dependent manner, suggesting that TLR8 activation may induce inflammasome activity. Indeed, the TLR8 agonist, imidazoquinoline, can activate monocyte IL-1β production via caspase-1 (Philbin et al., 2012), and viral ssRNA-mediated IL-1β release by monocytes is dependent on the Nalp3 inflammasome (Allen et al., 2011).

To further explore the mechanisms by which viral dsRNA and ssRNA induce FM cytokine/chemokine production, we determined the functional role of the TLR adapter proteins, MyD88 and TRIF in this response. TLR3 is known to signal through TRIF (Yamamoto et al., 2002; Oshiumi et al., 2003), while TLR8 signals through MyD88 (Han et al., 2012). In this study we found that Poly(I:C) induced MIP-1α, MIP-1β and RANTES production via MyD88, MIP-1α production was also mediated by TRIF, but TNFα production was dependent upon neither adapter protein. This suggests that in FMs, Poly(I:C), in addition
to activating the TLR3/TRIF pathway, may also be activating another receptor utilizing MyD88. Alternatively, in human FMs, TLR3 may be able to signal through both adapter proteins, similarly to TLR4 (Yamamoto et al., 2003). Indeed an early study reported that TLR3 could indeed utilize MyD88 (Alexopoulou et al., 2001). The lack of dependency for either MyD88 or TRIF for Poly(I:C)-induced TNFα could again suggest activation of an alternative, TLR3-independent pathway (Hoebe et al., 2003), such as the cytosolic RIG-like receptors (RLR) and non-RLR helicases (Vabret and Blander, 2013). We found a similar differential usage of MyD88 and TRIF in FMs exposed to viral ssRNA. Both adapter proteins played a role in the secretion of the majority of cytokotyos and chemokines induced by ssRNA, except for MCP-1β, which was only dependent upon TRIF. This suggests that in the FMs, TRIF can also utilize both MyD88 and TRIF, or that an additional TRIF-dependent pathway is activated. Indeed, Marshall-Clarke et al. (2007), reported that TRIF can sense both dsRNA and ssRNA. However, similarly to the Poly(I:C) response, TNFα was dependent upon neither adapter protein, as was MCP-1β production, again suggesting activation of a TLR-dependent pathway (Vabret and Blander, 2013).

Our last observation in this study was that Poly(I:C)-triggered the expression of the type interferon, IFNβ, and the IFN-inducible antiviral factors, OAS, MxA and APOBEC3G (Samuel, 2001; Turelli et al., 2004; Abrahams et al., 2006; Bonvin et al., 2006). However, the antimicrobial peptide SLPI, which is not regulated by type I IFNs, but has been shown to be regulated by TLR3 in other tissues (Schafer et al., 2005; Abrahams et al., 2006), was not induced in Poly(I:C)-treated FMs. Furthermore, and in contrast to the Poly(I:C)-induced response, treatment of FMs with viral ssRNA did not induce the expression of IFNβ or the antiviral factors. That FMs can generate this antiviral response to viral dsRNA is in keeping with observations that infection of human choriocarcinoma cells with influenza induces IFNβ (Uchide et al., 2002b), and treatment of FM explants with Poly(I:C) elevates the production of IL-29 (IFN-lambda3) (Nace et al., 2010), a virally induced type III IFN (Li et al., 2013).

In summary, we have demonstrated that viral dsRNA and ssRNA induce distinct pro-inflammatory cytokine and chemokine responses in an MyD88/TRIF-dependent and independent manner. Thus, FM inflammatory responses to viral dsRNA and ssRNA may be both TLR dependent and TLR independent. The FM response to viral dsRNA [Poly(I:C)] appears more protective since a strong type I IFN and antiviral response is also generated, while the ssRNA-induced response is predominantly pro-inflammatory, and FM sensing of viral ssRNA may activate the inflammasome giving rise to IL-1β secretion. Together these findings provide further evidence of how different pattern recognition receptors can generate distinct responses in the FMs through distinct mechanisms, and suggest a role for viruses in promoting inflammation of the chorioamnion.

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Authors’ roles

S.L.B and V.M.A. participated in the study design, data analysis and manuscript drafting. S.L.B, J.A.P. and M.H. performed the experiments. S.G. and C.S.H. facilitated tissue collection. S.G., E.R.N. and C.S.H. contributed to the final manuscript and critical discussion.

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Conflict of interest

None declared.

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