The activation of TLR 2 and 2/6 in the endometrium is detrimental for embryo implantation

By

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A thesis submitted for the degree of Doctor of Philosophy from the University of Sheffield

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June 2014

Academic Unit of Reproductive and Developmental Medicine

Abstract

The implantation of the embryo is a complex interaction between the maternal endometrium and the blastocyst. During the late secretory phase the endometrium will undergo phenotypical and morphological changes to guarantee this interaction and the continuation of pregnancy. The uterine cavity must provide a sterile environment for the implantation and embryo development. Hence, a tight regulation of the innate immune system is necessary so as to increase its effector mechanisms during the secretory phase. The toll-like (TLR) receptor family is a family of innate immune receptors in charge of recognising the presence of potential pathogens and alert the body to initiate the defence mechanisms. The innate immune system and specifically TLRs might play a very important role in the implantation and establishment of pregnancy.

In this thesis, we hypothesised that the activation of the endometrial TLR 2 and 2/6 will affect the capacity of the endometrium to receive an implanting embryo. We employed different approaches to study endometrial and implantation biology ranging from *in vivo* murine models to *in vitro* human endometrial-trophoblast interaction models. The results from our studies suggest that the activation of endometrial TLRs *in vivo* drastically reduces embryo implantation and *in vitro* is able to decrease trophoblast adhesion to the endometrial cells possibly by modifying the endometrial cell membrane morphology. It seems the endometrium is able to react against potential infectious agents and guarantee the protection of the mother but potentially compromising the chance of an embryo to implant.

Publications

Sánchez-López, J.A., Caballero, I., Montazeri, M., Maslehat, N., Elliott, S., Fernandez-Gonzalez, R., Calle, A., Gutierrez-Adan, A., and Fazeli, A. (2014). Local Activation of Uterine Toll-Like Receptor 2 and 2/6 Decreases Embryo Implantation and Affects Uterine Receptivity in Mice. *Biology of reproduction*, 90(4):87-87.

Manuscript in preparation:
The activation of epithelial endometrial cells Toll-like receptors is able to induce membrane microstructural modifications and alter the protein profile.

Abstracts and Presentations

Sanchez-Lopez JA, Aboussahoud W, Moezin-Vaziri N, Adalmarhi A, Almiñana-Brines C, Hobbs J and Fazeli A. A Systems biology approach to study the relationship between the implantation of the blastocyst and the uterine toll like receptors. Research Training School GEMINI COST Action FA0702, Systems Biology in Maternal Interactions with Gametes and Embryos, 9-13 May 2010, Opatija, Croatia, 2010 (Poster Presentation).

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Sanchez-Lopez JA, Montazeri M, Maslehat N, Caballero-Posadas I, Elliott S, Hobbs J, Fazeli A. Nano-scale membrane alterations are induced by the activation of TLR 2 and 2/6 in endometrial epithelial cells. 2nd Symposium of the Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, 23 November 2012, Sheffield, United Kingdom, 2012 (Poster Presentation, First Prize).

Sanchez-Lopez, J.A., Caballero, I., Montazeri, M., Maslehat, N., Elliott, S., Fernandez-Gonzalez, R., Calle, A., Gutierrez-Adan, A., Fazeli, A. (2014). Activation of Uterine innate immune system affects embryo implantation and endometrial receptivity. 11th Symposium of Mexican Students and Studies in the United Kingdom, 11 of July 2013 (Oral Presentation).

Sanchez-Lopez JA, Fazeli A. Activation of uterine innate immune system affects embryo implantation and endometrial receptivity. 3rd Symposium of the Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, 23 November 2013, Sheffield, United Kingdom, 2013 (Oral presentation).

Acknowledgements

Writing a PhD thesis is not an easy task. But I guess you know that you have finished it when you have to write the acknowledgements. I tried and got paralysed. Why? Because this means I reached the end. The end of a 4-year trip to the core of science and human life. A trip in which I have grown, matured and developed fundamental personal and scientific skills. I could not have done it on my own, and here is where I want to thank a number of people that walked with me, guided me and sometimes carried me along the way. As you are all as important to me, I will try to mention you in the order we crossed paths.

Going back almost 30 years, I want to thank my mother, Mónica and my father Arturo for starting this experiment. What a good set of genes you gave me! Family has always been an essential pillar for my life. I know I can always turn around and find you there for me. Andrea and Anaís, you are my infinite source of inspiration. Pedro, for giving me the logic to think. Gabriel, Daniel and Ángel my force to keep going. My Nan, uncles and aunts, all for your support and cheering me up and for reminding me how warm, sunny it is back at home and the good food you eat at the parties. My cousins, Soni, I'll be always looking up to you; young ones for your love and having me as an example – I am proud of you. Keep doing what you like to.

My path in science could have never happened without Armando teaching me who I am and who can I be. Lulú thanks for your showing me that "to become a scientist you need to develop tolerance to frustration". Specially, thank to both for introducing me to the reproductive world that brought my path to Alireza and Sheffield.

Alireza. It was a long ride. As in a roller coaster, full of ups, downs and 360° turns. I want to thank you for riding it besides me, showing me all this opportunities, the encouragement but especially for your trust. Thanks for showing me how to spread the seeds. I am sure we will spend some good time collecting the fruits from this. The Jessop Wing Team. Sarah, my dear surrogate mother. For teaching me how to speak Yorkshire and giving me your care and support. The whole Fazeli's group (even if they are not here anymore): Carmen, Nacho, Ahmed, Najmeh, Mehrnaz, Nasim,

Shaghayegh, Nurul, Tamer, Natalie and Laszlo for your company and showing me the diversity of the world. The whole AURDM: Gill, Katrina, Sarah, Mark, fellow lecturers and colleagues.

Thanks to Sheffield for showing me that no matter where we come from, we are all humans with the same feelings, thoughts and worries. To my first family: Claudia, Iwona, Blazej and Anika. Magdalena for your stories, your care and support. Thanks to the Mexican crew: Pueblita, Lore, Fer, Vero, Bianca, Javier, Martín, Osvaldo, Ramón and everyone for the familiar warmth we only know. Liz, thanks for your care, for showing me tolerance and to always look beyond the surface. Bert, for your complicity, in the music, on thoughts on life. Josh, for your care, for showing me how Mexican I am, getting me in touch with myself and for your immense wisdom. Daría, for always being there for me and sometimes keeping me alive. Scott for dada dadadada dadadaaaaa, and the scotch and good support and friendship in the last phase. Karen, for the inspiration and your example that there comes a time when comfort takes a second place. Tamer, for giving force to carry on and imagination to create. Jack, my little brother, for your energy, your support and care. Sofi, for your caring and loving when they were most needed, for your critical thinking and source of inspiration. Yael, for your wisdom and showing me to be patient.

Thanks to the people in Madrid. Alfonso for the opportunity of learning from you and your group. Sandra and Raúl for your guidance and help. All the kind people who took me and showed me the Spanish world: Ricardo, Pris, Ana, Miguel, Yosu, Angie. Special thanks to María for the new collaboration established, which form part of this thesis and Soraya for the help with the heavy load of work.

Thanks to the Euro-Uimiqs. Ale, Shiba and Cris. You my siblings in Europe for your good times, the creative thinking, the wine and the great support. Finally, thanks to my friends in México. You who would never give up on me. To Karla for our eternal friendship. Alfonso and Rodrigo, my brothers in arms. Cin and Alex for your care and support.

This PhD was partially funded by the Mexican government through a scholarship from CONACyT (Reg. No 211315, Scholarship 308175).

Grants and Awards

- COST GEMINI (Maternal communication with gametes and embryos) Short Term Scientific Mission (STSM). Madrid, Spain. October 2011, April-May 2012.

- COST EPICONCEPT STSM. Rehovot Israel. January 2014.

- CONACyT PhD Scholarship from the Mexican government 2010-2013. (Reg. No 211315, Scholarship 308175).

- First prize poster presentation. 2nd Symposium of the Academic Unit of Reproductive and Developmental Medicine, University of Sheffield, 23 November 2012, Sheffield, United Kingdom.

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Abbreviation list

ADAM – a disintegrin and metalloproteinase domain-containing protein

A_E – Area of the endometrium with lumen

AFM – Atomic force microscopy

A_L – Area of the lumen

AP-1 – Activating protein 1

A_S – Area of the endometrial stroma

A_T – Total area of the uterine horn

AFM – Atomic force microscopy

BCL – B-Cell chemo-attractant

CBA – Cytometric Bead Array

CD – Cluster of differentiation

CpG - Cytidine-phosphate-Guanosine repeats

CSF – Colony stimulating factor

Ct – Threshold cycle number

Ctrl – Control

DAMP – Damage associate molecular pattern

DC – Dendritic cell

DNA – Deoxyribonucleic acid

DPBS – Dulbecco's phosphate-buffered saline

ds – Double stranded

EBAF – Endometrial bleeding associated factor

eCG – Equine chorionic gonadotropin

ECM – Extracellular Matrix

EDTA – Ethylenediaminetetraacetatic acid

EEC – Endometrial epithelial cells

EGF – Epithelial growth factor

E_i – Endometrial index

ELISA – Enzyme linked immunosorbent assay

EMMPRIN – Extracellular matrix metalloproteinase inducer

ER – Estrogen receptor

ERK – Extracellular signal-reguated kinase

ESC – Endometrial stromal cells

FCS – Fetal calf serum

FITC – Fluorescein isothiocyanate

FRT – Female Reproductive Tract

FSH – Follicle-stimulating hormone

GCSF – Granulocyte colony stimulating factor

G_i – Gland index

GM-CSF – Granulocyte and Monocyte Colony stimulating factor

GnRH – Gonadotropin releasing hormone

hCG – Human chorionic gonadotropin

HAT – Heterodimeric amino acid transporter

HCV – Hepatitis C virus

HDMS - Hexamethyldisilazane

H&E – Haematoxylin Eosin

HMGB-1 – High mobility group box-1

HPG – Hypothalamic-pituitary-gonadal axis

HSP – Heat shock protein

IκB – Inhibitor of NF-κB

ICAM – Intercellular adhesion molecule

ICM – Inner cell mass

IETAC – Interferon-inducible T-cell alpha chemo-attractant

IFN-γ – Interferon gamma

Ig – Immunoglobulin

IGF – Insulin-like growth factors

IGFBP – IGF binding proteins

IKK – IκB kinase

IL – Interleukin

IL-1RA – IL-1 Receptor antagonist

INIA – Instituto Nacional de Investigación y Tecnología Agraria

i.p. – Intraperitoneal

IP-10 – Inflammatory protein-10

IRF – Interferon regulatory factor

IU – International units

JNK – c-jun terminal kinase

KC – Keratinocyte chemo-attractant

KO – Knock out

KSOM – K modified simplex optimised medium

LDL – Low-density lipoproteins

LH – Luteinising hormone

L_i – Luminal index

LIF – Leukaemia inhibitory factor

LPS – Lipopolysaccharide

LRR – Leucine rich repeats

LRT – Lower reproductive tract

LTA – Lipoteichoic acid

MALP-2 – Macrophage activated lipoprotein-2

MAPK – Mitogen-activated protein kinase

MCP-1 – Monocyte chemotactic protein-1

MCF-7 – Michigan Cancer Foundation-7

M-CSF – Monocyte colony stimulating factor

MHC-I – Major histocompatibility complex

M_i – Myometral index

MIF – Mean intensity of fluorescence

MIG – Monokine induced by gamma-interferon

MIP-1β – Macrophage inflammatory protein-1β

MMP – Matrix metalloproteinase

MRI – Magnetic resonance imaging

MyD88 – Myeloid differentiation primary gene 88

NK – Natural killer cell

NF-κB – Nuclear factor-κB

PAMP – Pathogen associated molecular pattern

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PE - Phycoerythrin

PGN – Peptidoglycan

PI – Propidium iodide

PID – Pelvic inflammatory disease

PIBF – Progesterone-induced blocking factor

PMSG – Pregnant mare's serum gonadotropin

Poly I:C - Poly inosinic-poly cytidylic acid

PRR – Pattern recognition receptor

P/S – Penicillin / Streptomycin

PSGL-1 – P-Selectin glycoprotein ligand-1

qPCR – Quantitative polymerase chain reaction

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

R_{RMS} – Root mean square roughness

RNA – Ribonucleic acid

SDF – Stromal cell derived factor

SEAP – Secreted placental alkaline phosphatase

sICAM – Soluble intercellular adhesion mollecule

SLPI – Secretory leukocyte protease inhibitor

SO – Superovulation

ss – Single stranded

STD – Sexually transmitted disease

STSM – Short term scientific mission

TARC – Thymus and activation regulated chemokine

TCA-3 – T cell activator-3

TER – Transepithelial resistance

TGF – Transforming growth factor

TIMP-1 – Tissue inhibitor of metalloproteinases

TIR – Toll/IL-1 receptor

TIRAP – TIR containing adapter-like MyD88

TLR – Toll-like receptor

TNF – Tumour necrosis factor

TR – Tandem repeat

TRAM – TRIF related adaptor molecule

TREM-1 – Triggering receptor expressed on myeloid cells -1

TRIF – TIR-related adaptor protein inducing interferon

UH – Uterine horn

uNK – Uterine Natural killer cell

URT – Upper reproductive tract

VCAM-1 – Vascular cell adhesion molecule -1

VEGF – Vascular endothelial growth factor

Chapter 1. Introduction

1.1 Overview

The incidence of infertility has increased in the last 50 years. Social and economic motors have limited the decisions of modern adults to have families. In rich countries the rate of fertility has fallen or is within the replacement rate, keeping a stable population. Adults are deciding to have families much later in life, as reaching stability will take longer than before. This means that at least for women, age related complications might interfere with their fertility. In addition to this, clinical factors in both women and men have also been found to increase. One of the main infertility mechanisms proposed is embryo implantation failure due to endometrial deficiencies or embryo abnormalities. The interaction of the maternal reproductive tract and embryo is a topic with a high interest for both reproductive medicine and developmental biology. Nevertheless, research in human implantation presents great challenges for the development of correct models for its study as well as moral and ethical implications. The establishment of *in vitro* human and *in vivo* animal models for studying implantation processes represents a fundamental aid in this respect. At a molecular level, implantation of the embryo is a complex process where a crosstalk of hormones, cytokines, chemokines and growth factors must be established between the mother and the embryo (Norwitz et al. 2001). Adhesion molecules in the blastocyst will interact with its ligands offered by a "receptive" endometrium to establish a firm adhesion. The female reproductive tract (FRT) should maintain an immune surveillance to guarantee a safe environment for the mother and embryo. At the same time, this surveillance should allow a semi-allogeneic embryo to implant and develop without rejecting it (Mor 2008).

The innate immune system is the first line of defence of the body against invasive pathogens such as bacteria, virus, fungi or parasites. It performs a surveillance role throughout the tissues to maintain the body homeostasis (Medzhitov & Janeway 2002). Almost all cells in the tissues of the body and the resident immune cells participate in this surveillance using the pattern recognition receptors (PRR). PRRs can sense pathogen associated molecular patterns (PAMP) that are shared between microorganisms (Beutler 2004). One family of PRR, the toll-like receptors (TLR) can sense a diverse range of PAMPs from bacteria like lipopolysaccharide (LPS) through TLR 4, peptidoglican by TLR 2-6 or 2-1, and flagellin by TLR 5 (Fitzgerald et al. 2001; Takeuchi et al. 1999). Viruses can be sensed by double stranded RNA and poly

inosinic-poly cytidylic acid (poly I:C) by TLR 3, single stranded RNA by TLR 7 and DNA with repeated cytidine-phosphate-guanosine (CpG) by TLR 9 (Barton 2007). The female reproductive tract (FRT) expresses these receptors differentially through the epithelia of its different parts. In this way TLR 1, 2, 3, 5 and 6 are present in the epithelia of most regions of the tract and TLR 4 is only found in the upper part of the tract the endocervix, endometrium and fallopian tubes (Fazeli et al. 2005). It has been found that the expression of TLR in the FRT is regulated by hormonal cycles, increasing it during the secretory phase of the menstrual cycle (Aflatoonian et al. 2007). It is possible that alterations in the immune homeostasis of the FRT can interfere with the receptivity of the endometrium and the implantation of the embryo (Sánchez López et al. 2014).

In the following chapters, I will present a short explanation and background to human embryonic implantation, followed by a description of mediation of innate immune system in the female reproductive tract. Then I will show our results for the effect of the stimulation of the innate immune TLR system at the time of implantation using an *in vivo* murine model. To extrapolate these observations to the human, we employed an *in vitro* model of adhesion of trophoblast spheroids to endometrial cells using human cell lines. Furthermore, using different approaches, I will try to decipher the mechanisms that TLR activation would have induced on the endometrial cells responsible for the interference with embryo implantation. These results include: a) Exploration of the nanometric changes on the membrane surface of the endometrial cells using atomic force microscopy. b) Profiling of the membrane proteins of the endometrial cells by flow cytometry. c) Assessment of the expression of genes related to mucosal immune responses by quantitative polymerase chain reaction (qPCR). d) Finally the signalling pathways of the endometrial TLRs and its influence on embryo implantation were assessed using reporter plasmids and signalling inhibitors. These findings will try to create a global picture on the role that activation of the innate immune system could have on the embryo implantation process. This research will hopefully open new perspectives on the study and diagnosis of infertility and biology of the endometrium and FRT.

1.2 The female reproductive tract

1.2.1 Anatomy and physiology of the tract

The female reproductive system is a compartmentalised tract, which performs a diversity of functions. The tract possesses the female gonads or ovaries that form part of the hormonal control axis of the body. The ovaries are the gamete reservoir that controls their maturation and release for its fertilisation in the fallopian tubes. The vagina receives the male reproductive organ during mating. The tract allocates the male gametes, the spermatozoa in the vagina and favours their transport through the cervix and uterus to the egg fertilisation site in the fallopian tubes. Finally, in the event of a fertilisation, the uterus will support the formation and development of the embryo and the future delivery of the child. For its study, we have divided the female reproductive tract in upper and lower reproductive tract (Fig. 1.1).

1.2.1.1 Lower reproductive tract

The lower tract (LRT) comprises the vagina and the ectocervix. The main particularity of this part is the presence of commensal microbiota that maintains the acidic conditions of the vagina.

The ***Vagina*** is a fibro muscular tube usually of 8 to 10 cm in depth and 4 cm in diameter. It is conformed by a muscular wall lined by a rugose epithelial surface of stratified squamous non-keratinised cells (Pernoll 2001). It is the section of the tract that will receive the penis during mating to collect the spermatozoa. It serves as the exit channel of the child during delivery. The pH of the vagina is around 4.5 and it is sustained by the lactic acid produced by the commensal microbiota which predominantly is formed by the Gramm positive genus *Lactobacillus* (Lidbeck & Nord 1993). The acidic conditions and production of H_2O_2 by the commensal microbiota will protect the tract from the colonisation by pathogenic bacteria (Witkin et al. 2007).

The ***Ectocervix*** is the external intravaginal portion of the cervix. It measures about 3 cm long and 2.5 cm wide with a convex and elliptical surface. Stratified squamous cells form its epithelium. The external opening of the cervix is called the “external Os” which will slightly open during the menstrual period to discard the endometrium. It is the gate

that controls the access of sperm and potential infectious microorganisms to the uterine cavity in the upper tract (Confino et al. 1987). The cervix possesses a great flexibility and will open almost 10 cm during delivery of the child.

1.2.1.2 Upper reproductive tract

The upper reproductive tract (URT) is formed by the endocervix, the uterus, the fallopian tubes and the ovaries. It is considered a microorganism-free tissue as it is the site where gametes maturate, fertilisation takes place and the development of the embryo happens. For this reason any microorganism found in this portion is considered to be pathogenic (Judlin & Thiebaugeorges 2009).

The ***Endocervix*** is the inner canal of the cervix, which connects the lower and upper portions of the tract. It is also called the "neck" of the uterus derived from its Latin original meaning. A lining of columnar ciliated secretory cells, a thin layer of smooth muscle cells and fibroblasts form the endocervix. The mucus produced by the epithelial and cryptic cells will vary due to hormonal stimulation. It thickens during the follicular phase to block the access to the URT and thins at the luteal phase to allow the sperm swim through it (Strauss et al. 2004). During pregnancy the muscular layer remains contracted to keep the fetus in place and relaxes during delivery. This is also due to the extracellular matrix (ECM) composition, where collagen fibres and elastin predominate giving the cervix a great flexibility (Westergren-Thorsson et al. 1998).

The ***Uterus*** is a muscular hollow pear shaped organ situated in the pelvic cavity. It measures about 7.5 cm in depth and 5 cm wide. It is divided in fundus, which is the dome shaped part in between the uterine tubes and the corpus or main body, linked in its lower portion to the cervix. Three layers of tissue form the endometrial walls: The *perimetrium* forms an envelope over the whole uterus and connects with the peritoneum forming the vesicouterine and rectouterine pouches. It also forms the broad ligament that connects the uterus to the lateral pelvic wall. The *myometrium* is the muscular layer that contracts during menstruation to expel the shedding endometrium and help the delivery of the offspring. The *endometrium* is the uterine inner layer responsible for implantation and menstruation. It is formed by two different layers (Waugh & Grant 2006; Strauss et al. 2004):

- Stratum Basalis. It is formed by the uterine mesenchymal stem cells and stromal fibroblasts. These cells will proliferate after the endometrial breakdown in menstruation and regenerate the functional layer. This layer never sheds from the endometrium.

- Functional layer. It forms the most upper layer and the uterus lining. It is covered by columnar and partially ciliated epithelial cells that form the lining and glands and contains the stromal fibroblasts. It is rich in blood vessels, derived from the spiral arteries, which develop during the first half of the menstrual cycle. The glands are embedded in the stromal tissue and their secretions are under hormonal control.

The ***Fallopian tubes*** are trumpet like structures situated in both sides of the uterus fundus and extend in about 10 cm long. The peritoneum forms an outer cover, followed by a smooth muscular layer and an inner ciliated epithelial layer that will propel the oocyte/embryo into the uterus. At their ends the fimbriae are located, which are finger like motile structures surrounding the tube opening or infundibulum that will receive the oocyte delivered from the ovaries. The next dilated part is the ampulla, site where the fertilisation of the oocyte takes place (Waugh & Grant 2006).

The ***Ovaries*** are the female gonads. They are the reservoir of the female gametes (oocytes) and are responsible for the production of steroid hormones (estradiol and progesterone). They are situated at both sides of the uterus body attached to it by the ovarian ligament. These structures are 2.5-3.5 cm long, 2 cm wide and 1 cm thickness and are formed by three major portions (Waugh & Grant 2006):

- The hilum. Support tissue containing the nerves, blood vessels and steroid producing hilus cells.
- The medulla. Inner portion highly innerved and irrigated by blood vessels.
- The cortex. The outer layer, mesothelium or tunica albuginea is formed by cuboidal cells. The germinal layer contains the immature primordial follicles with the ovum surrounded by stromal tissue. Each 28 days ovulation occur when a

Graffian follicle (the ovarian mature follicle) ruptures and expels one oocyte into the peritoneum that is received by the fallopian tubes fimbriae.

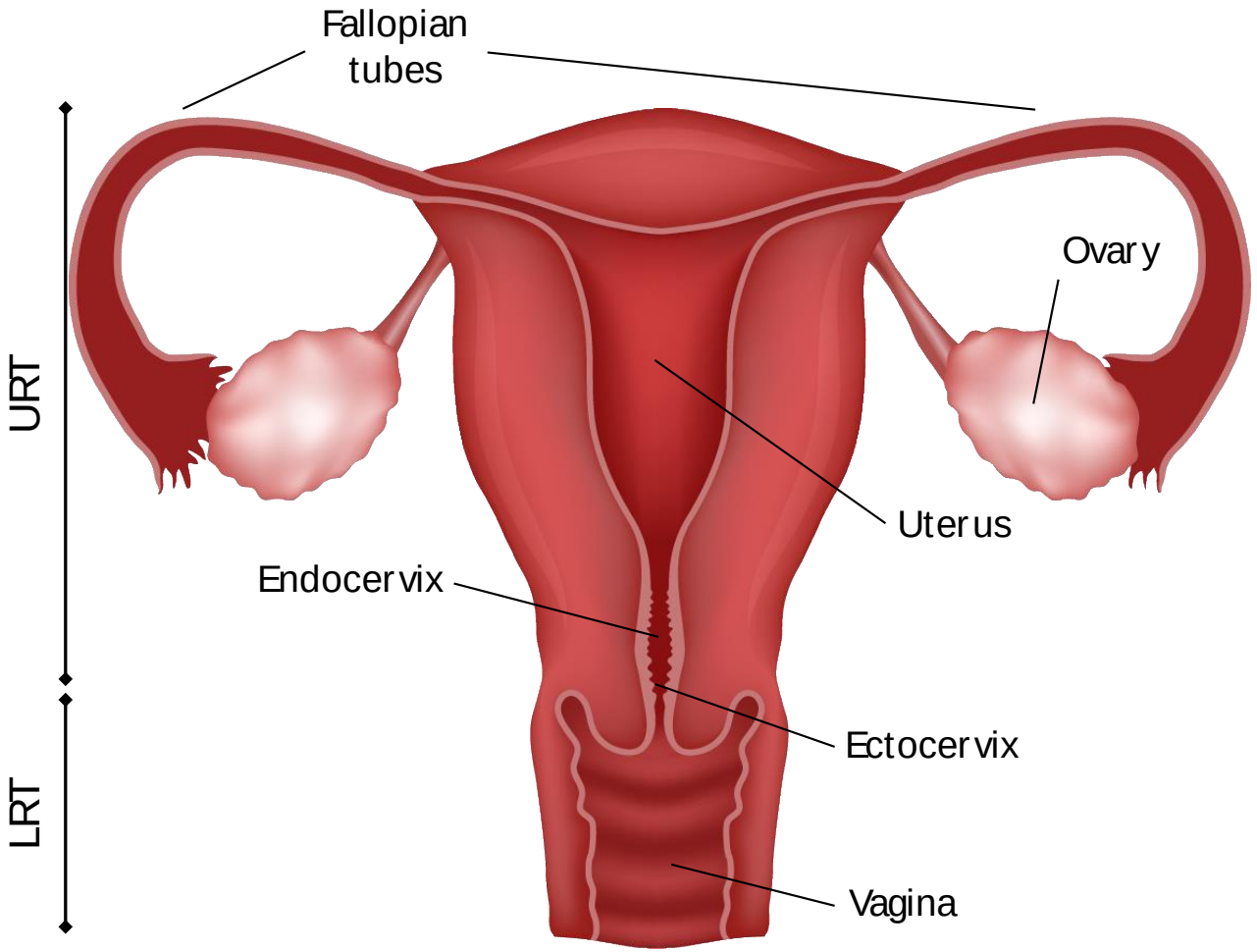

Figure 1.1. Anatomy of the Female reproductive tract. The human female reproductive tract comprises the lower reproductive tract (LRT) formed by the vagina and ectocervix. The upper tract (URT) is formed by the endocervix, uterus, fallopian tubes and ovaries.

1.2.2 Menstrual regulation of the female reproductive tract

1.2.2.1 Hormonal control of the tract

The main neuroendocrine axis controlling the functions of the FRT is the hypothalamic-pituitary-gonadal (HPG) axis. It includes the production and release of the gondadotropin-releasing hormone (GnRH) by the hypothalamus, the gonadotropic hormones follicle-stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary and the steroidal hormones estradiol and progesterone by the ovaries (Strauss et al. 2004)(Figure 1.2). The pulsatile secretion of GnRH initiates the general function of the HPG axis that governs the menstrual cycle. This stimulation promotes the production and secretion of the FSH and LH by the anterior pituitary. In response, the ovary increases the production of estradiol to favour the recruitment and maturation of follicles. As the cycle progresses, the estradiol level in blood increases and together with GnRH favour the LH surge. The peak of LH on day 14 of the menstrual cycle induces ovulation and an increase in progesterone production. High levels of estrogen and progesterone inhibit the production of GnRH at the hypothalamus and the pituitary response to estradiol, inhibiting overall the production of the gonadotropic hormones (Speroff & Fritz 2004).

The steroidal hormones dictate the behaviour of the different compartments that comprise the FRT. They are recognised by nuclear receptors that promote the expression of genes, co-activators and co-repressors specific for each cell type and tissue. The expression of these cell receptors follows a spatial and temporal pattern along the menstrual cycle that guarantees the control of processes like cell proliferation, tissue breakdown and factors necessary for gamete transport, embryo implantation and development (Lessey et al. 1988).

1.2.2.2 Ovarian function

As mentioned before, the ovaries are the storage sites of the oocytes. At birth, the ovaries contain around 1-2 million germ cells that can potentially differentiate into oocytes by undergoing meiosis. A cluster of germ cells gives rise to the primordial follicle. In it, the pre-granulosa cells surround the oocyte. During childhood the ovaries

remain active, inducing the development of follicles. The granulosa cells proliferate and the oocyte increases size forming the primary or pre-antral follicle. The lack of gonadotropin stimuli for these follicles will prevent them from reaching the full maturation and undergoing atresia. At the onset of puberty, there will only remain around 300,000 to 500,000 germ cells from which only around 400-500 will mature and be ovulated throughout the sexually mature life (P. Reddy et al. 2010).

In the pre-ovulatory follicle, the granulosa cells form two subsets, the mural cells that continue the steroid production and the cumulus cells that nourish the oocyte. The steroidogenic capacity of the ovaries starts at the onset of puberty. During the follicular phase of the ovarian cycle, estrogen is produced by cooperation between the granulosa and thecal cells upon FSH stimulation. On day 14 of the ovarian cycle, the LH surge initiates the luteal phase. Upon the LH surge, the granulosa cells of the now antral follicle continue their proliferation and differentiation to granulosa lutein cells characterised by a yellow appearance due to the pigment lutein and the surrounding stromal cells differentiate into theca lutein cells. This is when the ovulation occurs by the enzymatic degradation of the follicle basal lamina. The empty follicle or corpus luteum reorganises and create new blood vessels for the transport of cholesterol, substrate for the synthesis of the steroidal hormones. This change is followed by the expression of enzymes to initiate the production of progesterone in the luteal and theca cells. If the fertilisation of the oocyte fails, luteolysis occurs where the corpus is degraded by ischemia and cellular death (Johnson 2007; Strauss et al. 2004)(Figure 1.2).

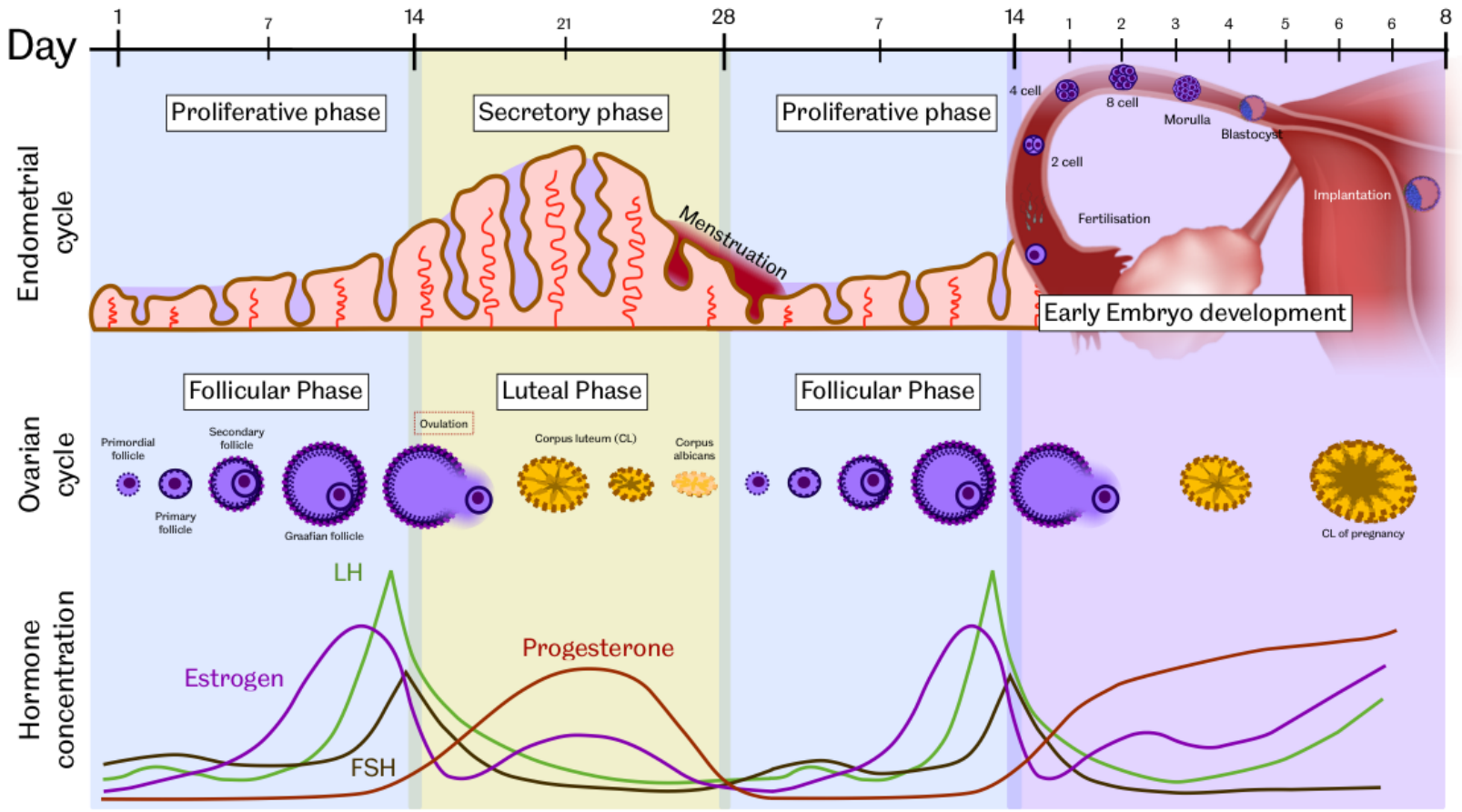

Figure 1.2. The menstrual cycle: Hormonal, Ovarian and Endometrial changes. The human menstrual cycle is orchestrated by the pituitary hormones Luteinising Hormone (LH) and the Follicle stimulating hormone (FSH). During the follicular phase of the ovary, the estrogen will induce the maturation of the primordial follicle. Upon the peak of LH and FSH the mature follicle will release the mature oocyte. The corpus luteum then will continue the follicular ovarian phase by increasing the production of progesterone and estrogen. The menstrual endometrium sensitive to the estrogen will regenerate during the proliferative phase. During the secretory phase, progesterone will induce changes in the endometrium that will allow the implantation of an embryo. After fertilisation the early development of the embryo takes place as it travels through the fallopian tube. It must arrive between days 5-8 post-fertilisation to guarantee an implantation on a receptive endometrium.

1.2.2.3 Fallopian tube function

The transport of gametes and embryo along the tubes is dependant on hormonal stimulation. During the follicular phase, the stimulation of the epithelial cells with estrogen induces the increase in cell height, cilia development and beating. Upon ovulation, together the fimbriae and the epithelial cilia beat creating a current that collects the released oocyte. A constant stimulation of the epithelial cells by progesterone induces atrophy and cilia loss. Once inside the tube, the egg will be transported to the ampulla where it resides in between the highly folded epithelium that guarantees nutrition while it waits for the potential arrival of the sperm. The sperm is temporarily allocated in the tubal isthmus where it presumably binds to the epithelial cells using lectins recognised by glycoconjugates of the epithelium. This interaction increases the secretion of tubal fluid, which contents favour sperm viability and induce their capacitation. The hyperactivated spermatozoa continue their journey towards the ampulla to fertilise the oocyte. The combined effect of progesterone and estrogen induce the movements of the myosalpinx or muscular layer of the tube. The isthmus epithelium has less tissue folds and less ciliated cells, hence the movement of the developing embryo or oocyte depends on the myosalpinx contraction and relaxation. By the time the embryo reaches the utero-tubal junction, five to six days after fertilisation, the differentiated trophoblasts produce human Chorionic gonadotropin (hCG). This hormone induces the opening of the sphincter that allows the access to the uterine cavity for further implantation and development (Strauss et al. 2004).

1.2.2.4 Uterine function

The endometrium behaviour is dictated by the ovarian steroidal hormones progesterone and estrogen. Based on the responses observed on the tissue, the uterine menstrual cycle is divided in proliferative, secretory and menstrual phase (Figure 1.2).

a. Early proliferative phase

It is characterised by a high proliferation of the stromal and epithelial (luminal and glandular) cells after the endometrial loss from the menstrual phase. The endometrium at this stage grows from 0.5 to 2 mm in thickness due to cell proliferation (Strauss et al. 2004). The proliferation of the stroma is characterised by a period of oedema and

observation of mitoses. Immersed in the stroma, the glands look narrow and tubular and the blood vessels start to extend towards the lumen. This phase corresponds to the follicular phase of the ovarian cycle, where estradiol is produced to stimulate the follicular growth. The estrogen receptors (ER) show their highest expression in the functionalis and basalis layers (Lessey et al. 1988). Activation of ERα by estradiol promotes the proliferative activity of the stromal cells as the knock out (KO) mouse for this receptor lacks endometrial development (Cooke et al. 1997). The ERβ on the other hand seems to control this process and its deficiency promotes hyperplasia of the endometrium in response to estradiol (Krege et al. 1998).

b. Late proliferative phase

During the second part of the proliferative phase, the endometrial growth reaches a maximum thickness of 3.5 – 5 mm. The expression of ER in the functionalis layer peaks around days 8-10 which corresponds to the peak of estradiol production. The glandular luminal cells increase in height and pseudostratify. The size of the stroma increases due to the synthesis of new ECM. The highest level of DNA and RNA synthesis is observed during this period in the functionalis layer, preparing for the secretory phase (Speroff & Fritz 2004). The presence of cilia and microvilli is observed on the membrane of the glandular and luminal epithelial cells. The beating of the cilia aids the distribution of the secretions along the uterine cavity (R. R. Masterton et al. 1975).

c. Early secretory phase

The production of progesterone after ovulation marks the start of this phase, which corresponds to the luteal ovarian phase. Cell proliferation ceases after 3 days of the progesterone surge and heterocromatin is observed in the nucleus of glandular and stromal cells. The glandular epithelial cells accumulate glycogen-rich vacuoles and a modest secretion towards the lumen is observed on days 17-18. The epithelial luminal cells enlarge their endoplasmic reticulum and mitochondria. The stromal cells synthesise a reticular network of type collagen fibres types I and III. The effect of progesterone antagonises the proliferative activity of estradiol on the cells. Progesterone inhibits transcription of oncogenes and activates the degradation of estradiol, which produces a metabolite with lower estrogenic activity.

d. Mid-secretory phase

The highest secretory level is observed during this phase, 7 days after ovulation. Histologically, it is possible to identify three different zones at this stage, the compact zone, the spongy zone and basal zone. Proliferation of the blood vessels and glands is confined to the compact zone increasing the coiling of the vessels and tortuosity of the glands. The intense secretory activity delivers glycoproteins into the endometrial cavity and the glands appear exhausted. Glandular and luminal epithelial cells acquire the nucleolar channel system that facilitates the export of mRNA from the nucleus (Strauss et al. 2004). Microvilli is found on the luminal cells, which is meant to help the endometrial – trophoblast interactions (Sarani et al. 1999). The spongy zone is full of enlarged stromal cells; the ones around blood vessels acquire an eosinophilic cytoplasm and produce membrane-bound tissue factor and plasminogen activator inhibitor type 1 to prevent possible haemorrhage due to trophoblast migration. Stromal cells form new peri-cellular ECM composed by laminin, fibronectin, heparan sulphate and type IV collagen. Finally, a very characteristic infiltrate of uterine natural killer (NK) cells is observed close to the arteries. They will favour embryo implantation and endometrial menstrual breakdown (Wilkens et al. 2013).

e. Menstrual phase

In the absence of a fertilisation, the progesterone and estrogen produced by luteal body of the ovary will drop, initiating the events that govern endometrial breakdown (Maybin & Critchley 2011). The lack of progesterone induces production of factors like the endometrial bleeding-associated factor (EBAF) that antagonises the transforming growth factor (TGF)-β that maintains endometrial integrity. The breakdown of the endometrium is regulated by a pro-inflammatory activity characterised by the production of pro-inflammatory cytokines that induces cell apoptosis and a high infiltration of neutrophils (J. Evans & Salamonsen 2012). The neutrophils are in part responsible for the production of matrix metalloproteinases (MMPs), which in turn degrades the ECM supporting the decidua. Vasoconstriction of the uterine arterioles and spiral arteries ceases the blood flow to the functional zone. This generates a hypoxic effect favouring the apoptosis of stromal and epithelial cells (Strauss et al. 2004). The

arteries eventually open up releasing blood breaking the damaged vessels, stroma and epithelium. This mix will flow through the uterine cavity without formation of clots. The basal layer remains intact, as it replenishes the lost tissue during the next proliferative cycle (Speroff & Fritz 2004).

f. Endometrial receptivity

The implantation of the embryo occurs during the days 20 – 24 of the mid-secretory phase. This lapse of 4 days is called the "window of implantation". Before or after these days, the endometrium is unfit to host the implantation of the embryo. By this time the endometrium measures 10 – 14 mm in thickness and the secretory activity peaks to bring the nutrients and the paracrine stimulation elements that the embryo needs to develop. It has also acquired a "receptive phenotype" which comprises the expression of functional biomarkers as proteins, genes and morphological changes of the endometrial epithelial (EECs) and stromal cells (ESCs) (Psychoyos 1986). Histologically, it is possible to identify apparent changes as the previously mentioned thickening and high vascularisation of the functional layer, edema of the stroma, convolution of the glands, pseudostratification of the luminal epithelium among others (Strauss et al. 2004). The research field in endometrial receptivity has been enriched along the years and the depth and complexity changed from a histological and hormonal perspective to a molecular and biochemical focus (Figure 1.3). Some of the markers of endometrial receptivity include the following:

- ***Epithelial cell membrane microstructure.*** The microstructure of the epithelial luminal cells changes in the mid-secretory phase. Cells enlarge, polarise and loose the glycocalix to form smooth and large membrane projections called pinopodes due to their pinocytotic activity (Nikas et al. 1999). It has been observed that the implantation site of the blastocysts is always surrounded by these structures (Bentin-Ley et al. 1999).

- ***Adhesion molecules.*** The endometrium during the receptive phase expresses a diversity of adhesion molecules. Membrane bound glycans, integrins, selectins, cadherins among other molecules mediate their adhesion with the trophoblast cells of the blastocyst (Campbell et al. 2000; H. Singh & Aplin 2009).

Glycoprotein ligands of L-Selectin like the von Willebrand factor are required for the initial attachment of the blastocyst, as the endometrium of infertile women express a low amount of them (Margarit et al. 2009). Another family of adhesion molecules with great value for implantation are the integrins. They are transmembrane proteins that form heterodimers with an α and a β subunits that binds to ECM proteins (K. V. R. Reddy & Mangale 2003). The expression of the integrins α1β1 and αvβ3 has been observed to cycle and reach its peak during the late secretory phase hence they have been proposed as basic adhesion molecules for embryonic implantation (H. Singh & Aplin 2009). Both endometrial cells and blastocyst produce laminin and fibronectin, which are ECM proteins where integrins attach providing a firm substrate for the blastocyst attachment (Burrows et al. 1996).

- ***Prostaglandins.*** The events surrounding endometrial receptivity and implantation resemble an inflammatory reaction. Prostaglandin E_2 is produced by the secretory endometrium and decreases during decidualisation. Its production is found particularly increased at the implantation site and may induce vascular permeability for the establishment of the new blood vessels (van der Weiden et al. 1991).

- ***Growth factors.*** A great variety has been implicated with endometrial receptivity. Factors like epithelial growth factor (EGF), TGF-α and β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) among others are found to increase during the mid-secretory phase and play a role in the acquisition of receptivity. TGF-β for example has been observed to inhibit endometrial cell proliferation in the secretory phase. It stimulates the production of collagen to favour decidualisation of the stroma and inhibits the production of MMPs that are able to degrade the endometrium (Bruner et al. 1995).

- ***Cytokines.*** As previously mentioned, mediators of implantation are shared with inflammation. Cytokines like interleukin (IL)-1β, IL-6, tumour necrosis factor (TNF)-α, macrophage migration inhibitory factor (MIF) and leukemia inhibitory

factor (LIF) participate actively in the implantation process. LIF for example is highly expressed in human endometrium at the time of implantation (Lédée-Bataille et al. 2002). Mutations in LIF gene have been co-related with human infertility and the LIF KO murine blastocysts are unable to implant (Králícková et al. 2005). MIF has been found expressed cyclically in the murine endometrium with an increase during mid-secretory phase. The i.p. administration of MIF increased the expression of integrins, VEGF and the von Willebrand factor (Bondza et al. 2008). The balance of IL-1 and its antagonist IL-1 receptor antagonist (RA) has been suggested to play an important role during embryo implantation. IL-1β produced by both embryo and endometrium is found to decrease apoptotic factors and increase the expression of multiple factors necessary for implantation as the β3 integrin, IL-6, LIF, CSF and placental metalloproteinases (Geisert et al. 2012). The i.p. administration of IL-1RA at implantation time, prevents the implantation of embryos (Simón et al. 1998) depicting the relevance of IL-1β to induce receptivity related genes.

- ***Anti-adhesive factors.*** Some membrane bound proteins like mucins have been associated with an anti-adhesive function. Mucins are highly glycosylated proteins that are present in all the epithelial mucosa of the body as membrane bound or gel forming proteins (M. C. Rose & Voynow 2006). The expression of mucin 1 has been observed to increase during secretory and menstrual endometrium (Gipson et al. 1997). In humans, the expression of MUC1 seems to increase during peri-implantation period (Achache & Revel 2006) and the presence of the embryo down-regulates its expression in the implantation zone (Meseguer et al. 2001; H. Singh et al. 2010).

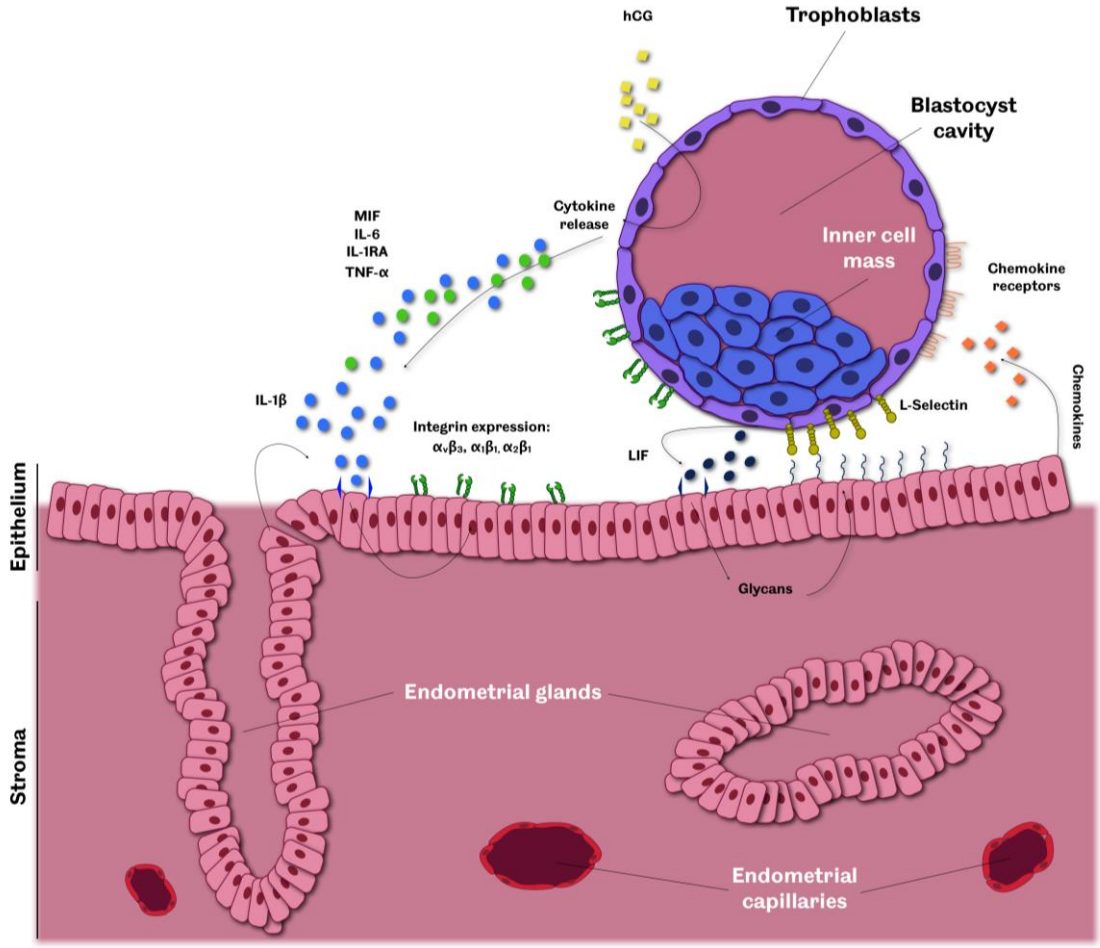

Figure 1.3. The complex interaction during blastocyst implantation. The crosstalk between the maternal endometrium and the implanting blastocyst is regulated by a series of paracrine and autocrine mediators. Chemokines released by the endometrium will guide the blastocyst to the implantation site. The apposition will be mediated by an initial binding of the trophoblast L-selectins to endometrial glycoproteins. The production of hCG will stimulate the secretion of embryonic mediators like leukaemia-inhibitory factor (LIF), interleukin (IL)-1β among others to induce the expression of integrins that will guarantee a firm adhesion to the endometrium.

1.2.3 Fertilisation, early embryo development and implantation

1.2.3.1 Fertilisation

After coitus, around 300 million spermatozoa are delivered in the vagina and need to travel through the cervix and the uterus to reach the oviduct. Only 1% of them are able to enter the cervix and start the long journey. It has been found that sperm can be detected in the ampulla as soon as 2 h post-coitus and only a few hundreds of sperm reach their destination (Ahlgren 1975). After their temporary storage on the oviduct isthmus, the sperm is capacitated by removal of some of the seminal and epididimal proteins that spermatozoa are coated with (Rodriguez-Martinez 2007). The capacitation process increases the motility and induces membrane morphological changes in the spermatozoa but at the same time decreases their longegivity. Upon the encounter with the oocyte zona pellucida, the sperm head undergoes the acrosome reaction. This consists on the delivery of the acrosomal vesicle contents, like proteolytic enzimes to digest the zona and access the oocyte membrane (Johnson 2007). The oocyte membrane protein "Juno" has recently been identified as the receptor for the sperm. It transiently binds Izumo1 on the sperm membrane. Juno is removed from the membrane as soon as 40 min after sperm binding to oocyte membrane to discard the possibility of another sperm fertilising the egg (E. Bianchi et al. 2014). Oocyte and sperm membrane fuses by interaction of integrins and the tetraspanin cluster of differentiation (CD)9 on the oocyte and the protein "a disintegrin and metalloproteinase domain-containing (ADAM)" (Johnson 2007). As the sperm enters, the haploid nucleuses fuse and form the diploid zygote. The oocyte then completes the meiotic process it was arrested in, expels the second polar body and continues a normal mitotic development. Besides half of the genetic information, the conceptus will inherit mitochondria, ribosomes, Golgi, cytoskeleton and the protein biosynthetic machinery from the mother egg. The centriole and the gene imprinting mechanisms that will dictate the way in which the genes will be expressed are inherited from the father through the sperm.

1.2.3.2 Early embryo development

The initiation of the mitotic divisions leads to the cleavage of the fertilised egg to the two-cell stage. By this time the developing embryo has a low metabolic activity based

on the maternal inherited organelles, proteins and mRNA. The developing embryo initiates its journey through the fallopian tube, propelled by the ciliated epithelia and the uterine muscle contractions. After 2 days, the embryo reaches the 4 - 8 cell-stage, degrading all maternal mRNA and synthesises its own RNA and protein production machinery (Edwards 1980). When the embryo reaches the 16 cell-stage it is called morulla and is characterised by the compaction of the blastomeres and its polarisation to form an apical and a basal layer of cells. After 4.5 days of development, the embryo reaches the blastocyst stage, in which two distinct layers of cells are found: The outer rim of trophoblasts that forms the extraembryonic tissue, a blastocoelic cavity and the inner cell mass (ICM) or pluriblast that will give rise to the fetus (Figure 1.4a). By this stage, the embryo is still covered by the zona pellucida, which hold the blastomeres together and protects it from the adhesion with other embryos and from a tubal or ectopic implantation. By days 5 – 8 post-fertilisation, the blastocyst reaches the uterine cavity and hatches from the zona pellucida to find a suitable spot for implantation. The uterine secretions support the early development of the embryo by diffusion of oxygen and carbon dioxide and active transport of nutrients. This is called the histiotrophic nutrition and it accompanies the pre-, peri- and early post-implantation phases (Johnson 2007).

1.2.3.3 Implantation

a. Implantation stages

Human embryonic implantation is a complex process that has been divided in stages based on the level of maternal-embryonic interaction. In order to achieve a successful implantation, the development of both blastocyst and endometrium should synchronise. The first contact with the endometrium as the blastocyst hatches from its zona is called the *apposition* (Fig. 1.4b). The production of chemokines by the endometrium is supposed to attract the embryo to a suitable implantation site. The recognition of these chemokines induces the expression of a diversity of intercellular adhesion molecules (ICAMs) that will fix the blastocyst to the epithelia. This is a weak interaction between the ICAMs in the blastocyst and glycoproteins in the endometrium to fix the blastocyst to the implantation site (H. Singh & Aplin 2009). This stage is followed by a firm *adhesion* mediated by the interaction between integrins of both blastocyst and

endometrium and the ECM ligands (Fig. 1.4c). During the stage of *invasion* (Fig. 1.4d), the trophoblasts penetrate the uterine epithelia and invade the decidua and even reach the basal endometrium to establish contact with the spiral arteries. The trophoblast migration is mediated by the release of matrix degrading enzymes as MMPs and adhesion molecules as integrins (Paria et al. 2000). The cytotrophoblasts are the invasive trophoblasts, which migrate through the uterine epithelia and stroma to establish a contact with the maternal vasculature and create new vascular conducts towards the new implanted embryo for its nourishment (Fig. 1.4e). Some of these trophoblasts fuse together to generate a syncythiotrophoblast, which produces hCG. This hormone extends the activity of the corpus luteum, which continues the production of progesterone and stop the menstrual endometrial decay. This reaction is the primary decidualisation reaction where stromal cells enlarge, increase mitotic activity and produce an ECM necessary for the trophoblast migration (Johnson 2007). The paracrine factors as insulin-like growth factors (IGFs) and IGF binding proteins (IGFBPs), TGF-β, relaxin, renin, prolactin among others are produced by the decidual stromal cells to keep the homeostasis of the endometrial decidua during implantation and early pregnancy and favour its vascularisation (Speroff & Fritz 2004).

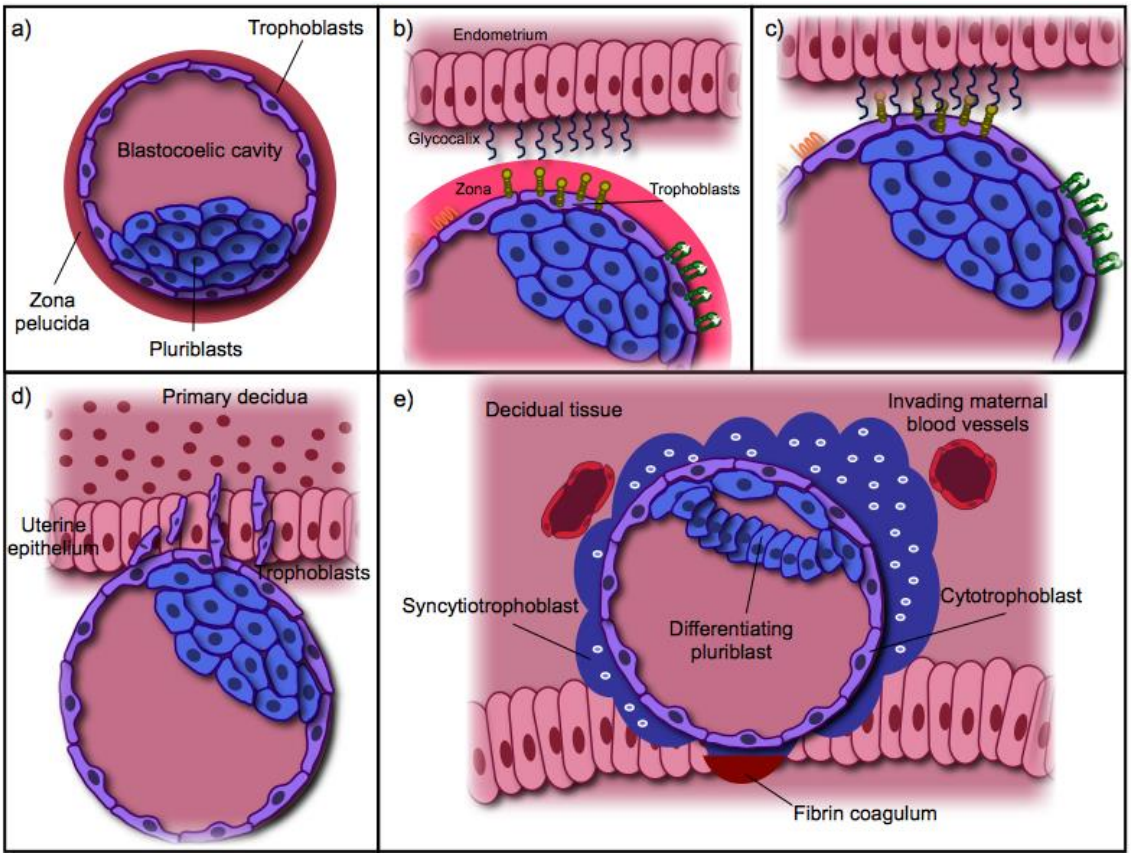

Figure 1.4. Implantation of the embryo. The blastocyst (a) is formed by a zona pelucida which forms an envelop around the trophoblast cells, a pluriblast inner cell mass and a blastocoelic cavity. During its migration through the oviduct, the zona pelucida interferes with the adhesion to the epithelia (b). When the blastocyst reaches the uterus, it hatches from the zona pelucida and the now exposed trophoblasts attach to the luminal epithelial cells of the endometrium (c). Once attached the trophoblasts start the invasion. They break through the surface of the epithelium to reach the underlying stroma and the maternal blood vessels (d). Once the blastocyst has invaded the stroma, the trophoblast will fuse and differentiate into syncythiotrophoblasts to form with the stroma cells the placenta, new blood vessels will nourish this process and the differentiating pluriblast start the development of the conceptus (e). Modified from Johnson MH, 2010.

1.3 Innate immune system in the female reproductive tract

The human body is a marvellous organism where systems and organs work together in homeostasis to perform all the physiologic functions that keep us alive. Different circumstances encountered through life like infections or physiological deficiencies can interfere with this balance and bring the system into a pathological state. The body possess highly specialised system of surveillance of all the organs in the body, the immune system. A diversity of effector mechanisms has been evolutionally designed to detect the body failure or pathogenic challenge and counteract to bring back homeostasis to the body.

The immune system is divided in innate and adaptive systems. The innate system is the first line of defence of the body. It initiates a quick reaction against pathogenic invasion or tissue damage by recognising non-specific patterns in the microorganisms or cell debris (Medzhitov & Janeway 2002). The adaptive system relies on the recognition of specific antigens present in the pathogens and counteracts with cellular and humoral responses as effector mechanisms (Murphy 2011).

1.3.1 The innate immune system

The innate immune system is the first line of surveillance and defence system of the body against invading microorganisms and tissue damage. To achieve this, the body elicits a series of effector mechanisms that lead to inflammation. Inflammation is a physiological response triggered by noxious stimuli and conditions, such as infections and tissue injury to regain the homeostasis of the body (Medzhitov 2008). The innate immune system is present in all the tissues of the body as epithelial barriers, tissue resident immune cells and specialised immune cells that migrate from the blood to the tissues. The innate system has the capacity of recognising bacterial, viral or parasite pathogen associated molecular patterns (PAMPs). These are structurally conserved molecules shared between similar families of pathogens, required for their survival and structurally different from any molecule present in our bodies (Medzhitov & Janeway 2002). Molecules such as lipopolysaccharide (LPS) of Gram-negative bacteria, lipopeptides in Gram-positive bacteria, single and double stranded RNA and DNA of virus and bacteria form part of these PAMPs (Medzhitov & Janeway 2002). These are

recognised by the cells through a group of membrane and soluble pattern recognition receptors (PRRs), which initiate a signal transduction cascade that activates a variety of defence mechanisms of the cell. Some of these mechanisms can be the synthesis of free radicals derived from oxygen and nitrogen, the increase of phagocytic activity of macrophages and the secretion of chemokines and cytokines by tissue and immune cells (Medzhitov & Janeway 1997). A good innate response is crucial for the establishment of an effective adaptive immune response that leads to a complete removal of the invading pathogen. This is necessary to recover tissue homeostasis and generate the memory of the immune system through antibody producing B lymphocytes and specific T lymphocytes. If the pathogen re-infects the host, the adaptive response can be quicker and more effective (Janeway 1989). In some situations where sterile tissue damage occurs, these PRRs are capable of sensing own molecules known as molecular patterns associated with damage (DAMPs). Example of DAMPs are fragmented ECM proteins and cellular proteins that in homeostatic conditions are not free in the extracellular space and are secreted as an alarm signal and cell death (M. E. Bianchi 2007; Matzinger 2007).

1.3.1.1 The Toll-like receptor family

One of the most studied families of PRRs is the TLR family. It has a very important role in the innate recognition of PAMPs and DAMPs and the activation of the effector mechanisms in the cell (Kawai & Akira 2007b). A homologue of these receptors was first discovered in Drosophila where a fungal infection triggers an anti-microbial response. The proteolytic cleavage of Spätzle activates Toll receptor which initiates a signalling cascade through the protein kinase Pelle (Lemaitre et al. 1996). A homologue of Toll, the 18 wheeler receptor was described to participate also as a receptor in the antibacterial responses (Williams et al. 1997). Both receptors degrade the inhibitor cactus to activate the transcription factor dorsal, responsible for the transcription of the antimicrobial peptides genes in Drosophila. Dorsal is part of the Rel family of transcription factors and homologue of the human nuclear factor (NF)-κB. It is known that in vertebrates it also has an important role in the transcription of genes involved with synthesis of defence related proteins such as antimicrobial peptides, chemokines and cytokines (Kopp & Ghosh 1995). Later, a homologue of toll was found in vertebrates with the same properties of pathogen recognition and immune activation,

therefore it was called the “toll-like receptor” (Medzhitov et al. 1997). The TLRs are a family of 11 identified receptors in human and 13 in the mouse with a unique ability of PAMPs or DAMPs recognition. They are type I membrane integral proteins with an extracellular domain containing leucine-rich repeat (LRR) motifs and an intracellular domain homologue to the IL-1 receptor termed Toll/IL-1R (TIR) domain. Some TLRs are mainly expressed on the cell surface (1, 2, 4, 5 and 6) to sense extracellular PAMPs. Others (3, 7 and 9) are found mainly in endosomes to sense intracellular pathogens and viral nucleic acids (Akira et al. 2006).

The interaction of TLR and its ligand starts a signalling pathway mediated by sequential phosphorylation of different proteins. The classic signalling pathway is initially mediated by the myeloid differentiation primary gene 88 (MyD88), which is an adaptor protein containing TIR domain. MyD88 is anchored to the TIR domain of the TLR it or to the TIR domain containing adaptor-like MyD88 (TIRAP or Mal) which promotes the signalling pathway (L. O'Neill 2006; Bernard & L. A. O'Neill 2013). This pathway ends up in the activation of NF-κB, which promotes the transcription of pro-inflammatory genes like the cytokines TNF-α, IL-1β, IL-6 and chemokines like IL-8. TLR 1, 2, 4, 5 and 6, mainly undertakes this classical activation pathway (Kawai & Akira 2007b). TLR activation is able to signal through the mitogen-activated protein kinase (MAPK) pathway. MAPK can also promote NF-κB activation or signal through the transcription factor activator protein (AP)-1 (Jeong & Joo Young Lee 2011). The activation of TLRs by intracellular PAMPs not only activates NF-κB but is also able to activate antiviral interferon (IFN) type I dependent immune responses following the activation of IFN regulatory factors (IRF) 3 and 7 (Matsumoto & Seya 2008). In the case of TLR 3 and TLR 4, this is mediated by the TIR-related adaptor protein inducing interferon (TRIF), using the TRIF related adaptor molecule (TRAM) (Yamamoto et al. 2003). TLR 7 and 9 signalling is classically mediated by MyD88 (Cros et al. 2010; Gowda et al. 2012).

1.3.1.2 TLRs and their ligands

As mentioned before, there is a great variety of molecules that can be recognized by TLRs, from exogenous pathogens as bacteria, virus or parasites to self-molecules released in physiological damage situations (Beutler 2009). Based on its primary

sequences they can be divided into subfamilies that can recognise related molecules (Fig. 1.5):

TLR 1, 2 and 6: This group of TLRs form dimers to recognise exogenous lipids. Acting as a homodimer, TLR2 recognise peptidoglycan (PGN) of gram-positive bacteria and discriminate between different long lipopeptides and mycoplasma lipopeptide (Schenk et al. 2009). It also interacts with the accessory molecules differentiation cluster (CD)14 and CD36 to sense lipoteichoic acid (LTA) (Shamsul et al. 2010). TLR2 can form heterodimers with TLR1 to recognise triacylated lipopeptides (Buwitt-Beckmann et al. 2006) and with TLR6 to recognise diacylated lipopeptides (W. A. Rose et al. 2009). TLR 2 can also recognise DAMPs as the extracellular matrix component hyaluronan (D. Jiang et al. 2005).

***TLR 3, 7 and 9*:** This group of TLRs are located in endosome vesicles and can recognise nucleic acids from intracellular pathogens.

- **TLR 3** is able to recognise foreign double stranded RNA of viruses and the synthetic ligand poly inosinic-poly cytidylic acid (poly I:C) (Barton 2007).
- **TLR 7** (in human) and **8** (in mouse) is able to recognise single stranded RNA viruses and the synthetic molecule imidazoquinoline used against papiloma virus (Gantier et al. 2008).
- **TLR 9** can recognise DNA with repeated cytidine-phosphate-guanosine (CpG) motifs present in bacteria and viruses (Bauer et al. 2001).

***TLR 4*:** This is the first TLR described in human. Its ligand is the LPS present in the outer membrane of gram-negative bacteria. In order to achieve this, TLR 4 needs the accessory molecules MD2 and CD14 (Q. Jiang et al. 2000; Wright et al. 1990).
This TLR can also recognise different DAMPs generated by cleavage of ECM components as hyaluronan (Voelcker et al. 2008), biglican (D. Jiang et al. 2005) and fibronectin (Morwood & Nicholson 2006). It can recognise intracellular DAMPs released only under stressful conditions where the cells increase the synthesis of heat shock proteins (HSP) 60 and 70 (Schmitt et al. 2007). In necrotic death, where the intracellular and nuclear content is released, it recognises the high mobility group box 1 (HMGB-1), a nuclear protein (Lotze & Tracey 2005). Recently a new association

between TLR 4 and TLR 6 forming a heterodimer has been found, which is regulated by the scavenger receptor CD36 to initiate the pro-inflammatory response when recognising LDL (low density lipoproteins) and amyloid-β (Stewart et al. 2010).

***TLR 5*:** This TLR has been found to recognise the flagella of high virulent bacteria through flagellin, which is its major component protein (Fitzgerald et al. 2001).

***TLR 11*:** This TLR is only expressed in mice although the genomic homologue has been found in human, but it has a stop codon in its sequence. This TLR prevents the murine bladder infection by uropathogenic bacteria (Blander & Medzhitov 2004).

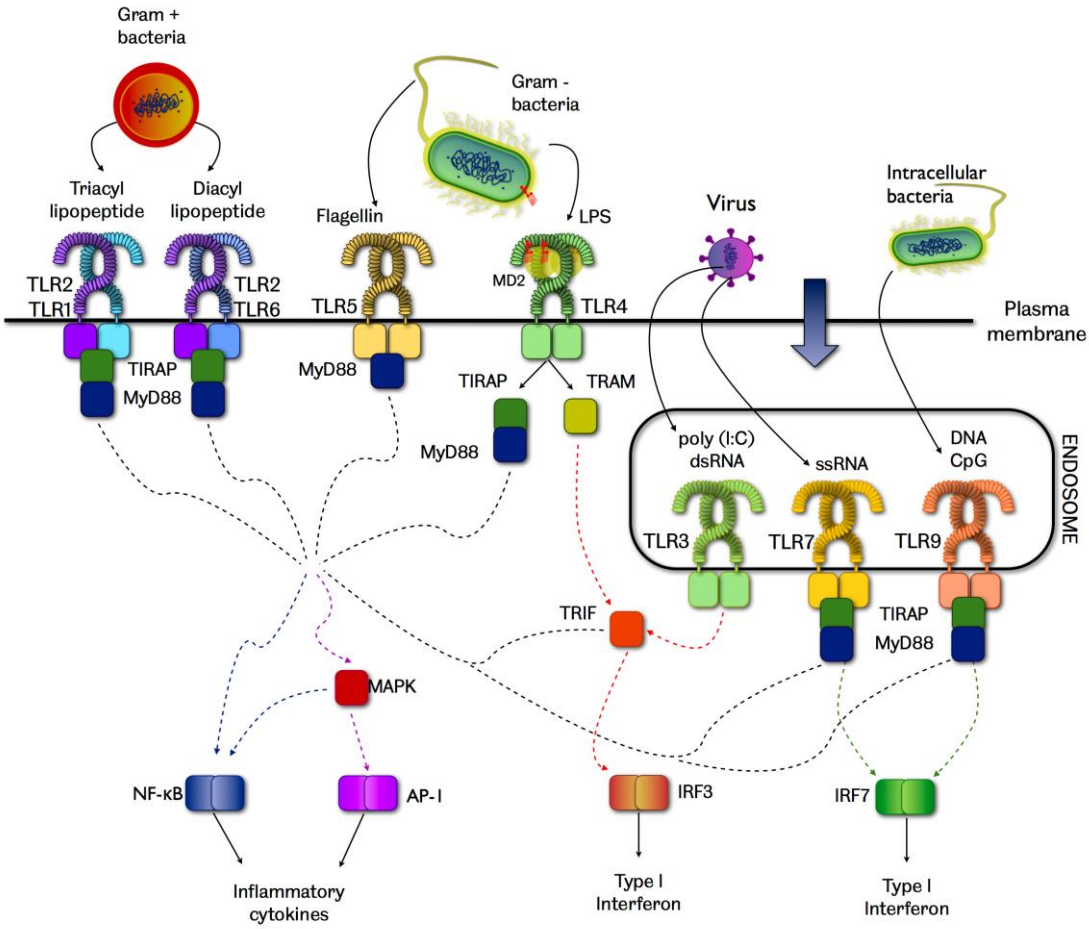

Figure 1.5. General activation pathway of the Toll-like receptors in response to pathogen stimulation. Immune system and epithelial cells can recognise a great variety of PAMPs by different TLRs on the membrane. For example Gram-negative bacterial lipopolysaccharide (LPS) through TLR 4, triacyl and diacyl lipopeptide of Gram-positive bacterial wall through TLR 2 – TLR 1 and TLR 2 – TLR 6 respectively and flagelin through TLR 5. Upon recognition a signalling pathway activates the MAP kinases pathway that can activate the nuclear transcription factor NF-κB or AP-1 to secrete inflammatory cytokines like TNF-α, IL-6 and IL-1β. TLR 4 can also activate in a late phase the IRF3 to produce IFN-γ. PAMPs from viral and intracellular pathogens can be recognised by TLR in endosomes. TLR 3 recognises double stranded (ds)RNA and poly(I:C) to activate IRF3, NF-κB or AP-1. TLR 7 recognises single stranded (ss)RNA and TLR 9 unmethylated 2'-deoxyribo CpG DNA motifs present frequently in bacteria and virus both to activate IRF7 and NF-κB.

1.3.2 Innate immunity of the uterus

The FRT is a mucosal epithelia comprised by an upper sterile part formed by the fallopian tubes, uterus and endocervix, and a lower part formed by vagina and ectocervix. As the intestinal tract mucosa, commensal bacteria like *Lactobacillus acidophilus* colonise the lower FRT, which helps to maintain the vaginal pH between 4.9 and 3.5. This acidity inhibits the growth of other pathogenic bacteria, which may enter from the perineum (Waugh & Grant 2006). The mucosa of the upper FRT must perform a tight immune surveillance to avoid microorganisms of the lower tract access the upper sterile tissue. A sum of diverse events such as menstrual cycle stages, pregnancy, use of hormonal contraceptive agents, frequency of sexual intercourse, sexual partners, vaginal douching and antibiotics that occur in the FRT, may change the environmental conditions of the tract and the composition of the inhabitant microbiota (Witkin et al. 2007).

The uterus is an immune-privileged organ as it is where most of these events take place. In particular pregnancy and development, where it will tolerate the invasion and damage derived from the implantation and the development of a support system for pregnancy and at the same time protect from potential infections (Geisert et al. 2012). In order to achieve this, the innate immune system has different mechanisms of action.

The FRT has a variety of weapons to face an infection, as any other mucosa. The epithelial cells can secret humoral innate mediators with antimicrobial activity like lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI) and α and β defensins (Hickey et al. 2013). The production of antimicrobial peptides has been found constitutive in the endometrium, changing along the menstrual cycle, with the highest peak at secretory phase (King et al. 2003). Also it counts with a constant surveillance of innate immune system cells like natural killer (NK) cells, monocytes, macrophages and neutrophils (Bélec 2002).

1.3.2.1 Progesterone as an immunomodulatory hormone

The uterine function is highly regulated by progesterone, initially released by the corpus luteum of the ovary after ovulation and later by the placenta. One of the suggested roles of this hormone is the immunosuppression of the normal uterine leukocyte function for

the establishment and maintenance of pregnancy (Pope 1990). Early studies in mice suggested that inhibition of progesterone during pregnancy lead to pre-term delivery and resorptions. Nevertheless, pregnancy could be rescued by transferring supernatant of progesterone treated splenocytes to modulate NK tolerance (Szekeres-Bartho et al. 1990). The NK inhibitory factor was later identified as the Progesterone-induced blocking factor (PIBF), an immunomodulatory peptide that can inhibit degranulation hence cytotoxicity of the NK cells (Faust et al. 1999) and prone the cytokine response of uterine leukocytes towards a Th2 profile (Szekeres-Bartho et al. 2001). Clinically, the importance of progesterone-induced fetal tolerance has been suggested as low serum levels of progesterone or PIBF correlate with abortions and pre-term deliveries (Arck et al. 2007). Progesterone can modulate the migration of macrophages, mast cells and dendritic cells to the uterine decidua and their activation and production of pro-inflammatory mediators as IL-1β and instead produce a Th2-type cytokine environment (Schumacher et al. 2014). Recently, the immunosuppressor effect of progesterone has been observed in the placenta response towards LPS. Progesterone downregulated TLR 4 and MyD88 expression and blunted the production of pro-inflammatory cytokines in the human amniotic epithelium (Flores-Espinosa et al. 2013).

1.3.2.2 Uterine leukocytes

The diversity of leukocytes infiltrating the uterus varies along the reproductive cycle. The surveillance increases along the cycle towards the secretory phase and being at its highest peak during the perimenstrual phase. This is required to control the breakdown, clear the endometrial tissue debris during menstruation and protect it from infections (J. Evans & Salamonsen 2012).

Uterine natural killer cells. The major population of cells present in the endometrium is the uterine natural killer (uNK) cells. The decidual secretions regulate the proportion of uNK cells in the endometrium reaching a 70% of the total leukocyte population during the mid-secretory phase and decreasing towards the end of the first trimester of pregnancy (Le Bouteiller & Piccinni 2008). They contribute towards the implantation of the embryo by producing cytokines and growth factors to regulate and facilitate it (Sentman et al. 2007). Also they promote the vascularisation of the uterus and trophoblast migration (Ji Yeong Lee et al. 2011).

Macrophages and dendritic cells. These are the two main populations of antigen presenting cells of the uterus. Macrophages perform the immune surveillance of the endometrium increasing in numbers during secretory phase (Ji Yeong Lee et al. 2011). They contribute towards the angiogenesis of the spiral arteries during implantation. They are found to modulate an anti-inflammatory environment to contribute to the feto-maternal tolerance. Also they are able to promote inflammation and protect the fetus from an infection during pregnancy (Nagamatsu & Schust 2010). Dendritic cells (DCs) are a small population of 5-10% of the total leukocytes in the endometrium. Nevertheless, their role in the establishment of pregnancy has been crucial as they will produce LIF necessary for embryo implantation and TGF-β for vascularisation (Ji Yeong Lee et al. 2011). The depletion of DCs from murine endometrium makes the mice infertile due to a deficiency of implantation, vascularisation and decidualisation of the endometrium (Plaks et al. 2008).

Neutrophils. As with the previous leukocytes, uterine neutrophil infiltrates vary with the menstrual cycle. The presence of the neutrophils in the uterus is constant until the proliferative phase where the production of the chemokine IL-8 increases, attracting the migration of more neutrophils to the tissue. Upon the drop in progesterone observed prior to menstruation, IL-8 production increases, attracting even more neutrophils (Kelly et al. 1994). Neutrophils are the first subset of cells found during inflammatory conditions to clear the wounds from possible pathogens. During menstruation it might be possible that they contribute to the endometrial shedding by production of MMPs. They will favour the activation of other neutrophils and clearing of the tissue debris by the production of IFN-γ (Salamonsen & Woolley 1999).

1.3.2.3 TLR in the female reproductive tract

The cells of the FRT can express TLRs and promote inflammatory reactions in response to the recognition of PAMPs. It has been also found that the TLRs are differentially expressed through the segments of the FRT. In humans, the TLRs 1, 2, 3, 5 and 6 are present in the epithelia of both upper and lower regions of the tract. TLR 4 was only found in the endocervix, endometrium epithelial and stromal cells and fallopian tubes (Hirata et al. 2005; Fazeli et al. 2005). This differential expression might be a

mechanism of tolerance in the lower FRT colonized by commensal bacteria, otherwise a constant activation of the TLR 4 pathway would generate an exacerbated inflammatory process through the tract (Fazeli et al. 2005). The expression of TLRs and its function has been studied in different endometrial cell lines such as RL95-2 and Ishikawa with the objective of establishing a suitable in vitro model of study (Aboussahoud, Aflatoonian, et al. 2010b; Young et al. 2004). It has been found that the human uterine epithelial cell line ECC1 can express all the TLRs (Schaefer et al. 2004).

1.3.2.4 Regulation of TLR expression during the menstrual cycle

One of the most notable findings is that the menstrual cycle can regulate the immunologic state of the FRT. It has been reported that estradiol and progesterone can control the proliferation, apoptotic rate, secretion and the ability to respond to pathogen invasion by controlling the migration and localisation of the immune cells. This activation can also modify the physical barrier through the trans-epithelial resistance (TER) to avoid the bacterial migration through the intercellular spaces (Fahey et al. 2005). These hormonal changes throughout the menstrual cycle can also affect the TLR expression in the epithelial cells of the human endometrium. The lowest level of expression of the TLR 2-6, 9 and 10 was found to be during the menstruation and proliferative phase and the highest levels were found to occur in the secretory phase (Aflatoonian et al. 2007). These findings support the idea that the FRT must comply with the defence against exogenous microorganisms, recognise commensal ones and allow the presence of allogenic spermatozoa and immunologically distinct foetus (Grossman 1985).

1.3.2.5 Functionality of TLRs in the uterus

As suggested before, a strict surveillance of the uterus is essential for implantation, pregnancy and menstruation. The highest expression of the TLRs during the secretory phase and pregnancy may suggest that they play an important role in the tissue defence and maternal and fetal protection. In order to achieve this, TLRs are able to activate several mechanisms upon activation. The production of pro-inflammatory chemokines like IL-1β, IL-6, TNF-α and IL-8 is one of the main effector mechanisms of the TLRs. It has been found that the EECs and ESCs are able to produce IL-8 in response to TLR

4 and CD14 stimulation with LPS (Hirata et al. 2005). In a similar way, the production of IL-8 has been observed when TLR 3 of primary EECs and RL95-2 epithelial cell line were activated with Poly I:C (Jorgenson et al. 2005). IL-8 will promote the migration of neutrophils, monocytes and macrophages to the tissue to deal with the potential infection. The endometrial cell line EEC-1 can produce IL-8, monocyte chemotactic protein (MCP-1) and IL-6 upon stimulation with zymogen and flagellin (TLR 2 and 5 agonist respectively) (Schaefer et al. 2004). The antiviral responses of endometrial cell lines mediated by TLR 3 have also been studied through the stimulation of the RL95-2 and Ishikawa cells with poly I:C, inducing IL-8 secretion and an IFN type 1 response by the secretion of IFN-β (Fahey et al. 2005; Jorgenson et al. 2005). Murine primary EECs also express all the TLRs. The stimulation of TLR 1/2, TLR 3, TLR 4 and TLR 2/6 in these cells are induce the production of MCP-1 (Soboll et al. 2006).

An exacerbated activation of TLRs during pregnancy has been observed to bring negative consequences and to even induce pre-term labour. The i.p. administration of LTA, LPS, PGN and Poly I:C (ligands for TLR 1/2, TLR 4, TLR 2/6 and TLR 3 respectively) to pregnant mice increases the chances of pre-term labour and the production of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and deficiencies in the vascularisation of the tissue (Kajikawa et al. 1998; Clark et al. 2004; Ilievski et al. 2007; Ilievski & Hirsch 2010; Koga et al. 2009; J. Zhang et al. 2007; Friebe et al. 2011).

1.3.3 Female reproductive tract infections and infertility

About 40-50% of the infertility cases in couples are related with female problems. In recent years, the incidence of these problems has increased proportional to the rate of infections by sexually transmitted diseases (STD) and the number of sexual partners (Pernoll 2001).

Infertility factors can be congenital (related with structure abnormalities) or acquired (related to infections and surgical treatments). These are classified in cervical factors, uterine-tubal factors, ovulatory factors and peritoneal or pelvic factors (Speroff & Fritz 2004). Within the infections of the URT we can find those related to the endometrium (endometritis), uterine wall (myositis), fallopian tubes (salpingitis), ovary (oophoritis), uterine serosa and broad ligaments (parametritis) and pelvic peritoneum (peritonitis).

99% of the URT infections follow a intraluminal entrance of pathogens in which they have accessed the FRT through the colonisation of the vagina, then they have infected the cervix and finally accessed the URT organs (Pernoll 2001).

One of the principal STD pathogens involved in these infertility problems is the gram-negative intracellular bacterium *Chlamydia trachomatis*. The detection and treatment of the *C. trachomatis* infections is difficult because between the 50-70% of the cases are asymptomatic (Carey & Beagley 2010). The infection is intraluminal and its complication in the URT can generate pelvic inflammatory disease (PID), which can lead to infertility problems. From the uterus it can reach the fallopian tubes (salpingitis) where it can cause tubal infertility (Pernoll 2001).

Another pathogen associated with infertility problems in women is *Trichomonas vaginalis*. It is the most common STD reaching epidemic levels with about 8-10 million cases in the United States and 11 million cases in Europe (Mcclelland 2008). *T. vaginalis* is an extracellular flagellated single cell parasite that colonises the vaginal epithelia and can persist asymptomatic for several years. It can colonise the URT causing endometritis, atypical PID, cervical cancer and infertility (Fichorova et al. 2006).

Some other infertility cases are those associated with genital micoplasma. *Mycoplasma hominis* and *Ureoplasma urealyticum* are strongly associated with cervicitis, endometritis, PID, salpingitis and infertility. These microorganisms are usually found associated with other pathogens of the URT in infertility cases and the prevalence of single infections causing infertility is low (Pellati et al. 2008).

Neisseria gonorrhoeae is a gram-negative diplococcus and is one of the most common STD pathogens. The primary infections reside in the endocervix and in the urethra. In 20% of the cases, it ascends into the URT causing endometritis, salpingitis, peritonitis and PID with possibilities of generating ectopic pregnancies, chronic abdominal pain and infertility (Pellati et al. 2008). The infections *N. gonorrhoeae* are commonly related with co-infections with *C. trachomatis* (Pernoll 2001).

The FRT must deal with the infections of these pathogens to return to homeostasis. The innate immune system of the tract is the first line of defence against infections. It is capable of recognizing these pathogens and acting in response with effector mechanisms to try to eliminate them.

1.4 Hypothesis and aims of the study

The implantation of the embryo in the uterus is a complex process in which a direct line of communication between the mother and the embryo must be established. The establishment of an optimum environment in this embryo maternal interface characterised by the presence of cytokines, chemokines, hormones, growth factors and adhesion molecules is vital for the success of the implantation process.

For this reason the main hypothesis of this work is that the activation of the endometrial TLR 2 would negatively affect the function of the endometrium at the time of implantation.

As for the particular aims of the work we followed a sequential set of aims trying to answer the main experimental question:
In chapter two, we aim to explore the *in vivo* effects that the endometrial TLR activation could inflict on the endometrial-embryonic interaction in a murine model.

On chapter 3, using an *in vitro* model of human endometrial-trophoblast interaction our aim was to describe how the activation of the endometrial TLR 2 and TLR 2/6 can be detrimental for the interaction with the trophoblasts.

On chapter 4, we aim to explore if the cell membrane morphology could be affected by the TLR stimulation. We used of atomic force microscopy for a topological characterisation and explored the membrane profile of different endometrial cell lines and a breast carcinoma cell with a powerful antibody panel to identify 23 different membrane surface proteins by flow cytometry.

On chapter 5, we aimed to study the role of the membrane on the lack of endometrial adhesiveness after TLR stimulation through the expression of two anti-adhesive mucins for the implantation of the embryo, MUC1 and MUC16.

Finally on chapter 6 our aim was to explore the capacity of the endometrial cells to activate the transcription factors NF-κB and AP-1 in response to the activation of different TLRs.

Together these results indicate that the activation of the endometrial TLRs can affect multiple elements involved in endometrial receptivity. These effects could be observed *in vivo* and *in vitro* affecting cell morphology, protein expression and transcription factor activation.

Chapter 2. The *in vivo* effects of TLR 2/6 activation on the uterine horn morphology and murine embryo implantation

2.1 Introduction

Embryonic implantation is a critical event leading to a successful pregnancy (Dekel et al. 2010). The implantation process requires a complex orchestration of cellular and molecular events that include: expression of adhesion molecules, remodelling of the uterine extracellular matrix and an intricate crosstalk of hormones, cytokines and growth factors between the embryo and the endometrium (Lessey 2000; Dimitriadis et al. 2010; H. Singh & Aplin 2009; Paria et al. 2000; McEwan et al. 2009). The immune system plays an important role in the modulation of the mechanisms involved in implantation. Pregnancy represents an immunological contradiction in which a semi-allogeneic foreign entity, the embryo, is not rejected by the maternal immune system but accepted and nourished. This is achieved by several mechanisms, including the modulation of the maternal immune system by the pre-implantation embryo (Walker et al. 2010). Consequently, any interference that may imbalance the immune system responses could result in embryo loss and infertility. In fact, many infertility problems in women are associated with infections of the URT compartments: endocervix, fallopian tubes and uterus. The pathogens responsible for STD such as *Mycoplasma genitalium, Neisseria gonorrhoeae* and *Chlamydia trachomatis* have been associated with infertility in women (Dekel et al. 2010; Pellati et al. 2008).

The FRT is able to respond against these pathogenic entities and initiate an immune response. The first recognition of pathogens in the FRT is mediated by the innate immune system of epithelial, resident dendritic and NK cells (Dimitriadis et al. 2010; Hickey et al. 2011; Walker et al. 2010). This innate response will later prime and instruct the adaptive immune system to initiate cellular and humoral responses against pathogens (Walker et al. 2010; Hickey et al. 2011; Wira et al. 2005). In order to detect the potential pathogens, endometrial epithelial cells express PRRs to common PAMPs. One of the main families of PRRs is TLR family (Hickey et al. 2011; Kawai & Akira 2010). They can recognize a great variety of PAMPs from bacterial, fungal, parasitical and viral origin. The TLR family is formed by 10 members where each respond to a specific ligand that promotes the secretion of a different subset of cytokines and chemokines (Horne et al. 2008; Aboussahoud, Aflatoonian, et al. 2010b; Fazeli et al. 2005; Koga & Mor 2008). TLR 2 and its heterodimers with TLR 1 and TLR 6 can sense a great variety of PAMPs from pathogens that might be present in the FRT and can be

activated by PGN from bacteria (*C. trachomatis, Staphylococcus aureus, N. gonorrhoeae, M. genitalium* lipoproteins) (O'Connell et al. 2006), yeast (*Candida albicans* phospholipomannan) (Gil & Gozalbo 2006) and parasites (*Trichomonas vaginalis* lipophosphoglycan) (Fichorova et al. 2006). The heterodimer of TLR 2 and TLR 6 has been found to recognize the macrophage activated lipoprotein derived from *Mycoplasma fermentans* (MALP-2) (Buwitt-Beckmann et al. 2005). FSL-1 is a specific and potent ligand for the heterodimer TLR 2/6 and was synthesized based on MALP-2 structure by changing its amino acid sequence (Okusawa et al. 2004). Upon this recognition, they initiate signalling pathways that end up in the activation of the transcription factors NF-κB or AP-1 that promote the activation of pro-inflammatory genes (Brown et al. 2011).

The expression of TLRs 1-10 has been observed in mammalian uterus and in different human endometrial epithelial cell lines (Aboussahoud, Aflatoonian, et al. 2010b). This expression follows the menstrual hormonal cycle. The highest level of TLR expression in the human endometrium has been observed during the late secretory phase, where implantation of the embryo takes place (Aflatoonian et al. 2007). Although TLRs have been observed to be involved in a number of pregnancy disorders such as preterm labour, early pregnancy loss and pre-eclampsia (Koga & Mor 2010), not much is known about their function during embryo implantation. Recent results from our laboratory have shown that activation of TLR5 by its ligand flagellin can interfere with trophoblast spheroids adhesion to epithelial cells in vitro (Aboussahoud, Bruce, et al. 2010a).

The study of the human embryo implantation process in vivo is limited due to the anatomical and the physiological restrictions and the ethical and the legal issues surrounding experimentation with human embryos. For these reasons, research on the embryo implantation has opted to employ different animal model species. Although the implantation of different mammals can vary on the degree of invasiveness; they all follow an initial phase of interaction with the maternal tract. The human embryo implantation and placentation is invasive. The primate embryo implantation is the closest model to human (Sharkey & Smith 2003) but the manipulation of these species is also limited due to ethical responsibility. It is for this reason that the murine model has been chosen to study implantation due to the similarities that shares with human embryo implantation and a certain degree of invasiveness (H. Wang & Dey 2006). It

also offers many advantages like the possibility to manipulate their gametes and oestrous cycle (between 4 to 5 days) and a short gestation (Approx. 3 weeks) (Silver 1995). Implantation of the murine embryo will take place on metestrus (day 4.5) the equivalent of the late secretory phase in human.

In the current investigation, we determined if the activation of the TLR 2 could influence the implantation of the embryo. We used an in vivo murine embryo implantation model, to assess if the activation of TLR 2/6 in the UH may affect uterine morphology and secretory profile during implantation period as well as embryonic implantation.

The experiments in this chapter has been published in the paper *Local activation of uterine toll-like receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity in mice* by the journal *Biology of Reproduction* (Sánchez López et al. 2014).

2.2 Materials and Methods

2.2.1 Embryo transfer experiments

2.2.1.1 Mice superovulation and mating

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

2.2.1.2 Foster mice preparation

The foster CD1 mice were obtained from Harlan Laboratories. The females bedding was replaced with male bedding to initiate the oestrous cycle. At the start of the estrus phase of the cycle (two days later), the females were mated with vasectomized males to induce the pseudo-pregnancy state and prepare the reproductive tract to receive the

embryos. The foster pseudo-pregnant mice were selected by the presence of a vaginal plug. On day 3.5 post-mating, females were anesthetized by inhalation of Isofluorane and kept on a warm plate during the surgery. The left uterine horn was exposed and the top of the utero-tubal junction was carefully pierced with the tip of a 30G needle where a transfer pipette loaded with the blastocysts was inserted. The pipette reached the uterine horn (UH) through the opened utero-tubal junction and delivered the blastocysts. The foster mice were kept in separate cages with tags, identifying the date of embryo transfer, number of embryos transferred and treatment. Food and water were supplied ad libitum.

2.2.2 Histological evaluation of the murine UHs

2.2.2.1 Sample collection and storage

The foster mice were euthanized by cervical dislocation. UHs were dissected and placed in petri dishes. The UHs were stored in 10% paraformaldehyde and dehydrated in a series of ethanol dilutions (70, 90 and 100%) for fixation. Finally UHs were sectioned in two and embedded in paraffin. The paraffin blocks were cut in transversal sections of 4 μm using a microtome and fixed to normal microscope slides. The sections on the slides were then stained using the conventional Haematoxylin & Eosin (H&E) method. Briefly, wax was removed by rinsing twice in xylene for 5 min. Tissue was hydrated in series of ethanol dilutions (Twice in 99, 95 and 70%) and tap water for 5 min. The slides were stained with Gill II Haematoxylin (VWR, Lutterworth, UK) for 1 min and rinsed with tap water for 3 min. The slides were stained in 1% aqueous Eosin (VWR) with 1% Calcium Carbonate (Sigma) for 5 min and rinsed in tap water for 30 sec. The sections were dehydrated in serial dilutions of ethanol (70 and 95% for 10 sec and twice 99% for 30 sec) and twice in Xylene (1 and 3 min). Finally the coverslip was fixed with DPX mountant (VWR). The cross sections in the slides were imaged using light microscopy (Olympus CKX41; Southend-on-sea, UK) and captured with the NIS elements software (Version F3.0 SP4; Nikon, Surrey, UK). The changes of the development of the uterine decidua due to estrus cycle were observed.

2.2.2.2 Morphometric analysis of the UH

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

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

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

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

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

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

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

$$G_i = nG \times E_i$$

2.2.3 Cytokine measurement

2.2.3.1 Sample collection and storage

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

2.2.3.2 Cytokine Arrays

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

2.2.3.3 Cytokines and chemokine quantification

The chemokine MCP-1 and cytokine IL-1β were evaluated in the UH flushing samples by flow cytometry using a Cytokine Bead Array (CBA, BD Biosciences, Oxford, UK). Briefly, 50 µl of the flushed media samples were incubated with 50 µl of different cytokine detection beads mix, 50 µl of the Phycoerythrin (PE) detection reagent and incubated for 3h. The samples were washed with 300 µl of the provided wash buffer and centrifuged at 200 x g for 5 min. The pellets were re-suspended in 300 µl of wash buffer and analysed in a FACSArray cytometer (BD Biosciences). The cytokine IL-1RA was measured using an ELISA kit (PeproTech; London, UK). Briefly 100 µl of the flushed media samples were incubated at room temperature in the anti-IL-1RA antibody pre-coated 96 well plate for 2h. The plate was rinsed 4 times with washing buffer and 100 µl of the secondary biotinylated antibody (500 ng/ml) were added and co-incubated

for 2 h. The plate was rinsed 4 times with the washing buffer and 100 µl of the avidin peroxidase (1:200) were added and incubated for 30 min. Finally the plate was rinsed 4 times with washing buffer and 100 µl of the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) liquid substrate were added. The optical density was measured in a plate reader at 405 nm with wavelength correction set at 650 nm.

2.3 Experimental Design

2.3.1 Evaluation of the murine UH after TLR 2/6 activation

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

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

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

The Swiss foster mice were prepared for embryo transfer as mentioned above. The utero-tubal junction of the left UH in each mice was pierced with a 30G needle to make an opening and using a transfer pipette infused with a drop of 10 µl of saline solution

(n=15) or FSL-1 (0.1 μg/μl; n=16) followed by a gap of air and a drop containing the 12 blastocysts. After 15 days the foster mice were euthanized by cervical dislocation. The uterine horns were dissected and the number of embryos implanted and foetuses resorbed was registered (Fig. 2.1).

2.3.2 Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). The comparison of two experimental groups was performed with the non-parametric Mann-Whitney U test. The individual cytokine measurement was log transformed to normalize distribution. Multiple groups were compared with a one-way ANOVA and a Bonferroni multiple comparison post-test. $P \leq 0.05$ was considered to be significant.

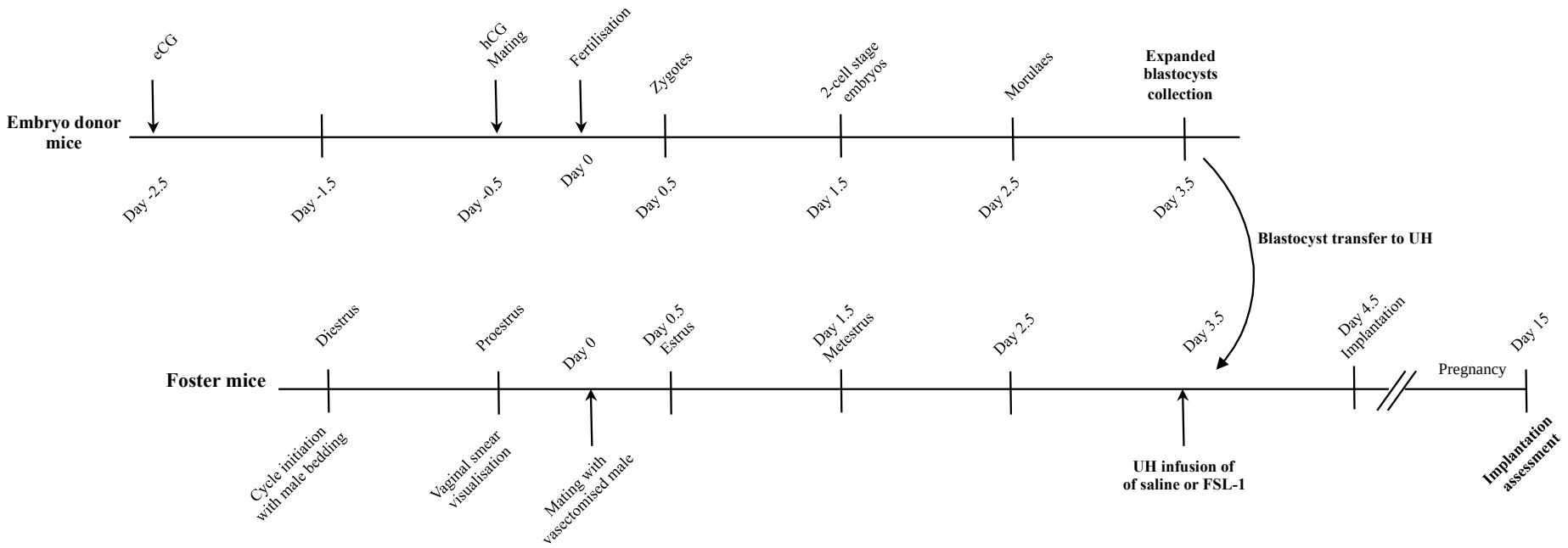

Figure 2.1. Experimental design of the embryo transfer and stimulation procedures. Time line of super-ovulation of embryo donor mice, foster mother preparation, administration of FSL-1 and blastocyst transfer. eCG (Equine Chorionic gonadotropin), hCG (Human Chorionic Gonadotropin) and UH (Uterine horns).

2.4 Results

2.4.1 FSL-1 administration induced morphological changes in the UH

The saline treated UHs on the pre-implantation day (4h post-treatment) retained the morphology of a normal UH. The myometrium was dense and endometrial stroma filled with secretory glands. The endometrial lumen was pseudo-stratified and convoluted, showing a small luminal space. Finally, an abundant secretory activity in the endometrial luminal cells and glands could be observed (Fig. 2.2a). In contrast to this, the FSL-1 treated UH presented an atrophic morphology. The thickness of the myometrium and stroma was reduced as well as the number of glands observed. The FSL-1 treated epithelial endometrial cells morphology changed from columnar pseudo-stratified to simple columnar/cuboidal shape. Nevertheless, some secretory activity could still be observed (Fig. 2.2a). The *Mi*, *Ei* and *Gi* were significantly higher in the saline group compared to the FSL-1 treated group (Fig. 2.2b). In contrast, the lumen was smaller and more convoluted in the saline than in the FSL-1 treated (Fig. 2.2b). In controls, the morphology of the UHs on the implantation day (24h post-treatment) was similar to the ones at estrus. In addition to this, the stroma was loose and filled with edema, a characteristic of a decidualised endometrium. The presence of vacuoles in the cytoplasm of luminal and glandular epithelial cells confirmed a high secretory activity at this stage. The 24 h FSL-1 treatment induced an atrophia of the UHs decidua (Fig. 2.3a). This could be observed in the reduction of the *Mi* and *Ei* of the FSL-1 treated UHs compared to the saline treated UHs (Fig. 2.3b).

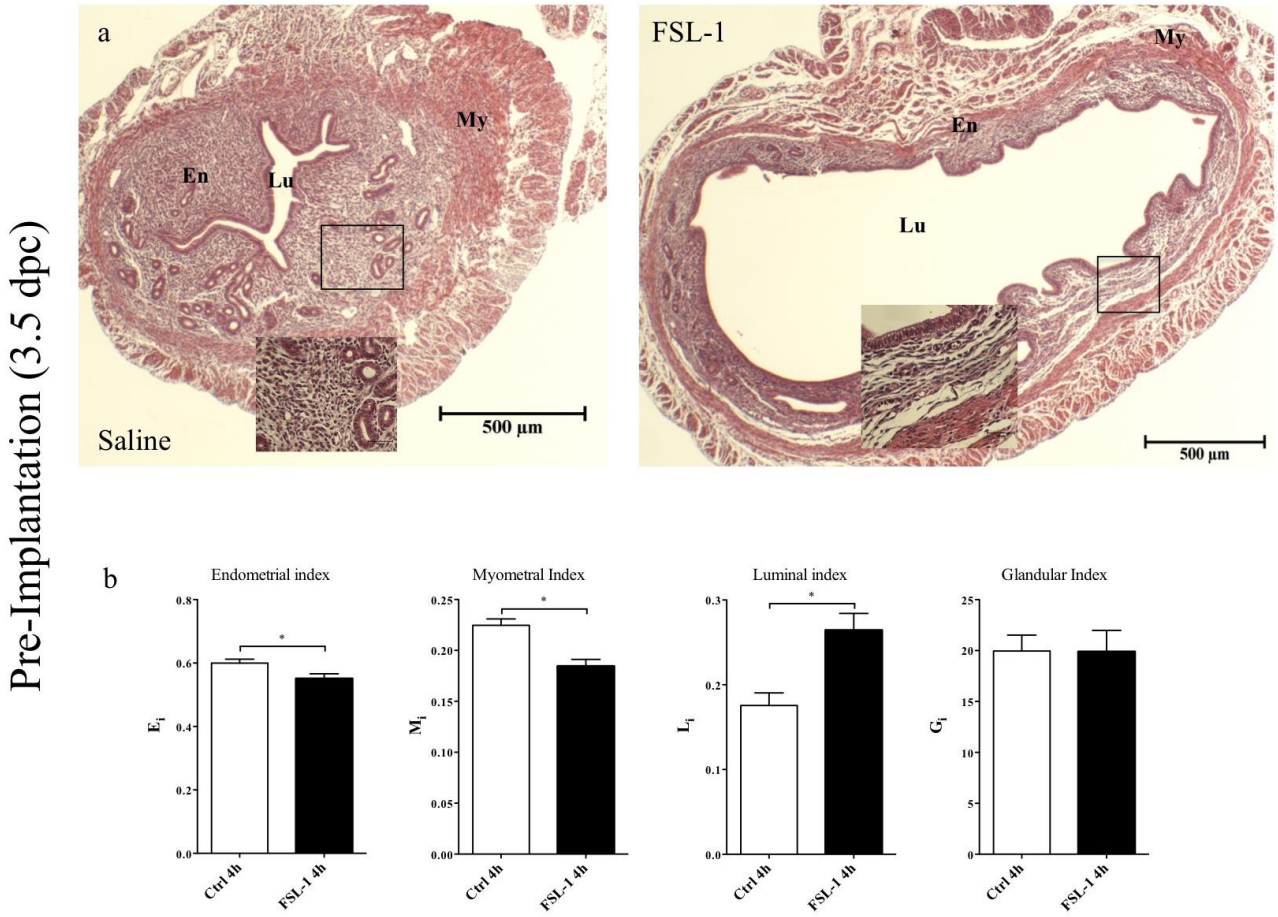

Figure 2.2. Effect of the TLR 6 activation on the murine uterine horn morphology on the Pre-Implantation day. Uterine horn morphology and morphometry after TLR 2/6 stimulation. Cross-section of the H&E stained murine UH treated with saline solution or FSL-1 (0.1 µg/µl) after 4h on pre-implantation day (a). Images were taken at 40x and 400x magnifications. Morphometric analysis of the different layers of saline and FSL-1 treated UHs: endometrium (Ei), myometrium (Mi), lumen (Li) and glands (Gi) on pre-implantation (b). The bars are representative of the mean ± SEM from 10 cross-sections of each UH. The statistical comparisons between saline control and FSL-1 treatments were performed with a Mann Whitney U-test where * p≤0.05. Lu: Lumen, En: Endometrium and My: Myometrium. As published before **(Sánchez López et al. 2014).**

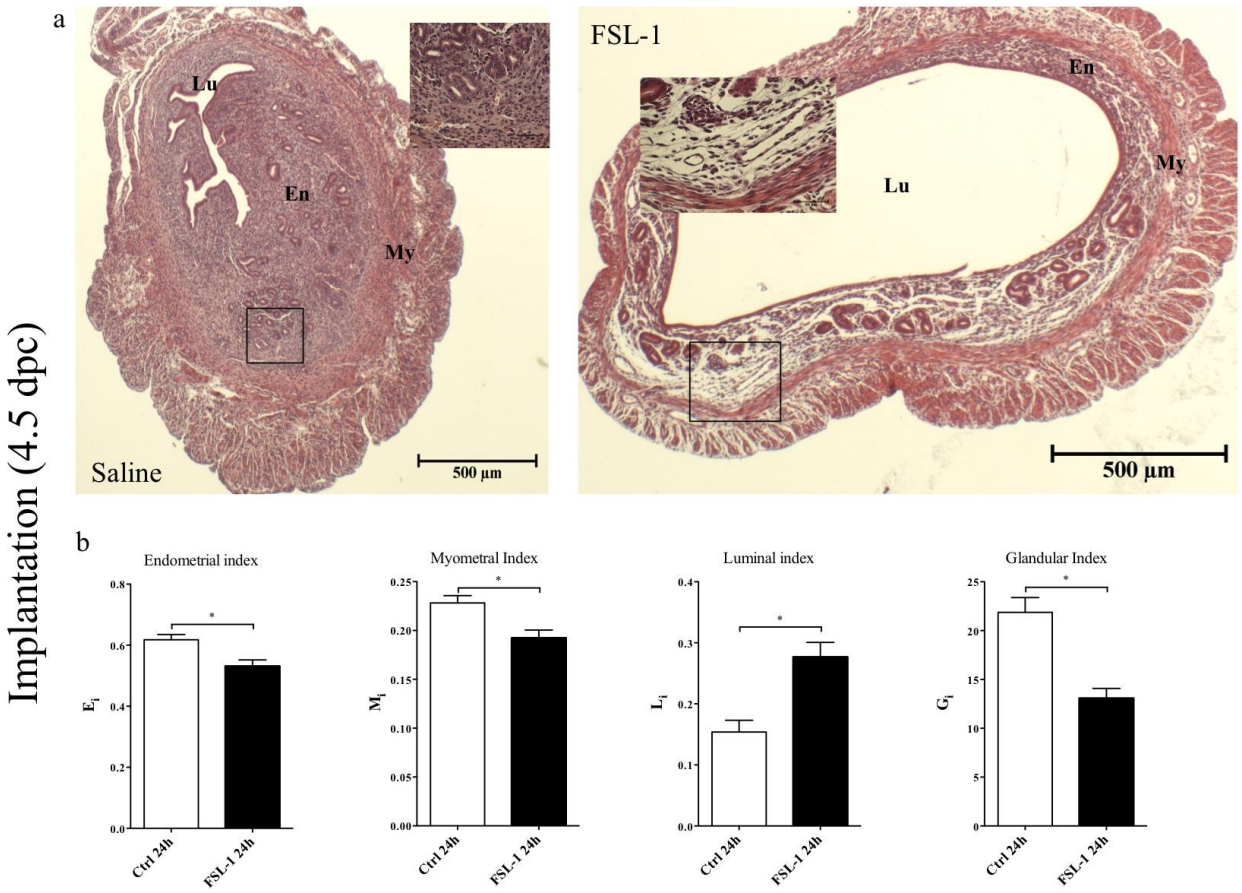

Figure 2.3. Effect of the TLR 6 activation on the murine uterine horn morphology at the Implantation day. Uterine horn morphology and morphometry after TLR 2/6 stimulation. Cross-section of the H&E stained murine UH treated with saline solution or FSL-1 (0.1 µg/µl) after 24h on implantation day (a). Images were taken at 40x and 400x magnifications. Morphometric analysis of the different layers of saline and FSL-1 treated UHs: endometrium (Ei), myometrium (Mi), lumen (Li) and glands (Gi) on pre-implantation (b). The bars are representative of the mean ± SEM from 10 cross-sections of each UH. The statistical comparisons between saline control and FSL-1 treatments were performed with a Mann Whitney U-test where * $p \leq 0.05$. Lu: Lumen, En: Endometrium and My: Myometrium. As published before **(Sánchez López et al. 2014).**

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

Sixteen proteins were detected 4h after treatments and 17 proteins were detected 24h after treatments in the cytokine array. The FSL-1 treatment of the UH on the pre-implantation day, increased the production of TNF-α, TIMP-1, MCP-1, M-CSF, KC, IL-16, IL-1ra, IL-1β and IFN-γ (Fig. 2.4a). From these proteins, the highest difference between FSL-1 and saline treatments was observed in MCP-1, IL-1ra and IL-1β. FSL-1 treatment for 24 hrs increased significantly the production of MCP-1, IL-17, IL-16, IL-13, IL-7, IL-4, IL-1ra, IL-1β, IL-1α and C5a (Fig. 2.4b). Based on these analyses, each individual sample was analysed again by CBA for the production of IL-1β, MCP-1 and by ELISA for IL-1RA. The FSL-1 treatment of the tissue for 4h considerably increased the production of IL-1β, MCP-1 however the change in IL-1RA production was minimal (Fig. 2.4c). The production of IL-1β and MCP-1 after 24h of FSL-1 treatment was lower and no difference was observed between the different treatments. In contrast, this treatment significantly increased IL-1RA production (Fig. 2.4d).

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

The activation of TLR 2/6 with FSL-1 severely reduced the number of total implantations in the UH compared to the saline treated horns (45.00 ± 6.49 vs 16.69 ± 5.01%, $p < 0.05$; Fig. 2.5a). The number of total implantations included the embryos that developed to foetuses and those that were resorbed (implanted embryos that could not develop; Fig. 2.5b). The percentage of developed foetuses decreased after the administration of FSL-1 to the UH (45.56 ± 6.79 vs 26.04 ± 6.84%, $p < 0.05$; Fig. 2.5c). On the other hand, we observed an increase in the percentage of resorbed foetuses in the FSL-1 treated group compared to the saline group (4.44 ± 1.79 vs 14.17 ± 3.45%, $p < 0.05$; Fig. 2.5d).

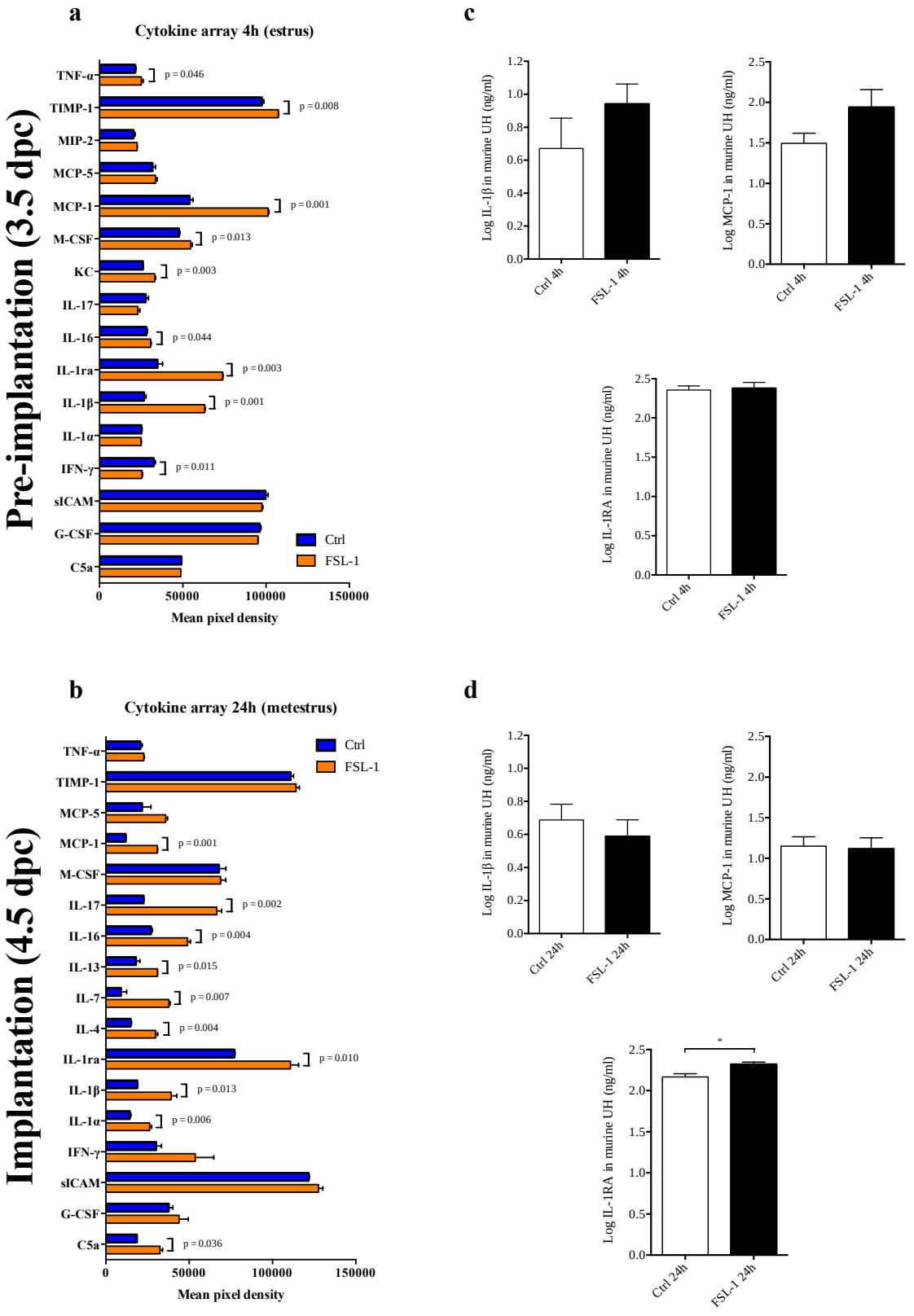

Figure 2.4. Expression of cytokines in the murine uterine horn after TLR 6 stimulation. Semi quantitative profiling of the luminal lavages after 4 (a) and 24 h (b) of saline or FSL-1 treatment (0.1µg/µl). Quantification of the production of IL-1β, MCP-1 and IL-1RA after 4 (c) or 24 h (d) of saline (white bars) or FSL-1 (black bars) treatment. The bars on the array represent the mean ± SEM of two technical replicates of the pooled flushing samples of saline (n=10) or FSL-1 (n=16) treated UHs. The bar charts of IL-1β, MCP-1 and IL-1RA represent the mean ± SEM of the logarithmic concentration of the different samples of the UH flushed media. Statistical differences between treatments were assessed using Mann Whitney U-test (*p≤0.05). As published before **(Sánchez López et al. 2014).**

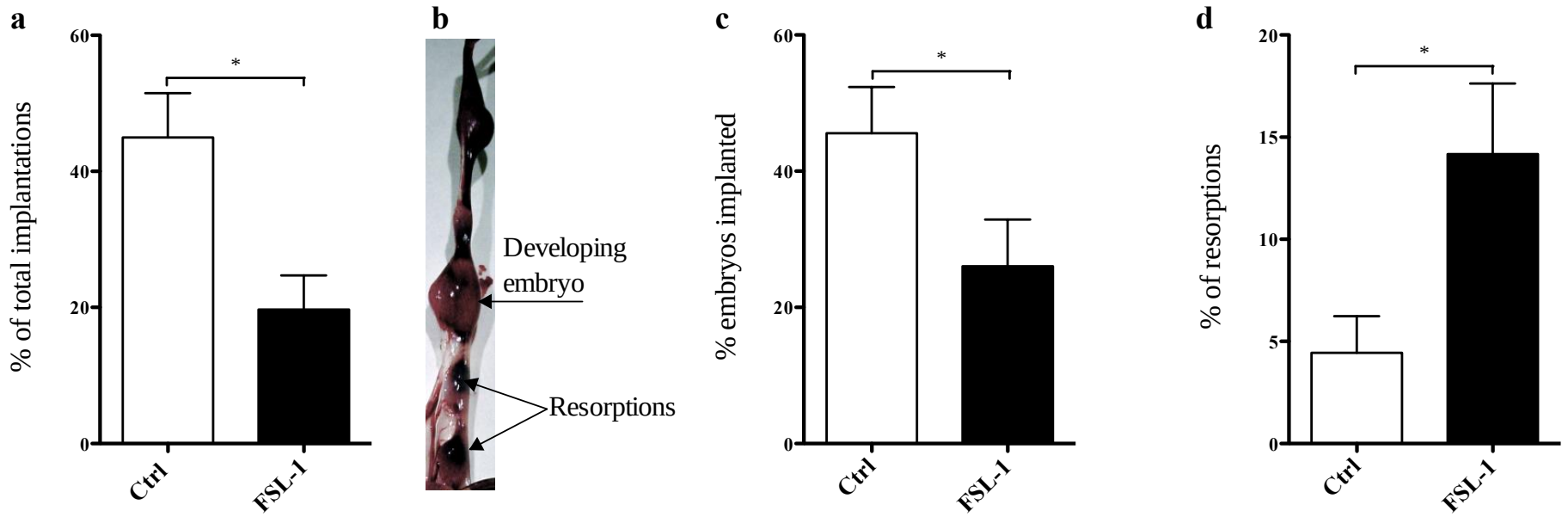

Figure 2.5. Effect of the TLR 6 stimulation of the murine uterine horns on embryo implantation. The percentage of total embryos implanted was assessed on day 16 on the control (n=15) and FSL-1 (0.1µg/µl; n=16) treated foster mothers (a). Representative image of implanted and resorbed embryos in the murine UH (b). The percentage of embryos implanted (c) and embryos resorbed (d) was estimated and compared between control and FSL-1 treated UHs. The bars are the mean ± SEM of the control and FSL-1 treated foster mice. The statistical comparisons between treatments were performed using a Mann Whitney U-test (*$p \leq 0.05$). As published before **(Sánchez López et al. 2014).**

2.5 Discussion

Embryo implantation is a complex event initiated by the adhesion of the trophectoderm to the endometrial epithelial cells. This is followed by the trophoblast invasion into the uterine decidua. Two major factors are involved in a successful implantation, a good embryo quality and proper endometrial receptivity. Any disturbance in the intricate crosstalk of hormones, cytokines, and adhesion factors during this critical period of time could lead to implantation failure (Norwitz et al. 2001; M. Singh et al. 2011).

In our study using an in vivo murine model, we explored the characteristics of the UH on pre-implantation and implantation day. We were able to create an inflammatory environment in the UH by stimulating the innate immune system via TLR 2/6 ligand and observe how it could affect the tissue structure and impair embryo implantation. The inflammatory insult in the UH strongly affected the architecture of the different layers of the tissue. This effect could be observed as soon as 4 h of the FSL-1 administration, when the endometrium is remodelling for suitable embryo receptivity. This might indicate that the epithelial and stromal cells of the uterus are sensitive to innate immune activation via TLR stimulation as a protective mechanism for the mother. Stromal cells have been found necessary to mediate immune responses in different body tissues. They offer a substrate for the migrating leukocytes and orchestrate part of the adaptive immune responses (Sato & Iwasaki 2004). During embryo implantation, the uterine stromal cells provide a scaffold for migrating cytotrophoblasts and establishment of the spiral arteries. The stromal sensitivity to respond to inflammatory stimuli has been studied, where administration of i.p. Poly I:C (TLR 3 ligand) to mice affects stromal decidualisation and vascularisation of the uterus (J. Zhang et al. 2007). In our experiments the innate immune activation was performed via FSL-1 which is a synthetic diacyl lipopeptide specific ligand for TLR 2/6 (Shibata et al. 2000). It is possible that the effect on the tissue remodelling will occur regardless of the type of TLR activated. In fact, the capacity of the endometrial cells to react to foreign entities has been assessed previously where the exposure of the UH to the toxic compound di-(2-ethylhexyl)-phtalate (DEHP) is able to affect tissue decidualisation and impair embryo implantation (R. Li et al. 2012). The current methods to evaluate the estrogenic effects of toxic compounds on the UH is based on the uterotrophic assay developed in the 1930s. It estimate the weight of the horns after treatment and gives a

description of the morphological characteristics of the uterine layers and cells (OECD 2007). In humans, the criteria to date the endometrium based on pathologist observations were established by Noyes in the 1950s. It estimates scores for the different morphological changes of the endometrial characteristics in gland, epithelia and stromal development due to the phase of the cycle (Fadare & Zheng 2005). In our morphometry assessment, we were able to measure accurately the areas of the different UH compartments with the help of the digital imaging tools. Also the possible differences due to the horn size and sampling along the length of the horn were normalized with the calculation of the indexes. This technique can potentially be used as an effective method of estimating toxic effects of chemical compounds on the uterine horn of different mammalian species, although more standardisation in its validity is required.

The implantation as mentioned before is an agreement between the maternal tract and the embryo. Cytokines produced by the maternal tract are fundamental effectors, which also peculiarly resemble an inflammatory reaction. By profiling the UH secretions, we were able to observe a disruption in the cytokine levels when the uterine TLR 2/6 was activated. After 4 h of stimulation (pre-implantation day) three proteins were up-regulated: MCP-1 (CCL2), IL-1β and IL-1ra. The three of them share important roles in both embryo implantation and in inflammation. The stimulation of the endometrium epithelia with IL-1β has been found induce the expression of endometrial adhesion molecules necessary for embryo apposition and adhesion (Geisert et al. 2012). MCP-1 has been proven to be inducible by in vitro stimulation of TLR 2/6 in murine uterine epithelial cells and TLR4 in uterine epithelial and stromal cells (Soboll et al. 2006). The increase in IL-1RA production after 24 h of TLR 2/6 stimulation could interfere with the embryo – mother crosstalk antagonizing IL-1β. The i.p. administration of IL-1RA to mice in the pre-implantation period reduces drastically embryo implantation (Simón et al. 1998). After 24 h of FSL-1 treatment we observed a distinctive change in pattern of expression of cytokines towards a response type Th17. This response is characterized by the production of IL-17 by T helper cells primed by active innate immune cells. The Th17 response can lead to autoimmune diseases, chronic inflammatory disorders and infectious diseases (Qu et al. 2013). In the endometrium they have been associated with cases of unexplained infertility (Nakashima et al. 2010; Jasper et al. 2006) and in response to C. muridarum, the murine homologue of *C. trachomatis* (Scurlock et al.

2011). This pathogen can initially be recognized by TLR 2 prior to an adaptive response (O'Connell et al. 2006).

The induction of the inflammatory effect was observed in the UH secretions as soon as 4 h after exposure to TLR 2/6 ligand as an increase in IL-1β and MCP-1 production. From a non-inflammatory perspective MCP-1 has been proven to be inducible by in vitro stimulation of TLR 2/6 in murine uterine epithelial cells and TLR4 in uterine epithelial and stromal cells (Soboll et al. 2006). One of the most remarkable observations of our study was the increase in IL-1RA production after 24 h of TLR 2/6 stimulation. It is likely that this overproduction on the implantation day could interfere with the embryo – mother crosstalk as IL-1RA antagonizes IL-1β. A crucial role of this cytokine in implantation has been demonstrated before. The i.p. administration of IL-1RA to mice in the pre-implantation period reduces drastically embryo implantation (Simón et al. 1998). The importance of these cytokines for implantation is more apparent as their secretion is regulated by menstrual cycle without the presence of an inflammatory insult (Hickey et al. 2013). It is during the estral phase in mouse or late secretory phase in human when their production in the uterus increases, which is also the phases where the endometrium changes for the arrival of the embryo. For this reason, a safe environment should be guaranteed. The balance of the mediators of implantation is also delicate. From one side pro-inflammatory factors as IL-1β mediates the implanting proces, but from the other, a tolerance to the trophoblasts of the blastocyst must exist. Studies have proposed that IL-10 participates in this tolerance as KO mice for it, have an increased trophoblast invasiveness and increased placentation and vascularisation (C. T. Roberts et al. 2003). The implanting trophoblasts and uNK cells participate actively in this tolerance as both are a source of IL-10 in the uterine stroma to modulate the infiltration and avoid the NK cytotoxic response against trophoblasts (Viganò et al. 2001). The influence of IL-10 can be observed not only in the implantation environment but also in the tolerance during pregnancy by T regulatory cells (Tregs). High levels of circulating IL-10 are found in blood and Tregs during pregnancy highly express IL-10 receptor, which contributes for their survival. This is suggested to be benefitial during pregnancy and for the future regulation of the immune system and development of tolerance and allergies (Santner-Nanan et al. 2013). 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 result to adversely affect the implantation of the embryo.

The reduction of embryo implantation confirmed a direct effect of the activation of uterine TLR 2/6 on embryo implantation. This activation was able to affect the development of the uterine tissue and the secreted protein profile necessary for implantation. As observed, the lack of a receptive epithelium to adhere would affect the apposition and adhesion of the blastocyst. It might be possible that the molecular characteristics of the endometrial cells could be also affected by this stimulation. It is unlikely that the treatment of the UH with the TLR 6 agonist could harm the embryos and could be the reason of embryo implantation loss. A previous report has demonstrated that the first trimester trophoblast lack the expression of TLR 6 and stimulation does not change blastocyst viability (Abrahams et al. 2008). The FRT has been proved to be sensitive to TLR stimulation in vivo, for example the infusion of LPS to the murine UH induces a TLR 4 dependent inflammatory pelvic disease (Sheldon & M. H. Roberts 2010). It has also been observed that the administration of i.p. LPS to mated mice on day 5.5 could increase foetal loss (Friebe et al. 2011). The atrophic stroma observed after TLR 2/6 stimulation, would offer a deficient soil for the embryo to invade and start its development. Similar to this study, it has been observed that administration of i.p. Poly I:C, TLR 3 ligand can decrease the implantation rate and increase the percentage of resorbed embryos by impairing stromal decidualisation and spiral arteries development (J. Zhang et al. 2007). Hence, the effect of the impairment of the uterine decidua and stromal cells and maybe also the development of vascular tissue is likely to be a contributing factor for the increase in embryo resorption observed in our investigation. a potential reason for the low success rate of implantation after embryo transfer is the simple manipulation of the uterine horn or endometrium during embryo transfer procedures in human or livestock that could damage the tissue. This damage could induce a sterile inflammatory process by the release of DAMPs molecules that are also recognized by the TLRs, initiating an inflammatory process and leading to embryo implantation failure. A potential way of clarifying these effects on the murine UH and a future scope of our research is to use a TLR6 KO mouse. In this way we would be able to test the effects of TLR activation on both tissue structure and embryo implantation.

Clinically, endometrial TLR 2 might be playing a very important role in the recognition of pathogens that cause STDs and infertility. The most common bacterial transmitted STD caused by the intracellular obligated gram-negative pathogen *C. trachomatis*. Chlamydia can cause a cervical infection that if ascends into the upper tract can generate PID and infertility (Weiss et al. 2009). Chlamydia can be recognized by TLR 2 in the intracellular inclusions and induce the production of IL-8 (O'Connell et al. 2006). Furthermore this recognition and the initiation of the immune responses is dependent on TLR 2 and not TLR 4 and mice lacking TLR 2 showed a reduced oviductal inflammation (Darville et al. 2003).

Determining the suitable endometrial conditions for the implantation of the embryo is a topic under constant research (Lessey 2000; Dimitriadis et al. 2010; Paria et al. 2000; McEwan et al. 2009; M. Singh et al. 2011). The correct diagnose of infertility can increase significantly the success of pregnancy for women with fertility problems and couples undergoing *in vitro* fertilisation (IVF) treatment. With further research on the mechanisms responsible for the effects observed here, it would become possible to design and direct a therapy targeting the endometrial innate immune system to increase the implantation success. Our data support the importance of TLRs during the time of implantation suggesting that they could be used as a potential target in management of infertility cases.

Chapter 3. Development of an *in vitro* model for embryo implantation failure

3.1 Introduction

The study of human embryo implantation represents a complicated research area due to ethical and moral implications. For this reason scientists have developed several different strategies and models for the study of the human embryo implantation. The use of primary human endometrial tissue is often employed but the limitation of the lifespan of the cells, the cellular differentiation that the cells undergo and female inter-variation makes this study more difficult. Many of the studies rely on the use of human cell lines from endometrium immortalised in different stages of their menstrual cycle so they keep the characteristics and behaviour of the endometrium like receptive endometrium (EEC-1, Ishikawa and HES cell lines), non-receptive endometrium (HEC-1A) and glandular epithelium (RL95-2 and Ishikawa) (Hannan et al. 2010). The human embryos are mainly used of in vitro fertilisation processes and have a limited access for research. For this reason human trophoblast cell lines have been developed for research simulating different trophoblast behaviour as syncytialisation (BeWo), migration and adhesion (AC1M-88, HTR-8/Svneo) and invasion (JEG-3, HTR-8/Svneo, JAr and BeWo) (Hannan et al. 2010).

The selection of the appropriate cell lines is a fundamental issue to be considered due to the differences in endometrial and trophoblast cell developmental stages (Hannan et al. 2010). In the current investigation, we decided to explore the interactions between human endometrial cells and trophoblasts an *in vitro* model of human embryo implantation. The endometrium was simulated using the RL95-2 cell line which has been reported to maintain the epithelial polarisation, express adhesion molecules and microfilaments in the apical surface and being responsive to hormones and secretion of cytokines (Way et al. 1983). The breast carcinoma epithelial cells MCF7 were also employed in our investigations to observe if the trophoblasts were able to interact with another epithelial cell line. MCF7 maintain epithelial cell characteristics and are responsive to hormonal stimulation (Levenson & Jordan 1997). Hence in our study, we employed them as another hormone responsive reproductive tissue from a non-endometrial origin. The human embryo was simulated with multi-cellular spheroids from choriocarcinoma trophoblast cell line JAr. This cell line has been employed in different studies for its ability to establish adhesive interactions with endometrial cell lines in short periods of co-incubation (Thie et al. 1998).

To complement the set of experiments performed *in vivo* in the murine implantation model, we optimized the above mentioned *in vitro* implantation model. In this way, we could assess if the activation of TLR 2 and 2/6 in the endometrial epithelial cells can affect the adhesion of the trophoblast spheroids.

As already mentioned before, the initial recognition of PAMPs is performed by the TLRs in epithelial cells or leukocytes (Dimitriadis et al. 2010; Hickey et al. 2011; Walker et al. 2010). The TLR family of PRRs plays a key role in this recognition for the initiation of immune responses (Hickey et al. 2011; Kawai & Akira 2010). The expression of these TLRs has been assessed before in the human endometrium and a diversity of endometrial cell lines. (Aboussahoud, Aflatoonian, et al. 2010b). This expression varies along the menstrual cycle with its highest expression on the late secretory phase (Aflatoonian et al. 2007). This will prepare a hospitable environment for the reception of the embryo and its further implantation and development.

In our experiments we simulated the infectious insult by activating TLR 2 and TLR 2/6. TLR 2 and its heterodimers with TLR 1 and TLR 6 can sense a diversity of pathogens responsible for STDs like *C. trachomatis, Staphylococcus aureus*, *N. gonorrhoeae*, *M. genitalium, Candida albicans* phospholipomannan or *Trichomonas vaginalis* (O'Connell et al. 2006; Gil & Gozalbo 2006; Fichorova et al. 2006). TLR 2 and TLR 6 can be specifically activated by the diacyl-lipopeptide FSL-1 (Okusawa et al. 2004). The activation will trigger a pathway that classically will promote the pro-inflammatory gene expression NF-κB or AP-1 mediated (Brown et al. 2011).

We determined if the activation of TLR 2 in endometrial epithelial cells could interfere with the binding of human trophoblast spheroids to the epithelial cells. We optimised the previously reported model where TLR 5 stimulation also decreased the adhesion of the trophoblast spheroids to the endometrial cells (Aboussahoud, Bruce, et al. 2010a).

Part of the experiments in this chapter has been published in the paper *Local activation of uterine toll-like receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity in mice* by the journal *Biology of Reproduction* (Sánchez López et al. 2014).

3.2 Material and methods

3.2.1 *In vitro* adhesion assay of trophoblast spheroids to the epithelial cell monolayer

3.2.1.1 Epithelial cell lines culture

a. Human endometrial epithelial cell line RL95-2

The human endometrial adenosquamous carcinoma cell line (RL95-2) was obtained from ATCC and employed to mimic the receptive endometrium. The RL95-2 cells were cultured in T75 flasks at 37°C in DMEM/F-12 (Sigma) supplemented with 10% of heat inactivated fetal bovine serum (FBS; Sigma), 5 μg/ml Insulin (human recombinant insulin from Gibco, cat. No. 12585-014), and 1% L-glutamine (Invitrogen) in 5% CO_2 atmosphere until confluency. The media was changed every second day till confluence of the cells. At confluence, cells were washed with Dulbecco's phosphate-buffered saline (DPBS) (Sigma) and were harvested using 1 ml of trypsin-EDTA (Sigma). The cells were incubated for 3 min at 37°C and then pelleted by centrifugation at 300x g for 4 min. Finally, the pellet was resuspended in 3 ml of media and the cells were either seeded in another T75 flask or any other container for its use. One confluent T75 flask of RL95-2 cells gives around $1x10^7$ cells.

b. Breast cancer epithelial cell line MCF-7

The Michigan Cancer Foundation (MCF)-7 cells were cultured in T75 flasks at 37°C in DMEM (F12) (Invitrogen) supplemented with 1% penicillin/streptomycin (P/S) (Sigma), 10% fetal calf serum (FCS) (Invitrogen), 160 ng/ml Insulin (human recombinant insulin from Gibco, cat. No. 12585-014), and 1% L-glutamine (Invitrogen) in 5% CO_2 atmosphere till confluency. The media was changed every second day till confluence of the cells. At confluence, cells were washed with Ca^{2+} and Mg^{2+} free Dulbecco's phosphate-buffered saline (DPBS) (Sigma) and were harvested using trypsin-EDTA (Invitrogen), the cells were incubated for 3 min and then pelleted by centrifugation at 300 g for 4 min. The pellet was washed twice and the cells were transferred to a T75 culture flask and or cell culture plates.

3.2.1.2 Formation of JAr cells spheroids

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

3.2.1.3 Adhesion of JAr spheroids to endometrial cells

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

3.2.1.4 Viability assessment of the epithelial cells

The RL95-2 and MCF7 cells were grown in 12-well plates until total confluence. The media was replaced with serum free media before they were either activated or not with PGN and FSL-1 (Invivogen, Toulouse, France). The cells were harvested with 50 µl of trypsin EDTA to detach them from the wells, collected in 500 µl of media and pelleted by centrifuging at 300 g for 5 min. The cells were then resuspended in 200 µl of PBS

and divided in two 5 ml cytometry tubes. One sample was used as autofluorescence and the other used stained with propidium iodide (PI; Life technologies, Paisley, UK) 3 µM and captured immediately. The samples were read in a FACSCalibur cytometer (BD) capturing $1x10^4$ events and the percentage of PI positive events (death cells) was registered. The results are expressed as percentage of live cells.

3.3 Experimental design

3.3.1 Standardising the JAr spheroid formation

To create multicellular spheroids from the monolayers JAr cells we used a previously reported method in which $2x10^5$ cells were cultured in a spinner (John et al. 1993; Hohn et al. 2000). To standardise the method and assess if the performance could be increased, different amount of cells were counted with Haemocytometer ($2x10^5$, $5x10^5$ and $1x10^6$ cells). The cells were seeded in 5 ml of RPMI 1640 media in 60 x 15mm Petri dishes (CellStar tissue culture dishes, greiner bio-one, GmbH/Germany). They were incubated overnight in a humid atmosphere containing 5% CO_2 at 37°C on a gyratory shaker, set at 250 rpm.

3.3.2 Influence of the number of JAr spheres on the adhesion percentage to the MCF7 monolayer

To find out if the initial amount of JAr spheroids delivered to the epithelial cells influenced final number of spheroids attached to the epithelial cell monolayer, we co-cultured 10, 30, 50, 100 and 300 JAr spheroids with the confluent MCF7 cells at 37°C for 24h. Plates were washed mildly and the number of spheroids attached was counted under the fluorescence microscope.

3.3.3 Effect of the activation of endometrial TLR 2 and TLR 2/6 on the adhesion of trophoblast spheroids

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

To determine whether TLR2 and TLR 2/6 activation is affecting the trophoblast adhesion to the endometrium; we evaluated the effect of different concentrations of PGN and FSL-1 on the endometrial epithelial cells in vitro. The RL95-2 and MCF7 cells were grown in 12-well plates until confluency. The media was replaced with serum free media before they were either activated or not with PGN and FSL-1 (Invivogen, Tolouse, France). Then the epithelial cells were stimulated with different concentrations of PGN (0, 5, 10 and 20 μg/ml) or FSL-1 (0, 1, 10 and 100 ng/ml) for 24 h. JAr spheroids were delivered on the confluent monolayers and co-incubated for 1h. Adhesion was assessed as described.

3.3.3.2 Adhesion of trophoblast spheroids with stimulated endometrial epithelial cells at different time intervals

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

3.3.3.3 Effect of the inhibition of endometrial TLR activation on trophoblast spheroid adhesion

To understand the effect of TLR2 inhibition in endometrial cells on trophoblast spheres binding to endometrial cells, the epithelial cells were either pre-treated or not with a monoclonal antibody against TLR2 (2 μg/ml, neutralizing IgA monoclonal, Invivogen) or anti-TLR6 monoclonal antibody (mouse IgG1, 10 ng/ml, Invivogen, mabg-htlr6) 1 h before the addition of PGN (10 μg/ml) or FSL-1 (10 ng/ml) respectively. After 24 h, 50 JAr spheroids were delivered on the monolayer and co-incubated for 30 min. Non-

adherent spheroids were removed and the percentage of adhered spheroids was determined.

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

To determine whether TLR 2 and TLR 2/6 activation is affecting the viability of the epithelial cells; we treated the cells with different concentrations of PGN and FSL-1. The epithelial cells were then stimulated with different concentrations of PGN (0, 5, 10 and 20 μg/ml) or FSL-1 (10, 100 and 200 ng/ml) for 24 h. The viability of the cells was verified by PI staining in the FACS Calibur cytometer.

3.3.5 Statistical analysis

The results were expressed as mean ± SEM. Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). Relationship between the initial numbers of spheroids co-incubated and the final number of spheres adhered was performed with a linear regression analysis. Multiple groups were compared with a one-way ANOVA and a Bonferroni multiple comparison post-test. The effects of FSL-1 and PGN treatments on the co-incubation kinetics were compared with a two-way ANOVA test and a Bonferroni post-test to compare effects between non-stimulated control and treated cells. $P \leq 0.05$ was considered to be significant.

3.4 Results

3.4.1 Standardizing the JAr spheroid formation

The method for spheroid formation was tested previously in the lab. We standardise the process in order to characterize and optimise the spheroid formation method for the following experiments. The initial results varied from huge amorphous cell clusters measuring more than 1 mm to spheroids with a constant shape in between 200-250 μm. Different amount of trophoblast cells seeded in each petri dish was found to be crucial for obtaining homogeneous spheroids. In figure 3.1a it can be observed that by using $2x10^5$ cells we obtained small sized spheroids (between 100-150 μm). When the amount of cells used was increased to $5x10^5$, the spheroids obtained were bigger (between 150-200 μm) (Fig. 3.1b). Finally using $1x10^6$ JAr cells, we obtain a constant spheroid size between 200-250 μm (Fig. 3.1c). We determined that $1x10^6$ JAr cells should be used in the spheroid formation process.

3.4.2 The effect of trophoblast sphere concentration on the number of spheres attached to the endometrial monolayer

The number of spheroids bound to the endometrial cells increased proportionally to the amount of spheres delivered ($r2 = 0.9991$; $p < 0.0001$). We observed that even 300 spheroids, the highest amount of spheroids delivered, could still adhere to the monolayer (287.2 ± 1.74 spheres; Fig. 3.2).

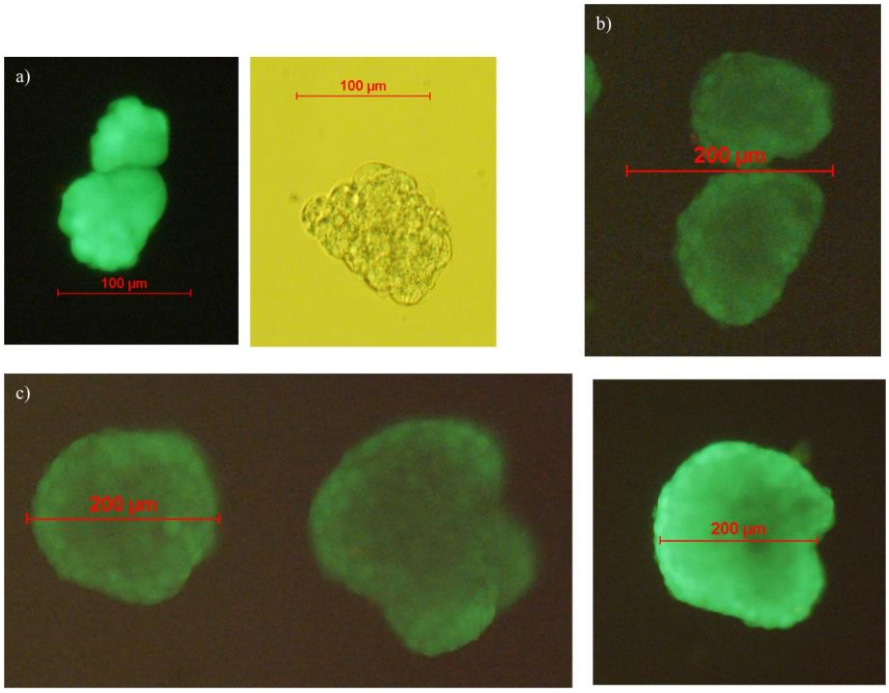

Figure 3.1. Standardisation of the JAr spheroid formation. The method for the formation of spheres was standardised by analysing the effect of the incubation of a) $2x10^5$, b) $5x10^5$ and c) $1x10^6$ JAr cells on the number, size and shape of the spheres. The spheres were stained with calcein to observe the viable cells under the fluorescent microscope.

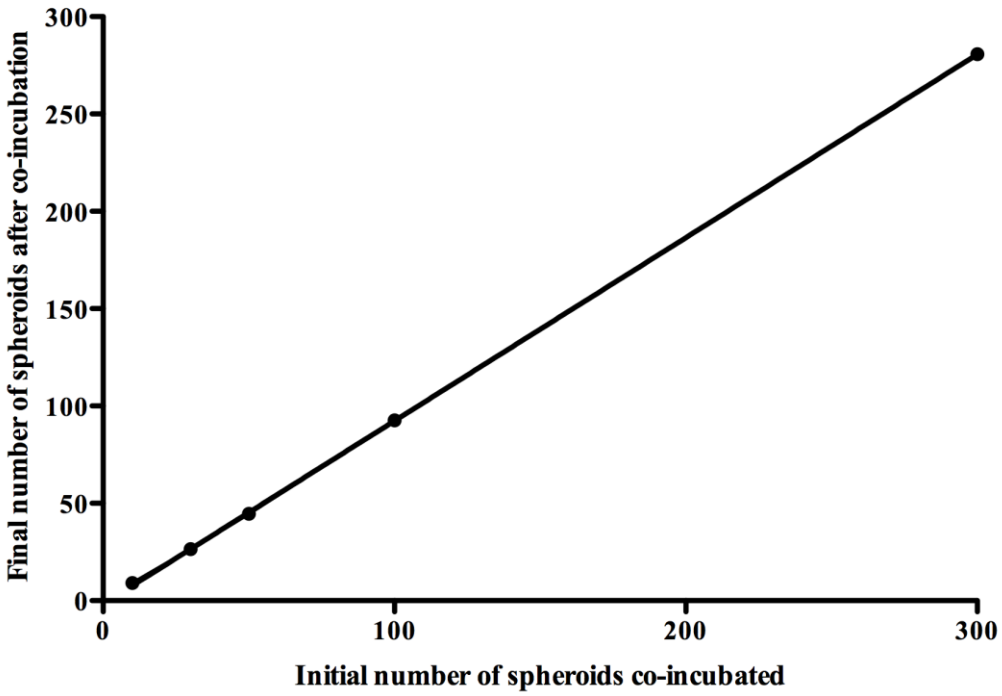

Figure 3.2. Effect of the initial number of JAr spheroids co-incubated, on their adhesion to the MCF7 cells. Different numbers of JAr spheres (10, 30, 50, 100 and 300) were co-incubated with the MCF7 monolayer. The final amount of spheres attached to it is shown in the graph per initial number of spheres delivered. A linear regression analysis was performed where the r^2=0.9991.

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

The trophoblast spheroids adhered to the cells as soon as 30 min after their introduction to the wells and this adhesion was always higher in the RL95-2 cells (63.4 ± 4.38% and 37.35 ± 3.02% to MCF7 cells). The effect of the treatment of the epithelial cells with 100 ng/ml of FSL-1 for 24 h on the adhesion of the JAr spheroids was significant for RL95-2 ($p < 0.0022$) and MCF7 cells ($p < 0.0001$) as well as the effect of the co-incubation time ($p < 0.0001$ for RL95-2 and $p < 0.0001$ for MCF7). The FSL-1 stimulation was consistent throughout the time as no interaction was observed in both cell lines ($p = 0.6473$ for RL95-2 and $p = 0.8718$ for MCF7 cells; Fig. 3.3a and b). The JAr spheroid adhesion was found significantly different between the non-stimualted control and FSL-1 stimulated MCF7 cells at 30 min (37.35 ± 3.02 vs 26.56 ± 2.59%, $p < 0.01$) and 4 h (73.01 ± 2.23 vs 62.28 ± 2.053%, $p < 0.01$). The treatment of the cells with PGN was also consistent throughout the stimulation time as no interaction was observed ($p = 0.5527$ for RL95-2 and $p = 0.5663$ for MCF7). We observed a significant effect of the treatment of the cells with 10 μg/ml of PGN on the adhesion of JAr spheroids adhered in both RL95-2 ($p < 0.0001$) and MCF7 cells ($p < 0.0001$). The effect of time on the adhesion of the spheroids was also found to be significant in both cell lines ($p < 0.0001$ for both RL95-2 and MCF7; Fig. 3.3a and b). The adhesion of the JAr spheroids to the non-treated RL95-2 cells differ significantly from the PGN treated cells at 1 h (73.79 ± 2.85 vs 64.1 ± 3.9, $p < 0.05$) and 2h of co-incubation (89.05 ± 2.02 vs 76.39 ± 2.54, $p < 0.01$). The same effect was observed in the MCF7 cells at 1 h (73.01 ± 2.43 vs 67.95 ± 3.23, $p < 0.05$) and 2 h of co-incubation with the JAr spheroids (84.19 ± 3.11 vs 71.34 ± 4.26, $p < 0.01$).

3.4.4 Trophoblast spheroid decreased adhesion to the epithelial monolayer proportionally to the TLR ligand concentration

The 24h stimulation of RL95-2 and MCF7 cells with 100 and 200 ng/ml of FSL-1 (68.11 ± 4.83 and 61.07 ± 4.52 % for RL95 and 41.66 ± 2.82 and 40.21 ± 3.36 % for MCF7)

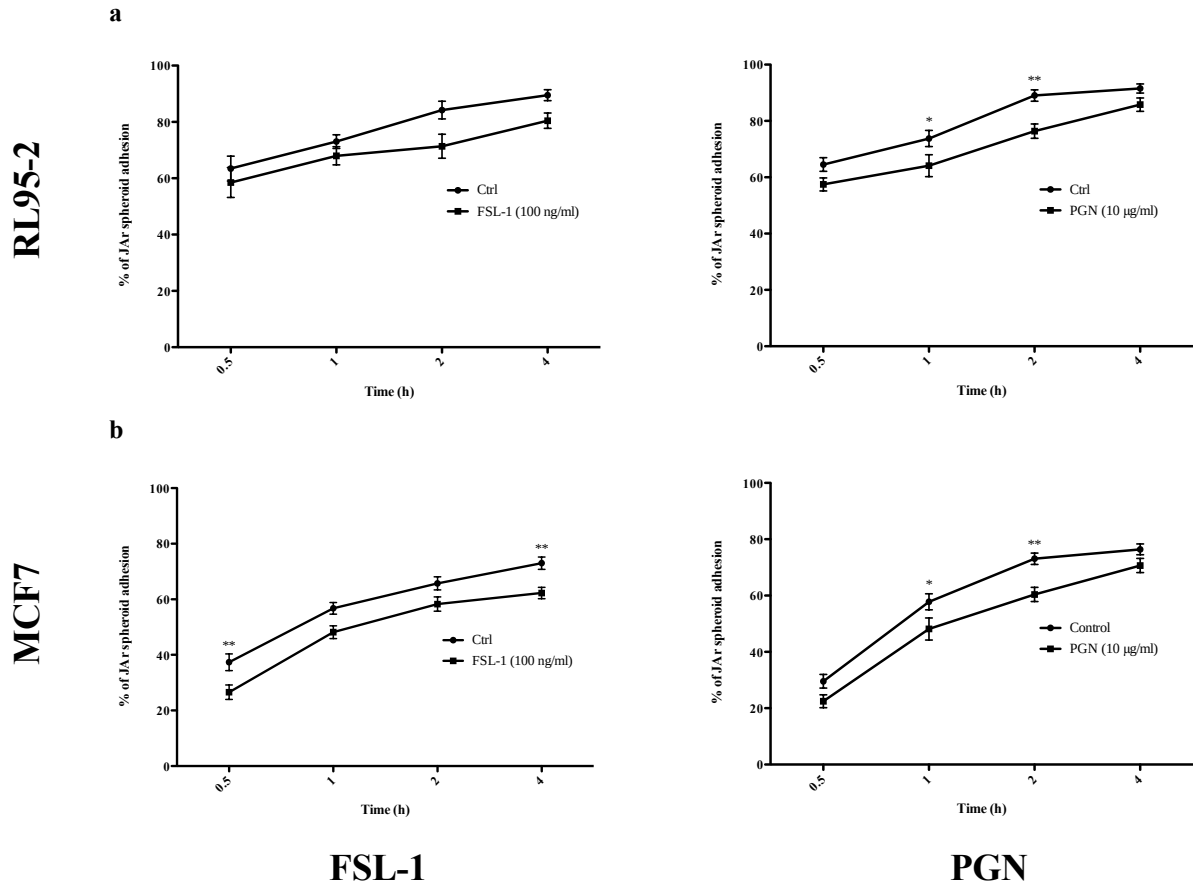

Figure 3.3. Kinetics of adhesion of the trophoblast spheroids to the TLR 2 and 2/6 stimulated epithelial cells. The percentage of adhesion of JAr spheroids to A) endometrial or B) MCF7 cells was determined after a kinetic of adhesion (0.5, 1, 2 and 4h). The adhesion of the spheroids to endometrial untreated cells (●) was compared to the endometrial cells treated (■) with FSL-1 100 ng/ml treated or PGN 10 µg/ml treated. Each point represents the mean ± SEM of four independent experiments treatments were compared by a two-way ANOVA to test interaction between effects, treatment and co-incubation time. Furthermore the difference between control and treated groups per individual time points was assessed with a Bonferroni multiple comparisons post-test (*$p \leq 0.05$ was considered significant). As published before **(Sánchez López et al. 2014).**

significantly decreased the adhesion of the JAr spheroids to the monolayer compared to the non-stimulated control (83.46 ± 2.685 %, p = 0.0012 for RL95 and 58.58 ± 2.43 %, p = 0.0002 for MCF7) after one hour of co-incubation (Fig 3.4a and b). A similar effect was observed with PGN stimulation. The RL95-2 PGN stimulation with 20 µg/ml significantly decreased JAr spheroid adhesion compared to the non-stimulated control (57.09 ± 3.83 vs 72.92 ± 3.74 %, p = 0.0435; Fig. 3.4a). In MCF7 5, 10 and 20 µg/ml of PGN significantly decreased JAr spheroid adhesion (57.05 ± 3, 47.04 ± 1.23 and 50.19 ± 2.86 % respectively) compared to the non-stimulated control (68.25 ± 2.71 %, p = 0.0001; Fig. 3.4b).

3.4.5 The pre-treatment of the epithelial cells with TLR blocking antibodies could restore the spheroid adhesion

Furthermore, we confirmed the specificity of the TLR effect by blocking their activation with monoclonal antibodies. The pre-treatment with a monoclonal anti-TLR 6 antibody followed by FSL-1 stimulation, restored the percentage of adhered JAr spheroids to RL95-2 cells (59.71 ± 1.91%) and MCF7 cells (30.70 ± 4.54%) compared to non-treated cells (63.26 ± 2.74% and 31.48 ± 4.087% respectively; Fig. 3.5a and b). The pre-treatment of the epithelial cells with a monoclonal anti-TLR 2 blocking-antibody before the PGN stimulation, allowed JAr spheroids to adhere to the monolayer to similar levels (58.15 ± 4.14% for RL95-2 and 33.15 ± 4.44% for MCF7) to those observed in the non-stimulated control (61.81 ± 2.65% for RL95-2 and 35.01 ± 3.78% for MCF7; Fig. 3.5a and b). The treatment with the antibodies did not show an effect on the adhesion of JAr spheroids to the monolayers (Anti TLR 6: 63.35 ± 2.48% for RL95-2 and 33.08 ± 4.28% for MCF7; Anti TLR 2: 61.96 ± 3.25% for RL95-2 and 36.72 ± 2.45% for MCF7) whereas the stimulation with FSL-1 and PGN decreased significantly the adhesion of the JAr spheroids (FSL-1: 45.70 ± 1.91%, p = 0.0001 for RL95-2 and 18.20 ± 3.66%, p = 0.0004 for MCF7; PGN: 45.34 ± 3.51%, p = 0.0001 for RL95-2 and 24.00 ± 2.87%, p = 0.0001 for MCF7).

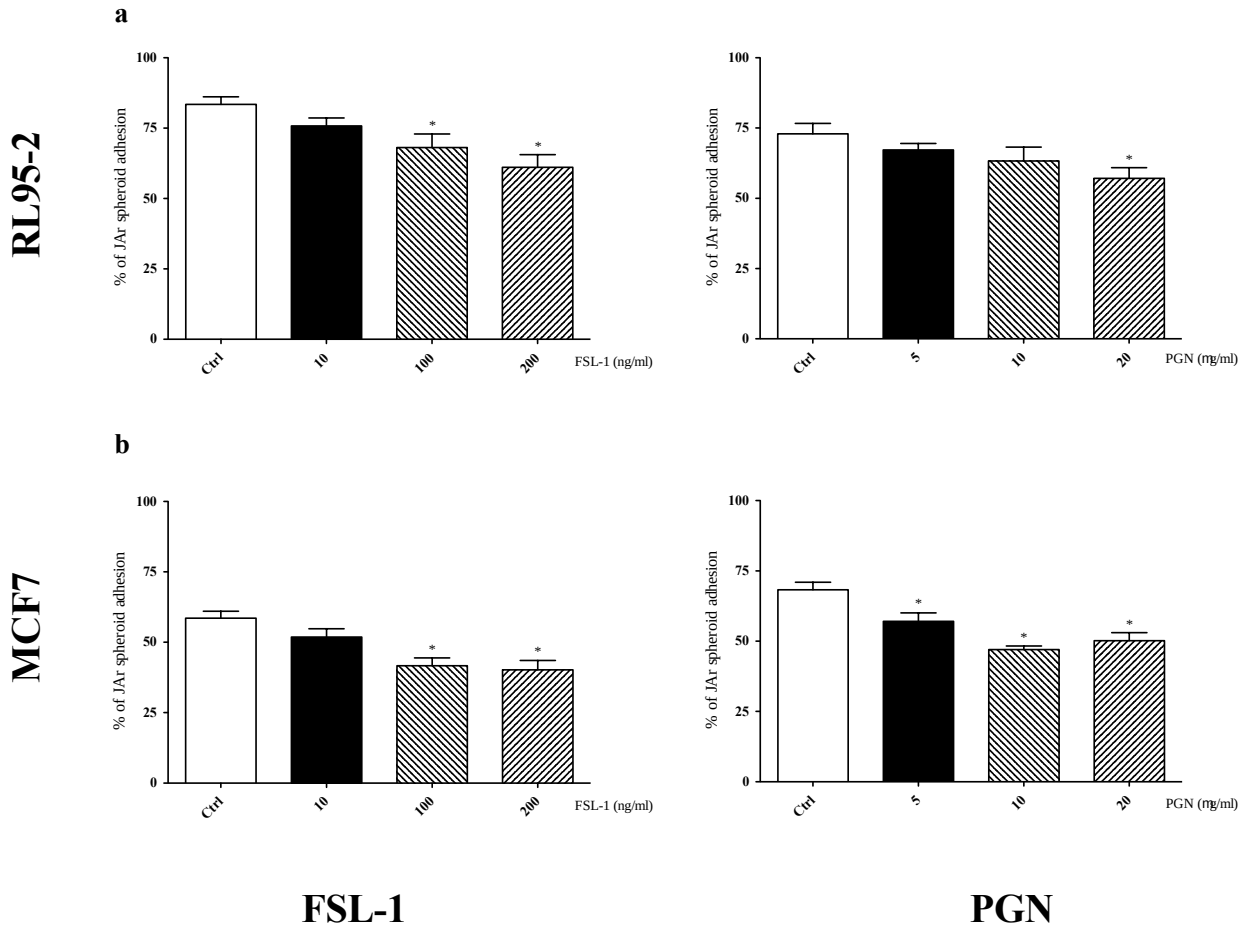

Figure 3.4. Effect of the concentration of FSL-1 and PGN on JAr spheroid adhesion. The adhesion of JAr spheroids to the a) endometrial or b) MCF7 cells was determined after their pre-treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 µg/ml). The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups (*$p \leq 0.05$). As published before **(Sánchez López et al. 2014).**

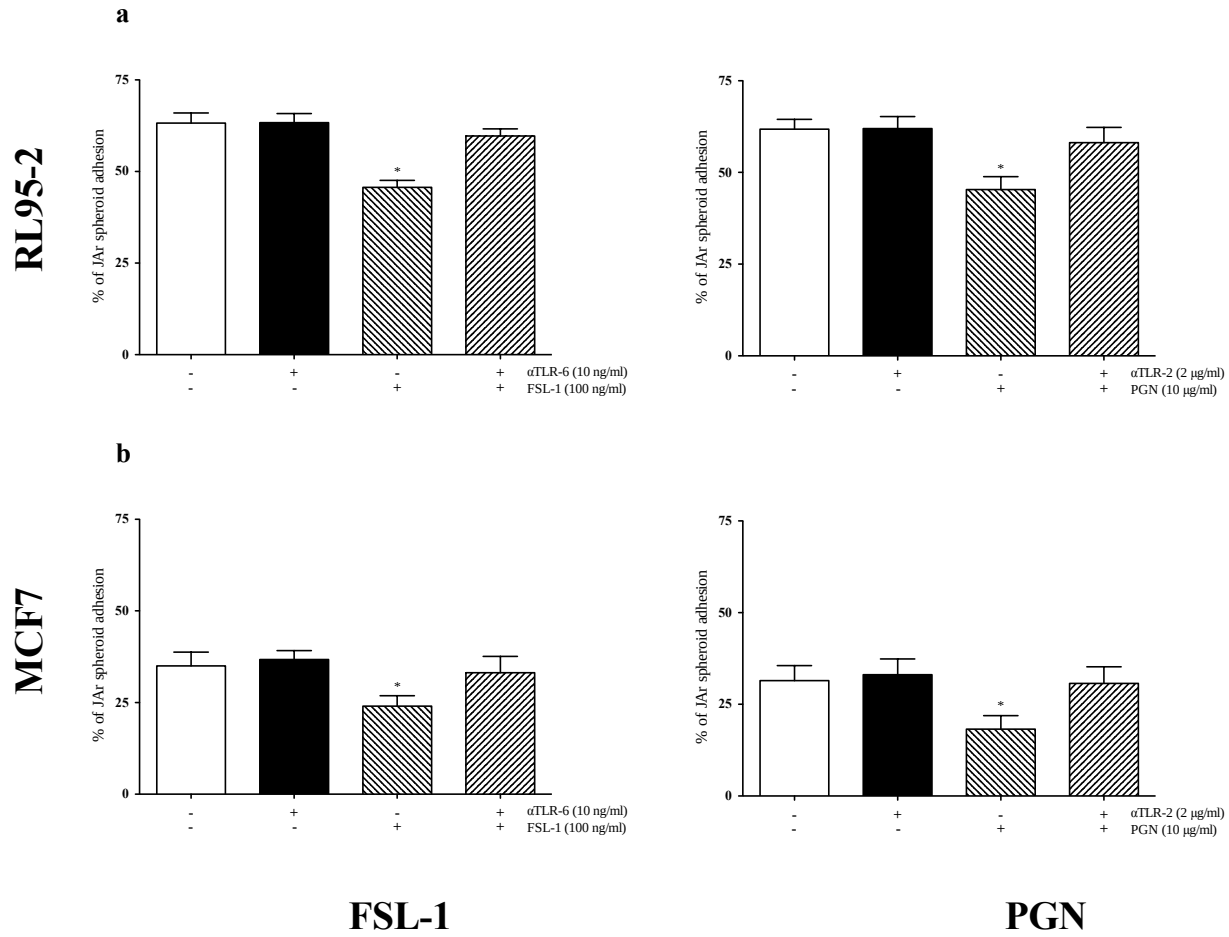

Figure 3.5. The inhibition of TLR 6 and 2 activation restores trophoblast spheroid binding to the epithelial cells. The a) RL95-2 or b) MCF7 cells were pre-treated or not with an IgG anti-TLR6 antibody (10 ng/ml) or IgA monoclonal anti TLR-2 antibody (2 μg/ml) and stimulated with FSL-1 (10 ng/ml) or PGN (10 μg/ml) respectively. The percentage of adhesion to the non-treated or different treated groups was assessed. The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni post-test was employed to compare groups, where * denotes statistical difference compared to the rest of the conditions ($p < 0.05$). As published before **(Sánchez López et al. 2014).**

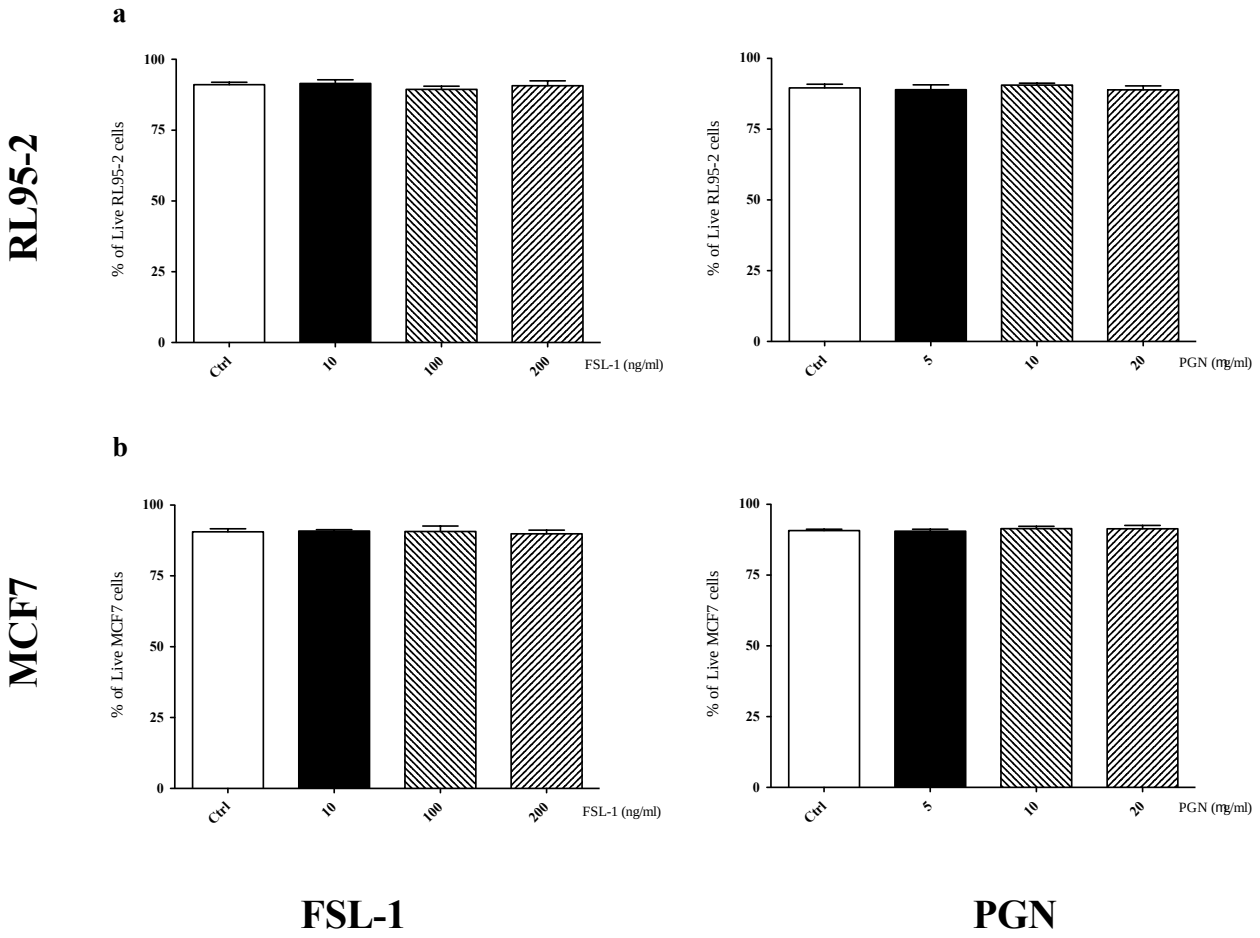

Figure 3.6. Effect of FSL-1 and PGN on the RL95-2 and MCF7 cell viability. The viability of a) RL95-2 or b) MCF7 cells was determined after their treatment with different concentrations of FSL-1 (50, 100 and 200 ng/ml) or PGN (5, 10 and 20 μg/ml). The data represents the mean of 3 independent experiments ± SEM. A multifactorial ANOVA with Bonferroni multiple comparison test was used to compare the stimulated vs. non-stimulated groups (*$p \leq 0.05$). As published before **(Sánchez López et al. 2014).**

3.4.6 The viability of the epithelial cells was unaltered after TLR activation

The stimulation of the RL95-2 and MCF7 cells with both FSL-1 and PGN did not affect the viability of the cells. Around 90% of the non-stimulated cells remain viable after harvesting from the wells. The viability of the cells stimulated with FSL-1 or PGN was also around 90% of the total population (Figure 3.6).

3.5 Discussion

The establishment of a correct maternal-embryo interface is crucial for the implantation and development of the embryo. Several factors as hormones, cytokines, growth factors and adhesion molecules must be regulated perfectly to set the appropriate environment for the implantation of the embryo (Paria et al. 2000; McEwan et al. 2009). It is possible that the presence of pathogens in the endometrium, sensed by the endometrial epithelial TLRs, influence the establishment of the suitable implantation conditions (Aboussahoud, Bruce, et al. 2010a).

Previously in our research group, it has been identified that the epithelial endometrial cells can express the TLRs differentially through the FRT (Fazeli et al. 2005). This expression is regulated by the menstrual cycle with an increased expression of TLRs 2-6, 9 and 10 during the secretory phase (Aflatoonian et al. 2007). Also, we characterised that the immortalized endometrial epithelial cell lines Ishikawa and Ishikawa 3H-12 express TLRs 1-3 and 5-9 and have functional activity (Aboussahoud, Aflatoonian, et al. 2010b). To assess the role of the innate immune system in the implantation process of the embryo, an *in vitro* assay was developed. It proved that the flagellin, ligand of TLR 5 negatively influenced the attachment of the trophoblast spheres in the endometrium (Aboussahoud, Bruce, et al. 2010a). In this chapter, we optimised the model to continue the exploration of innate immunity in the female – embryo interface. By optimising the cell concentration assays we have found that the variation of the cell number seeded in the petri dish influences sphere morphology. This optimised method makes the collection of the spheres easier and the type of sphere more homogeneous.

The study of human embryo implantation is difficult to perform *in vivo*. Thus, *in vitro* models have been developed. The selection of the appropriate cell lines is a fundamental issue to be taken into account (Hannan et al. 2010). We simulated the endometrium with the RL95-2 cell line. This cell line has been proposed for the study of the embryo implantation due to its ability to maintain the epithelial polarisation, expression of adhesion molecules in the apical surface, presence of microfilaments on its apical membrane, responsiveness to hormones and secretion of cytokines (Hohn et al. 2000; John et al. 1993). The embryo was represented by cellular spheroids from a choriocarcinoma trophoblast cell line JAr, were formed to mimic the embryo contact

with the RL95-2 cells. JAr cells have been described before as an invasive trophoblast cell line that can be used for migration assays (Hannan et al. 2010). It has been demonstrated that they can interact with different endometrial cell lines and establish adhesive interactions in short periods of co-incubation times (Thie et al. 1998). The percentage of spheroids adhered increased proportionally with time, as observed previously (Hohn et al. 2000; John et al. 1993). The JAr cells have been described as a very invasive choriocarcinoma cell line (Hohn et al. 2000). The interaction of MCF7 cells with first trimester placental explants has been observed before. The trophoblast explants affected MCF7 proliferation and induced apoptosis in long co-incubation times (Tartakover-Matalon et al. 2010). Our experiments were carried out in short co-incubation times where we were able to observe adhesion of trophoblast to both epithelial cells. Nevertheless a better interaction was observed with the endometrial cells as trophoblast cells adhered to them in a higher percentage and faster than the MCF7 cells.

The kinetics of adhesion between the RL95-2 cells and the JAr spheroids showed that more than the 60% of the spheres were already adhered to the monolayer after 1 h of co-incubation with a gradual increase in the percentage of adhesion in a time-dependent manner up to 4 h. These results are similar to those observed previously where JAr spheroids adhere in similar percentages to RL95-2 cells (Hohn et al. 2000; Aboussahoud, Bruce, et al. 2010a; John et al. 1993).

It is important to take into account that TLR2 is a particular receptor of the TLR family. It can form homodimers and heterodimers with either TLR1 or TLR6. This increases the range of ligands that TLR 2 can recognise, ranging from bacterial PGN to diacyl and triacyl lipopeptides (Schwandner et al. 1999; Yoshimura et al. 1999; Takeuchi et al. 1999; Buwitt-Beckmann et al. 2005; Kang et al. 2009). To assess if the activation of TLR2 and the heterodimer TLR2/6 could influence the adhesion of the spheroids, the RL95-2 cells with FSL-1 and PGN. FSL-1 is a synthetic diacyl lipopeptide based in the *Mycoplasma salivarium* lipoprotein that signals specifically through TLR 2/6 (Shibata et al. 2000). Activation of TLR 2/6 by FSL-1 and TLR2 by PGN decreased the number of attached spheroids to the endometrial monolayer at all co-incubation times. Initially, this effect was concentration dependant but no further decrease in the percentage of adhered spheres was observed when FSL-1 concentration was increased beyond 100

ng/ml. The reason why no further effect is observed is not clear but it could be related to saturation of TLR 2 receptors or any of the signalling molecules involved in the pathway.

To confirm the specificity of TLR 2/6 and TLR 2 competitive inhibition assay was performed using TLR 6 and TLR 2 blocking antibodies. TLR6 blocking antibody restored the ability of the JAr spheroids to bind to both FSL-1 and PGN stimulated RL95-2 cells. TLR2/6 functionality is dependent on the dimerisation and the recognition of the ligand by both TLRs. If one of them is absent, then the activation by the diacylated lipopeptides will not occur (Buwitt-Beckmann et al. 2005; Kang et al. 2009). The innate immune system can use another set of receptors to sense PGN like Nod2 (Muller-Anstett et al. 2010). In our study we showed the specificity of endometrial cells TLR 2 activation by PGN and how this activation reduce the adhesion of the JAr spheroids in the endometrial monolayer by blocking TLR 2 with a specific function blocking antibody. This restored the ability of the JAr spheroids to bind to the monolayer.

Several studies have stressed the importance of TLRs during implantation and pregnancy. For example, the activation of TLR5 in epithelial cells decreased trophoblast cell adhesion to epithelial cells *in vitro* (Aboussahoud, Bruce, et al. 2010a). Different reports have shown that the stimulation of different TLRs in the FRT, such as TLR4, TLR3 and TLR9, produced implantation failure, fetal losses and preterm delivery using *in vivo* mice models (Sheldon & M. H. Roberts 2010; Koga et al. 2009; Kwak-Kim et al. 2010; Friebe et al. 2011). It seems that the activation of TLRs will have a detrimental effect on implantation. But it is not clear how TLR activation can influence endometrial receptivity and if this effect is extended to the embryo. For example, it is known that after activation, TLRs trigger a sequence of downstream signalling events that leads to NF-κB and MAPK activation and the secretion of pro-inflammatory cytokines (Lien et al. 1999; Mele & Madrenas 2010; Q. Wang et al. 2001; Xu et al. 2001). Some of these cytokines are also involved in the modulation of endometrial receptivity by regulating the expression of several adhesion molecules (M. Singh et al. 2011).

Determining the suitable endometrial conditions for the implantation of the embryo is a topic under constant research (Lessey 2000; Dimitriadis et al. 2010; Paria et al. 2000;

McEwan et al. 2009; M. Singh et al. 2011). The correct diagnose of infertility can increase significantly the success of pregnancy for women with fertility problems and couples undergoing IVF treatment. With further research on the mechanisms responsible for the effects observed we could possible design and direct a therapy targeting the endometrial innate immune system to increase the implantation success.

Dealing with the course of an infection in the reproductive tract represents an energy expenditure that the mother must invest. Allowing the development of an embryo at the same time represents an extra investment of energy and a great risk for both the mother and the embryo. It might be possible that the recognition of the pathogens through the endometrial TLRs induce a whole range of changes of phenotype from a receptive endometrium to a non-receptive and defensive endometrium.

In the previous chapter, we have demonstrated that the activation of TLR 2/6 in the murine UH results in tissue atrophia and induction of a pro-inflammatory cytokine environment. These effects might be responsible for the decrease in embryo implantation. Using the *in vitro* human trophoblast adhesion model, we demonstrated that the presence of PGN or FSL-1, both components of Gram-positive bacteria, at the time of implantation results in a significant decrease of JAr spheroids adhesion to the endometrial cells. This effect seems to be mediated through, TLR2 dimer and TLR2/6 heterodimer. Their inhibition restored the adhesion of JAr spheres to normal levels. In conclusion, our data support previous studies regarding the importance of TLRs during the time of implantation suggesting that they could be used as a potential target in management of infertility cases.

Chapter 4. Understanding how TLR activation affects endometrial cell membrane surface morphology and surface protein profile

4.1 Introduction

One of the main characteristics of the female reproductive tract (FRT) cells is their plasticity to respond to the hormonal changes along the cycle. Endometrial stromal fibroblasts and epithelial cells at the late secretory phase will proliferate and change their respective phenotypes to prepare the tissue for the possible implantation of an embryo (T. C. Li et al. 1991). At the arrival of the developing embryo to the uterus, the endometrial epithelial cells will have the first encounter with the embryonic trophoblast cells. The success of this event determines the possibility of implantation and pregnancy. Micro-structural analysis of the endometrial cell morphology has characterised the development of microvilli and pinopodes on the surface of endometrial cells at the time of implantation (More & R. G. R. Masterton 1975). These are membrane microstructures that are required for a proper interaction between endometrium and trophoblasts (Petersen et al. 2005; Bentin-Ley et al. 1999).

The atomic force microscopy (AFM) is a powerful tool that allows investigation of the surface ultrastructure of virtually any compound. AFM can provide information regarding the atomic composition, sample structure properties and even the forces of structural bonding in the same molecular structure and between two different interacting bodies (Binnig et al. 1986). The initial design of the AFM was used to image molecular structures of solid materials like inorganic compounds, alloys, polymers, minerals and metals (Giessibl 2003). In biological research, AFM has been employed to study structures of biomolecules such as nucleic acids, proteins, lipid bilayers or membranes, cells and bacterial walls (Santos & Castanho 2004; Alonso & Goldmann 2003). Using the particular features of AFM, it has also been possible to measure interaction forces between a cell fixed on a cantilever with another cell or a solid substrate such as extracellular matrix proteins (Benoit et al. 2000). AFM can be used to observe microstructural characteristics of cells without the complicated procedures used for sample preparation by electronic microscopy. For example, it was possible to detect the presence of microvilli and cytoskeletal proteins in the images of fibroblasts obtained by AFM with the same precision obtained with electron microscopy (Braet et al. 1998). The membrane microstructure of the endometrial cell lines Ishikawa, HEC-1a and 1b has also been imaged by AFM before. In this study, it was possible to characterise the influence of progesterone on the cell membrane acquisition of microstructures such as

microvilli and pinopodes (Francis et al. 2009). The presence of these structures on the endometrial cells is related to the acquisition of a receptive morphology necessary for the implantation of the embryo (Francis et al. 2009).

The cell membrane is a dynamic organelle that compartmentalises the cell and preserves the cellular internal structure and content. It also mediates the social interaction of the cells regulating the localisation of the necessary proteins for intercellular adhesion. The endometrial epithelial cells will form a monolayer of polarised cells tightly bound to each other. It is during the late secretory phase that their phenotype will change. They will express a subset of glycans, integrins, selectins, cadherins among other molecules to mediate their adhesion with the trophoblast cells of the blastocyst (Campbell et al. 2000; H. Singh & Aplin 2009). It is possible that the lack of one of these molecules may affect the quality of endometrial interaction with the embryonic cells (Aplin & H. Singh 2008). For example the endometrium of patients with unexplained infertility have been found to have a lower expression of L-Selectin ligands (Margarit et al. 2009). The characterisation of the expression of a subset of surface molecules by endometrial cells has been performed before trying to detect a surface marker necessary for embryo implantation (T. C. Li et al. 1991; Domínguez et al. 2010). According to this study, the surface molecules integrin β1, CD147, CD9 and CD98 were highly expressed in primary and immortalised cell lines originated from endometrium. The integrins are transmembrane heterodimeric proteins composed of α and β subunits that mediate inter-cellular and extracellular matrix (ECM) interactions (More & R. G. R. Masterton 1975; K. V. R. Reddy & Mangale 2003). A high expression of different subsets of integrins in the endometrium like α1β1 and αvβ3 has been observed during the late secretory phase hence they have been related to the endometrium plasticity to change and allow the implantation of the embryo (Petersen et al. 2005; H. Singh & Aplin 2009; Bentin-Ley et al. 1999). β1 integrin has a possibility to dimerise with a great variety of α subunits. This grants a good heterogeneity of potential ligands from ECM proteins like type I collagen, fibronectin and laminin (α1, α2 and α3) that are produced by the embryo at the time of implantation (Binnig et al. 1986; Lessey 1998). In fact the treatment of human endometrial decidual cells with a β1 blocking antibody allowed the attachment of murine hatched blastocyst but decreased their spreading capacity leading to embryonic death (Shiokawa et al. 1996). Another membrane bound protein that has been associated with the implantation process is the cluster of differentiation (CD) 147 molecule also

called Extracellular matrix metalloproteinase inducer (EMMPRIN) or basignin. CD147 has been found to be expressed in the endometrial stroma and epithelium surounding the site of embryo implantation in mouse and rat. This expression might facilitate trophoblast migration through the stroma (Xiao, Diao, et al. 2002b; Alonso & Goldmann 2003; Xiao, Chang, et al. 2002a). CD147 has a physiological role of inducing the expression of matrix metalloproteinases (MMPs) during tissue remodelling in development and tissue repair (Yurchenko et al. 2010). Its expression facilitates the migration of the metastatic cells in endometrial carcinoma mediating the secretion of MMP-2 and MMP-9 (K. Nakamura et al. 2012). CD9 is a member of the tetraspanin membrane proteins. Its expression has been observed to be constant along the menstrual cycle in the human endometrium forming clusters with the integrins α_6, α_3 and β_1 (Park, Inoue, Ueda, Hirano, Higuchi, Maeda, et al. 2000b). CD9 seems to regulate the migration of cells through the endometrial epithelium. It has been demonstrated that the inhibition of CD9 with a specific antibody enhanced murine embryo implantation (Liu et al. 2006) and the migration of endometrial cancer cells *in vitro* (Park, Inoue, Ueda, Hirano, Higuchi, Konishi, et al. 2000a). CD98 is the heavy chain of the heterodimeric amino acid transporter (HAT). In this function, CD98 has been linked with the adhesion capacity of integrins like β1. CD9 is localised within tetraspanin in actin rich domains such as microfilaments and is associated with CD147 and CD98 to aid cellular adhesion linking β1 and β3 integrins to tetraspanin complexes (Sala-Valdés et al. 2006; Iacono et al. 2007). In the endometrium, CD98 expression is increased at the late secretory phase induced by hormonal stimulation of the endometrial cells (Domínguez et al. 2010). These multi-protein complexes might have the possibility to mediate the adhesion of trophoblasts to the endometrium epithelial cells and facilitate the implantation and invasion process of the embryo.

The Toll-like receptors (TLR) are a family of pattern recognition receptors (PRR) of the innate immune system that recognise pathogen associated molecular patterns (PAMPs) of bacterial, viral or parasite origin (Kawai & Akira 2010). They are present in almost all the tissues of the body and expressed cyclicaly in the female reproductive tract (Fazeli et al. 2005). Upon their ligation with specific PAMPs, they initiate a signalling network that classically activates the nuclear factor (NF)-κB or a network regulated by the mitogen-activated protein kinase (MAPK) (Brown et al. 2011). In previous reports originated from our laboratory, we have demonstrated that the activation of endometrial

TLR 5, 2 and 6 can impair trophoblast adhesion *in vitro* and TLR 6 activation affect the implantation of murine embryo *in vivo* (Aboussahoud, Bruce, et al. 2010a; Sánchez López et al. 2014). The exact mechanism that activation of TLR would lead to faulty trophoblast/endometrial interactions is unknown. In the present investigation, we speculated that TLR stimulation could interfere with the acquisition of the cellular morphology and membrane protein profile necessary for establishing a proper contact between endometrial cells with the embryo. In order to do this, we employed AFM to image the endometrial cell membrane ultra-structure and observe if the TLR stimulation elicited any changes. Using an antibody panel of 22 different surface molecules related with cell adhesion we tried to characterise the membrane profile of three different endometrial cell lines (Ishikawa, Ishikawa 3H-12 and RL95-2) and a breast carcinoma cell line (MCF7).

4.2 Materials and methods

4.2.1 Cell culture and sample preparation

4.2.1.1 RL95-2 cells

The human endometrial adenosquamous carcinoma cell line (RL95-2) was obtained from ATCC and employed to mimic the receptive endometrium. The RL95-2 cells were cultured in T75 flasks at 37°C in DMEM/F-12 (Sigma) supplemented with 10% of heat inactivated fetal bovine serum (FBS; Sigma), 5 μg/ml Insulin (human recombinant insulin from Gibco, cat. No. 12585-014), and 1% L-glutamine (Invitrogen) in 5% CO_2 atmosphere until confluency. The media was changed every second day till confluence of the cells. At confluence, cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Sigma) and were harvested using 1 ml of trypsin-EDTA (Sigma). The cells were incubated for 3 min at 37°C and then pelleted by centrifugation at 300x g for 4 min. Finally, the pellet was resuspended in 3 ml of media and the cells were either seeded in another T75 flask or any other container for its use. One confluent T75 flask of RL95-2 cells gives around $1x10^7$ cells.

4.2.1.2 Ishikawa cells

The human Asian endometrial adenocarcinoma cell line Ishikawa was cultured in T75 flasks at 37°C in MEM (Sigma) supplemented with 10% of FBS (Sigma) and 1% L-glutamine (Invitrogen) in 5% CO2 atmosphere until they reached total confluence.

4.2.1.3 Ishikawa 3H-12

The human Asian endometrial adenocarcinoma cell line Ishikawa 3H-12 was cultured in T75 flasks at 37°C in EMEM (Sigma) supplemented with 10% of FBS (Sigma) and 1% L-glutamine (Invitrogen) in 5% CO2 atmosphere until they reached total confluence.

4.2.1.4 MCF7 breast carcinoma cells

The MCF7 cell line was employed as an epithelial cell line from non-endometrial origin. The cells were cultured in DMEM/F-12 supplemented with 10% of heat

inactivated FBS (Sigma), 160 ng/ml Insulin (Gibco), and 1% L-glutamine (Invitrogen) in 5% CO2 atmosphere until total confluence.

4.2.2 Analysis of RL95-2 endometrial cell morphology by atomic force microscopy

4.2.2.1 Fixation of the cells for AFM

The RL95-2 cells concentration was adjusted to $5x10^4$ cells/ml and 1 ml of cell suspension was seeded in each well of a 4 well chamber slide (BD Biosciences, Oxford, UK). The cells were cultured for a couple of days, until they regained the epithelial morphology and started spreading on the slide. Cells were rinsed twice with PBS for 5 min. They were fixed using 3% glutaraldehyde in cacodilate buffer 0.1M for 10 min and rinsed with deionised water for 10 min. The cells were then dehydrated rinsing them in serial steps of ethanol (70, 85, 95 and 100%) for 5 min each. Finally the cells were rinsed with HDMS (hexamethyldisilazane) for 5 min and dried at room temperature for 20 min (Francis, Lewis et al. 2009).

4.2.2.2 Cell imaging by atomic force microscopy and analysis

Fixed cells were imaged using a Dimension 3100 AFM with Nanoscope IV controller (Bruker Ltd.; Coventry, UK). Ten individual cells were selected using the integrated optical microscope by their appearance. A good cell for imaging had a typical epithelial morphology i.e. being spread and having a stellate shape. The stacked cells were avoided as the height of the cells may affect the cantilever movements during scanning. Measurements were performed in air tapping mode using standard non-conductive silicon probes (With nominal spring constant of 40 N/m, Bruker Ltd). The scanning parameters were 30 μm in size, 512 samples/line and a tip velocity of 40 μm/s. The height and phase data were collected from each scan.

The free software Gwyddion (Nečas & Klapetek 2012) was used to carry out the analysis of the AFM images of the endometrial cells. The maximum height of each cell was determined using the measuring tool. The root mean square roughness (R_{RMS}) value was obtained by tracing a line over the transversal segment of each cell. To be able to

distinguish the micro-structural details of the membrane from the overall shape of the cell, a cut-off value of 0.03 was set to obtain the waviness of the membrane (micrometric irregularities of the membrane profile). A cut-off value of 0.09 registered the roughness of the membrane (irregularities of nanometric size like microvilli) (D Antonio et al. 2012). Three random sections were obtained from each cell by cropping an area of 5 μm. Cell height, waviness and roughness were obtained from each cropped section of the cell to have a consistent measure along the whole cell (Fig 4.1).

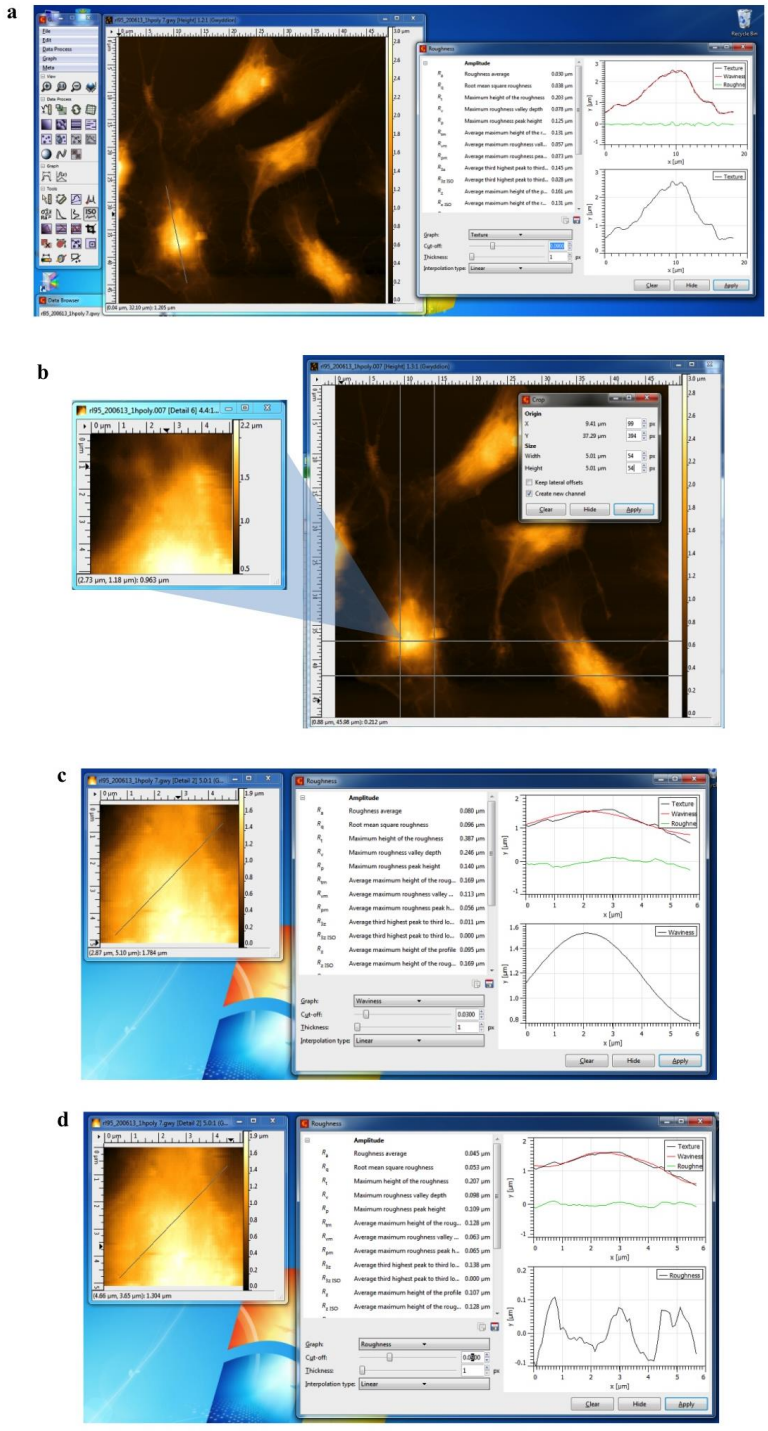

Figure 4.1. Analysis of the cell membrane characteristics using Gwyddion. The RL95-2 cells were fixed and imaged using intermittent contact mode atomic force microscopy. The height image was obtained and the profile of the membrane measured tracing a line across the cell. The waviness and roughness (R_{RMS}) were determined using the cut-off values 0.03 and 0.09 respectively (a). To estimate a consistency of these values in the same cell, two random sections of 5x5 µm were cropped (b). A diagonal line was traced along the new sections and the waviness (c) and roughness (d) estimated.

4.2.3 Flow cytometry analysis of the endometrial cell surface

4.2.3.1 Sample preparation for flow cytometry

a. Preparation of the samples

The cells were cultured to complete confluence, subcultured and $1x10^6$ cells were transferred per well of a 6 well plate. Thereafter the cells were grown to total confluence and harvested using trypsin-EDTA (Sigma) at 37°C for 3 min and diluted in 1 ml of PBS. Cells were pelleted by centrifugation at 300 g for 4 min and resuspended in 1 ml of PBS. $1x10^5$ cells were incubated with 50 µl of the different monoclonal antibodies (5-10 µg/ml) for 30 min (Table 4.1). The antibodies were produced in mouse hybridoma cell lines and were received as a kind gift of Dr. María Yáñez-Mo (Membrane Microdomains in Immunity Laboratory, Instituto de Investigación Sanitaria Princesa, Madrid, Spain) and were used as described before (T. C. Li et al. 1991; Barreiro et al. 2005; Yáñez-Mó et al. 1998). The myeloma protein pX63 (IgG1 kappa) was used as isotype control. Cells incubated with different antibodies were rinsed in 100 µl of PBS, centrifuged at 300 g for 4 min. The supernatants were discarded and the pelleted cells resuspended in 50 µl of the fluorescein isothiocyanate (FITC) labelled secondary antibody (Polyclonal rabbit anti-mouse IgG, Dako, Cambridge, UK) and incubated for 30 min. Cells were rinsed and pelleted as before and resuspended in 100 µl of PBS.

b. Analysis by flow cytometry

The stained cells were analysed using a FACSCalibur (BD Biosciences) cytometer. The main cell population (live cells) was gated and $1x10^4$ events were recorded per sample (Figure 4.2). The FITC tagged antibodies were excited using the 488 nm laser and the emission signal recorded using the FL1 detector (530 nm). The mean intensity of fluorescence (MIF) was recorded and used for the analysis of the expression of the selected proteins.

4.3 Experimental Design

4.3.1 Assessment of the FSL-1 stimulated endometrial epithelial cell membrane morphology alterations by atomic force microscopy

4.3.1.1 Imaging of the RL95-2 cells stimulated with FSL-1

To assess if the activation of TLR 2/6 can modify the RL95-2 cell morphology, we studied the microstructure of the cell membrane by AFM. The RL95-2 cells were grown in chamber slides. The cells were stimulated with 100 ng/ml of the synthetic diacylated lipoprotein FSL-1 (InvivoGen, Tolouse, France) for 24 h. The cells were fixed, imaged and analysed as described above.

4.3.1.2 AFM imaging of the kinetics of stimulation of RL95-2 cells with FSL-1

Once we observed that the stimulation with FLS-1 induced microstructural changes in the membrane, we studied RL95-2 cells responses after different intervals of stimulation. The RL95-2 cells were cultured in chamber slides and stimulated with 100 ng/ml of FSL-1 (InvivoGen) for 30 min, 1, 2, 4, 8 and 24 h. The cells were fixed, imaged by AFM and analysed to obtain the height, waviness and roughness of the cell membrane.

4.3.1.3 AFM imaging of the Poly I:C stimulated RL95-2 cells

To verify if the changes observed after TLR 2/6 stimulation could be induced when another TLR was activated, the RL95-2 cells in chamberslides were stimulated with 100 ng/ml of Poly I:C (Ligand for TLR 3, InvivoGen) for 1, 2 and 4 h. The cells were fixed, imaged by AFM and analysed to obtain the height, waviness and roughness of the cell membrane.

4.3.1.4 AFM imaging of the RL95-2 cells treated with the inhibitor of NF-κB activation

To assess if the inhibition of NF-κB, one of the main mediators of the TLR signalling pathways could block the effects of FSL-1 on the cell morphology, the RL95-2 cells in chamber slides were pre-treated with Bay 11-7082 10 μM (IκBα inhibitor, InvivoGen)

for 1 h and followed by the treatment with 100 ng/ml of FSL-1 (InvivoGen) for 4 h. The cells were fixed, imaged by AFM and analysed to obtain the height, waviness and roughness of the cell membrane.

4.3.2 Profiling the cell surface proteins by flow cytometry

4.3.2.1 Screening of the epithelial cells for surface marker expression

The epithelial cells Ishikawa, Ishikawa 3H-12 and MCF-7 were grown in 6 well plates until confluence. The cells were then processed as described above to assess the level of expression of 22 different surface proteins (Table 4.1).

4.3.2.2 Cell surface protein expression after TLR stimulation

The epithelial cells were grown in 6 well plates until confluence. Thereafter, the cells were stimulated with the ligands for TLR 2/6 (FSL-1, 100 ng/ml), TLR 3 (Poly I:C, 10 μg/ml), TLR 5 (Flagellin 100 ng/ml), TLR 9 (10 nM) and a combination of the ligands for 4 and 24 h. The cells were then processed as described before to assess the expression of surface proteins using a panel of different antibodies.

4.3.3 Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). The effects of FSL-1 and Poly I:C cell stimulation on the co-incubation kinetics were compared with a two-way ANOVA test and a Bonferroni post-test to compare effects between non-stimulated control and treated cells. $P \leq 0.05$ was considered to be significant.

Table.4.1. Antibody panel for flow cytometry analysis of the epithelial cells.

	Protein detected (antigen)	Hibridoma clone name
1	**Isotype control IgG1**	**pX63**
2	**β1 Integrin**	**TS2/16**
3	**α1 Integrin**	**TS2/7**
4	**α2 Integrin**	**Tea1/41**
5	**α3 Integrin**	**VJ1/6.1**
6	**α4 Integrin**	**HP1/7**
7	**α5 Integrin**	**P1D6**
8	**Intercellular adhesion molecule (ICAM)-1**	**HU5**
9	**Differentiation cluster (CD)43**	**TP1/36**
10	**CD44**	**HP2/9**
11	**CD59**	**VJ1/12**
12	**Major histocompatibility complex (MHC)-I**	**W6/32**
13	**CD147**	**VJ1/9**
14	**CD98**	**FG1/10**
15	**CD9**	**VJ1/20**
16	**CD81**	**5A6**
17	**CD151**	**Lia1/1**
18	**β2 Integrin**	**TS1/18**
19	**P-Selectin glycoprotein ligand (PSGL)-1**	**PL-1**
20	**CD15**	**My1**
21	**ICAM-3**	**HP2/19**
22	**Vascular cell adhesion molecule (VCAM)-1**	**YBA**
23	**CD31**	**TP1/15**

4.4 Results

4.4.1 The activation of TLR 2/6 in RL95-2 cells induced microstructural membrane changes

A normal RL95-2 cell analysed by AFM measured around 15-20 μm in length and had a clear nuclear region with a maximum height of 2.1 ± 0.16 μm. The surface of the cell membrane was covered in fine extensions or microvilli, characteristic of an endometrial cell (Fig. 4.2a). In contrast 24 h FSL-1 stimulation of the cells induced several changes in the membrane microstructure. There was a loss of the surface microvilli. The most striking change in membrane appearance was observed as an induction of bleb like structures of the membrane (formation of round protrusions; Fig. 4.2b).

4.4.2 The microstructural changes in membrane topology were proportional to the TLR 2/6-stimulation time

The height of the cells, R_{RMS} of the waviness (micrometric) and roughness (nanometric) profile of the cell was quantified using Gwyddion (Nečas & Klapetek 2012). There was a significant effect of the FSL-1 stimulation on the endometrial cell height ($p < 0.0001$; Fig. 4.3a). This effect on cell height could have been influenced by duration of time the cells were exposed to ligand ($p < 0.001$) due to the significant interaction observed between the FSL-1 treatment and time ($p < 0.001$). We observed significant differences between the height of control and FSL-1 treated cells after 1 and 24 h of stimulation (3.4 ± 0.2; $p \leq 0.001$).

The waviness of the cell membrane increased proportionally with the stimulation time, starting as soon as 30 min. There was a significant effect of FSL-1 on the waviness of the cells ($p < 0.0001$; Fig. 4.3b), which was not influenced by the stimulation time as no interaction was observed ($p > 0.05$). The time had no influence on the endometrial cell waviness ($p > 0.05$). The waviness increased from 60.5 ± 4.5 nm in the control to 90 to 110 nm up to 8h of FSL-1 stimulation. All the values were significantly different from the control ($p \leq 0.05$). The highest waviness increase was observed at 24 h of FSL-1 stimulation compared to the control (139.7 ± 9.3 vs 60.9 ± 5.2 nm; $p < 0.001$).

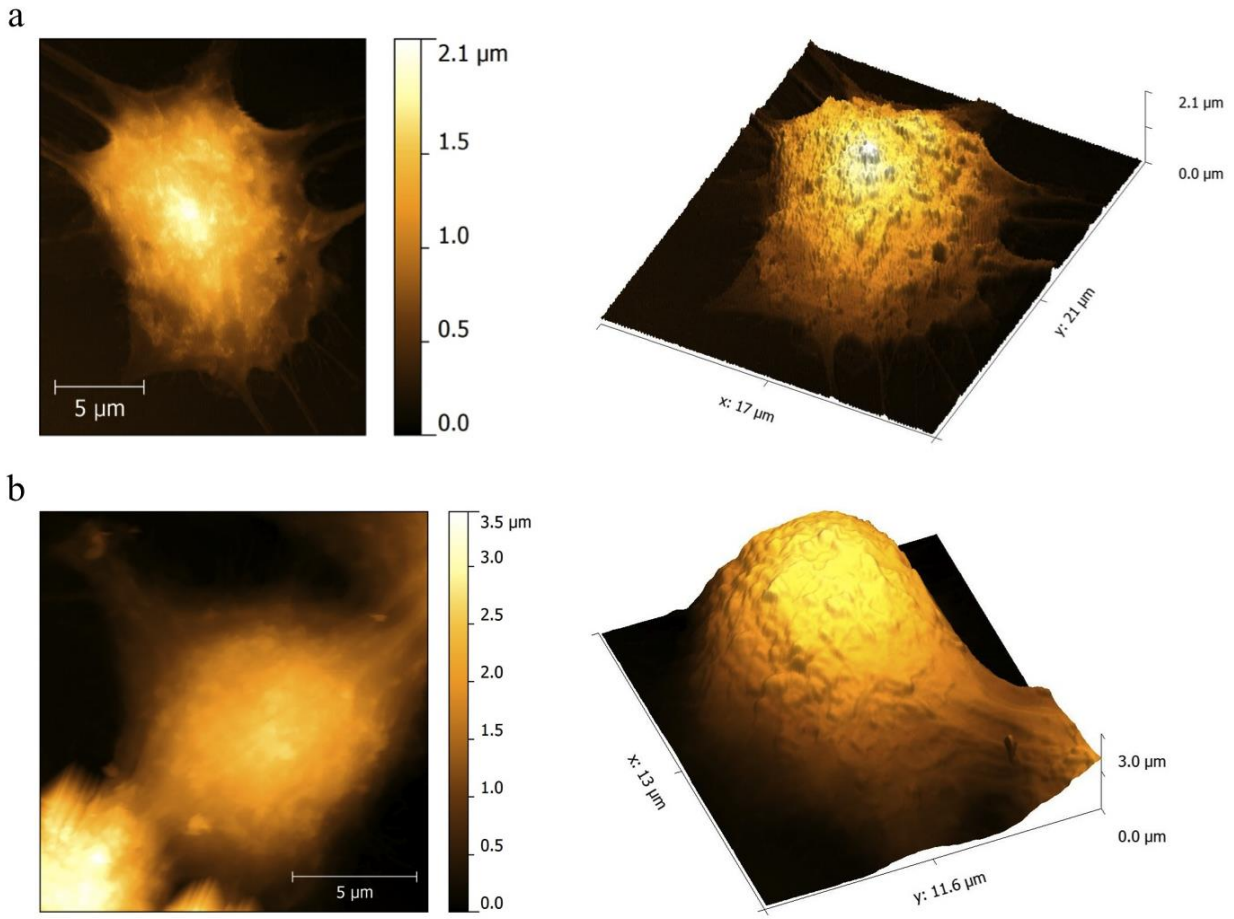

Figure 4.2. Height 2D and 3D images of the untreated or FSL-1 stimulated RL95-2 cells. The free image analysis software Gwiddion was used to generate 2D (left) and 3D (right) images of the RL95-2 endometrial epithelial cells non-stimulated (a) or 24 h treated with 100 ng/ml FSL-1 (b). The brightness of the image is proportional to the cell height.

As shown in figure 4.3c, the roughness of the membrane followed a similar pattern, where the FSL-1 treatment had a significant effect ($p < 0.0001$). This effect significantly increased along the time ($p < 0.001$) and the highest increase was observed after 24 h (66.6 ± 7.2 vs 25.1 ± 1.9 nm). We can say that the effect considerably increased along the stimulation time as the interaction between time and treatment was found significant ($p < 0.05$). These changes could also be visualised on the AFM images, where compared to the non-stimulated control, the surface morphology of the cell changed drastically proportionally with the stimulation time (Fig 4.4). We were able to observe an active blebbing process and a loss of microvilli as described before.

4.4.3 Inhibition of NF-κB activation in RL95-2 cells before FSL-1 stimulation was not able to stop the membrane changes

The RL95-2 cells were pre-treated with the inhibitor BAY11-7082 to inactivate NF-κB signalling. The cell membrane increased waviness and roughness after 1 h of FSL-1 administration even when pre-treated with BAY11-7082 (Fig. 4.5). This was significantly different from the non-stimulated and only BAY11-7082 treated cells ($p < 0.001$).

4.4.4 The activation of TLR 3 in RL95-2 cells induced microstructural changes at the cell membrane

To test if the membrane changes were unique to TLR2/6 ligation or could be induced by another TLR activation or ligand, the RL95-2 cells were treated with the TLR 3 ligand, the double stranded RNA analogue Poly I:C. TLR 3 activation was also able to induce the microstructural changes and blebbing of the membrane similar to that of TLR 2/6 (Figure 4.6a and b). An increase in the cell membrane waviness (Fig. 4.6c), roughness (4.6d) and height (4.6e) was observed starting at 1 h of stimulation compared to the untreated control ($p \leq 0.05$). These membrane changes were present at 2 and 4 h of Poly I:C stimulation compared with their non-treated controls ($p \leq 0.05$).

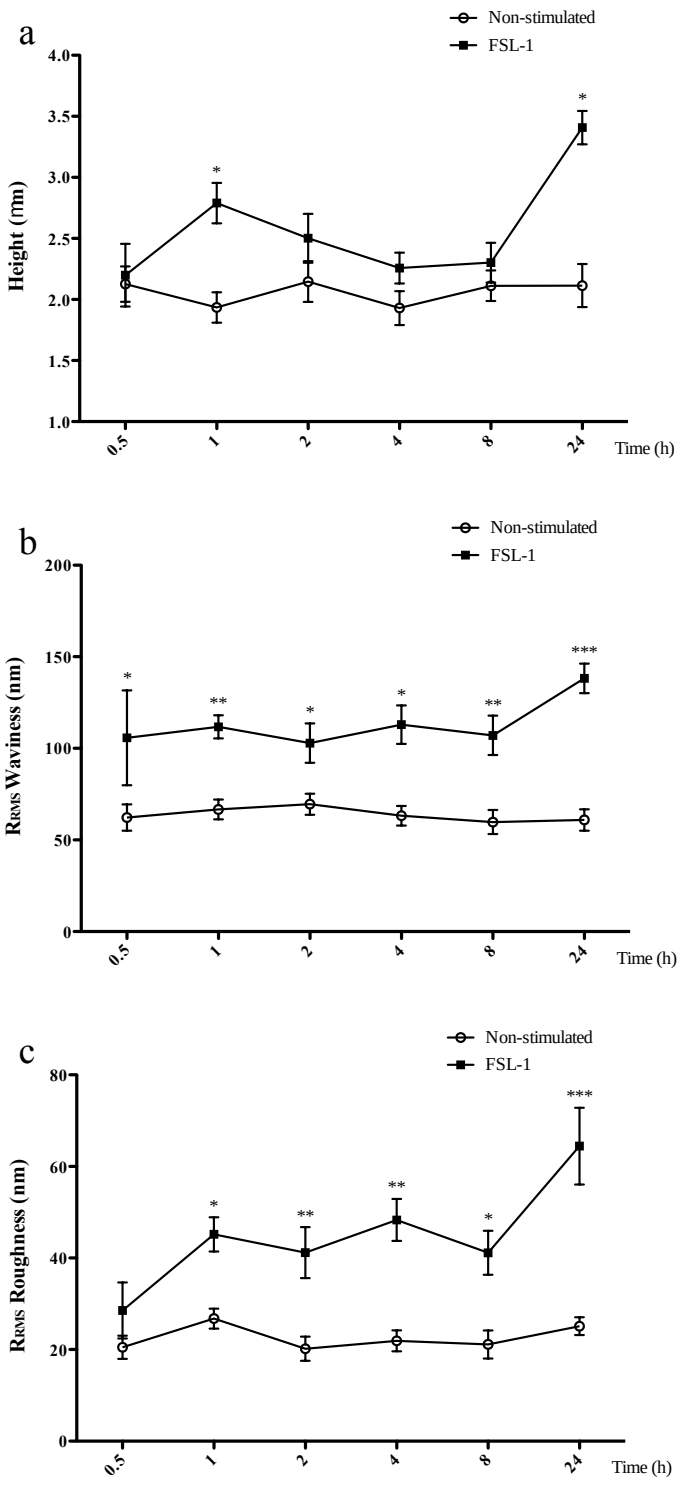

Figure 4.3. Measurement of RL95-2 height, membrane waviness and roughness. The endometrial cell line RL95-2 was treated or not with FSL-1 (100 ng/ml) for 30 min, 1, 2, 4, 8 and 24 h. The cells were fixed and imaged by intermittent contact AFM. The cell height (a), membrane waviness (b) and roughness (c) were determined in Gwyddion using a cut-off value of 0.03 for waviness and 0.09 for roughness. The effects between non-stimulated versus FSL-1 were compared using a two-way ANOVA with Bonferroni post-test to compare the different timepoints. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

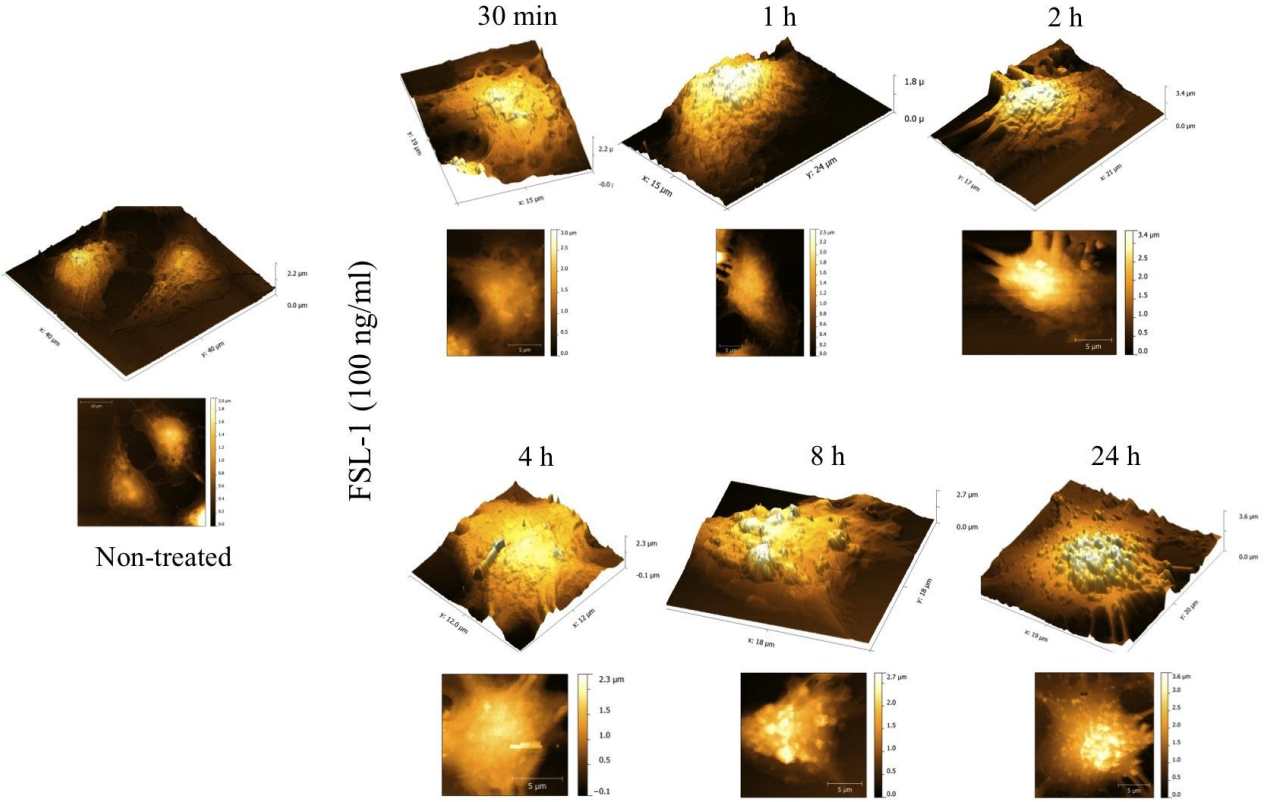

Figure 4.4. 2D and 3D height images of the RL95-2 cells non-stimulated or FSL-1 stimulated. Representative 3D (top) and 2D (bottom) images of the RL95-2 endometrial epithelial cells non-stimulated or treated with 100 ng/ml FSL-1 for 30 min, 1, 2, 4, 8 and 24 h were generated in Gwiddion. The brightness of the image is proportional to the cell height.

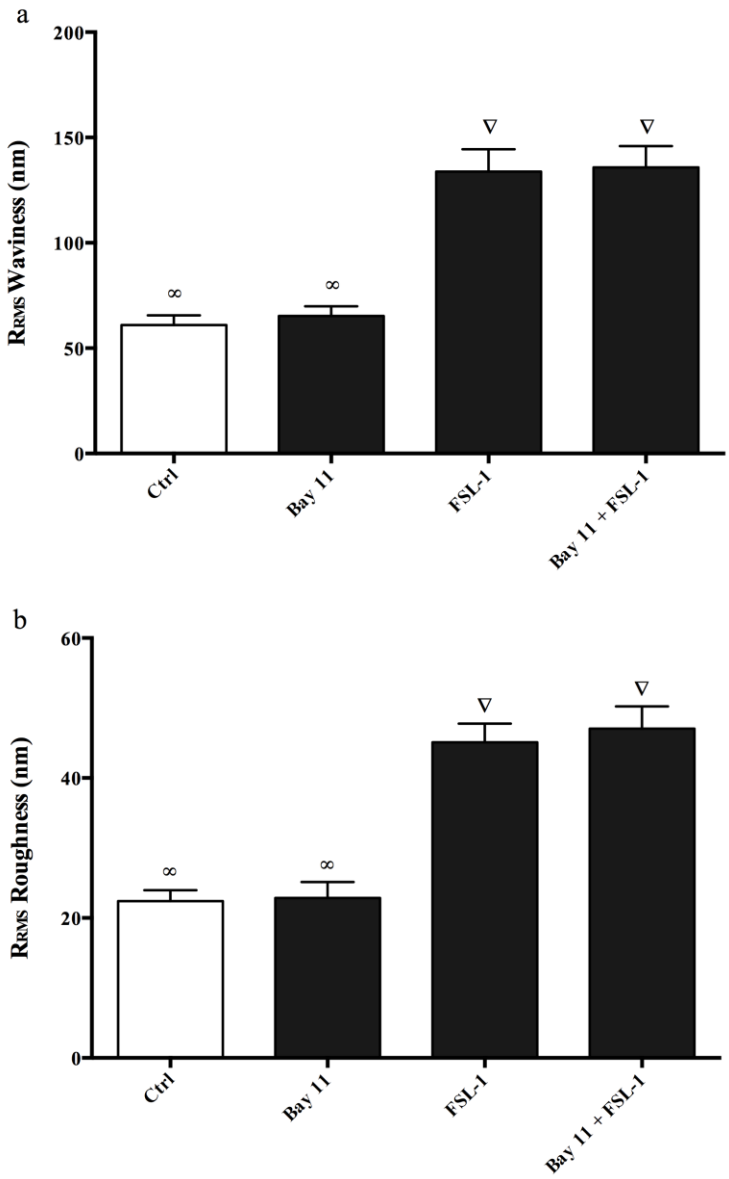

Figure 4.5. Membrane waviness and roughness of NF-κB inhibited RL95-2 cells. The endometrial cell line RL95-2 was pre-treated or not with 10 μM BAY11-7082 for 1 h and then treated with FSL-1 (100 ng/ml) for 1 h. The cells were fixed and imaged by intermittent contact AFM. The membrane waviness (a) and roughness (b) were determined in Gwyddion using a cut-off value of 0.03 for waviness and 0.09 for roughness. The effects were compared using a one-way ANOVA with Bonferroni post-test to compare non-treated versus FSL-1 treated cells per time-point. The symbols ° and □ denote significant differences of Ctrl and Bay 11 with FSL-1 and Bay 11 + FSL-1 groups $p < 0.001$.

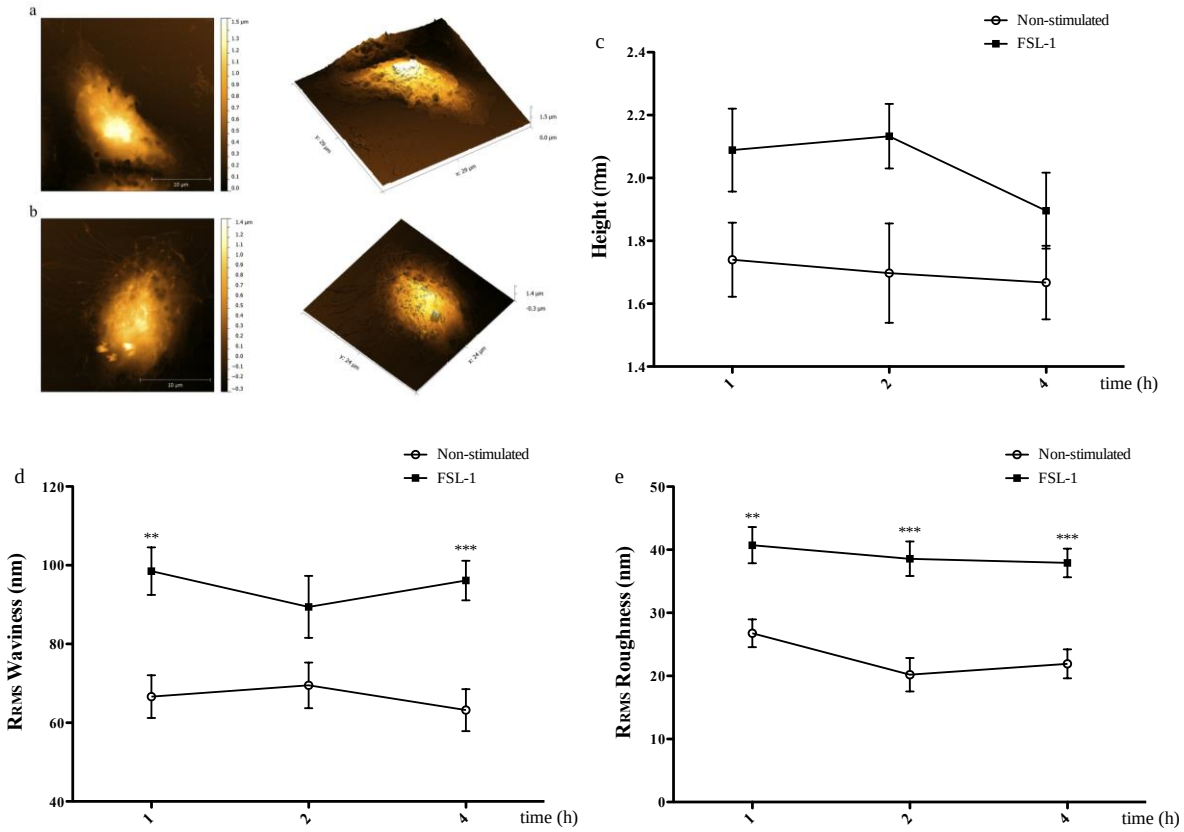

Figure 4.6. Changes on RL95-2 cell height and membrane waviness and roughness after TLR 3 stimulation. The endometrial cell line RL95-2 was treated or not with Poly I:C (100 ng/ml) for 1, 2 and 4 h. The cells were fixed and imaged by intermittent contact AFM. Representative 2D (left) and 3D (right) images of the RL95-2 endometrial epithelial cells non-stimulated or treated with 100 ng/ml Poly I:C for 1 h were generated in Gwiddion. The brightness of the image is proportional to the cell height The cell height (a), membrane waviness (b) and roughness (c) were determined in Gwyddion using a cut-off value of 0.03 for waviness and 0.09 for roughness. The effects between non-stimulated versus Poly I:C were compared using a two-way ANOVA with Bonferroni post-test to compare the different timepoints. ** $p < 0.01$ and *** $p < 0.0001$.

4.4.5 Flow cytometry evaluation of the expression of different membrane proteins in the endometrial cells after TLR stimulation

4.4.5.1 A high expression of nine molecules from the panel was observed in the epithelial cells

After performing a general screening of the with the whole expression panel, we observed that the expression of 13 of the surface proteins was similar to the isotype control antibody in the three cell lines (X63 IgG, Median fluorescence intensity MFI = 3.5, Fig. 4.7). Hence these 13 markers were excluded for the subsequent analysis. The final panel of analysis was: β1 Integrin, α2 Integrin, CD59, MHC-I, CD147, CD98, CD9, CD81 and CD151. The selection was based on the expression of the molecules in the three epithelial cell lines Ishikawa 3H-12, Ishikawa and MCF7. After its analysis we observed that only the expression of β1 integrin, CD147, CD9, and CD98 change after the treatment with different TLR ligands. The changes observed in their expression are presented as follows:

a. β1 integrin

The expression of β1 integrin in both Ishikawa 3H-12 and Ishikawa showed a tendency to increase after 24h of stimulation (Fig 4.8). The CpG treatment of Ishikawa at 24h was different from the control ($p < 0.05$). Significant differences were only observed in both Poly I:C and all ligands treatment compared to the control at 24h ($p < 0.05$). The treatment of the RL95-2 cells with Poly I:C and the combination of all TLR ligands was able to increase this protein expression significantly ($p < 0.05$).

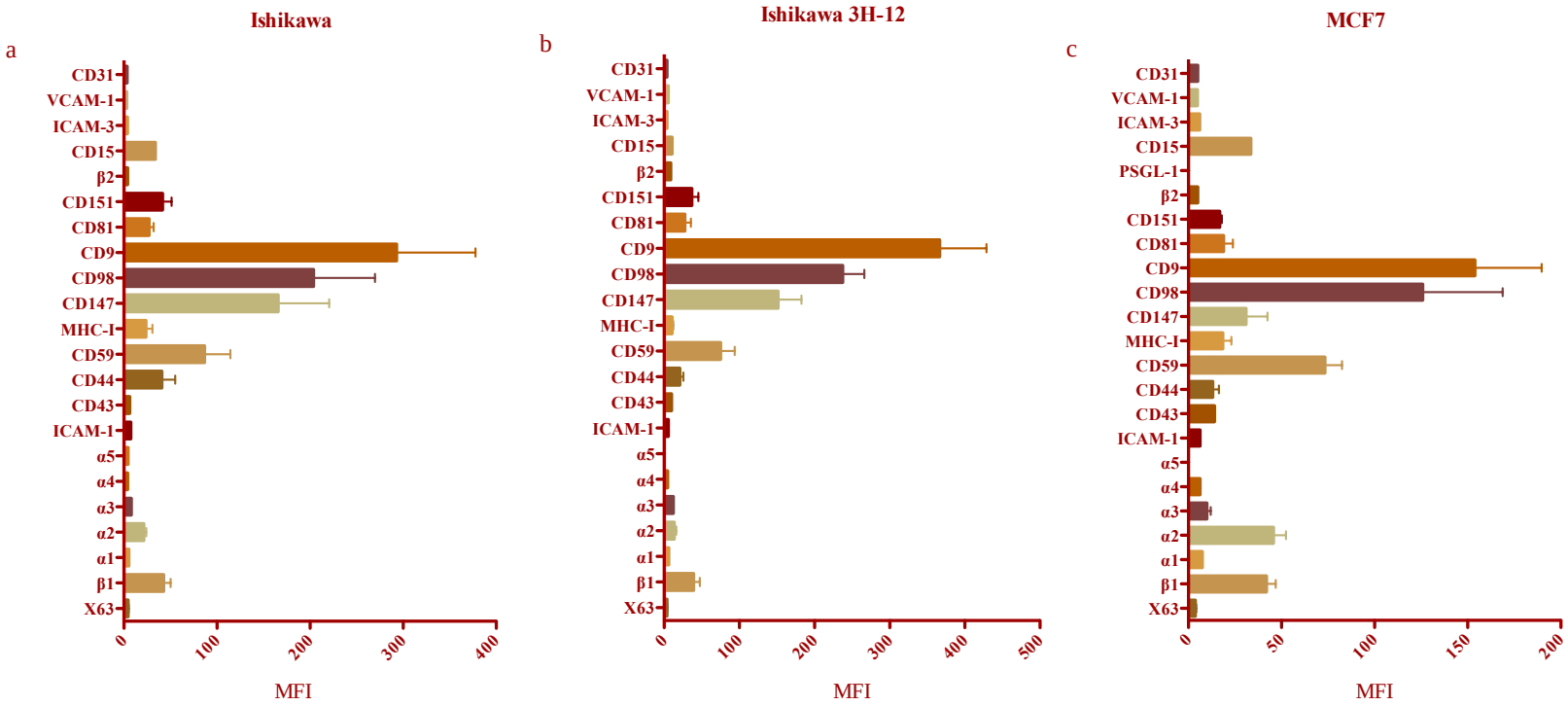

Figure 4.7. Panel of expression of 22 different surface markers in endometrial and breast carcinoma cell lines. The expression of 22 different membrane surface markers was assessed by flow cytometry on the endometrial cell lines Ishikawa (a) and Ishikawa 3H-12 (b) and the breast carcinoma cell line MCF7 (c). The isotype control is X63 a murine IgG antibody. The results of expression are shown as median fluorescence intensity (MIF).

b. CD147

The expression of CD147 increased after treatment of Ishikawa 3H-12 with TLR ligands in both 4 and 24h compared to the untreated control (Fig 4.9). The 24h treatment with FSL-1 was significantly different from the control and 4h treatments with Poly I:C and combined ligands ($p < 0.05$). Compared to this, the Ishikawa treatment at 4 and 24h showed a decrease in the expression of CD147 compared to the control. This reduction was found to be significant at 24h treatment with flagellin ($p < 0.05$, Fig. 4.9). The treatment of MCF7 cells for 24 h increased CD147 expression in all the conditions compared to the control ($p < 0.05$). In the RL95-2 cells, the treatment with Poly I:C was able to induce an increase in CD147 expression ($p < 0.05$).

c. CD9

The expression of CD9 in the Ishikawa 3H-12 and the Ishikawa cells remained constant after the stimulation (Fig. 4.8a and 4.10). MCF7 cells showed a slight increase after the stimulation for 24h with the TLR ligands. This increase was significant only in the CpG and Poly I:C stimulated groups compared to the control ($p < 0.05$). In the RL95-2 cells, the expression of CD9 was increased by the treatment with Poly I:C and in the combination of ligands ($p < 0.05$).

d. CD98

The expression of CD98 in Ishikawa cells was not modified by the TLR treatment, however a great variability was observed (Fig 4.11). The same expression in Ishikawa 3H-12 had a tendency to increase after 4 and 24h of TLR stimulation. The stimulation of the MCF7 cells with Poly I:C decreased substantially this expression after 24 h. In contrast, the stimulation of RL95-2 with Poly I:C and the combination of the ligands increased the expression of CD98 considerably.

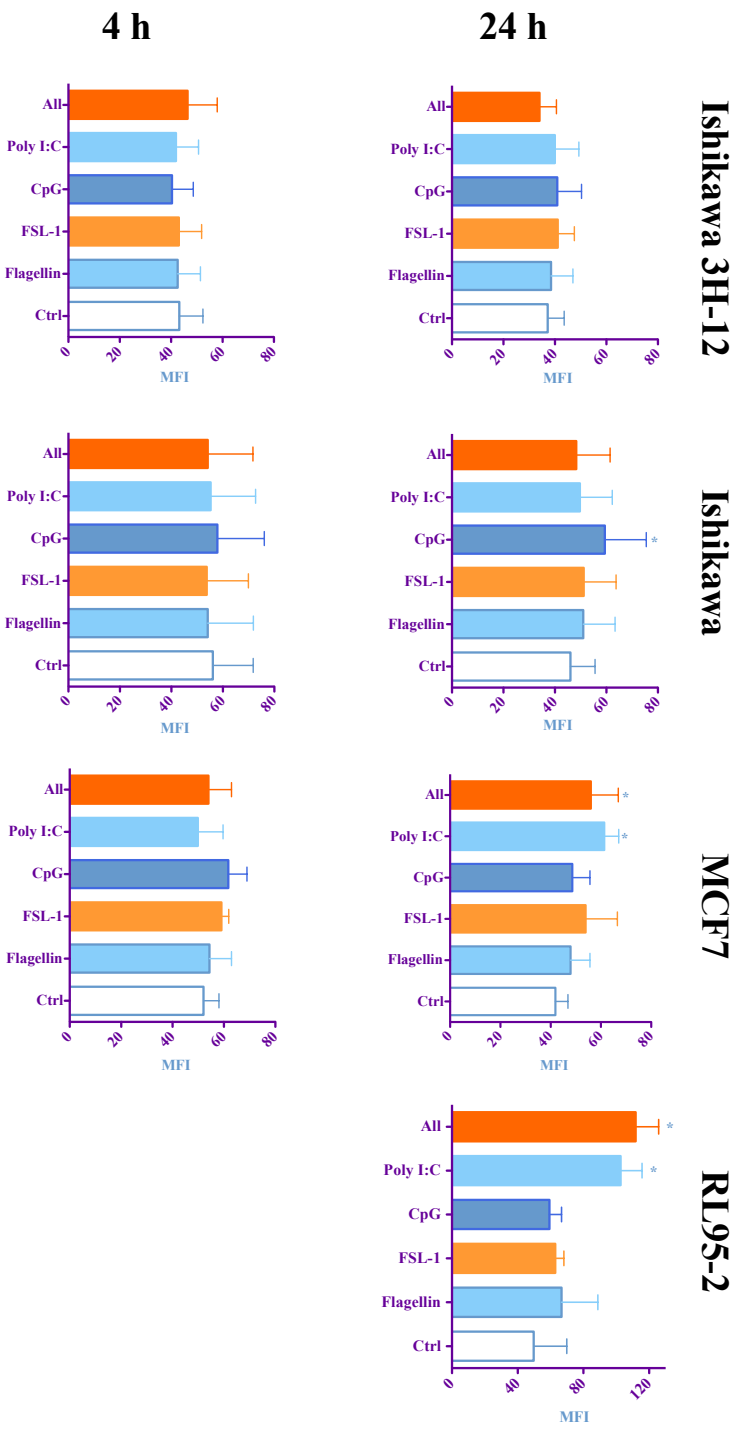

Figure 4.8. Expression of Beta-1 integrin in different epithelial cell lines after TLR stimulation. The expression of β1 integrin was assessed in the epithelial cell lines Ishikawa 3H-12, Ishikawa, MCF7 and RL95-2. Cells were stimulated or not (Ctrl) with the ligands for TLR 5 (Flagellin 100 ng/ml), TLR 2/6 (FSL-1, 100 ng/ml), TLR 9 (10 nM), TLR 3 (Poly I:C, 10 µg/ml) and a combination of all for 4 and 24 h. The expression is registered as median intensity of fluorescence (MFI) and differences between treatments and control estimated using a One-Way ANOVA with Bonferroni post-treatment. $P < 0.05$ was considered to be significant.

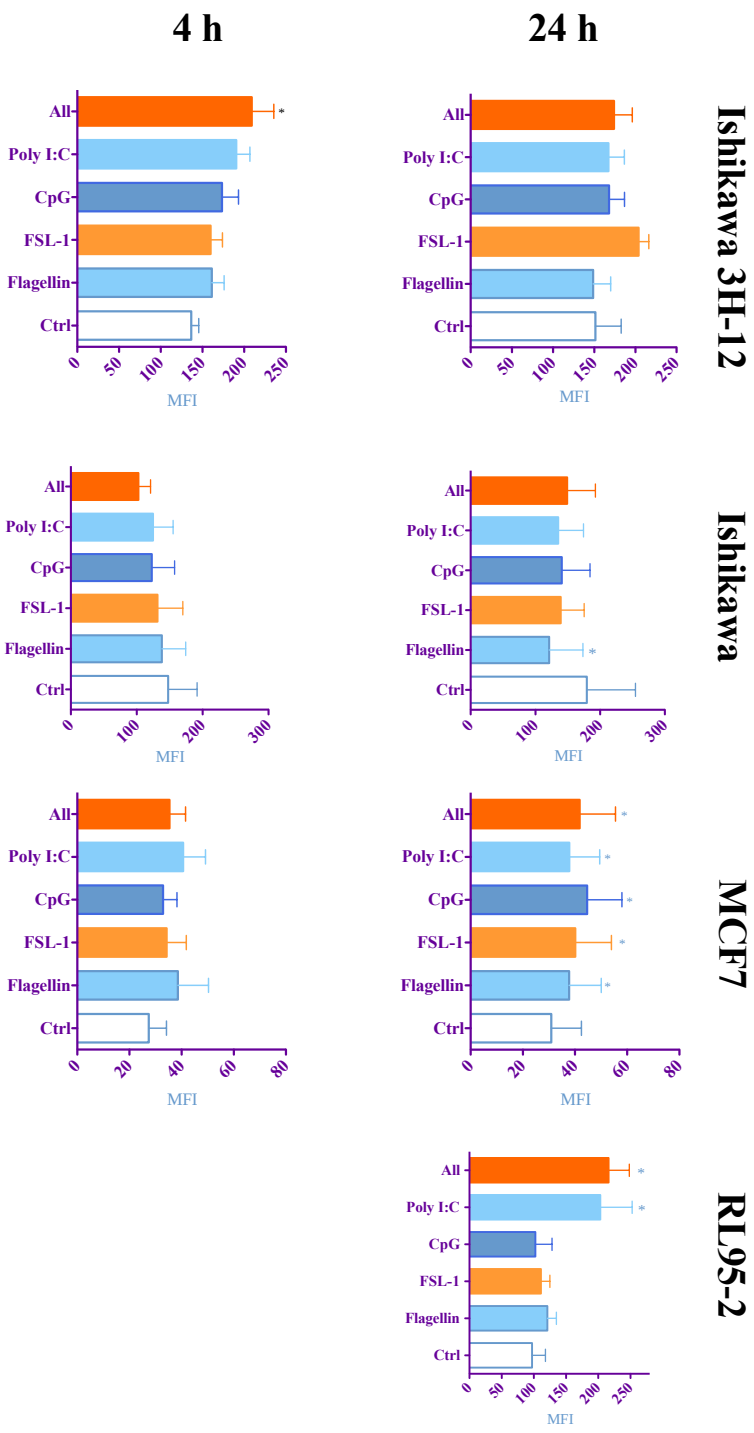

Figure 4.9. Expression of CD147 in different epithelial cell lines after TLR stimulation. The expression of CD147 was assessed in the epithelial cell lines Ishikawa 3H-12, Ishikawa, MCF7 and RL95-2. Cells were stimulated or not (Ctrl) with the ligands for TLR 5 (Flagellin 100 ng/ml), TLR 2/6 (FSL-1, 100 ng/ml), TLR 9 (10 nM), TLR 3 (Poly I:C, 10 μg/ml) and a combination of all for 4 and 24 h. The expression is registered as median intensity of fluorescence (MFI) and differences between treatments and control estimated using a One-Way ANOVA with Bonferroni post-treatment. $P < 0.05$ was considered to be significant.

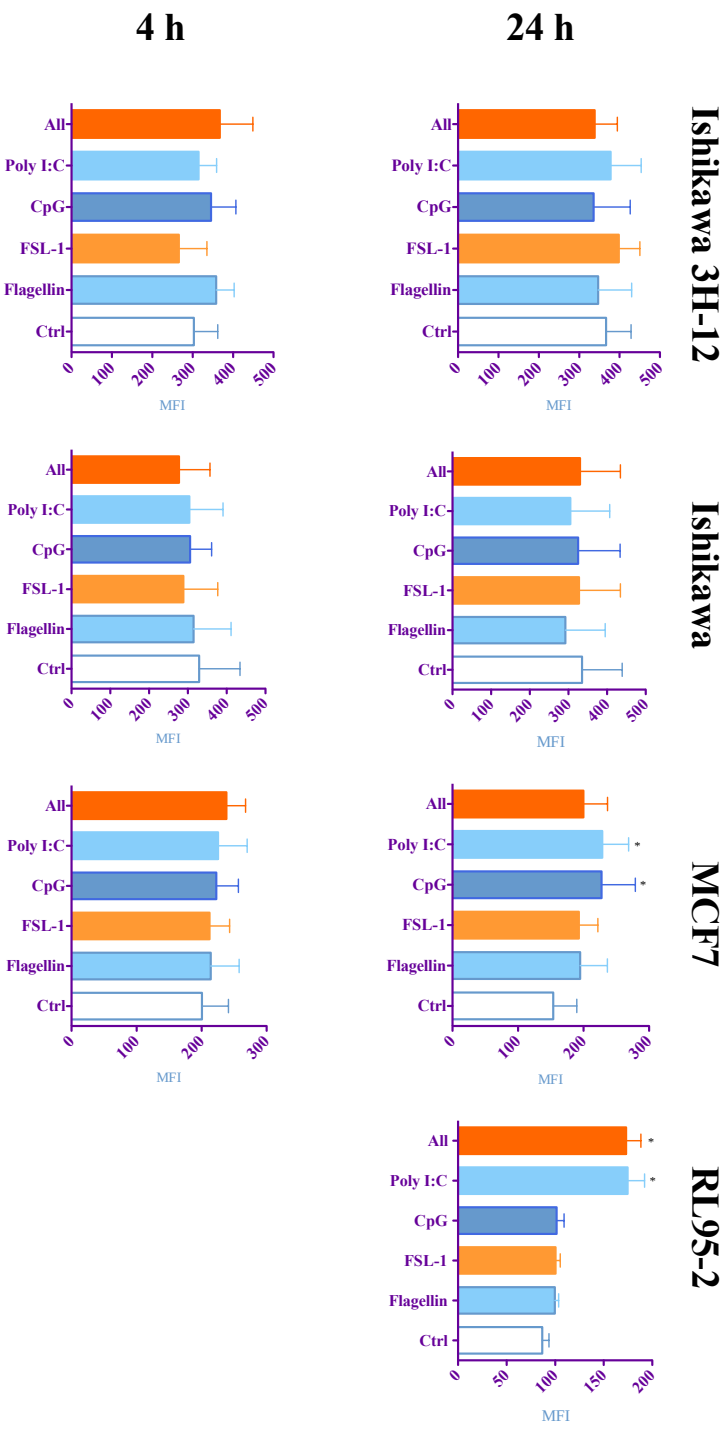

Figure 4.10. Expression of CD9 in different epithelial cells lines after TLR stimulation. The expression of CD9 was assessed in the epithelial cell lines Ishikawa 3H-12, Ishikawa, MCF7 and RL95-2. Cells were stimulated or not (Ctrl) with the ligands for TLR 5 (Flagellin 100 ng/ml), TLR 2/6 (FSL-1, 100 ng/ml), TLR 9 (10 nM), TLR 3 (Poly I:C, 10 µg/ml) and a combination of all for 4 and 24 h. The expression is registered as median intensity of fluorescence (MFI) and differences between treatments and control estimated using a One-Way ANOVA with Bonferroni post-treatment. $P < 0.05$ was considered to be significant.

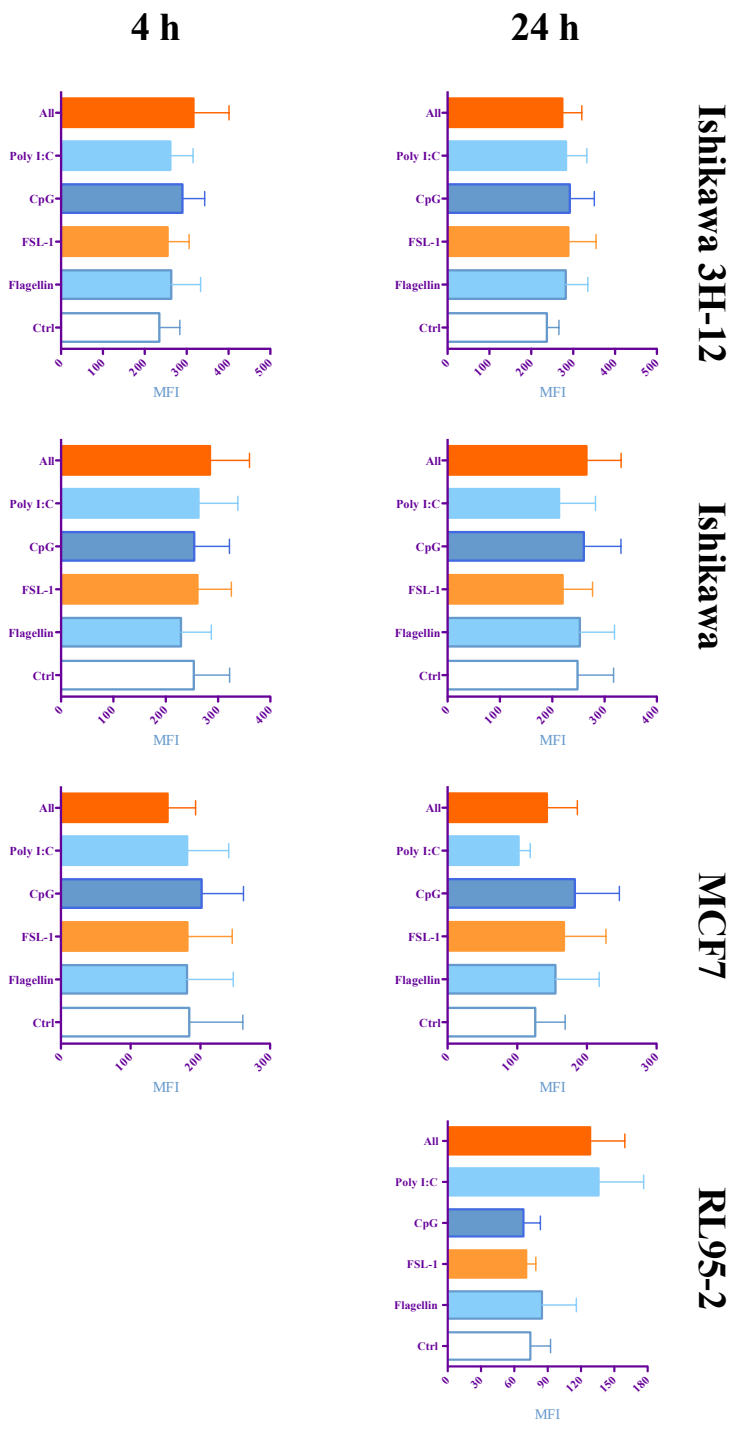

Figure 4.11. Expression of CD98 in different epithelial cell lines after TLR stimulation. The expression of CD98 was assessed in the epithelial cell lines Ishikawa 3H-12, Ishikawa, MCF7 and RL95-2. Cells were stimulated or not (Ctrl) with the ligands for TLR 5 (Flagellin 100 ng/ml), TLR 2/6 (FSL-1, 100 ng/ml), TLR 9 (10 nM), TLR 3 (Poly I:C, 10 µg/ml) and a combination of all for 4 and 24 h. The expression is registered as median intensity of fluorescence (MFI) and differences between treatments and control estimated using a One-Way ANOVA with Bonferroni post-treatment. $P < 0.05$ was considered to be significant.

4.5 Discussion

Endometrial receptivity is a term designed to characterise the changes undergone by the uterine cells at late secretory phase to favour the implantation of the embryo. These changes among others, encompass proliferation of stromal and epithelial cells, cell morphology alterations, increase of the protein secretory profile and polarisation of the adhesive proteins expressed on the apical side of the cell membrane (Achache & Revel 2006; Bentin-Ley et al. 1999).

It is known that during the proliferative phase of the cycle, some of the cells membrane get covered in microvilli but only few endometrial cells will develop cilia (More & R. G. R. Masterton 1975). During the late-secretory phase the endometrial cells get their microvillous surface and pinopodes ready to interact with the trophoblasts of an implanting embryo (Bentin-Ley et al. 1999; Bartosch et al. 2011). These morphological changes are induced by progesterone promoting the receptiveness of the endometrium towards embryo. In fact, the use of an anti-progestagen compound reduces the presence of swollen microvilli and pinopodes on the endometrial surface (Petersen et al. 2005). It is for this reason that the development of uterine microstructures has been used in different studies to date the endometrium and as a marker for receptivity prognosis. Normally the endometrium would develop pinopodes around day 20 of the cycle being day 18 the earliest and day 23 the latest. Nevertheless this developments seems to be heterogeneous and different for each women (Nikas et al. 1999). It has been found that the controlled ovarian hyper-stimulation or a hormonal replacement therapy, common procedures during *in vitro* fertilisation (IVF), reduces and increases this development respectively.

As shown in our results the microvilli found in the non-stimulated cells were lost after the stimulation with both FSL-1 and Poly I:C ligands. It is possible that this defence mechanism against inflammatory stimuli impairs drastically the efficiency of trophoblast-endometrial adhesion and affect cell receptivity. The loss of villi on the membrane surface would suggest a decrease in the roughness values but in contrast, we observed a gradual increase. This is likely to be due to the increase in membrane protrusions found in many of the cells imaged. The retraction of microvilli in response to stimuli has been studied by AFM using pulmonary ATII cells, where the microvilli

play an important role in the release of pulmonary surfactant (Hecht et al. 2011). ATII cells were treated with the Ca+2 channel modulator capsazepine, which promoted microvilli retraction, decreasing membrane roughness. The stimulation of dendritic cells with Pam3CSK (TLR 2 ligand) was able to decrease the podosomes, surface microstructures and focal contacts of the cells, all regulated by F-actin. This presumably allowed the cells to increase their phagocytic activity by relocating the actin used before for migration (West et al. 2008). This decrease in podosome activity is induced as soon as 20 min of TLR stimulation and it is independent from MyD88 signalling, an initial adapter protein for TLR signalling. Nevertheless, the authors were able to observe that blocking ERK1/2 and p38, adaptors of the MAPK pathway, an additional TLR signalling pathway, could partially recover this podosomal loss (West et al. 2004).

In our study, we were able to observe changes in cell membrane. By measuring the R_{RMS} of the membrane and using two cut-off values, we could distinguish between the overall cell profile (waviness) and the nanometrical structures (roughness) as microvilli on the membrane (D Antonio et al. 2012). The endometrial cell membrane has been previously studied by AFM. Francis and colleagues were able to observe the microvillus membrane surface of the endometrial epithelial cells (Francis et al. 2009; Francis et al. 2010). In our observations we were unable to find pinopodes in the endometrial cells. This is likely to be due to the lack of a progesterone stimulation, which is known to induce the pinopode development in the apical surface of the cells (Campbell et al. 2000; Nikas 1999; H. Singh & Aplin 2009).

The changes in the membrane roughness induced by TLR activation seemed to be bigger than the microvilli size, therefore we measured the waviness of the cells. It is possible that the changes observed in the cells that appear as protrusions correspond to membrane blebbing induced by the TLR stimulation. Cell blebbing is a type of membrane extension where it protrudes in an spheroidal shape dependant on the actin polymerisation (Hagmann et al. 1999). It has been suggested that membrane blebbing occurs during cell motility, exocytosis and apoptosis (Fackler & Grosse 2008). In our observations, we could discard the possibility of the cells undergoing apoptosis as a result of the TLR stimulation. The morphology of the TLR stimulated cells differed from that of an apoptotic cell: flattened membrane with hole-like structures in it. AFM studies have shown the morphology of an apoptotic endometrial cell (Ishikawa cell line)

or murine macrophage cells showing the above mentioned characteristics (K. S. Kim et al. 2012; D.-C. Wang et al. 2011). It has been proposed that cell blebbing would come as a defence mechanism against different pathogens. For example, the contact of the bacterial pore-forming toxin (PFT) O streptolisin with the cells, induced the blebbing of the CHO cell membranes in a Ca+2 channel dependent way. This blebbing prevented cell damage and apoptosis (Keyel et al. 2011). In a similar way, the vaginolysin of Gardnerella vaginalis a PFT, induced the blebbing of vaginal and cervical epithelial cells in a CD59 dependent manner (Randis et al. 2013; Xiao, Chang, et al. 2002a). It has been observed that the stimulation of P2X7R, another receptor of the innate immunity that recognises ATP, can induce membrane blebbing and IL-1beta secretion in macrophages (Verhoef et al. 2003). In our observations, the activation of TLRs was sufficient to induce a similar process. The mechanisms mediating initiation of blebbing in cells remain unknown and membrane blebbing has never been linked to TLR activation before. Nevertheless it is logical to think that it would be induced as an innate defence mechanism of the cell to release pathogens from the cell surface.

The fact that the inhibitor Bay-117082 was unable to stop the membrane changes induced by TLR stimulation shows that the effect over the membrane morphology is NF-κB independent. Although the exact mechanism is unknown, we have previously observed that the FSL-1 stimulation of the RL95-2 endometrial epithelial cells has a functional effect by decreasing the adhesion of trophoblast spheroids to the monolayer (Sánchez López et al. 2014). Previous experiments have shown that the treatment of endometrial cells with flagellin (TLR 5) ligand is also able to reduce the adhesion of trophoblast spheroids (Aboussahoud, Bruce, et al. 2010a). Pre-treatment of Ishikawa endometrial cells with 10 μM BAY-117082 is able to increase the adhesion of trophoblast spheroids to flagellin stimulated cells (Caballero et al. 2013). It might be possible that the observed effects of TLR stimulation over trophoblast binding and membrane morphology are independent from each other. It also might be possible that the endometrial cells have a differential response depending on the stimulated TLR as the production of IL-8, a cytokine produced after TLR stimulation, is mainly induced by flagellin (Aboussahoud, Aflatoonian, et al. 2010b). Membrane changes due to external stimuli are commonly induced rapidly by enzyme phosphorylation at membrane level without the need of inducing initial gene activation. As an example, we can observe that the stimulation of murine lungs with lipopolysaccharide (LPS, TLR 4 ligand) has been

shown to phosphorylate Src, a tyrosine kinase that mediates cytoskeletal assembly with the cell membrane. The use of a Src inhibitor before LPS stimulation decreased neutrophil migration, production of TNF-α, MMPs and NF-κB activation (Hui S Lee et al. 2007). This shows that the membrane associated tyrosine kinases might have an early role in the initiation of immune responses and might also mediate the responses observed in our experiments. Further investigation is required to determine the mediators of the cell membrane morphological changes.

We explored a set of 23 different surface expressed proteins as integrins, selectins, CAMs and other transmembranal glycoproteins. We found that the epithelial cells had a low expression of most of these molecules or the expression was absent. For this reason we continued the assays with molecules that comply with two main characteristics: 1) membrane molecules highly expressed in the cells and 2) markers that were previously suggested in literature to be related with implantation, such as: β1 integrin, CD147, CD9, and CD98 (Domínguez et al. 2010). The expression of these membrane molecules was assessed before in primary epithelial endometrial cells and the cell lines HEC-1A and RL95-2. These molecules were also found highly expressed in the cells, suggesting they could have an important role in intercellular adhesion and endometrial receptivity (Domínguez et al. 2010).

The expression of β1 integrin increased after 24 hrs of TLR stimulation in all the cell lines tested. Similar observations were reported in colorectal cancer cells where the activation of TLR 4 by LPS has been found to increase β1 integrin expression and increasing their metastatic potential (J. H. Wang et al. 2003; Hsu et al. 2011). Integrin β1 has been found to be important for TLR activation, for example in macrophages the attachment to ECM proteins has been found to increase TLR mediated responses (Monick et al. 2002). Furthermore, a pathogen recognition capacity of integrins has been suggested where α3β1 integrin mediates the recognition of bacteria by chondrocytes (Behera et al. 2006) and facilitates the phagocytosis of lipopeptides for TLR 2/1 recognition in endosomes (Marre et al. 2010). Integrin β1 is also involved in a diversity of inflammatory processes. It helps the migration of leukocytes to the inflammation site, remodelling of the tissue in wound healing and cartilage destruction in arthritis (Al-Jamal & Harrison 2008; de Fougerolles et al. 2000). From the reproductive perspective β1 integrin is important in embryo development as the knock

out mice shows developmental impairment and problems to invade the endometrial decidua (Stephens et al. 1995). Blocking of human endometrial β1 integrin with specific antibodies of peptides *in vitro* allows murine embryos to attach but not to spread in the decidua (Shiokawa et al. 1999; Hanashi et al. 2003). It may be possible that the functional role of β1 integrin in the endometrium after TLR stimulation changes from modulating the trophoblast attachment to modulating the recognition of pathogen for the initiation of immune responses.

The endometrium is an organ that undergoes constant remodelling. The expression of CD147 has been found in epithelial cells and stroma and might aid this remodelling process by priming the production of MMPs necessary for the menstrual breakdown and favouring the trophoblast invasion to the uterine decidua (Xiao, Chang, et al. 2002a). We observed a high expression of CD147 in all the endometrial cell lines and the MCF7 cells highlighting its importance for a normal epithelial function and adhesion with trophoblast cells. In fact, the knock out mice for CD147 is infertile due to embryo implantation defects (Igakura et al. 1998). Arthritis is an inflammatory disease related to an excessive remodelling of the synovial tissue. The synovial cells during this process overexpress CD147 that facilitates cartilage degradation (Treese et al. 2008). Inflammatory processes in the reproductive tract, such as endometriosis have been also linked with CD147 overexpression (Braundmeier et al. 2010). In our results, we observed that the expression of CD147 tended to increase after TLR stimulation in MCF7, Ishikawa 3H-12 and RL95-2 cells but a tendency towards reduction of this molecule in the Ishikawa cells. This might be related to the malignancy of the cells, as they are all carcinoma-derived cells. Changes on the expression of CD147 in the endometrium has been related before with endometrial carcinomas and the level of malignancy and patient survival (K. Nakamura et al. 2012). A correlation with TLR activation and cancer development has been observed where TLR 4 activation increased the colorectal tumoural cell invasiveness capacity (Hsu et al. 2011). If a similar effect is happening in the TLR stimulated endometrial cells, it might be possible that the stimulation favour the malignancy of the cells. It might be necessary to assess this same effect on the endometrial stromal cells to see what the effect might have on the modulation of trophoblast migration.

The expression of CD9 in the endometrial cells was variable and remained unchanged in the endometrial cell lines Ishikawa and Ishikawa 3H-12. Nevertheless this expression increased after 24 h of stimulation of all the TLRs in MCF7 breast carcinoma cells and with Poly I:C in RL95-2 endometrial cells. The inhibition of CD9 has been observed to facilitate the migration of endometrial carcinoma cells and a low expression of this molecule is associated with the carcinoma development and metastasis (Park, Inoue, Ueda, Hirano, Higuchi, Konishi, et al. 2000a). Hence it might be possible that changes in its expression with the cell line correlates again with malignancy of the cell line similar to what was observed with CD147. In macrophages stimulated with LPS, the expression of CD9 decreases and the inhibition or silencing of CD9 facilitates production of pro-inflammatory mediators. CD9 is able to exert this immune-modulatory effect by mediating the association of CD14 and TLR4 in lipid rafts necessary for LPS sensing (Suzuki et al. 2009). A similar function for CD9 has been observed in the recognition and induction of anti-viral responses by hepatocytes infected by hepatitis C virus (HCV). In this study, the tetraspanins CD9 and CD81, receptors for HCV, mediated the uptake and viral recognition by TLR 7 and production of interferon alpha (S. Zhang et al. 2013).

The expression of CD98 tended to increase in almost all of the cell lines when stimulated with the TLR ligands for 24 h, however there was a lack of significant difference due to the great variability. The association of CD98 and β1 integrin has been found to mediate adhesion processes and polarisation of the cells and is normally found in the basolateral compartment of epithelial cells (Cai et al. 2005). Up to this moment, no direct link between expression of CD98 and TLRs has been suggested. Nevertheless, CD98 and β1 has been observed to participate during inflammatory responses in the epithelial cells of the intestine relocating to the apical membrane. In this study CD98 was found to bind directly to the enterophatogenic *Escherichia coli* and mediate the expression of proinflammatory genes (Charania et al. 2013). It has also been found to mediate the endocytosis of Vaccinia virus (Schroeder et al. 2012). The importance of CD98 in embryo implantation was proposed as it was found expressed in the apical portion of the endometrium epithelium during late secretory phase and the silencing of this molecule in endometrial cells decreased the adhesion capacity to murine blastocysts (Domínguez et al. 2010). Furthermore CD98 deletion in murine embryos is lethal depicting its importance during embryogenesis (Tsumura et al. 2003). Whether the TLR

stimulation of endometrial cells directly influences CD98 adhesive functions and changes to facilitate pathogen recognition, still needs to be determined. Together these observations suggested that the complexes of β1 integrin, CD147, CD9 and CD98 might regulate immune responses by facilitating the pathogen sensing to TLRs. These complexes also mediate the intercellular adhesion of endometrial cells and trophoblasts during implantation. The variation of expression of these markers observed in the endometrial cells after TLR activation might be related to the epithelial immune responses. This might affect their adhesion capacity during implantation. Nevertheless knowing the cellular localisation of these molecules after TLR stimulation would help us to understand their roles in the epithelium.

The heterogeneity of the endometrial cell membrane phenotype has been suggested previously. Graham et al were able to describe that in a normal endometrial epithelium some endometrial cells are covered in microvilli but only subpopulations of epithelial cells were able to develop cilia (R. R. Masterton et al. 1975; More & R. G. R. Masterton 1975). The distribution of glycan markers in the cell membrane has also been found heterogeneous within the endometrial population (Campbell et al. 2000). This suggests that the endometrial cells respond differentially to the cyclic changes. This might suggest that specialised subsets of cells are highly sensitive to the cyclical changes and responsible for the interaction with the embryo. The observation of cell subtypes with specific functions is found in the epithelial layer of the intestine, where enterocytes, microfold (M) cells, goblet cells and Paneth cells interact with each other to guarantee a proper intestinal function (Rescigno 2011; Maldonado-Contreras & Mccormick 2011). In our work, we were able to measure the changes that TLR activation elicited in the membranous microstructures by AFM and alterations in surface molecule expression. It is likely that the highly heterogenic endometrial population expressing the membrane markers could mask any small effect induced by the TLR stimulation. Another possibility is that the TLR stimulation only induced a rearrangement of the membrane microdomains through actin cytoskeleton or the effect was direct over the rearrangements of the cytoskeleton scaffolding. Further experiments are required to verify the role of the TLR activation on the endometrial cell cytoskeleton and its relation with membrane structure. These membrane changes could contribute to the loss of receptivity of the endometrial cells influencing the adhesive surface exposed to the implanting embryo and reduce the possibility of attachment.

In conclusion the results presented are indicative of an immediate change in the surface structure and molecular composition of endometrial cells in response to TLR ligands. It remains to be determined how does these alterations might help these cells to gain protection from infection and/or reduce their ability to interact with embryo and achieve a successful implantation.

Chapter 5. The expression of MUC1 and MUC16 in response to endometrial TLR stimulation as a mucosal immune mechanism of interference with blastocyst adhesion

5.1 Introduction

The implantation of the embryo is a process that requires a special coordination between the developing embryo and the cycling uterus of the mother. The embryo will take around 5-7 days to reach the uterus. By then it must have reached the blastocyst stage of development and hatched from the zona pellucida. Meanwhile the endometrium now in the luteal or late-secretory phase would have undergone a series of morphological and molecular changes to facilitate the allocation of the embryo (Norwitz et al. 2001). The "window of implantation" is the period where the functional layer of the endometrium has acquired a "receptive phenotype" (Lessey 2000). This includes the stratification of the epithelial cells, decidualisation of the stromal compartment, development of spiral arteries and glands and secretory activity of epithelial glandular and stromal cells, among others. At the molecular level, the endometrial epithelial cells express a series of glycans that will serve as attachment molecules for integrins of the blastocyst (H. Singh & Aplin 2009). The embryo implantation is not only regulated by the expression of factors important for its attachment but also anti-adhesive factors as the glycoprotein mucin 1 (Simón et al. 2001).

Mucins are glycoproteins with O-linked long carbohydrate chains to a core rich in threonine/serine tandem repeats (TR) that are present in all the epithelial mucosa of the body. They are found on the apical side of the epithelial cells as membrane linked or secreted forming gel in the lumen of different organs (M. C. Rose & Voynow 2006). They can be found in the respiratory, digestive and reproductive tracts as well as in the eye cornea and the mouth's mucosa (C. M. Evans & Koo 2009). There are 10 different membrane linked mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17, MUC20) and 8 gel forming mucins from which 5 belong to the cysteine-rich TR (MUC6, MUC2, MUC5AC, MUC5B and MUC19) and 3 to the cysteine-poor TR (MUC7, MUC8 and MUC9) (M. C. Rose & Voynow 2006).

The expression of the membrane bound mucin 1 was found in secretory and menstrual endometrium whereas gel forming mucins were detected only in the lower female reproductive tract (Gipson et al. 1997). Mucin 1 has been characterised to be expressed in the apical portion of the endometrial cells and its expression varies during the menstrual cycle (Achache & Revel 2006). Its presence has been reported to interfere

with the accessibility of the adhesion molecules of the endometrium, hence with the implantation of the embryo (Simón et al. 2001). Mucin 1 is constitutively expressed in the endometrium of various species, such as mice, rats, sheep and pigs. The progesterone surge down-regulates its expression during receptive phase of the cycle (Aplin & Kimber 2004; Braga & Gendler 1993; Spencer et al. 2004). In contrast, in other species such as humans, baboons and rabbits, the expression of MUC1 seems to increase during peri-implantation period (Achache & Revel 2006; Gipson et al. 1997; Hoffman et al. 1998). Interestingly the local regulation of MUC1 in the human endometrium seems to be dependant on the presence of the embryo, where the epithelial cells in the embryo surroundings down-regulate its expression (Meseguer et al. 2001; H. Singh & Aplin 2009).

Anomalies in the production of mucin 1 in the reproductive tract has been related with, tumour development and fertility problems. For example, endometrial adenocarcinomas show a high expression of MUC1, MUC5B and MUC8, which seems to be a common feature of tumours as it can be also observed in cervical cancerous tissue (Hebbar et al. 2005). Endometriosis is another disease where high levels of mucin 16 are found in the serum but the expression remains constant in the endometriotic tissue (Dharmaraj et al. 2014). It has been reported that the detection of low levels of soluble MUC1 in endometrial flushing has been found in patients with miscarriages as this mucin should shed from the endometrium to facilitate pregnancy (Hey et al. 1995). Another example of MUC1 anti-adhesive properties can be observed in the cells of the fallopian tubes where its highest expression level is observed during the luteal phase of the menstrual cycle. This will prevent the embryo from implanting in the fallopian tubes as it has been observed that patients with ectopic pregnancies lack MUC1 expression in the tubal cells (Al-Azemi et al. 2009).

The production of mucus is an innate immune mechanism of defence that acts as a physical barrier against invading microorganisms (Moncada et al. 2003). The epithelial cells of the female reproductive tract (FRT) are able to recognise microorganisms and initiate immune responses through the pattern recognition receptor family of the Toll-like receptors (TLR) (Koga & Mor 2008). The TLR family in human is formed by 10 members that are able to recognise bacterial, fungal, parasitical and viral pathogen associated molecular patterns (PAMPs) (Fazeli et al. 2005). Ligation of the TLRs will

initiate a signalling cascade that will activate the transcription factors nuclear factor (NF)-κB or AP-1 responsible for promoting the expression of inflammatory genes (Takeuchi & Akira 2001). TLR 2 form heterodimers with TLR 1 and 6 to recognise PAMPS from pathogens like *Chlamydia trachomatis or N. gonorrhoeae* responsible for sexually transmitted and pelvic inflammatory diseases (O'Connell et al. 2006). Particularly in the endometrium it has been observed that the TLR expression varies along the menstrual cycle (Aflatoonian et al. 2007). Recently the activation of endometrial TLRs has been found detrimental for embryonic implantation. The *in vitro* activation of TLR 5 by flagellin and TLR 2 and 2/6 by peptidoglycan and the synthetic diacyl lipopeptide FSL-1 respectively, decreased the adhesion capacity of trophoblast spheroids to the stimulated endometrial cells (Aboussahoud, Bruce, et al. 2010a). The stimulation of TLR 2/6 in the murine uterine horn has also been observed to reduce embryo implantation affecting uterine morphology and cytokine secretion (Sánchez López et al. 2014). The exact mechanisms for this decrease of trophoblast adhesion and embryo implantation remain unknown. We can speculate that the activation of this endometrial TLRs is able to affect endometrial receptivity and activate mucosal innate immune defences. In the epithelial airway, the activation of TLR 2 has been reported to increase the production of mucin 5AC in response to *Mycoplasma pneumoniae* (Chu et al. 2005). It is possible that a similar response could prime the production of mucins in the endometrium as a defence mechanism. While protecting the endometrium from a pathogenic invasion, this mucin production would form an anti-adhesive factor for the embryonic implantation.

In the present experiments, we tested *in vitro* if the activation of endometrial TLRs could affect the expression of two membrane bound mucins, mucin 1 and mucin 16. The endometrial cell line RL95-2 and the breast carcinoma cell line MCF7 were used as epithelial cells. They were stimulated with the synthetic diacylated lipopeptide FSL-1, specific ligand of TLR 2/6 and flagellin ligand of TLR 5 for 4 and 24 h.

5.2 Materials and methods

5.2.1 Analysis of the expression of MUC1 and MUC16 in the endometrial epithelial cells

5.2.1.1 Preparation of epithelial cells

The human endometrial adenosquamous carcinoma cell line (RL95-2) was obtained from ATCC and employed to mimic the receptive endometrium. The RL95-2 cells were cultured in T75 flasks at 37°C in DMEM/F-12 (Sigma) supplemented with 10% of heat inactivated foetal bovine serum (FBS) (Sigma), 5 μg/ml Insulin (human recombinant insulin from Gibco), and 1% L-glutamine (Invitrogen) in 5% CO_2 atmosphere until total confluency. The MCF7 cell line was employed as an epithelial cell line from non-endometrial origin. The cells were cultured in DMEM/F-12 supplemented with 10% of heat inactivated FBS (Sigma), 160 ng/ml Insulin (Gibco), and 1% L-glutamine (Invitrogen) in 5% CO2 atmosphere until total confluency.

The media was changed every second day till confluence of the cells. At confluence, cells were washed with DPBS (Sigma) and were harvested using 1 ml of trypsin-EDTA (Sigma). The cells were incubated for 3 min at 37°C and then pelleted by centrifugation at 300x g for 4 min. Finally, the pellet was resuspended in 3 ml of media and the cells were seeded in a T75 flask.

5.2.1.2 RNA extraction, purification and cDNA synthesis

The endometrial cells were grown in T25 culture flasks until total confluency. Then the cells were homogenised and collected in 1 ml of TRI reagent (Ambion, Huntingdon, UK). RNA was extracted using the conventional chloroform – ethanol extraction. Briefly, 200 μl of chloroform (Sigma) were added to the TRI reagent cell homogenate, mixed and incubated at room temperature for 15 min. The mix was centrifuged at 12000x g for 10 min at 4°C. The aqueous transparent upper layer was transferred to a tube with 500 μl of isopropanol (Sigma), vortexed 30 seconds and incubated 10 min at room temperature. The sample was then centrifuged at 12000x g for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of 75% ethanol (Sigma) and centrifuged at 7500x g for 5 min at 4°C. The supernatant was carefully

discarded and the pellet was resuspended in 40 µl of RNase free water (Sigma). The DNA-free™ (Ambion) kit was used for RNA purification following the manufacturer instructions. Briefly, the 0.1 volumes of 10x DNase buffer and 1 µl of DNase were added to the sample and incubated for 30 min at 37°C. Then, 0.1 vol of DNase inactivation reagent was mixed in and incubated for 2 min at room temperature. The samples were centrifuged at 10000x g for 1.5 min and the supernatant was transferred to a clean tube. The procedure was repeated twice. Finally, the concentration of RNA was determined with the Nanodrop spectrophotometer (Labtech, UK). The ratio of absorbance between 260 and 280 nm was used as a standard of purity (A_{260}/A_{280} = 1.8 – 2). The first-strand complementary DNA (cDNA) was synthetized from 2 µg of RNA with the RNA to cDNA kit (Applied biosystems, Life Technologies; Paisley, UK) following the instructions from manufacturer. Briefly 2 µg of RNA were mixed with 10 µl of 2x RT buffer, 1 µl of 20x enzyme mix and enough nuclease-free water to complete 20 µl of the total mix. Negative control was prepared for each sample where no enzyme was added. The mix was incubated in a thermocycler (Eppendorf, Stevenage UK) for a cycle of 37°C for 60 min, followed by 95°C for 5 min and finally 4°C. Samples were stored at -20°C for further analysis.

5.2.1.3 Primer design, optimisation and validation for qPCR

The forward (fwd) and reverse (rev) primers for the human mucin (MUC) 1 (Accession no. NM_001204296.1) and MUC16 (Accession no. NM_024690.2) were created with the tool Primer-BLAST (NCBI website). The genes for human β-Actin (BACT) and β-2 microglobulin (B2M) were used as reference genes (Table 5.1). The primers were between 18-23 nucleotides, with a G/C content of 45 – 57% and a similar melting temperature (T_m) for fwd and rev primers. Both were designed to bridge an exon-exon junction to avoid genomic DNA amplification. Their specificity was verified with BLAST (NCBI website) against human RNA and DNA databases. The possibilities for a primer-dimer formation and secondary structures were analysed with the Oligo Analyser tool of the Integrated DNA technology (IDT) website and ordered from the same company (Leuven, Belgium). The efficiency of the primers for mucin and reference genes was verified by quantitative real-time PCR (qPCR). The variation of the threshold cycle number (C_t) was estimated during the exponential phase. A standard curve was generated using serial dilutions of the samples of cDNA (1:5, 1:15, 1:45,

1:135 and 1:405) and plotted using the logarithm of the cDNA dilution versus the average C_t of three replicates. The efficiency for each set of primers was calculated as follows:

$$E = 10^{[-1/slope]}$$

And the percentage estimated with the equation:

$$E(\%) = [-1+ (10^{[-1/slope]})]*100$$

An efficiency of 80 to 120% for each set of primers was considered acceptable for further analysis of gene expression.

Table 5.1. Gene names and symbols, accession numbers, sequences and product size expected after amplification.

Accession number	Gene	Symbol	Sequence (5'-3')	Product size (bp)
NM_001204296.1	Mucin 1	MUC1	F: CCGCCGAAAGAACTACGG	179
			R: CCTGCAGAAACCTTCTCATAG	
NM_024690.2	Mucin 16	MUC16	F: GCCTCTACCTTAACGGTTACAATGAA	114
			R: GGTACCCCATGGCTGTTGTG	
NM_001101.3	β-Actin	BACT	F: CCTCCCTGGAGAAGAGCTAC	152
			R: CTTCATGATGGAGTTGAAGGT	
NM_004048.2	β-2 Microglobulin	B2M	F: TATGCCTGCCGTGTGAACCA	98
			R: GCGGCATCTTCAAACCTCCA	

5.2.1.4 Quantitative real-time PCR and gel analysis

The qPCR reaction was performed using the SYBR Green Jump Start *Taq* Ready mix® (Sigma) containing 20 mM Tris-HCl, 100 mM KCl, 0.4 mM dNTP (dCTP, dTTP, dGTP, dATP), 7 mM $MgCl_2$, stabilisers, JumpStart Taq antibody, SYBR Green and 0.05 unit/µl of Taq DNA polymerase. A master mix of 20 µl of total volume was prepared with 1 µl of each primer (100 nM), 1 µl of sample cDNA, 10 µl of the SYBR green buffer mix and 7 µl of RNase free water. Each experiment included the experimental samples, one positive control (1 µl of the pooled experimental samples) and two negative controls (no template control using 1 µl of water instead of cDNA and no-RT control). The qPCR was performed in triplicates of each biological replicate in 96 well plates using a Stratagene 3005x real time PCR machine (Stratagene,

Waldbronn, Germany). The amplification cycle was set as follows: 95°C for 30 sec for DNA denaturation, 56°C (MUC1) or 57°C (MUC16) for 1 min for primer annealing and 72°C for 1 min for primer extension. The amplification was set to 40 cycles and the final extension was at 72°C for 3 min. The products of amplification were resolved by electrophoresis in an agarose 2% gel at 90V for 1 h. The products of amplification were compared to a MiniSizer ladder (Norgen Biotek; Ontario, Canada) to corroborate the expected size according to table 5.1.

5.3 Experimental design

5.3.1 Evaluation of the MUC1 and MUC16 gene expression after TLR stimulation

The RL95-2 and MCF7 cells were cultured in T25 flasks to total confluence. The media was replaced with serum-free DMEM/F-12 and the cells stimulated with FSL-1 (100 ng/ml) and flagellin (100 ng/ml) for 4 and 24h. One flask was used as positive control and was stimulated with phorbol 12-myristate 13-acetate and ionomycin (PMA/I) for 4h as this compound can be toxic when the cells are exposed to it for more than 6 h. The cells were then collected in TRI-reagent, total RNA extracted, cDNA synthesised and the expression of the MUC1 and MUC16 genes was determined by qPCR using BACT and B2M as reference genes.

5.3.2 Statistical analysis

Each experiment is comprised by 9 samples formed by three samples (experimental replicates) performed in three different days (biological replicates). The $\Delta\Delta C_t$ method was used to analyse the collected expression data and was calculated as follows: The average of the C_t values for each reference gene (C_{tBACT} and C_{tB2M}) is calculated and averaged (C_{tRef}). Then the C_t of each sample of the gene of interest (C_{tGene}) is normalised to the C_{tRef} to obtain the ΔC_t:

$$\Delta C_t = C_{tGene} - C_{tRef}$$

Then, the ΔC_t was normalised with the ΔC_t of the standard sample (pool of the cDNA of all the samples):

$$\Delta\Delta C_t = \Delta C_t - \Delta C_{t\,STD}$$

A relative mRNA expression for the samples was calculated as $2^{(-\Delta\Delta Ct)}$. The results were expressed as mean ± SEM. Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). Multiple groups were compared with a one-way ANOVA and a Bonferroni multiple comparison post-test. $P \leq 0.05$ was considered to be significant.

5.4 Results

5.4.1 Expression of mucin 1 and 16 in the epithelial cells after TLR stimulation

5.4.1.1 Primer optimisation

The efficiency and specificity of each set of primers was confirmed using a standard and a dissociation curves. The set of primers for the reference genes BACT and B2M had an efficiency of 96.1 and 94.7% respectively and one amplification product observed both in the dissociation curve (Fig. 5.1 and 5.2). The primers for MUC1 and MUC16 had an efficiency of 95.9% and 109.4% with only one amplification product (Fig. 5.3 and 5.4). The amplification products resolved on an electrophoretic agarose gel were found to be of 147 bp for MUC1, 114 for MUC16 and 152 bp for BACT and 98 bp for B2M (Fig. 5.5).

5.4.1.2 The activation of the TLRs in the epithelial cells increased the expression of MUC1

The stimulation of the MCF7 cells with FSL-1 and PMA/I for 4 h was able to increase significantly the expression of MUC1 compared to the non-stimulated control ($p < 0.05$). Flagellin only increased slightly this expression (Figure 5.6a). The 24 h stimulation of the endometrial cells with the TLR ligands had a small influence in MUC1 expression increasing it slightly but not significantly compared with the 24 h non-stimulated cells (Figure 5.6b).

In contrast with this, the stimulation of RL95-2 with flagellin for 4h significantly increased MUC1 expression compared to the non-stimulated control ($p < 0.05$). The FSL-1 stimulation was unable to increase MUC1 expression but the unspecific activity of PMA/I increased significantly this expression compared to the non-stimulated control ($p < 0.05$; Figure 5.6c). After 24 h of stimulation, we observed a small increase in MUC1 expression with FSL-1 but not with flagellin compared to the respective non-stimulated control. PMA/I stimulated cells had a significantly higher expression of MUC1 than the 24 h non-stimulated cells ($p < 0.05$; Figure 6d).

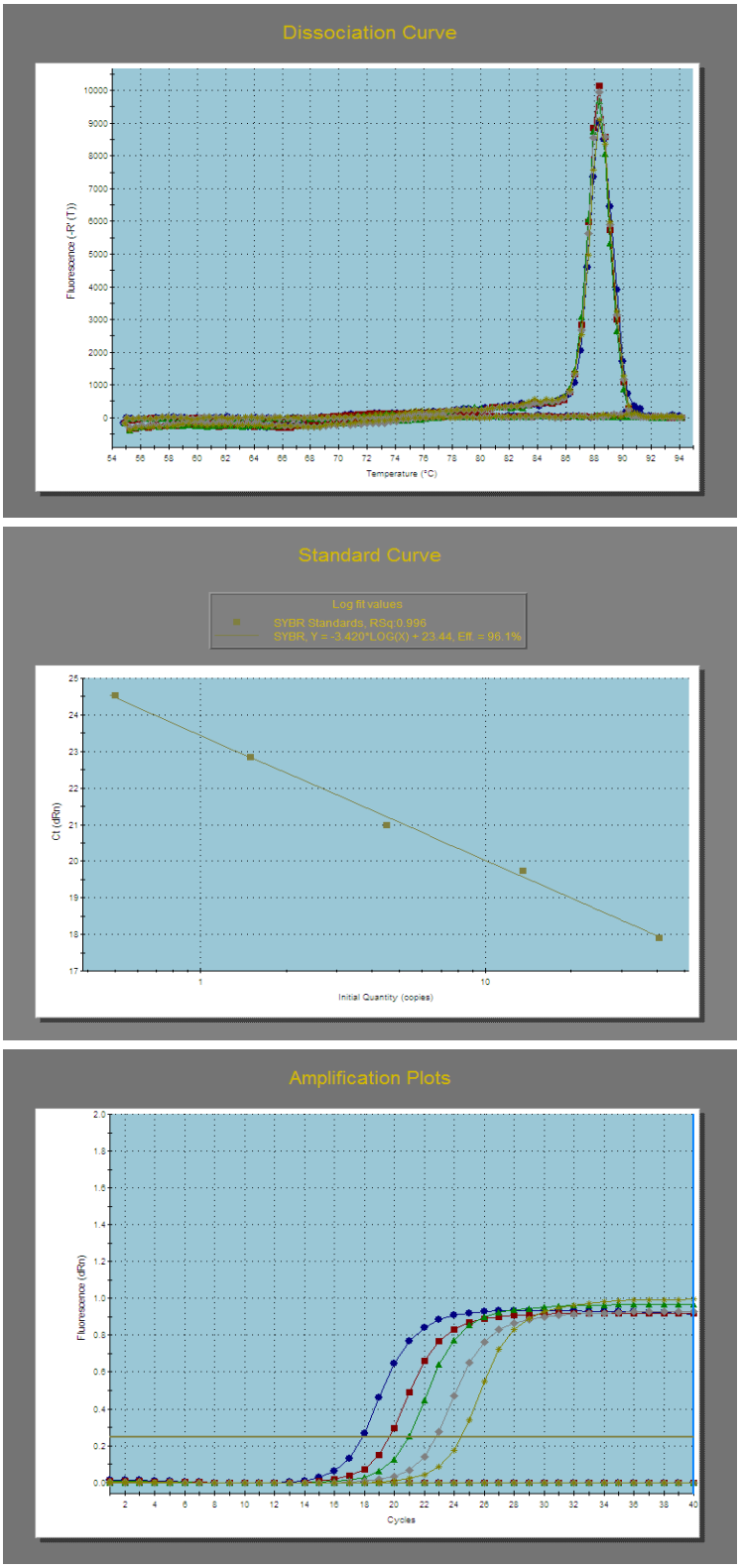

Figure 5.1. Standardisation of β-actin primers. The specific primers for β-actin (BACT) amplification were standardised at 57° C. The specificity of the amplification product was verified on the melting curve. The primers had an efficiency of 96.1% estimated with the standard curve. The cDNA concentration was estimated with the amplification curves.

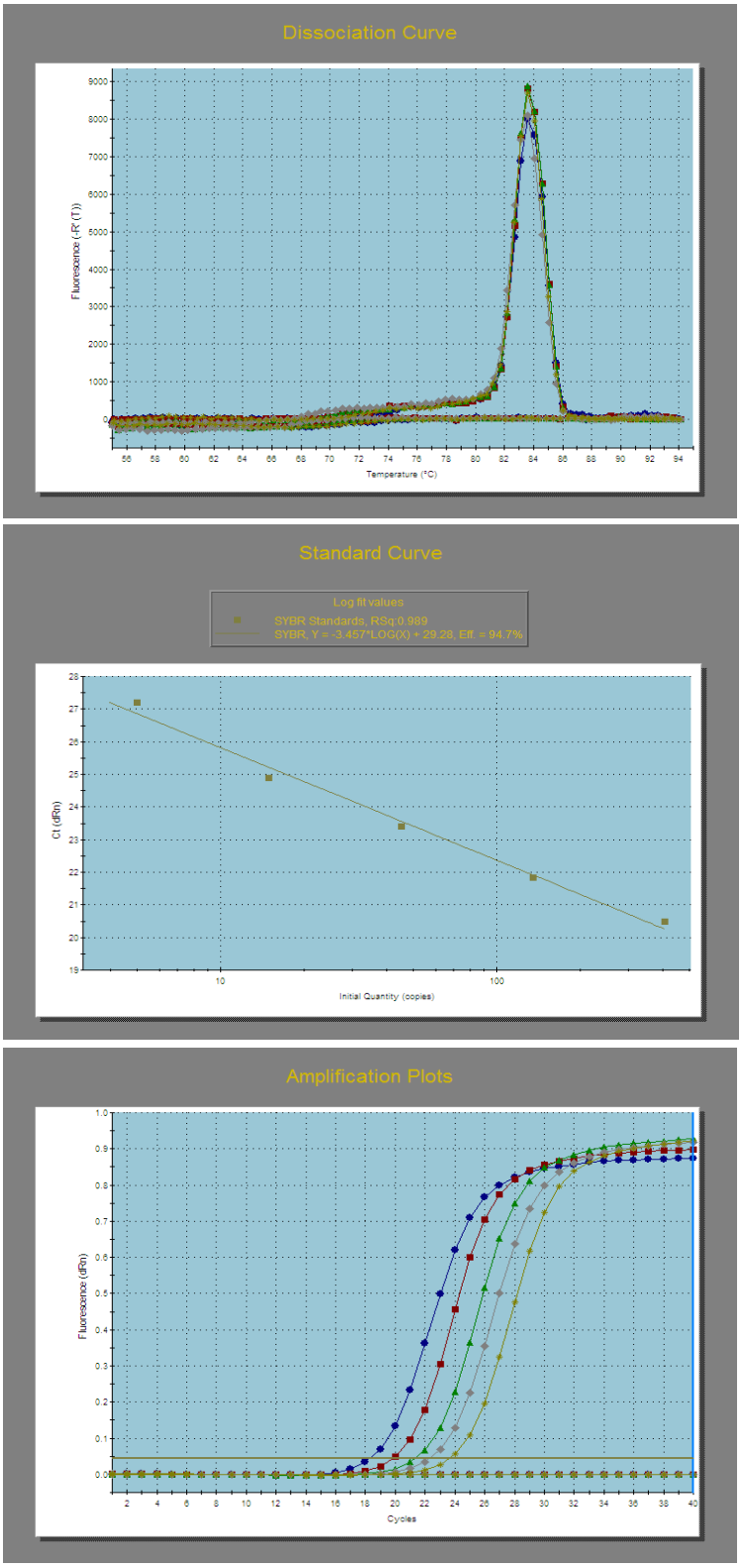

Figure 5.2. Standardisation of Beta 2 Microglobulin primers. The specific primers for β2 microglobulin (B2M) amplification were standardised at 57° C. The specificity of the amplification product was verified on the melting curve. The primers had an efficiency of 94.7% estimated with the standard curve. The cDNA concentration was estimated with the amplification curves.

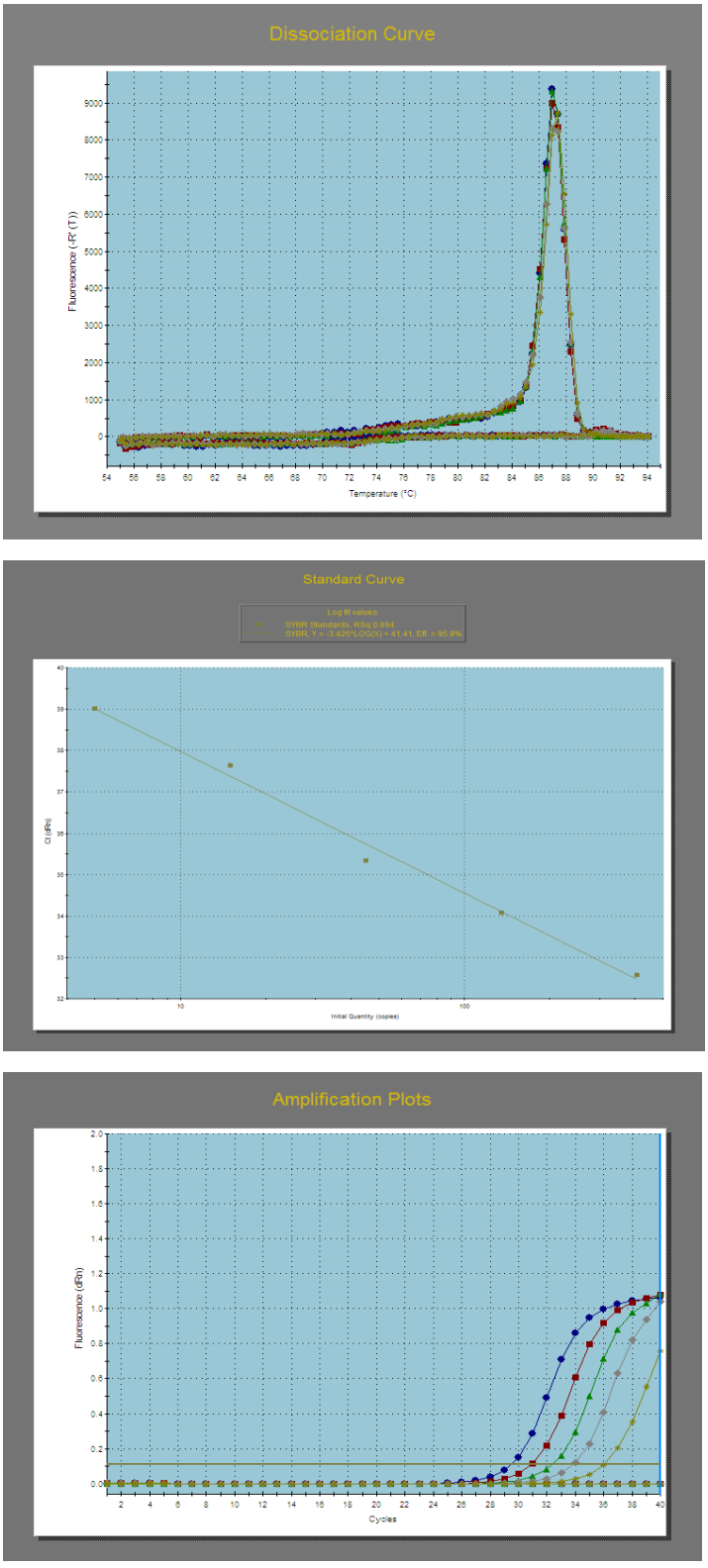

Figure 5.3. Standardisation of the Mucin 1 primers. The specific primers for MUC1 amplification were standardised at 55° C. The specificity of the amplification product was verified on the melting curve. The primers had an efficiency of 95.9% estimated with the standard curve. The cDNA concentration was estimated with the amplification curves.

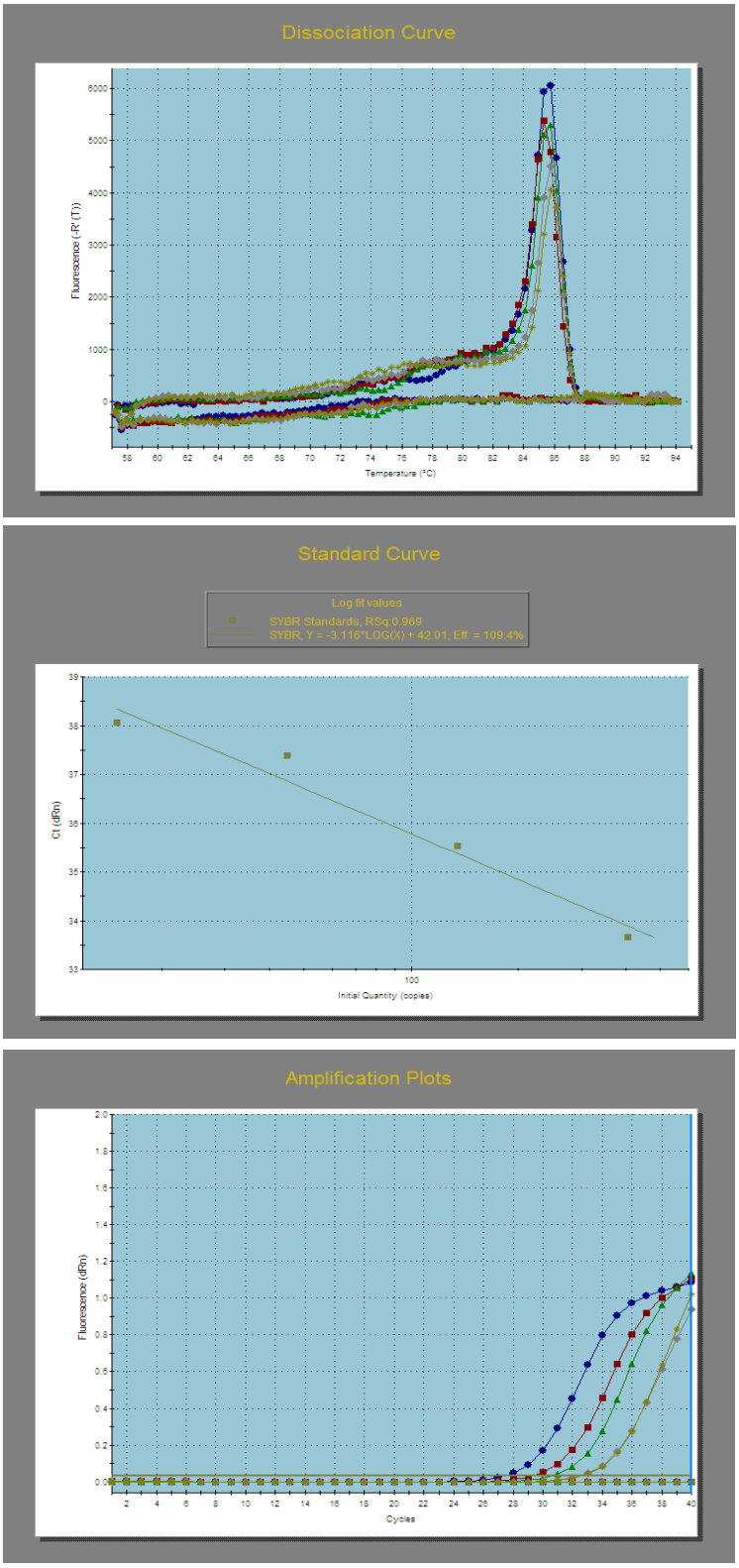

Figure 5.4. Standardisation of the MUC16 primers. The specific primers for mucin 16 (MUC16) amplification were standardised at 55° C. The specificity of the amplification product was verified on the melting curve. The primers had an efficiency of 109.4% estimated with the standard curve. The cDNA concentration was estimated with the amplification curves.

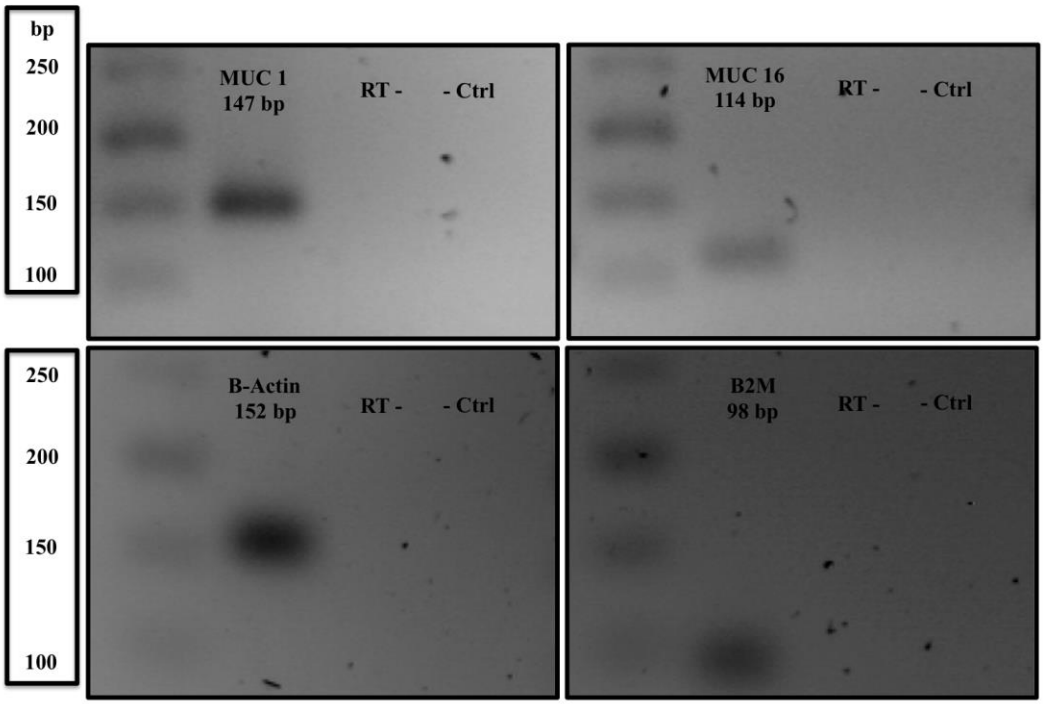

Figure 5.5. Products of amplification of MUC1, MUC16, Beta-Actin and Beta 2 Microglobulin . The products of the qPCR amplification of the genes MUC1 and MUC16 and their respective negative RT (-RT) and negative controls (-Ctrl) were resolved by electrophoresis in a 2.5% agarose gel. The specific amplification product for MUC1, MUC16, B-Actin and B2M measured 147, 114, 152 and 98 bp respectively.

5.4.1.3 The expression of MUC16 is increased after TLR activation only in the endometrial cells

The 4 h treatment of MCF7 cells with FSL-1 and flagellin failed to affect the MUC16 expression compared with the non-treated control. (Figure 5.7a). The treatment of these cells with FSL-1 for 24 h increased MUC16 expression compared to the 24 h non-stimulated control ($p < 0.05$). The stimulation for 24 h with flagellin failed to increase this expression (Figure 5.7b). Treatment of the MCF7 cells with PMA/I increased significantly MUC16 expression compared with both 4 and 24 h non-stimulated controls.

The treatment of the endometrial RL95-2 cells with the different stimuli for 4 h failed to increase the expression of MUC16 compared to the non-stimulated control. In fact, the flagellin and PMA/I treatment significantly decreased MUC16 expression ($p < 0.05$; Figure 5.7c). In contrast, after 24 h of stimulation, both FSL-1 and flagellin were able to significantly increase MUC16 expression compared to the non-stimulated control ($p < 0.05$; Figure 5.7d). There were no observed effects of PMA/I stimulation on MUC16 expression.

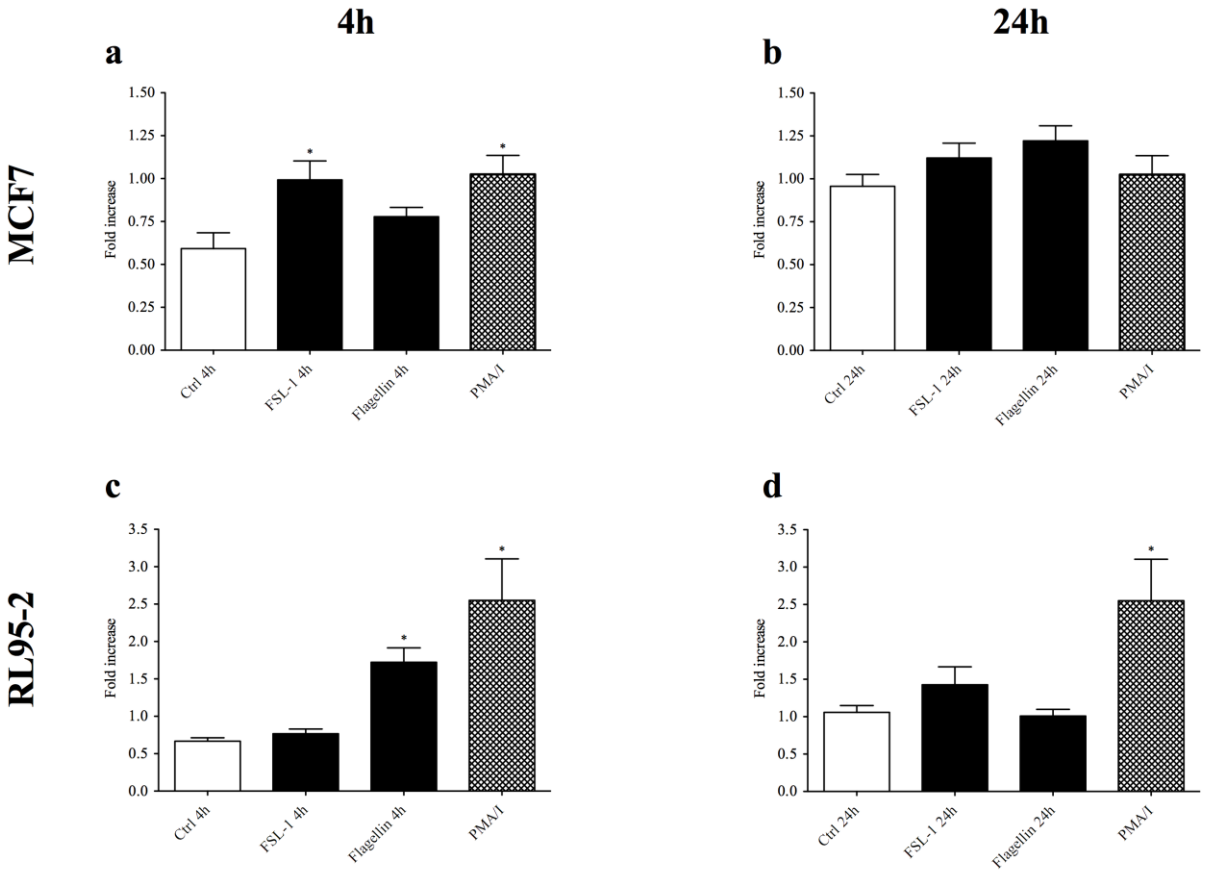

Figure 5.6. Expression of MUC1 in MCF7 and RL95-2 cells after TLR 2 and 5 stimulation. The expression of MUC1 was assessed in MCF7 cells a breast carcinoma cell line (a and b) and the endometrial epithelial cells RL95-2 (c and d). The cells were stimulated with were stimulated with the ligands for TLR 2/6 (FSL-1; 100 ng/ml) and for TLR 5 (flagellin; 100 ng/ml) for 4 and 24h. The cells were stimulated with the unspecific activator PMA/Ionomycin for 4 h. The expression of MUC1 was normalised using BACT and B2M as reference genes and reported as fold of increase. The data is presented as mean ± SEM and one-way analysis of variance with Dunnett's multiple comparison test performed to compare the multiple conditions; $p < 0.05$ was considered significant.

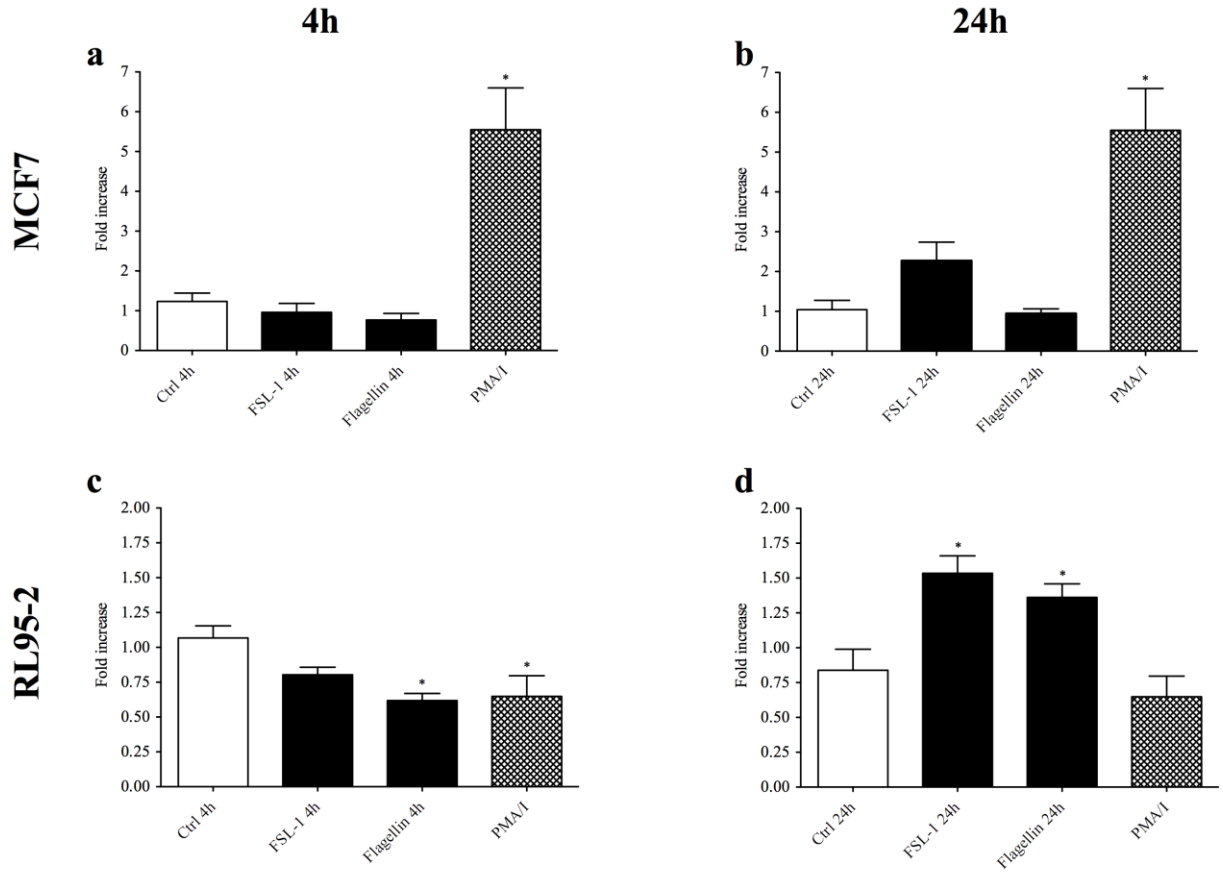

Figure 5.7. Expression of MUC16 in MCF7 and RL95-2 cells after TLR 2 and 5 stimulation. The expression of MUC16 was assessed in MCF7 cells a breast carcinoma cell line (a and b) and the endometrial epithelial cells RL95-2 (c and d). The cells were stimulated with were stimulated with the ligands for TLR 2/6 (FSL-1; 100 ng/ml) and for TLR 5 (flagellin; 100 ng/ml) for 4 and 24h. The cells were stimulated with the unspecific stimuli PMA/Ionomycin for 4 h. The expression of MUC16 was normalised using BACT and B2M as reference genes and reported as fold of increase. The data is presented as mean ± SEM and one-way analysis of variance with Dunnett's multiple comparison test performed to compare the multiple conditions; $p < 0.05$ was considered significant.

5.5 Discussion

The epithelial mucosa of the body forms a tight barrier of cells that will impede any microorganisms from breaching the luminal space and initiate innate immune responses. Epithelial cells of the female reproductive tract are known to modulate the immune mechanisms throughout the menstrual cycle as an innate mechanism to prevent any possible infection for example the secretion of anti-microbial peptides (King et al. 2003). This innate immune mechanisms increase their activity at the late secretory phase and are inducible by microbial stimulation (Hickey et al. 2011). Another mucosal innate immune mechanism is the production of mucin.

In our work, we were able to detect the expression of mucin 1 and 16, membrane bound mucins, in response to TLR stimulation. The increase in MUC1 expression was induced rapidly, finding an increase after 4 h of stimulation of TLR 2/6 in MCF7 cells and TLR5 in RL95-2 cells. In a similar way, the expression of MUC1 in reproductive tissues has been found to be fast in response to bacterial ligands. For example in the hen oviduct, the stimulation with LPS for 3 h could induce the overexpression of Muc-1 (Ariyadi et al. 2013). In a human cervical 3D model, MUC1 expression was increased by the stimulation with both FSL-1 and flagellin and greatly enhanced by Poly I:C (TLR 3) ligand (Radtke et al. 2012). The induction of the expression of MUC16, another membrane bound mucin was observed after 24 h of TLR stimulation. This increase was specific to TLR 2/6 and 5 in the endometrial cells and TLR 5 in the breast carcinoma epithelial cells. In the same 3D cervical model, MUC16 was also induced by the activation of TLRs. In particular the activation of TLR 3 increased significantly its expression, but TLR 2/6 was also capable of increasing MUC16 expression (Radtke et al. 2012). Mucin 16 has been found expressed constantly throughout the cycle in the endometrial epithelial cells with an overexpression of 4 folds at late secretory phase. The silencing of MUC16 has shown to increase trophoblast cells adhesion to the endometrial cells, compared to the normal endometrial cells and to MUC1 silenced endometrial cells (Gipson et al. 2008).

In our experiments, we found that the expression of MUC1 in the endometrial cells was different than that of the breast carcinoma MCF7 cells. The presence of MUC1 in breast epithelial cells has been only associated with tumour development. It has been used as a

prognostic marker for breast carcinoma and a potential adjuvant for cancer chemotherapy (Sinn et al. 2013). The expression of MUC1 has been observed in MCF7 as a potential anti-adhesive molecule that aids the metastasis of the cells by helping migration and inhibiting firm adhesion (Richard Bennett et al. 2001). Although this expression has not been related to the TLR stimulation, it is possible that the activation of TLRs could enhance the metastatic capacity of the cells. This has been observed in colorectal cancer cells where LPS-mediated activation of TLR 4 induces overexpression of β1 integrin increasing the metastatic potential of the cells (Ariyadi et al. 2013; Hsu et al. 2011).

As observed, the initial TLR activation of the epithelial cells is enough to stimulate the production of mucin. If the inflammatory reaction persists, many soluble inflammatory mediators are also able to induce the expression of mucin in the endometrial cells. For example Tumour necrosis factor (TNF)-α can increase MUC1 expression and shedding in the uterine epithelial cell line HES (Thathiah et al. 2004). A similar induction has been observed promoted by TNF-α and interferon (IFN)-γ. The MUC1 gene sequence possesses Nuclear Factor (NF)-κB and progesterone binding regions. NF-κB is activated by cytokines signalling which will then together with the progesterone receptor modulate MUC1 expression (Dharmaraj et al. 2010). The activation of the MAPK and NF-κB pathways in gut and lung epithelia, both involved in TLR-mediated responses, has been reported to increase the expression of MUC2, MUC5B and MUC5AC (D. Li et al. 1998; Jono et al. 2002; Chu et al. 2005). In a previous report from our group, we have demonstrated that the stimulation of Ishikawa 3H-12 endometrial cells with flagellin is able to induce the activation of NF-κB (Caballero et al. 2013). In another report, the stimulation of RL95-2 cells with flagellin was able to induce the production of the pro-inflammatory cytokine IL-8 which can be mediated by NF-κB activation (Young et al. 2004). It is likely that the increases in MUC1 and MUC16 expression observed are also related to this TLR mediated NF-κB activation.

The role of mucin 1 overexpression has been suggested as a regulatory mechanism to avoid exacerbated inflammatory responses mediated by the activation of TLRs (Ueno et al. 2008). This activation primed by flagellin for example could generate a negative feedback through the epithelial growth factor receptor to phosphorylate mucin 1 intracellular domain. This in term will bind to TLR 5 and inhibit its signalling via

MyD88 and the perpetuation of the inflammatory reaction (Kato et al. 2012). The increase in mucin expression in the reproductive tissue in response to microbial stimuli implies a challenge to the tract. This will protect the tract from the potential infection but as it has been proposed, the expression of mucin 1 will also affect and decrease embryo attachment to the epithelia (Meseguer et al. 2001; Hoffman et al. 1998; Braga & Gendler 1993). Mucin 1 has been found to be conserved between different mammalian species as human, mice, rat, baboon, rhesus monkey, wallaby, marmoset, horse, rabbit, cow, dog and even whales making it a suitable candidate to exert its anti-inflammatory / anti-implantation effects among these species (Pemberton et al. 1992).

In summary, the mucosal innate immune mechanisms of the endometrium are diverse and unspecific, as they need to protect against pathogens of different nature. We explored the production of mucins in the endometrium as an innate defence mechanism that will form a physical barrier against the invasion of microorganisms that could potentially reach and breach the epithelial barrier. This is the first time that the expression of endometrial mucins has been linked to the activation of TLR 2 and TLR 5. The anti-adhesive characteristics of MUC1 and MUC16 and its pattern of expression along the reproductive cycle make them suitable effector molecules on both immune responses and embryo implantation. An enhanced expression due to TLR activation during implantation or due to a chronic inflammatory process will guarantee the protection of the endometrium but at the same time seems to prevent an embryo from implanting.

Chapter 6. The effects of TLR activation on the endometrial function and embryo implantation

6.1 Introduction

Inflammation is one of the best-studied strategies of the innate immune system. Its initial activation mechanisms are based on the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Medzhitov & Janeway 1997). The activation of the PRRs induces the production of pro-inflammatory cytokines and surface molecules that will promote inflammatory reactions. The most studied and better-characterised PRRs are the Toll-like receptors (TLRs) (Medzhitov & Janeway 2002). They can recognise a significant variety of PAMPs from gram-negative bacterial lipopolysaccharides (LPS, by TLR 4), gram-positive peptidoglycans (PGN by TLR 2), diacyl and triacyl lipopeptides (by TLR 2/6 and 2/1 respectively) and bacterial flagellum (flagellin by TLR 5) (J. H. Wang et al. 2003; Buwitt-Beckmann et al. 2006; Fitzgerald et al. 2001; Schwandner et al. 1999). Pathogen genetic material can also be sensed like intracellular bacterial DNA (CpG by TLR 9), double stranded (ds)RNA (Poly I:C by TLR 3) and single stranded (ss)RNA (TLR 7 and 8) (Matsumoto & Seya 2008; Brown et al. 2011; Bauer et al. 2001). Even synthetic ligands as imidazoquinoline-like molecules (TLR 7) and parasitical ligands from Toxoplasma gondi (profilin by TLR 11) are recognised by this system (Kawai & Akira 2010). The ligation of the TLRs will initially induce the formation of homo-dimers using the homologue conserved intracellular domain, the Toll/IL-1 receptor (TIR) or heterodimers of TLR 2 with TLR 1 and TLR 6 (Kang et al. 2009). Once the dimer is formed two options of signalling will proceed depending on the TLR activated, one dependent on the activation of the myeloid differentiation factor 88 (MyD88) and the other MyD88-independent (X. Li & Qin 2005). The activation of TLR 2 or 4 will recruit the TIR adapter protein (TIRAP) prior to MyD88 but the activation by TLR 5, 7 and 9 will directly recruit MyD88. The MyD88-independent pathway is mediated by the TIR domain-containing adaptor inducing interferon-β (TRIF) and is initiated by the activation of TLR 3 (Z. Jiang et al. 2004) and by TLR 4 using the TRIF-related adaptor molecule (TRAM or TICAM-2) (Oshiumi et al. 2003).

The classical TLR signalling cascade will promote the activation of the Nuclear Factor (NF)-κB that binds to the promoter κB regions of a great variety of genes (Fig. 6.1). The NF-κB transcription factors are formed by homo or hetero-dimers that regulate different cellular processes like proliferation, adhesion, cell cycle and immune responses (Perkins

2007). Their function depends on the subunits that conform the dimer. For example, the dimer p50/p65 has been found to induce the expression of pro-inflammatory cytokine genes as IL-8, TNF-α, IL-6, IL-1β and IL-12 (Chen & Greene 2004). In contrast, the homo-dimer p50/p50 has been found to promote anti-inflammatory type genes or repress gene transcription (Perkins 2007). The main TLR signalling pathway activates a series of kinases that will phosphorylate the inhibitor of NF-κB (IκB) and degrade the IκB kinase (IKK) complex formed by IKKγ/NEMO, IKKα and IKKβ (Karin & Ben-Neriah 2000). The phosphorylation of IκB in the cytoplasm will then release the NF-κB dimer that will translocate to the nucleus and promote the pro-inflammatory gene transcription (Tian & Brasier 2003). An alternative route of signalling is the activation of the mitogen activated protein kinases (MAPK) p38, extracellular signal-regulated kinase (ERK) or c-jun terminal kinase (JNK) (Dong et al. 2002). The TLR stimulation of these MAPK would in term activate the activating protein (AP)-1, composed of the proteins Jun and Fos, capable to dimerise and transcribe pro-inflammatory genes (Kawai & Akira 2007a). Finally, besides the above mentioned transcription factors, TLRs like 3, 7 or 9 that are activated by intracellular pathogens as viruses and bacteria, promote the activation of the interferon (IFN) regulatory factors 3 and 7 that will promote the secretion of type I IFN (IFN-α and -β) to mediate anti-viral responses (Kawai & Akira 2006).

The TLRs have been found differentially expressed throughout the different components of the FRT (Fazeli et al. 2005). A constitutive production of pro-inflammatory cytokines and anti-microbial peptides is observed throughout the FRT and varies along the cycle to protect it from potential infections (King et al. 2003; Fahey et al. 2005; Hickey et al. 2013). The activation of TLRs in murine uterine cells has been found to increase the production of pro-inflammatory cytokines (Soboll et al. 2006; Sheldon & M. H. Roberts 2010). The production of cytokines in response to TLR stimulation of different human endometrial epithelial cell lines has been observed to be mainly in response to TLR 3 and 5 activation (Aboussahoud, Aflatoonian, et al. 2010b; Young et al. 2004; Schaefer et al. 2004). The expression of TLRs in the endometrium is related to the stage of the menstrual cycle being the highest expression at the secretory phase, preparing the tissue for a possible embryo implantation (Aflatoonian et al. 2007). The implantation of the embryo is a process that requires a special coordination between the developing embryo and the cycling uterus of the mother. The endometrium in late-

secretory phase would have acquired morphological and molecular “receptive” changes to facilitate the allocation of the embryo (Norwitz et al. 2001). Implantation of the embryo resembles a pro-inflammatory process as cytokines like IL-1β, IL-6, IFN-γ and MCP-1 are produced by embryo and endometrium (Robertson et al. 2011). Using an *in vitro* model of adhesion of trophoblast spheroids to endometrial cells, we have characterised that the activation of endometrial TLRs 5, 2 and 2/6 can decrease the percentage of trophoblast spheroid adhesion to endometrial cells (Aboussahoud, Bruce, et al. 2010a). Furthermore, a direct activation of the murine TLR 2/6 at implantation time has been found to significantly affect the endometrial architecture, cytokine profile and decrease the possibility of embryos to implant (Sánchez López et al. 2014).

As described, TLR activation could induce the production of pro-inflammatory cytokines that could potentially effect embryonic implantation. It its uncertain if the NF-κB and AP-1 mediated pathway of TLR activation would directly influence the capacity of endometrial cells to receive the implanting embryo. For this reason, the aim of the following chapter is to characterise the role of these two pathways in the endometrial TLR responses.

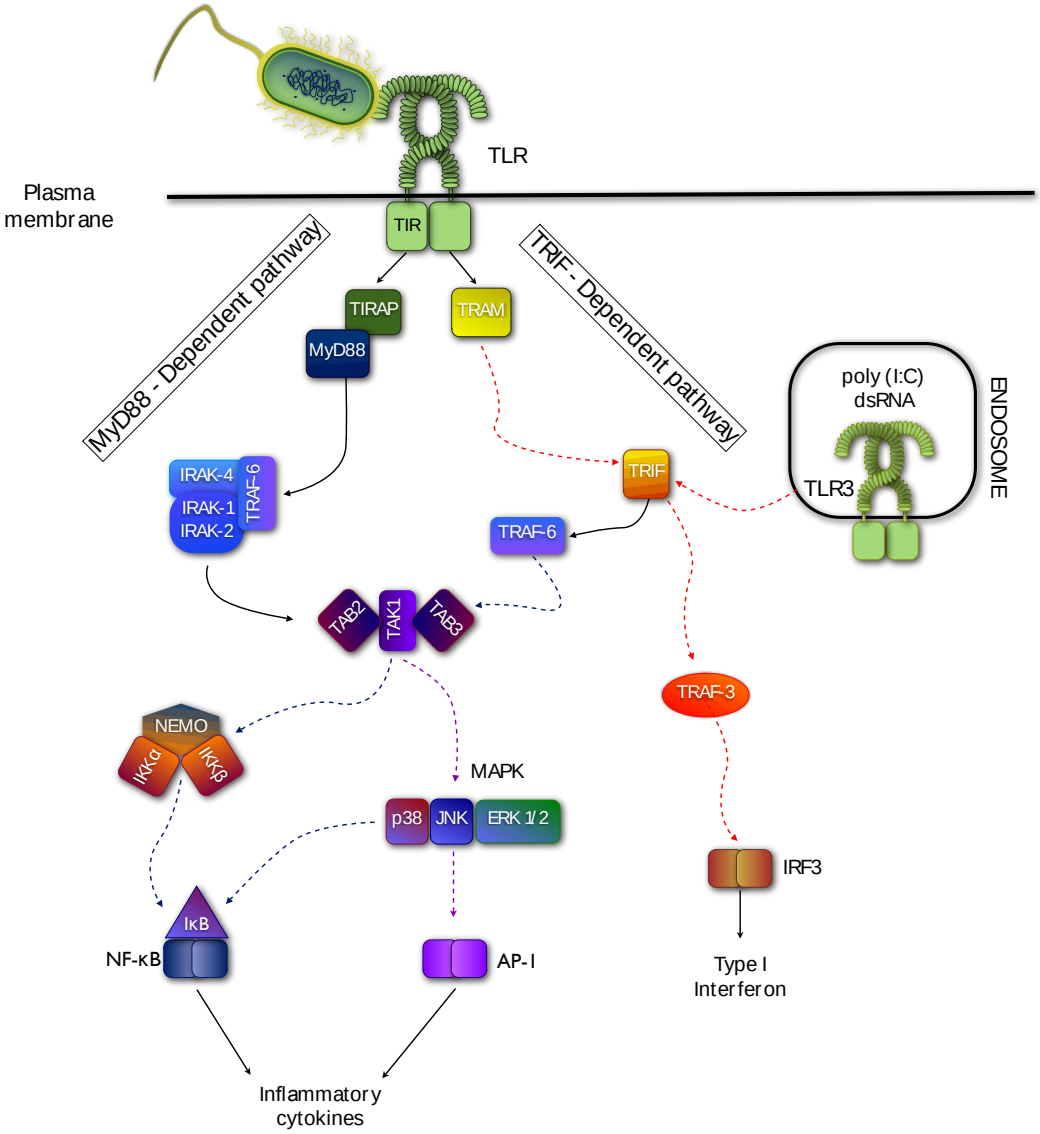

Figure 6.1. The TLR signalling pathway. The classical TLR signalling cascade will promote the activation of the Nuclear Factor (NF)-κB that binds to the promoter κB regions of a great variety of genes. Upon activation, a series of kinases will be phosphorylated to activate the inhibitor of NF-κB (IκB) and degrade the IκB kinase (IKK) complex formed by IKKγ/NEMO, IKKα and IKKβ. The phosphorylation of IκB in the cytoplasm will then release the NF-κB dimer that will translocate to the nucleus and promote the pro-inflammatory gene transcription. An alternative route of signalling is the activation of the mitogen activated protein kinases (MAPK) p38, extracellular signal-regulated kinase (ERK) or c-jun terminal kinase (JNK). This will activate the activating protein (AP)-1 or the same NF-κB. Besides the above mentioned transcription factors, TLRs like 3, 7 or 9 that are activated by intracellular pathogens as viruses and bacteria, promote the activation of the interferon (IFN) regulatory factors 3 and 7 that will promote the secretion of type I IFN (IFN-α and -β) to mediate anti-viral responses.

6.2 Materials and Methods

6.2.1 Assessment of the TLR activation pathway in RL95-2 cells and its influence on trophoblast spheroid binding

6.2.1.1 Epithelial cell lines culture

The endometrium was simulated with the human endometrial epithelial cell line RL95-2. The cells were cultured in T75 flasks at 37°C in DMEM/F-12 (Sigma, Irvine, UK) supplemented with 1% Penicillin/Streptomycin (P/S; Sigma), 5 μg/ml Insulin (human recombinant insulin; Gibco Invitrogen, Denmark), 1% L-glutamine (Sigma) and 10% fetal bovine serum (FBS; Sigma) at 37ºC in 5% CO2 atmosphere until confluency. The breast carcinoma cell line MCF7 was used as a non-specific epithelial cell line. The MCF7 cells were grown in DMEM/F-12, supplemented with 10% FBS, 1% P/S, 1% L-glutamine and 160 ng/ml insulin in a T75 flask at 37ºC in 5% CO2 atmosphere. At confluency, cells were washed with Dulbecco's phosphate-buffered saline (DPBS; Sigma), harvested using trypsin-EDTA (Sigma) and pelleted by centrifugation at 300 x g for 4 min. About $5x10^5$ RL95-2 cells were transferred to each well of a 12-well plate and incubated at 37°C for 3-4 days.

6.2.1.2 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) in a ratio of 1 μg of pNifty2 or 2 μg of pNifty3 per 1 μl of reagent. Briefly, each well of the 24-well plate was 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) detected using QUANTI-blue™ (InvivoGen) according to manufacturers protocol.

6.2.2 Adhesion of the trophoblast spheroids to the RL95-2 monolayer

6.2.2.1 Epithelial cell lines culture

RL95-2 cells were cultured in T75 flasks at 37°C in DMEM/F-12 (Sigma, Irvine, UK) supplemented with 1% P/S (Sigma), 5 μg/ml Insulin (Gibco Invitrogen), 1% L-glutamine (Sigma) and 10% FBS (Sigma) at 37ºC in 5% CO2 atmosphere until confluency. Then $5x10^4$ cells were transferred per well of a 96 black-welled, clear bottom plate.

6.2.2.2 Formation of JAr cells spheroids

JAr cells were grown in RPMI 1640 (Sigma), supplemented with 10% FBS, 1% P/S and 1% L-glutamine in a T75 flask at 37ºC in 5% CO2 atmosphere. At confluency, cells were washed with DPBS (Sigma), harvested using trypsin-EDTA (Sigma) and pelleted by centrifugation at 300 x g for 4 min. To mimic the blastocyst, multicellular spheroids were formed from the JAr cells (approximately of 200 μm diameter). One million cells were counted with a haemocytometer and cultured in 5 ml of supplemented RPMI in 60 x 15 mm Petri dishes. They were cultured overnight on a gyratory shaker at 280 rpm (JKA, MTS 2/4 digital, Staufen Germany) in a humid atmosphere and 5% CO2 and 37°C. On the next day, the spheroids were transferred to a 35 x 10 mm Petri dish and stained with 0.4 μM Calcein (Life technologies, Molecular Probes, Paisley, UK) in 2 ml of serum-free RPMI 1640 media for 30 min.

6.2.2.3 Co-incubation of JAr spheroids with endometrial cells and assessment of the adhesion with a fluorescence plate reader

The JAr spheroids were collected in a 1.5 ml tube, allowed to sediment by gravity and the supernatant was discarded. They were re-suspended in 1 mL of PBS and the equivalent volume containing 50 spheroids was delivered to each well with a confluent monolayer of the epithelial cells. They were co-cultured for 45 min in supplemented DMEM-F12 at 37°C and 5% CO2. After co-incubation, the initial fluorescence (Fi) was registered using a microplate fluorescence reader (Spectra Max Gemini XS, Molecular Devices) between the wavelengths of 495 and 515 nm (Excitation and emission for calcein). To remove non-adherent JAr spheroids from the endometrial cell monolayers,

plates were washed using a horizontal shaking device (Labman Automation LTD, Stokesley, UK). The plate was set to shake for 4 min at 200 rpm and the media was discarded. Each well was filled with 50 µl of PBS containing Ca2+ and Mg2+ (PBS/Ca-Mg; Gibco Invitrogen) and then set to shake for 4 min at 200 rpm. The PBS/Ca-Mg was discarded and 50 µl of PBS/Ca-Mg were added. The final fluorescence (Ff) was recorded using the microplate reader and the percentage of adhesion was estimated as follows:

$$\% \text{ of spheroid adhesion} = (Ff / Fi) * 100$$

6.3 Experimental design

6.3.1 Determining activation of NF-κB and AP-1 as a result of endometrial TLR activation

The RL95-2 cells were transfected with either the pNifty-2 or 3 plasmids. On the next day, the culture media was replaced with serum-free DMEM/F-12 and the cells were stimulated with the different TLR ligands: 100 ng/ml of FSL-1 (TLR 2/6), 10 μg/ml of PGN (TLR 2), 100 ng/ml of flagellin (TLR 5) or 10 μg/ml of Poly I:C for 4 and 24 h. The supernatants were collected and the SEAP was measured with QUANTI-blue™ (InvivoGen). Data were reported as the fold induction of SEAP activity over the non-stimulated control.

6.3.2 Assessment of the inhibitiors of NF-κB and AP-1 in the endometrial cell 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 then the cells were stimulated with 100 ng/ml of flagellin for 24 h. The pNifty3 transfected RL95-2 cells were pre-treated or not with 10, 20 and 30 μM of SB203580 (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-blue™ (InvivoGen).

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

To study the relevance of the NF-κB activation in response to TLR2 and 2/6 activation and how can this affect the adhesion of the trophoblast spheroids to the endometrial monolayer, the RL95-2 cells were pre-treated or not with the NF-κB and AP-1 inhibitors. NF-κB activation was blocked with 20 μM of Bay11-7082. The inhibitor for p38 MAPK SB203580 (30 μM) was used to inhibit AP-1 activation. The RL95-2 cells were pre-treated with the inhibitors for 30 min. Thereafter, the RL95-2 were either

stimulated or not with 100 ng/ml of FSL-1, 10 μg/ml of PGN or 100 ng/ml of flagellin for 4 and 24 h. Then, 50 calcein stained JAr spheroids were gently delivered into each well and co-incubated for 1 h at 37ºC. Fluorescence was determined in the microplate reader. Non-adherent spheroids were removed and the percentage of adhesion was determined.

6.3.4 Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using Statistica (V7; Statsoft UK, Letchworth, UK). The data for NF-κB or AP-1 activation was normalised assuming a value of 1 for the non-stimulated control. The results were compared with one-way ANOVA test and a multiple comparison post-test of Dunnett to compare differences against the control group and a multiple comparison post-test of Bonferroni to compare effects between different groups. $P \leq 0.05$ was considered to be significant.

6.4 Results

6.4.1 Activation of the TLR signalling pathway in endometrial cells after stimulation

6.4.1.1 The activation of NF-κB in the endometrial cells was induced by flagellin treatment

The treatment of the endometrial cells with flagellin was able to increase significantly NF-κB activity after 24 h (1.9 ± 0.2; $p < 0.05$). After 4 h of flagellin stimulation, we found a significant increase compared to the resto of the treatments (5.8 ± 0.8; $p < 0.05$). The treatment of the cells with FSL-1 or PGN was unable to increase this activation in either 4 or 24 h of stimulation (Fig. 6.2).

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

The treatment of the RL95-2 cells with 10 and 20 μM of Bay11-7082 was unable to induce the NF-κB activation. The pre-treatment of the cells with 10 μM of Bay11-7082 and activation with flagellin was not effective nor significan as NF- κB decreased slightly compared to the flagellin treatment (2.1 ± 0.1 vs 2.317 $p < 0.204$). In contrast, the pre-treatment of the cells with 20 μM of Bay11-7082 significantly decreased the flagellin induced NF-κB activation (1.4 ± 0. 1; $p < 0.05$; Fig. 6.3).

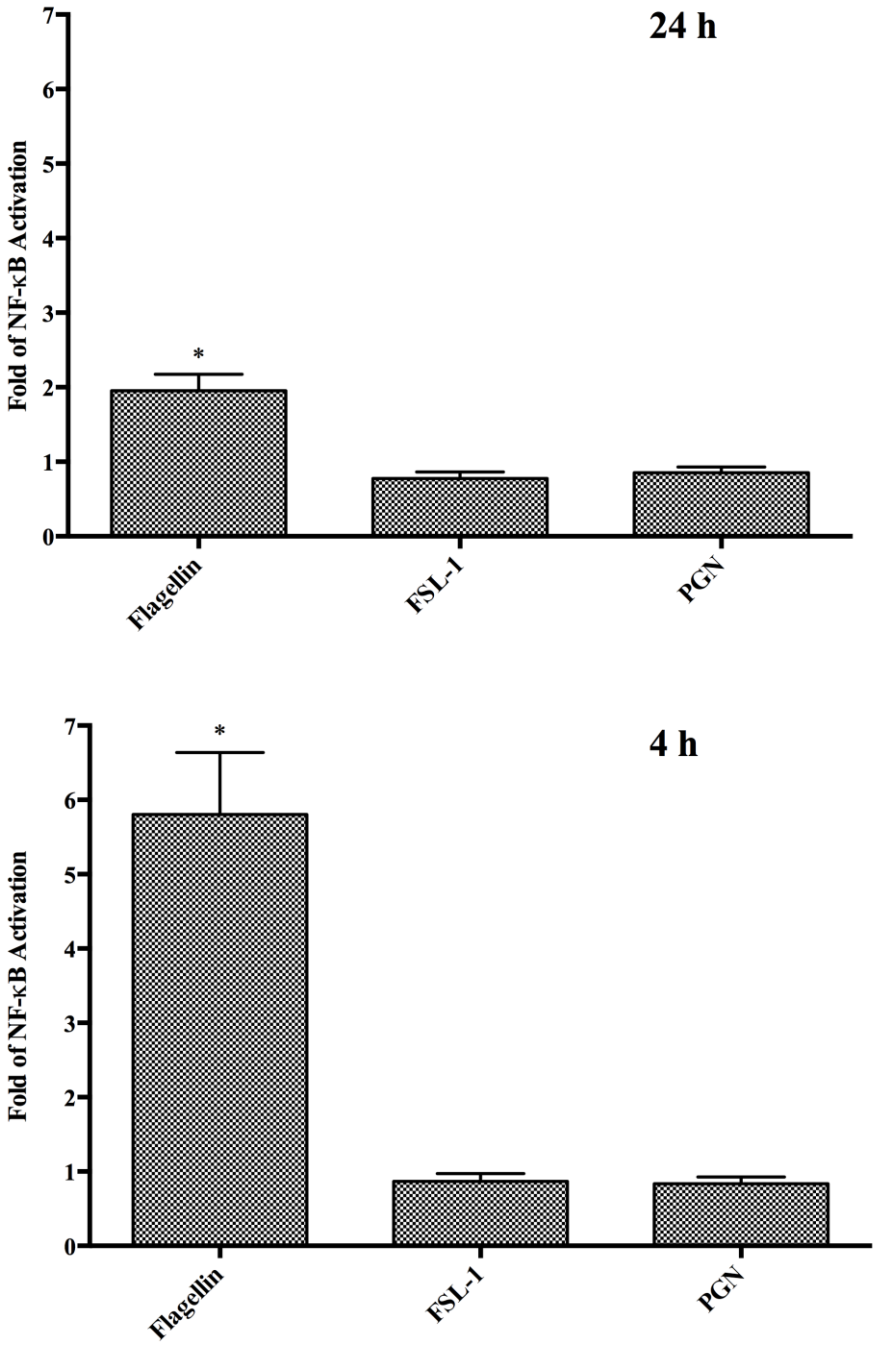

Figure 6.2. Activation of NF-κB in RL95-2 cells after TLR stimulation. The RL95-2 epithelial endometrial cells were transfected with the pNifty2 plasmid containing a secreted alkaline phosphatase (SEAP) reporter promoted by 5 κB binding regions. The cells were stimulated with the ligands for TLR 2/6 (100 ng/ml FSL-1), TLR 2 (10 μg/ml PGN) and TLR 5 (100 ng/ml flagellin) for 24 and 4 h. SEAP production was measured and results represented as fold of NF-κB change when the untreated control is one. Results were compared with a One-Way ANOVA followed by a Bonferroni post-test. * $p < 0.05$ was considered significant compared to the rest of the conditions.

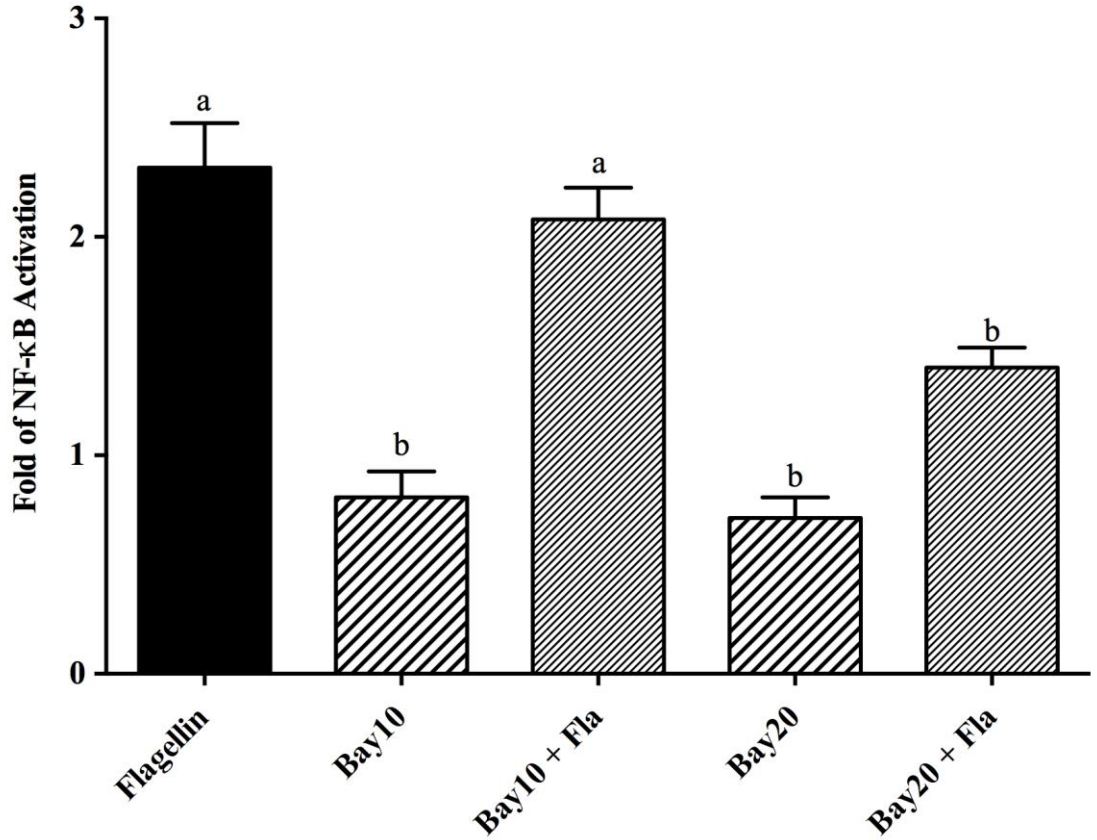

Figure 6.3. Pre-treatment of the RL95-2 cells with Bay11-7082 inhibited the flagellin-induced NF-κB activation. The RL95-2 epithelial endometrial cells were transfected with the pNifty2 plasmid containing a secreted alkaline phosphatase (SEAP) reporter promoted by 5 κB binding regions. The cells were pre-treated with different concentrations of Bay11-7082 (10 and 20 μM) for 1h and then treated with the TLR 5 ligand (100 ng/ml flagellin) for 4h. SEAP production was measured and results represented as fold of NF-κB activation change, considering the untreated control as one. Results were compared with a One-Way ANOVA followed by a Bonferroni post-test. The conditions marked in a are significantly different from the conditions marked as b ($p < 0.05$).

6.4.1.3 The treatment of the RL95-2 cells with different TLR ligands was able to modify the activation of AP-1

The treatment of the endometrial cells with Poly I:C was able to increase significantly the activity of AP-1 in both 24 and 4 h (1.7 ± 0.1 and 1.8 ± 0.4; $p < 0.05$) compared with the stimulation with Flagellin, FSL-1 and PGN (Fig. 6.4).

6.4.1.4 The TLR 3-mediated activation of AP-1 was ablated by the p38 MAPK inhibitor SB203580

The pre-treatment of the RL95-2 cells with the p38 inhibitor SB203580 was able to decrease the Poly I:C activation of the endometrial cells. The pre-treatment of the cells with 10 μM of SB203580 significantly reduced the AP-1 activation compared to the Poly I:C stimulated control (1.6 ± 0.1 vs 2.1 ± 0.1; $p < 0.05$). The treatment of the cells with 20 μM and 30μM of SB203580 was able to significantly reduce AP-1 activation to a basal level close to 1. The treatment of the cells with different concentrations of SB did not modify AP-1 activation (Figure 6.5).

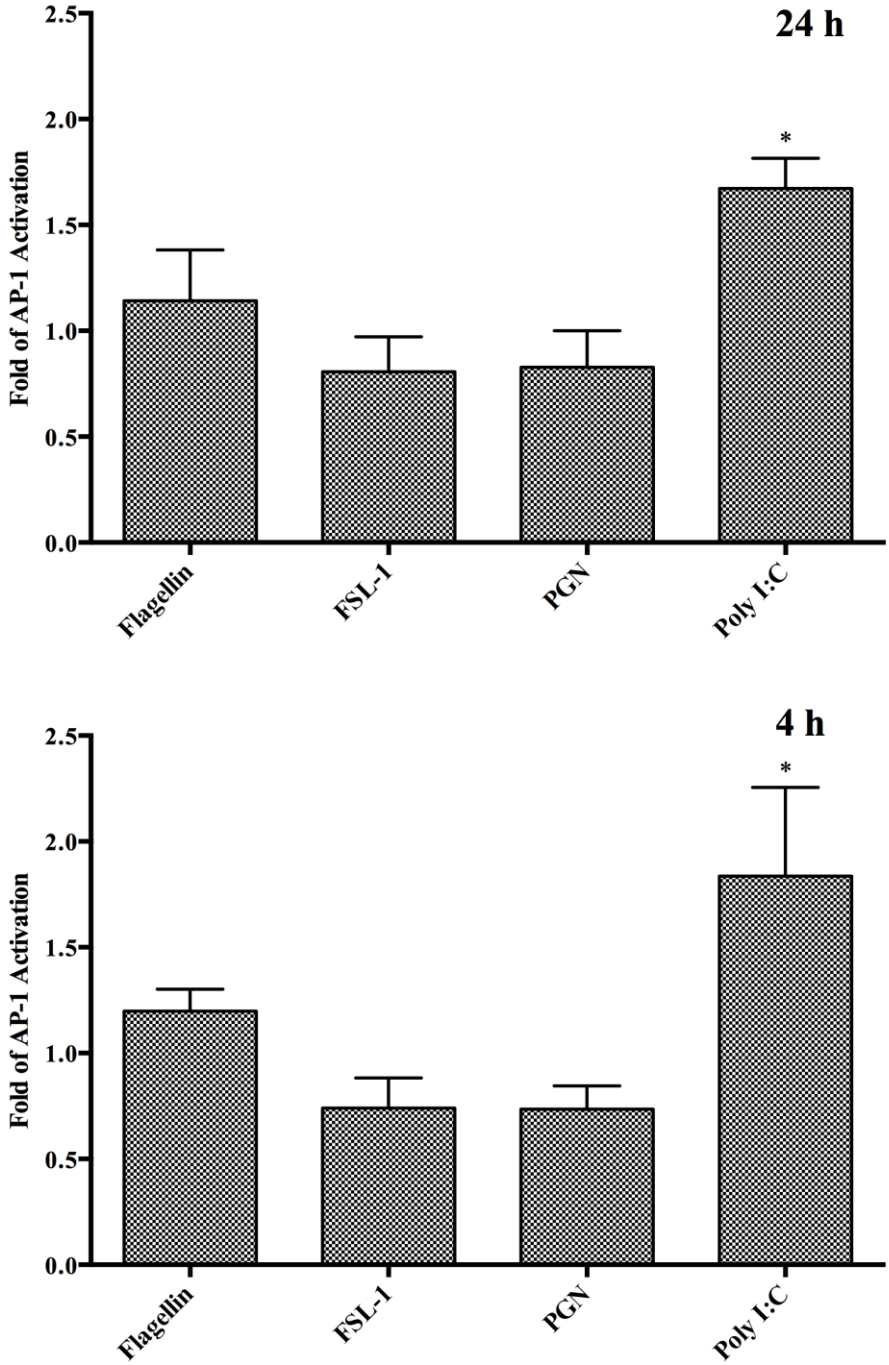

Figure 6.4. Activation of AP-1 in RL95-2 cells after TLR stimulation. The RL95-2 epithelial endometrial cells were transfected with the pNifty3 plasmid containing a secreted alkaline phosphatase (SEAP) reporter promoted by 5 AP-1 binding regions. The cells were stimulated with the ligands for TLR 2/6 (100 ng/ml FSL-1), TLR 2 (10 µg/ml PGN), TLR 5 (100 ng/ml flagellin) and TLR 3 (10 µg/ml Poly I:C) for 24 and 4 h. SEAP production was measured and results represented as fold of AP-1 change when the untreated control is one. Results were compared with a One-Way ANOVA followed by a Bonferroni post-test. * $p < 0.05$ was considered significant compared to the rest of the conditions.

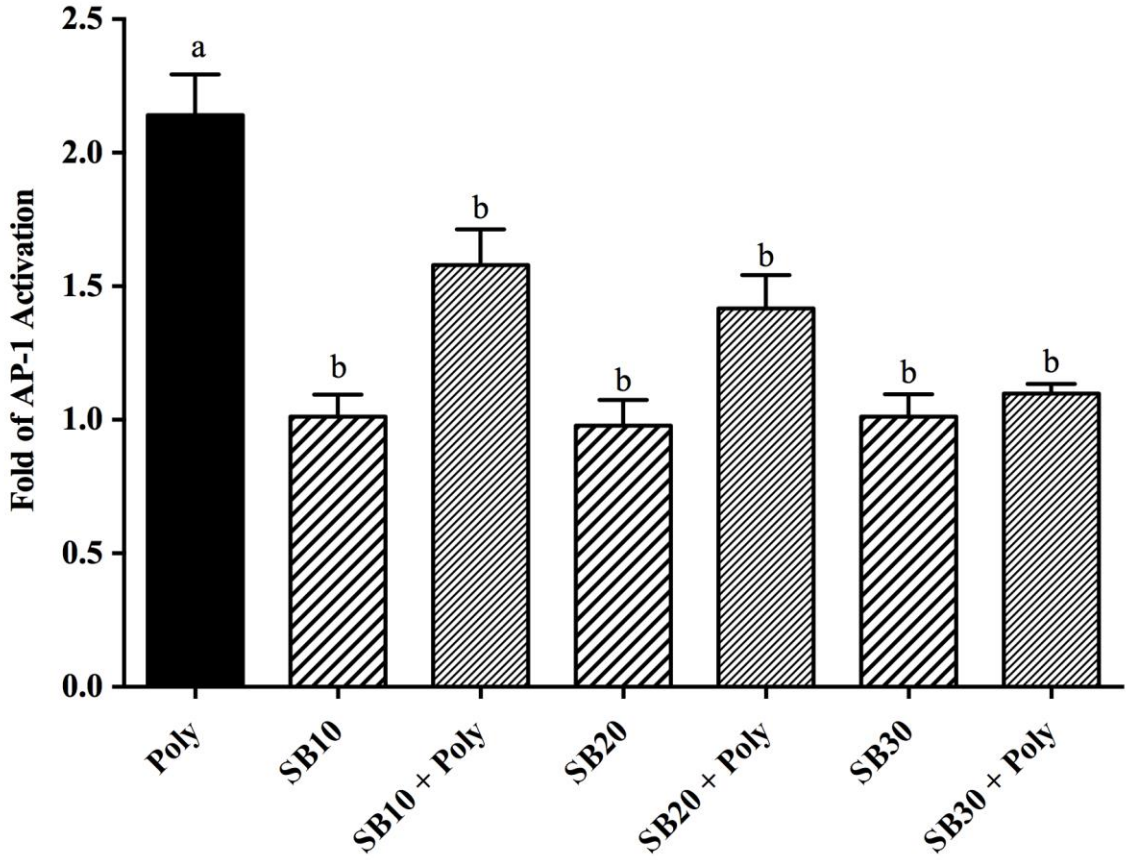

Figure 6.5. Pre-treatment of the RL95-2 cells with SB203580 inhibited the Poly I:C mediated activation of AP-1. The RL95-2 epithelial endometrial cells were transfected with the pNifty3 plasmid containing a secreted alkaline phosphatase (SEAP) reporter promoted by 5 AP-1 binding regions. The cells were pre-treated with different concentrations of SB203580 (10, 20 and 30 μM) for 1h and then treated with the TLR 3 ligand (Poly I:C 10 μg/ml) for 4h. SEAP production was measured and results represented as fold of AP-1 change where the untreated control is considered one. Results were compared with a One-Way ANOVA followed by a Bonferroni post-test. The conditions marked in a are significantly different from the conditions marked as b ($p < 0.05$).

6.4.2 The pre-treatment of the endometrial cells with TLR signalling inhibitors affected the binding of the trophoblast spheroids

The 24 h TLR stimulation of the endometrial cells with FSL-1 and PGN decreased the adhesion of the JAr spheroids but only the effect of flagellin stimulation was significantly different from the untreated control (51.8 ± 5.8% vs 71.3 ± 2.8%; $p < 0.05$). The pre-treatment of the cells with Bay11-7082 and SB203580 decreased the adhesion of the spheroids compared to the untreated cells. This reduction was significantly different between the SB203680 treated cells and the untreated control (52.7 ± 4 %; $p < 0.05$). The SB203580 inhibition of the TLR stimulated endometrial cells was unable to restore the adhesion of the spheroids compared to the untreated, Bay11-7082 and SB203580 controls (Fig. 6.6).

The stimulation of the endometrial cells with the TLR 2/6, 2 and 5 ligands for 4 h significantly decreased the adhesion of the spheroids to the endometrial monolayer compared to the untreated control cells (57.7 ± 3.1%, 49.8 ± 3.7% and 47.9 ± 4.4% vs 71.1 ± 3.2% respectively; $p < 0.0001$). The pre-treatment of the cells with Bay11-7082 and SB203580 was unable to significantly affect the adhesion of the spheroids compared to the untreated control.

The pre-treatment of the cells with Bay11-7082 was unable to restore the adhesion of the spheroids to the FSL-1, PGN or flagellin treated cells compared to the only Bay11-7082 pre-treated control (72.1 ± 4.0). Nevertheless, an increase in spheroid adhesion was observed when cells were pre-treated with Bay11-7082 and then treated with PGN and flagellin, compared to the only ligand treated cells (55.8 ± 3.1% vs 49.8 ± 3.7% and 57.4 ± 3.2% vs 47.4 ± 4.4% respectively).

The SB203580 pre-treatment was unable to restore the JAr spheroid adhesion to the FSL-1 and flagellin stimulated cells compared to the only SB203580 pre-treated control (46.9 ± 4.1% and 45.9 ± 4.9% vs 64.6 ± 3.6 %; $p < 0.005$). In contrast, the adhesion of the spheroids increased when the endometrial cells were pre-treated with SB203580 and then treated with PGN (59.7 ± 5.6 %) although no significant difference was observed with the only-PGN treated cells (49.8 ± 3.7%; Fig. 6.6).

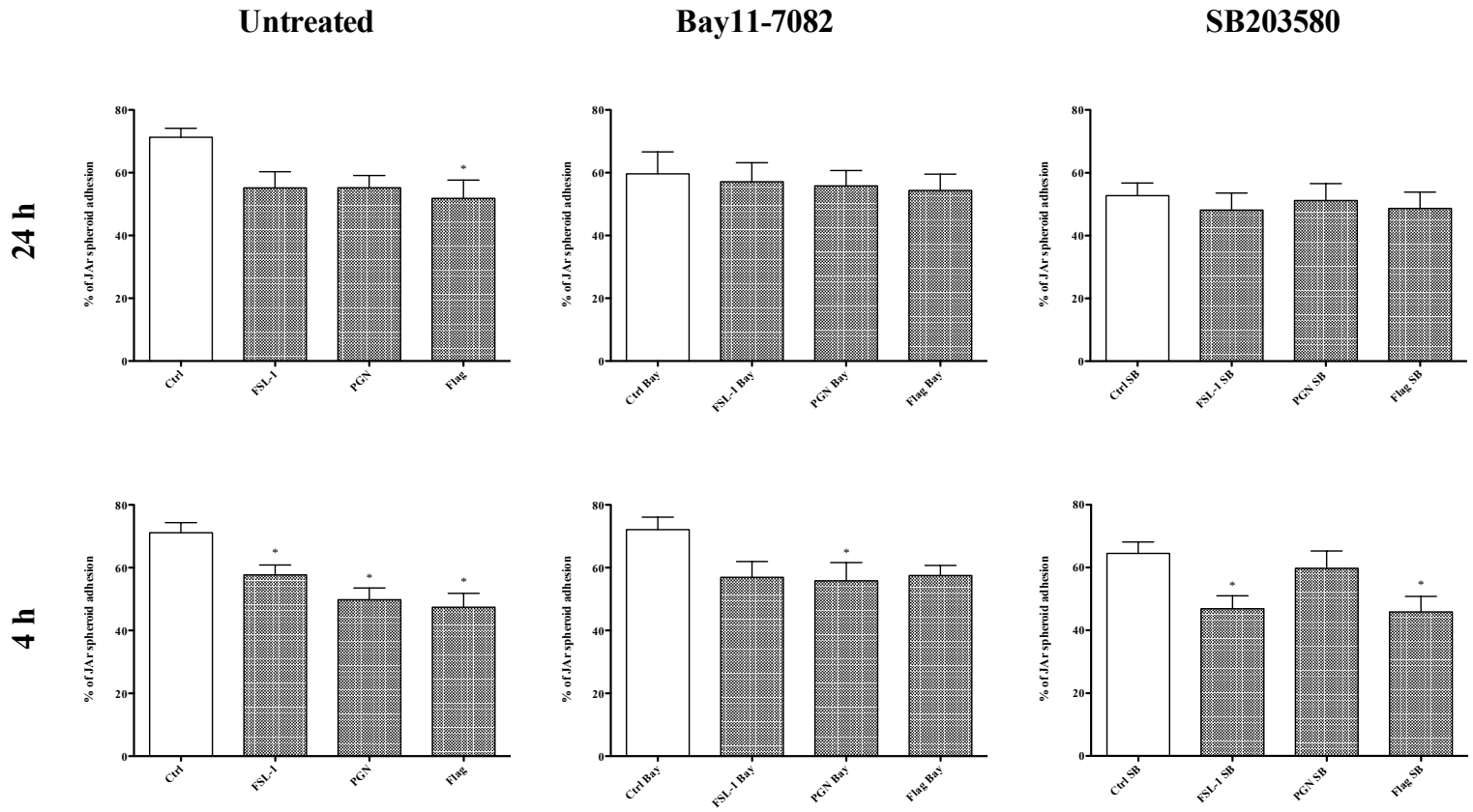

Figure 6.6. Pre-treatment of the RL95-2 cells with NF-κB and AP-1 inhibitors affected the TLR-induced reduction of JAr spheroid adhesion. RL95-2 cells were pre-treated with Bay11-7082 (20 μM) and SB203580 (30 μM) for 1 h. The cells were stimulated with the ligands for TLR 2/6 (100 ng/ml FSL-1), TLR 2 (10 μg/ml PGN) and TLR 5 (100 ng/ml flagellin) for 24 and 4 h. 50 JAr spheroids were stained with calcein, delivered and co-cultured with the endometrial cells for 1 h. The fluorescence of the spheroids was measured, the plate was rinsed and final fluorescence determined. The results are expressed as percentage of attachment. Differences were assessed with a One-way ANOVA followed by a post-test of Dunnett for differences between treatment and controls. * $p < 0.05$ was considered significant.

6.5 Discussion

The activation of NF-κB and AP-1 are the main functional effectors of TLR stimulation that will promote inflammatory reactions in response to PAMPs (Kawai & Akira 2007b). Human endometrium has the possibility of recognising pathogens through TLRs and initiate signalling that will mediate immune responses (Aflatoonian & Fazeli 2008). A disturbance of the homeostasis of the embryo implantation environment through TLR activation at implantation time would interfere with the endometrial receptiveness and inhibit embryo implantation (Sánchez López et al. 2014).

In our work, we assessed the activity of NF-κB in the endometrial cells in response to TLR stimulation *in vitro*. We observed a differential activation depending on the TLR ligand used to stimulate the cells. Using our alkaline phosphatase reporter system, FSL-1 and PGN were unable to activate NF-κB and flagellin was the only ligand able to activate it significantly. The treatment of the endometrial cells with flagellin for 4 h was enough to detect this activation. In a previous report, we showed that TLR 5 activation in 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). It has been observed that an active NF-κB complex (mainly formed by p65 and p50 subunits) can be found as early as 30 min of LPS stimulation of macrophages. Its highest peak of transcriptional activity of pro-inflammatory genes was observed after 4 h of this activation (Sharif et al. 2007). It is possible that in our model, the endometrial cells follow a similar activity that peaked at 4 h of TLR 5 stimulation but TLR 2 or TLR 2/6 followed a different signalling pathway.

To verify this, we tested the activity of AP-1 in response to different TLR ligands. As in our NF-κB observations AP-1 activation was induced by different TLR stimulation. It increased when the endometrial cells were stimulated with Poly I:C and slightly with flagellin but the stimulation with FSL-1 and PGN only showed a small decrease. The primary endometrial cells and endometrial cell lines have been proven very susceptible to the TLR 3 stimulation with Poly I:C as they can produce a variety of pro-inflammatory cytokines like IL-8, IFN-β and interferon induced genes (Patel et al. 2012; Jorgenson et al. 2005). This might be related with the danger associated with the presence of dsRNA viruses that would represent a danger for the FRT. Upon the

activation of TLRs, the signalling mechanisms are able to stimulate the MAPK pathway that in term could mediate the activation of the AP-1 transcription factor via p38, JNK or ERK (Kawai & Akira 2006). The classical TLR signalling pathways promote the activation of NF-κB and AP-1 transcription factors. In previous studies, the endometrial TLR responsiveness of the endometrial cell lines Ishikawa 3H-12 cells (formerly HEC-1B) and RL95-2 has been assessed. Only the stimulation of the cells with flagellin and Poly I:C induced the secretion of IL-8, cytokine which is mainly dependant upon NF-κB activation (Aboussahoud, Aflatoonian, et al. 2010b; Young et al. 2004). The MAPK activation pathway is also important for the induction of inflammatory responses by Poly I:C. For example, the natural killer (NK) cells are able to respond to Poly I:C stimulation, activating p38 to produce pro-inflammatory cytokines like IFN-y or CXCL10 chemokine (Pisegna et al. 2004).

As observed in our results, the responsiveness of the endometrial cells to TLR stimulation was particular for flagellin and Poly I:C activating the classical signalling pathways. Nevertheless, using an *in vitro* trophoblast spheroid adhesion model, we have demonstrated that the treatment of RL95-2 endometrial epithelial cells with FSL-1 and PGN (TLR 2/6 and TLR 2) ligands is able to reduce the attachment of the spheroids to the treated cells (Sánchez López et al. 2014). In addition to this, using atomic force microscopy we have observed that the FSL-1 and Poly I:C stimulation of the cells is able to induce morphological changes in the microstructure of the cell membrane that might be vital for the adhesion of the trophoblast spheroids to the endometrial cells (Manuscript in preparation, Chapter 4). This may signify that in the endometrial epithelial cells are able to respond to TLR stimulation but do not engage the classical activation pathways reported.

To assess 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. As expected the pre-treatment of the cells with Bay11-7082 reduced significantly NF-κB activity induced by flagellin treatment. It has been reported that 1 h of Bay11-7082 pre-treatment of human umbilical vein endothelial cells is enough to reduce NF-κB activation by TNF-α without affecting cell viability (Pierce et al. 1997). In the same way, 1 h pre-treatment of the endometrial cells with SB203580 was able to reduce the Poly I:C induced AP-1 activation. The effectiveness of SB203580 to activate

inflammation in response to TLR stimulation has been proven before, where the pre-treatment of HeLa cells with SB203580 is able to reduce p38 phosphorylation and TLR and cytokine expression induced by the stimulation with *Trichomonas vaginalis* (Chang et al. 2006).

In our trophoblast spheroid adhesion experiments, the 24 h treatment of the endometrial cells with both inhibitors Bay11-7082 and SB203580 was enough to reduce the adhesion of the spheroids to the cells. This effect was not observed when the treatment was reduced to 4 h. It is possible that the extended treatment of the cell with both inhibitors affected other vital endometrial cell functions, which as a result reduced the spheroid adhesion rate. It has been reported that the prolonged treatment of HBL-1 lymphoma cells with Bay11-7082 reduced cell proliferation and when cells were treated over 24 h of treatment increased cellular death (Strickson et al. 2013). It is very likely that the reduction in spheroid adhesion after a prolonged exposure to the inhibitors affected morphogenic characteristics and the viability of the endometrial cells hence spheroid adhesion.

In a similar way, the activation of MAPK has been observed an important requirement for the maintenance of epithelial cell integrity. In mammary epithelial cells, the pharmacological inhibition of p38 and JNK is able to increase expression of claudins, proteins that regulate tight junction formation, and increase the transepithelial resistance (TER) (Carrozzino et al. 2009). TER measures the resistance to electricity conductance of a tissue and can be correlated to the integrity of the cell monolayer. The TER of the epithelial cells of the FRT can change according to the hormonal cycle stimulation, for example estrogen stimulation of human cervical cells can reduced the TER of the monolayer (Gorodeski 1998). The effect of p38 inhibition has also been studied in the interaction between endometrial cells and trophoblasts. The direct activation of p38 in the endometrial cells has been observed after the attachment and expansion of trophoblast spheroids. The treatment of the endometrial cells with SB203580 reduced trophoblast outgrowth (H.-Y. Li et al. 2003). These observations might be related with our lack of trophoblast adhesion induced by the SB203580 treatment in our model. If the inhibition of p38 in the endometrial cells induced a tightening of the monolayer it might be possible that the trophoblast adhesion and migration resulted impaired.

The inhibition of TLR activity with Bay11-7082 was not able to restore the spheroid binding to the endometrial cells completely, only partial increase was observed when Bay11-7082 pre-treated cells were exposed to PGN and flagellin compared to those cells that were not pre-treated. Previously it has been observed that the flagellin-induced reduction on the adhesion of trophoblasts spheroids to the endometrial Ishikawa 3H-12 cells can be increased by the pre-treatment with Bay11-7082 (Caballero et al. 2013). This observation was similar to the one in our work but was unable to completely restore the adhesion of the spheroids, suggesting the participation of a different mechanism on the TLR-mediated reduction of trophoblast spheroid adhesion to the endometrial cells.

The pre-treatment of the endometrial cells with the p38 inhibitor SB203580 was able to increase slightly the spheroid adhesion to the PGN treated cells but left unaffected this adhesion to the FSL-1 and flagellin treated cells. It has been observed that the stimulation of TLR 2 in the intestinal epithelial cell lines Caco-2 and HT-9 with Pam3CysSK4, is able to increase the TER of the cells by a mechanisms mediated by the protein kinase C α and δ. This activation induced the tightening of the cells through an increase in expression of ZO-1 a protein that mediates tight junction formation as a defence mechanism against intestinal bacterial infection (Cario et al. 2004). It is possible that the endometrial cells could follow a similar activation pathway that could modify cellular adhesion and interfere with trophoblast adhesion.

It has been described that the human endometrium has a characteristic constitutive NF-κB activation along the menstrual cycle that may aid the morphological changes that the epithelial cells undergo and in preparation for an embryo implantation. The immunohistochemical detection of p65 sub-unit has shown that it is localised in the nucleus of the endometrial epithelial cells along the menstrual cycle increasing at mid-secretory phase and peaking on the late secretory phase (S. H. Kim et al. 2013). The direct quantitation of p65 by DNA-binding immunodetection confirmed that it has its highest peak during the proliferative phase. This activation is presumably constitutive as it occurs without TLR stimulation. Hence it might be related to the morphogenic development of the endometrium (González-Ramos et al. 2012). In respect to the p50 sub-unit of NF-κB, two peaks of activation have also been observed. The first one during the proliferative phase and the second one at mid-secretory phase. Following

each p50 peak, the activity of IκBα was found to increase acting as a negative regulator of its activity (Page et al. 2002). Supporting this evidence, a study has shown that progesterone induces the expression of IKKα at mid-secretory phase and represses IKKβ, responsible for the pro-inflammatory activity of NF-κB, which again, suggests a morphogenic activity of NF-κB during this phase (King et al. 2001). The cross-talk between embryo and mother might be also mediated by NF-κB as suggested by a previous report from our group, where the contact of JAr trophoblasts with endometrial Ishikawa 3H-12 cells *in vitro* increased NF-κB activity (Caballero et al. 2013). In the murine endometrium, the activity of p50 and p65 increases at pro-estrus and estrus, the equivalent for the human late-proliferative and secretory phases. If fertilisation is achieved this NF-κB activity increases from the day 1.5 post-coitus peaking at day 3.5 when embryo normally implants (H. Nakamura, Kimura, Ogita, Nakamura, et al. 2004b). A similar observation was found in the endometrium of gilts preparing the endometrium for pregnancy (Ross et al. 2010).

As stated before, there is a differential activity between an NF-κB destined to influence the morphogenic activity of the endometrial cells from an NF-κB that regulates inflammatory responses. The activity of NF-κB that we observed in our experiments might be totally related to the inflammatory response of the cells as it is induced by the activation of the TLRs. More evidence is required to characterise if the inhibition of NF-κB used in our experiments can also affect the morphogenic activity of the endometrial cells in response to the hormonal stimulation. If we desire to block the activation of NF-κB during the menstrual cycle as an anti-inflammatory therapy, we would necessarily require targeting its inflammatory activity. One of the main pathways shared in both cell adhesion and TLR activation is the MAPK pathway. Upon the adhesion of the integrins to their substrate, they will promote the signalling to processes like cell proliferation and survival (Short et al. 2000). The transcription factor AP-1 is activated by different stimuli that trigger the MAPK activity during a variety of processes like embryogenesis, inflammation, cell migration, differentiation and death, among others (Pearson et al. 2001). As observed in this work, an additional mechanism of activation of the endometrial cells must exist as not all the TLR signal through similar pathways; nevertheless, in all of them we observe an influence on the receptive capacity of the epithelial cells for trophoblasts. Epithelial cells form the lining of all the tissues and will be the first cells to encounter external stimuli. Intestinal epithelial cells

are able to recognise pathogens through TLRs and react in response to this. In particular, TLR 2 activation can modify the TER of intestinal epithelial cells to tighten the inter-cellular adhesion junctions and in this way avoid the pass of bacteria to the subepithelial compartment (Cario et al. 2004). It is possible that the endometrium posses a similar mechanism that will protect it from bacterial invasion but this would compromise the adhesion of trophoblast cells for blastocyst implantation. At the same time, the TLR responses seem to be selective as not all TLR would initiate classical inflammatory reactions mediated by cytokines and even the secretion of cytokines seem to be discrete probably to avoid exacerbated inflammatory reactions.

As observed, a delicate balance between immune surveillance and inflammation should be preserved during implantation period. The increase in the innate immune recognition at time of implantation and during pregnancy is necessary for the maternal health and success of pregnancy (Koga & Mor 2008). The TLRs are expressed along the FRT and are capable of initiating immune responses when they sense PAMPs. NF-κB and AP-1 are the main transcription factors activated during TLR signalling. These two transcription factors are also active during physiological processes undergone in the endometrium. Implantation and pregnancy can be compromised by TLR activation as observed in many *in vitro* and *in vivo* models (Aboussahoud, Bruce, et al. 2010a; Sánchez López et al. 2014; Friebe et al. 2011; J. Zhang et al. 2007). If a possibility exists of targeting TLRs for a therapeutic approach, precautions need to be taken and select a mediator that only plays a role in inflammatory cascades of these transcription factors. Otherwise we could compromise the morphogenic activity of the endometrial cells during the cycle and interfere with acquisition of receptivity and embryo implantation or even development.

Chapter 7. General discussion

7.1 Visions of reproductive biology research

The research area of reproductive biology is diverse and therefore we are forced with multiple challenges to overcome. It is an exciting area of research that brings us close to the core of human life and origins of life.

I can recall the work of Master & Johnson during the 50s on the physiology of the sexual act. They were proving the existence of the "female orgasm", an event that up to the time was considered as non-existent. Their first approaches had to be performed in secret by a cohort of volunteers that were curious about the act. They were massively criticised by the medical community and their ideas rejected. But as in all scientific breakthroughs, good and solid proof for your hypothesis will convince the most stubborn mind. Personally I believe that this is what science is all about, creating novel ways to explain and understand the curious world that surround us.

7.2 Summarising discussion

Scientists through years of research have tried to understand the menstrual transitions the endometrium undergoes to acquire the receptive phenotype necessary for embryo implantation. Nevertheless we still lack a clear understanding of the endometrial biology. Perhaps through the study of the endometrial behaviour in disease we could get a better picture of the particularities the tissue must acquire in order to host an embryo. In the present work, by using both *in vivo* and *in vitro* models of embryo implantation, we were able to describe mechanisms of endometrial behaviour in response to TLR activation that can interfere with embryo implantation. From a molecular perspective we observed novel cellular responses to TLR stimulation, such as cell morphology changes and endometrial mucosal activity. In this last chapter, I summarise the major findings of my work, elaborate possible implications for these findings and propose further ideas to clarify and develop forward the research initiated.

7.2.1 Thesis findings

The main objective pursued in the development of the current work was to identify if the endometrial innate immune responses are able to interfere with embryo

implantation. In order to answer this question, a different number of approaches were taken. These varied from *in vitro* and animal embryo implantation models, gene expression, characterisation of cell surface proteins by flow cytometry, arrays for protein profiling, cellular transfection, light and fluorescent microscopy and even atomic force microscopy. To obtain the right answers to our research questions, an in depth understanding of analysis techniques, coupled with creativity is fundamental. The work performed during my PhD and presented in this thesis, give some insights of the biology of human endometrial cells and with some further development may lead to discovery of new therapies in reproductive and cellular biology.

One of the main satisfactions I obtained during the development of this PhD is the opportunity to collaborate with different areas of knowledge, like the Physics department of the University of Sheffield. We went even further and established a couple of international collaborations with two research institutes in Spain for the further development of our research.

My interest to work in the lab of Alireza Fazeli was at first related to his investigations on the characterisation of innate immune system in the female reproductive tract. The background for my work came from a publication arising from Fazeli's laboratory in 2005. They described how different sections of the FRT could differentially express TLRs. According to this, the lower components of the tract, which are in contact with commensal bacteria, lack the ability to express TLR 4 as a regulatory mechanism to avoid constant activation. In contrast, the sterile upper tract expresses TLR 4 and the rest members of the TLR family. In that paper they introduce the idea of a differential function of the tract that guarantees a complete protection from pathogens and a safe environment for the development of the embryo at the upper parts of the female reproductive (Fazeli et al. 2005). Following this work, the group was able to describe that the expression of TLRs not only varied depending on the part of the tract but also was dependent on the phase of the menstrual cycle. This cyclical expression of TLR 1-10 in the endometrium peaked at the late secretory phase, when the tract is ready for the reception of the embryo (Aflatoonian et al. 2007). Two last publications dictated the direction of the present work. The first indicated that the endometrial TLRs are expressed in endometrial cell lines and they could be activated to provide a functional response (Aboussahoud, Aflatoonian, et al. 2010b). The second adapted an *in vitro*

model of human endometrium – trophoblast interactions to study the implantation of the embryo in response to endometrial TLR 5 activation. This paper suggested a relevant role of the TLRs in controlling the homeostasis of the uterus at the time of embryo implantation. The activation of the endometrial TLR 5 would affect the endometrial cells and reduce the chances for an embryo to implant (Aboussahoud, Bruce, et al. 2010a). Having this background to support my investigations, we decided to focus my research on the role of TLR 2 and 2/6 on endometrial innate immune responses and their effects on embryo implantation.

The study of the human embryo has always been challenging, as its use in experimentations is very limited and protected by strict ethical and legal frames. To avoid the need of human embryos for the research on implantation and development, the reproductive research community has followed a couple of strategies: The use of trophoblast and endometrial cell lines (mostly choriocarcinomas and endometrial carcinomas) and the use of animal models for its study (Hannan et al. 2010).

In our first investigation to study the relationship of reproduction and the innate immune function we used a murine embryo implantation model. The collaboration between Alfonso Gutiérrez-Adán at the INIA in Madrid and our research group made this approach possible. Many studies have been performed trying to characterise the functional role of the TLRs in the FRT using murine models. Some have focused on the inflammatory reaction in the uterine horns and the roles of the different subset of cells that compose the uterus (Sheldon & M. H. Roberts 2010). Others have explored the production of soluble mediators of inflammation along the oestral cycle or during TLR mediated responses (Hickey et al. 2013) and even some in responses against sexually transmitted murine pathogens like *Chlamydia muridarum* and the importance of the TLRs in the resolution of the infection (Nagarajan et al. 2011). The role of TLR inflammatory responses and pregnancy has been suggested in studies where activation of TLR 3 or TLR 4 during pregnancy leads to pre-term birth and deficient decidualisation and vascularisation of the uterus (J. Zhang et al. 2007; Friebe et al. 2011). Our study introduced for the first time the concept of a possible failure of the initiation of pregnancy due to the activation of TLR 2/6 at the time of implantation (Chapter 2). We described that this activation could modify the type of cytokines produced and negatively affect tissue architecture, which both are required for embryo

implantation. Furthermore, the activation of TLR 2/6 decreased the percentage of embryos implanted in the uterine horn and increased embryonic resorptions. This confirmed the existence of a direct effect of uterine TLR activation on embryo implantation (Sánchez López et al. 2014).

As suggested by the murine model, TLR 2 could be playing an important role in uterine protection. To go beyond the murine model and try to verify these observations in human cells, we employed our *in vitro* human implantation model. The trophoblast cell line JAr was selected to simulate the embryo by forming small tri-dimensional spheroid-shaped cell clusters. The JAr trophoblast cell line is derived from a first trimester choriocarcinoma. They have been shown to interact with endometrial cells and form firm adhesions with endometrial cell lines like RL95-2 and HEC-1A (John et al. 1993; Thie et al. 1998). The telomerase-immortalised human endometrial epithelial cell line (hTERT-EEC) simulated the human endometrium. These cells were originally described as a non-tumoral, receptive endometrial cell line (Hombach-Klonisch et al. 2005). Unfortunately, during the development of our experiments the cells were found to be contaminated with the breast carcinoma cell line MCF7 (Korch et al. 2012). In addition a report showed that HEC-1B cells were mislabelled and they were found to be a subset of Ishikawa cells (named now Ishikawa 3H-12). These issues represented a challenge for our work. We had to select a new cell line for our studies and to characterise all the cell lines we had in our bank to make sure we had the correct lines. We selected the RL95-2 cells as an embryo receptive cell line (Way et al. 1983) and repeated most of the previously performed experiments using cell lines such as HTERT-EEC and HEC1B. Hence, the experiments where we described the effects of TLR 2 on the interaction between the trophoblast and endometrial cells were performed using two different epithelial cells (Chapter 3).

We observed that the interactions of trophoblast with the RL95-2 endometrial cells were faster and the adhesion higher than that with breast carcinoma MCF7 cells. Nevertheless the activation of TLR 2 and TLR 2/6 in all cases reduced the percentage of adhering spheroids. In a normal TLR response, the epithelial and immune cells would produce the pro-inflammatory cytokine IL-8. In the case of the endometrial epithelial cell lines, the functionality of the TLR activation by producing IL-8 seems to be limited. For example, only the stimulation of TLR 2 and 5 in ECC-1 cells and TLR 3 and 5 in RL95-

2 and Ishikawa cells has been observed to induce a low secretion of IL-8 (Schaefer et al. 2004; Aboussahoud, Aflatoonian, et al. 2010b; Young et al. 2004; Schaefer et al. 2004). It has also been suggested that the contact with stromal cells is necessary and fundamental for the production of cytokines and initiation of immune responses (Sheldon & M. H. Roberts 2010). These results suggested that the TLR activation of the epithelial cells used in our *in vitro* model was different from a classical response mediated by cytokines. It might be possible that the response could affect a cell feature that mediates their inter-cellular adhesion capacity.

With the last statements in mind, the evident question to answer was: How does the activation of endometrial TLR affect the adhesion of the trophoblast spheroids? Endometrial receptivity is a concept used to describe morphological and phenotypical changes that the endometrium undergoes every menstrual cycle to allow the implantation of the embryo. Several parameters such as expression of αvβ3 and β1 integrins, glycans, mucin 1, cadherins, among others have been proposed to favour endometrial receptivity (Lindhard et al. 2002). Some membrane structures like microvilli, cilia and pinopodes are also described to appear during late secretory phase and have been found to be a key component of endometrial receptivity (T. C. Li et al. 1991; Bentin-Ley et al. 1999). Collaboration established between our unit and Jamie Hobbs from the Physics and Astronomy department allowed us to observe these micro-structural characteristics by Atomic Force Microscopy (AFM; Chapter 4). The treatment of RL95-2 cells with a TLR 2/6 ligand was found to affect membrane morphology. It reduced the microvilli in the membrane and increased its roughness. The effect was also induced by TLR 3 stimulation and was not mediated by NF-κB, the main transcription factor activated by TLRs. These last observations made evident that the cell membrane was being affected by the TLR activation. To further develop a possible answer for this question, we came across a paper where the expression of a variety of endometrial cell membrane markers was evaluated. This report suggested the importance of new membrane molecules such as CD98 in the acquisition of endometrial receptivity (Domínguez et al. 2010). During my time in Madrid, I was lucky enough to come in contact with María Yañéz-Mo, the main author of this work. We met and agreed to start a new collaboration to try to find an explanation to our observations on endometrial TLR responses. We performed a cell surface proteome profile analysis using a panel of antibodies to profile expression of 22 different membrane markers in 3 endometrial cell

lines (Ishikawa, Ishikawa 3H-12 and RL95-2) and one breast carcinoma cell line (MCF7). From the total panel, 13 markers were discarded as no expression of these markers on the cell surface was found. In the end, 9 of the markers were highly expressed in the epithelial cells and only the expression of 4 of them: β1 integrin, CD147, CD9 and CD98 was affected by the TLR stimulation with diverse ligands. These molecules have been found to form membrane complexes around integrins in focal adhesion points that mediate inter-cellular adhesion and migration (Al-Jamal & Harrison 2008). These complexes have been also found to mediate the formation of membrane-cytoskeleton linking complexes (Sala-Valdés et al. 2006; Iacono et al. 2007). In immunological studies β1 integrin and CD98 have been found to associate around the endocytic complex to facilitate the movement of the endosome and favour recognition of PAMPs by TLRs (Charania et al. 2013; Schroeder et al. 2012; S. Zhang et al. 2013; Hsu et al. 2011). We concluded that potentially in the endometrial membrane complexes, these molecules aid the adhesion of the blastocyst during implantation as their absence makes the implantation impossible (Hanashi et al. 2003; Igakura et al. 1998; Domínguez et al. 2010). As suggested, these molecules are also used for regulation of immune responses. It might be possible that during TLR signalling, these complexes initially used for blastocyst attachment are relocated to the immune signalling and endocytic compartments. In this way they would lose their intercellular adhesion function and impair trophoblastic adhesion.

Another membrane protein that negatively influences endometrial receptivity is the glycoprotein mucin 1 (Simón et al. 2001). The expression of MUC1 in the endometrium of humans, several primates and a diversity of mammals has been found to be higher during the implantation period (Meseguer et al. 2001; H. Singh et al. 2010) compared to the other phases of the reproductive cycle. Its expression is suggested to have an anti-adhesive role for the blastocyst. Nevertheless, immunofluorescence analysis have shown that MUC1 is lost from the implantation site (H. Singh et al. 2010). In our experiments, the expression of MUC1 messenger was induced rapidly by TLR 5 in RL95-2 cells and by TLR 2/6 in MCF7 cells. In contrast, the increase in MUC16 expression was observed after a long stimulation of TLR 2/6 and 5 and was particularly expressed only by the endometrial cells. Our results suggested that these membrane-bound mucins could be expressed in response to TLR stimulation for the epithelial

defence. This mucin expression might be able to decrease the chances of an embryo to implant.

To further increase our knowledge of the endometrial cell activity in response to TLR activation, we assessed the activation of the classical transcription factors NF-κB and AP-1 (Chapter 6). Classically NF-κB and AP-1 are activated in response to TLR stimulation to promote production of pro-inflammatory mediators like IL-8, IL-1β, TNF-α, among others (Takeuchi & Akira 2001). In the endometrium, NF-κB is activated following the menstrual cycle activity to promote differentiation of the stromal and epithelial cells (González-Ramos et al. 2012; King et al. 2001). NF-κB has also been significantly increased during the implantation phase and in response to the trophoblast contact (H. Nakamura, Kimura, Ogita, Koyama, et al. 2004a; Caballero et al. 2013). The MAPK activation pathway is involved in inter-cellular adhesion events, like integrin-extracellular matrix or cell proliferation for wound healing (Pearson et al. 2001). Our assays were sensitive enough to detect a high activity of NF-κB in response to TLR 5 stimulation but it remained unaffected by TLR 2 or 6 stimulation. TLR 5-mediated activation of NF-κB was inhibited by Bay11-7082. AP-1 was only detected when TLR 3 was stimulated with Poly I:C. Blocking the p38 activation pathway decreased this TLR 3 mediated activation. We decided to verify if the decreased adhesion of trophoblast spheroids to TLR stimulated endometrial cells could be ameliorated by these inhibitors. We were unable to observe a positive effect on spheroid adhesion by pre-treating the endometrial cells with the inhibitors. It might be possible that the decrease in trophoblast adhesion to the monolayer mediated by TLR 2 and 2/6 is independent from the classical TLR activation pathways. Bay11-7082 could be affecting viability of the cells and SB203580 might interfere not only with the TLR activation pathway but affect the intercellular adhesion (H.-Y. Li et al. 2003). If the inhibition of TLR signalling is intended to be used as a therapy, it is desirable to use specific inhibitors that leave the endometrial menstrual behaviour unaltered as both NF-κB and MAPK are involved in normal endometrial development and implantation.

7.3 Implication of the major thesis findings

7.3.1 The inflammatory responses of the endometrium to inflammatory stimuli and its effects on embryo implantation

Our *in vitro* implantation model was able to describe the capacity of the tissue to react to foreign pathogenic stimuli. Upon recognition, the activation of uterine TLR 2/6 was able to modify the tissue architecture and the profile of cytokines produced. The endometrium must be fit to receive an implanting embryo. This will require the proliferation of epithelial and stromal cells to form a solid substrate for the invasion of the blastocyst (Norwitz et al. 2001). Our findings suggest that the pre-implantation period is critical for the acquisition of these tissue characteristics and it is very sensitive to the presence of inflammatory stimuli. TLR 2/6 activation during implantation period was able to reduce embryo implantation and increase resorption as could be demonstrated in chapter 2. This is the first report implicating TLR activation in implantation. Previous reports have focus on the induction of foetal loss by activation of TLR 4 and TLR 3 (J. Zhang et al. 2007; Friebe et al. 2011).

Furthermore, we propose a novel method and index to evaluate the development of the endometrial estral changes and effects of toxic – inflammatory compounds in the uterine horn. The uterotrophic assay is currently the method of choice for evaluation of these type of compounds by weighing the whole horns after being treated with toxic substances (Ashby 2001). Our method for evaluating the endometrial morphometry is able to distinguish the effects on each layer of the uterine horn. By calculating an index that relates each region of the uterine horn (myometrium, endometrium and lumen) with the full area of the cross section, we avoid the animal-size variation. Also the processing of the digital image using ImageJ would increase the sensitivity of the assay and can eliminate the user bias. Although our uterine indexing method proved to be effective in determining the effects of TLR 2/6 activation on the uterine horn, further standardisation is required to validate it.

The application of our findings to be used as a tool for diagnostic purposes of human endometrial pathology might sound very far fetched. But in fact this is possible and necessary. Pathologies like thin endometrium or endometriosis can have different

effects like infertility or high-risk pregnancy. This could be avoided by a proper pre-evaluation of the endometrial development. Image analysis by ultrasound or magnetic resonance imaging (MRI) of the uterus can potentially reach this level of detail and make an objective prognosis without the need of tissue sampling.

The evaluation of the uterine secretions can also relate to the endometrial status. Many cytokines like IL-1β, IL-1RA or MCP-1 have been proposed to participate in both implantation and inflammatory process (Lindhard et al. 2002). A proper determination of their production in normal conditions or in response to pathogenic stimuli could also be implemented as a diagnostic method. Determination cytokines like IL-1RA as a predictor of embryo implantation has been suggested before (Simón et al. 1998). Our observations remark the importance of endometrial lumen cytokines in health and disease. It is possible that introducing the measurement of cytokine levels as a pre-implantation diagnosis would bring benefits to patients who are currently under IVF treatment and increase our knowledge on the role they play in implantation.

7.3.2 Evaluation of the cellular morphology in response to TLR activation

We have mentioned several times that the female reproductive tract is extremely plastic as undergoes constant changes every month along the menstrual cycle. Example of this is the morphological changes of the endometrial epithelial cell membrane. Cells acquire microvilli, cilia and swollen pinopodes during late secretory phase to allow embryo implantation. These morphological changes have been used as prognostic criterion of endometrial receptivity and a deficiency of these structures with infertility (Nikas et al. 1999). As presented in chapter 4, the novel observation that TLR stimulation can influence cell membrane morphology suggests that epithelial cells can undergo a different defence mechanism from a conventional immune cell response. The membrane microstructure of the endometrial cells initially covered by microvilli will be affected by the TLR stimulation. The cells will lose the microvilli and form blebs. Whether the blebbing corresponds to the secretion of newly synthesised proteins, like inflammatory mediators is matter of further study. It might be possible that the cytoskeleton of the cell, responsible for the formation of these membrane protrusions (microvilli and blebs), becomes a mediator of the immune recognition of foreign entities and the changes are part of the defence mechanism. Membrane blebbing has been proposed to participate in

mechanisms of cell survival. For example, the cell contact with the bacterial pore-forming toxin (PFT) O streptolisin induces cell blebbing that will release the toxin bound blebs preventing cell damage and apoptosis (Keyel et al. 2011).

The fact that these changes are not limited to TLR 2/6 but also induced by TLR 3, suggests that the effect might be shared by several TLRs. We are unsure if the effect will occur in epithelial cells of a tract different from the reproductive tract. However, one point that becomes clear is that at least the changes elicited in the endometrial cells could affect their receptive morphology. This might be one of the effects leading to infertility originated by pre-existent or current subclinical infections and might be important to help with the clinical diagnosis of the symptoms of the disease.

7.3.3 The endometrial membrane protein profile might be altered as a result of TLRs activation

As suggested by our observations in chapter 5, the expression of a diversity of surface molecules that are highly expressed in the endometrial cell lines might result modified by the TLR stimulation. Molecules like β1 integrin, CD147, CD9 and CD98 are involved in the formation of focal-adhesion complexes (Al-Jamal & Harrison 2008). They might be necessary for the interaction with the trophoblast during the initial apposition. As suggested by the murine models which lack these molecules, they are considerably important for proper implantation (Hanashi et al. 2003; Igakura et al. 1998; Domínguez et al. 2010). On the other hand, they have also been found to form part of immune complexes to recognise and process foreign entities (Charania et al. 2013; Schroeder et al. 2012; S. Zhang et al. 2013; Hsu et al. 2011). We were able to observe changes on the expression of these surface markers in response to TLR stimulation. These reaction, similar to the one observed in the membrane morphology would affect the embryo implantation. We are unsure if the cell will modify their expression or only the reorganisation of the complexes in the cell membrane. A possibility is that the TLR stimulation would modify their adhesion capacity and facilitates the immune function for cell protection compromising the function of endometrial – trophoblast interaction. The use of endometrial carcinoma-derived cell lines represents a limitation in the understanding of these complexes. It has been

proposed that they play an important role on carcinoma cell metastasis by aiding their migration (K. Nakamura et al. 2012; Park, Inoue, Ueda, Hirano, Higuchi, Maeda, et al. 2000b). Further studies on the microscopical localisation of these molecules on the endometrial cell membrane are required in order to completely characterise our observations.

A new mechanism of endometrial defence was observed when the TLR stimulation increased the expression of MUC1 and MUC16. The membrane-bound mucin 1 and 16 have been proposed as an anti-adhesive molecule expressed by the endometrium during the secretory phase (Gipson et al. 2008; Brayman et al. 2004). In different mucosal epithelial cells like lungs, eye or gut, these mucins and secreted mucins are expressed in response to TLR activation forming a physical barrier for pathogens (C. M. Evans & Koo 2009). In the reproductive tract, the expression of these mucins in response to TLR stimulation has been observed in the cervix (Radtke et al. 2012). In our observations, MUC16 and MUC1 increased their expression in response to TLR stimulation in both endometrial and breast carcinoma cells. Furthermore, MUC16 expression was observed only in the endometrial cells and might have a higher relevance for immune responses. As proposed, these mucins may protect the endometrium from infections but make the implantation of the embryo difficult and less possible.

7.3.4 Classical activation pathways of the TLRs in endometrial cells might not be involved in the decrease of trophoblast adhesion

The classical pathway of TLR signalling in most of the cases ends up in the activation of the transcription factors NF-κB or AP-1 (Takeuchi & Akira 2001). The pathway might vary for different cell types and types of stimulation. In our observations, the TLR 5 was the main activator of NF-κB and TLR 3 of AP-1 and TLR 2 and 2/6 left unaffected this activation. This might be a case of differential activation of TLR depending on the cell line studied. It is also a fact that the method of assessing this activation was indirect through alkaline phosphatase by using specific promoters for these transcriptions factors. Hence we are only able to detect them if they promote alkaline phosphatase gene transcription. But it is a fact that the activation of NF-κB not only promotes gene transcription but a different dimer combination can also repress it (Ghosh & Hayden 2008). Nevertheless, the inhibition of these pathways was unable to

recover the total adhesion of trophoblast spheroids from the TLR stimulated endometrial cells. This might signify that a different activation mechanism is responsible for the reduction of spheroid adhesion and it is independent of gene expression. A couple of reports have shown that TLR 3 and TLR 4 have alternative pathways that can affect cellular adhesion and migration (Yamashita et al. 2012; Yi et al. 2012). TLR 2 stimulation of intestinal cells can modify focal intercellular adhesion by a mechanism mediated by protein kinase C (Cario et al. 2004). There is a diversity of methods to detect the subunits of NF-κB that are activated like western blot, flow cytometry or electrophoretic mobility shift assay (EMSA). These could be used to understand which signalling mechanisms might be affecting the endometrial cells capacity of binding trophoblast cells.

7.3.5 Summary of achievements

The *in vivo* murine model of embryo implantation presented in chapter 2 highlighted the importance of a sterile environment in the uterine horn at time of embryo implantation. If this requirement is not met, in this case simulated by TLR 2/6 stimulation, detrimental effects are observed in the uterine horn architecture and pro-inflammatory environment that will ultimately neglect the implantation of the blastocyst (Sánchez López et al. 2014). The results in chapter 3 confirmed the *in vivo* observations using an *in vitro* human trophoblast adhesion model. The stimulation of endometrial TLR 2 and 2/6 decreased the endometrial cell capacity to receive trophoblast spheroids. The following experimental chapters were designed to decipher the mechanisms that TLR activation induced in the endometrial cells to mediate this decrease in trophoblast adhesion. The atomic force microscopy experiments in chapter 4 gave a microstructural perspective of the endometrial cell membrane reaction towards TLR 2/6 and 3 stimulation. This TLR activation is able to influence the cell membrane profile, roughness, loss of microvilli and bleb-like structures formation. Continuing the experiments on membrane focus, a profiling of 22 markers was performed by flow cytometry. We were able to detect that TLR stimulation could modify the expression of β1 integrin, CD147, CD9 and CD98. These molecules are important mediators of adhesive intercellular complexes that might function during implantation but can also promote the immune recognition of pathogens to initiate inflammatory responses. In chapter 5, the mucosal innate immunity of the endometrium was tested with the

expression of MUC1 and 16, which are anti-implantation factors. The stimulation of TLR 2/6 and 5 was able to promote the expression of the membrane bound MUC1 in epithelial endometrial and breast cancer cells. Interestingly, the expression of MUC16, another membrane bound mucin was only increased in the endometrial cells after TLR 2/6 and 5 stimulation. The function of both mucins is to avoid bacterial colonisation but has been observed to affect also embryo implantation. Finally, in chapter 6 we described how the activation of the endometrial TLR follows a differential signalling pathway depending on the ligand employed. This might differ from the conventional NF-κB and MAPK activation pathways, because in our experiments, the use of inhibitors for both pathways were unable to restore the TLR-mediated decrease of trophoblast adhesion.

Together these results show the importance of the immune regulation the endometrial tissue must exert at the time of implantation. The effects that TLR stimulation has over the endometrial cell biology are diverse but most of them point towards a morphological – phenotypical modification of the characteristics that endometrial cells must possess to receive an implanting blastocyst. As pregnancy and support of a developing embryo represents a huge expenditure of energy, it might be possible that upon the encounter of an infection, the endometrial cells would acquire a defensive phenotype that would avoid the implantation of the embryo and a potential risk for the health of the mother.

7.4 Directions for future study

7.4.1 Early evaluation of the endometrial characteristics

The results observed in this work have suggested that the endometrial tissue is highly plastic and sensitive enough to change as a response to the presence of unknown or non-self entities. As it has been mentioned before, the remodelling of the endometrium to host an embryo is crucial. From a basic science point of view, I am very interested in further investigating the behaviour of the stromal cells towards TLR stimulation. It might be possible that as a defence mechanism, the cells react negatively compromising the cell proliferation for decidualisation of the endometrium. It has been observed that i.p. administration of poly I:C, TLR 3 ligand, to gestating mice impairs the vascularisation of endometrium (J. Zhang et al. 2007) increasing fetal loss. The importance of the stromal cell pro-inflammatory activity has been suggested as the cooperation of stromal and epithelial cells is necessary for a complete inflammatory response to TLR 4 activation (Sheldon & M. H. Roberts 2010). The problem with these studies is that they focus on the effects of the activation of TLRs once pregnancy is established. From the immunological point of view, a good characterisation of the endometrial responses at time of implantation is required. We know that the endometrium is infiltrated with a particular subset of NK cells that regulate immune tolerance to favour implantation and pregnancy (Fu et al. 2013). Would it be possible that the TLR mediated inflammation of the uterus modify the activity of the NK cells and favour the rejection of trophoblasts and embryo?

From a clinical perspective, several pathologies are linked with the absence of a suitable decidua for the invasion and implantation of the embryo. For example, placenta accreta is a pregnancy complication in which due to the lack of endometrial decidua, the placental villi (invading trophoblasts) penetrate the myometrium and increase the size of the newly created blood vessels in the placental bed (Chantraine et al. 2012). In most of the cases this abnormal invasion only reach the myometrium but in severe complications the placental villi can reach the peritoneal cavity and even the bladder (Bauer & Bonanno 2009). Placenta accreta pregnancies can proceed with minor complications. It is during delivery when serious care is required as the blood vessels from the mother might not be able to dilate as they would in normal pregnancy, causing

severe haemorrhages (Khong 2008). In general, the establishment of the placental bed is a crucial moment for the development of pregnancy. An abnormal decidua might influence the placentation process and increase the development of conditions like pre-eclampsia (Pijnenborg 1998).

Based on our observations, inflammatory conditions might lead to complications related to decidualisation deficiencies. Current imaging techniques used for evaluation of the course of pregnancy like ultrasound and MRI could be included as a diagnostic tool. A good evaluation of the endometrium, from infections to tissue morphology might be required in patients with recurrent miscarriage history and even for management of patients undergoing IVF treatments.

7.4.2 Endometrial tissue remodelling

The cycling endometrium represents a challenge for our understanding of tissue healing, regeneration and inflammation. Every month we observe that the endometrium proliferates and regenerates the tissue forming new decidua, blood vessels and glands. Without fertilisation, the tissue will degenerate and the functional stromal and epithelial layers will shed. In this process, degradation of the tissue will produce cellular debris, ECM fragments, blood from the arteries and many pro-inflammatory mediators (Maybin & Critchley 2011). Immune cells like neutrophils will be present at the time to favour this tissue remodelling, clear the tissue from the debris and regulation of bleeding (Wilkens et al. 2013). Nevertheless, we would not find scars or formation of thick connective tissue as a result of this remodelling process. It might be possible that abnormal tissue regeneration could initiate inflammatory conditions by the release and recognition of damage associated molecular patterns (DAMPs). Molecules like ATP, fragments of ECM proteins like laminin, fibronectin or collagen, heat-shock protein 90 (HSP90), high-mobility group box 1 (HMGB1) are released in events like cell death, tissue remodelling and damage (Rosin & Okusa 2011). The innate immune system is able to recognise them through different PRRs and initiate immune responses to clear the damaged tissue.

In the last few years, a technique called “endometrial scratch” has been introduced to the IVF treatments. The procedure consists in performing a small biopsy in the fundus

of the endometrium at a menstrual cycle prior to the planned for embryo transfer (Barash et al. 2003). This procedure has reported a significant increase in the chances of achieving a clinical pregnancy after IVF (Nastri et al. 2013). So far the mechanisms that mediate the success of this procedure is unknown but presumably have to do with the increase in production of cytokines, growth factors and adhesive factors related to the healing of the wound. This idea is similar to the pathology of placenta accreta due to c-sections. It seems that the implanting blastocyst and in particular the invading trophoblasts prefer a tissue where a lesion has been previously found. In a c-section the amount of connective tissue available will increase. This tissue lacks of endometrial cells that will represent a barrier much difficult to traverse than the ECM of the scar, which they could easily degrade with enzymes like matrix metalloproteinases (MMPs) and facilitate their migration. It is possible that DAMPs release from the endometrium, promote sterile tissue inflammation that could be beneficial for trophoblasts and embryo implantation.

7.4.3 Cellular changes and activation of cell signalling pathways in response to TLR activation

One of the main findings that I would like to follow in further research is the observation of cellular morphology change in response to TLR stimulation. Whether this behaviour is particular of the endometrial cell or could happen to any epithelial or immune cell remains to be characterised. It is logical to think that this would interfere with the endometrial receptivity as it affects the cell membrane but could occur in any cell as a protective mechanism. In fact, the collaboration with María Yañez-Mo, where we performed a characterisation of the cell surface marker profile, allowed us to get a better insight to this topic. The localisation of membrane proteins that mediates the interaction of cytoskeleton with the cell membrane like the ezrin, radixin, moesin, (ERM) complex may be a possible way to explain the changes in cell morphology after TLR stimulation.

Understanding the activation pathway of the cell in response to the TLR stimulation is an important topic where more investigation is required, as currently there is no obvious explanation to our observations through the classical activation pathways.

7.5 Conclusion

The interaction of the maternal tract and the implanting embryo is an event surrounded by multiple factors. The influence of external events could easily interfere with this interaction and lead to failure. The endometrial tissue is highly plastic and undergoes constant remodelling. Evidences from this work indicate that it can be affected *in vivo* by TLR 2/6 stimulation, generate architectural and protein changes that will lead to embryo implantation failure. At the molecular level, the endometrial cells would respond to the TLR stimulation in a diversity of ways from changing membrane morphology to altering protein expression. Potentially these changes will favour the protection of the maternal tissue from infectious agents but will compromise the implantation of an embryo. These observations could be brought to the clinic and aid the diagnosis and therapy of infertile women.

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