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Title: Local activation of uterine Toll-Like Receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity

Running title: Tlr2 and 2/6 activation affects uterine receptivity

Summary sentence: The activation of uterine innate immune system through TLR 2 and 2/6 affects the endometrial receptivity and embryo implantation in vivo in mice and in vitro in a human trophoblast-epithelial cells adhesion model.

Key words: Endometrium, Female Infertility, Implantation, Toll-Like Receptors (TLR), Trophoblast Spheroids.

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Abstract:

Embryo implantation is a complex interaction between maternal endometrium and embryonic structures. Failure in implantation is highly recurrent and impossible to diagnose. Inflammation and infections in the female reproductive tract are common causes of infertility, embryo loss and preterm-labor. The current work describes how the activation of endometrial Toll-like receptor (TLR) 2 and 2/6 reduces embryo implantation chances. We developed a morphometric index to evaluate the effects of the TLR 2/6 activation along the uterine horn (UH). TLR 2/6 ligation reduced the endometrial myometrial and glandular indexes and increased the luminal index. Furthermore, TLR 2/6 activation increased the pro-inflammatory cytokines such as Interleukin (IL)-1β and Monocyte Chemotactic Protein (MCP)-1 in UH lavages in the pre-implantation day and IL-1 Receptor Antagonist in the implantation day. The engagement of TLR 2/6 with its ligand in the uterine horn during embryo transfer severely affected the rate of embryonic implantation (45.00 ± 6.49 vs 16.69 ± 5.01%, $p < 0.05$, control vs test; respectively). Furthermore, this interference with the embryo

implantation process was verified using an in vitro model of human embryo implantation where trophoblast spheroids failed to adhere to a monolayer of TLR 2 and TLR 2/6 activated endometrial cells. The inhibition of TLR receptors 2 and 6 in the presence of their specific ligands, restored the ability of the spheroids to bind to the endometrial cells. In conclusion, the activation of the innate immune system in the uterus at the time of implantation interfered with the endometrial receptivity and reduced the chances of implantation success.

Introduction

Embryonic implantation is a critical event leading to a successful pregnancy[1]. The implantation process requires a complex orchestration of cellular and molecular events that include: expression of adhesion molecules, remodeling of the uterine extracellular matrix and an intricate crosstalk of hormones, cytokines and growth factors between the embryo and the endometrium[2-6]. Immune system plays an important role in the modulation of the mechanisms involved in implantation. Pregnancy represents an immunological contradiction in which a semi-allogeneic foreign entity, the embryo, is not rejected by the maternal immune system but accepted and nourished. This is achieved by several mechanisms, including the modulation of the maternal immune system by the pre-implantation embryo[7]. Consequently, any interference that may imbalance the immune system responses could result in embryo loss and infertility. In fact, many infertility problems in women are associated with infections of the upper female reproductive tract (FRT) compartments: endocervix, fallopian tubes and uterus. The pathogens responsible for sexually transmitted diseases (STD) such as Mycoplasma genitalium, Neisseria gonorrhoeae and Chlamydia trachomatis have been associated with infertility in women[1,8].

The FRT is able to respond against these pathogenic entities and initiate an immune response. The first recognition of pathogens in the FRT is mediated by the innate immune system of epithelial, resident dendritic and natural killer (NK) cells[3,7,9]. This innate response will later prime and instruct the adaptive immune system to initiate cellular and humoral responses against pathogens[7,9,10]. In order to detect the potential pathogens, endometrial epithelial cells express pattern recognition receptors (PRRs) to common

pathogen-associated molecular patterns (PAMPs). One of the main families of PRRs is TLR family[9,11]. They can recognize a great variety of PAMPs from bacterial, fungal, parasitical and viral origin. The TLR family is formed by 10 members where each respond to a specific ligand that promotes the secretion of a different subset of cytokines and chemokines[12-15]. TLR 2 and its heterodimers with TLR 1 and TLR 6 can sense a great variety of PAMPs from pathogens that might be present in the FRT and can be activated by peptidoglycans (PGN) from bacteria (C. trachomatis, Staphylococcus aureus, N. gonorrhoeae, M. genitalium lipoproteins)[16], yeast (Candida albicans phospholipomannan)[17] and parasites (Trichomonas vaginalis lipophosphoglycan)[18]. The heterodimer of TLR 2 and TLR 6 has been found to recognize the macrophage activated lipoprotein derived from Mycoplasma fermentans (MALP-2)[19]. FSL-1 is a specific and potent ligand for the heterodimer TLR 2/6 and was synthesized based on MALP-2 structure by changing its amino acid sequence[20]. Upon this recognition, they initiate signaling pathways that end up in the activation of the transcription factors nuclear factor (NF)-κB or AP-1 that promote the activation of pro-inflammatory genes[21].

The expression of TLRs 1-10 has been observed in mammalian uterus and in different human endometrial epithelial cell lines[13]. This expression follows the menstrual hormonal cycle. The highest level of TLR expression in the human endometrium has been observed during the late secretory phase, where implantation of the embryo takes place[22]. Although TLRs have been observed to be involved in a number of pregnancy disorders such as preterm labor, early pregnancy loss and pre-eclampsia[23], not much is

known about their function during embryo implantation. Recent results from our laboratory have shown that activation of TLR5 by its ligand flagellin can interfere with trophoblast spheroids adhesion to epithelial cells in vitro[24].

The study of the human embryo implantation process in vivo is limited due to the anatomical and the physiological restrictions and the ethical and the legal issues surrounding experimentation with human embryos. For these reasons, research on the embryo implantation has opted to employ different animal model species. The human embryo implantation is an invasive process, which makes it different from other mammalian species. The closest in vivo model to human is the primate embryo implantation[25] but the manipulation of these species is also limited due to ethical responsibility. The murine model is a good animal model that shares similar implantation characteristics with human embryo implantation such as its invasiveness[26]. It also offers many advantages like the possibility to manipulate their gametes and estrous cycle (between 4 to 5 days) and a short gestation (Approx. 3 weeks)[27]. Implantation of the murine embryo will take place on metestrus (day 4.5) the equivalent of the late secretory phase in human. Another alternative for the study of embryo implantation are the in vitro models where different cell lines, primary cells or tissue explants mimic both endometrium and embryo[28]. The selection of the appropriate cell lines is a fundamental issue to be considered due to the differences in endometrial and trophoblast cell developmental stages[28]. In the current investigation, we employed an in vitro model of human implantation, where the endometrium was simulated using the RL95-2 cell line which has been reported to maintain the epithelial polarization, express adhesion molecules and microfilaments in the apical surface and being responsive to hormones and

secretion of cytokines[29]. The breast carcinoma epithelial cells MCF7 were also employed in our investigations to observe if the trophoblasts were able to interact with another epithelial cell line. MCF7 maintain epithelial cell characteristics and are responsive to hormonal stimulation, hence they were used as another hormone responsive reproductive tissue from a non-endometrial origin[30]. The human embryo was simulated with multi-cellular spheroids from choriocarcinoma trophoblast cell line JAr. This cell line has been employed in different studies for its ability to establish adhesive interactions with endometrial cell lines in short periods of co-incubation[31].

In the current investigation, we determined if the activation of the TLR 2 could influence the implantation of the embryo. The problem was explored using two different approaches. Initially we used an in vivo murine embryo implantation model, to assess if the activation of TLR 2/6 in the UH may affect uterine morphology and secretory profile during implantation period as well as embryonic implantation. Thereafter, we corroborated these observations in an in vitro human implantation model. In summary, the activation of TLR 2/6 in vivo decreased the murine embryo implantation rate with significant changes in tissue morphology and protein secretion. In addition, the in vitro adhesion of trophoblast spheroids to the endometrial cells was affected specifically by the activation of the endometrial TLRs.

Materials and Methods

Embryo transfer experiments

Mice superovulation and mating

C57/CBA mice were obtained from Harlan Laboratories. All mice used in the experiments were housed under controlled temperature conditions of 22°C with a 14/10 h light/dark cycle, 40-60% relative humidity and free access to water and food. Females were kept in cages of 1000 cm^2 in groups of 8-10. Males were kept individually in 250 cm^2 cages. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee guidelines from the INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria) and in adherence with guidelines established in the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the Society for the Study of Reproduction. Females were injected i.p. with 7.5 IU of equine chorionic gonadotropin (eCG; Foligon 500, Intervet), followed 48 h later by 7.5 IU of human chorionic gonadotropin (hCG; Veterin Corion, Equinvest). Thereafter each female was placed in a male cage (2:1) for mating. On the next day (day 0.5 8:00-10:00 h) the females with vaginal plug were separated from the males. The selected mice were euthanized by cervical dislocation 3.5 days post-mating (embryos reached blastocyst stage). Both uterine horns were dissected and placed in a petri dish in pre-warmed M2 media. The lumen of each horn was rinsed with M2 media (M7167, Sigma, Dorset, UK), using a 1ml syringe with a 30G needle. The blastocysts were collected under a stereoscopic microscope using a glass micropipette and rinsed twice in M2 media. Finally they were rinsed once in pre-warmed K^+ modified simplex optimized medium (KSOM, KSOMaa Evolve, ZEKS-050, Zenith Biotech) for at least 30 min until

embryo transfer. The blastocysts were rinsed in warmed up M2 media and placed in a transfer pipette.

Foster mice preparation

The foster CD1 mice were obtained from Harlan Laboratories. The females bedding was replaced with male bedding to initiate the estrous cycle. At the start of the estrus phase of the cycle (two days later), the females were mated with vasectomized males to induce the pseudo-pregnancy state and prepare the reproductive tract to receive the embryos. The foster pseudo-pregnant mice were selected by the presence of a vaginal plug. On day 3.5 post-mating, females were anesthetized by inhalation of Isofluorane and kept on a warm plate during the surgery. The left uterine horn was exposed and the top of the utero-tubal junction was carefully pierced with the tip of a 30G needle where a transfer pipette loaded with the blastocysts was inserted. The pipette reached the uterine horn (UH) through the opened utero-tubal junction and delivered the blastocysts. The foster mice were kept in separate cages with tags, identifying the date of embryo transfer, number of embryos transferred and treatment. Food and water were supplied ad libitum.

Histological evaluation of the murine UHs

Sample collection and storage

The foster mice were euthanized by cervical dislocation. UHs were dissected and placed in petri dishes. The UHs were stored in 10% paraformaldehyde and dehydrated in a series of ethanol dilutions (70, 90 and 100%) for fixation. Finally UHs were sectioned in two and embedded in paraffin. The paraffin blocks were cut in transversal sections of 4 μm

using a microtome and fixed to normal microscope slides. The sections on the slides were then stained using the conventional Haematoxylin & Eosin (H&E) method. Briefly, wax was removed by rinsing twice in xylene for 5 min. Tissue was hydrated in series of ethanol dilutions (Twice in 99, 95 and 70%) and tap water for 5 min. The slides were stained with Gill II Haematoxylin (VWR, Lutterworth, UK) for 1 min and rinsed with tap water for 3 min. The slides were stained in 1% aqueous Eosin (VWR) with 1% Calcium Carbonate (Sigma) for 5 min and rinsed in tap water for 30 sec. The sections were dehydrated in serial dilutions of ethanol (70 and 95% for 10 sec and twice 99% for 30 sec) and twice in Xylene (1 and 3 min). Finally the coverslip was fixed with DPX mountant (VWR).

The cross sections in the slides were imaged using light microscopy (Olympus CKX41; Southend-on-sea, UK) and captured with the NIS elements software (Version F3.0 SP4; Nikon, Surrey, UK). The changes of the development of the uterine decidua due to estrus cycle were observed.

Morphometric analysis of the UH

The images of 10 cross-sections were captured using the 20x objective and measured using the software ImageJ (NIH, Bethesda, MD, USA). This step was taken to ensure the consistency of the measurements along the length of the horn. The scale for ImageJ area measurements was set using the scale bar (500 µm) given by the capture software. Each respective area was measured by outlining it with the free hand selection tool and the area obtained using the measure option. For each UH cross-section the total area (A_T; myometrium, endometrial stroma and epithelium and lumen), endometrial area (A_{el};

including lumen), luminal area (A_L) was measured and the number of glands (nG) was counted. The endometrial area (A_E) and the myometral area (A_M) were calculated as follows:

$$A_E = A_{el} - A_L$$

$$A_M = A_T - A_{el}$$

Then the myometral index (Mi), endometrial index (Ei), luminal index (Li) and gland index (Gi) were calculated as follows:

$$M_i = \frac{A_M}{A_T}$$

$$E_i = \frac{A_E}{A_T}$$

$$L_i = \frac{A_L}{A_T}$$

$$G_i = nG \quad E_i$$

Cytokine measurement

Sample collection and storage

The foster mice were euthanized by cervical dislocation. UHs were dissected and placed in petri dishes. The UHs were rinsed with 200 µl of M2 media using a 1 ml syringe. The media was collected in 0.5 ml centrifuge tubes and stored at -80°C.

Cytokine Arrays

The flushed media samples were analyzed using the Mouse Cytokine Array Panel A kit (R&D Systems, Abingdon, UK) for the production of 40 soluble proteins: B-Cell Chemo-attractant (BCL), Complement component 5a (C5a), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte – Macrophage Colony Stimulating Factor (GM-CSF), T-Cell Activation-3 (TCA-3), Eotaxin, Soluble Intercellular Adhesion Molecule-1 (sICAM-1), Interferon (IFN)-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12 p70, IL-16, IL-17, IL-23, IL-27, Inflammatory Protein (IP)-10, Interferon-inducible T-cell Alpha Chemo-attractant (I-TAC), Keratinocyte Chemo-attractant (KC), Macrophage Colony-stimulating Factor (M-CSF), MCP-1, MCP-5, Monokine Induced by Gamma-interferon (MIG), Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, MIP-2, Regulated upon Activation Normal T-cell Expressed and presumably Secreted (RANTES), Stromal cell Derived Factor (SDF)-1, Thymus and Activation Regulated Chemokine (TARC), Tissue Inhibitor of Metalloproteinases (TIMP)-1, Tumor Necrosis Factor (TNF)-α and Triggering Receptor Expressed on Myeloid cells (TREM)-1. The samples were incubated overnight with the membranes and the detection antibodies at 8°C on a rocking platform. The membranes were rinsed with wash buffer twice for 10 min and incubated with 2 ml of Streptavidin-HRP for 30 min. The membranes were rinsed twice in wash buffer and 0.5 mL of chemiluminescent reagent was used to develop the array. Images of the membranes were captured in an image analyzer after 5 min exposure.

Cytokines and chemokine quantification

The chemokine MCP-1 and cytokine IL-1β were evaluated in the UH flushing samples by flow cytometry using a Cytokine Bead Array (CBA, BD Biosciences, Oxford, UK). Briefly, 50 µl of the flushed media samples were incubated with 50 µl of different cytokine detection beads mix, 50 µl of the Phycoerythrin (PE) detection reagent and incubated for 3h. The samples were washed with 300 µl of the provided wash buffer and centrifuged at 200 x g for 5 min. The pellets were re-suspended in 300 µl of wash buffer and analyzed in a FACSArray cytometer (BD Biosciences). The cytokine IL-1RA was measured using an ELISA kit (PeproTech; London, UK). Briefly 100 µl of the flushed media samples were incubated at room temperature in the anti-IL-1RA antibody pre-coated 96 well plate for 2h. The plate was rinsed 4 times with washing buffer and 100 µl of the secondary biotinylated antibody (500 ng/ml) were added and co-incubated for 2 h. The plate was rinsed 4 times with the washing buffer and 100 µl of the avidin peroxidase (1:200) were added and incubated for 30 min. Finally the plate was rinsed 4 times with washing buffer and 100 µl of the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) liquid substrate were added. The optical density was measured in a plate reader at 405 nm with wavelength correction set at 650 nm.

In vitro adhesion assay of trophoblast spheroids to the endometrial cells

Epithelial cell lines culture

RL95-2 cells were cultured in T75 flasks at 37°C in Dulbecco Modified Eagle Medium (DMEM)/F-12 (Sigma) supplemented with 1% Penicillin/Streptomycin (P/S; Sigma), 5 µg/ml Insulin (human recombinant insulin; Gibco Invitrogen, Denmark; cat. No. 12585-

014), 1% L-Glutamine (Sigma) and 10% Fetal Bovine Serum (FBS; Sigma) at 37ºC in 5% CO_2 atmosphere until reaching confluency.

MCF7 cells grown in DMEM/F-12, supplemented with 10% FBS, 1% P/S, 1% L-Glutamine and 160 ng/ml Insulin in a T75 flask at 37ºC in 5% CO_2 atmosphere. At confluency, cells were washed with Dulbecco Phosphate-Buffered Saline (DPBS; Sigma), harvested using Trypsin-EDTA (Sigma) and pelleted by centrifugation at 300 x g for 4 min. Approximately $5x10^5$ RL95-2 cells and $2.5x10^5$ MCF7 cells were transferred to each well of a 12-well plate and incubated at 37°C for 3-4 days.

Formation of JAr cells spheroids

JAr cells were grown in RPMI 1640 (Sigma), supplemented with 10% FBS, 1% P/S and 1% L-Glutamine in a T75 flask at 37ºC in 5% CO_2 atmosphere. At confluency, cells were washed with DPBS (Sigma), harvested using Trypsin-EDTA (Sigma) and pelleted by centrifugation at 300 x g for 4 min. To mimic the blastocyst, multicellular spheroids were formed from the JAr cells. One million JAr cells were counted with a haemocytometer and transferred to 5 ml of supplemented RPMI media in 60 x 15 mm Petri dishes. The spheroids were formed when the cell suspension was spun overnight on a gyratory shaker (JKA, MTS 2/4 digital, Staufen, Germany) at 280 rpm in a humid atmosphere and 5% CO_2 at 37°C. Approximately 2000 spheroids were obtained per petri dish (approximately 150-200 μm diameter).

Adhesion of JAr spheroids to endometrial cells

Fifty JAr spheroids were picked and gently delivered to each well of a 12-well plate with a confluent monolayer of the epithelial cells. They were co-cultured in supplemented DMEM-F12 at 37°C and 5% CO_2. After co-incubation, the initial number of JAr spheroids was confirmed by visual examination under a stereoscopic microscope. To remove non-adherent JAr spheroids from the epithelial cell monolayers, the 12-well culture plate was washed using a horizontal shaking device (Labman Automation LTD, Stokesley, UK). The plate was incubated for 4 min at 200 rpm and the media was discarded. Each well was filled with 1 ml of PBS containing Ca^{2+} and Mg^{2+} (PBS/Ca-Mg; Gibco Invitrogen; UK) and then set to shake for 4 min at 200 rpm. Finally, the PBS/Ca-Mg was discarded. The final number of spheroids was counted under the microscope and the results expressed as the percentage of spheroids from the initial number of spheroids counted before washing.

Viability assessment of the epithelial cells

The RL95-2 and MCF7 cells were grown in 12-well plates until total confluence. The media was replaced with serum free media before they were either activated or not with PGN and FSL-1 (Invivogen, Toulouse, France). The cells were harvested with 50 µl of trypsin EDTA to detach them from the wells, collected in 500 µl of media and pelleted by centrifuging at 300 g for 5 min. The cells were then resuspended in 200 µl of PBS and divided in two 5 ml cytometry tubes. One sample was used as autofluorescence and the other used stained with propidium iodide (PI; Life technologies, Paisley, UK) 3 µM and captured immediately. The samples were read in a FACSCalibur cytometer (BD) capturing $1x10^4$ events and the percentage of PI positive events (death cells) was

registered. The results are expressed as percentage of live cells and were compared using a one-way ANOVA and $p < 0.05$ was considered significant.

Experimental Design

Evaluation of the murine UH after TLR 2/6 activation

Morphological and morphometrical assessment and evaluation of the UH protein secretion after TLR 2/6 activation

The foster Swiss mice were mated with vasectomized males to induce the pseudo-pregnancy state. On day 3.5 post-mating, the utero-tubal junction of the left UH was pierced with the tip of a 30G needle and a transfer pipette was inserted through the opening to deliver 10 µl of FSL-1 (0.1 µg/µl; n = 16) or saline solution as a control group (n = 10). Mice were sacrificed 4h later (pre-implantation day) or on day 4.5 (implantation day) and the uterine horns collected. The inflammatory reaction in the UH was assessed by morphological and morphometric evaluation of the H&E stained samples. The flushed media samples from the UHs were pooled in four different groups: Ctrl 4h, FSL-1 4h, Ctrl 24h and FSL-1 24h. The protein production was assessed using the semi-quantitative Mouse Cytokine Array Panel A kit (R&D Systems) for the presence of 40 different cytokines. The profile of protein production was compared between the experimental groups. Furthermore, the concentrations of the cytokines IL-1β, and MCP-1 were assessed in each individual sample by CBAs (BD) and IL-1RA with an ELISA kit (PeproTech) and data were log transformed to normalize distribution.

Evaluation of the effect of TLR 2/6 activation on embryo implantation

The Swiss foster mice were prepared for embryo transfer as mentioned above. The utero-tubal junction of the left UH in each mice was pierced with a 30G needle to make an opening and using a transfer pipette infused with a drop of 10 µl of saline solution (n=15) or FSL-1 (0.1 µg/µl; n=16) followed by a gap of air and a drop containing the 12 blastocysts. After 15 days the foster mice were euthanized by cervical dislocation. The uterine horns were dissected and the number of embryos implanted and fetuses resorbed was registered (Fig. 1).

Effect of the activation of epithelial TLR 2 and TLR 2/6 on the adhesion of trophoblast spheroids.

Adhesion of trophoblast spheroids to epithelial cells treated with different concentrations of TLR 2 and 2/6 ligands

To determine whether TLR 2 and TLR 2/6 activation is affecting the trophoblast adhesion to the epithelial cells, we evaluated the effect of different concentrations of Peptidoglycan (PGN) and FSL-1 on the epithelial cells. The RL95-2 and MCF7 cells were grown in 12-well plates until total confluence. The media was replaced with serum free media before they were either activated or not with PGN and FSL-1 (Invivogen, Toulouse, France). The epithelial cells were then stimulated with different concentrations of PGN (0, 5, 10 and 20 µg/ml) or FSL-1 (10, 100 and 200 ng/ml) for 24 h. JAr spheroids were delivered onto the confluent monolayers and co-incubated for 1h. Adhesion was assessed as described.

Adhesion of trophoblast spheroids to stimulated epithelial cells at different time intervals

To understand if co-incubation duration is affecting the outcome of trophoblast binding to the epithelial cells, the cells were pre-incubated with 10 μg/ml of PGN or 100 ng/ml of FSL-1 for 24h. There after 50 JAr spheroids were delivered onto the confluent epithelial cell monolayers in 12-well plates and co-incubated for 0.5, 1, 2 or 4 h. The adhesion was assessed as described before.

Effect of the inhibition of epithelial TLR activation on trophoblast spheroid adhesion

To understand the effect of TLR 2 inhibition in endometrial cells on trophoblast spheroids binding to endometrial cells, the epithelial cells were either pre-treated or not with a monoclonal antibody against TLR 2 (2 μg/ml, neutralizing IgA monoclonal, Invivogen) or anti-TLR 6 monoclonal antibody (mouse IgG1, 10 ng/ml, Invivogen) 1 h before the addition of PGN (5 μg/ml) or FSL-1 (10 ng/ml) respectively. After 24 h, 50 JAr spheroids were delivered onto the monolayer and co-incubated for 30 min. Non-adherent spheroids were removed and the percentage of adhered spheroids was determined.

The effect of TLR activation on the viability of the epithelial cells

To determine whether TLR 2 and TLR 2/6 activation is affecting the viability of the epithelial cells; we treated the cells with different concentrations of PGN and FSL-1. The epithelial cells were then stimulated with different concentrations of PGN (0, 5, 10 and

20 μg/ml) or FSL-1 (10, 100 and 200 ng/ml) for 24 h. The viability of the cells was verified by PI staining in the FACS Calibur cytometer.

Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). The comparison of two experimental groups was performed with the non-parametric Mann-Whitney U test. The concentration of the cytokines measured by CBA and ELISA were log transformed to normalize distribution. Multiple groups were compared with a one-way ANOVA and a Bonferroni multiple comparison post-test. The effects of FSL-1 and PGN treatments on the co-incubation kinetics were compared with a two-way ANOVA test and a Bonferroni post-test to compare effects between non-stimulated control and treated cells. $P \leq 0.05$ was considered to be significant.

Results:

Local FSL-1 administration in uterus induced morphological changes in UH.

The saline treated UHs on the pre-implantation day (4h post-treatment) retained the morphology of a normal UH. The myometrium was dense and endometrial stroma filled with secretory glands. The endometrial lumen was pseudo-stratified and convoluted, showing a small luminal space. Finally, an abundant secretory activity in the endometrial luminal cells and glands could be observed (Fig 2A). In contrast to this, the FSL-1 treated UH presented an atrophic morphology. The thickness of the myometrium and stroma was

reduced as well as the number of glands observed. The FSL-1 treated epithelial endometrial cells morphology changed from columnar pseudo-stratified to simple columnar/cuboidal shape. Nevertheless, some secretory activity could still be observed (Fig 2A). The Mi, Ei and Gi were significantly higher in the saline group compared to the FSL-1 treated group (Fig. 2B). In contrast, the lumen was smaller and more convoluted in the saline than in the FSL-1 treated (Fig. 2B). In controls, the morphology of the UHs on the implantation day (24h post-treatment) was similar to the ones at estrus. In addition to this, the stroma was loose and filled with edema, a characteristic of a decidualized endometrium. The presence of vacuoles in the cytoplasm of luminal and glandular epithelial cells confirmed a high secretory activity at this stage. The 24 h FSL-1 treatment induced an atrophia of the UHs decidua (Fig. 2C). This could be observed in the reduction of the Mi and Ei of the FSL-1 treated UHs compared to the saline treated UHs (Fig. 2D).

FSL-1 treatment of the UHs increased the production of pro-inflammatory cytokines.

Sixteen proteins were detected 4h after treatments and 17 proteins were detected 24h after treatments in the cytokine array. The FSL-1 treatment of the UH on the pre-implantation day, increased the production of TNF-α, TIMP-1, MCP-1, M-CSF, KC, IL-16, IL-1ra, IL-1β and IFN-γ (Fig. 3A). From these proteins, the highest difference between FSL-1 and saline treatments was observed in MCP-1, IL-1ra and IL-1β. FSL-1 treatment for 24 hrs increased significantly the production of MCP-1, IL-17, IL-16, IL-13, IL-7, IL-4, IL-1ra, IL-1β, IL-1α and C5a (Fig. 3B). Based on these analyses, each individual sample was analyzed again by CBA for the production of IL-1β, MCP-1 and by ELISA for IL-1RA.

The FSL-1 treatment of the tissue for 4h considerably increased the production of IL-1β, MCP-1 however the change in IL-1RA production was minimal (Fig. 3C). The production of IL-1β and MCP-1 after 24h of FSL-1 treatment was lower and no difference was observed between the different treatments. In contrast, this treatment significantly increased IL-1RA production (Fig. 3D).

Activation of TLR 2/6 in the murine uterine horn affects embryo implantation.

The activation of TLR 2/6 with FSL-1 severely reduced the number of total implantations in the UH compared to the saline treated horns (45.00 ± 6.49 vs 16.69 ± 5.01%, $p < 0.05$; Fig. 4A). The number of total implantations included the embryos that developed to fetuses and those that were resorbed (implanted embryos that could not develop; Fig. 4B). The percentage of developed fetuses decreased after the administration of FSL-1 to the UH (45.56 ± 6.79 vs 26.04 ± 6.84%, $p < 0.05$; Fig. 4C). On the other hand, we observed an increase in the percentage of resorbed fetuses in the FSL-1 treated group compared to the saline group (4.44 ± 1.79 vs 14.17 ± 3.45%, $p < 0.05$; Fig. 4D).

The activation of epithelial TLR 2 and TLR 2/6 decreased the adhesion of trophoblasts spheroids in vitro regardless of the co-incubation time.

The trophoblast spheroids adhered to the cells as soon as 30 min after their introduction to the wells and this adhesion was always higher in the RL95-2 cells (63.4 ± 4.38% and 37.35 ± 3.02% to MCF7 cells). The effect of the treatment of the epithelial cells with 100 ng/ml of FSL-1 for 24 h on the adhesion of the JAr spheroids was significant for RL95-2 ($p < 0.05$) and MCF7 cells ($p < 0.0001$) as well as the effect of the co-incubation time (p

406 < 0.0001 for RL95-2 and p < 0.0001 for MCF7). The FSL-1 stimulation was consistent
407 throughout the time as no interaction was observed in both cell lines (p = 0.6473 for
408 RL95-2 and p = 0.8718 for MCF7 cells; Fig. 1A and B). The JAr spheroid adhesion was
409 found significantly different between the non-stimualted control and FSL-1 stimulated
410 MCF7 cells at 30 min (37.35 ± 3.02 vs 26.56 ± 2.59%, p < 0.01) and 4 h (73.01 ± 2.23 vs
411 62.28 ± 2.053%, p < 0.01). The treatment of the cells with PGN was also consistent
412 throughout the stimulation time as no interaction was observed (p = 0.5527 for RL95-2
413 and p = 0.5663 for MCF7). We observed a significant effect of the treatment of the cells
414 with 10 μg/ml of PGN on the adhesion of JAr spheroids adhered in both RL95-2 (p <
415 0.0001) and MCF7 cells (p < 0.0001). The effect of time on the adhesion of the spheroids
416 was also found to be significant in both cell lines (p < 0.0001 for both RL95-2 and
417 MCF7; Fig. 5A and B). The adhesion of the JAr spheroids to the non-treated RL95-2
418 cells differ significantly from the PGN treated cells at 1 h (73.79 ± 2.85 vs 64.1 ± 3.9, p <
419 0.05) and 2h of co-incubation (89.05 ± 2.02 vs 76.39 ± 2.54, p < 0.01). The same effect
420 was observed in the MCF7 cells at 1 h (73.01 ± 2.43 vs 67.95 ± 3.23, p < 0.05) and 2 h of
421 co-incubation with the JAr spheroids (84.19 ± 3.11 vs 71.34 ± 4.26, p < 0.01).
422
423 **Trophoblast spheroid decreased adhesion to the epithelial monolayer proportional to**
424 **the TLR ligand concentration.**
425 The 24h stimulation of RL95-2 and MCF7 cells with 100 and 200 ng/ml of FSL-1 (68.11
426 ± 4.83 and 61.07 ± 4.52 % for RL95 and 41.66 ± 2.82 and 40.21 ± 3.36 % for MCF7)
427 significantly decreased the adhesion of the JAr spheroids to the monolayer compared to
428 the non-stimulated control (83.46 ± 2.685 %, p = 0.0012 for RL95 and 58.58 ± 2.43 %, p

< 0.0005 for MCF7) after one hour of co-incubation (Fig 6A and B). A similar effect was observed with PGN stimulation. The RL95-2 PGN stimulation with 20 µg/ml significantly decreased JAr spheroid adhesion compared to the non-stimulated control (57.09 ± 3.83 vs 72.92 ± 3.74 %, $p < 0.05$; Fig. 6A). In MCF7 5, 10 and 20 µg/ml of PGN significantly decreased JAr spheroid adhesion (57.05 ± 3, 47.04 ± 1.23 and 50.19 ± 2.86 % respectively) compared to the non-stimulated control (68.25 ± 2.71 %, $p < 0.0001$; Fig. 6B).

The pretreatment of the endometrial cells with TLR blocking antibodies could restore the spheroid adhesion.

Furthermore, we confirmed the specificity of the TLR effect by blocking their activation with monoclonal antibodies. The pre-treatment with a monoclonal anti-TLR 6 antibody followed by FSL-1 stimulation, restored the percentage of adhered JAr spheroids to RL95-2 cells (59.71 ± 1.91%) and MCF7 cells (30.70 ± 4.54%) compared to non-treated cells (63.26 ± 2.74% and 31.48 ± 4.087% respectively; Fig. 7A and B). The pre-treatment of the epithelial cells with a monoclonal anti-TLR 2 blocking-antibody before the PGN stimulation, allowed JAr spheroids to adhere to the monolayer to similar levels (58.15 ± 4.14% for RL95-2 and 33.15 ± 4.44% for MCF7) to those observed in the non-stimulated control (61.81 ± 2.65% for RL95-2 and 35.01 ± 3.78% for MCF7; Fig. 7A and B). The treatment with the antibodies did not show an effect on the adhesion of JAr spheroids to the monolayers (anti TLR 6: 63.35 ± 2.48% for RL95-2 and 33.08 ± 4.28% for MCF7; anti TLR 2: 61.96 ± 3.25% for RL95-2 and 36.72 ± 2.45% for MCF7) whereas the stimulation with FSL-1 and PGN decreased significantly the adhesion of the

JAr spheroids (FSL-1: 45.70 ± 1.91%, $p < 0.0001$ for RL95-2 and 18.20 ± 3.66%, $p < 0.0005$ for MCF7; PGN: 45.34 ± 3.51%, $p < 0.0001$ for RL95-2 and 24.00 ± 2.87%, $p < 0.0001$ for MCF7).

The viability of the epithelial cells was unaltered after TLR activation

The stimulation of the RL95-2 and MCF7 cells with both FSL-1 and PGN did not affect the viability of the cells. Around 90% of the non-stimulated cells remain viable after harvesting from the wells. The viability of the cells stimulated with FSL-1 or PGN was also around 90% of the total population (Figure 8).

Discussion

Embryo implantation is a complex event initiated by the adhesion of the trophectoderm to the endometrial epithelial cells. This is followed by the trophoblast invasion into the uterine decidua. Two major factors are involved in a successful implantation, a good embryo quality and proper endometrial receptivity. Any disturbance in the intricate crosstalk of hormones, cytokines, and adhesion factors during this critical period of time could lead to implantation failure[32,33].

In our study using an in vivo murine model, we explored the characteristics of the UH on pre-implantation and implantation day. We were able to create an inflammatory environment in the UH by stimulating the innate immune system via TLR 2/6 ligand and observe how it could affect the tissue structure and impair embryo implantation. The inflammatory insult in the UH strongly affected the architecture of the different layers of

the tissue. This effect could be observed as soon as 4 h of the FSL-1 administration, when the endometrium is remodeling for suitable embryo receptivity. This might indicate that the epithelial and stromal cells of the uterus are sensitive to innate immune activation via TLR stimulation as a protective mechanism for the mother. Stromal cells have been found necessary to mediate immune responses in different body tissues. They offer a substrate for the migrating leukocytes and orchestrate part of the adaptive immune responses[34]. During embryo implantation, the uterine stromal cells provide a scaffold for migrating cytotrophoblasts and establishment of the spiral arteries. The stromal sensitivity to respond to inflammatory stimuli has been studied, where administration of i.p. Poly I:C (TLR 3 ligand) to mice affects stromal decidualization and vascularization of the uterus[35]. In our experiments the innate immune activation was performed via FSL-1 which is a synthetic diacyl lipopeptide specific ligand for TLR 2/6[36]. It is possible that the effect on the tissue remodeling will occur regardless of the type of TLR activated. In fact, the capacity of the endometrial cells to react to foreign entities has been assessed previously where the exposure of the UH to the toxic compound di-(2-ethylhexyl)-phtalate (DEHP) is able to affect tissue decidualization and impair embryo implantation[37]. The current methods to evaluate the estrogenic effects of toxic compounds on the UH is based on the uterotrophic assay developed in the 1930s. It estimate the weight of the horns after treatment and gives a description of the morphological characteristics of the uterine layers and cells[38]. In humans, the criteria to date the endometrium based on pathologist observations were established by Noyes in the 1950s. It estimates scores for the different morphological changes of the endometrial characteristics in gland, epithelia and stromal development due to the phase of the

cycle[39]. In our morphometry assessment, we were able to measure accurately the areas of the different UH compartments with the help of the digital imaging tools. Also the possible differences due to the horn size and sampling along the length of the horn were normalized with the calculation of the indexes. This technique can potentially be used as an effective method of estimating toxic effects of chemical compounds on the uterine horn of different mammalian species, although more standardization in its validity is required.

The implantation as mentioned before is an agreement between the maternal tract and the embryo. Cytokines produced by the maternal tract are fundamental effectors, which also peculiarly resemble an inflammatory reaction. By profiling the UH secretions, we were able to observe a disruption in the cytokine levels when the uterine TLR 2/6 was activated. After 4 h of stimulation (pre-implantation day) three proteins were up-regulated: MCP-1 (CCL2), IL-1β and IL-1ra. The three of them share important roles in both embryo implantation and in inflammation. The stimulation of the endometrium epithelia with IL-1β has been found induce the expression of endometrial adhesion molecules necessary for embryo apposition and adhesion[40]. MCP-1 has been proven to be inducible by in vitro stimulation of TLR 2/6 in murine uterine epithelial cells and TLR4 in uterine epithelial and stromal cells[41]. The increase in IL-1RA production after 24 h of TLR 2/6 stimulation could interfere with the embryo – mother crosstalk antagonizing IL-1β. The i.p. administration of IL-1RA to mice in the pre-implantation period reduces drastically embryo implantation[42]. After 24 h of FSL-1 treatment we observed a distinctive change in pattern of expression of cytokines towards a response

type Th17. The Th17 response can lead to autoimmune diseases, chronic inflammatory disorders and infectious diseases[43]. In the endometrium they have been associated with cases of unexplained infertility[44,45] and in response to C. muridarum, the murine homologue of C. trachomatis[46]. This pathogen can initially be recognized by TLR 2 prior to an adaptive response[16].

The reduction of embryo implantation confirmed a direct effect of the activation of uterine TLR 2/6 on embryo implantation. This activation was able to affect the development of the uterine tissue and the secreted protein profile necessary for implantation. As observed, the lack of a receptive epithelium to adhere would affect the apposition and adhesion of the blastocyst. It might be possible that the molecular characteristics of the endometrial cells could be also affected by this stimulation. It is unlikely that the treatment of the UH with the TLR 6 agonist could harm the embryos and could be the reason of embryo implantation loss. A previous report has demonstrated that the first trimester trophoblast lack the expression of TLR 6 and stimulation does not change blastocyst viability[47]. The FRT has been proved to be sensitive to TLR stimulation in vivo, for example the infusion of LPS to the murine UH induces a TLR 4 dependent inflammatory pelvic disease [48]. It has also been observed that the administration of i.p. LPS to mated mice on day 5.5 could increase fetal loss[49]. The atrophic stroma observed after TLR 2/6 stimulation, would offer a deficient soil for the embryo to invade and start its development. This is likely to be a contributing factor for the increase in embryo resorption observed in our investigation. Similar to this study, it has been observed that administration of i.p. Poly I:C, TLR 3 ligand can decrease the

implantation rate and increase the percentage of resorbed embryos by impairing stromal decidualization and spiral arteries development[35]. Hence, a potential reason for the low success rate of implantation after embryo transfer is the simple manipulation of the uterine horn or endometrium during embryo transfer procedures in human or livestock that could damage the tissue. This damage could induce a sterile inflammatory process by the release of danger associated molecular patterns (DAMPs) molecules that are also recognized by the TLRs, initiating an inflammatory process and leading to embryo implantation failure. A potential way of clarifying these effects on the murine UH and a future scope of our research is to use a TLR6 KO mouse. In this way we would be able to test the effects of TLR activation on both tissue structure and embryo implantation.

To corroborate our observations in the murine model of embryo implantation, we used an in vitro human trophoblast spheroid adhesion model as mentioned before. The percentage of spheroids adhered increased proportionally with time, as observed previously[50,51]. The JAr cells have been described as a very invasive choriocarcinoma cell line[50]. The interaction of MCF7 cells with first trimester placental explants has been observed before. The trophoblast explants affected MCF7 proliferation and induced apoptosis in long co-incubation times. [52]. Our experiments were carried out in short co-incubation times where we were able to observe adhesion of trophoblast to both epithelial cells. Nevertheless a better interaction was observed with the endometrial cells as trophoblast cells adhered them in a higher percentage and faster than the MCF7 cells.

Stimulation of TLR 2 and 2/6 in the epithelial cells decreased the percentage of attached spheroids to the monolayers. It is unclear how the TLR activation can influence endometrial receptivity. The fact that we observed a decrease in trophoblast spheroid adhesion in both epithelial cell lines after TLRs activation might suggest that the TLR activation effect relies on modification of cellular adhesion capability. Expression of surface adhesion molecules, cell polarity or even cytoskeletal arrangement necessary for the cell-cell interactions might be involved. In our study it was clear that the decrease in trophoblast adhesion to epithelial cells came from the TLR activation on the epithelial cells. The inhibition of TLR 6 and TLR 2 with specific antibodies restored the ability of the JAr spheroids to bind to both FSL-1 and PGN stimulated RL95-2 cells. It is important to consider that TLR 2 is a particular receptor of the TLR family. It can form homodimers and heterodimers with either TLR 1 or TLR 6 increasing the range of ligands that TLR 2 can recognize, from bacterial PGN to diacyl and triacyl lipopeptides[53,54]. TLR 2/6 functionality is dependent on the dimerization and the recognition of the ligand by both TLRs. If one of them is absent, then the activation by the diacylated lipopeptides will not occur[19,55]. A previous report from our laboratory explored how the stimulation of TLR 5 with Flagellin can induce a similar effect on the adhesion of trophoblast cells to endometrial epithelial cells[24]. Finally, as shown in our results (Figure 8), the decrease in spheroid adhesion to the endometrial cells cannot be attributed to the effect of the TLR stimulation on the viability of the cells. Together these results suggest that the activation of TLRs will have a detrimental effect on implantation and this might happen regardless of which TLR molecule is being stimulated.

589 Clinically, endometrial TLR 2 might be playing a very important role in the
590 recognition of pathogens that cause Sexually Transmitted Diseases (STDs) and infertility.
591 The most common bacterial transmitted STD caused by the intracellular obligated gram-
592 negative pathogen C. trachomatis. Chlamydia can cause a cervical infection that if
593 ascends into the upper tract can generate Pelvic Inflammatory Disease (PID) and
594 infertility[56]. Chlamydia can be recognized by TLR 2 in the intracellular inclusions and
595 induce the production of IL-8[16]. Furthermore this recognition and the initiation of the
596 immune responses is dependent on TLR 2 and not TLR 4 and mice lacking TLR 2
597 showed a reduced oviductal inflammation[57].
598
599 Determining the suitable endometrial conditions for the implantation of the
600 embryo is a topic under constant research[2,3,5,6,33]. The correct diagnose of infertility
601 can increase significantly the success of pregnancy for women with fertility problems and
602 couples undergoing IVF treatment. With further research on the mechanisms responsible
603 for the effects observed here, it would become possible to design and direct a therapy
604 targeting the endometrial innate immune system to increase the implantation success. Our
605 data support the importance of TLRs during the time of implantation suggesting that they
606 could be used as a potential target in management of infertility cases.
607
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609
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List of figures

MCP-1 and IL-1RA represent the mean ± SEM of the logarithmic concentration of the different samples of the UH flushed media. Statistical differences between treatments were analyzed by Mann Whitney t-test (*p≤0.05).

Figure 4. Murine embryo implantation on FSL-1 treated uterine horns. The percentage of total embryos implanted was assessed on day 16 on the control (n=15) and FSL-1 (0.1μg/μl; n=16) treated foster mothers (A). Representative image of implanted and resorbed embryos in the murine UH (B). The percentage of embryos implanted (C) and embryos resorbed (D) was estimated and compared between control and FSL-1 treated UHs. The bars are the mean ± SEM of the control and FSL-1 treated foster mice. The statistical comparisons between treatments were performed using a Mann Whitney t-test (*p ≤ 0.05).

Figure 5. Kinetics of adhesion of trophoblast spheroids on epithelial cells stimulated with FSL-1 and PGN. The percentage of adhesion of JAr spheroids to A) endometrial or B) MCF7 cells was determined after a kinetic of adhesion (0.5, 1, 2 and 4h). The adhesion of the spheroids to endometrial untreated cells (•) was compared to the endometrial cells treated (■) with FSL-1 100 ng/ml treated or PGN 10 μg/ml treated. Each point represents the mean ± SEM of four independent experiments treatments were compared by a two-way ANOVA to test interaction between effects, treatment and co-incubation time. Furthermore the difference between control and treated groups per individual time points was assessed with a Bonferroni multiple comparisons post-test (*p ≤ 0.05 was considered significant).

Figure 6. Effect of the concentration of FSL-1 and PGN on the adhesion of the JAr spheroids to the epithelial monolayers. The adhesion of JAr spheroids to the A) endometrial or B) MCF7 cells was determined after their pre-treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 μg/ml). The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups (*$p \leq 0.05$).

Figure 7. Effects of the inhibition of epithelial TLR 6 and TLR 2 activation on the adhesion capacity of the JAr spheroids. The A) RL95-2 or B) MCF7 cells were pre-treated or not with an IgG anti-TLR6 antibody (10 ng/ml) or IgA monoclonal anti TLR-2 antibody (2 μg/ml) and stimulated with FSL-1 (10 ng/ml) or PGN (10 μg/ml) respectively. The percentage of adhesion to the non-treated or different treated groups was assessed. The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the different groups (*$p < 0.05$).

Figure 8. Effect of the concentration of FSL-1 and PGN on the viability of the epithelial cells. The viability of A) RL95-2 or B) MCF7 cells was determined after their treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 μg/ml). The data represents the mean of 3 independent experiments ± SEM. A

multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups ($*p \leq 0.05$).