Activation of Toll-like receptor 3 reduces actin polymerization and adhesion molecule expression in endometrial cells, a potential mechanism for viral-induced implantation failure

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STUDY QUESTION: Does activation of endometrial Toll-like receptor 3 (TLR 3) affect cell receptivity to trophoblast adhesion?

SUMMARY ANSWER: TLR 3 activation in vitro reduces the attachment of trophoblast cells to endometrial cells by altering the cell cytoskeleton and reducing the expression of adhesion molecules in human endometrial cells.

WHAT IS KNOWN ALREADY: It is well documented that the presence of an infection at the time of implantation can lead to implantation failure. The female reproductive tract recognizes invading micro-organisms through the innate pathogen recognition receptors such as the TLRs.

STUDY DESIGN, SIZE, DURATION: Poly I:C was used as a TLR 3-specific ligand and endometrial cells were either treated or not with Poly I:C (treated versus control) in vitro. The experiments were performed in three replicates on three separate days.

PARTICIPANTS/MATERIALS, SETTING, METHODS: An in vitro assay was developed using RL95-2 (a human endometrial cell line) and JAr (a human trophoblast cell line) cells. Initially, the percentage of attached JAr spheroids to RL95-2 was measured in response to TLR 3 activation. Next, actin polymerization in RL95-2 cells was assessed in response to TLR 2/6, 3 and 5 activation. Phalloidin was used to assess the mean fluorescence intensity of F-actin by flow cytometry or confocal microscopy. Secondly, the influence of TLR 2/6, 3 and 5 activation on the expression of cluster of differentiation 98 (CD98) and β3 integrin was determined. To further understand through which pathways the TLR 3-induced alterations occur, inhibitors were applied for Toll/interleukin-1 receptor domain-containing adaptor inducing interferon-beta (TRIF), myeloid differentiation primary response 88 (MYD88), mitogen-activated protein kinases (MAPK) and nuclear factor pathways.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed that stimulation of TLR 3 in endometrial cells with different concentrations of Poly I:C led to a reduction in the percentage of trophoblasts attached to the endometrial cells in a dose-dependent manner (P < 0.05). This decrease was consistent in the Poly I:C treated group regardless of the co-incubation time (P < 0.05). In addition, our results demonstrated that actin polymerization and CD98 expression significantly decreased only in response to TLR 3 activation (P < 0.05). Activation of endometrial cells with TLR 2/6, 3 and 5 significantly reduced β3 integrin expression (P < 0.05). These alterations were shown to work via MYD88-MAPK pathways (P < 0.05).

LIMITATIONS, REASONS FOR CAUTION: This study has been performed in vitro. Future in vivo studies will be required in order to confirm our data.

WIDER IMPLICATIONS OF THE FINDINGS: This is a novel discovery which extends our current knowledge concerning diagnosis and treatment of viral-induced infertility cases.
Introduction

Embryo implantation requires communication between the endometrium (mother) and the embryo (Paria et al., 2002; Wang and Dey, 2006). Although the embryo should be regarded as an immunologically foreign entity, it is not rejected by the maternal immune system. It survives and forms a close physical contact with the endometrial cells throughout pregnancy. Thus a balanced reaction of the immune system is required not only to maintain the immune tolerance of the maternal–fetal immune tolerance system is required not only to maintain the immune tolerance of the maternal–fetal interface but also to protect the uterus from potential infections. Several factors are involved in the maternal–fetal immune tolerance during pregnancy, including hormones such as estrogen, progesterone and hCG (Schumacher et al., 2014), regulatory T-cells (Alijotas-Reig et al., 2014), cytokines such as interleukin (IL)-35 (Mao et al., 2013) and mucins (Redzovic et al., 2013). In addition, it seems that the pre-implanting embryo also has the ability to modulate the maternal tract immune system (Walker et al., 2010; Alminana et al., 2012). Consequently, any disturbance of the initial tolerance of the immune system may be responsible for early pregnancy failure.

A growing body of literature has shown that the activation of innate immune system in response to genital tract infection leads to infertility and pregnancy loss (Pellati et al., 2008; Dekel et al., 2010). The innate immune system in the female reproductive tract recognizes the invading micro-organisms through pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997, 2002). To date, 10 members of TLR family have been recognized in human (TLR1 to 10) and each of them responds to a specific ligand (Beutler, 2004). Between different TLRs, TLR 3 plays a crucial role in the antiviral responses by responding to double-stranded RNA (Schumacher et al., 2014). It has been shown that TLR 3 recognizes viruses such as cytomegalovirus and herpes simplex virus 1 (Matsumoto et al., 2011), whose strong association with female infertility has been shown in many studies (Yang et al., 1995; El Borai et al., 1997; Medvedev et al., 2009). Upon recognition of viruses by TLR 3, a cascade of signalling pathways is initiated that results in the activation of transcription factors such as nuclear factor (NF)-kB or activating protein (AP)-1 that promote the activation of pro-inflammatory genes (Carty et al., 2006). There is a considerable body of evidence that TLR 3 is expressed in the primary uterine epithelial cells (Schafer et al., 2005; Aflatoonian et al., 2007) and endometrial cell lines including ECC-1 (Schafer et al., 2004), Ishikawa (Aboussahoud et al., 2010a) and RL95-2 (Jorgenson et al., 2005).

Although TLRs have been shown to be involved in a number of pregnancy disorders, such as early pregnancy loss, preterm labour and preeclampsia (Koga et al., 2009), we know very little about their role in embryo implantation. The fact that the expression of all TLR family members increases at the late secretory phase, when the implantation of the embryo takes place (Aflatoonian et al., 2007), indicates the potential role of TLRs in regulating endometrial receptivity and embryo implantation. Our earlier results have demonstrated that the stimulation of TLR 2/6 and 5 in the maternal tract can reduce implantation chances in vivo and in vitro (Aboussahoud et al., 2010b; Sanchez-Lopez et al., 2014). In addition, other reports have demonstrated that during murine pregnancy, activation of TLR 3 by Poly I:C led to fetal losses (Zhang et al., 2007; Koga et al., 2009). Implantation failure is likely to be a major cause of infertility (Carver et al., 2003), thus understanding the role of TLR family members in implantation failure would shed light on treating infertility.

Ethical concerns regarding experimentation with primary human tissue do not allow wide usage of human embryos to study implantation disorders. To overcome these limitations, scientists have resorted to the use of cell lines and in vitro models of implantation (Hannan et al., 2010). In order to understand the molecular and cellular events involved in human implantation, an in vitro model was established, where the endometrium was simulated using the RL95-2 cell line. These cells are known to maintain the epithelial polarization, express adhesion molecules and microfilaments in the apical surface, and are responsive to hormones and secrete cytokines (Way et al., 1983). The human embryo was simulated with multi-cellular spheroids from the choriocarcinoma trophoblast cell line, JAr. This cell line is derived from term trimester trophoblast cells and is regarded as an invasive trophoblast cell line (Pattillo et al., 1971).

In the current investigation, we determined whether the activation of TLR 3 could affect the binding of trophoblast cells to endometrial cells. To achieve this aim, an in vitro human implantation model was employed. We also determined if TLR 3 activation could affect actin polymerization or the expression of adhesion molecules such as β3 integrin (Lesse et al., 1992; Illera et al., 2003) and cluster of differentiation 98 (CD98) (Dominguez et al., 2010) in endometrial cells, since these changes could represent the molecular mechanism responsible for TLR 3 suppression of trophoblast cells adhesion to endometrial cells. Our results showed that the activation of TLR 3 in endometrial cells decreases the adhesion of trophoblast cells to endometrial cells in vitro. In addition, TLR 3 stimulation leads to alterations in actin polymerization, CD98 and β3 integrin expression, through signal transduction pathways involving myeloid differentiation primary response 88 (MYD88) and mitogen-activated protein kinases (MAPK). These data open a new perspective for finding new prognostic markers for endometrial receptivity, which may facilitate better selection or treatment of affected individuals and may provide insight into the involvement of MAPK pathways in the pathogenesis of implantation failure caused by viral pathogens.

Methodology

Cell lines and cell culture

RL95-2 cell line was used as a model to mimic endometrial cells in vitro. They were grown in DMEM:F12 HAM supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Poole, UK), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen, Paisley, UK).
and 0.005 mg/ml Insulin (Sigma) in T75 flasks at 37 °C at 5% CO₂ atmosphere. The human choriocarcinoma cell line, JAr, was obtained from ATCC (catalog NO. HTB-144) and used as a model for trophoblast cells. JAr cells were grown in RPMI 1640 (Sigma), supplemented with 10% FCS (Invitrogen), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma), and 2 mM L-glutamine (Invitrogen). At confluency, cells were washed with Ca²⁺- and Mg²⁺-free Dulbecco’s phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). All experiments were performed in three replicates.

**Ligands, inhibitors and antibodies**

All ligands and inhibitors used in the current study were obtained from Invivogen (Inviogen, Toulouse, France). Poly Inosinic Poly Cytidilic Acid (Poly I:C), TLR 3 synthetic ligand, was used to stimulate TLR 3. Flagellin and FSL-1 were used to stimulate TLR 5 and 2/6, respectively. The Inhibitors used were Pepinh-TRIF and Pepinh-MYD88 to inhibit TRIF and MYD88 signalling pathways, respectively. SP600125 (JNK) was applied to inhibit the MAPK pathway. BAY11-7082 was used to inhibit NF-kB pathway. Anti-CD98h (FG1/10) and anti-β3 integrin (P97) mAb have been previously described (Dominguez et al., 2010).

**In vitro human implantation assay**

RL95-2 cells were cultured in T75 flasks until 100% confluency, thereafter cells were harvested by trypsin-EDTA. The cells were then counted and 3 × 10⁵ endometrial cells were cultured in each well of a 12-well plate. They were incubated for 4 days until confluency. To create multi-cellular spheroids from the monolayers of JAr cells, 10⁶ cells were counted with a Haemocytometer, and cultured in 5 ml of media in 60 × 15 mm Petri dishes (CellStar tissue culture dishes, greiner bio-one, GmbH/Germany) in a humid atmosphere containing 5% CO₂ at 37 °C on a gyratory shaker, set at 280 rpm overnight. The trophoblasts were gently transferred into each well of confluent endometrial cells in 12-well plates, and the co-culture was maintained in DMEM-F12, with supplements as mentioned above. Non-adherent spheres were removed from the monolayer using an automatic horizontal shaker to detach loosely bound or unbound spheres. In brief, once the trophoblast spheres were co-cultured with endometrial cells, the number of spheres was counted under the microscope and each plate was shaken at 200 rpm for 4 min. The cells were washed twice and then the number of attached spheres was counted under the microscope. The results were expressed as the percentage of spheres attached from the total number of spheres used to initiate the co-incubation experiments. All the experiments were performed in three replicates.

**Flow cytometry analysis**

RL95-2 cell monolayers were treated with the different stimuli, trypsinized and fixed with 2% paraformaldehyde and dispensed onto 96-well plates pre-loaded with the different primary mAbs at a concentration of 5 – 10 μg/ml, followed by FITC-labelled Rabbit anti-mouse IgG (DAKO). Labelled cells were analysed by flow cytometry in a Cytomics FC 500 MPL (Beckman Coulter).

**Actin polymerization assay**

RL95-2 cell monolayers, treated or not with the different stimuli, were fixed with 4% formaldehyde, scraped and loaded onto 96 wells. Cells were permeabilized with 0.5% Triton X-100 (5 minutes) and stained with Phalloidin Alexa Fluor 488 (Invitrogen). Mean fluorescence intensity of F-actin staining was analysed in a Cytomics FC 500 MPL (Beckman Coulter).

**Experimental design**

**The effect of Poly I:C concentrations on the binding of JAr spheroids to endometrial cells**

To investigate the effect of TLR 3 stimulation with different Poly I:C concentrations on the ability of JAr spheroids to bind to the endometrial cells, RL95-2 cells were cultured in 12-well plates and the media replaced with serum-free media. Poly I:C was then added to each well at four concentrations (0, 5, 10 and 25 μg/ml). RL95-2 spheroids were then treated with Poly I:C for 4 h. The effect of Poly I:C concentrations on the binding of JAr spheroids to endometrial cells was assessed as described.

**The effect of incubation time of Poly I:C on the binding of JAr spheroids to endometrial cells**

To understand if co-incubation duration is affecting the outcome of trophoblast binding to the endometrial cells, RL95-2 cells were pre-incubated with 10 μg/ml of Poly I:C for 4 h. 30 JAr spheroids were then delivered onto the confluent endometrial cell monolayers in 12-well plates and co-incubated for 1 h. The adhesion was assessed as described.

**The effect of TLR 3 activation on the viability of the endometrial cells**

To determine whether TLR 3 activation is affecting the viability of RL95-2 cells, they were stimulated with different concentrations of Poly I:C (0.5, 10 and 25 μg/ml) for 4 h. The viability of the cells was verified by PI staining in the FACS Calibur cytometer.

**Viability assessment of endometrial cells**

In order to check the viability of RL95-2 cells, they were grown in 12-well plates to 100% confluency. The media was replaced with serum-free media before they were either treated or not with Poly I:C for 4 h. The cells were then harvested using trypsin-EDTA and collected in 500 μl of media and pelleted by centrifugation at 300 g for 5 min. The cells were then resuspended in 200 μl of DPBS and divided into two 5 ml cytometry tubes. One sample was used as autofluorescence and the other stained with propidium iodide (PI; Life technologies, Paisley, UK) 3 × 10⁵ events and the percentage of PI positive events (dead cells) was registered. The results were expressed as percentage of live cells and were compared using a one-way ANOVA and P < 0.05 was considered significant.
Effect of TLR 2/6, 3 and 5 activation on actin polymerization, CD98 and β3 integrin expression in endometrial cells

RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. They were then incubated with 10 μg/ml of Poly I:C for various time points (30 min, 1, 2 and 4 h). In addition, to identify the specificity of Poly I:C effect on altering actin polymerization, CD98 and β3 integrin expression, the influence of some other TLR ligands such as Flagellin and FSL-1 on these alterations was tested. RL95-2 cells were treated with Flagellin (100 ng/ml) and FSL-1 (100 ng/ml) for 4 h. The incubation was further extended to 24 h to detect any potential responses of RL95-2 cells to these ligands. Actin polymerization, CD98 and β3 integrin expression were assessed by flow cytometry.

These results were further confirmed on RL95-2 monolayers of Poly I:C treated cells by confocal microscopy. RL95-2 monolayers were stained for F-actin, CD98 or β3 integrin. Briefly, RL95-2 cells were cultured in 24-well plates on the coverslips and the media replaced with serum-free media. Poly I:C was then added to each well at a concentration of 10 μg/ml in various time points (1, 2 and 4 h) and F-actin, CD98 and β3 staining was performed as described and optical sections were acquired by confocal microscopy.

Analysis of the signalling pathways downstream TLR 3 ligation on actin polymerization, CD98 and β3 integrin expression in RL95-2 cells

To determine the signalling pathways involved in the effect observed after TLR 3 ligation, different specific inhibitors were used. In order to assess the involvement of TRIF pathway RL95-2 cells were exposed to Pepinh-TRIF, an efficient TRIF signalling pathway inhibitor. The endometrial cells were pretreated with Pepinh-TRIF (20 μM for 6 h) at 37°C. Poly I:C was then added to each well at a concentration of 10 μg/ml and incubated for 4 h. Similarly, other signalling pathways such as MYD88 and its downstream targets the NF-kB and MAPK pathways, were assessed by treating RL95-2 cells with Pepinh-MYD88 (10 μM for 6 h), BAY11-7082 (20 μM for 1 h) or SP600125 (50 μM for 1 h) at 37°C. Poly I:C was then added to each well at a concentration of 10 μg/ml and incubated for 4 h. Actin polymerization, CD98 and β3 integrin expression were assessed by flow cytometry. The activity of the inhibitors had been validated before (Yoshizawa et al., 2008; Caballero et al., 2013) and only their functional dose was applied in this set of experiments.

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed using ANOVA (Statistica: Statsoft UK, Letchworth, UK) with Fisher’s multiple comparison test. P < 0.05 was considered to be significant.

Results

Poly I:C presence decreased trophoblast spheroid attachment to endometrial cells in a dose-dependent manner

To understand if Poly I:C concentration influenced the outcome of trophoblast binding to the endometrial cells, Poly I:C was added to RL95-2 cells at various concentrations (0, 5, 10 and 25 μg/ml). The treatment of RL95-2 (Fig. 1a) with different Poly I:C concentrations significantly decreased the percentage of the attached JAr spheroids to the endometrial cells compared with the control group (P < 0.05). The adhesion decreased as the concentration of Poly I:C increased.

Addition of Poly I:C had a negative effect on trophoblast spheroid binding to endometrial cells

To investigate the effect of co-incubation time of Poly I:C treated RL95-2 cells with JAr spheroids on the binding ability of trophoblast cells, RL95-2 cells were pre-incubated with 10 μg/ml of Poly I:C for 4 h. JAr spheroids were then delivered onto the confluent endometrial cell monolayers and co-incubated for various durations (0.5, 1, 2 or 4 h). Addition of Poly I:C to RL95-2 cells reduced the percentage of attached JAr spheroids to endometrial cells (Fig. 1b). This decrease was consistent in the Poly I:C treated group regardless of the co-incubation time.

The viability of the endometrial cells was unaltered after TLR 3 activation

To assess whether the viability of RL95-2 cells is affected by TLR 3 stimulation, they were stimulated with different concentrations of Poly I:C (0, 5, 10 and 25 μg/ml) for 4 h and the viability of the cells was verified by PI staining. The stimulation of the RL95-2 cells with Poly I:C did not affect the viability of the cells. Around 90% of the non-stimulated cells remained viable after harvesting from the wells. The viability of the cells stimulated with Poly I:C was also around 90% of the total population (Fig. 1c).

Actin polymerization was decreased in response to TLR 3 but not TLR 2/6 or 5 activation in RL95-2 cells

To determine the effect of TLR 2/6, 3 and 5 activation on actin polymerization, RL95-2 cells were treated with TLR ligands and actin polymerization was assessed as described. As can be seen from Fig. 2a, the mean fluorescent intensity of F-actin gradually decreased in Poly I:C treated RL95-2 cells compared with the control (P < 0.05). Poly I:C significantly reduced actin polymerization even after 30 min. However, addition of Flagellin (Fig. 2b) and FSL-1 (Fig. 2c) to RL95-2 cells did not alter the levels of intracellular filamentous actin.

CD98 expression was decreased only in response to TLR 3 activation in RL95-2 cells

To assess whether the activation of TLR 2/6, 3 and 5 affected CD98 expression, RL95-2 cells were treated with TLR ligands and CD98 expression was measured as described. It is apparent from Fig. 3a that CD98 expression was gradually decreased in Poly I:C treated RL95-2 cells compared with the control (P < 0.05). This effect started after 30 min of Poly I:C administration. However, this effect was not observed when Flagellin (Fig. 3b) and FSL-1 (Fig. 3c) were added to RL95-2 cells.

β3 integrin expression was decreased in response to TLR 2/6, 3 and 5 activation in RL95-2 cells

To clarify the influence of TLR 2/6, 3 and 5 on β3 integrin expression, the endometrial cells were treated with TLR ligands and β3 integrin...
expression was assessed as described. As shown in Fig. 4a, β3 integrin expression was gradually decreased in Poly I:C treated RL95-2 cells compared with the control (P < 0.05). Poly I:C reduced β3 integrin expression significantly after 30 min. In contrast to F-actin or CD98, stimulation with Flagellin (Fig. 4b) and FSL-1 (Fig. 4c) also significantly decreased β3 integrin expression, as soon as 4 h of TLR ligand treatment (P < 0.05).

F-actin and CD98’s content fluorescent intensity was decreased in response to TLR 3 activation

To further corroborate flow cytometry results in non-disturbed cell monolayers, confocal analyses were performed. As shown in Fig. 5a, confocal microscopy revealed that control cells showed the typical distribution of F-actin in cell–cell contacts, and actin cables. Stimulation of cells with Poly I:C resulted in an overall decrease in F-actin content with redistribution of F-actin and concomitant morphological changes. This effect was observed after 1 h of Poly I:C treatment (P < 0.05). Similarly, addition of Poly I:C significantly reduced CD98 content fluorescence intensity compared with the control (Fig. 5b) (P < 0.05). This effect was observed after 1 h of Poly I:C treatment. β3 integrin staining was however too faint to be assessed by confocal microscopy as reported before (Dominguez et al., 2010).

MYD88 inhibitor restored the levels of F-actin polymerization, CD98 and β3 integrin expression in response to TLR 3 activation in RL95-2 cells

To assess whether the TLR 3-induced alteration in F-actin content and CD98 and β3 integrin expression occurred via TRIF, RL95-2 cells were treated with TRIF signalling inhibitor. Addition of a functional dose of Pepinh-TRIF (20 μM) to RL95-2 cells was not able to reverse the effects of Poly I:C treatment on actin polymerization (Fig. 6a), CD98 (Fig. 6b) and β3 integrin expression (Fig. 6c). Since the TRIF pathway did not seem to be involved in the effects observed upon TLR 3 ligation, we assessed the role of the MYD88 signalling cascade. Inhibition of the MYD88 pathway by pretreatment of the endometrial cells with Pepinh-MyD88 recovered the effect of Poly I:C on actin polymerization (Fig. 6a), CD98 (Fig. 6b) and β3 integrin expression (Fig. 6c) (P < 0.05).
Figure 2  The effect of TLR 2/6, 3 and 5 activation on actin polymerization in endometrial cells. RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. RL95-2 cells were then incubated with 10 μg/ml of Poly I:C for various time points (30 min, 1, 2 and 4 h) (a). In addition, RL95-2 cells were treated with Flagellin (100 ng/ml) (b) and FSL-1 (100 ng/ml) (c) for 4 and 24 h. Actin polymerization was assessed by flow cytometry. Each experiment was performed in triplicate on different experimental days. The data are the average of three independent experiments. MIF: mean fluorescent intensity. Different letters denote significant differences. P < 0.05 was considered to be significant. The results were presented as the mean ± SEM. ANOVA was used to compare the fluorescent intensity in ligand treated RL95-2 cells and control group.

Figure 3  The effect of TLR 2/6, 3 and 5 activation on cluster of differentiation 98 (CD98) expression in endometrial cells. RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. RL95-2 cells were then incubated with 10 μg/ml of Poly I:C for various time points (30 min, 1, 2 and 4 h) (a). In addition, RL95-2 cells were treated with Flagellin (100 ng/ml) (b) and FSL-1 (100 ng/ml) (c) for 4 and 24 h. CD98 expression was assessed by flow cytometry. The data are the average of three independent experiments. Different letters denote significant differences. P < 0.05 was considered to be significant. The results were presented as the mean ± SEM. ANOVA was used to compare the fluorescent intensity in ligand treated RL95-2 cells and control group.
Inhibition of MAPK but not NF-κB restored the levels of F-actin, CD98 and β3 integrin expression in response to TLR 3 activation in RL95-2 cells

To determine the signalling pathways involved in the effect observed after TLR 3 ligation, various specific inhibitors were used for NF-κB and MAPK pathways. Addition of SP600125 to RL95-2 cells significantly restored the Poly I:C-induced reduction in actin polymerization (Fig. 7a), CD98 (Fig. 7b) and β3 integrin expression (Fig. 7c) (P < 0.05). However, addition of Bay11-7082 was unable to recover these alterations in RL95-2 cells (Fig. 7a–c).

Discussion

Infertility is being treated by a variety of assisted reproductive techniques (ART). However, embryo implantation remains the rate-limiting factor for the success of ART and in particular ART therapies involving IVF (Stafford-Bell and Copeland, 2001; Salamonsen et al., 2002; Dekel et al., 2010). To overcome this problem, different markers have been proposed as a determinant of endometrial receptivity, but at the moment none of the functional studies have demonstrated any clinical application for them (Horcajadas et al., 2007). Thus there is a great need for understanding the molecules that can affect the endometrial receptivity so that they can be used as diagnostic tools in clinic. Furthermore, endometrial factors which may be pivotal for the improvement of endometrial receptivity could act as targets for a new range of therapies to enhance endometrial receptivity (Salamonsen et al., 2002). The current study was designed as an approach to explore how the activation of TLR 3 could affect embryo implantation. Furthermore, it provided insight into the molecules whose alterations via TLR 3 activation could affect the adhesion of trophoblast cells to endometrial cells. This information will help us to better understand the process of endometrial receptivity and potential impact of immune system on this process.

The results from our experiments clearly showed that TLR 3 activation in RL95-2 cells reduced the adhesion of trophoblast cells to endometrial cells in vitro. Moreover, TLR 3 stimulation led to alterations in cell cytoskeleton arrangements and expression of adhesion molecules at least in vitro. The treatment of RL95-2 cells with Poly I:C significantly induced actin depolymerization and decreased the expression of β3 integrin and CD98. Moreover, MYD88–MAPK signalling seemed to be involved in the Poly I:C-induced decrease in actin polymerization and expression of adhesion molecules, as blocking their activation with specific inhibitors restored the actin depolymerization and disruption of adhesion molecules expression in TLR 3-stimulated endometrial cells.

The plasma membrane of epithelial endometrial cells undergoes morphological changes at the time of implantation, which is known as ‘plasma membrane transformation’ (Murphy, 1993, 1995). Some of these
alterations include shortening and flattening of the microvilli and loss of surface negative charge. Another aspect of plasma membrane transformation involves the large apical protrusions that are found in a wide variety of species during the peri-implantation period (Martel et al., 1991) and are called pinopodes (Enders and Nelson, 1973; Parr and Parr, 1974). The pinopodes appear to be the most reliable morphological marker of receptive endometrium at the time of implantation (Murphy and Dwarte, 1987; Nikas et al., 1995) and represent sites of preferential blastocyst attachment (Murphy, 1995, 2000). Since actin microfilaments are the major components of the cell cytoskeleton and play a crucial role in cell morphology and rearrangement, it is reasonable to believe that these filaments can also influence the process of plasma membrane transformation at the window of implantation. This assumption has been further supported by many studies, where it has been shown that adhesion of trophoblast cells to endometrial epithelial cells depends on the actin cytoskeleton (Thie et al., 1997; Heneweer et al., 2002). Moreover, it is well established that pinopodes are richly invested with a dense network of F-actin (Luxford and Murphy, 1992). TLR 3 activation in RL95-2 cells led to a decrease in, and disruption of, actin polymerization (also known as actin depolymerization) that can be regarded as a potential mechanistic pathway of implantation failure via innate immune activation. This decrease in F-actin content was further confirmed by confocal microscopy. This is consistent with the results of West et al., where addition of LPS and Poly I:C to dendritic cells (DCs) induced F-actin disassembly soon after 30 min of TLR ligand treatment (West et al., 2004). Hsu et al. also reported that F-actin filaments were disassembled and decreased in Poly I:C-treated neuroblastoma cell lines (Hsu et al., 2013).

The disassembly in F-actin content in dendritic cells or other members of the immune system in response to TLR activation might be desirable, as upon detection of an infectious stimulus, DCs would initiate a transient phase of enhanced actin-dependent capture of local antigens. In contrast, in endometrial cells this disruption of F-actin formation could affect the receptivity of the endometrium and interfere with the process of embryo attachment to the endometrial cells. This has been further supported by Thie and his colleagues (Thie et al., 1997), where they found that inhibition of actin polymerization by cytochalasin D impaired JAr spheroid attachment to RL95-2 cells in vitro. Different pathways have been proposed as the mechanisms through which TLR activation leads to F-actin disruption. West et al. has shown that TLR signalling induces activation of ADAM17, which then cleaves key surface proteins required for cell-substrate contact and podosome formation (West et al., 2008). Other studies have shown that TLR-induced actin reorganization signals through routes such as MYD88, TRIF and MAPK, whose signalling might affect these alterations (West et al., 2004; Kong and Ge, 2008; Shin et al., 2009). Hence assessing the involvement of these pathways in TLR3-induced actin depolymerization would shed light on the mechanisms of cell cytoskeleton alterations in response to Poly I:C.
Adhesion molecules play a crucial role in the attachment of embryo to endometrial cells at the window of implantation (Aplin et al., 1996; Aplin, 1997; Aplin and Kimber, 2004; Kaneko et al., 2013). Among adhesion molecules, β3 integrin (Kuno et al., 1998) and CD98 (Dominguez et al., 2010) expression increases significantly at the window of implantation. Investigations in human (Kaneko et al., 2011) and mice (Illera et al., 2000) have shown that the blockade of β3 integrin expression in the endometrium led to failure of blastocyst attachment to the endometrial epithelial cells. Moreover, aberrant patterns of β3 integrin expression have been correlated with certain diagnoses in infertile women such as unexplained infertility (Lessey and Castelbaum, 1995). Despite all this evidence that clearly confirm the importance of β3 integrin in the process of embryo implantation, its value as a prognostic indicator of successful implantation remains uncertain (Creus et al., 1998; Gonzalez et al., 1999; Lessey et al., 2000). Likewise CD98 seems to play a crucial role in the implantation process, as its blockade in RL95-2 cells remarkably impairs mouse blastocyst attachment to endometrial cells (Dominguez et al., 2010).

In the current study, we characterized the role of TLR 3 activation in the expression of CD98 and β3 integrin in RL95-2 cells. Expression of both molecules was suppressed. This can be another potential indication of the mechanism of action of TLR 3 activation in reducing cycle fecundity in women.

CD98 is a type II integral membrane protein (Dong and Hughes, 1997) and it appears to play a pivotal role in regulation of integrin-mediated cell adhesion (Fenczik et al., 1997), cell differentiation, growth, transformation and apoptosis (Warren et al., 1996). Involvement of different subunits of the MAPK pathway including p38 and ERK has been shown in CD98 functions such as integrin-mediated cell adhesion and aggregation (Melchior et al., 2008). Moreover, inhibition of the ERK and p38 pathways with specific inhibitors resulted in suppression of CD98-mediated induction of tyrosine-protein kinase Src (c-Src) (Miyamoto et al., 2000). TLR 3 activation by Poly I:C significantly induced MAPK pathway activity by increasing the production of AP-1 in Fibroblast-like synoviocytes (FLS) (Yoshizawa et al., 2008). Hence, it is logical to assume that TLR 3-induced reduction of CD98 expression in our experiments is mediated through the MAPK pathway, which would result in a negative feedback to limit the overall activation of the CD98 pathway. Further work is required to establish the role of MAPK pathway in this system.

It is a well-established dogma in the innate immunity-related literature that TLR 3 signalling follows the TRIF-dependent pathway. Once TLR 3 binds to its ligand it recruits Toll/IL-1 receptor domain-containing...
adaptor inducing IFN-beta (TRIF). TRIF triggers an intracellular cascade of molecular reactions, which leads to stimulation of the transcription factors NF-κB and Mitogen-activated protein kinases (MAPKs). MAPKs are important family of protein kinases involved in transmitting signals from the cell membrane to the nucleus. The MAPK signalling cascade regulates a variety of cellular activities, including cell growth, differentiation, survival and cell death (Zhou et al., 2013). These transcription factors are translocated to the nucleus after activation, which results in induction of type I IFN, pro-inflammatory cytokines and chemokines and maturation of dendritic cells (Matsumoto et al., 2011). To understand the pathways through which TLR 3 activation leads to alterations in the cell cytoskeleton and a decrease in expression of β3 integrin and CD98 and involvement of TRIF in mediating this process, we employed an inhibitor of TRIF pathway, Pepinh-TRIF. This led to some unexpected observations. Addition of a functional dose of Pepinh-TRIF was unable to restore the reduction in actin polymerization or CD98 or β3 integrin expression. Hence, TRIF is unlikely to be involved in TLR3 activation leading to alterations in cell cytoskeleton and a decrease in expression of β3 integrin and CD98. Thus, we sought to determine the effect of other pathways such as myeloid differentiation primary response 88 (MYD88) on mediating this effect. Surprisingly, MYD88 inhibition recovered the alteration in cell cytoskeleton and adhesion molecule expression. This finding on the one hand corroborates the ideas of Brenner et al. (2012), who showed that MYD88 knock down, but not TRIF, inhibited Poly I:C induced cytokine production in adipocytes (Brenner et al., 2012), which shows the importance of MYD88 signalling pathway in TLR3-induced cytokine production. Similarly, the TLR 3-induced production of cytokines such as MCP-1 and IL-6 was significantly suppressed in MYD88−/− mice, which were inoculated with Poly I:C (Chun et al., 2010). This finding differs from many published descriptions of TLR 3 signalling in human model systems, in which TRIF has been shown to be the specific and the only adaptor molecule for the TLR 3 signalling pathway (Matsumoto et al., 2011, Yu and Levine, 2011). The disparity between these studies and our finding could probably be due to the fact that TLR 3 signalling is cell type or tissue-specific.

Kutsuna et al. (2004) found that the reduction in F-actin content in human neutrophils induced by tumour necrosis factor (TNF), Granulocyte macrophage colony-stimulating factor (GM-CSF), and Granulocyte colony-stimulating factor (G-CSF) was restored by inhibition of MAPK pathway activation (Kutsuna et al., 2004). Consistent with these

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**Figure 7** The effect of inhibition of mitogen-activated protein kinases (MAPK) and NF-κB signalling pathways on actin polymerization, CD98 and β3 integrin expression in RL95-2 cells. RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. RL95-2 cells were pre-treated with SP600125 (50 μM) and BAY11-7082 (20 μM) for 1 h in separate wells. They were then incubated with 10 μg/ml of Poly I:C for 4 h. Actin polymerization (a), CD98 (b) and β3 integrin expression (c) were assessed by flow cytometry. The data are the average of three independent experiments. Different letters denote significant differences (P < 0.05). Each experiment was performed in triplicate on different experimental days. The results were presented as the mean ± SEM. ANOVA was used to compare the mean fluorescent intensity in different groups.
finding, West et al. showed LPS-induced disassembly of F-actin formation in dendritic cells was recovered after addition of MAPK inhibitors (West et al., 2004). To the best of our knowledge, there are no published data examining the effect of MAPK pathway after implantation failure. Nevertheless, involvement of the MAPK pathway in the trophoblast spheroid outgrowth on a RL95-2 monolayer has been demonstrated before (Li et al., 2003; Hsu et al., 2008), where the activation of p38 MAPK/JNK induced apoptotic pathways (Fas/Fasl) in endometrial epithelial cells (EECs). This facilitated trophoblast-induced apoptosis and displacement of EECs. In addition, many studies have reported the role of MAPK pathway in the pathogenesis of endometriosis, as one of the known causes of infertility in women (Yoshino et al., 2004; Zhou et al., 2010; Lee et al., 2012; Huang et al., 2013; Li et al., 2013). Based on the novel data obtained in the current study, blocking and regulating the MAPK pathway by its inhibitors can be a potential new strategy to prevent and treat viral-induced infertility cases undergoing ART techniques.

Taken together, the identification of MYD88/MAPK as a key pathway for the TLR 3-mediated defect in actin polymerization, and CD98 and β3 integrin expression, provides a new insight into the process of viral-induced implantation failure. These findings may not only have important implications in diagnosis of viral-induced implantation defects, but also may be used as new therapeutic approaches in treatment of infertility cases. Application of inhibitors for MYD88/MAPK pathway may make several noteworthy contributions to treating infertility.

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Authors’ roles

Each author made substantial contributions to the design of the study, the interpretation of the data and the drafting and revising of the submitted manuscript. A.F. and M.Y.-M. designed the original study and M.M. designed and operated the experimental work. J.A.S.-L also participated on the design of the study. M.M., I.C., J.A.S.-L., N.M., S.E., S.L.-M., M.Y.-M. and A.F. all contributed to the follow-up study design, data collection and manuscript preparation. All authors have seen and approved the final submitted manuscript.

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Conflict of interest

None declared.

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