The University Of Sheffield.

Activation of endometrial innate immune system; a potential mechanism for human infertility

Mehrnaz Montazeri

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Abstract

Embryo implantation is a complex process in which an intact cross talk is required between maternal endometrium and embryonic structures. During each menstrual cycle, the endometrium undergoes morphological changes to govern this communication and the progression of pregnancy. The presence of infection at the site of implantation can lead to implantation failure. Hence, tight regulation of the innate immune system is required so as to provide a sterile environment for implantation and embryo development. This is achieved by a family of innate immune receptors, called toll-like receptors, which recognize the presence of potential pathogens and react against potential infection at the site of implantation. The innate immune system and specifically TLRs might play an important role in the providing the proper endometrial receptivity, embryo implantation and establishment of pregnancy.

In this thesis, we hypothesized that activation of the endometrial TLR 3 will influence the ability of the endometrium to receive an implanting embryo. We applied different approaches to study endometrial and implantation biology ranging from *in vitro* human endometrial-trophoblast interaction to time lapse experiments. The results obtained from our work indicate that activation of endometrial TLR 3 *in vitro* is able to reduce trophoblast binding to the endometrial cells, possibly by altering the morphology of the endometrial cells. It seems the endometrium is able to initiate a defence mechanism against invading microorganisms and protect the mother from the potential infection, but this potentially compromises the chance of an embryo to implant.

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Abbreviation list

ART – Assisted reproductive technologies

CAMs – Cell adhesion molecules

CD – Cluster of differentiation

CGR – Chorionic gonadotropic hormone receptor

CMV – Cytomegalovirus

CpG – Cytidine-phosphate-Guanosine repeats

CSF – Colony stimulating factor

Cq – Quantification cycle

Ctrl – Control

DAMP – Damage associate molecular pattern

DC – Dendritic cell

DNA – Deoxyribonucleic acid

DPBS – Dulbecco's phosphate-buffered saline

Ds – Double stranded

ECC – Endometrial carcinoma cell line

ECM – Extracellular Matrix

EDTA – Ethylene diaminetetra acetatic acid

EEC – Endometrial epithelial cells

EGF – Epithelial growth factor

ELISA – Enzyme linked immunosorbent assay

ER – Estrogen receptor

ERK – Extracellular signal-regulated kinase

ESC – Endometrial stromal cells

EVT – Extravillous trophoblast

FCS – Fetal calf serum

FITC – Fluorescein isothiocyanate

FLS – Fibroblast-like synoviocytes

FRT – Female Reproductive Tract

GFP – Green fluorescent protein

hCG – Human chorionic gonadotropin

HESC – Human endometrial stromal cell line

HLAG – Human leucocyte antigen-G

HNEC- Human nasal epithelial cells

HSP – Heat shock protein

HSV-1 – Herpes simplex virus

IkB – Inhibitor of NF-κB

ICM – Inner cell mass

IFN ɣ – Interferon gamma

IGF – Insulin-like growth factors

IKK – IkB kinase

IL – Interleukin

IL-1RA – IL-1 Receptor antagonist

IL-1R tI – IL-1 Receptor type I

IL-1RtII – IL-1 Receptor type II

IP-10 – Inflammatory protein-10

IRF3 – Interferon regulatory factor 3

IRF7 – Interferon regulatory factor 7

IVF – *in vitro* fertilisation

JAMs – Junctional adhesion molecules

JNK – c-jun terminal kinase

KO – Knock out

KRT - Cytokeratins

LH – Luteinising hormone

LIF – Leukaemia inhibitory factor

LPS – Lipopolysaccharide

LRR – Leucine rich repeats

LRT – Lower reproductive tract

LTA – Lipoteichoic acid

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemotactic protein-1

MIF – Mean intensity of fluorescence

MMP – Matrix metalloproteinase

MMR – Macrophage mannose receptor

MSR – Macrophage scavenger receptor

MTX – Methotrexate

Muc1 – mucin 1

MyD88 – Myeloid differentiation primary gene 88

NK – Natural killer cell

NF-κB – Nuclear factor-kB

NLR – NOD-like receptor

NOD – Nucleotide-binding oligomerization domain

PAMP – Pathogen associated molecular pattern

PCR – Polymerase chain reaction

PGN – Peptidoglycan

PI – Propidium iodide

Poly I:C – Poly inosinic-poly cytidylic acid

PRR – Pattern recognition receptor

P/S – Penicillin / Streptomycin

QPCR – Quantitative polymerase chain reaction

RANTES – Regulated upon activation normal T-cell expressed and presumably secreted

RHD – Rel-homology domain

RIG – Retinoic acid-inducible gene-1

RLR – RIG-like receptor

RNA – Ribonucleic acid

SEAP – Secreted placental alkaline phosphatase

SEM – Scanning electron microscopy

Ss – Single stranded

STD – Sexually transmitted disease

STSM – Short term scientific mission

TE - Trophectoderm

TGF – Transforming growth factor

TIR – Toll/IL-1 receptor

TIRAP – TIR containing adaptor-like MyD88

TLR – Toll-like receptor

TLR 3KO – TLR 3 knock out mice

TNF – Tumor necrosis factor

TRAM – TRIF related adaptor molecule

Treg – Regulatory T-cells

TRIF – TIR-regulated adaptor protein inducing interferon

VEGF – Vascular endothelial growth factor

VT – Villous cytotrophoblast

Chapter 1. Introduction

1.1. Overview

Ovulation of the mature Graafian follicle occurs 17 h after the surge of the luteinizing hormone (LH). When the oocyte is released into the Fallopian tube, fertilisation may occur in the presence of a sperm. The zygote migrates towards the uterus, and 4-5 days after ovulation it reaches the endometrium. Implantation occurs between Day 20 and day 24 of the menstrual cycle. The process consists of three stages; the first stage is initiated by apposition of blastocyst trophoblast cells to the epithelial cells of the endometrium. The attachment stage will start where adherence of the trophoblast cells to the free surface of the endometrium occurs. The final stage is invasion, where the trophoblast cells penetrate the epithelium and the underlying endometrial stroma (Johnson and Everitt, 2007). The process of embryo implantation is known also to be partially regulated by the immune system. Particularly, the innate immune system plays an important role in providing proper endometrial receptivity for embryo implantation (Koga et al., 2009a).

The immune system can be divided into two domains in vertebrates: the adaptive and innate immune systems. The innate immune system is the human body's first line of defence, which recognizes the invading microorganisms through pathogen recognition receptors (PRRs) in the female reproductive tract (FRT) (Medzhitov and Janeway, 1997, Medzhitov and Janeway, 2002). One of the main members of the PRR family is the Toll-like receptors (TLRs). They are trans-membrane receptors and play a crucial role in mediating innate immunity in the FRT (Fazeli et

al., 2005, Aflatoonian et al., 2007, Aflatoonian and Fazeli, 2008). To date, 10 members of the TLR family have been identified in humans, which are known as TLR 1 to 10. Among the different TLR family members, TLR 3 recognizes double stranded RNA of viruses. TLR 3 plays a pivotal role in the recognition of viruses such as herpes simplex virus 1 (HSV-1) and cytomegalovirus (CMV), whose correlation with female infertility has previously been established (el Borai et al., 1997, Medvedev et al., 2009, Yang et al., 1995). In addition to detecting invading pathogens in the human body, TLRs also participate in remodelling of the endometrium and embryo implantation (Koga and Mor, 2008). It has been recently shown that stimulation of some members of the TLR family (TLR 2/6 and 5) in the FRT at early stages of pregnancy may lead to implantation failure *in vivo* and *in vitro* (Aboussahoud et al., 2010b, Sanchez-Lopez et al., 2014). Limited research data are available about the role of other TLRs in the process of implantation and even the pregnancy process. Understanding the role of TLR family members in implantation failure may be useful in treating infertility.

Due to the ethical issues regarding working with embryos in humans, it is nearly impossible to study trophoblast implantation into the endometrium *in vivo*. Hence, laboratories resort to the use of cell lines and *in vitro* models for investigating the early events of human implantation (Hannan et al., 2010).

This chapter will explain the different stages of human implantation, as well as various factors that affect the process of embryo implantation and endometrial receptivity, including cytoskeletal rearrangement, adhesion molecules, growth

factors and cytokines. Then the different model systems which are used to simulate human embryonic implantation will be described. Finally, various Toll-like receptor family members and their role in embryonic implantation and pregnancy complications will be discussed. The final section will describe the overall hypothesis, aims and objectives of the current thesis.

1.2. Embryonic implantation

After the fertilisation of the oocyte with the sperm in the Fallopian tube, a conceptus is formed (Johnson and Everitt, 2007). The conceptus is transported through the oviductal isthmus and enters the uterus. During this period, the two-cell conceptus goes through cell divisions at a rate which varies in different species. Each cell or blastomere undergoes a series of cleavage divisions while the total size of the conceptus remains nearly the same. Morula is formed at around the 8-16-cell stage, and blastocyst is formed at the 32 to 64-cell stage in most species. The precise form of the blastocyst varies in different species but in nearly all of them it can be divided to two distinct regions: (1) The outer cell mass, also called trophectoderm (TE); (2) an inner cell mass (ICM) which is eccentrically surrounded by the blastocoelic cavity (Johnson and Everitt, 2007). TE gives rise to the trophoblasts and extra-embryonic structures such as placenta; the ICM gives rise to the embryo.

As soon as the embryo enters the uterus, the conceptus is positioned to implant at the site (or sites) of implantation.

In the following section, various types of implantation that occur in different mammalian species are described.

1.2.1. Classification of implantation in various species

Implantation is classified into two main groups as invasive and non-invasive implantation (Johnson and Everitt, 2007) (Fig 1.1). In invasive implantation, the trophoblast invades the endometrium, whereas in non-invasive implantation no erosion of the underlying epithelium occurs at the time of implantation.

1.2.1.1. Invasive implantation

Invasive implantation is divided to interstitial and non-interstitial implantation. In the interstitial type of implantation the blastocyst completely invades the endometrium, whereas in eccentric implantation partial erosion of the maternal tissues occurs (Johnson and Everitt, 2007).

1.2.1.1.1. Interstitial

The conceptus invades maternal tissues in varying depths. When the conceptus invades the stroma deeply and the surface epithelium is eroded, this is called interstitial implantation (eg humans, chimpanzee and guinea-pig).

1.2.1.1.2. Eccentric

When the conceptus partially invades the stroma and partially projects into the uterine lumen, this is called eccentric implantation (eg rhesus monkey, mice, dog, cat and rat).

1.2.1.2. Non-invasive implantation (Centric implantation)

The conceptus does not invade the epithelium and no erosion occurs in this centric implantation type (eg sow, mare, ewe and cow). The pre-attachment period is longer in this type of implantation. The free-living embryo utilizes secretions of uterine glands and grows to a much larger pre-implantation size than the human or rodent invasive conceptus. This large surface area allows the exchange of metabolites and extensive attachment to uterine epithelial cells.

In Humans, implantation is invasive in type. The rest of this section will describe different stages/phases of Human implantation.

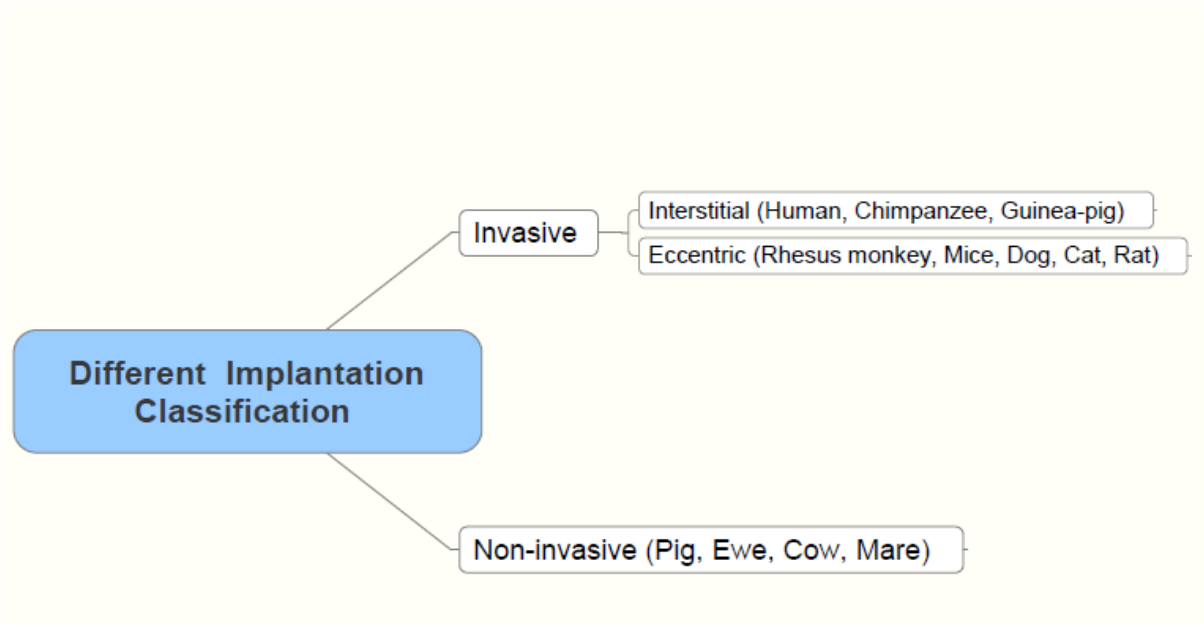

Figure 1.1 Classification of implantation in various species (Johnson and Everitt, 2007).

1.2.2. Implantation stages in humans

Implantation in human consists of three phases: apposition, attachment and invasion. A brief description of each phase now follows (Johnson and Everitt, 2007) (Fig 1.2):

1.2.2.1. Apposition

At this phase the blastocyst hatches from the *zona pellucida* and initiates the first contact with the endometrium.

1.2.2.2. Attachment

Attachment is characterized by adherence of the trophoblast cells to the endometrial epithelial cells. After trophoblast attachment, changes are induced in both endometrial epithelium and the endometrial stromal tissue. This is the stromal reaction. The stromal reaction is characterized by edema, with increased vascular permeability and progressive growth of capillaries of stromal tissue underlying the conceptus. This reaction is particularly well observed in primates and rodents, where it is called primary decidualization.

1.2.2.3. Invasion

Within a few hours of attachment, the trophoblast cells start to invade the endometrial epithelial cells and digest them. Some trophoblast cells fuse together and form a syncytium (syncytiotrophoblast), while others retain their cellularity (cytotrophoblast) and generate more trophoblast cells. As a result of the destruction of the glandular and decidual tissue of the uterus, some histiotrophic metabolic substrates are released. These will give nutritional support to the growing conceptus. The uterine glands adjacent to the implantation site provide sustained support at least in the first 10 weeks of human pregnancy.

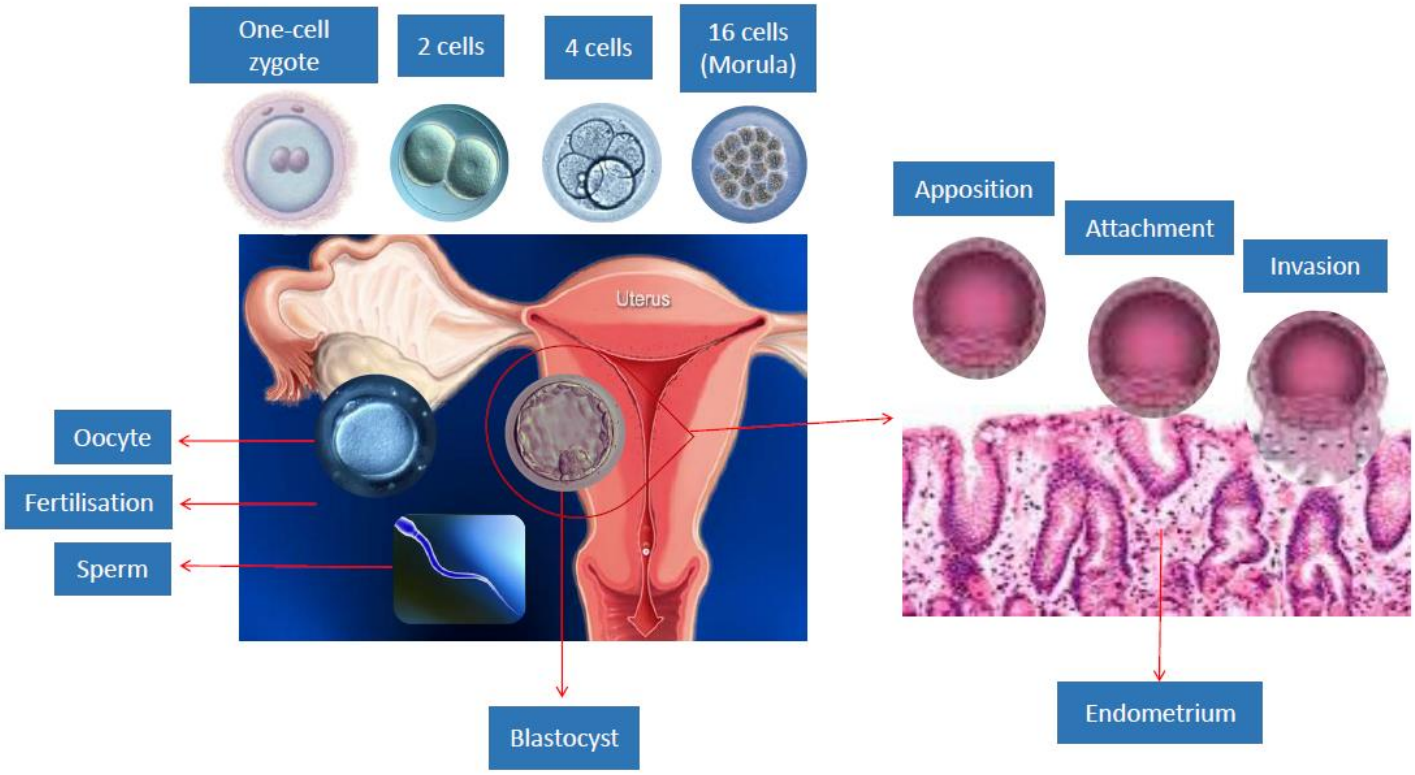

Figure 1.2 A schematic of fertilisation and stages of implantation in humans.

1.2.3. Endometrial receptivity

To achieve successful implantation, a healthy embryo and a receptive endometrium are required. Endometrial receptivity can be defined as the capacity of the endometrium to accelerate successful embryonic implantation. The endometrium is known to be receptive only during a very short time period in the mid-secretory phase, which is called the “window of implantation” (receptive phase) (Aghajanova et al., 2008) and is limited to days 19-24 of the menstrual cycle (Johnson and Everitt, 2007). Beyond this period, the embryo is unable to establish any contact with the endometrium. Therefore, the timely arrival of an embryo in a receptive endometrium is extremely crucial for successful implantation.

1.2.3.1. Plasma membrane transformation

While the type of implantation differs widely among species, the process starts with the attachment of trophoblast cells to the uterine epithelial cells (Johnson and Everitt, 2007). Since the epithelial layer of endometrial cells is the first site of contact between the embryo and maternal cells, it is logical to assume that the plasma membrane of these cells plays a crucial role in the implantation process. During the window of implantation, the endometrium undergoes structural rearrangements and biochemical modifications. These membrane alterations are important morphological sign of endometrial receptivity at the time of implantation and are termed “plasma membrane transformation” (Murphy, 1993).

Some of the plasma membrane transformation changes are described below. These include shortening of the microvilli, formation of the pinopodes, thinning of the mucin layer and loss of surface negative charge.

1.2.3.1.1. Shortening of the microvilli

In the non-receptive phase, the endometrium is covered with long, thin and regular microvilli, whereas during the window of implantation, these microvilli are gradually replaced by shorter and less regular forms of villi (Johnson and Everitt, 2007, Murphy, 1993). It is conceived that these different forms of highly irregular and flattened projections prime the endometrial cells for the embryo's attachment.

1.2.3.1.2. Formation of the pinopodes

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), later known as pinopodes (Enders and Nelson, 1973). The pinopodes appear to be the most reliable morphological marker of receptive endometrium at the time of implantation (Nikas et al., 1995, Murphy and Dwarte, 1987) and represent sites of preferential blastocyst attachment (Murphy, 1995, Murphy, 2000a).

1.2.3.1.3. Thinning of the mucin layer

Endometrial epithelial cells are covered with a thick layer of a trans-membrane glycoprotein called mucin1 (Muc1) during the non-receptive phase (Johnson and Everitt, 2007). The expression of Muc1 increases during the early pre-receptive phase at the epithelial surface, but it then decreases at the receptive phase, either globally (mouse) or locally (rabbit, human) (Braga and Gendler, 1993, Hey et al., 1994, Johnson and Everitt, 2007). In humans, it is believed that the conceptus produces an enzyme that can locally cleave Muc1's carbohydrate side chains, thereby removing its repulsive properties (Johnson and Everitt, 2007).

1.2.3.1.4. Loss of surface negative charge

Several reports have shown a generalized reduction in overall cell surface negativity in the uterine epithelial cells in human and other species around the time of implantation (Jansen et al., 1985, Navot et al., 1989). This probably assists the endometrium in making intimate contact with the embryo as well as the attachment of the trophoblast to the endometrial cells at the time of implantation.

1.2.3.2. Different factors involved in regulating endometrial receptivity

In addition to the physical contact of the embryo with the endometrium, the process of embryo implantation is undoubtedly influenced by various factors secreted from both maternal and embryonic tissues (Singh et al., 2011). Some of

the most important factors in increasing and regulating endometrial receptivity are listed below (Fig. 1.3):

1.2.3.2.1. Cytoskeletal rearrangement

The involvement of cell cytoskeleton organization in the establishment and progression of plasma membrane transformation has been reported in many studies. It has been shown that plasma membrane transformation is more directly related to alterations in microfilament (F-actin) organization than to microtubules (Luxford and Murphy, 1993). In this regard, it is well established that pinopodes are richly invested with a dense network of F-actin (Luxford and Murphy, 1992). Moreover, the loss of surface negative charge on human uterine epithelial cells (Psychoyos and Mandon, 1971), as well as flattening of the microvilli (Murphy, 1995) at the time of implantation has been correlated with F-actin dissociation and reorganizations. In addition, the importance of F-actin on the adhesion phase of embryo implantation has been shown (Heneweer et al., 2002), where a significant increase in signal of F-actin has been observed by confocal microscopy at the contact site between RL95-2 cells and JAr cells.

1.2.3.2.2. Adhesion molecules

Apart from F-actin, expression of cell adhesion molecules (CAMs) has also been shown to play an important role at the time of implantation (Wang and Dey, 2006, Aplin et al., 1999, Aplin, 1997, Aplin and Kimber, 2004, Kaneko et al., 2013). CAMs include integrins, selectins and cadherins, whose association with trophoblast

adhesion to the endometrium at the time of implantation has previously been shown (Campbell et al., 2000, Singh and Aplin, 2009). Recent research has tended to suggest that between different members of CAMs**, integrins** contribute to cell surface rearrangement during plasma membrane transformation (Lessey, 1994, Lessey et al., 1992). Moreover, it has been found that pinopodes are integrin-enriched structures (Nardo et al., 2003). In vertebrates, the integrin family consists of two subunits, α (24 types) and β (9 types). Among integrin subunits, **β3 integrin** is known as the best characterized adhesion molecule on the luminal surface of the endometrium (Lessey et al., 1992), as its expression increased in the endometrium only at the time of implantation in both human (Lessey et al., 1992) and animal models (Illera et al., 2003). Because β3 integrin up-regulation coincides with the window of implantation, this has led to the conclusion that β3 integrin could serve as one of the markers of endometrial receptivity. Moreover, animal studies showed a reduction in implantation sites following functional blockade of integrin β3 integrin in mice (Illera et al., 2000) and human endometrial epithelial cell line (Ishikawa) (Kaneko et al., 2011). Other integrins whose role in regulating the endometrial receptivity has been reported include α9β1, αvβ1, α1β1, α3β1, α6β1, αvβ5, and αvβ6 (van Mourik et al., 2009).

CD98 is another important adhesion molecule whose role on embryo implantation has recently been demonstrated. CD98 is a multifunctional protein that plays a role in amino-acid transport in different cell types such as renal and intestinal cells (Chillaron et al., 2001), integrin regulation (Fenczik et al., 1997) and embryogenesis (Tsumura et al., 2003). It is one of the important molecules for cell

development, differentiation, proliferation and regulation of cellular function (Tsumura et al., 2003). CD98 was found to be absent in the human endometrium in the proliferative phase, whereas its expression was increased at the window of implantation (Dominguez et al., 2010). Moreover, CD98 was localized to the apical surface of endometrial epithelium in the mid-secretory phase, which corresponds to the window of implantation (Dominguez et al., 2010). This evidence confirms CD98's role as an important determinant of endometrial receptivity during the window of implantation (Dominguez et al., 2010).

1.2.3.2.3. Growth factors

Transforming growth factor (TGF)

The TGF superfamily are expressed in the human endometrium and have vital roles in modulating endometrial functions such as proliferation, decidualization, and implantation process (Jones et al., 2006). Moreover, TGF-βs have been shown to play a role in human implantation via stimulation of fibronectin (Feinberg et al., 1994) as well as promotion of attachment of trophoblast cells to the extracellular matrix (ECM) (Irving and Lala, 1995).

Epidermal growth factor (EGF)

Addition of exogenous EGF to rat blastocysts significantly improved the rate of implantation compared with the control blastocyst *in vitro* (Aflalo et al., 2007). In addition, EGF is thought to be important for embryo development, as it is present in human pre-implantation embryos (Chia et al., 1995).

Vascular endothelial growth factor (VEGF)

VEGF regulates the growth of vascular membranes, angiogenesis and the formation of the placenta (Huang, 2006). VEGF has been detected in the human endometrium throughout the menstrual cycle at the mRNA and protein levels. Its highest expression was detected during the secretory phase, suggesting VEGF plays a central role during embryo implantation (Huang, 2006).

1.2.3.2.4. Cytokines

In the humans and primates, implantation is known to serve as an inflammatory type response. During the apposition stage of implantation, cytokines are the first wave of molecules produced locally by the endometrium. Some of these cytokines include IL-1β, IL-6, IL-8 and leukemia inhibitory factor (LIF), whose production was conceived to regulate and control the functions of endometrial cells during the menstrual cycle (Tabibzadeh and Sun, 1992, Tabibzadeh, 1994). Moreover, aberrant patterns of expression of some of the cytokines have been correlated with an absolute or partial failure of implantation and abnormal placental formation (Guzeloglu-Kayisli et al., 2009).

Leukemia inhibitory factor (LIF)

LIF is present in the human endometrium and its expression was found to be enhanced at the time of implantation, suggesting its role in embryo implantation (Kralickova et al., 2005). This assumption is further supported by the fact that

mutations in the LIF gene lead to unexplained infertility and repeated implantation failures in women (Steck et al., 2004).

Interleukin 1 (IL-1)

IL-1 is a key regulator of the inflammatory response and plays a crucial role in implantation (Bankers-Fulbright et al., 1996). The IL-1 system is a family of polypeptides comprising two agonists, IL-lα and IL-1β (Dinarello, 1988), and an inhibitor, IL-1 receptor antagonist (IL-1RA). Two IL-1 receptors have been identified and characterized: IL-1 receptor type I (IL-1R tI) (Sims et al., 1988) and IL-1 receptor type II (IL-1R tII) (Horuk and McCubrey, 1989). The available information indicates that IL-1R tII is not functional and IL-1 signalling occurs exclusively via type I receptors (Sims et al., 1993). **IL-1RA** can block the binding of IL1α and IL1β to its receptor and its binding to the receptor does not result in signal transduction (Bankers-Fulbright et al., 1996).

The expression of IL-1β is significantly increased in the human endometrium at the time of implantation (Simon et al., 1993a). In addition, it seems that IL-1β enhances endometrial receptivity by increasing the expression of various parameters required for successful implantation, such as α1, α4 and β3 integrin integrin (Simon et al., 1997). The balance of IL-1 and IL-1RA has been shown to play an important role in the embryo implantation process (Huang et al., 2001). In this regard, injection of IL-1RA in mice at the time of implantation reduced the implantation rate of embryos, presumably by disrupting the IL-1/IL-1RA balance at

the fetal-maternal interface. These data indicate that IL-1RA plays an anti-implantation role at the time of implantation (Simon et al., 1998).

Interleukin 6 (IL-6)

IL-6 is involved in tissue remodelling, decidualization, and placenta/trophoblast development (Jasper et al., 2007). IL-6 expression in the endometrium is increased at the time of implantation (Perrier d'Hauterive et al., 2004). Moreover, low IL-6 mRNA production has been correlated with recurrent miscarriages (Jasper et al., 2007). Hence, IL-6 may play a role in the implantation of the embryo. The crucial role of IL-6 in embryo implantation has been further defined using IL-6-deficient mice, characterized by reduced implantation sites and infertility (Robertson et al. 2000).

Interleukin 8 (IL-8)

IL-8 is expressed in the human endometrium and its staining intensity was reported to be significantly enhanced at the secretory phase of the menstrual cycle, indicating that it has a role in embryo implantation. Furthermore, IL-8 mRNA and protein were up-regulated in endometrial epithelial cells (EECs) co-cultured with human embryos compared to EECs without embryos (Caballero-Campo et al., 2002). In addition, human blastocyst induces the expression and polarization of CXCR1 (IL-8 receptor) in endometrial epithelial cells (Dominguez et al., 2003). IL-8 is also thought to act as a signal for receptor polarization and activation of endometrial adhesion molecules (Dominguez et al., 2003).

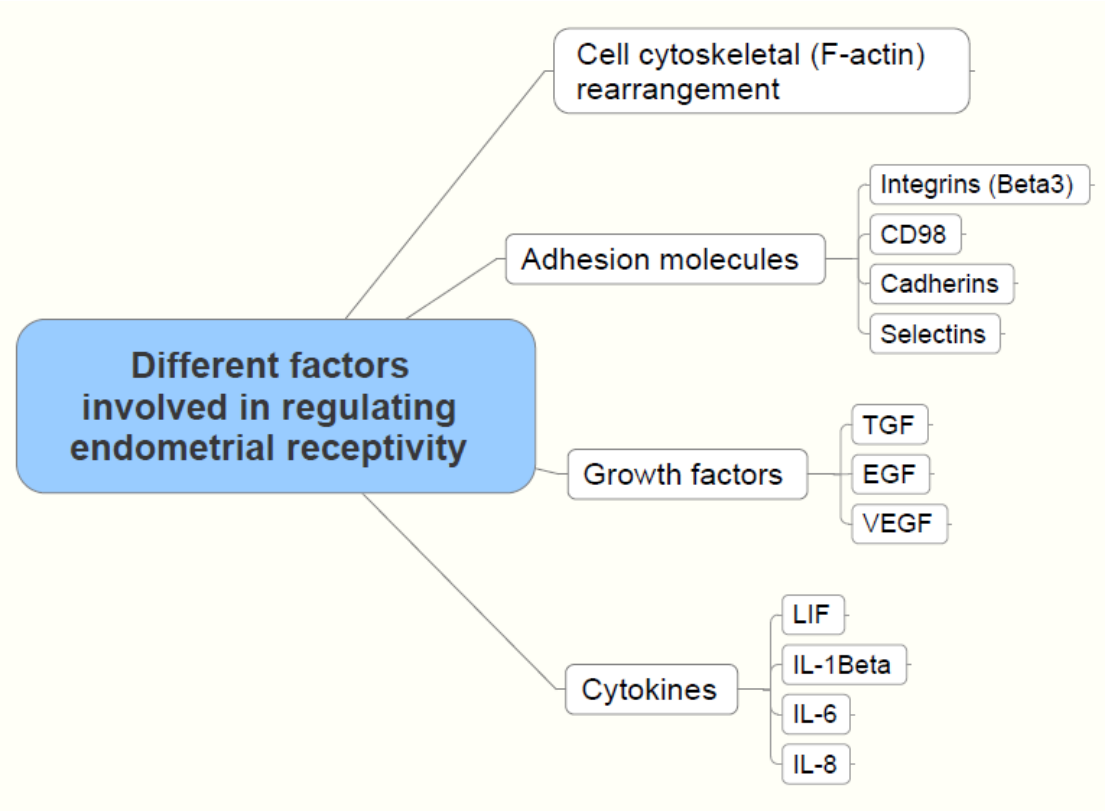

Figure 1.3 Different factors involved in regulating endometrial receptivity.

1.3. Model systems of implantation

An *in vivo* research on human implantation is ethically not acceptable, it is very difficult to study the implantation process in humans. Several model systems have therefore been developed for studying implantation in humans. These models can be divided into two main categories: human cell-based and animal-based systems. Human cell-based models can apply either primary cell culture or cell lines, whilst animal-based models use mice, rabbits, pigs, cows, primates and sheep. In the rest of this chapter, each of these model systems is described in details.

1.3.1. *In vitro* Human cell-based models of implantation

After successful accomplishment of *in vitro* fertilisation (IVF) procedure in 1978, the field of reproductive medicine was radically changed and human implantation research received new impetus (Steptoe and Edwards, 1978). After 1978, human embryos were created by routine IVF procedure without ethical issues and without the invasive technique of removing blastocysts from post-conception women (Hertig and Rock, 1973). The development of human embryos was a breakthrough in reproductive medicine research, which enabled the scientists to establish new *in vitro* implantation models and investigate various parameters regulating the process of embryo implantation.

Current model systems study the process of human implantation and molecular interactions between the embryo and the endometrium by using multiplex assays, proteomics, and microarrays. In the following chapter, various types of human cell-based models are described.

1.3.1.1. Primary cell culture

In this model, endometrium explants are used to imitate the environment of the uterus during implantation. This kind of model can be divided into two groups, monolayer and multilayer (Hannan et al., 2010). In the monolayer model, only the epithelial or the stromal layer of endometrium is cultured (Fig 1.4), whereas in the multilayer model, both epithelial and stromal cells are grown. In the following section, some examples for each of these model systems are given.

1.3.1.1.1. Monolayer primary cell culture

Landgren and colleagues used organ level explants of the endometrium. With this technique they closely imitated the *in vivo* environment, because the three-dimensional structure of the endometrium was preserved (Landgren et al., 1996). Endometrial biopsies were obtained 4, 5, and 6 days after the luteinizing hormone (LH) peak, from healthy women with normal menstrual cycles. The samples were taken from the secretory phase, which was confirmed with histological dating and placed in tissue culture medium RPMI 1640. After three hours, 1-3 embryos, obtained by IVF, were co-cultured with the endometrial biopsies. The biopsies

were analysed morphologically by light microscopy before and after co-culture. Their investigations showed that the embryos produced 4 days after IVF invaded the lining of *endometrial* epithelial cells (EECs) obtained 4 days after the LH peak (Landgren et al., 1996).

This model was able to study embryo development beyond day 6 of blastocyst stage, but it also preserved endometrial interaction with the developing embryo. However, the human embryos obtained from IVF treatments did not represent natural embryo tissue.

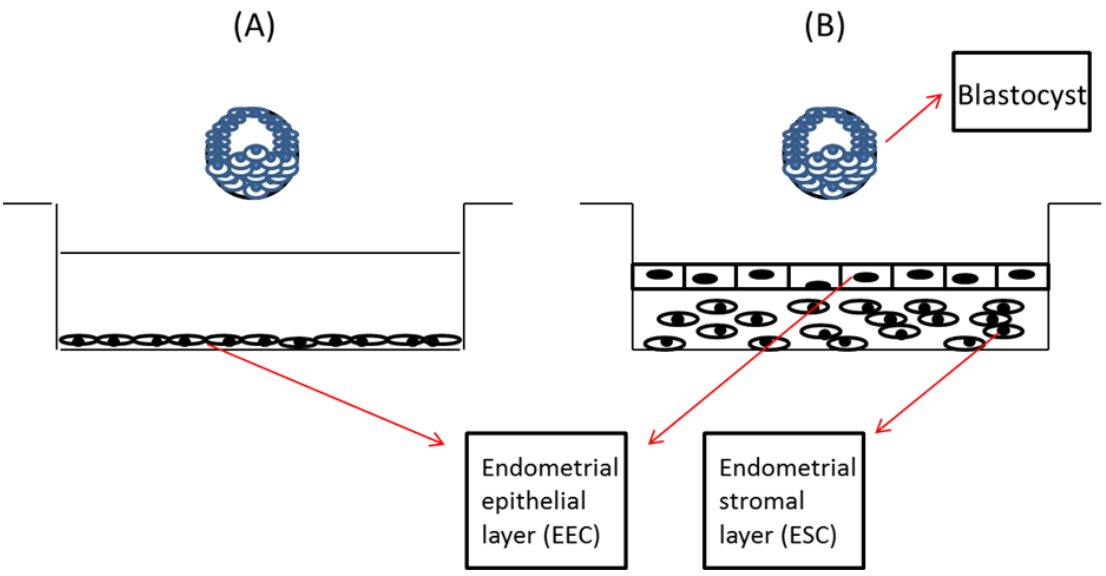

Figure 1.4 Comparison of monolayer and multilayer models.

A) Monolayer model: Epithelial explants of endometrium are cultured.

B) Multilayer model: Both epithelial and stromal layers of endometrium are co-cultured.

1.3.1.1.2. Complex and multilayer primary cell culture

In the previously discussed studies only one layer of endometrium was employed. This failed to represent the *in vivo* uterine environment. To overcome this limitation, a number of groups have worked on the development of three-dimensional co-culture models in which both endometrial epithelial cells (EECs) and endometrial stromal cells (ESCs) were co-cultured. In this way ESCs and EECs not only interacted with the embryo, but also with each other (Fig 1.4). Some examples of these models are the Bentin and Pedersen models. The following will describe these models in more details.

Bentin-Ley model

Bentin and colleagues managed to simulate the three-dimensional architecture of the endometrium (Lopata et al., 2002, Bentin-Ley et al., 1999, Bentin-Ley et al., 2000). They spread EECs on an artificial matrigel-coated basement membrane and ESC matrix was co-cultured underneath (Bentin-Ley et al., 1994). Later, they used this model to study the ultrastructure of embryo–endometrial interactions at *in vitro* implantation sites by scanning electron microscopy (SEM), with focus on endometrial changes in the presence of a blastocyst (Bentin-Ley et al., 1999). Three hatched embryos obtained from IVF treatments were co-cultured with three endometrial multilayer cell cultures. The co-cultures were examined for implantation sites every morning through a stereo microscope. The adherence of the blastocysts was assessed by gentle shaking of the culture dish. All three blastocysts were fully hatched and attached 48 h after co-culture.

Later, this model was used to study the effect of contraceptives on the endometrium and embryo attachment (Petersen et al., 2005, Lalitkumar et al., 2007, Meng et al., 2009). Although this model cannot completely imitate the *in vivo* environment of endometrium, it allows investigations of specific processes which are involved in human embryo-endometrium interactions.

1.3.1.2. Cell lines

Embryo implantation cannot be studied *in vivo*, and because of the limited availability of human blastocysts, the use of cell lines and *in vitro* models is vital to implantation research. Each cell line has a unique phenotype. A cell line may be useful to study one particular parameter but it may be entirely unsuitable for studying another. The trophoblast cell line JAr, for example, is a suitable model to mimic invasion phase of human implantation but not a suitable model for earlier stages of pregnancy. The trophoblast cell line AC-1M88, meanwhile, is an excellent model for studying earlier stages in implantation and can simulate the adhesion phase of human implantation (Hannan et al., 2010).

Epithelial and trophoblast cell lines are generally used for investigating the implantation process. In the following section, the phenotypic characteristics of these cell lines are discussed and comparison between their *in vitro* and *in vivo* phenotypes is drawn. The diagram (Fig 1.5) summarises cell line models and their applications in studying various aspects of implantation in humans.

1.3.1.2.1. Endometrial cell lines

Most endometrial epithelial cell lines originated from endometrial adenocarcinomas. They are classified into five main groups (Table 1).

HEC-1 (human endometrial carcinoma)

This cell line was established more than 40 years ago (Kuramoto et al., 1972), and was the first readily available endometrial cell line. It has low adhesive properties, and was used as a model for non-receptive endometrial cell lines.

RL95 (receptive endometrial cell line)

As this cell line is highly adhesive to both trophoblast cell lines and mouse blastocysts, it is generally used as a suitable model system for receptive endometrial cells (Hohn et al., 2000). The cells are characteristically epithelioid, as they are α-keratin positive (Way et al., 1983). They are hormonal responsive and express both estrogen (Way et al., 1983) and progesterone receptors (Tamm et al., 2009). Expression of various adhesion molecules makes them suitable for models to study the adhesion of trophoblast cells to the endometrial cells.

Ishikawa

The Ishikawa cell line (Nishida et al., 1985) is a good model for studying normal endometrial function. It shows mixed characteristics of glandular and luminal epithelium (Mo et al., 2006). Ishikawa cells possess apical adhesiveness to JAR cells (Heneweer et al., 2005). They are particularly useful for studying endocrine signalling in the endometrium. They express steroid receptors (estrogen,

progesterone, and androgen) and mucin 1 (Muc1) similarly to *in vivo* endometrial epithelial cells.

ECC-1 (endometrial carcinoma cell line)

The ECC-1 cells express cytokeratins (KRTs) 13 and 18, which are characteristic of luminal epithelium. They also hold estrogen receptors α and β, progesterone receptors A and B, and androgen receptors (Mo et al., 2006). The ECC-1 cell line is stable and highly responsive.

HES

The HES cells are particularly useful cell lines for studying early events and interactions between the endometrium and the trophectoderm. They express Muc1 occurring in normal endometrium (Wang et al., 2008), and they have embryotrophic characteristics (Desai et al., 1994).

1.3.1.2.2. Trophoblast cell lines

Trophoblast stem cells of the placenta differentiate into two major lineages: cytotrophoblast and syncytiotrophoblast. Some trophoblast cells fuse together and form syncytiotrophoblast, while others retain their cellularity and form cytotrophoblast. They can be used as a proliferative source for generating trophoblast cells (Johnson and Everitt, 2007). They differentiate into extravillus trophoblast (EVT) and form cell columns in anchoring villi. EVTs in the distal cell columns invade the decidua. Trophoblast cell lines are generated from a variety of

sources and, like endometrial epithelial cell lines, most trophoblast cell lines originate from placenta choriocarcinomas (King et al., 2000) (Table 1). In addition, some of general markers of human trophoblast cell lines are summarised in Table 2.

Trophoblast adhesion, attachment and invasion are crucial events of human implantation. Cell adhesion molecules such as integrins and trophoblast integrin receptors in trophoblast cells play a vital role in the adhesion and attachment stages of a successful implantation. Proteolytic enzymes are required for the invasion stage. HTR-8/SVneo and AC-1M88 cells are useful in adhesion experiments, while JEG, JAR and HTR-8/SVneo cells are mainly applied in invasion studies (Hannan et al., 2010).

Cell line	Origin	Implantation functions	Phenotype	References
	Endometrial cell lines			
ECC-1	Adenocarcinoma		Luminal	(Satyaswaroop and Tabibzadeh, 1991)
Ishikawa	Adenocarcinoma	Adhesive	Glandular & luminal	(Heneweer et al., 2005, Nishida et al., 1985)
HEC-1	Adenocarcinoma	Poorly adhesive		(Kuramoto et al., 1972)
HES		Embryotrophic	Luminal	(Desai et al., 1994)
RL95-2	Moderately differentiated adenosquamous carcinoma	Highly adhesive		(Hohn et al., 2000, Thie et al., 1997, Way et al., 1983)
	Trophoblast cell lines			
HTR-8/SVneo	First trimester villous explants	Adhesion Migration Invasion Proliferation	EVT, VT	(Paiva et al., 2009)
AC1M-88	Fusion of JEG-3 and term trophoblast	Adhesion Migration	EVT	(Hannan et al., 2006, Hannan and Salamonsen, 2008, Paiva et al., 2007)
JEG-3	Choriocarcinoma explants	Invasion	EVT	(Grummer et al., 1999)
JAR	Gestational Choriocarcinoma	Invasion	VT/EVT	(Mandl et al., 2006)
BeWo	Choriocarcinoma	Syncytialization	EVT	(Grummer et al., 1999, Mandl et al., 2006)
SGHPL-4	Primary extravillous trophoblast	Adhesion Migration Invasion Proliferation	EVT	(Cartwright and Balarajah, 2005)

Table 1. Characteristics of endometrial and trophoblast cell lines.

EVT: extravillous trophoblast; **VT**: villous cytotrophoblast (Hannan et al., 2010).

General Markers	KRT7	CG	CGR	HLAG	CD9
HTR-8/SVneo	+	+		+	
AC1M-88	+	+		+	+
JEG-3	+	+	+	+	
JAR	+	+		–	
BeWo	+	+		+/–	+
SGHPL-4	+	+		+	+
References	(Choy and Manyonda, 1998)	(Choy and Manyonda, 1998	(Handschuh et al., 2007)	(Choy and Manyonda, 1998)	(Choy and Manyonda, 1998)

Table 2. General markers of human trophoblast cells.

KRT7: cytokeratin 7; expressed throughout the trophoblast lineage *in vivo*, but not by any other cells in placental villous or maternal deciduas; **CG**: human chorionic gonadotrophic hormone; is first detected in the embryo as early as the eight-cell stage; **CGR**: human chorionic gonadothrophic hormone receptor; **HLAG**: human leucocyte antigen-G, expressed by trophectoderm in pre-implantation conceptus

during the blastocyst stage; **CD9**: cluster of differentiation antigen 9 (Hannan et al., 2010).

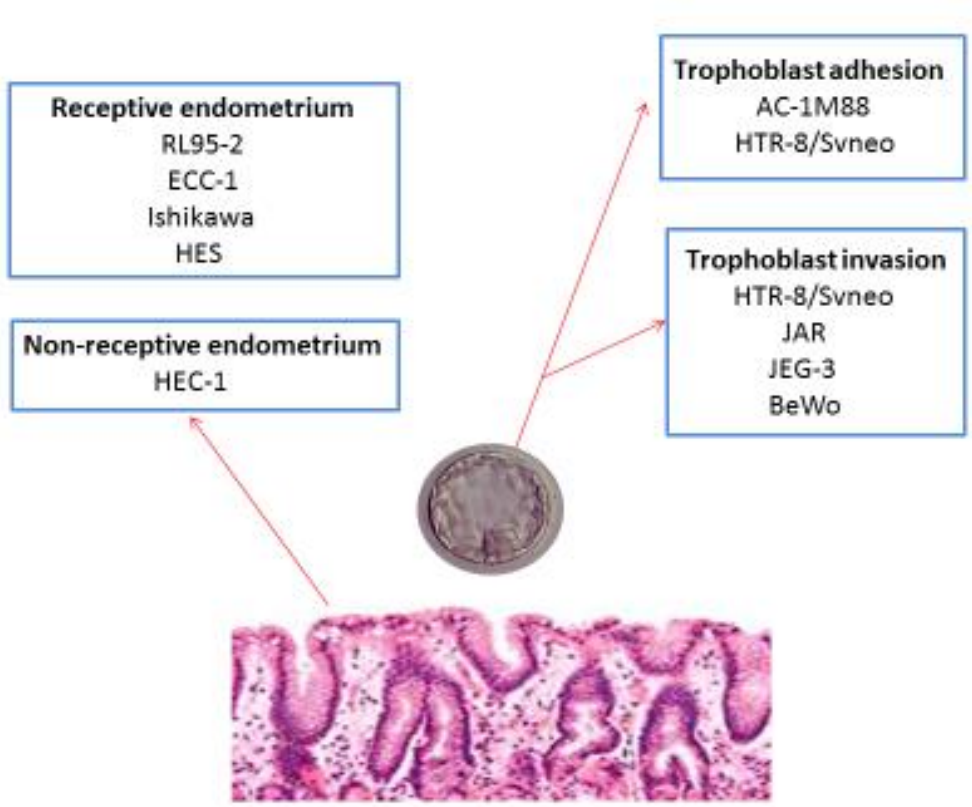

Figure 1.5 Cell line models and their applications in studying human implantation.

1.3.2. Animal-based models

Because of ethical issues it is close to impossible to carry out *in vivo* experiments on implantation in humans. Animal models are used to simulate the molecular and mechanical events of implantation. Although the mechanism of implantation varies among species, the fertilisation process and early events of implantation are very similar (Lee and DeMayo, 2004). Animal models used for studying human implantation are outlined in the following section.

1.3.2.1. Mouse

Mouse is not a suitable candidate to investigate early events of implantation as all three stages of implantation occur within 6 hours of fertilisation. However, gene ablation in genetically engineered mice made it possible to understand the function of individual genes which are involved in embryo implantation (Dey et al., 2004). In addition, mouse models were utilized in delayed implantation, pseudopregnancy and artificial induction of decidualization (McLaren, 1969, Finn and Martin, 1972, Cooke et al., 1986). These investigations revealed many uterine implantation and pre-implantation events as well as implantation disorders.

1.3.2.2. Rabbits

Rabbit is an excellent model for investigating apical cell adhesion (blastocyst adhesion) because it is easy to detect the exact time of ovulation (10 h after

mating), as well as the time of apposition and attachment (day 6.5). In addition, the trophoblasts adhere very well to *in vitro* cultured epithelium (Hoffman et al., 1998, Lee and DeMayo, 2004).

1.3.2.3. Pigs, sheep and cows

Since the type of implantation in these animals is non-invasive, and characterized by a long pre-implantation phase consisting of apposition and attachment (Gray et al., 2001, Wooding, 1992), they are suitable candidates to study early events of implantation.

1.3.2.4. Primates

Studying the process of implantation in animal models, such as mice and pigs, has distinct advantages. But, their ability to simulate the mechanisms of human implantation is limited. However, non-human primates have similar implantation processes to humans (Enders, 2000). Marmosets and rhesus monkeys are good models for investigating early stages of implantation. In contrast, the macaque monkey was shown to be a good model to study late stages of implantation (Lee and DeMayo, 2004, Enders, 2000).

The above are some of the model systems used to study implantation. Each of these models may be used to investigate specific aspects of implantation in humans.

The next sections give an overview of the innate immune system and its role in providing protection against pathogens in the FRT. In addition, a comprehensive explanation is developed of how the innate immunity in the endometrium regulates endometrial receptivity and affects embryo implantation.

1.4. Toll-like receptors in the female reproductive tract

1.4.1. The innate immune system

The innate immune system is the first line of defence against invading pathogens and provides an immediate response. It evolved to detect foreign structures which are normally not found in the host (Medzhitov and Janeway, 2000a). The innate immune system plays a crucial role in the development of antigen-specific acquired immunity (Bulmer et al., 1988).

There are several pathogen recognition receptors (PRRs) in the innate immune system and their major role is to recognize non-self-infectious molecules and eradicate these invading pathogens. It is well-documented that PRRs are expressed either on the cell surface, in the intracellular compartments or secreted into the blood stream or tissue fluids (Medzhitov and Janeway, 1997). Some examples of intracellular PRRs are nucleotide-binding oligomerization domain NOD-like receptor (NLRs), retinoic acid-inducible gene-1, RIG-1-like receptors (RLRs) and the protein kinase receptor (PKR). NLRs respond to bacterial stimuli, whereas RLRs and PKR recognize viral stimulation (Creagh and O'Neill, 2006, Meylan et al., 2006). The cell surface receptors include Macrophage mannose receptors (MMR) and Macrophage scavenger receptors (MSR). MMR and MSR play a role in phagocytosis of gram-positive and gram-negative bacteria and fungal

infection (Gordon, 2002). Finally, the most important family of trans-membrane PRRs are Toll-like receptors (TLRs), which recognize the presence of a wide range of stimuli known as pathogen-associated molecular pattern sequences (PAMPs). Some examples of PAMPs are molecules such as lipopolysaccharide (LPS) of gram-negative bacteria, or single and double stranded RNA and DNA in viruses (Janeway, 1989). When PRRs bind to PAMPs, a series of inflammatory responses are triggered against the invading pathogen (Medzhitov and Janeway, 1997). PRRs are capable of sensing endogenous molecules generated from tissue damage and free of pathogens. These damage associated molecular patterns (DAMPs) are not found in the extracellular space in a homeostatic state. One example of this family is fragmented extracellular matrix. They are secreted as an alarm signal for cell death (Bianchi, 2007).

1.4.2. The Toll-like receptor family

Toll-like receptors (TLRs) are a family of innate immune receptors which have a vital role in the recognition of PAMPs and DAMPs (Bianchi, 2007, Kawai and Akira, 2007). Innate immune responses are triggered by the detection of PAMPs in insects and mammals (Lemaitre et al., 1996). Their humoral immune responses are cascaded in the (NF)-κB signalling pathway (Williams et al., 1997). In 1996, *Drosophila* Toll was initially identified as a receptor responsible for dorso-ventral polarity during development. It was also shown that it participates in anti-fungal innate immune responses (Lemaitre et al., 1996). The 18 wheeler receptor, as a homologue of this Toll, increases antibacterial response in *Drosophila* (Williams et

al., 1997). Both receptors stimulate nuclear factor (NF)-κB, a transcription factor responsible for the antimicrobian peptides genes in *Drosophila* and humans (Kopp and Ghosh, 1995). In subsequent years, numerous homologues of Toll, known as Toll-like receptors (TLRs), were identified in mammals. They were shown to be able to detect PAMPs and to activate innate immune responses (Akira et al., 2006).

Toll-like receptors are trans-membrane proteins. They have an extracellular domain which consists of leucine-rich repeats (LRR) and binds to ligands. They also have a cytoplasmic domain which is known as the Toll/IL-1 receptor (TIR) domain. This binds to TIR-containing adaptor molecules which regulate downstream signalling (Akira and Takeda, 2004). To date, 13 members of the TLR family have been identified in mammals, 10 of which are expressed in humans and 12 in mice (Beutler, 2004). TLR 1, TLR 2, TLR 4, and TLR 6 are cell-surface TLRs. They recognize microbial membrane lipids. TLR 3, TLR 7, and TLR 9 are expressed inside the cell and recognize pathogen nucleotides in intracellular compartments (Saitoh and Miyake, 2009).

1.4.3. Signalling pathways of Toll-like receptors

Recognition of PAMPs by TLRs induces intracellular signalling pathways. This entails the induction of inflammatory cytokines genes such as tumor necrosis factor (TNFα), IL-6, IL-1β, IL-12, and in the final step, type 1 interferon (IFN), which plays a role in antiviral responses. (Carty et al., 2006) (Fig 1.6).

When PAMPs bind to TLRs, a set of cytoplasmic TIR-domain-containing adaptors is stimulated by TIR-TIR interactions. This includes MyD88, TIRAP (also known as MAL), TRIF (also known as TICAM1) and TRAM (also known as TICAM2) (Akira and Takeda, 2004). There are two main inflammatory pathways induced by TLRs: MyD88-dependent and TRIF-dependent pathways.

Myeloid differentiation primary response 88 (MyD88) is recruited to all TLRs except TLR 3, and it activates mitogen-activated protein kinases (MAPKs) and nuclear factor (NF-κB) to control the expression of inflammatory cytokine genes. TIRAP controls the downstream activation of TLR 2 and TLR 4 in the MyD88-dependent pathway.

TLR 3 and TLR 4 share Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) in the TRIF-dependent pathway. They activate an alternative pathway that leads to the activation of NF-κB, MAPKs and the transcription factor IRF3. IRF3 is a critical adaptor for induction of type1 IFN, particularly IFNβ. TRAM is involved in the activation of TLR 4 downstream but not TLR 3 (Carty et al., 2006).

1.4.4. Ligands of Toll-like receptors

Each TLR recognizes and binds to ligands from various types of PAMPs including bacteria, viruses, fungi and parasites (Janeway and Medzhitov, 2002, Zhang and Ghosh, 2002).

TLR 2 and its associated receptors **TLR 1** and **TLR 6** are mainly involved in the detection of molecules from mycobacteria and gram-positive bacteria (Medzhitov and Janeway, 2000a). For detection of some PAMPs, such as lipoteichoic acid, only TLR 2 is required (Schwandner et al., 1999). In contrast, detection of triacylated lipoproteins is due to interactions between TLR 1 and TLR 2 (Takeuchi et al., 2002), whereas recognition of diacylated lipoproteins and peptidoglycans requires interactions of TLR 2 and TLR 6 (Wetzler, 2003).

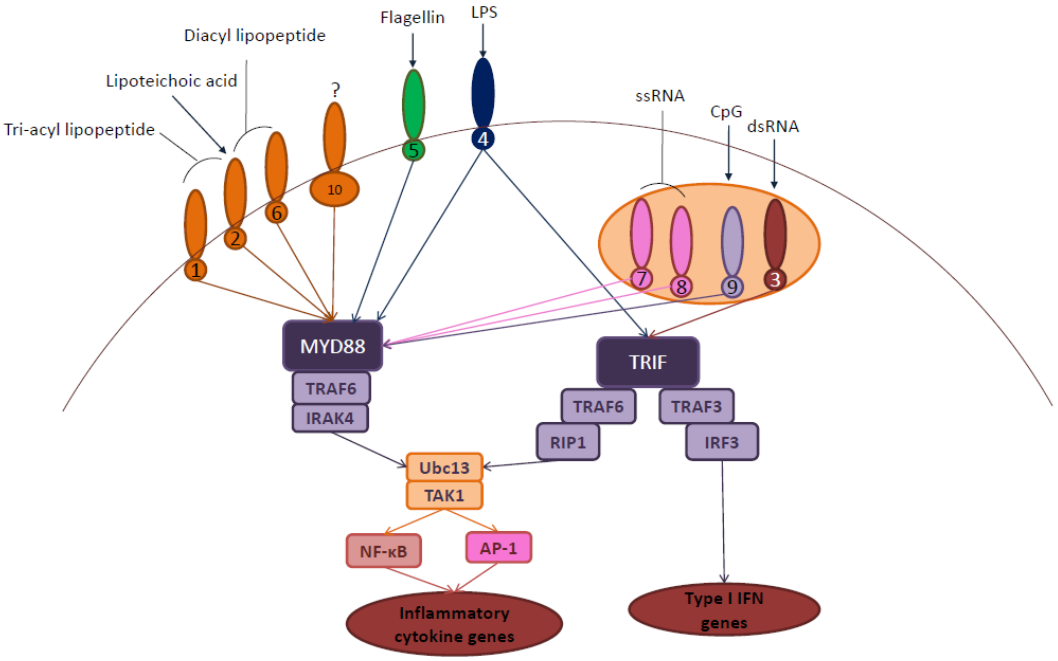

Figure 1.6 Signalling pathways of Toll-like receptors.

TLRs (except TLR 3) react with their specific ligands through MyD88 and activate NF-κB and AP-1, leading to inflammation. TLR 3 utilizes TRIF pathway to activate NF-κB and AP-1 adaptor molecules. TLR 4 can react through both MyD88 and TRIF pathways. In addition to TRIF pathway, TLR 3 induces anti-viral responses through the IRF-3 pathway.

TLR 2 also recognizes lipopeptidephosphoglycan from *Entamoeba histolytica* (Maldonado-Bernal et al., 2005) and lipophosphoglycan from *Leishmania major* (Becker et al., 2003). TLR 2 can in addition recognize DAMPs in the extracellular matrix component hyaluronan (Noble and Jiang, 2006).

TLR 3 is able to detect foreign double-stranded RNAs of viruses and the synthetic ligand polyinosinic-polycytidilic acid (Poly I:C) (Barton, 2007).

TLR 4, the first identified TLR family (Medzhitov and Janeway, 1997), recognizes Gram-negative bacterial lipopolysaccharide (LPS), expressed in the outer membrane of Gram-negative bacteria. To achieve this goal, the presence of the accessory molecules MD2 and CD14 is also required (Jiang et al., 2000). TLR 4 also recognizes other classes of DAMPs such as hyaluronan (Voelcker et al., 2008), biglican (Jiang et al., 2005) and fibronectin (Morwood and Nicholson, 2006).

TLR 5 is able to detect flagellin, which is the major component protein of bacteria (Akira et al., 2006).

TLR 7 (in human) and **TLR 8** (in mouse) are able to recognize single-stranded RNA viruses and imidazoquinoline, which is a synthetic molecule used against papilloma virus infections (Barton, 2007).

TLR 9 can recognize DNA with repeated cytidine-phosphate-guanosine (CpG) motifs present in bacteria and viruses (Barton, 2007).

TLR 10, similarly to TLR 1 and TLR 6, is highly homologous to TLR 2 and is probably another TLR 2-associated receptor. Its function and ligand have not been identified yet (Chuang and Ulevitch, 2001).

1.4.5. Toll-like receptors in the female reproductive tract

Although the developing fetus is immunologically distinct from the mother (semi-allogenic), it is not rejected by the maternal immune system and establishes intimate physical contact with the endometrial cells throughout pregnancy. The mother has to adapt to the physiological events of fertilisation, implantation and pregnancy. The female reproductive tract also has to deal with sexually-transmitted bacterial and viral infections at the same time (Abrahams et al., 2005, Grossman, 1985). In order to achieve this adaptation, the FRT evolved into different compartments such as ovaries, Fallopian tubes, uterus, cervix, and vagina, which belong to the mucosal immune system. The pH of the FRT is between 3.5 and 4.9, due to commensal colonized bacteria such as *Lactobacillus acidophilus*. This pH range inhibits the growth of other bacteria which may enter from the perineum (Waugh 2001).

TLRs are expressed on the epithelial cells of the FRT and detect non-self-infectious pathogens (Schaefer et al., 2004). These epithelial cells play an essential defence

role against pathogens, and regulate immunity in order to maintain the health of the mother and the allogenic fetus (Wira 2002).

The expression of TLRs varies in the compartments of the FRT in humans. TLR 1, TLR 2, TLR 3, TLR 5 and TLR 6 are expressed in the epithelia of the upper and the lower parts of the tract, while TLR 4 has only been found in the upper compartments (endocervix, endometrium and fallopian tubes). A secretory form of TLR 4 was found in the endocervical glands. This pattern of expression suggests a possible tolerance mechanism in the lower FRT, where the continuous activation of the TLR 4 pathway would generate exacerbated inflammatory processes (Fazeli et al., 2005). All TLR molecules with the exception of TLR 10 are expressed in endometrial cell lines (ECC-1) (Schaefer et al., 2004) and primary cell cultures of EECs (Schaefer et al., 2005).

The sex hormones control the endometrial environment during the menstrual cycle. TLRs are expressed throughout the menstrual cycle with an alternating gene expression pattern for TLR 2, TLR 3, TLR 4, TLR 5, TLR 6, TLR 9 and TLR 10. The lowest levels of gene expression occur during the menstrual and proliferative phases while the highest levels of gene expression have been found in the secretory phase. This differential expression of TLRs throughout the menstrual cycle may indicate the regulatory effect of sex hormones on TLR expression. In addition, it suggests that TLRs may play a pivotal role in tissue remodelling of the endometrium, which could prepare the endometrial epithelial cells for implantation of the embryo (Aflatoonian et al., 2007).

1.4.6. The role of Toll-like receptors in implantation failure

One of the reasons for implantation failure and fetal loss may be the activation of TLRs in the FRT at early stages of pregnancy (Aboussahoud et al., 2010b). Aboussahoud and colleagues have shown that trophoblast cells' attachment to epithelial cells can fail due to TLR 5 activation caused by *in vitro* ligation of TLR 5. In addition, activation of TLR 2/6 has been shown to decrease the rate of trophoblast adhesion to the endometrial cells *in vivo* and *in vitro* (Sanchez-Lopez et al., 2014). These findings may contribute to the diagnosis and treatment of infertility cases induced by activation of the innate immune system in the endometrium. Still more investigations are needed to fully understand the role of the TLR family members in implantation failure.

1.5. Aims of the study

The implantation of human embryo into the uterine endometrium is a complex process in which a direct line of communication between the mother and the embryo is required. The presence of cytokines, chemokines, hormones, growth factors and adhesion molecules are crucial to successful implantation (McEwan et al., 2009).

The presence of pathogenic microorganisms in the FRT can affect female fertility (Pellati et al., 2008). They are recognized by receptors of the innate immune system such as TLRs. It has been shown that stimulation of TLR 2/6 and 5 by their specific ligands reduced the adhesion rate of trophoblast cells to the endometrial cells *in vivo* and *in vitro* (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014). Activation of TLRs in the FRT which is caused by infection may initiate an inflammatory response and imbalance in cytokine and chemokine production. This could lead to impairment of the implantation process. Earlier reports have identified an association between production of some pro-inflammatory cytokines such as IL-6, TNF-α, IFN-ɤ, IL-1 and pregnancy loss (Kwak-Kim et al., 2010). The role of TLR signalling in the FRT is not clear yet and it is still unknown whether the ligands of these receptors can directly affect endometrial receptivity.

To address this, the current study focused on understanding the role of the activation of TLR 3 in human embryo implantation and the potential mechanisms and pathways involved in implantation failure caused by innate immune activation

in the FRT. We hypothesized that the activation of endometrial TLR 3 negatively affects endometrial function and receptivity.

To test the validity of this hypothesis, first, the presence of different TLRs in different endometrial cell lines was characterized and an assay was developed for quantifying trophoblast cells' attachment to endometrial cells *in vitro* (Chapter 1). We used this model to detect the potential effect of TLR 3 on trophoblast interaction with endometrial cells (Chapter 2). Furthermore, in determining the potential route of action of TLR 3 in implantation failure our focus was on mechanistic studies (Chapters 3, 4 and 5).

Together, our results indicated that the activation of the endometrial TLR 3 influences various parameters required for endometrial receptivity and successful implantation. These effects could be observed *in vitro* affecting cytokine production, transcription factor activation, cell cytoskeleton organization and adhesion molecule expression.

Chapter 2. Establishment of an *in vitro* model to investigate the effect of TLR 3 activation on implantation

2.1. Introduction

Approximately 70 % of pregnancies end up with fetal loss (Chard, 1991, Wilcox et al., 1988, Zinaman et al., 1996) and despite many advances in assisted reproductive technologies (ART), embryo implantation rates are still low (Diedrich et al., 2007). The high incidence of pregnancy failure in women undergoing *in vitro* fertilisation (IVF) after replacement of high-quality embryos indicates the significance of implantation failure as a major cause of fetal loss in IVF (Carver et al., 2003). Failure of implantation after natural mating or IVF may be caused by embryo-related or endometrial related factors. Two thirds of implantation failures are due to problems in endometrial receptivity (Ledee-Bataille et al., 2002). The immune system plays an important role in providing proper endometrial receptivity (Koga et al., 2009a). Sub-clinical infections, inflammation or malfunction of the immune system in the female reproductive tract (FRT) may all contribute to implantation failure (Pellati et al., 2008). The innate immune system recognizes the invading microorganisms through pathogen recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997, Medzhitov and Janeway, 2002). Up to now, 10 members of the TLR family have been recognized in humans (Beutler, 2004).

The expression of TLR 1-10 has been shown in endometrial cell lines (ECC-1) (Schaefer et al., 2004) and primary cell cultures of endometrial epithelial cells (EECs) (Schaefer et al., 2005). This expression was menstrual cycle-dependent (varies along the menstrual cycle), with the highest level of TLR expression in the

human endometrium during the late secretory phase, when the implantation of the embryo takes place (Aflatoonian et al., 2007). This will prepare a hospitable environment for the reception of the embryo and its further implantation and development. Although TLRs have been shown to be involved in a number of pregnancy disorders, such as early pregnancy loss, preterm labour and pre-eclampsia (Koga et al., 2009b), we know very little about their role in embryo implantation. Previous experiments in our lab have shown that stimulation of TLR 2/6 and 5 leads to reduced binding of trophoblast cells to endometrial cells *in vivo* and *in vitro* (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014). 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.

There is a wealth of literature supporting the evidence that TLR 3 is expressed in the uterine endometrial epithelial cells (Schaefer et al., 2005) and different cell lines such as ECC-1 (Schaefer et al., 2004) and Ishikawa (Aboussahoud et al., 2010a). It has been also shown that TLR 3 is expressed in the human endometrial epithelial cell line, RL95-2, and responds to double-stranded RNA leading to production of interleukin (IL)-6, IL-8, regulated on activation normal T cell expressed and secreted (RANTES), and interferon β (IFN-β) (Jorgenson et al., 2005), and plays a crucial role in antiviral responses in the FRT (Yu and Levine, 2011b). TLR 3 recognizes cytomegalovirus (CMV) and herpes simplex virus 1 (HSV-1) (Matsumoto et al., 2011). A growing body of evidence suggests that there is a significant association between the presence of HSV 1 and 2 and CMV infection in

the female reproductive tract and infertility in women (el Borai et al., 1997, Medvedev et al., 2009). Similarly, it was found that seroprevalence and genital viral shedding of CMV were relatively high in infertile women (Yang *et* al 1995). This suggests that CMV infection in the FRT may have an effect on female infertility and as such may involve TLR 3 dysfunction (Yang et al., 1995).

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 epithelial polarization, express adhesion molecules and microfilaments in the apical surface and to be responsive to hormones and secretion of cytokines (Way et al., 1983).

Embryo implantation consists of three stages known as apposition, adhesion and invasion of the trophoblast cells to the epithelium and the underlying endometrial stroma (Johnson and Everitt, 2007). In the current study, we aimed to investigate the effect of TLR 3 activation on embryo implantation and as each implantation stage has different characteristics, this effect was investigated both in early (apposition-adhesion) and late (adhesion-invasion) stages of implantation. To address this, we used two different trophoblast cell lines: AC-1M88 and JAr. AC-1M88 is a hybridoma cell line which is a mixture of choriocarcinoma cell line (Jeg-

3) and Primary Extravillous Trophoblast. This cell line has been derived from earlier stages of pregnancy and used widely in trophoblast adhesion assays (Hannan et al., 2006, Hannan and Salamonsen, 2008, Paiva et al., 2007). On the other hand, JAr cell line is derived from term trimester trophoblast cells and is regarded as an invasive trophoblast cell line (Pattillo et al., 1971). Both trophoblast cell lines were used to form spheroid bodies, which imitate human embryos *in vivo* (Heneweer et al., 2005). AC-1M88 and JAr spheroids were delivered onto the monolayers of RL95-2 cells to simulate the early (apposition-adhesion) and late (adhesion-invasion) stages of embryo implantation respectively.

In the present investigation we investigated the effect of TLR 3 activation on the attachment of trophoblast cells to the endometrial cells as well as testing the hypothesis that inhibition of TLR 3 signalling pathway could restore the binding ability of trophoblast cells to endometrial cells *in vitro*. In summary, our results with either JAr cells or AC-1M88 cells showed that TLR 3 stimulation by Poly I:C decreased the attachment of trophoblast cells to the endometrial epithelial cells, suggesting that activation of TLR 3 at both early and late stages of implantation may be detrimental and leading to implantation failure.

2.2. Methods

2.2.1. Cell lines and cell culture

RL95-2 was obtained from ATCC and was used to mimic endometrial cells. RL95-2 cells were grown in DMEM-F12 HAM supplemented with 10% FCS (Invitrogen, Paisley, UK), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, Poole, UK), and 2 mM L-glutamine (Invitrogen) in a T75 flask. 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). AC-1M88 (DSMZ–Germany) was cultured in DMEM (F12) (Invitrogen, Paisley, UK), supplemented with 10% FCS (Invitrogen), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma), and 2 mM L-glutamine (Invitrogen). All cell lines were cultured in T-75 flasks at 37°C at 5 % CO2 atmosphere. Media were changed every second day. At confluence, the cells were washed with Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). The cells were then incubated for 3 min, pelleted by centrifugation at 300 g for 4 min and the supernatant was discarded. The cells were diluted with 3 ml of media and suspended with pipetting 5-6 times in order to ensure a homogenised solution. While suspending, it was necessary to remove any clot of considerable size from the solution, otherwise, formation of the spheroids would have been compromised.

2.2.2. Ligands and inhibitors

All ligands and inhibitors used in the current study were obtained from Invivogen Company (Invivogen, Toulouse, France). Poly Inosinic Poly Cytidilic Acid (Poly I:C) (Invivogen, tlrl-pic, Toulouse, France), TLR 3 synthetic ligand was used to stimulate TLR 3 (Alexopoulou et al., 2001). Pepinh-TRIF (Invivogen, tlrl-pitrif, Toulouse, France) and Pepinh-MYD88 (Invivogen, tlrl-pimyd, Toulouse, France) were used to inhibit the TRIF and MYD88 signalling pathways respectively (Toshchakov et al., 2005).

2.2.3. *In vitro* Human implantation assay

RL95-2 cells were cultured in T75 flasks until 100% confluence, thereafter cells were harvested using trypsin-EDTA. The cells were then counted and 3 x 10^5 endometrial cells were cultured in each well of a 12-well plate. They were incubated for 4 days until confluence (Sanchez-Lopez et al., 2014).

To create multicellular spheroids from the monolayers of JAr cells and AC-1M88 cells, 10^6 cells and 4×10^6 cells were counted respectively with a Haemocytometer, and cultured in 5 ml of media in 60×15mm Petri dishes (CellStar tissue culture dishes, greiner bio-one, GmbH/Germany) in a humid atmosphere containing 5% CO_2 at 37°C on a gyratory shaker, set at 110 rpm for 24 h (Sanchez-Lopez et al., 2014). AC-1M88 and JAr spheroids were then stained with 0.4 µM Calcein (Life

technologies, Molecular Probes, Paisley, UK) and captured by fluorescent microscope (OLYMPUS CKX41).

Once the trophoblast spheroids were formed on the shaker, they were gently transferred onto each well of confluent endometrial cells in 12-well plates, and the co-culture was maintained in DMEM-F12 HAM, with supplements as mentioned above (Sanchez-Lopez et al., 2014).

Non-adherent spheroids were removed from the monolayer using an automatic horizontal shaker to detach loosely bound or unbound spheroids. In brief, once the trophoblast spheroids were co-cultured with endometrial cells, the number of spheroids was counted under the microscope and each plate was placed on a shaker, which was set at 200 rpm for 4 min. The cells were washed with PBS twice and then the number of attached spheroids was counted under the microscope. The results were expressed as the percentage of spheroids attached from the total number of spheroids used to initiate the co-incubation experiments. All the experiments were performed in three replicates.

2.2.4. Methotrexate assay

Methotrexate (MTX) was used as one of the most effective chemicals for differentiating trophoblast cells (Hohn et al., 2000). AC-1M88 cells were treated with 5 µM MTX (Fluka, UK. CAS number: 59-05-2). Once the AC-1M88 cells had been trypsinised and 4×10^6 cells added to 5 ml of media in petridishes, MTX was

added to AC-1M88 cells and incubated for 24 h on a gyratory shaker. To confirm the differentiation of AC-1M88 cells upon addition of MTX, the production of human chorionic gonadotropin (hCG) was determined by radioimmunoassay.

2.2.5. Viability assessment of endometrial cells

In order to check the viability of RL95-2 cells, they were grown in 12-well plates until 100 % confluence. The media were replaced with serum free media before they were either treated or not with Poly I:C. The cells were then harvested using trypsin-EDTA and 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 an autofluorescence control sample and the other was used for staining with 3 µM propidium iodide (PI; Life technologies, Paisley, UK). The samples were read immediately in a FACSCalibur cytometer (BD), capturing $1x10^4$ events and the percentage of PI positive events (death cells) was registered. The results were expressed as percentage of live cells and were compared using a one-way ANOVA, with $p < 0.05$ considered significant (Sanchez-Lopez et al., 2014).

2.2.6. RNA isolation and cDNA synthesis

For endometrial cell lines genomic studies, RL95-2 cells were washed with DPBS without Ca^{2+} and Mg^{2+} and one milliliter of TRIreagent (Sigma) was added to the flask. Thereafter total RNA from cells was extracted following a standard protocol

supplied by the manufacturer. Total RNA obtained from RL95-2 cells was treated three times with DNase I (DNA-freeTM, Ambion Austin, TX, USA) to remove genomic DNA contamination from the samples. First strand cDNA synthesis was performed using oligo dT primers (Metabion, Martinsried, Germany) and reverse transcription by Super-Script II (200 U/µl; Invitrogen). Negative controls were prepared without inclusion of the enzyme (no RT controls) (Aboussahoud et al., 2010a).

2.2.7. Quantitative Real-Time PCR (QPCR)

QPCR was carried out with the cDNA prepared from RL95-2 cells as described. Intron-spanning IRF7 primers had previously been designed for QPCR to exclude amplification from genomic DNA template. The IRF7 primer sequence was 5'-CTGTGCTGGCGAGAAGGC-3' for the forward sequence and 5'-GGAGTCCAGCATGTGTGTGT-3' for the reverse sequence. For normalization purposes, expression of the reference genes β-actin and B2M was also quantified (Sanchez Lopez et al. 2014b). The sequence of their primers was as follows: β-actin forward sequence was 5'-CAAGATCATTGCTCCTCCTG-3' and the reverse sequence was 5'-ATCCACATCTGCTGGAAGG-3'. The B2M forward sequence was 5'-TATGCCTGCCGTGTGAACCA-3' and the reverse sequence was 5'-GCGGCATCTTCAAACCTCCA-3'. SYBR Green Jump Start (Sigma) master mix (containing 10µl SYBR Green, 7µl H2O, 1µl of test or reference gene primers and 1µl cDNA) was added to each well of PCR plate and amplification was performed under the following conditions: 40 cycles of 95◦ for 30 s, 62◦ for 30 s and 72◦ for

30 s. All experiments included RT controls and negative controls (no cDNA). QPCR was performed using Mx3005P QPCR (Stratagene, Waldbronn, Germany) and results were analysed using MxPro QPCR software version 4.01. The amplified QPCR products were sequenced to confirm the identity of the amplified product.

The QPCR data were analysed using the comparative quantification cycle (May et al.) method. Briefly, the difference in cycle time (ΔCq) was determined as the difference between the number of cycles required for amplification of the test gene and reference genes β-actin and B2M. ΔΔCq was then obtained by finding the difference between groups. The results were expressed as mean ± SEM. Statistical analysis was performed by using ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant (Aflatoonian et al., 2007).

2.2.8. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-8 was determined in culture supernatants with the commercially available IL-8 ELISA kit from R&D Systems (R&D Systems, DY208, Minneapolis, USA). The ELISA was performed according to the manufacturer's instructions with 100µl of cell-free supernatant. ELISA assays in our hands had a sensitivity of 31 pg/ml for IL-8. Sample concentrations were determined with interpretation from the standard curve (Aboussahoud et al., 2010a).

2.3. Experimental design

2.3.1. Standardizing the AC-1M88 spheroid formation

The method for JAr spheroid formation was tested previously in the lab. To optimise the method and assess whether this performance could be also applied for AC-1M88 cells, different amounts of cells were counted by haemocytometer (2×10^6, 3×10^6 and 4×10^6). The cells were seeded in 5 ml of DMEM (F12) media in 60 x 15 mm petri dishes (CellStar tissue culture dishes, greiner bio-one, GmbH/Germany). They were incubated overnight in a humid atmosphere containing 5% CO_2 at 37C on a gyratory shaker, set at 110 rpm.

2.3.2. Optimising implantation assay using AC-1M88 cells

To optimise our implantation assay using AC-1M88 cells, the effects of (A) co-incubation time (0.5, 1, 2 and 4 h), (B) AC-1M88 spheroid concentration (10, 30, 50 and 100), and (C) AC-1M88 differentiation on the number of AC-1M88 spheroids attached to endometrial cells was investigated. The adhesion were assessed as described.

2.3.3. The effect of TLR 3 stimulation on early and late implantation stages

In order to investigate the effect of TLR 3 activation on early and late stages of implantation, we used spheroids produced from AC-1M88 and JAr cell lines respectively.

2.3.3.1. The effect of incubation time of Poly I:C treated endometrial cells on the binding of trophoblast spheroids to endometrial cells

To understand if co-incubation duration was affecting the outcome of trophoblast binding to endometrial cells, RL95-2 cells were pre-incubated with 10 µg/ml of Poly I:C for 4 h. 30 AC-1M88 and JAr spheroids were then delivered onto the confluent endometrial cell monolayers in separate 12-well plates and co-incubated for 0.5, 1, 2 or 4 h. The adhesion was assessed as described.

2.3.3.2. The effect of Poly I:C concentrations on the binding of trophoblast spheroids to endometrial cells

To determine whether TLR 3 activation was affecting trophoblast adhesion to endometrial cells, we evaluated the effect of different concentrations of Poly I:C

on the endometrial cells. The RL95-2 cells were grown in 12-well plates until total confluence. The media were replaced with serum free media before they were either activated or not with Poly I:C. RL95-2 cells were then stimulated with different concentrations of Poly I:C (0, 5, 10 and 25 μg/ml) for 4 h. AC-1M88 and JAr spheroids were delivered onto the separate confluent monolayers and co-incubated for 1 h. Adhesion was assessed as described.

2.3.4. The effect of inhibition of TRIF pathway on Poly I:C treated endometrial cells on the binding of trophoblast cells

The activity of the inhibitor of TRIF (Pepinh-TRIF) was verified in RL95-2 cells (Hosmane et al., 2012). The cells were pre-treated with various concentrations of Pepinh-TRIF (0, 5, 10 and 20 μM) for 6 h and then stimulated with 10 μg/ml of Poly I:C for 4 h. The RNA was extracted and cDNA synthesized and the gene expression of IRF7 was compared in different groups by real-time QPCR.

To determine through which pathway implantation failure was induced by TLR 3 activation, endometrial cells were exposed to Pepinh-TRIF. RL95-2 cells were pretreated with Pepinh-TRIF at a concentration of 20 μM for 6 hours at 37°C. Poly I:C was then added to each well of RL95-2 cells at a concentration of 10 μg/ml and incubated for 4 h. JAr spheroids were delivered onto the monolayers of endometrial cells in separate 12-well plates and co-cultured for 1 h.

2.3.5. The effect of inhibition of MYD88 pathway on Poly I:C treated endometrial cells on the binding of trophoblast cells

The functionality of the inhibitor of MYD88 (Pepinh-MYD88) was verified in RL95-2 cells. The cells were pre-treated with Pepinh-MYD88 (10 μM) for 6 h and then stimulated with 10 μg/ml of Poly I:C for 4 h. The production of IL-8 was compared in different groups by using IL-8 ELISA kit as described.

To determine an alternative signalling pathway via which TLR 3 might work in RL95-2 cells, endometrial cells were treated with MYD88 signalling pathway inhibitor, Pepinh-MYD88, at a concentration of 10 μM for 6 h at 37°C. Poly I:C was then added to each well of RL95-2 cells at a concentration of 10 μg/ml and incubated for 4 h. JAr spheroids were delivered onto the monolayers of endometrial cells in separate 12-well plates and co-cultured for 1 h.

2.3.6. The effect of TLR 3 activation on the viability of the endometrial cells

To determine whether TLR 3 activation was 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.

2.3.7. Statistical Analysis

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

2.4. Results

2.4.1. Standardizing the AC-1M88 spheroid formation

As shown in Fig. 2.1A and B, by using 2×10^6 and 3×10^6 of AC-1M88 cells small sized spheroids were obtained. When the amount of cells was increased to 4×10^6, a constant and bigger spheroid size was obtained (Fig. 2.1C), similar in size to JAr spheroids (Fig. 2.1D). We determined that 4×10^6 AC-1M88 cells should be applied in the spheroid formation process.

2.4.2. AC-1M88 spheroids attach and adhere to RL95-2 cells

As the time of incubation of AC-1M88 spheroids increased, the percentage of spheroids attached to epithelial cells did not vary (around 93.5 %) ($P > 0.05$) (Fig. 2.2A). Regardless of the number of AC-1M88 spheroids added to endometrial cells, the same percentage of cells attached to RL95-2 monolayer (Fig. 2.2C). Finally, treatment of AC-1M88 cells with MTX resulted in increased secretion rates of hCG (51 IU/l) compared to the control group (29 IU/l). Addition of MTX to AC-1M88 cells significantly reduced the percentage of AC-1M88 spheroids attached to the confluent monolayer of RL95-2cells ($P < 0.05$) (Fig. 2.2D).

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

Addition of Poly I:C to RL95-2 cells suppressed the percentage of AC-1M88 (Fig. 2.3A) and JAr spheroids (Fig. 2.4A) attached to endometrial cells. This decrease was consistent in the Poly I:C treated group regardless of the co-incubation time.

2.4.4. Poly I:C presence decreased attachment of trophoblast spheroids to endometrial cells in a dose-dependent manner

The treatment of RL95-2 with different Poly I:C concentrations significantly decreased the percentage of AC-1M88 (Fig. 2.3B) and JAr spheroids (Fig. 2.4B) attached to endometrial cells compared to the control group ($P < 0.05$). Adhesion decreased as the concentration of Poly I:C increased.

2.4.5. Inhibition of TRIF signalling pathway did not restore the attachment of trophoblast spheroids to RL95-2 cells

As can be seen in Fig. 2.5A, Poly I:C was able to significantly enhance gene expression of IRF7 in RL95-2 cells ($P < 0.05$). Pepinh-TRIF was unable to suppress gene expression of IRF7 at 5 and 10 μM in the presence of Poly I:C, but it was functional at 20 μM in reducing IRF7 expression (Fig. 2.5A) ($P < 0.05$). Hence, 20 μM of Pepinh-TRIF was applied for the next set of inhibition experiments.

Pretreatment of RL95-2 cells with Pepinh-TRIF did not restore the attachment of JAr spheroids in the presence of Poly I:C (Fig. 2.5B). Treatment of endometrial cells

with only Pepinh-TRIF had no significant effect on the attachment of trophoblast spheroids to endometrial cells.

2.4.6. Inhibition of MYD88 signalling pathway significantly restored the attachment of trophoblast spheroids to RL95-2 cells

As can be seen in Fig. 2.5C, addition of Poly I:C to RL95-2 cells significantly increased production of IL-8 compared to the control group ($P < 0.05$). Pepinh-MYD88 was able to suppress production of IL-8 in the presence of Poly I:C (Fig.2.5C) ($P < 0.05$).

Pretreatment of RL95-2 cells with Pepinh-MYD88, recovered the ability of JAr spheroids in attaching to RL95-2s in the presence of Poly I:C (Fig. 2.5D) ($P < 0.05$).

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

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. 2.6).

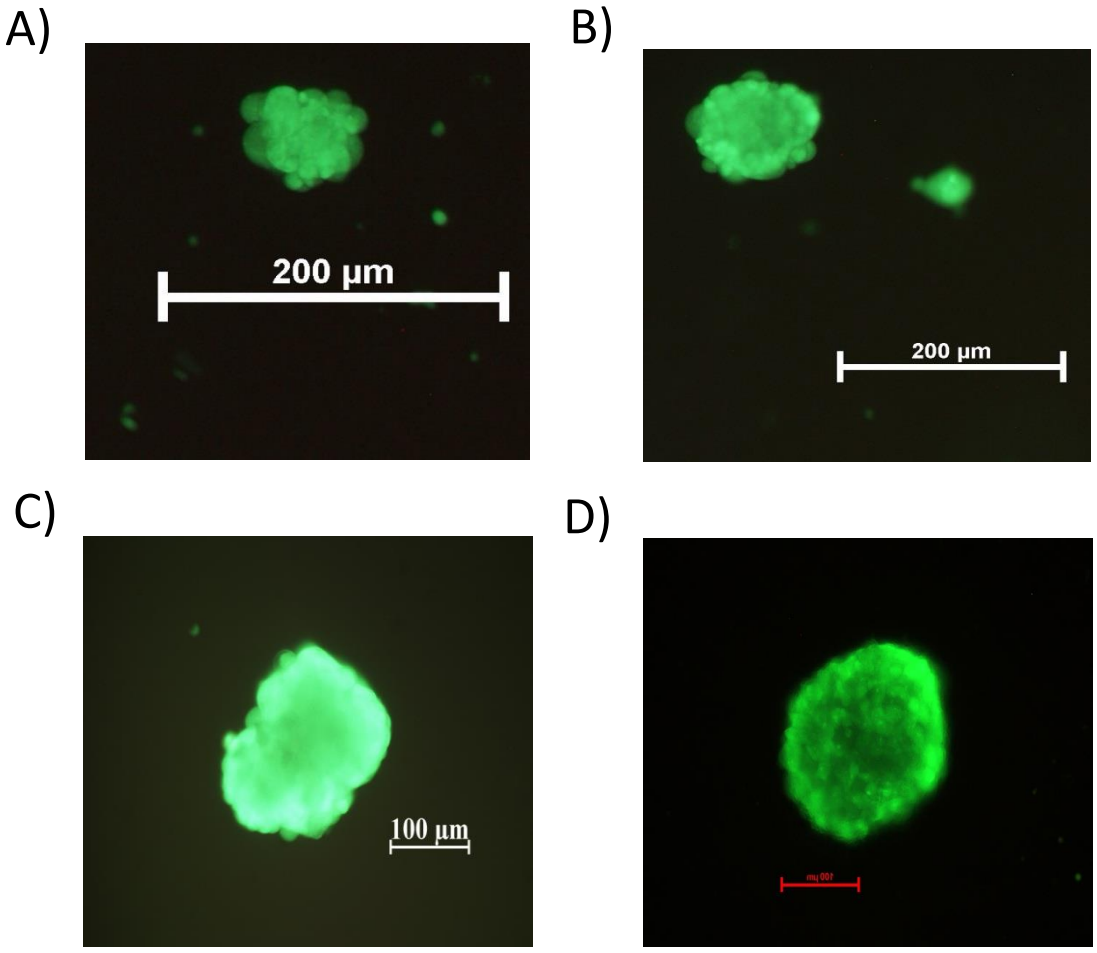

Figure 2.1 Standardisation of the AC-1M88 spheroid formation.
The method for the formation of spheroids was standardised by assessing the effect of incubation of AC-1M88 cells on the number, size and shape of the spheroids. AC-1M88 spheroids with 2 x 10^6 (A), 3 x 10^6 (B) and 4 x 10^6 (C) cells, and JAr spheroids (D) were stained with Calcein and visualized by fluorescence microscopy.

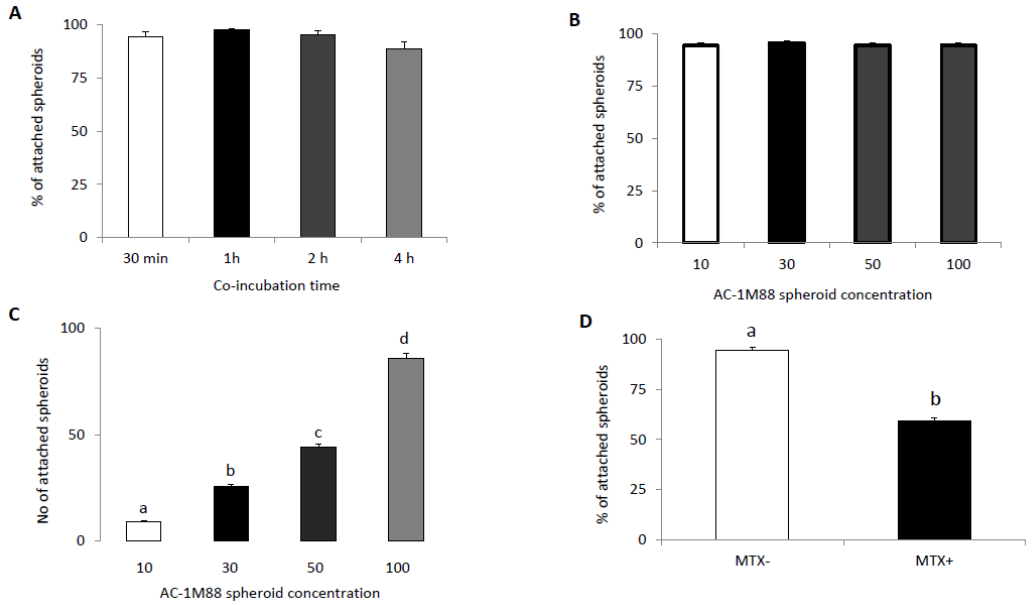

Figure 2.2 Standardising the *in vitro* implantation assay using AC-1M88 cells.
To optimise our implantation assay using AC-1M88 cells, the effects of (A) co-incubation time (0.5, 1, 2 and 4 h) and (B) AC-1M88 spheroid concentration (10, 30, 50 and 100) on the percentage of AC-1M88 spheroids attached to endometrial cells were investigated. In addition, the influence of AC-1M88 spheroid concentration on the number of trophoblast spheroids attached was verified (C). Finally, the effect of AC-1M88 spheroid differentiation on the percentage of trophoblast spheroid attached was assessed by treatment of AC-1M88 cells with MTX (5 µM) for 24 h (D). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids at each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

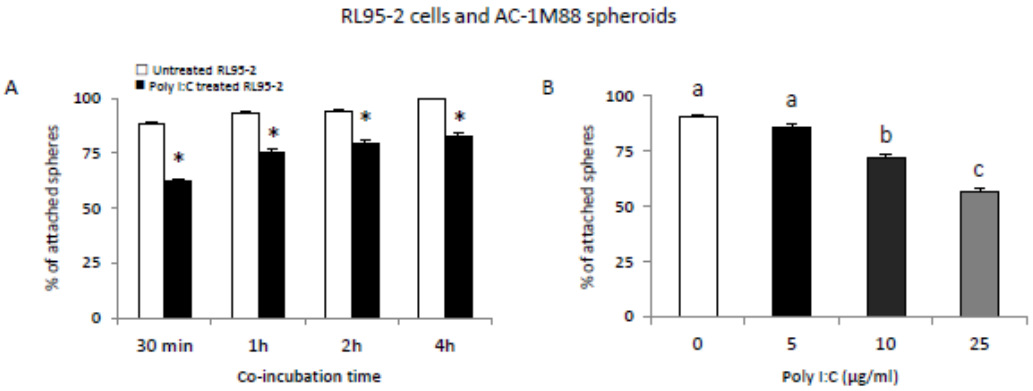

Figure 2.3 The effect of TLR 3 stimulation on early stage of trophoblast adhesion to the endometrial cells.

RL95-2 cells were treated with 10 µg/ml of Poly I:C for 4 h. Firstly, the effect of co-incubation times of Poly I:C-treated endometrial cells with AC-1M88 cells on the number of attached AC-1M88 spheroids (A) was investigated. Secondly, the influence of treatment of various Poly I:C concentrations on the binding of AC-1M88 spheroids to RL95-2 cells (B) was reported. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids at each co-incubation time and Fischer's multiple comparison test was applied as the post-hoc test. * indicates significant differences from control. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

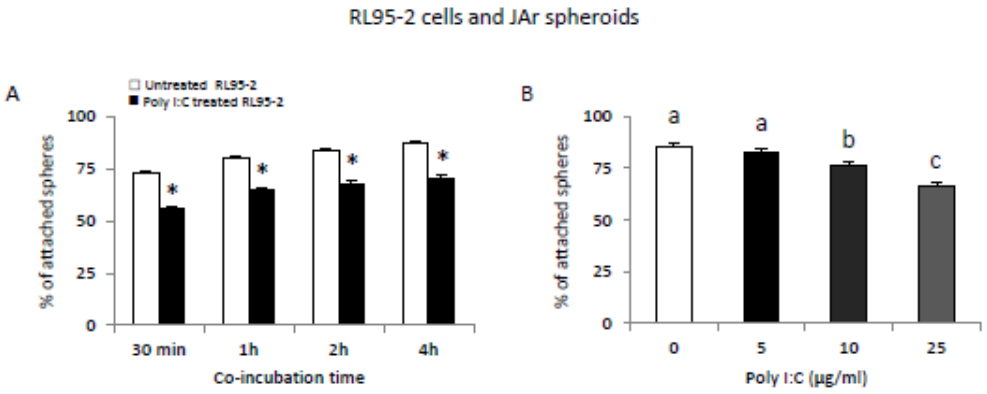

Figure 2.4 The effect of TLR 3 stimulation on late stage of trophoblast adhesion to the endometrial cells.

RL95-2 cells were treated with 10 µg/ml of Poly I:C for 4 h. Firstly, the effect of co-incubation times of Poly I:C-treated endometrial cells with JAr cells on the number of attached JAr spheroids to RL95-2 (A) was investigated. Secondly, the influence of treatment of various Poly I:C concentrations on the binding of JAr spheroids to RL95-2 cells (B) was reported. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids at each co-incubation time. * indicates significant differences from control and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

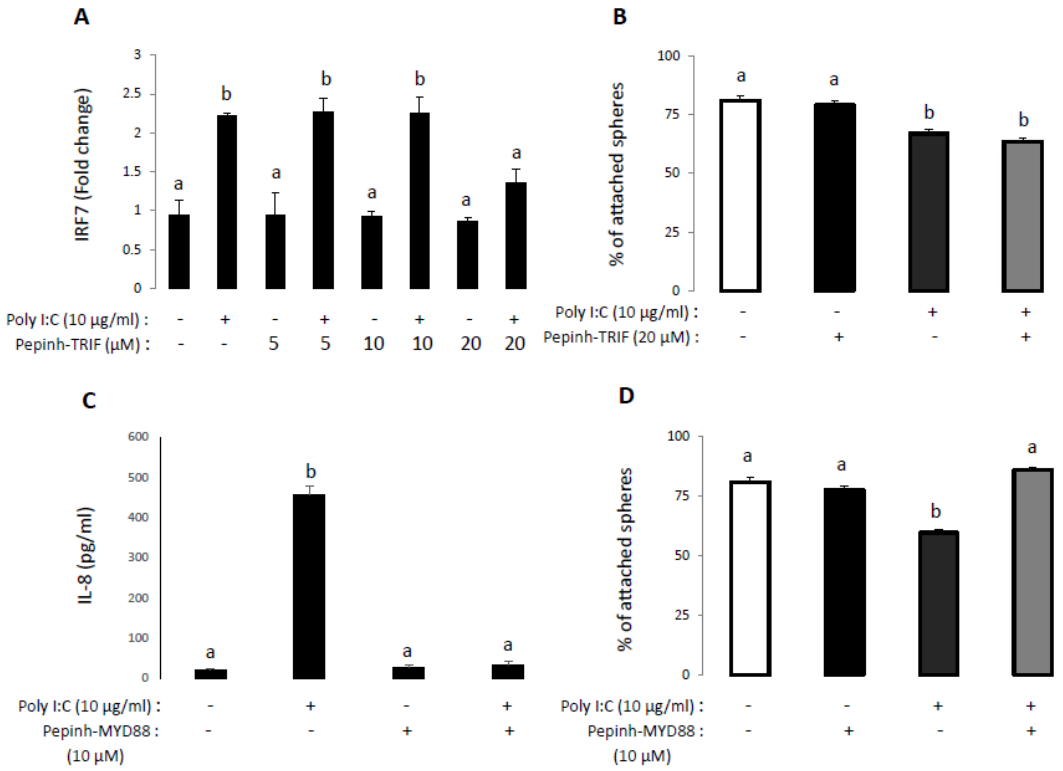

Figure 2.5 The effect of Inhibition of TRIF and MYD88 pathways in endometrial cells on binding of trophoblast spheroids to endometrial cells.

First, the functionality of Pepinh-TRIF was assessed (A) by measuring its effect on the gene expression of IRF7 in the presence of Poly I:C by QPCR. Addition of functional dose of Pepinh-TRIF to RL95-2 cells (20 µM) after 6 h did not restore the percentage of attached JAr spheroids to RL95-2 cells in the presence of Poly I:C (B). Functionality of Pepinh-MYD88 was also verified by investigating its effect on the production of IL-8 in the presence of Poly I:C (C). Addition of Pepinh-MYD88 (10 µM) to RL95-2 cells after 6 h recovered the inhibitory effect of Poly I:C on trophoblast cells' binding to RL95-2 cells (D). Treatment of endometrial cells with only Pepinh-TRIF or Pepinh-MYD88 had no significant effect on the attachment of trophoblast spheroids to epithelial cells. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids at each co-incubation time and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

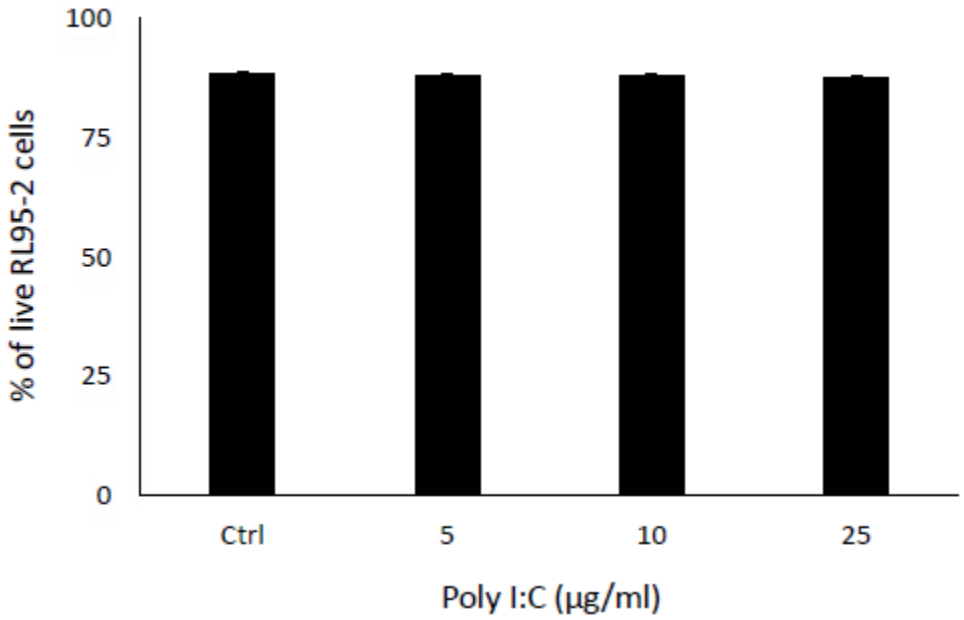

Figure 2.6 The effect of concentration of Poly I:C on the viability of the RL95-2 cells.

The viability of RL95-2 cells was determined after their treatment with different concentrations of Poly I:C (0, 5, 10 and 25 µg/ml) for 4 h. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids at each co-incubation time and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

2.5. Discussion

We demonstrated that stimulation of TLR 3 in endometrial epithelial cells by Poly I:C significantly reduced the percentage of attachment of trophoblast spheroids (AC-1M88 cells and JAr cells) to the endometrial cells. This reduction was irrespective of using trophoblast cells (AC-1M88 or JAr) to represent early (apposition-adhesion) and late (adhesion-invasion) stages of embryo implantation respectively. From these data we would speculate that stimulation of TLRs and, broadly speaking, the activation of the innate immune system at any time during the process of implantation might lead to implantation failure. Further experiments using *in vivo* models are essential to confirm and corroborate this idea.

We were able to create spheroids from AC-1M88 cells, similar in size to human blastocysts (200 μm) and JAr spheroids (200 μm). They adhere and attach to endometrial cells irrespective of incubation times between AC-1M88 cells and RL95-2s. In addition, our results showed that treatment of AC-1M88 cells with MTX reduced the percentage of attached spheroids to endometrial cells. This finding is in agreement with the Hohn et al. results, where the authors showed that addition of MTX to trophoblast choriocarcinoma cells (JAr, Jeg-3 and BeWo) decreased the binding of trophoblast cells to RL95-2 cells with variable levels (Hohn et al., 2000). It is of note that due to some issues regarding culturing AC-1M88 cells, they were not used more extensively and JAr cells were chosen for later studies. Generally speaking, JAr cells were growing faster compared to AC-1M88 cells and it was

easier to keep them in the culture for the longer time intervals. In addition, less JAr cells were required to form spheroids (10^6 cells) compared to AC-1M88 cells (4×10^6 cells).

Many factors are involved in the embryo implantation process, including growth factors, cytokines and chemokines, adhesion molecules and proteases (Singh et al., 2011). During the adhesion stage, mainly adhesion molecules are involved, whereas during the invasion stage, mainly proteases are produced (Johnson and Everitt, 2007). Expression of various adhesion molecules in AC-1M88 cells makes them a suitable model to investigate earlier stages of implantation such as the adhesion phase (Ferretti et al., 2007, Hannan et al., 2006, Hannan and Salamonsen, 2008), whereas the invasive properties of JAr cells make them suitable model systems to study the last stage of implantation, where invasion of the endometrium by the trophoblast takes place. In the same line of evidence, we observed that AC-1M88 spheroids adhered more to endometrial cells compared to JAr cells. Activation of TLR 3 by Poly I:C decreased the percentage of attached AC-1M88 and JAr spheroids to RL95-2s at all co-incubation times and this effect was concentration dependent. Previous reports from our laboratory explored how the activation of TLR 2/6 and 5 with their specific ligands can induce a similar effect on the adhesion of trophoblast cells to endometrial epithelial cells (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014).

To date, no investigation has shown that TLR 3 activation in the FRT can lead to implantation failure in humans. However, TLR 3 stimulation with Poly I:C during

pregnancy was able to increase fetal losses in mice (Zhang et al., 2007b). One such study used C57B/6 wild type and TLR 3 knockout (TLR 3KO) mice. Despite treating both mice varieties with Poly I:C, pre-term labour was only induced in wild type mice within 24 h of Poly I:C treatment and not in TLR 3KO mice (Koga et al., 2009b). Together all the data obtained in the current study points toward a decrease in trophoblast cells' attachment to endometrial cells in response to TLR 3 activation, but the mechanism through which these alterations happen is still unknown. Finding the pathways involved in TLR 3-induced implantation failure will help us to better understand the process of endometrial receptivity and potential effect of the immune system on this process. Jorgenson and colleagues (Jorgenson et al., 2005) have shown that the addition of Poly I:C to the endometrial cell line RL95-2 and primary endometrial cells leads to a TLR 3-dependent increase in the production of pro-inflammatory cytokines IL-6 and IL-8. In a similar study, it was demonstrated that the presence of Poly I:C increased interferon ɤ (IFN- ɤ) and tumor necrosis factor α (TNF-α) production by natural killer (NK) cells of murine uterine cells (Zhang et al., 2007a). The cytokine milieu plays an essential role in normal functions of the endometrium such as uterine cycle progression, epithelial proliferation and shedding, and embryo implantation, thus any impairment of the balance of cytokine production could affect the process of embryo implantation. This has been further supported by Kwak-Kim *et al.* (2010), where they found that there is an association between pro-inflammatory cytokines such as IL-6, TNF-α, IFN-ɤ, IL-1, and pregnancy losses (Kwak-Kim et al., 2010). It has also been found that secretion of IL-6 is increased by endometrial epithelial cells (EECs) in women suffering from endometriosis (Piva et al., 2001), recurrent abortion and

unexplained infertility (Tseng et al., 1996, von Wolff et al., 2002). In addition, stimulation of TLR 3 by Poly I:C in human nasal epithelial cells (HNECs) significantly increased the production of IL-8 and decreased the expression of junctional adhesion molecules (JAMs) (Ohkuni et al., 2011). Thus, it is reasonable to assume that one of the potential mechanisms of TLR 3-induced implantation failure could be the effect it has on impairment of the balance in cytokine production, which may result in the alteration of adhesion molecule expression required for successful implantation. Hence, determining the cytokine and chemokine production triggered by TLR 3 activation will help us further understand the mechanism of stimulation of TLR 3 in relation to implantation failure. Moreover, measurement of the expression of the cell adhesion molecules family (CAMs) in the embryo-maternal surface during TLR 3 activation will shed light on the causes of virus-induced implantation failure in the FRT.

To further investigate the mechanism underlying TLR 3-induced reduction of spheroid binding to endometrial cells, a competitive inhibition assay was performed using a TRIF signalling pathway inhibitor. There are two inflammatory pathways induced by TLRs: the myeloid differentiation (MyD88)-dependent and the Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF)-dependent pathways (Akira and Takeda, 2004). TLR 3 signalling follows the TRIF-dependent pathway. Upon detection of PAMPs by TLR 3, TRIF triggers an intracellular cascade of molecular reactions which leads to stimulation of the transcription factors NF-κB, IRF-3 and AP-1. These transcription factors are translocated to the nucleus after activation, which results in induction of type 1

IFN, pro-inflammatory cytokines and chemokines (Matsumoto et al., 2011). Since TLR 3 is expressed inside the cell and the antibodies can only block the antigens expressed on the cell surface in intact viable cells and are unable to access inside intact cells, we proposed the application of TRIF signalling inhibitors that were peptide based. Peptide based inhibitors have the following advantages: They are cell permeable and no permeabilization reagent is required. Moreover, they are non-toxic and cell culture compatible and they block without knocking down genes. In addition, peptide based inhibitors possess functional specificity, as well as various applications in *in vitro* and *in vivo* studies (Toshchakov et al., 2005, May et al., 2000).

The inhibitor of choice was Pepinh-TRIF, a 30 amino acid peptide which is able to pass through the cell membrane and suppress TRIF signalling by interfering with TLR-TRIF interaction (Toshchakov et al., 2005). Application of Pepin-TRIF did not restore the JAr spheroids' attachment to the endometrial cells. This suggested that TRIF is unlikely to be involved in TLR 3 activation leading to decrease in trophoblast cells adhesion to endometrial cells. Thus, we sought to determine the effect of other pathways, such as MYD88. Surprisingly, MYD88 inhibition restored the defect in binding of trophoblast cells to endometrial cells in RL95-2 cells. Moreover, we were able to show that inhibition of MYD88 reduced TLR 3-induced IL-8 production in the presence of Poly I:C, which shows the importance of MYD88 signalling pathway in TLR 3-induced cytokine production. These findings, on one hand, corroborate the ideas of Brenner and Simmonds (2012), who showed that MYD88 knock down, but not TRIF knock down, inhibited Poly I:C induced cytokine

production in adipocytes (Brenner et al., 2012). 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). On the other hand, 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 adaptor molecule for the TLR 3 signalling pathway (Matsumoto et al., 2011, Yu and Levine, 2011a). The disparity between these studies and our finding could probably be due to the fact that TLR 3 signalling is cell-specific and differs from one cell to another. Another explanation could be that no other study has investigated the mechanistic pathways of TLR 3-induced implantation failure in humans so far, and as a result of that the effect of MYD88 on this process has not been identified. In this regard, we were able to show that in RL95-2 cells, TLR 3 signals via both the TRIF and the MYD88 route, of which only MYD88 is involved in TLR 3-induced implantation failure.

The main goal of the current study was to determine the effect of TLR 3 activation on trophoblast cells' binding to endometrial cells *in vitro*. In conclusion, our hypothesis that stimulation of TLR 3 reduces the adhesion rate of trophoblast cells to endometrial cells was supported in this chapter and one of the more significant findings to emerge from this study is that TLR 3 activation has a detrimental effect on the attachment of trophoblast cells to endometrial epithelial cells. However, further research should be done to investigate the potential mechanisms involved in this process. Finding the molecule involved in implantation failure induced by

TLR 3 activation will open new diagnostic and therapeutic approaches in treating infertility caused by viral infections.

Chapter 3. Investigation of the TLR 3 signalling pathway

3.1. Introduction

The innate immune system plays a crucial role in defence against invading microorganisms. It detects the presence of infectious microorganisms and controls activation of adaptive immunity by inducing a set of endogenous signals such as inflammatory cytokines and chemokines (Medzhitov and Janeway, 2000b). The innate immune system recognizes the invading microorganisms through receptors known as pathogen recognition receptors (PRRs) (Medzhitov and Janeway, 2000b, Medzhitov and Janeway, 1997). PRRs recognize microbial components known as pathogen-associated molecular pattern sequences (PAMPs), such as lipopolysaccharide (LPS) of gram-negative bacteria, or double stranded RNA in viruses (Janeway, 1989). The best characterized examples of PRRs are members of the Toll-like receptor (TLR) family. TLRs participate in orchestrating innate immune responses to various microbial organisms. TLRs have been implicated in recognition of a vastly diverse range of microbial pathogens, including bacteria, fungi, viruses, and protozoa (Barton, 2007).

Up to now, 10 members of TLR family have been recognized in humans and each of them binds to its specific ligand (Beutler, 2004). Upon activation of TLRs by PAMPs, a downstream signalling cascade is initiated, which leads to the activation of various adaptor molecules. These interactions result in appropriate immune responses required for host defence (Carty et al., 2006). Myeloid differentiation factor 88 (MYD88) is required for signalling by all TLRs except TLR 3 and it activates nuclear factor (NF)-κB and mitogen activated protein kinases (MAPKs), which

control the gene expression of inflammatory cytokines and chemokines. TLR 3 signals through TIR-domain-containing adapter-inducing interferon-β (TRIF), which leads to the activation of NF-κB, MAPK as well as IRF3, which plays a crucial role in the induction of type 1 interferons (Carty et al., 2006).

The NF-κB family consists of dimeric transcription factors that contain Rel-homology domains (RHDs) that bind to discrete DNA sequences known as κB sites. There are five members of the NF-κB family: RelA (p65), RelB, C-Rel, p105 (NF-κB1; a precursor of p50) and p100 (NF-κB2; a precursor of p52) (Karin and Greten, 2005). NF-κB proteins form homo- or hetero-dimers and regulate expression of distinct but overlapping genes involved in innate and adaptive immunity, inflammation, anti-apoptosis, proliferation, stress responses and cancer progression. The most frequently activated form of NF-κB in TLR signalling is a heterodimer composed of RelA and p50 (Kawai and Akira, 2007). Under unstimulated conditions, the RelA-p50 complex is inactive in the cytoplasm by interaction with NF-κB inhibitor IκB proteins. Upon TLRs stimulation with their specific ligands, IκB is phosphorylated. Subsequently, phosphorylation of IκB proteins allows NF-κB to move into the nucleus. This pathway is called the 'canonical pathway' and is responsible for TLR-mediated induction of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-6 (Hayden et al., 2006, Kawai and Akira, 2007).

An alternative route of signalling is the activation of the MAPK pathway. MAPKs are an important family of protein kinases, which are activated in response to an

extraordinary diverse range of stimuli from growth factors and cytokines to irradiation and osmolarity (Widmann et al., 1999). MAPKs are 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). In mammalian cells, there are at least three MAPK subtypes, including extracellular signal-regulated kinase ERK, p38 MAPK, and c-Jun NH2-terminal kinase (JNK) (Kutsuna et al., 2004). TLR stimulation of these MAPKs would in turn activate the activating protein (AP)-1 composed of the proteins Jun and Fos, capable of dimerising and transcribing pro-inflammatory genes. Each MAPK subtype is activated by phosphorylation on threonine and tyrosine residues by upstream kinases, such as MAPK/ERK kinase, MAPK kinase-3 or -6 (MKK3/6), and MKK4/7. Activation of the distinct MAPK subtype cascade is dependent on the types of cells and the stimuli used, and the functional role of each MAPK subtype may be different according to the types of cells (Kutsuna et al., 2004).

Embryonic implantation is a dynamic process of paracrine interactions between the maternal compartment and the embryo and requires a receptive endometrium and a healthy blastocyst. Different parameters are required to control the process of implantation, including hormones, cytokines, adhesion molecules and growth factors (Singh et al., 2011). In humans and primates, implantation is known to serve as an inflammatory type response. Different cytokines have been identified in the human endometrium, such as interleukin 8 (IL-8), IL-1β, Transforming growth factor α (TGF-α) and IL-6, whose production was

conceived to regulate the functions of endometrial cells during the menstrual cycle and embryo implantation process (Tabibzadeh, 1994, Tabibzadeh and Sun, 1992). It is of note that the production of different cytokines has been shown in response to TLR stimulation in the endometrial epithelial cells (EECs) and cell lines (Aboussahoud et al., 2010a, Jorgenson et al., 2005, Schaefer et al., 2004, Young et al., 2004, Zhang et al., 2007a). However, aberrant pattern of cytokine production in the endometrium has been correlated to problems such as pregnancy loss, endometriosis, recurrent abortion and unexplained infertility (Kwak-Kim et al., 2010, Piva et al., 2001, Tseng et al., 1996, von Wolff et al., 2002). Hence, it is essential that TLR expression and as a result of that, cytokine production and immune responses be tightly controlled in the endometrium. In fact, activation of TLR 2/6 and 5 in the endometrium has been shown to reduce the rate of trophoblast adhesion to the endometrial cells *in vitro* and *in vivo* (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014). In addition, in the earlier chapter it was shown that TLR 3 stimulation could suppress the attachment of trophoblast cells to the endometrial cells *in vitro* (Montazeri et al., 2015), although it remains unclear through which pathways (NF-κB or MAPK) these alterations happen.

In view of this overwhelming evidence that TLR 3 activation could induce the production of pro-inflammatory cytokines that could potentially effect embryonic implantation, the current study was designed. We hypothesized that the activation of NF-κB and MAPK pathways could mediate the TLR 3-induced impairment of trophoblast cells adhesion to the endometrial cells. The *in vitro* model of trophoblast spheroid adhesion to endometrial cells was applied to mimic

embryo implantation. Activation of NF-κB and AP-1 was detected by transfecting the cells with secreted placental alkaline phosphatase (SEAP) reporter plasmids bearing promoter sequences for each transcription factor. The inhibitors for NF-κB (Bay11-7082) and AP-1 (JNK inhibitor SP600125) were used to block signalling. It was then investigated whether addition of these inhibitors could restore the TLR 3-induced impairment of trophoblast attachment to the endometrial cells. Finally, to assess the end product of TLR 3 signalling pathway, IL-8 production was measured in RL95-2 cells upon TLR 3 stimulation. Activation of NF-κB was mainly achieved by the activation of TLR 5 and slightly by TLR 3 and it was inhibited by Bay11-7082. Activation of AP-1 was only induced by TLR 3 activation and it was inhibited by SP600125. Of these inhibitors, only SP600125 could achieve restoration of spheroid adhesion, indicating the role of MAPK pathway in mediating TLR 3-induced implantation failure.

3.2. Methodology

3.2.1. Cell lines and cell culture

RL95-2 was obtained from ATCC and was used to mimic endometrial cells. RL95-2 cells were grown in DMEM-F12 HAM supplemented with 10% FCS (Invitrogen, Paisley, UK), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, Poole, UK), and 2 mM L-glutamine (Invitrogen) in a T75 flask. 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). All cell lines were cultured in T-75 flasks at 37°C at 5 % CO2 atmosphere. Media were changed every second day. At confluence, the cells were washed with Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). The cells were then incubated for 3 min, pelleted by centrifugation at 300 g for 4 min and the supernatant was discarded. The cells were diluted with 3 ml of media and suspended with pipetting 5-6 times in order to ensure a homogenised solution. While suspending, it was necessary to remove any clot of considerable size from the solution, otherwise, formation of the spheroids would have been compromised.

3.2.2. Ligands, inhibitors and antibodies

All ligands and inhibitors used in the current study were obtained from Invivogen Company (Invivogen, Toulouse, France). Poly Inosinic Poly Cytidilic Acid (Poly I:C), TLR 3 synthetic ligand, was used to stimulate TLR 3 (Alexopoulou et al., 2001). Flagellin was used to stimulate TLR 5 (Hayashi et al., 2001). SP600125 (JNK) was applied to inhibit the mitogen-activated protein kinases (MAPK) pathway (Bennett et al., 2001). BAY11 -7082 was used to inhibit the NF-κB pathway (Saraiva et al., 2005).

3.2.3. *In vitro* Human implantation assay

RL95-2 cells were cultured in T75 flasks until 100% confluence, thereafter cells were harvested using trypsin-EDTA. The cells were then counted and $3x10^5$ endometrial cells were cultured in each well of a 12-well plate. They were incubated for 4 days until confluence (Sanchez-Lopez et al., 2014).

To create multicellular spheroids from the monolayers of JAr cells, 10^6 cells were counted respectively with a Haemocytometer, and cultured in 5 ml of media in 60×15mm Petri dishes (CellStar tissue culture dishes, greiner bio-one, GmbH/Germany) in a humid atmosphere containing 5% CO_2 at 37°C on a gyratory shaker, set at 110 rpm for 24 h (Sanchez-Lopez et al., 2014).

Once the trophoblast spheroids were formed on the shaker, they were gently transferred onto each well of confluent endometrial cells in 12-well plates, and the co-culture was maintained in DMEM-F12 HAM, with supplements as mentioned above (Sanchez-Lopez et al., 2014).

Non-adherent spheroids were removed from the monolayer using an automatic horizontal shaker to detach loosely bound or unbound spheroids. In brief, once the trophoblast spheroids were co-cultured with endometrial cells, the number of spheroids was counted under the microscope and each plate was placed on the new shaker, which was set at 200 rpm for 4 min. The cells were washed with PBS twice and then the number of attached spheroids was counted under the

microscope. The results were expressed as the percentage of spheroids attached from the total number of spheroids used to initiate the co-incubation experiments. All the experiments were performed in three replicates.

3.2.4. Transfection of RL95-2 cells with SEAP plasmids containing NF-κB and AP-1 binding regions

The RL95-2 cells were grown in 24-well plates ($2x10^4$ cells) until 70% confluency and transiently transfected with pNifty2-SEAP for NF-κB expression (InvivoGen, Tolouse, France) or the pNifty3-SEAP for AP-1 expression using X-tremeGENE HP DNA transfection reagent (Roche. Briefly, the media in each well of the 24-well plate were replaced with fresh supplemented media. The mix was prepared using a 1:3 ratio plasmid – transfection reagent in 25 µl of serum-free DMEM/F-12. Supernatant samples were collected and secreted placental alkaline phosphatase (SEAP) was detected using the Phospha-Light™ SEAP Reporter gene assay system (Life Technologies, Applied Biosystems, Paisley, UK) according to the manufacturer's protocol (Sanchez Lopez et al., 2014b).

3.2.5. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-8 was determined in culture supernatants with the commercially available IL-8 ELISA kit from R&D Systems (R&D Systems, DY208, Minneapolis, USA). The ELISA was performed according to the manufacturer's instructions with 100µl of cell-free supernatant. ELISA assays in our hands had a sensitivity of 31 pg/ml for IL-8. Sample concentrations were determined with interpretation from the standard curve (Aboussahoud et al., 2010a).

3.3. Experimental design

3.3.1. Activation of NF-κB and AP-1 as a result of endometrial TLR activation

The RL95-2 cells were transfected with either the pNifty2 (NF-κB) or pNifty3 (AP-1) plasmids. On the next day, the culture media were replaced with serum-free DMEM/F-12 and the cells were stimulated with 100 ng/ml of flagellin (TLR 5) or 10 µg/ml of Poly I:C (TLR 3) for 4 h. The supernatants were collected and the SEAP was measured with QUANTI-blueTM (InvivoGen). Data were reported as the fold induction of SEAP activity over the non-stimulated control.

3.3.2. Assessment of the functionality of inhibitors of NF-κB and AP-1 in suppressing the TLR activation pathways

The activity of the inhibitors was verified in the endometrial cells transfected with pNifty2 (NF-κB) and pNifty3 (AP-1) plasmids (InvivoGen). The pNifty2 transfected RL95-2 cells were pre-treated or not with 10 and 20 µM of Bay11-7082 (InvivoGen) for 1 h and the cells were then stimulated with 100 ng/ml of flagellin for 4 h. The pNifty3 transfected RL95-2 cells were pre-treated or not with 50 µM of SP600125 (JNK) (InvivoGen) for 1 h and then stimulated with 10 µg/ml of Poly I:C for 4 h. The supernatants were collected and the SEAP production evaluated with the QUANTI-blueTM (InvivoGen).

3.3.3. Determining the effect of NF-κB and AP-1 on the adhesion of JAr spheroids to the endometrial cells

To assess whether NF-κB or AP-1 pathways could mediate TLR 3-induced impairment of trophoblast adhesion to endometrial cells, the RL95-2 cells were pre-treated or not with the NF-κB and AP-1 inhibitors. The activation of NF-κB and MAPK pathways was inhibited by using BAY11 (20 µM) and SP600125 (50 µM) respectively. The RL95-2 cells were pre-treated with the inhibitors for 1 h.

Thereafter, the RL95-2 were either stimulated or not with 10 µg/ml of Poly I:C for 4 h. 30 JAr spheroids were then gently delivered into each well and co-incubated for 1 h at 37°C. Adhesion was assessed as described.

3.3.4. Determining the effect of TLR 3 activation on the production of IL-8 in the endometrial cells

To assess the end product of the TLR 3 signalling pathway, IL-8 production was measured in RL95-2 cells. Briefly, Poly I:C was added to RL95-2 cells at a concentration of 10 µg/ml for various time intervals (2, 4, 6 and 24 h) and supernatants were collected. The production of IL-8 was then compared in different groups by using IL-8 ELISA kit as described.

3.3.5. Statistical analysis

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

3.4. Results

3.4.1. Activation of the TLR 3 signalling pathway in endometrial cells after TLR 3 stimulation

The activation of NF-κB in the endometrial cells was induced by flagellin treatment (Fig. 3.1A). The treatment of the endometrial cells with flagellin was able to significantly increase NF-κB activity after 4 h ($P < 0.05$). The treatment of the cells with Poly I:C was unable to increase this activation (Fig. 3.1A). The treatment of the endometrial cells with Poly I:C was able to significantly increase the activity of AP-1 after 4 h ($P < 0.05$) compared with the non-stimulated control (Fig. 3.1B).

3.4.2. TLR 5 mediated activation of NF-κB was prevented by the pre-treatment of the endometrial cells with Bay11-7082

The pre-treatment of RL95-2 cells with 10 μM of Bay11-7082 in the presence of Flagellin slightly decreased NF-κB activation compared with the flagellin treatment (Fig. 3.2), but activation was still significantly higher than in the non-stimulated control ($P < 0.05$). In contrast with this, the pre-treatment of the cells with 20 μM of Bay11-7082 significantly decreased the flagellin-induced NF-κB activation ($P < 0.05$).

3.4.3. TLR 3-mediated activation of AP-1 was ablated by the JNK inhibitor SP600125

The pre-treatment of the RL95-2 cells with the JNK inhibitor SP600125 was able to decrease the Poly I:C-induced AP-1 activation of the endometrial cells (Fig. 3.3. The pre-treatment of the cells with 50 µM of SP600125 significantly reduced the AP-1 activation compared with the Poly I:C stimulated control ($P < 0.05$. It reduced AP-1 activation to a level similar to that in the non-stimulated control ($P < 0.05$. The treatment of the cells with different concentrations of SP600125 did not modify AP-1 activation.

3.4.4. Inhibition of the MAPK JNK pathway affected the binding of the trophoblast spheroids to the endometrial cells

The pre-treatment of the RL95-2 cells with Bay11-7082 was unable to significantly restore the binding of trophoblast cells to endometrial cells in the presence of Poly I:C (Fig. 3.4). In contrast, addition of SP600125 to endometrial cells significantly recovered the percentage of trophoblast cells attached to RL95-2 cells (Fig. 3.4).

3.4.5. Stimulation of TLR 3 in the endometrial cells enhanced the production of IL-8

As shown in Fig. 3.5, the production of IL-8 was significantly increased in response to Poly I:C ($P < 0.05$). This effect started soon after 2 h of Poly I:C treatment, although it was not significant compared with the non-stimulated control. It was a clear trend of increase in IL-8 production, reaching nearly 1300 pg/ml at 24 h.

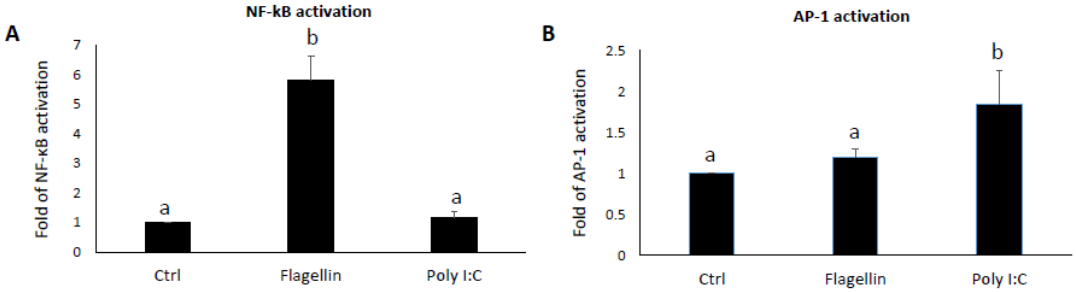

Figure 3.1 Activation of NF-κB and AP-1 in RL95-2 cells after TLR stimulation. RL95-2 cells were transfected with the pNifty2 (NF-κB) and pNifty3 (AP-1) plasmids containing a secreted alkaline phosphatase (SEAP) reporter. The cells were stimulated with the ligands for TLR 5 (100 ng/ml Flagellin) and TLR 3 (10 µg/ml Poly I:C) for 4 h. SEAP production was measured and results represented as fold of NF-κB and AP-1 activation with untreated control. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the fold of NF-κB and AP-1 production in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

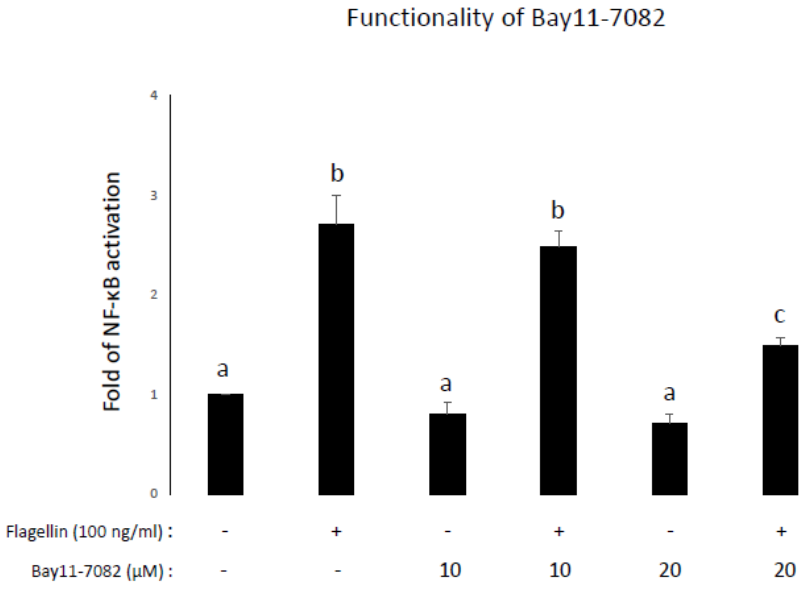

Figure 3.2 Pre-treatment of the RL95-2 cells with Bay11-7082 inhibited the flagellin-induced NF-κB activation.

RL95-2 cells were transfected with the pNifty2 plasmid containing a secreted alkaline phosphatase (SEAP) reporter. The cells were pre-treated with different concentrations of Bay11-7082 (10 and 20 μM) for 1 h and then treated with the TLR 5 ligand (100 ng/ml flagellin) for 4 h. SEAP production was measured and results represented as fold of NF-κB activation with untreated control. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the fold of NF-κB production in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant. Reproduced with permission (Sanchez Lopez et al., 2014b).

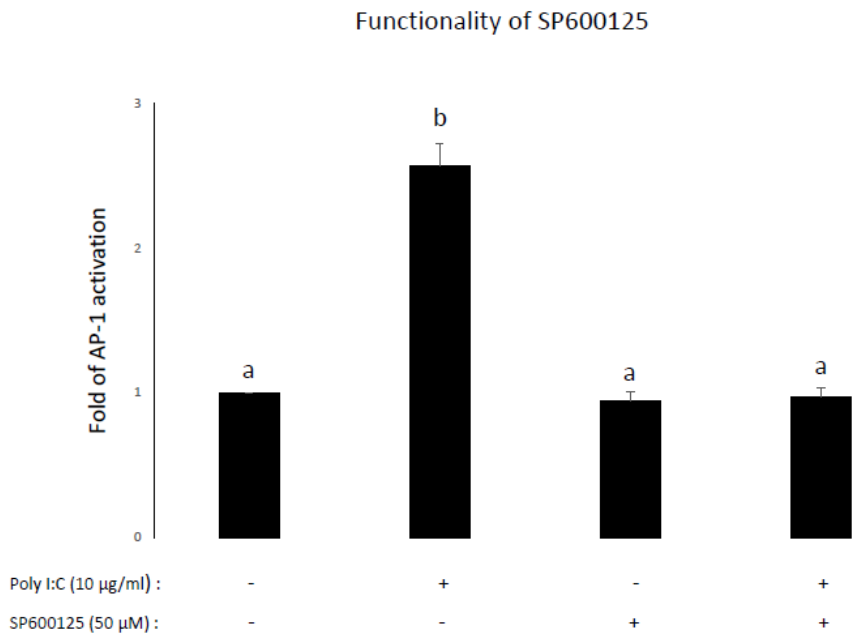

Figure 3.3 Pre-treatment of the RL95-2 cells with SP600125 inhibited the Poly I:C-induced AP-1 activation.

RL95-2 cells were transfected with the pNifty3 plasmid containing a secreted alkaline phosphatase (SEAP). The cells were pre-treated with SP600125 (50 µM) for 1 h and then treated with the TLR 3 ligand (10 µg/ml Poly I:C) for 4 h. SEAP production was measured and results represented as fold of AP-1 activation with untreated control. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the fold of AP-1 production in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

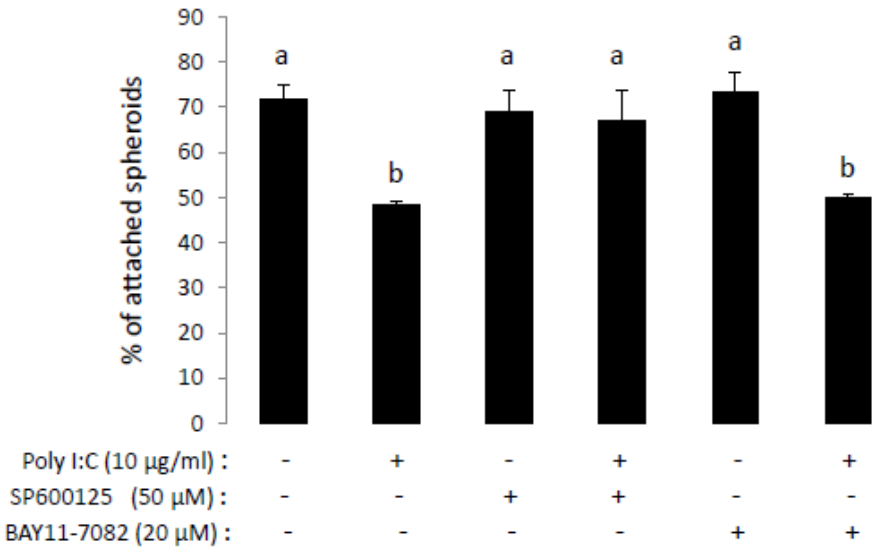

Figure 3.4 Pre-treatment of the RL95-2 cells with AP-1 inhibitor restored the TLR 3-induced reduction of JAr spheroid adhesion.
RL95-2 cells were pre-treated with Bay11-7082 (20 µM) and SP600125 (50 µM) for 1 h. The cells were stimulated with TLR 3 ligand (10 µg/ml Poly I:C) for 4 h. 30 JAr spheroids were then delivered and co-cultured with the endometrial cells for 1 h. The plate was rinsed and the results are expressed as the percentage of attachment spheroids. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of the attached spheroids in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

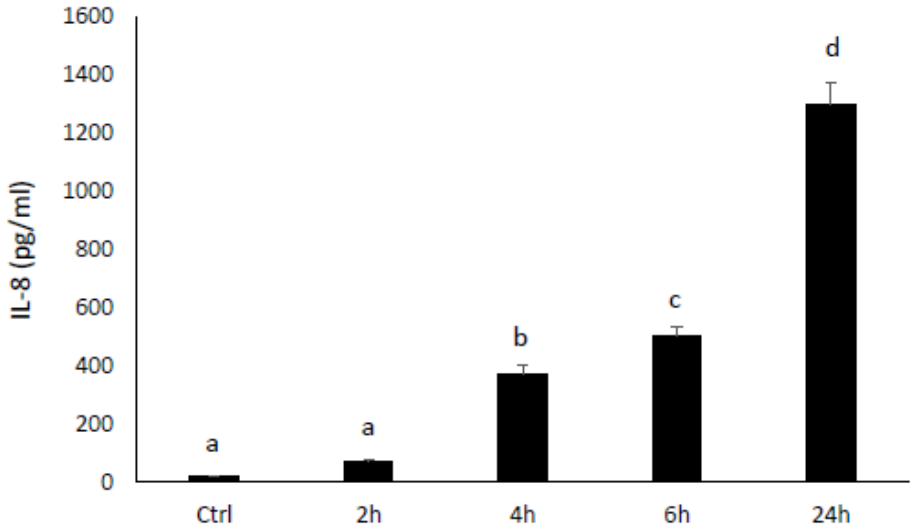

Figure 3.5 The production of IL-8 was significantly increased in RL95-2 cells in response to TLR 3 activation.

RL95-2 cells were pre-treated with TLR 3 ligand (10 µg/ml Poly I:C) in various time intervals (2, 4, 6 and 24 h). The supernatants were collected and the production of IL-8 was assessed by IL-8 ELISA kit. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the IL-8 production at each time interval and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

3.5. Discussion

In the current study, we assessed the activity of NF-κB and AP-1 in RL95-2 cells in response to TLR 3 stimulation *in vitro*. Using our alkaline phosphatase reporter system, Poly I:C was able to activate AP-1 and not NF-κB. Moreover, MAPK signalling seemed to be involved in TLR 3-induced impairment of trophoblast adhesion to endometrial cells, as blocking its activation with specific inhibitor restored the attachment of trophoblast cells to Poly I:C-stimulated endometrial cells. In addition, the production of IL-8 was significantly increased in the endometrial cells in response to TLR 3 activation.

The treatment of the endometrial cells with flagellin for 4 h significantly induced NF-κB activity. In the same line of evidence, it was shown that the addition of Flagellin to the endometrial cell line Ishikawa 3H-12 cells increased NF-κB activity as early as 4 h, using the same reporter system (Caballero et al., 2013). The addition of Poly I:C to the endometrial cells, however, did not increase NF-κB activity.

To verify whether TLR 3 could signal through an alternative pathway, we assessed the activity of AP-1 in response to TLR 3 activation. We could observe that AP-1 activity was significantly increased only in response to Poly I:C. These data are coupled with those obtained from Yoshizawa, where the authors showed that AP-1 activity was significantly induced in response to Poly I:C (20 μg/ml) in fibroblast-like synoviocytes (FLS) (Yoshizawa et al., 2008). The fact that primary endometrial

cells and endometrial cell lines are sensitive to Poly I:C has been further supported by Jorgenson and colleagues (Jorgenson et al., 2005), who showed that TLR 3 stimulation by Poly I:C in RL95-2 and primary endometrial cells led to the production of pro-inflammatory cytokines such as IL-6, IL-8, regulated on activation normal T cell expressed and secreted (RANTES) and interferon β (IFN-β). Similarly, it was shown that the addition of Poly I:C to natural killer cells of murine uterine enhanced the production of interferon ɤ (IFN- ɤ) and tumor necrosis factor α (TNF-α) (Zhang et al., 2007a). The reason why RL95-2 cells are highly sensitive to Poly I:C is still a matter of debate. One explanation could be that since the uterus is exposed to a diverse range of microorganisms, it should be immune-tolerant to some commensal bacteria to prevent any exacerbated inflammatory response. This is achieved by the hypo responsiveness of TLRs to stimuli such as LPS, thus requiring a higher sensitivity to other stimuli such as dsRNA. This is not unprecedented as TLR 4 hypo responsiveness to LPS has been shown in intestinal epithelial cells, which must tolerate exposure to commensal microorganisms to prevent inflammatory bowel disease (Abreu et al., 2001, Cario and Podolsky, 2000). In this regard, dysregulation of TLR 3 and 4 expression in intestinal cells has been correlated to the loss of tolerance to enteric bacteria and consequently to pathogenesis of idiopathic inflammatory bowel disease (IBD) (Abreu et al., 2001, Cario and Podolsky, 2000). A possible tolerance mechanism to TLR 4 expression has also been shown in the female reproductive tract, where TLR 4 was absent in the lower compartments of the FRT (Fazeli et al., 2005).

To check the functionality of the NF-κB and AP-1 inhibitors over the TLR activation, the RL95-2 cells were pre-treated with the inhibitors 1 h before TLR activation. The pre-treatment of the cells with Bay11-7082 at a concentration of 20 μM significantly reduced NF-κB activity in the presence of Flagellin. In the same line of evidence, it was reported that 1 h of Bay11-7082 pre-treatment of human umbilical vein endothelial cells could reduce TNF-α induced NF-κB activation (Pierce et al., 1997). 1 h pre-treatment of the endometrial cells with SP600125 significantly decreased the Poly I:C-induced AP-1 activation. The effectiveness of SP600125 to block JNK activity in response to TLR 3 stimulation has been proven before, where the pre-treatment of keratinocyte cells with SP600125 was able to block TLR 3-induced expression of IL-1β, TNF-α, IL-12p70 and IFN-β (Zhao et al., 2010). In addition, inhibition of JNK MAPK with SP600125 (20 μM) for 1 h in fibroblast-like synoviocytes (FLS) was able to decrease AP-1 production in the presence of Poly I:C (Yoshizawa et al., 2008).

The inhibition of NF-κB activity with Bay11-7082 was unable to restore the Poly I:C-induced reduction in trophoblast cells adhesion to endometrial cells. This is in contrast with the previous finding where it was shown that Bay11-7082 was able to restore the flagellin-induced impairment of the attachment of trophoblasts cells to the endometrial Ishikawa 3H-12 cells (Caballero et al., 2013). It is possible that in our model the TLR 3-mediated reduction of trophoblast spheroid adhesion to the endometrial cells could signal through a different route such as MAPK pathway. This assumption was further confirmed where the pre-treatment of RL95-2 cells with the JNK inhibitor SP600125 was able to recover the spheroid

adhesion to endometrial cells in the presence of Poly I:C, confirming the inhibitory role of MAPK JNK pathway on trophoblast spheroid adhesion to endometrial cells in RL95-2 cells.

The mechanism through which MAPK pathway activation alters the adhesion of trophoblast cells to the endometrial cells is still unknown. The effects of TLR 3 ligation in endometrial epithelium could be significant as the endometrium is a significant site for viral entry and infection (Jorgenson et al., 2005). Additionally, the cytokine milieu plays an essential role in normal functions of the endometrium such as embryo implantation and uterine cycle progression (Tabibzadeh, 1994, Tabibzadeh and Sun, 1992), and cytokine imbalances and immune dysfunction have been implicated in problems such as spontaneous abortion, endometriosis and unexplained infertility (Kwak-Kim et al., 2010, Piva et al., 2001, Tseng et al., 1996, von Wolff et al., 2002). Because TLR 3 activation impacts production of cytokines such as IL-8 and IL-6 (Jorgenson et al., 2005, Zhang et al., 2007a), recognition of dsRNA in the endometrial epithelium could be a critical event in endometrial dysfunction and failure of embryo implantation. Moreover, the involvement of MAPK pathway in IL-8 production has been shown before, where increase of IL-8 expression was accompanied by a significant increased phosphorylation of p38 MAPK, ERK, and JNK in response to IL-1β in Human retinal Müller cells (MIO-M1 cell line) (Liu et al., 2014). Similarly, we were able to show that enhanced levels of AP-1 production were coordinated by increased IL-8 production upon TLR 3 stimulation in RL95-2 cells. Hence, it is logical to believe that increased MAPK activity in response to TLR 3 activation could alter the

production of IL-8, which can result in suppressing trophoblast adhesion to the endometrial cells. More investigations are required to demonstrate this possibility.

No other study has shown that the MAPK pathway can alter the binding of trophoblast cells to endometrial cells. Nevertheless, the involvement of the MAPK pathway in trophoblast spheroid outgrowth on RL95-2 monolayer has been demonstrated before (Hsu et al., 2008, Li et al., 2003), 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. It is possible that in our model the activation of the MAPK pathway upon TLR 3 activation induced the same apoptotic pathway in the endometrial cells, which could lead to impairment of the adhesion of trophoblast cells to the endometrial cells.

To conclude, our hypothesis that the MAPK pathway mediates the TLR 3-induced impairment of trophoblast adhesion to the endometrial cells *in vitro* was supported in this chapter. Based on the novel data obtained in the current study, blocking and regulating the MAPK pathway by its inhibitors can be used as a new strategy to prevent and treat virus-induced infertility cases in ART techniques.

Chapter 4.

Defining the reaction of the endometrial cells to TLR 3 ligation: measurement of cytoskeletal and adhesion molecule changes in RL95-2 cells in response to TLR 3 activation

4.1. 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 maintains 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 tract, 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 human chorionic gonadotropin (hCG) (Schumacher et al., 2014), regulatory T-cells (Treg) (Alijotas-Reig et al., 2014), cytokines such as 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 the 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 (FRT) recognizes invading microorganisms through pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997, Medzhitov and Janeway, 2002). To date, 10 members of TLR family

have been recognized in humans (TLR 1 to 10) and each of them responds to a specific ligand (Beutler, 2004). Among the different TLRs, TLR 3 plays a crucial role in antiviral responses by responding to double-stranded RNA (Jorgenson et al., 2005, Schaefer et al., 2005, Yu and Levine, 2011a). It has been shown that TLR 3 recognizes viruses such as cytomegalovirus (CMV) and herpes simplex virus 1 (HSV-1) (Matsumoto et al., 2011), whose strong association with female infertility has been shown in many studies (el Borai et al., 1997, Medvedev et al., 2009, Yang et al., 1995). Upon recognition of viruses by TLR 3, a cascade of signalling pathways is initiated that end up 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 (Schaefer et al., 2005, Aflatoonian et al., 2007) and endometrial cell lines including ECC-1 (Schaefer 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 pre-eclampsia (Koga et al., 2009b), 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 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, 3 and 5 in the maternal tract can reduce implantation chances *in vivo* and *in vitro* (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014, Montazeri et

al., 2015). In addition, other reports have demonstrated that during murine pregnancy activation of TLR 3 by Poly I:C led to fetal losses (Koga et al., 2009b, Zhang et al., 2007b). 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 epithelial polarization, express adhesion molecules and microfilaments in the apical surface and to be responsive to hormones and secretion of cytokines (Way et al., 1983). The human embryo was simulated with multi-cellular spheroids from 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 whether TLR 3 activation could affect actin polymerization or the expression of adhesion molecules such as β3 integrin (Lessey et al., 1992, Illera et al., 2003) and 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 transaction pathways involving the MYD88-MAPK pathway. These data offer 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.

4.2. Methodology

4.2.1. Cell lines and cell culture

RL95-2 cell line was obtained from ATCC and used as a model to mimic endometrial cells *in vitro*. They were grown in DMEM-F12 HAM supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK), penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma-Aldrich, Poole, UK), 2 mM L-glutamine (Invitrogen) and 0.005 mg/ml Insulin (Sigma) in T75 flasks at 37°C at 5 % CO2 atmosphere. At confluence, the cells were washed with Ca^{2+} and Mg^{2+}-free

Dulbecco's phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). All experiments were performed in three replicates.

4.2.2. Ligands, inhibitors and antibodies

All ligands and inhibitors used in the current study were obtained from Invivogen Company (Invivogen, Toulouse, France). Poly Inosinic Poly Cytidilic Acid (Poly I:C), TLR 3 synthetic ligand, was used to stimulate TLR 3 (Alexopoulou et al., 2001). Flagellin and FSL-1 were used to stimulate TLR 5 and 2/6 respectively (Hayashi et al., 2001). The inhibitors used were Pepinh-TRIF and Pepinh-MYD88 to inhibit TRIF and MYD88 signalling pathways respectively (Toshchakov et al., 2005). SP600125 (JNK) was applied to inhibit the mitogen-activated protein kinases (MAPK) pathway (Bennett et al., 2001). BAY11 -7082 was used to inhibit the NF-κB pathway (Saraiva et al., 2005).

Anti-CD98hc (FG1/10) and anti-β3 integrin integrin (P97) mAb have been previously described (Dominguez et al., 2010).

4.2.3. 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 preloaded 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 flowcytometry in a Cytomics FC 500 MPL (Beckman Coulter).

4.2.4. Actin polymerization assay

RL95-2 cell monolayers, treated or not with the different stimuli, were fixed with 4% formaldehyde, scrapped 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).

4.2.5. Immunofluorescence and confocal analysis

RL95-2 cells were grown to confluence on fibronectin-coated coverslips (5 µg/ml), fixed with 4% formaldehyde and permeabilized for 5 minutes with 0.5% Triton X-100. They were stained with the appropriate combination of mAb and secondary antibodies and mounted with Prolong (Invitrogen). Confocal images were obtained with a Leica TCS-SP5 confocal scanning laser microscope with an HCX PL APO lambda blue 63×/1.4 oil immersion objective and analysed with Leica LAS confocal image processing software. The quantification of fluorescent in each image was analysed by Image J software.

4.2.6. Cell transfection

RL95-2 cells, at 70-80 % confluence, were transfected with actin green fluorescent protein (GFP) DNA using X-treme GENE HP (Roche). In brief, 200 μl of Opti-MEM was placed in a sterile tube. 6 μl of X-tremeGENE HP was added to the vial and pipetted gently. 2 μg of DNA (3:1 ratio of reagent to DNA) was then added and incubated at room temperature for 30 min. 200 μl of X-treme:DNA mix was added to different areas of one well of a 6-well plate and incubated for 24 h.

4.2.7. Viability assessment of endometrial cells

In order to check the viability of RL95-2 cells, they were grown in 12-well plates until 100 % confluence. The media were replaced with serum free media before they were either treated or not with Poly I:C. The cells were then harvested using trypsin-EDTA and 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 for staining with 3 μM propidium iodide (PI; Life technologies, Paisley, UK). 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 were expressed as percentage of live cells and were compared using a one-way ANOVA, with $p < 0.05$ considered significant.

4.3. Experimental design

4.3.1. 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 (0.5 h, 1 h, 2 h 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 and the media replaced with serum-free media. Poly I:C was then added to each well at a concentration of 10 μg/ml at various time points (1, 2 and 4 h) and F-actin, CD98 and beta3 staining was performed as described and optical sections were acquired by confocal microscopy.

4.3.2. Analyses of 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 TL3 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 NF-κB and MAPK pathways, were assessed by treating RL95-2 cells with Pepinh-MYD88 (10 μM for 6 h), BAY11 -7082 (20μM for 1 h) and 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 earlier (Chapters 2 and 4) and only their functional dose was applied in this set of experiments.

4.3.3. The effect of TLR 3 activation on the viability of the endometrial cells

To determine whether TLR 3 activation was affecting the viability of the 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.

4.3.4. *In vitro* imaging of living cells expressing actin GFP in response to TLR 3 activation in RL95-2 cells

RL95-2 cells were cultured in 6-well plates and the media were replaced with serum-free media. The cells were then transfected with actin GFP as described. 10 µg/ml of Poly I:C was then added to each well and incubated for 4 h. *in vitro* imaging of living cells expressing actin GFP was checked by wide-field fluorescence Leica DMIRE2 microscope coupled to a monochromator (Polychrome IV; Till Photonics, Munich, Germany) and a CCD camera (CoolSNAP HQ; Photometrics, Tuc- son, AZ). The data were analysed by Metamorph software. The cells were maintained at 37°C in a 5% CO_2 atmosphere. The videos of time lapse experiments are attached in a separate file.

4.3.5. Statistical Analysis

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

4.4. Results

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

As can be seen from the Fig. 4.1A, the mean fluorescent intensity of F-actin was gradually decreased in Poly I:C treated RL95-2 cells compared to the control ($P < 0.05$). Poly I:C significantly reduced actin polymerization even after 30 min. However, addition of Flagellin (Fig. 4.1B) and FSL-1 (Fig. 4.1C) to RL95-2 cells did not alter the levels of intracellular filamentous actin.

To further corroborate these results in non-disturbed cell monolayers, confocal analyses were performed. As shown in Fig. 4.1D, 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 ($P < 0.05$).

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

It is apparent from Fig. 4.2A that CD98 expression was gradually decreased in Poly I:C treated RL95-2 cells compared to 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. 4.2B) and FSL-1 (Fig. 4.2C) were added to RL95-2 cells.

Similarly, this effect was observed in immunostainings of RL95-2 cell monolayers. Addition of Poly I:C significantly reduced CD98 content fluorescence intensity compared with the control (Fig. 4.2D) ($P < 0.05$). This effect was observed after 1 h of Poly I:C treatment.

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

As shown in Fig. 4.3A, β3 integrin expression was gradually decreased in Poly I:C treated RL95-2 cells compared to the control ($P < 0.05$). Poly I:C reduced β3

integrin expression significantly after 30 min. In contrast to F-actin or CD98, addition of Flagellin (Fig. 4.3B) and FSL-1 (Fig. 4.3C) also significantly decreased β3 integrin expression, as soon as 4 h of TLR ligand treatment ($P < 0.05$). β3 integrin-staining was however too faint to be assessed by confocal microscopy (Dominguez et al., 2010).

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

Addition of a functional dose of Pepinh-TRIF (20 μM) to RL95-2 cells was not able to revert the effects of Poly I:C treatment on actin polymerization (Fig. 4.4A), CD98 (Fig. 4.4B) and β3 integrin expression (Fig. 4.4C).

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 pre-treatment with Pepinh-MyD88 recovered the effect on actin polymerization (Fig. 4.4A), CD98 (Fig. 4.4B) and β3 integrin expression (Fig. 4.4C) ($P < 0.05$).

4.4.5. 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.

Addition of SP600125 to RL95-2 cells significantly restored the reduction in actin polymerization (Fig. 4.5A), CD98 (Fig. 4.5B) and β3 integrin expression (Fig. 4.5C) in the presence of Poly I:C ($P < 0.05$). However, addition of Bay11-7082 was unable to recover these alterations in RL95-2 cells in response to Poly I:C (Fig. 4.5A, 4.5B and 4.5C).

4.4.6. TLR 3 activation leads to actin filaments re-organisation and decrease in F-actin content.

As can be seen in the time lapse videos, after 4 h of Poly I:C treatment actin filaments start to re-organise in RL95-2 cells and the total F-actin content clearly decreases as the time of Poly I:C treatment increases.

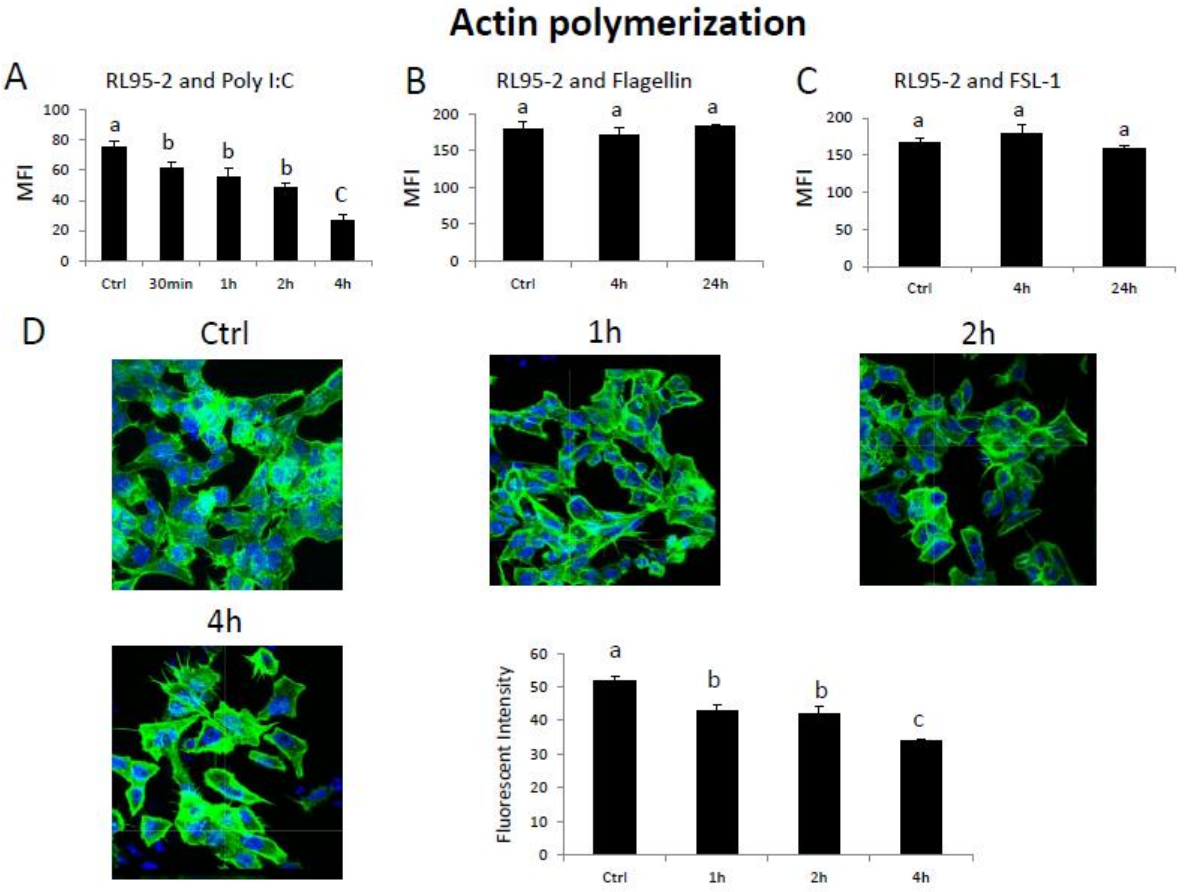

Figure 4.1 The effect of TLR 2/6, 3 and 5 activation on actin polymerization in endometrial cells.

RL95-2 cells were cultured in 24-well plates and the media were replaced with serum-free media. RL95-2 cells were then incubated with 10 µg/ml of Poly I:C for various time points (30min, 1h, 2 h 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 h 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. These results were further confirmed on RL95-2 monolayers of Poly I:C treated cells by confocal microscopy (D). RL95-2 monolayers were stained for F-actin. Briefly, RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. Poly I:C was then added to each well at a concentration of 10 µg/ml at various time points (1, 2 and 4 h) and F-actin staining was performed as described and optical sections were acquired by confocal microscopy. Confocal images were obtained with a Leica TCS-SP5 confocal scanning laser microscope. The quantification of fluorescent in each image was analysed by Image J software. 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 and Fischer's multiple comparison test was applied as the post-hoc test.

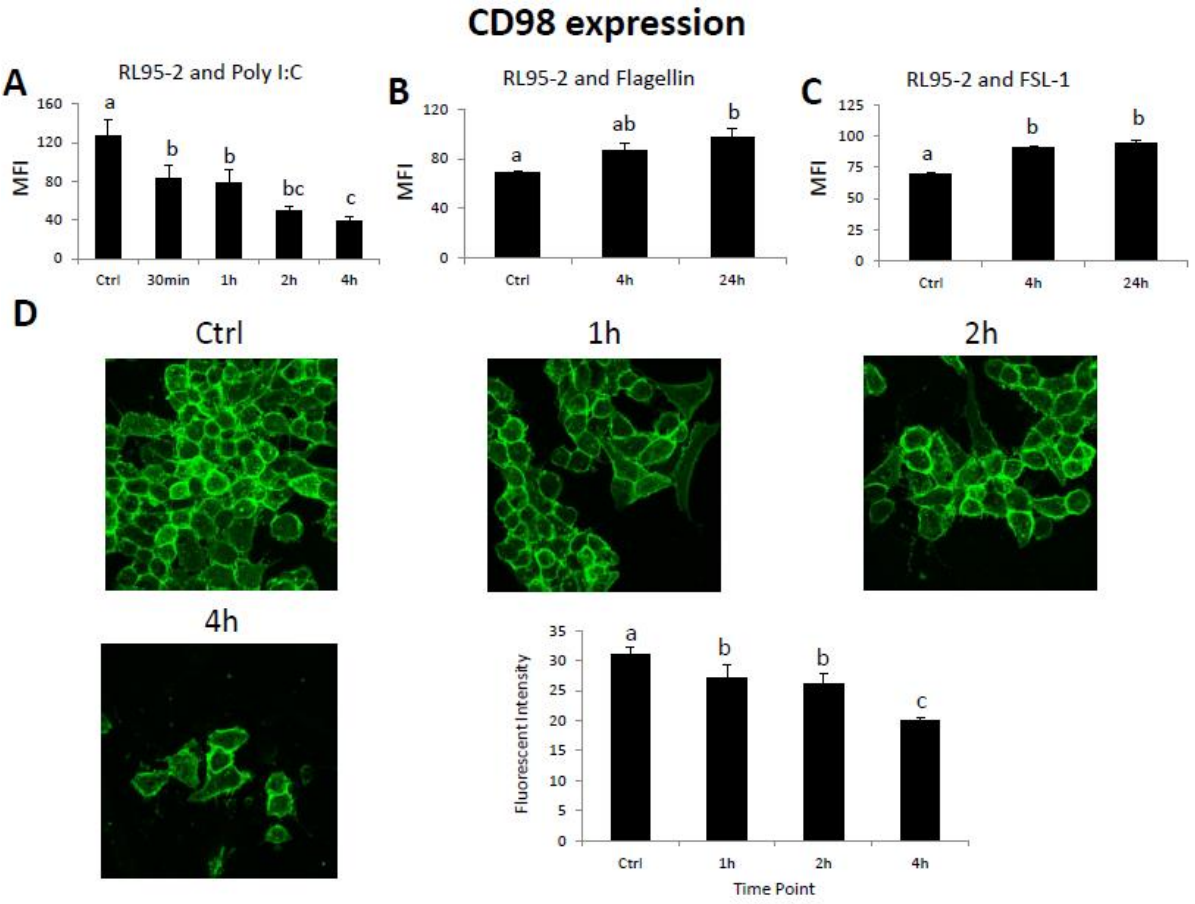

Figure 4.2 The effect of TLR 2/6, 3 and 5 activation on CD98 expression in endometrial cells.

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 (30min, 1h, 2 h 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 h and 24 h. CD98 expression was assessed by flow cytometry. The data are the average of three independent experiments. These results were further corroborated on RL95-2 monolayers of Poly I:C treated cells by confocal microscopy (D). RL95-2 monolayers were stained for CD98. Briefly, RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. Poly I:C was then added to each well at a concentration of 10 µg/ml at various time points (1, 2 and 4 h) and CD98 staining was performed as described and optical sections were acquired by confocal microscopy. Confocal images were obtained with a Leica TCS-SP5 confocal scanning laser microscope. The quantification of fluorescent in each image was analysed by Image J software. 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 and Fischer's multiple comparison test was applied as the post-hoc test.

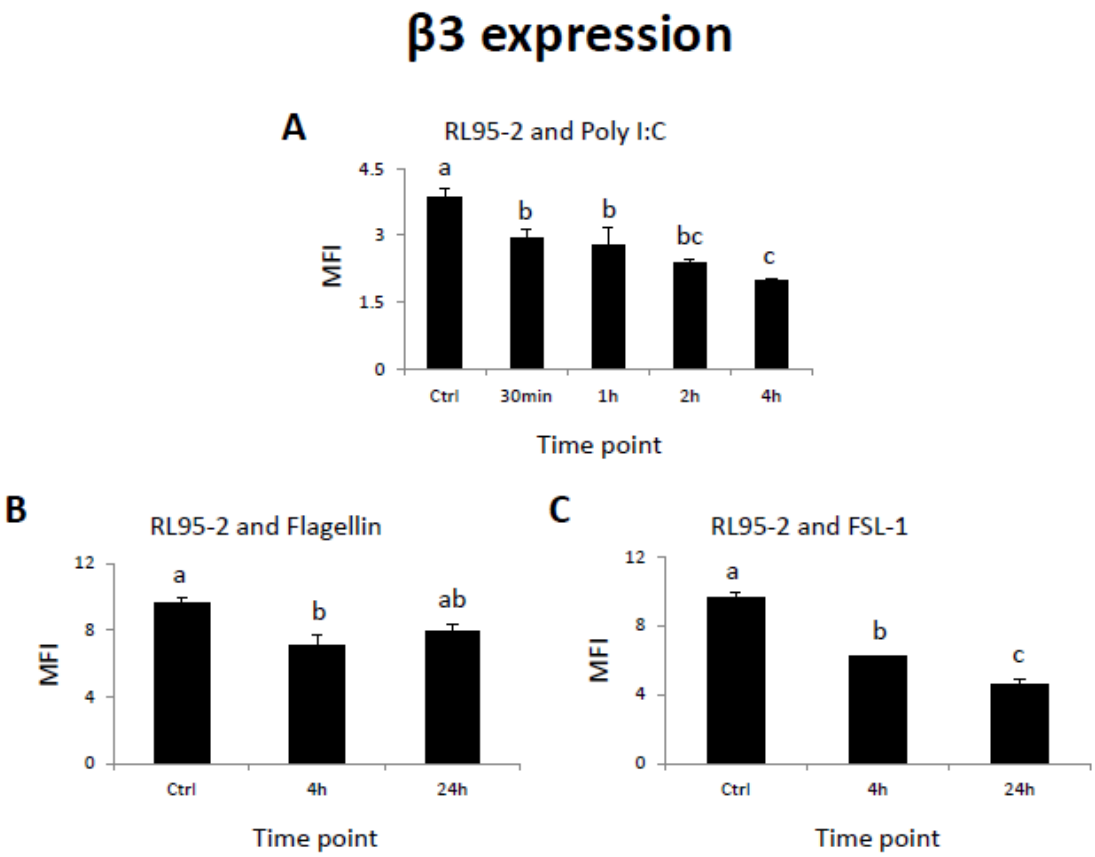

Figure 4.3 The effect of TLR 2/6, 3 and 5 activation on β3 integrin expression in endometrial cells.

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 (30min, 1h, 2 h 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 h and 24 h. β3 integrin expression was 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 ligand treated endometrial cells and control group and Fischer's multiple comparison test was applied as the post-hoc test.

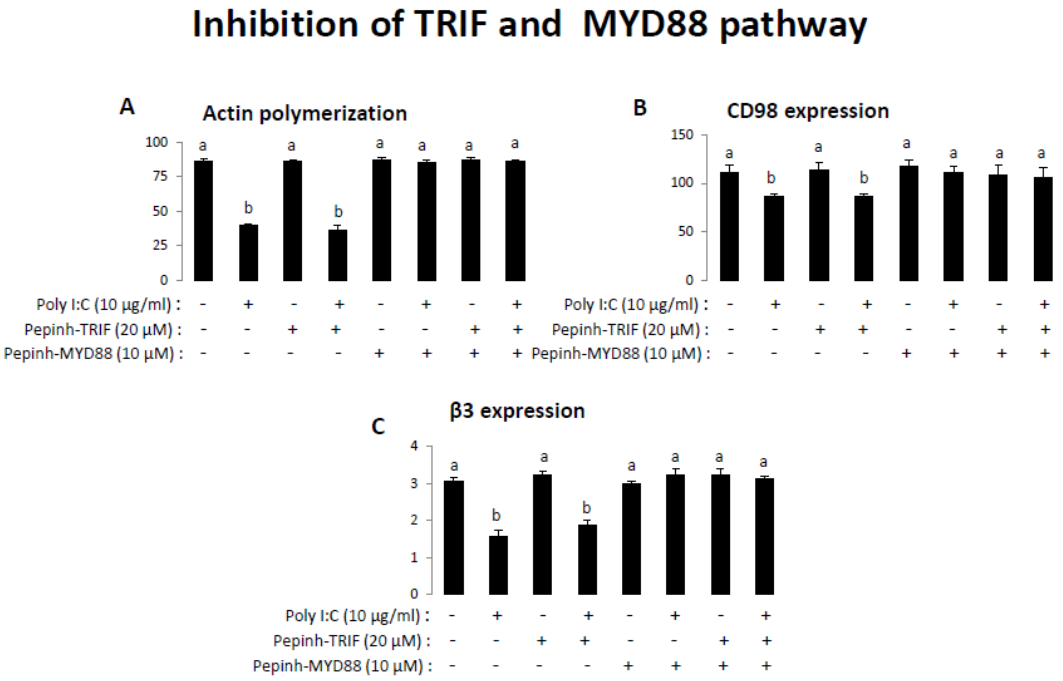

Figure 4.4 The effect of inhibition of TRIF and MYD88 signalling pathways on actin polymerization, CD98 and β3 integrin expression in RL95-2 cells.
RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. They were pre-treated with efficient and functional Pepinh-TRIF (20µM) and Pepinh-MYD88 (10 µM) for 6 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 and Fischer's multiple comparison test was applied as the post-hoc test.

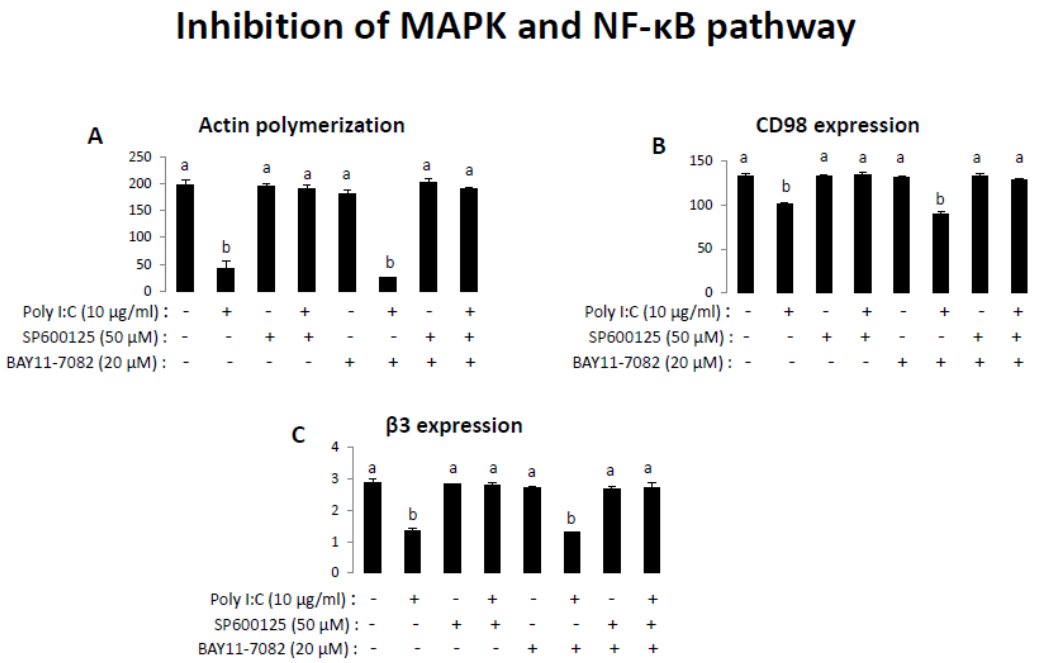

Figure 4.5 The effect of inhibition of MAPK and NF-κB signalling pathways on actin polymerization, CD98 and β3 integrin expression in RL95-2 cells.
RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media. RL95-2 cells were pre-treated with SP600125 (50µM) and BAY11-7082 (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 and Fischer's multiple comparison test was applied as the post-hoc test.

4.5. Discussion

Infertility is being treated by a variety of assisted reproductive techniques (ART). But embryo implantation remains the rate-limiting factor for the success of ART and in particular ART therapies involving *in vitro* fertilisation (IVF) (Dekel et al., 2010, Salamonsen et al., 2002, Stafford-Bell and Copeland, 2001). To overcome this problem, different markers have been proposed as determinants of endometrial receptivity, but at the moment none of the functional studies have demonstrated any appropriate clinical applications (Horcajadas et al., 2007). Thus there is a great need for understanding the molecules that can affect 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 the 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 Poly I:C-induced decrease in actin polymerization and expression of adhesion molecules, as blocking their activation with specific inhibitors restored actin depolymerization and disruption of adhesion molecule expression in TLR 3-stimulated endometrial cells.

The plasma membrane of epithelial endometrial cells undergoes morphological changes at the time of implantation, in a process known as "plasma membrane transformation" (Murphy, 1993, Murphy, 1995, Murphy, 2000b, Murphy et al., 2000, Murphy and Shaw, 1994). 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). Pinopodes appear to be the most reliable morphological marker of receptive endometrium at the time of implantation (Nikas et al., 1995, Murphy and Dwarte, 1987) and represent sites of preferential blastocyst attachment (Murphy, 1995, Murphy, 2000a). Since actin microfilaments are the major components of 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 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 decrease and disruption of actin polymerization (also known as actin depolymerization) 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 destructed in Poly I:C-treated neuroblastoma cell lines (Hsu et al., 2013).

The disassembly in F-actin content in dendritic cells or members of the immune system in general 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. Whereas, 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 colleagues (Thie et al., 1997), who found that inhibition of actin polymerization by cytochalasin D impaired attachment of JAr spheroids 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. have 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 (Kong and Ge, 2008, Shin et al., 2009, West et al., 2004). Hence assessing the involvement of these pathways in TLR 3-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 the embryo to endometrial cells at the window of implantation (Aplin, 1997, Aplin and Kimber, 2004, Aplin et al., 1996, Kaneko et al., 2013). Among adhesion molecules, β3 integrin integrin (Kuno et al., 1998) and CD98 (Dominguez et al., 2010) expression increases significantly at the window of implantation. Investigations in humans (Kaneko et al., 2011) and mice (Illera et al., 2000) have shown that the blockade of β3 integrin expression in the endometrium has 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 these evidences 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 (Gonzalez et al., 1999, Lessey et al., 2000, Creus et al., 1998). Likewise CD98 seems to play a crucial role in the implantation process, as its blockage in RL95-2 cells remarkably impaired 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 a 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 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 their 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 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 an 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 1 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 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 TLR 3 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 molecules expression. This finding, on one hand, corroborates the ideas of Brenner and Simmonds (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 the MYD88 signalling pathway in TLR 3-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, 2011b). 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 and Suzuki (2004) found that the reduction in F-actin content in human neutrophils induced by tumor 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 findings, 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 on implantation failure. Nevertheless, the involvement of MAPK pathway in the trophoblast spheroid outgrowth on RL95-2 monolayer has been demonstrated before (Hsu et al., 2008, Li et al., 2003), 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, Huang et al., 2013, Li et al., 2013, Zhou et al., 2010, Lee et al., 2012). Based on the novel data obtained in the current

study, blocking and regulating of the MAPK pathway by its inhibitors could be a potential new strategy to prevent and treat viral-induced infertility cases in ART techniques.

Taken together, the hypothesis that TLR 3 activation leads to decrease in actin polymerization and expression of β3 integrin and CD98 was supported. The identification of MYD88/MAPK as a key pathway for TLR 3-mediated defects in actin polymerization, CD98 and β3 integrin expression, provides a new insight into 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.

Chapter 5. Defining the effect of TLR 3 ligation on IL-1RA production; a potential pathway for implantation failure by TLR 3 activation

5.1. Introduction

Despite all the improvements in assisted reproductive technology (ART), which have led to major developments in the treatment of infertility and increasing successful pregnancy outcomes, implantation failure is still the major problem affecting the outcome of ART (Carver et al., 2003). Successful implantation relies on a high quality embryo, a receptive uterus and a series of tightly regulated interactions between the blastocyst and the endometrium. It has been shown that activation of the innate immune system in the female reproductive tract (FRT) in response to genital tract infection can affect this communication (Pellati et al., 2008, Dekel et al., 2010).

The innate immune system in the FRT recognizes infectious microorganisms through pathogen recognition receptors (PRRs) such as the Toll-like receptors (TLRs) (Medzhitov and Janeway, 1997, Medzhitov and Janeway, 2002). To date, 10 members of the TLR family have been identified in humans (TLR 1 to 10), each of which recognizes and binds to a specific ligand (Beutler, 2004). Among the various TLR members, TLR 3 recognizes double-stranded RNA and plays an important role in the recognition of infectious viruses (Jorgenson et al., 2005, Schaefer et al., 2005, Yu and Levine, 2011a). There is a considerable body of evidence that TLR 3 is expressed in the primary uterine epithelial cells (Schaefer et al., 2005, Aflatoonian et al., 2007) and endometrial cell lines including ECC-1 (Schaefer et al., 2004), Ishikawa (Aboussahoud et al., 2010a) and RL95-2 (Jorgenson et al., 2005). It has also been shown that TLR 3 recognizes viruses such as cytomegalovirus

(CMV) and herpes simplex virus 1 (HSV-1) (Matsumoto et al., 2011), whose strong association with female infertility has been shown in many studies (el Borai et al., 1997, Medvedev et al., 2009, Yang et al., 1995).

The effect of activation of some members of TLR family on embryo implantation has been shown before, with stimulation of TLR 2/6, 3 and 5 leading to impairment of trophoblast cells' attachment to endometrial cells *in vivo* and *in vitro* (Aboussahoud et al., 2010c, Sanchez-Lopez et al., 2014, Montazeri et al., 2015). Implantation failure is likely to be the major cause of infertility, thus deep insight into the molecular mechanisms that impact the process of embryo implantation in response to TLR 3 activation may provide new opportunities for improving the implantation rate in virus-induced infertility cases.

Emerging evidence suggests that in addition to adequate hormonal priming, successful embryonic implantation relies on an appropriate interaction between cytokines produced and received by the blastocyst and endometrium (Cross et al., 1994). The interleukin-1 (IL-1) system seems to be relevant to the implantation process (Kauma et al., 1990). The IL-1 family consists of two agonists, IL-lα and IL-1β (Dinarello, 1988), and an inhibitor, IL-1 receptor antagonist (IL-1RA). Two IL-1 receptors have been recognized: IL-1 receptor type I (IL-1R tI) (Sims et al., 1988) and IL-1 receptor typeII (IL-1R tII) (Horuk and McCubrey, 1989) (Fig. 5.1A). The available information indicates that IL-1R tII is not functional and IL-1 signalling occurs exclusively via type I receptors (Sims et al., 1993).

The presence of the IL-1 system in human endometrium (Tabibzadeh and Sun, 1992, Kauma et al., 1990, Simon et al., 1995, Simon et al., 1993a, Simon et al., 1993b), human embryos (De los Santos et al., 1996) and embryo-maternal interface (Simon et al., 1994) has been demonstrated. Furthermore, it has been shown that IL-1 expression significantly increases at the late secretory phase, when the implantation of the embryo takes place (Simon et al., 1993a). This evidence suggests that IL-1 system has a pivotal role in controlling and regulating the process of embryo implantation. IL-1RA can inhibit the binding of IL1α and IL1β to IL-1R tI and its binding to the receptor does not result in signal transduction (Fig. 5.1B) (Bankers-Fulbright et al., 1996). It is well documented that IL-1RA is regarded as an anti-implantation factor and as Simon and colleagues have shown, IL-1RA prevented embryonic implantation in mice by direct effect on the endometrial epithelium, which was related to the alteration of expression of α4, αv and β3 integrin adhesion molecules (Simon et al., 1998).

In view of this overwhelming evidence for the relation of IL-1RA to implantation failure, the current study was designed to assess whether IL-1RA could be involved in TLR 3-induced implantation failure. We hypothesized that IL-1RA mediates TLR 3-induced impairment of trophoblast adhesion to endometrial cells. The *in vitro* model of attachment of trophoblast spheroid (JArs) to endometrial cells (RL95-2) was applied to simulate embryo implantation. The production of IL-1RA in RL95-2 cells in response to TLR 3 activation was measured. The initial rise in IL-1RA levels reflected the effect of TLR 3 activation on increasing IL-1RA levels in endometrial cells. Next, to determine whether the Poly I:C-induced implantation failure was

mediated through IL-1RA, the effect of IL-1RA on the attachment of trophoblast cells to endometrial cells was determined. Secondly, to directly assess whether IL-1RA was able to suppress trophoblast attachment to endometrial cells, IL-1RA gene expression was knocked down by IL-1RA siRNA. The efficiency of IL-1RA siRNA was validated using QPCR and ELISA for IL-1RA. The functionality of IL-1RA siRNA was then tested by addition of IL-1β to RL95-2 and measuring the production of IL-8. Finally, by application of the efficient and functional IL-1RA siRNA, we examined whether the suppression of IL-1RA production in RL95-2 cells could restore the rate of JAr spheroids' adhesion to the endometrial cells in the presence of Poly I:C. Our results showed that suppression of IL-1RA gene expression in RL95-2 cells significantly restored trophoblast cells' attachment to endometrial cells in the presence of Poly I:C, confirming IL-1RA's role in TLR 3-induced implantation failure.

5.2. Methodology

5.2.1. Cell lines and cell culture

RL95-2 was obtained from ATCC and was used to mimic endometrial cells. RL95-2 cells were cultured in T75 flasks at 37º C in DMEM (F12) HAM (Invitrogen, Paisley, UK), supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Sigma, Poole, UK), 10% FCS (Invitrogen), 160 ng/ml Insulin (human recombinant insulin from Gibco (Invitrogen), catalog # 12585-014, and 2mM L-glutamine

(Invitrogen), in 5% CO2 atmosphere until confluence was reached. 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 confluence, the cells were washed with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS; Sigma) and harvested using trypsin-EDTA (Invitrogen). The cells were then incubated for 3 min, pelleted by centrifugation at 300 g for 4 min and the supernatant was discarded. The cells were diluted with 3 ml of media and suspended with pipetting 5-6 times in order to ensure a homogenised solution. While suspending, it was necessary to remove any clot of considerable size from the solution.

5.2.2. Ligands and inhibitors

Poly Inosinic Poly Cytidilic Acid (Poly I:C) (Invivogen, tlrl-pic, Toulouse, France), TLR 3 synthetic ligand was used to stimulate TLR 3 (Alexopoulou et al., 2001). Recombinant human interleukin-1beta (InvivoGen, rhil-1b, Toulouse, France) was used to induce IL-8 production. IL-1 receptor antagonist (IL-1RA) (PeproTech, 200-01RA, London, UK) was applied to block IL-1 signal transduction.

5.2.3. *In vitro* human implantation assay

The RL95-2 cells were cultured in T75 flasks until 100% confluence, cells were then harvested using trypsin-EDTA. The cells were counted and $3x10^5$ endometrial cells were cultured in each well of a 12-well plate. They were incubated at 37°C and 5% of CO2 for 4 days until confluence. The media were replaced every second day (Sanchez-Lopez et al., 2014).

To create spheroids from JAr cells monolayers, 10^6 cells/ml were counted with a Haemocytometer, and cultured in 5 ml of RPMI 1640 media in 60 × 15 mm Petridishes (CellStar tissue culture dishes, Greiner Bio-One, GmbH/Germany) in a humid atmosphere containing 5% CO_2 at 37°C on a gyratory shaker, set at 110 rpm for 24 h (Sanchez-Lopez et al., 2014).

Once the JAr spheroids were formed on the shaker, they were gently transferred onto each well of confluent RL95-2s in 12-well plates, and the co-culture was maintained in DMEM-F12 HAM, with supplements as mentioned above and incubated for 1 h at 37°C (Sanchez-Lopez et al., 2014).

Non-adherent spheroids were removed from the monolayer using an automatic horizontal shaker to detach loosely bound or unbound spheroids. In brief, once the trophoblast spheroids were co-cultured with endometrial cells, the number of

spheroids was counted under the microscope and each plate was placed on a shaker, which was set at 200 rpm for 4 min. The cells were washed with DPBS twice and then the number of attached spheroids was counted under the microscope. The results were expressed as the percentage of spheroids attached from the total number of spheroids used to initiate the co-incubation experiments. All the experiments were performed in three replicates.

5.2.4. RNA isolation and cDNA synthesis

For endometrial cell lines genomic studies, RL95-2 cells were washed with DPBS without Ca^{2+} andMg^{2+} and one milliliter of TRIreagent (Sigma) was added onto the flask. Thereafter total RNA from cells was extracted following a standard protocol supplied by the manufacturer. Total RNA obtained from RL95-2 cells was treated three times with DNase I (DNA-freeTM, Ambion Austin, TX, USA) to remove genomic DNA contamination from the samples. First strand cDNA synthesis was performed using oligo dT primers (Metabion, Martinsried, Germany) and reverse transcription by Super- Script II (200 U/µl; Invitrogen). Negative controls were prepared without inclusion of the enzyme (no RT controls) (Aboussahoud et al., 2010a).

5.2.5. Primer design, optimization and validation for QPCR

The forward and reverse primers for the human IL-1RA (Accession no. NM_001204296.1) were created with the tool Primer-BLAST (NCBI website).

The IL-1RA forward primer was 25 bp, with a G/C content of 48 % and a similar melting temperature (Tm) for forward and reverse primers (59.9°C). The IL-1RA reverse primer was 22 bp, with a G/C content of 59 %. The IL-1RA primer sequence was 5'-CCAGCAAGATGCAAGCCTTCAGAAT-3' for the forward sequence and 5'-CCAGACTTGACACAGGACAGGC-3' for the reverse sequence, and product size was 199 bp. They were designed to bridge an exon-exon junction to exclude amplification from genomic DNA template. Their specificity was verified with PLAST (NCBI website) against human RNA and DNA database. The possibilities for a primer-dimer formation and secondary structures were analysed with the Oligo Analyser tool from Integrated DNA Technologies (IDT website) and ordered from the sae company (Leuven, Belgium). The efficiency of the IL-1RA primer was verified by quantitative real-time PCR (QPCR). The variation of the quantification cycle number (May et al.) was estimated during the exponential phase. A standard curve was generated using serial dilutions of the samples of cDNA (1, 1/5, 1/15, 1/45, 1/135 and 1/405) and plotted using the logarithm of the cDNA dilution versus the average Cq of three replicates.

An efficiency of 80 to 120 % for each set of primers was considered acceptable for further analysis of gene expression (Sanchez Lopez et al., 2014b).

5.2.6. Quantitative Real-Time PCR (QPCR) and gel analysis

QPCR was carried out with the cDNA prepared from RL95-2 cells as described. For normalization purposes, expression of the reference genes β-actin and B2M were also quantified. The sequence of their primers was as follows: β-actin forward sequence was 5'-CAAGATCATTGCTCCTCCTG-3' and reverse sequence was 5'-ATCCACATCTGCTGGAAGG-3', and product size was 152 bp (Sanchez Lopez et al., 2014b). B2M forward sequence was 5'-TATGCCTGCCGTGTGAACCA-3' and reverse sequence was 5'-GCGGCATCTTCAAACCTCCA-3', and product size was 98 bp (Sanchez Lopez et al., 2014b). SYBR Green Jump Start (Sigma) master mix (containing 10µl SYBR Green, 7µl H2O, 1µl of test or reference gene primers and 1µl cDNA) was added to each well of PCR plate and amplification was performed under the following conditions: 40 cycles of 95◦ for 30 s, 62◦ for 30 s and 72◦ for 30 s. All experiments included RT controls and negative controls (no cDNA). QPCR was performed using Mx3005P QPCR (Stratagene, Waldbronn, Germany) and results were analysed using MxPro QPCR software version 4.01. The amplified QPCR products were sequenced to confirm the identity of the amplified product. After the QPCR amplification of IL-1RA gene with various cDNA concentrations (1,

1/5, 1/15, 1/45, 1/135, 1/405), the products were resolved by electrophoresis in a 1 % agarose gel.

The QPCR data were analysed using the comparative Cq method. Briefly, the difference in cycle time (ΔCq) was determined as the difference between the number of cycles required for amplification of the test gene and reference genes β-actin and B2M. ΔΔCq was then obtained by finding the difference between groups. The results were expressed as mean ± SEM. Statistical analysis was performed by using ANOVA with Tukey's multiple comparison test. $P < 0.05$ was considered significant (Aflatoonian et al., 2007).

5.2.7. Enzyme-linked immunosorbent assay (ELISA)

The concentration of IL-1RA was determined in culture supernatants with the commercially available IL-1RA Development ELISA kit (PEPROTECH, 900-K474, London, UK). The ELISA was performed according to the manufacturer's instructions with 100µl of cell-free supernatant. IL-8 concentration was also measured in the conditioned media analysed by ELISA, using a kit from R&D Systems (R&D Systems, DY208, Minneapolis, USA). ELISA assays in our hands had a sensitivity of 16.5 and 31 pg/ml for IL-1RA and IL-8 respectively. Sample concentrations were determined with interpretation from the standard curve (Aboussahoud et al., 2010a).

5.2.8. IL-1RA gene expression Knock down in RL95-2 cells

IL-1RA was knocked down by Accell Human IL1RN siRNA (SMARTpool) (ThermoScientific, E-007966-00-0005, USA). Briefly, IL-1RA siRNA was diluted with Accell Delivery Media (ThermoScientific, B-005000, USA) to reach a concentration of 1 µM. The growth media were removed from the cells and 100 µl of delivery mix was added to each well of 96-well plate. RL95-2 cells were incubated with IL-1RA siRNA at 37 with 5 % CO2 for 72 h. mRNA of knock down cells was assessed by QPCR as described. The proteomic sample of knock down cells was assessed by IL-1RA ELISA kit as described.

5.2.9. Viability assessment of endometrial cells

In order to check the viability of RL95-2 cells treated with either recombinant human IL-1RA or IL-1RA siRNA, RL95-2 cells were grown in 96-well plates until 100 % confluence. The media were replaced with serum free media before they were either treated or not with IL-1RA or IL-1RA siRNA. The cells were then harvested using trypsin-EDTA and 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 an autofluorescence control sample and the other was used for staining with 3 µM propidium iodide (PI; Life technologies, Paisley, UK) 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 were expressed as percentage of live cells and were compared using a one-way ANOVA, with $p < 0.05$ considered significant.

5.3. Experimental design

5.3.1. The effect of TLR 3 activation on the production of IL-1RA in RL95-2 cells

To clarify whether TLR 3 activation in RL95-2 cells could alter IL-1RA production at the gene and protein level, RL95-2 cells were cultured in 12-well plates and the media replaced with serum-free media before they were either activated or not with Poly I:C at a concentration of 10 µg/ml at various time points (1, 2, 4, 6, 8 and 24 h). IL-1RA QPCR and ELISA were performed as described.

5.3.2. Validation of IL-1RA siRNA efficiency in suppressing the production of IL-1RA in RL95-2 cells

The efficiency of siRNA transfection was assessed using Accell Green Non-targeting siRNA (ThermoScientific, D-001950-01-05, USA) and the number of transfected cells (GFP^+) was measured by FACSCalibur cytometer (BD). To validate the efficiency of IL-1RA siRNA to suppress IL-1RA production, RL95-2 cells were transfected by IL-1RA siRNA at various concentraions (0, 0.5, 1 and 2 μM) and time points (0, 2, 4, 8 and 24 h) respectively. In the case of time intervals, 72 h of IL-1RA siRNA treatment was regarded as 0. At each concentration and time interval, IL-1RA gene expression and protein production were evaluated using QPCR and ELISA for IL-1RA respectively. In parallel, the viability of RL95-2 cells upon siRNA treatment was assessed by Propidium Iodide staining as described, to exclude detrimental effects of IL-1RA siRNA on endometrial cells viability and hence unpredicted interference with the final results.

5.3.3. Validation of ability of IL-1RA siRNA in suppressing the functionality of IL-1RA in RL95-2 cells

To validate the ability of IL-1RA siRNA in suppressing the functionality of IL-1RA, RL95-2 cells were transfected by IL-1RA siRNA as described. IL-1β, known as the inducer of IL-8 production (Bersinger et al., 2011), was added or not to RL95-2 cells at a concentration of 0.4 ng/ml for 24 h. The production of IL-8 in response to addition of IL-1β was measured and compared in IL-1RA siRNA-transfected and non-transfected RL95-2 cells using IL-8 ELISA kit as described.

5.3.4. The effect of IL-1RA on binding of trophoblast cells to endometrial cells

5.3.4.1. The effect of exogenous recombinant human IL-1RA administration to RL95-2 cells on the binding of trophoblast cells to endometrial cells

In order to determine the influence of the treatment of RL95-2 cells with IL-1RA on the number of trophoblast cells binding to the endometrial cells, RL95-2 cells were cultured in 24-well plates and the media replaced with serum-free media before they were either activated or not with IL-1RA at various concentrations (5,

10, 20 and 40 ng/ml) for 4 h. In parallel, the viability of RL95-2 cells was assessed by Propidium Iodide staining as described, to exclude detrimental effects of IL-1RA on endometrial cells viability. 30 JAr spheroids were then gently delivered to the endometrial cells in each well and co-incubated for 1 h. Adhesion was assessed as described.

5.3.4.2. The effect of knock down of IL-1RA gene expression in RL95-2 cells on the binding of trophoblast cells to endometrial cells

To clarify whether the detected response to treatment of RL95-2 cells with Poly I:C was mediated through IL-1RA, we reduced IL-1RA expression in RL95-2 cells by IL-1RA siRNA as described. 30 JAr spheroids were then delivered onto the RL95-2 monolayer and co-cultured for 1 h. Adhesion was assessed as described.

5.3.5. Statistical Analysis

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

5.4. Results

5.4.1. Primer optimization

The efficiency and specificity of IL-1RA primer were confirmed using standard and dissociation curves. Only one product for IL-1RA's primer was amplified (Fig. 5.2B), with an efficiency of 92 % (Fig. 5.2C). The amplification products resolved on an electrophoretic agarose gel were found to be of 199 bp (Fig. 5.3).

5.4.2. Production of IL-1RA was increased in RL95-2 cells in response to Poly I:C.

As shown in Fig. 5.4A, addition of Poly I:C to RL95-2 cells significantly increased the gene expression of IL-1RA. This effect was observed as soon as 2 h of Poly I:C treatment. A similar effect was observed in the protein level of IL-1RA (Fig. 5.4B), where there was a clear trend of increase in the IL-1RA levels in response to Poly I:C even after 1 h, despite a slight but significant decline after 4 h.

5.4.3. IL-1RA siRNA efficiently suppressed IL-1RA production and functionality.

As shown in Fig. 5.5B, all of the RL95-2 cells that were transfected by Accell Green Non-targeting siRNA were GFP^+, indicating its 100 % transfection efficiency.

Addition of IL-1RA siRNA to RL95-2 cells significantly decreased the gene expression of IL-1RA, reaching nearly 74 % reduction at a concentration of 2 μM (Fig. 5.6A). Similarly, application of IL-1RA siRNA at various time points significantly decreased IL-1RA gene expression even after 96 h (Fig. 5.6B). This effect was also confirmed in the protein level (Fig. 5.6C).

As can be seen in Fig. 5.6D, addition of IL-1β to RL95-2 cells led to a considerable rise in IL-8 levels compared to control, indicating its role as an IL-8 inducer. Interestingly, administration of IL-1RA siRNA to RL95-2 cells in the presence of IL-1β significantly increased IL-8 levels compared to IL-1β only treated cells, confirming the ability of IL-1RA siRNA in suppressing IL-1RA functionality. IL-1RA siRNA did not affect the viability of RL95-2 cells (Fig. 5.7B).

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5.4.4. Addition of IL-1RA to RL95-2 cells significantly decreased percentage of attachment of JAr spheroids to endometrial cells.

As shown in Fig. 5.7A, recombinant human IL-1RA was not toxic to RL95-2 cells. The percentage of attachment of JAr spheroids to endometrial cells was significantly suppressed in the IL-1RA treated group compared to control in a dose-dependent manner (Fig. 5.8A).

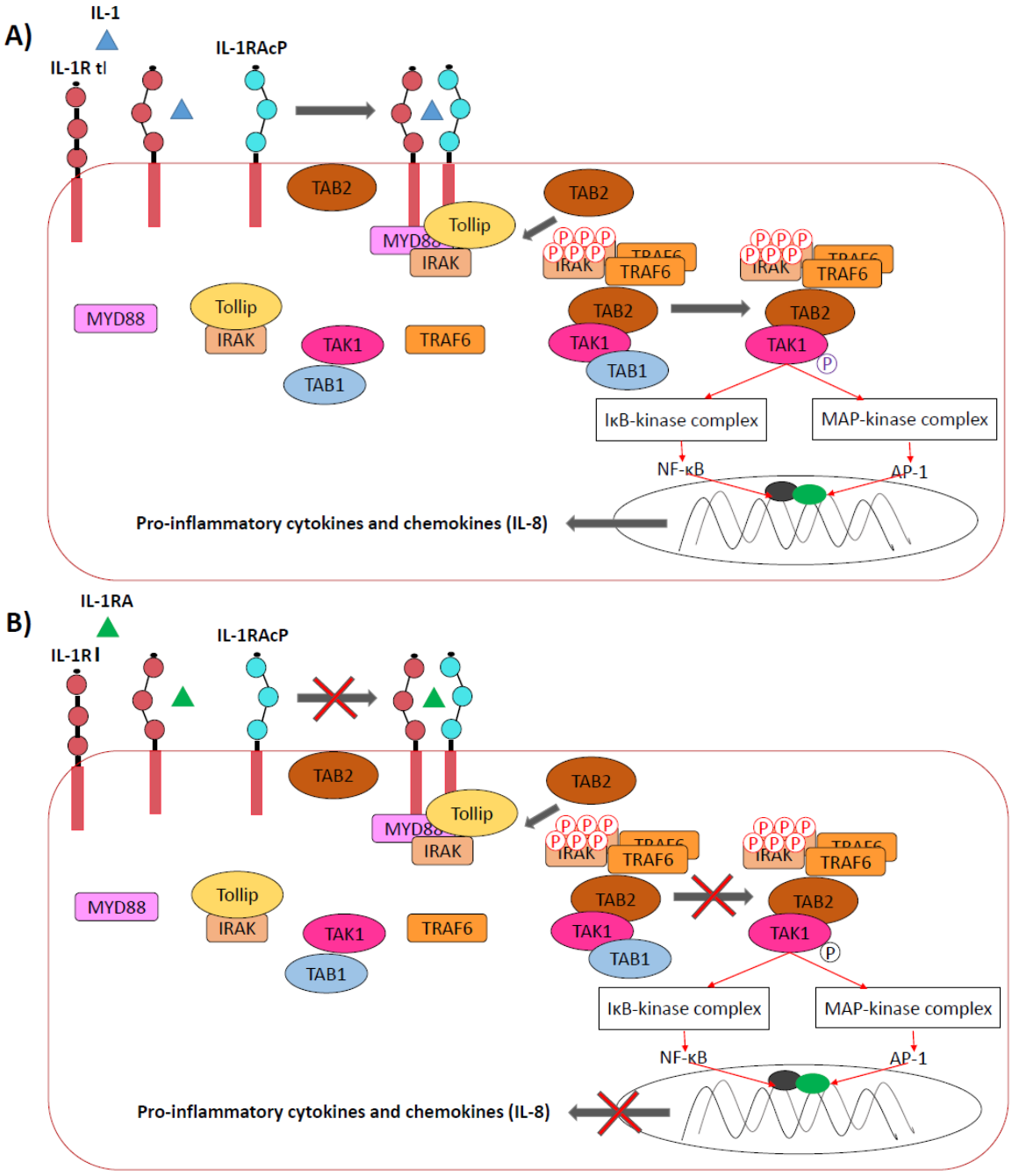

Figure 5.1 IL-1 signal transduction.

The IL-1 system is a family of polypeptides comprising two agonists, IL-lα and IL-1β, and an inhibitor, IL-1 receptor antagonist (IL-1RA). Two IL-1 receptors have been identified: IL-1 receptor type I (IL-1R tI) and IL-1 receptor type II (IL-1R tII). Upon ligation of IL-1 to IL-1R tI and its respective co-receptor (IL-1RAcP), a set of intracellular signalling is initiated. This activates the production of NF-κB and AP-1, which leads to the production of pro-inflammatory cytokines and chemokines such as IL-8 (A). IL-1RA can block the binding of IL1α and IL1β to IL-1R tI and its binding to the receptor does not result in signal transduction and as such production of cytokines (B).

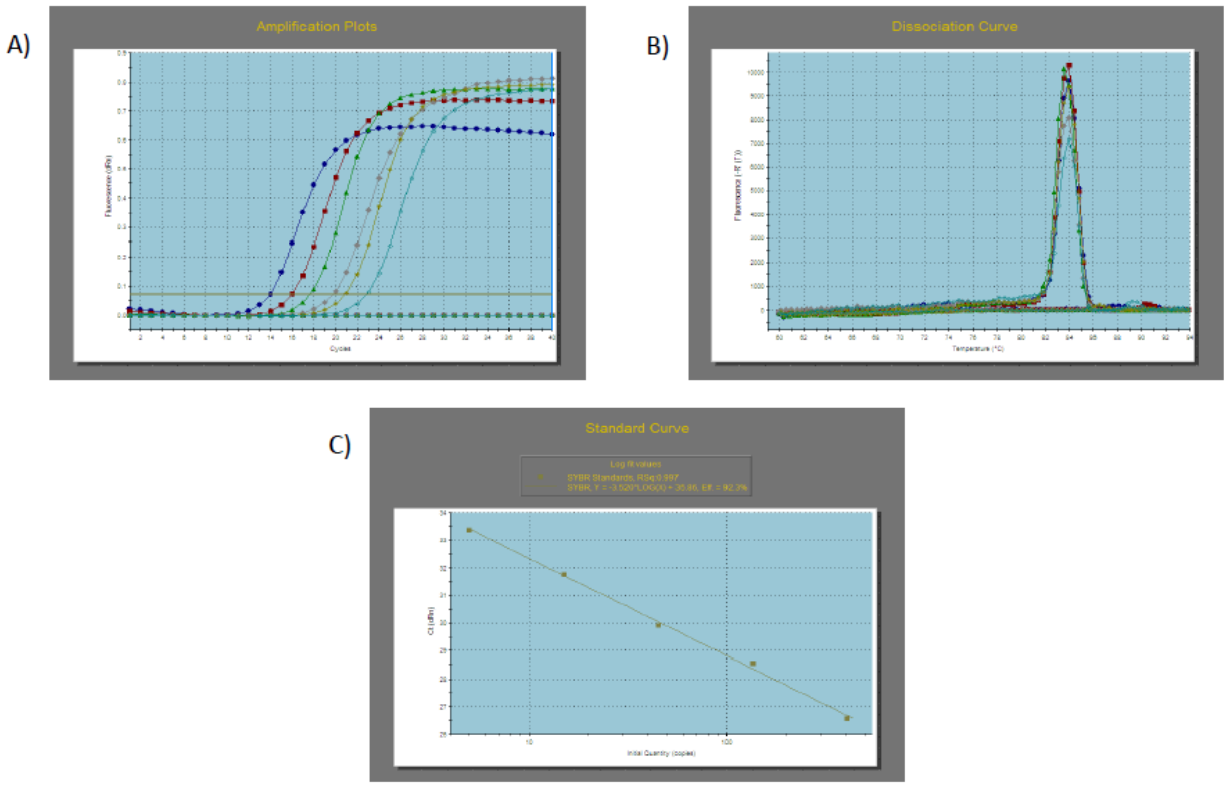

Figure 5.2 Standardization of the IL-1RA primers.
The specific primers for IL-1RA amplification were standardized at 60°C. The cDNA concentration was estimated with the amplification curves (A). The specificity of the amplification product was verified on the melting curve (B). The primers had an efficiency of 92 % estimated with the standard curve (C).

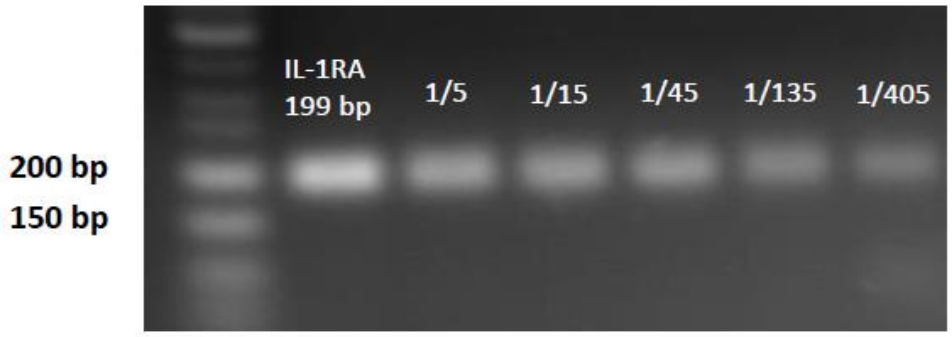

Figure 5.3 Product of amplification of IL-1RA.
After the QPCR amplification of IL-1RA gene with various cDNA concentrations (1, 1/5, 1/15, 1/45, 1/135, 1/405), the products were resolved by electrophoresis in a 1 % agarose gel. The specific amplification product for IL-1RA measured 199 bp.

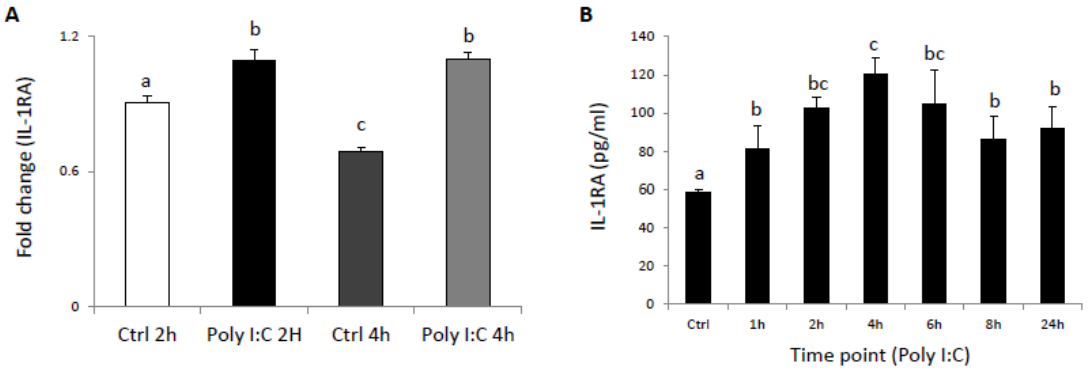

Figure 5.4 The effect of TLR 3 stimulation on IL-1RA production in RL95-2 cells.

RL95-2 cells were treated with 10 μg/ml of Poly I:C for various time points. Firstly, the gene expression of IL-1RA was assessed by QPCR (A). Secondly, the IL-1RA production at the protein level was investigated by IL-1RA ELISA (B). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the gene expression and production of IL-1RA at each time interval and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

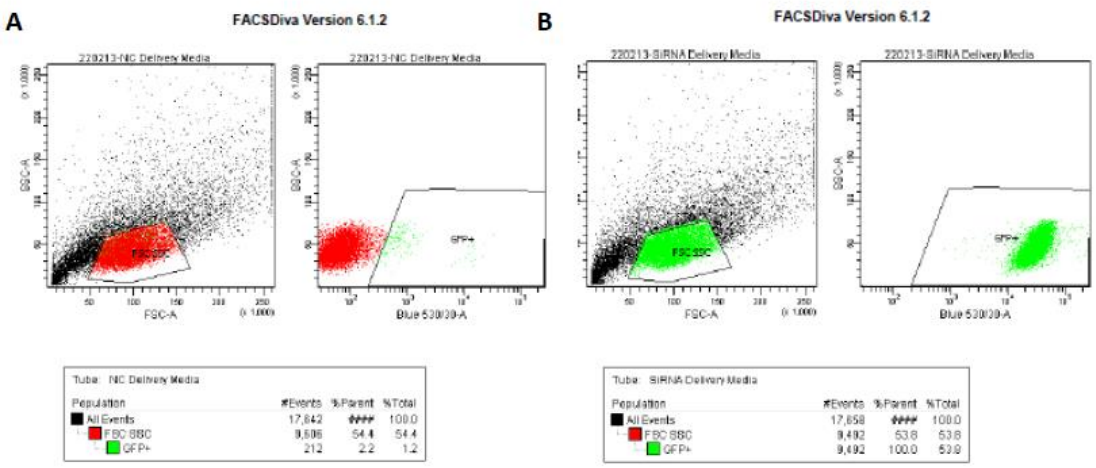

Figure 5.5 Validation of the efficiency of IL-1RA siRNA transfection in RL95-2 cells.

RL95-2 cells were either transfected by Accell Green Non-targeting siRNA (B) or not (A) at a concentration of 1 µM for 72 h. Thereafter, the number of GFP^+ cells was measured by FACSCalibur cytometer (BD). The percentage of GFP^+ cells was regarded as the efficiency of transfection.

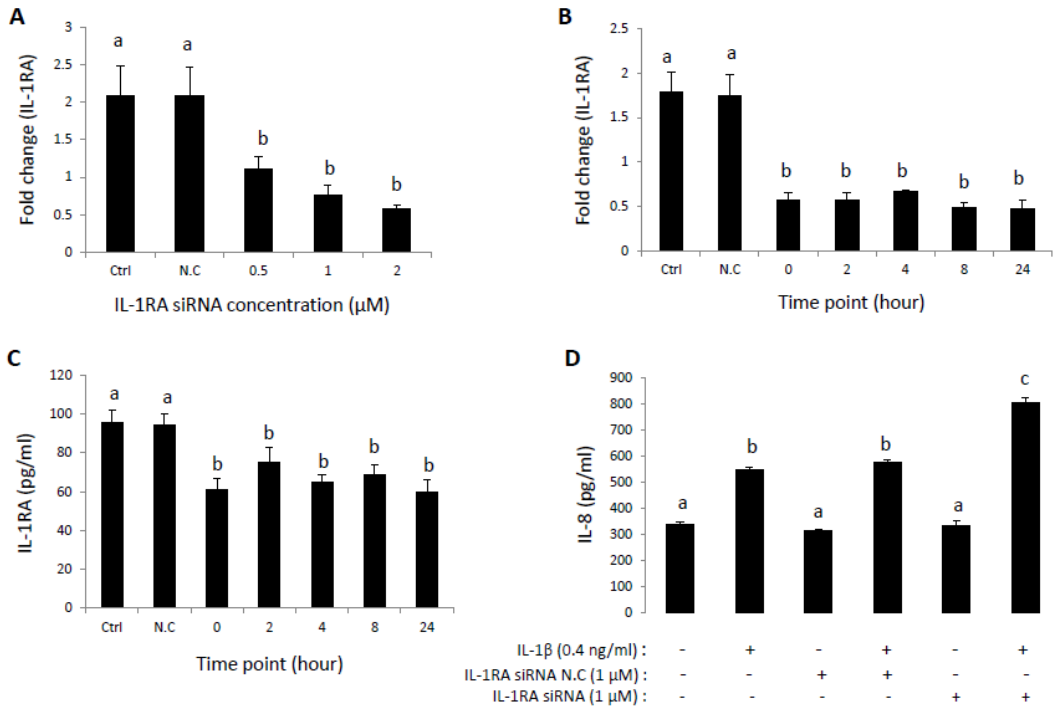

Figure 5.6 Validation of IL-1RA siRNA efficiency and functionality in suppressing the production of IL-1RA in RL95-2 cells.

RL95-2 cells were treated with various IL-1RA siRNA concentrations at different time points. Firstly, the gene expression of IL-1RA with various IL-1RA concentrations at 72 h (A) and different time intervals (B) was assessed by QPCR. Secondly, the IL-1RA production in the protein level was compared in different time intervals (C). Finally, the ability of IL-1RA siRNA in suppressing IL-1RA functionality was assessed by measuring IL-8 production by ELISA in the presence of IL-1β (0.4 ng/ml for 24 h) and IL-1RA siRNA (1 µM for 72 h) (D). NC: IL-1RA negative control (non-targeting) siRNA. The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the gene expression and production of IL-1RA and IL-8 in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

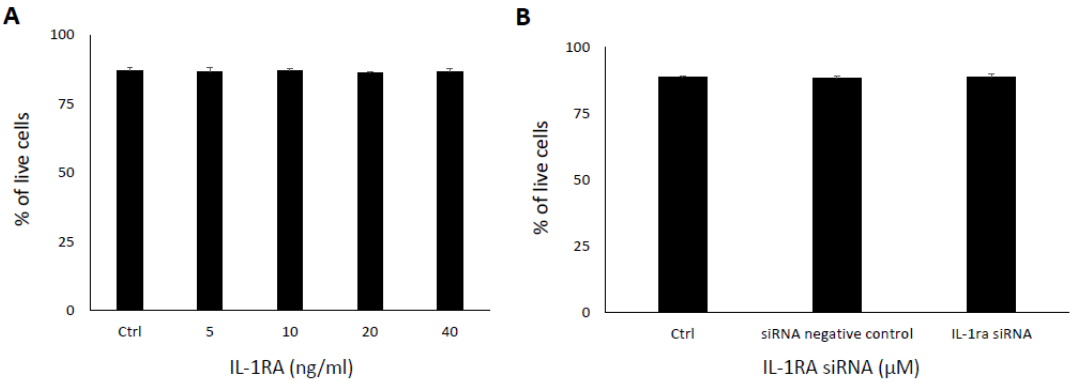

Figure 5.7 The effect of IL-1RA and IL-1RA siRNA on the viability of the RL95-2 cells.

The viability of RL95-2 cells was determined after their treatment with different concentrations of IL-1RA (0, 5, 10 and 20 and 40 ng/ml) for 4 h (A). In addition, the effect of addition of IL-1RA siRNA to RL95-2 cells on the viability of endometrial cells was assessed by addition of 1 µM of IL-1RA siRNA for 72 h (B). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of live cells in each group and Fischer's multiple comparison test was applied as the post-hoc test. $P < 0.05$ was considered to be significant.

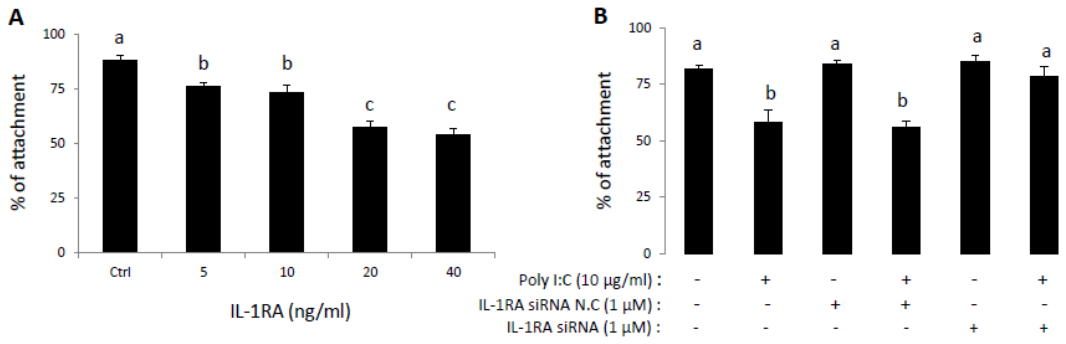

Figure 5.8 The effect of IL-1RA on trophoblast cells binding to endometrial cells.

RL95-2 cells were treated with IL-1RA at various concentrations (0, 5, 10, 20 and 40 ng/ml) for 4 h. The effect of IL-1RA was then investigated on the trophoblast adhesion to endometrial cells (A). RL95-2 cells were then transfected by IL-1RA siRNA at a concentration of 1 µM for 72 h and the influence of IL-1RA knock down on trophoblast binding to RL95-2 cells was assessed (B). The experiments were performed in three replicates on three separate days. The data are the average of three independent experiments. The results were presented as the mean ± SEM. ANOVA was used to compare the percentage of attached JAr spheroids to endometrial cells in each group and Fischer's multiple comparison test was applied as the post-hoc test. Different letters denote significant differences. $P < 0.05$ was considered to be significant.

5.5. Discussion

In the previous chapter, one of the highlights of our investigation was the novel finding indicating the ability of TLR 3 activation in the FRT to impair implantation chances in endometrial cells (Montazeri et al., 2015). Following that our major focus has been dedicated to mechanistic approaches towards describing the molecular details of the observed effect. The results of the current study demonstrated that TLR 3-induced impairment of trophoblast adhesion to endometrial cells seems to be mediated at least in part by IL-1RA.

Different parameters are required to control the process of implantation, including hormones, cytokines, adhesion molecules and growth factors (Singh et al., 2011). In humans and primates, implantation is known to serve as an inflammatory type response. Different cytokines has been identified in the human endometrium, such as IL-1, whose production was conceived to regulate and control the functions of endometrial cells during the menstrual cycle (Tabibzadeh and Sun, 1992, Tabibzadeh, 1994). IL-1 is a key regulator of the inflammatory response and plays a crucial role in implantation (Bankers-Fulbright et al., 1996). In accordance with the assumption that the IL-1 system plays crucial role in implantation, it was shown that IL-1β was expressed in human endometrium (Simon et al., 1993a, Tabibzadeh and Sun, 1992, Kauma et al., 1990) throughout the menstrual cycle and its staining progressively increased in the secretory phase in comparison to the proliferative phase. This rise was coordinated with the increase in the messenger levels of IL-1 receptor, where IL-1R tI mRNA levels

increased significantly in the luteal phase versus the follicular phase (Simon et al., 1993a, Simon et al., 1993b).

IL-1RA is also present in the human endometrial epithelial cells throughout the entire menstrual cycle (Tabibzadeh and Sun, 1992). IL-1RA's staining increases significantly during the follicular phase versus the early and mid-to-late luteal phase (Simon et al., 1995). Moreover, the IL-1 system was found in the human embryo (De los Santos et al., 1996), and at the maternal-embryonic interface (Simon et al., 1994). Embryonic IL-1 release occurred only when embryos were co-cultured with human endometrial epithelial cells (EECs) (Spandorfer et al.). It is noteworthy that IL-1 produced from human embryos can regulate and increase endometrial receptivity in EEC cells by increasing the expression of α1, α4 and β3 integrin (Simon et al., 1997). Moreover, successful implantation after *in vitro* fertilisation has been correlated to high concentrations of both IL-1α and IL-1β in the culture medium of human embryos (Karagouni et al., 1998). On the other hand, it is well-established that IL-1RA can suppress embryo implantation in mice, by decreasing endometrial receptivity (Simon et al., 1998). Accordingly, the assumption that IL-1RA serves as an anti-implantation factor led us to search for the possibility that TLR 3-induced impairment of trophoblast adhesion to endometrial cells could be mediated through IL-1RA. This prompted us to investigate whether TLR 3 activation in RL95-2 cells could alter the gene expression of IL-1RA.

Our results showed that the gene expression of IL-1RA was increased significantly even after 2 h of Poly I:C administration. These data were confirmed by IL-1RA ELISA in the protein level. This is consistent with the findings of Lee and colleagues, who showed that the production of IL-1RA was significantly increased in response to Poly I:C in microglial cells (Lee et al., 2007, Rabehi et al., 2001). In addition, activation of other TLR members such as TLR 4 by LPS has been shown to increase production of IL-1RA in monocytes (Rabehi et al., 2001, Rehani et al., 2009). Although these findings clearly document the stimulatory effect of TLR activation on IL-1RA production, little is known regarding the cellular mechanisms regulating the production of IL-1RA in response to TLR activation. Involvement of MAPK pathway in IL-1RA production has been shown in many studies, where the blocking of p38 and ERK, as subunits of MAPKs, dramatically decreased the production of IL-1RA upon LPS stimulation (Rabehi et al., 2001). This is in agreement with the findings of Rehani et al., in which it was shown that ERK pathway activity is required to augment IL-1RA production upon TLR 4 activation in monocytes (Rehani et al., 2009). Moreover, it has been shown that 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). Thus, it seems reasonable that induced MAPK pathway activity upon TLR 3 stimulation can lead to observed increased levels of IL-1RA production in response to TLR 3 activation. Further investigations are needed to demonstrate this possibility.

We could observe that addition of IL-1RA to RL95-2 cells reduced the adhesion of trophoblast cells to endometrial cells in a dose-dependent manner. Moreover, the

fact that the inhibitory effect of Poly I:C on spheroid binding was restored consequent to knock down of IL-1RA was a reassuring step in concluding that IL-1RA definitely mediated the observed Poly I:C effect. This is consistent with the previous studies, where it was shown that IL-1RA injection in mice prevented embryonic implantation through direct effect on the transformation of plasma membrane of epithelial endometrial cells at the time of implantation (Simon et al., 1998). Moreover, the concept that IL-1RA could have inhibitory effect on embryo adhesion at the time of implantation is further supported by the findings of Simon and Frances (1995), in which they showed that IL-1RA staining significantly decreased towards the secretory phase, which corresponds to the "window of implantation" (Simon et al., 1995). Together with the experimental data presented here, one can speculate that IL-1RA can lead to inhibition of trophoblast cells' binding to endometrial cells *in vivo* and *in vitro*, but the mechanisms through which this alteration happens remain unclear.

Since IL-1 is such a potent inflammatory cytokine, it is critical that its biological effects be precisely controlled. In this regard, IL-1RA acts as a regulator of IL-1 biological effects and when it binds to IL-1R tI on the cell surface, it blocks the IL-1 signal transduction (Bankers-Fulbright et al., 1996). The balance of the IL-1/IL-1RA levels is crucial. Indeed, it is well documented that the relative levels of IL-1 and the endogenous IL-1RA correlate with the pathogenesis of many diseases and an excess amount of IL-1, for instance, can develop inflammatory and autoimmune diseases such as diabetes and rheumatoid arthritis (Bankers-Fulbright et al., 1996, Arend, 2002). In addition, increased levels of IL-1RA have been found in the

circulation of patients with a variety of inflammatory, infectious, and post-surgical conditions (Arend et al., 1998). This indicates the importance of hepatic production of IL-1RA as an acute phase protein, which diffuses into the tissues and influences the local ratio of IL-1RA to IL-1 (Gabay et al., 1997). Accordingly, treatment of these diseases has been carried out by injection of recombinant IL-1RA protein or using gene therapy approaches (Arend, 2002). An appropriate ratio of IL-1 to IL-1RA is also pivotal to initiate and maintain successful implantation at the local fetal-maternal interface (Huang et al., 2001), and as shown in mice and the current study, increased levels of IL-1RA can interfere with the process of trophoblast cells binding to endometrial cells and may result in implantation failure. This can be due to the fact that in the presence of excess amount of IL-1RA, the stimulatory effect of IL-1β on endometrial receptivity (Simon et al., 1997) is blocked and as a result of that, the adhesion of embryo to endometrial cells is impaired. In the same line of evidence, the fact that IL-1RA expression significantly decreased in the secretory phase (Simon et al., 1995), when embryo implantation is taking place suggests the existence of specific inhibition of IL-1RA production at the time of implantation, which facilitates IL-1 pre-implantation actions on endometrial receptivity. The window of implantation is when the endometrium changes for the arrival of the embryo. For this reason, a safe environment should be guaranteed. If the innate immune system is activated at this time, the uterine tissue is able to respond actively. On one hand, the response will defend the maternal tract from a potential infection, but on the other hand this defence strategy might have the result of adversely affecting implantation of the embryo.

Many factors produced by the endometrium during the window of implantation have been proposed as molecular markers of endometrial receptivity, such as LIF and mucin 1 (Sharkey and Smith, 2003), but little attention has been paid to their application in the treatment of infertility. In the current study the discovery that increased levels of IL-1RA upon TLR 3 activation directly impair trophoblast adhesion to endometrial cells could be used as a significant diagnostic and therapeutic tool for the treatment of virus-induced infertility cases.

To conclude, our hypothesis that IL-1RA mediates TLR 3-induced impairment of trophoblast cells' adhesion to endometrial cells *in vitro* was supported. This is a novel discovery which extends current knowledge concerning diagnostic approaches towards underlying reasons for infertility cases. The findings of this study have a potential clinical application in introducing IL-1RA as one of the diagnostic infertility markers in the endometrium, which can affect the process of embryo adhesion at the time of implantation.

Chapter 6. General discussion

6.1. Summarising discussion

In recent years, research has focused increasingly on the role of endometrium as a determinant of reproductive outcomes, and understanding endometrial receptivity has become one of the most important challenges in the field of reproductive medicine (Murray et al., 2004). Nevertheless, endometrial biology and the factors involved in regulating endometrial receptivity are still a matter of debate. An interesting approach to assess the functionality of genes involved in human endometrial receptivity is to study the endometrial behaviour in refractory conditions leading to implantation failure. In the current investigation, by using *in vitro* models of embryo implantation, we were able to describe mechanisms of endometrial behaviour in response to TLR 3 activation that can interfere with embryo implantation. From a molecular perspective we observed novel cellular responses to TLR 3 stimulation, such as alteration in cell cytoskeleton and expression of adhesion molecules. The aim of this final chapter is to summarise the major findings of this thesis, consider their potential implications and propose further ideas for future investigations.

6.1.1. Thesis findings

The main aim pursued in the development of the current work was to identify whether endometrial innate immune responses are able to interfere with embryo implantation. To achieve this aim, a different number of approaches were taken. These included *in vitro* implantation models, gene expression, characterization of

cell cytoskeletal arrangement and adhesion molecule expression by flow cytometry, cellular transfection, light and fluorescent microscopy and even confocal microscopy.

Due to ethical and practical reasons, it is nearly impossible to study the human implantation process *in vivo* and experimentation with embryos is legally restricted (Hannan et al., 2010). To tackle these limitations, researchers in the reproductive area have followed two particular strategies: *in vitro* implantation models (the use of trophoblast and endometrial cell lines) and *in vivo* implantation models (the use of animal models) (Hannan et al., 2010).

In order to understand the molecular and cellular events involved in human implantation, we established and optimised an *in vitro* implantation model. In this model, the endometrium was simulated using the RL95-2 cell line. The expression of numerous adhesion molecules, microfilaments in the apical surface and estrogen and progesterone receptors makes them a suitable model to investigate early stages of implantation (Way et al., 1983).

As previously described, embryo implantation consists of three stages known as apposition, adhesion and invasion of the trophoblast cells to the endometrium (Johnson and Everitt, 2007). In the current study, we aimed to investigate the effect of TLR 3 activation on embryo implantation and as each implantation stage has different characteristics, we investigated this effect at both early (apposition-adhesion) and late (adhesion-invasion) stages of implantation. To address this, we

used two different trophoblast cell lines, AC-1M88 and JAr. AC-1M88 is derived from earlier stages of pregnancy and used widely in trophoblast adhesion assays (Hannan et al., 2006, Hannan and Salamonsen, 2008, Paiva et al., 2007). On the other hand, JAr cell line is derived from term trimester trophoblast cells and is regarded as an invasive trophoblast cell line (Pattillo et al., 1971). Both AC-1M88 and JAr cell lines were applied to mimic the embryo by forming multi-cellular spheroid-shaped cell clusters and were delivered onto the monolayers of RL95-2 cells to simulate the early (apposition-adhesion) and late (adhesion-invasion) stages of embryo implantation respectively.

The early phase of the study, described in Chapter 2, was dedicated to characterization of the role of TLR 3 activation in the embryo implantation *in vitro*. This included identifying the effect of TLR 3 stimulation on the binding of trophoblast cells to endometrial cells at both the adhesion and invasion phases of implantation. We observed that the activation of TLR 3 reduced the percentage of adhering AC-1M88 and JAr spheroids. This was the first study to show that activation of TLR 3 in the human endometrial cells can decrease implantation chances at both stages of implantation.

When faced with such an interesting observation, our major focus was dedicated to investigating the pathways involved in TLR 3-induced impairment of trophoblast binding. The involvement of TRIF pathway in TLR 3 signalling has been reported in many studies, in which TLR 3 signalling was shown to follow the TRIF-dependent pathway (Akira and Takeda, 2004, Carty et al., 2006).

With the last statements in mind, the question to answer was: Does the TRIF pathway mediate TLR 3-induced inhibition of trophoblast adhesion to the endometrial cells *in vitro*? To answer this question, specific inhibitor (Pepinh-TRIF) was applied to block the TRIF signalling pathway. However, our findings revealed that the suppression of the TRIF pathway with Pepinh-TRIF did not restore the binding of trophoblast cells upon TLR 3 stimulation, making it unlikely to be the pathway involved in this process. Hence, we sought to determine the role of other pathways such as myeloid differentiation primary response 88 (MYD88) in mediating this effect. In our findings, the fact that inhibition of the MYD88 pathway recovered the adhesion rate of trophoblast binding was intriguing as this result was different from many published data.

As an approach to provide insight into the mechanisms via which TLR 3-induced impairment of trophoblast adhesion occurs, the experiments described in the rest of the chapters were designed (Chapters 3, 4 and 5).

IL-1RA is known to negatively affect endometrial receptivity by having a direct effect on the expression of adhesion molecules such as α4, α5 and β3 integrin (Simon et al., 1998). This, in conjunction with supporting evidence that the expression of IL-1RA in the human endometrium was higher at the proliferative phase compared to the other phases of the menstrual cycle (Simon et al., 1995), was suggestive of IL-1RA's anti-implantation properties at the time of implantation. This speculation directed us to the next level of investigations, which established the ground for the forthcoming mechanistic approaches. Accordingly,

the experimental data presented in Chapter 3 were dedicated to clarifying whether the observed effect of TLR 3 stimulation on trophoblast attachment to endometrial cells was directly mediated by IL-1RA.

In our experiments, elevated levels of IL-1RA in RL95-2 cells were detected by ELISA after exposure to Poly I:C (Chapter 3), suggesting its possible role in TLR 3-induced implantation failure. The direct involvement of IL-1RA in trophoblast adhesion was assessed by means of two experiments: 1) Addition of recombinant human IL-1RA to RL95-2 cells 2) Knocking down the expression of IL-1RA in RL95-2 cells and measuring the percentage of attached JAr spheroids to the endometrial cells in the presence of Poly I:C. The fact that suppression of IL-1RA's production recovered the TLR 3-induced inhibition of trophoblast binding, confirmed the inhibitory effect of IL-1RA on spheroid adhesion to the endometrial cells.

Experimental data presented in Chapter 4 were dedicated to discovering the intracellular pathways which mediated Poly I:C effects on the reduction of implantation rates and examined whether the observed decrease in the adhesion of trophoblast to the endometrial cells might be the result of the classical transcription factors, nuclear factor (NF-κB) or Mitogen-activated protein kinases (MAPK) signalling activity. A significant rise in MAPK activity after Poly I:C treatment indicated the possible involvement of MAPK pathway in TLR 3-mediated inhibition of trophoblast adhesion. To directly assess whether MAPK and NF-κB mediated the observed effect of Poly I:C on the attachment of trophoblast cells, inhibitors were applied for each of these pathways. After validation of the

functionality of the inhibitors and application of functional dose of them, the obtained results clarified that inhibition of MAPK pathway significantly restored the decrease in the adhesion rate of trophoblast to the endometrial cells. This confirmed the inhibitory role of MAPK in the attachment of trophoblast cells to the endometrial cells upon TLR 3 activation.

Among various molecules involved in successful embryo implantation, F-actin was of particular interest to the current research, as it plays a crucial role in cell cytoskeleton rearrangement and adhesion of trophoblast cells to the endometrium at the time of implantation (Heneweer et al., 2002, Thie et al., 1997). Moreover, pinopode formation, as one of the most remarkable morphological determinants of plasma membrane transformation, seems to rely on F-actin reorganization (Luxford and Murphy, 1992). Hence, disruption of F-actin content under Poly I:C effect seemed a highly likely scenario of the mechanism of action of TLR 3-induced implantation failure in our investigation. A new investigation to determine the effect that TLR 3 stimulation may have on cell cytoskeleton arrangement and F-actin content was established. This allowed us to pursue the next set of experiments described in Chapter 5. Examination of actin polymerization, using Phalloidin, revealed a considerable decrease in F-actin content in response to TLR 3 activation in RL95-2 cells. This presented a potential mechanism for TLR 3-induced impairment of trophoblast adhesion to endometrial cells.

Considering the role of CD98 and β3 integrin in increasing endometrial receptivity led to a new series of experiments (described in Chapter 5) to explore the effect of TLR 3 stimulation on the expression of CD98 and β3 integrin in endometrial cells. The fact that TLR 3 activation significantly suppressed the expression of CD98 and β3 integrin revealed another possible mechanism of action of TLR 3-induced inhibition of trophoblast adhesion.

Having conclusively established that TLR 3 activation decreased F-actin content and expression of CD98 and β3 integrin in endometrial cells, our attention was directed to identifying the morphological changes of F-actin organization and CD98 and β3 integrin expression upon TLR 3 stimulation. We chose to use confocal microscopy to precisely localize and detect these morphological alterations. Confocal images revealed a significant decrease in F-actin content and CD98 expression in endometrial cells in response to TLR 3 activation. This further supported the data obtained from flow cytometry experiments, where TLR 3 activation significantly decreased and disrupted actin polymerization and the expression of CD98 in endometrial cells. β3 integrin staining was however too faint to be assessed by confocal microscopy, as reported earlier (Dominguez et al., 2010).

To further understand via which pathways TLR 3 activation altered cell cytoskeleton and expression of adhesion molecules, inhibitors were applied for the adaptor molecules involved in the TLR 3 signalling pathway, including TRIF, MYD88, NF-κB and MAPK. Our results demonstrated that MYD88/MAPK pathway

mediated the disruption in F-actin content and adhesion molecule expression upon TLR 3 stimulation (Fig. 6.1).

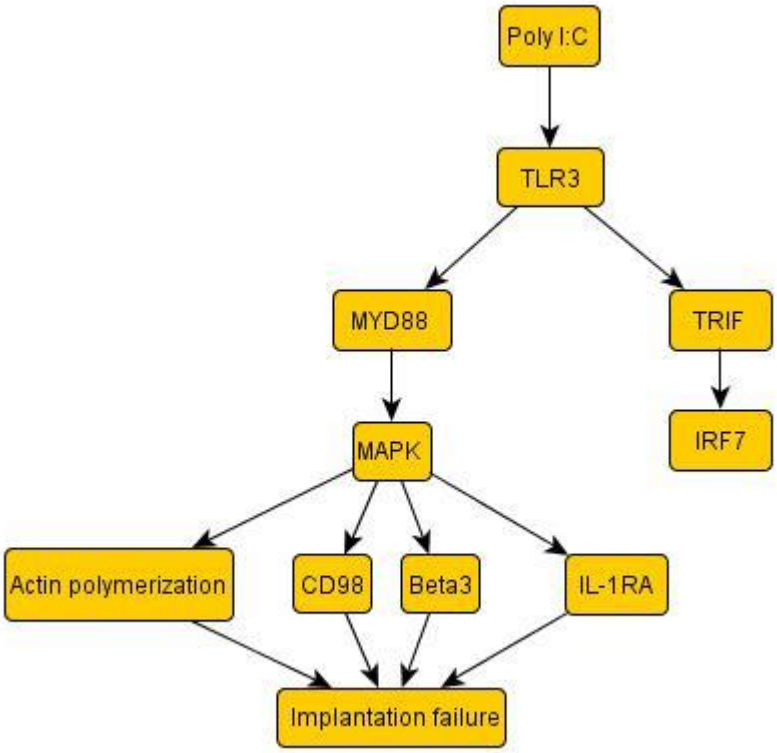

Figure 6.1 A schematic of TLR 3 signalling pathway in RL95-2 cells.

6.2. Implication of the major thesis findings

6.2.1. Inflammatory responses of the endometrium to inflammatory stimuli and the effect on embryo implantation

Applying our *in vitro* implantation model, we were able to show how activation of TLR 3 in the endometrium could negatively affect the process of trophoblast cells binding to the endometrial cells. This reduction was irrespective of using trophoblast cells (AC-1M88 or JAr) representing early (apposition-adhesion) and late (adhesion-invasion) stages of embryo implantation respectively, suggesting that activation of TLR 3 at both early and late stages of implantation may be detrimental and leading to implantation failure.

In contrast to many published data that TLR 3 signals via the TRIF pathway, we showed that TLR 3-mediated inhibition of trophoblast binding signals via the MYD88 pathway. This can be explained as the differential activation of pathways depending on the type of cell line applied.

Our findings raise the possibility of clinical significance of screening for viral infections among pre-pregnant women to identify a high-risk population for virus-induced implantation failure.

This will improve not only the embryo implantation rate in ART techniques but also will deepen our understanding of several factors involved in endometrial receptivity. In addition, the identification of MYD88 as a key pathway for TLR 3-induced defects in trophoblast adhesion to the endometrial cells will increase our knowledge on implantation biology and may enable novel clinical approaches to implantation-related infertility.

6.2.2. IL-1RA mediates the TLR 3-induced inhibition of trophoblast adhesion to the endometrial cells

The importance of cytokines such as IL-1β in the establishment and progression of successful embryo implantation has been reported in many studies (Simon et al., 1997, Karagouni et al., 1998). Moreover, the role of IL-1R antagonist as an anti-implantation cytokine has previously been suggested (Simon et al., 1998). In our findings, the fact that the knock down of IL-1RA in endometrial cells could recover the adhesion of trophoblast cells in the presence of Poly I:C confirms the direct involvement of IL-1RA in TLR 3-induced reduction of trophoblast adhesion to the endometrial cells.

It is possible that introducing IL-1RA as a potential prognostic marker of implantation failure and measuring IL-1RA levels in the blood samples or endometrial biopsies could bring benefits to infertile women who are currently

under IVF treatment and provides deeper insights into the role it has on embryo implantation.

6.2.3. Activation of classical pathways of TLR 3 is involved in the impairment of trophoblast adhesion

The signalling pathways of TLRs in most cases terminates in the activation of transcription factors NF-κB or AP-1. Depending on the type of cell line or stimuli, TLRs might signal through different pathways. In our observations, NF-κB was activated by TLR 5 and AP-1 by TLR 3. This could be due to the differential activation of signalling pathways depending on the stimuli employed. In addition, the inhibition of AP-1 pathway restored the adhesion rate of trophoblast cells in the presence of Poly I:C, indicating the inhibitory role of AP-1 pathway in the binding of trophoblast cells to the endometrial cells.

The identification of MAPK as a key pathway for TLR 3-mediated defects in trophoblast adhesion to the endometrial cells provides a new insight into the diagnosis of virus-induced implantation defects, as well as therapeutic approaches in treatment of infertility cases. Moreover, blocking of the MAPK pathway by its inhibitor may make several noteworthy contributions to treating infertility.

6.2.4. The cytoskeleton organization of endometrial cells is disrupted upon TLR 3 activation

The highly dynamic nature of the human endometrium is well-documented, as it undergoes a complex series of organized proliferative and secretory changes in each menstrual cycle (Murphy, 2000b). These changes include the flattening of the microvilli, thinning of the Muc-1 layer and formation of pinopodes at the late secretory phase. These alterations favour implantation of the embryo in the endometrium and have been applied as markers of endometrial receptivity (Nikas et al., 1995, Murphy and Dwarte, 1987). As shown in Chapter 5, the novel finding that TLR 3 activation altered cell cytoskeletal arrangement (F-actin) indicated that endometrial epithelial cells may undergo a possible defence mechanism in response to viral infection, which is a characteristic of the cells belonging to the immune system. In fact upon detection of PAMPs by TLRs, dendritic cells undergo a series of cell biological changes including F-actin reorganizations. This enables them to initiate defence mechanisms such as phagocytosis, antigen capture, processing, and presentation against the pathogens (Trombetta and Mellman, 2005, Watts et al., 2007). It is possible that the endometrial epithelial cells follow the same defence mechanisms to recognize and respond to the viruses invading the female reproductive tract. However, this will alter the cell morphology and endometrial receptivity required for successful implantation.

This novel observation could be used as a potential diagnostic marker in IVF protocol in patients suffering from sub-clinical viral infections and subsequently implantation-based infertility.

6.2.5. The endometrial adhesion molecule profile might be altered as a result of TLR 3 activation

As shown by our observations in Chapter 5, the expression of adhesion molecules such as β3 integrin and CD98 was altered upon TLR 3 stimulation. Molecules like β3 integrin integrin (Kuno et al., 1998) and CD98 (Dominguez et al., 2010) are thought to be the best characterized adhesion molecules of endometrial receptivity as their expression increases only at the window of implantation. Moreover, β3 integrin (Kaneko et al., 2011, Illera et al., 2000) and CD98's (Dominguez et al., 2010) blockade in the endometrium has led to failure of embryo implantation, indicating their crucial role in embryo implantation. Despite all these evidences that clearly confirm the importance of β3 integrin and CD98 in the process of embryo implantation, their value as a prognostic indicator of successful implantation remains uncertain (Gonzalez et al., 1999, Lessey et al., 2000, Creus et al., 1998). The novel discovery that TLR 3 activation decreases the expression of β3 integrin and CD98 could be regarded as another potential mechanism of action of TLR 3-induced impairment of trophoblast adhesion.

In addition to providing new insights into the complex process of endometrial receptivity, implementation of CD98 and β3 integrin as specific markers of receptive endometrium and measuring their expression levels in the endometrial biopsies could be a useful tool to predict success in an IVF cycle and promote the targeted drug treatment of implantation-based infertility.

6.2.6. Summary of achievements

The *in vitro* model of embryo implantation presented in Chapter 2 highlighted the importance of a sterile environment in the endometrium at the time of implantation. If this requirement is not met, in this case stimulated by TLR 3, detrimental effects are observed in the endometrium that will ultimately suppress the implantation of the embryo. The following experimental chapters were designed to decipher the mechanisms through which TLR 3 activation reduced the adhesion rate of trophoblast cells to the endometrial cells. In Chapter 3, the cytokine profile of the endometrium was assessed with the expression of IL-1RA, known as an anti-implantation factor. Stimulation of TLR 3 was able to enhance the expression of IL-1RA in epithelial endometrial cells. Moreover, the suppression of IL-1RA's functionality in the endometrial cells restored the TLR 3-mediated defect in the binding of trophoblast cells. In Chapter 4, we described how the activation of the endometrial TLRs follow a differential signalling pathway depending on the ligand applied. In addition, the application of MAPK pathway inhibitor was able to recover the TLR 3-induced impairment of trophoblast attachment. Finally, the flow cytometry experiments in Chapter 5 gave a

morphological perspective of the endometrial cells' reaction towards TLR 3 stimulation. This TLR activation is able to influence the cell cytoskeleton arrangement and F-actin content. Continuing the experiments in Chapter 5, the expression of some of the adhesion molecules was assessed by flow cytometry. We were able to detect that TLR 3 stimulation could alter the expression of β3 integrin and CD98. These molecules are an important determinant of endometrial receptivity and therefore play a vital role in implantation of the embryo.

Taken together, these findings indicate the significant immune regulatory role that the endometrial tissue must exert at the time of implantation. TLRs have diverse roles over endometrial biology, but most of them point towards the necessity for the endometrial cells to undergo morphological alteration in order to initiate defence mechanisms against the invading microorganisms. It might be possible that upon encountering an infection, the endometrial cells would undergo defence mechanisms that would alter the receptive status of the endometrium and subsequently prevent implantation of the embryo.

6.3. Directions for future study

6.3.1. Three-dimensional implantation model

Despite the determination of the role of TLR 3 stimulation in the disruption of trophoblast attachment to the epithelial endometrial cells in Chapter 2, the question still remains as to whether these changes could also be observed in

endometrial cells in interaction with stromal cells. In our model, only endometrial epithelial cells were applied, whereas in the *in vivo* environment the interaction of both epithelial and stromal layers is required.

The endometrial receptivity at the window of implantation highly depends on paracrine signals secreted from endometrial stromal cells (ESCs) underlying the luminal epithelium (Achache and Revel, 2006). In fact, the ESCs play an important role in endometrial functions such as tissue remodelling, endometrial regeneration, decidualization, embryo implantation and trophoblast invasion. ESCs also play a role as gatekeepers of different immune cells at the implantation site as well as triggering the expression of key receptivity genes at the embryo-maternal interface (Weimar et al., 2013).

One of the main findings that I would like to follow up in future research is to further characterize the effect of TLR 3 activation on trophoblast binding, using a three-dimensional (3D) implantation model, in which both EECs and ESCs are cultured. In this regard, an *in vitro* 3D implantation model has previously been established, where both endometrial epithelial cell line (HEC-1A and Ishikawa) and stromal cell line (HESCs) were cultured (Wang et al., 2012). In addition, to simulate the embryo-maternal interaction *in vivo*, an assay was developed to examine the adhesion of trophoblast cell line (JArs) to the 3D cell culture.

In practice, the application of a 3D implantation model will allow us to further mimic the *in vivo* environment of the endometrium, where the interaction of

stromal cells with the epithelium is crucial for successful implantation. In addition, development of 3D cell culture to study the effect of TLR 3 stimulation on trophoblast adhesion will enable us to better define the interactions existing between the molecules involved in virus-induced implantation failure.

6.3.2. Evaluation of IL-1RA levels in human blood samples

As mentioned before, an appropriate ratio of IL-1/IL-1RA is crucial for the maintenance of immune function (Bankers-Fulbright et al., 1996, Arend, 2002), as well as successful implantation at the embryo-maternal interface (Huang et al., 2001). Different mechanisms have been proposed to maintain a balance in the relative levels of IL-1 and IL-1RA, including hepatic production of IL-1RA and regulation of the expression of IL-1 receptors. In fact, IL-1RA is known as an acute phase protein, as it has been found at increased levels in the circulation of patients with a variety of inflammatory, infectious, and post-surgical conditions (Arend et al., 1998). This indicates the importance of hepatic production of IL-1RA as an acute phase protein, which diffuses into the tissues and influences the local ratio of IL-1RA to IL-1 (Gabay et al., 1997). Accordingly, treatment of some auto-immune diseases has been carried out by injection of recombinant IL-1RA protein or using gene therapy approaches (Arend, 2002).

In Chapter 3 of this thesis, a finding of enhanced levels of IL-1RA in response to TLR 3 stimulation was reported. In addition, the direct involvement of IL-1RA in the TLR 3-induced inhibition of trophoblast cells binding to the endometrial cells was shown. However, these data were obtained from *in vitro* models, which are not the representative of the *in vivo* situation.

An interesting approach to corroborate our findings obtained from *in vitro* work could be to measure the serum levels of IL-1RA in patients suffering from implantation-based infertility. This would provide deeper insight into the application of IL-1RA as a new potential prognostic marker of infertility and may benefit the human implantation field in the development of infertility treatments. This includes targeted therapies, which could be introduced to enhance the implantation process and potentially minimize implantation disorders.

6.3.3. Investigation of the effect of TLR 3 stimulation on embryo implantation in mice model and TLR 3 knock out mice

A major obstacle for studying human embryo implantation is that it cannot be studied *in vivo,* due to anatomical and the physiological limitations. Moreover, the embryos obtained from IVF can only be assessed indirectly, foremost on morphological criteria, and over a legally restricted period (Hannan et al., 2010). To overcome these hurdles, research on embryo implantation has opted to

employ different *in vitro* implantation models and *in vivo* animal models. In our investigations, we were able to show that the activation of endometrial TLR 3 reduced the adhesion rate of trophoblast cells. However, as these observations were conducted only *in vitro*, further experiments using *in vivo* models are essential to confirm and corroborate this idea.

The process of embryo implantation in non-human primates is known to show high fidelity to human implantation. However, the manipulation of these species is restricted due to ethical responsibility. The similarity of murine implantation characteristics to human implantation, such as invasiveness, makes the murine model a suitable animal model to study the process of human embryo implantation. It also offers advantages such as the possibility to manipulate the expression of genes in genetically engineered mice (Dey et al., 2004) and to induce delayed implantation, pseudopregnancy and artificial induction of decidualization (McLaren, 1969, Finn and Martin, 1972, Cooke et al., 1986). These properties have assisted researchers in the reproductive field to discover many uterine implantation and pre-implantation events as well as implantation disorders.

Further investigation of characterizing the role of TLR 3 activation on embryo implantation in mice will enable us to establish and further confirm the findings obtained from our *in vitro* observations. Moreover, application of mice model will give insights about the *in vivo* innate immune system and its effect on murine embryo implantation.

Another important point that must be taken into account is that due to the intracellular pattern of TLR 3 expression, we were not able to use any antibody to directly suppress the functionality of TLR 3 (Chapter 2). Hence, we had to apply inhibitors of the TLR 3 signalling pathway like TRIF inhibitor, which is not specific enough to block TLR 3 functionality. One of the approaches to assess the direct involvement of TLR 3 in implantation failure is to apply TLR 3 knock out mice (TLR 3KO). In a study carried out by Koga and colleagues, TLR 3 knockout mice (TLR 3KO) and wild type mice were applied and despite treating both mice groups with Poly I:C, pre-term labour was only induced in wild type mice within 24 h of Poly I:C treatment and not in TLR 3KO mice (Koga et al., 2009b). However, it is important to keep in mind that in this study only pre-term labour was assessed. The same approach can be used by injecting Poly I:C to the sites of fertilisation (uterine horns) in both wild type and TLR 3KO mice and assessing and comparing the implantation sites in the two mice varieties.

Harnessing the TLR 3 knock out mice is a challenging but worthwhile endeavour as it may not only enable us to directly assess the involvement of TLR 3 in implantation failure, but also to extrapolate our *in vitro* observation to the *in vivo* situation.

6.3.4. Measurement of the gene expression pattern of the endometrium in refractory conditions

For more than 50 years, histological evaluation of the endometrium, known as Noyes criteria, has been the gold standard for clinical diagnosis of endometrial abnormalities leading to implantation failure and female infertility (Noyes et al., 1975). In this method, different phases of the menstrual cycle were distinguished on the basis of morphological observations and histological features. However, in recent years, accuracy of the Noyes criteria as predictors of endometrial receptivity and diagnosis of endometrial abnormalities has been questioned and they have failed to guide clinical management of women with implantation failure (Coutifaris et al., 2004, Murray et al., 2004). The research in reproductive area, therefore, was directed towards deciphering the functional, rather than the morphological, characteristics of endometrial receptivity. In this regard, the discovery of markers of endometrial receptivity was a breakthrough in reproductive medicine, which assisted researchers in the development of specific diagnostic measures in infertility cases (Sharkey and Smith, 2003). However, none of the markers was shown to be efficient in increasing implantation or pregnancy rates (Horcajadas et al., 2007).

An interesting approach to assess the functionality of genes involved in human endometrial receptivity is to measure the gene expression pattern of the

endometrium in refractory conditions induced either pharmacologically or mechanically. It has been demonstrated that the intrauterine device (IUD) prevents, at a molecular level, the normal transition to a receptive genomic status and can be used for identifying the genes that are responsible for the refractory status of the human endometrium (Horcajadas et al., 2007). Using microarray analysis, the gene expression profile of receptive versus refractory endometrium could be analysed in the same patient in the presence of an inert IUD.

The knowledge acquired from this line of research will surely generate valuable information about the physiology of the receptive endometrium and provides a number of potential targets for the development of IVF techniques.

6.4. Conclusion

Successful embryo implantation is the result of a well-orchestrated sequence of events including a receptive endometrium, a normal and functional embryo and a synchronized dialogue between maternal and embryonic tissues (Simon et al., 2000). Embryo implantation represents the most critical step of the reproductive process in many species and external factors can easily interfere with this interaction and lead to failure. Evidences from this work indicate that embryo implantation can be affected *in vitro* by TLR 3 stimulation, which generates morphological and protein profile changes that will lead to implantation failure. At the molecular level, the endometrial cells respond to TLR 3 activation in a diversity of ways, from changing cell cytoskeleton to altering adhesion molecule expression. Potentially these changes will favour the protection of the maternal tissue from infectious agents but will compromise the implantation of an embryo. The knowledge acquired from this work could improve the ability of clinicians in the development of specific therapeutic tools that will optimise embryo implantation in ART techniques.

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