ALTERATIONS IN ENDOMETRIAL IMMUNE CELL POPULATIONS AFFECT ENDOMETRIAL CELL FUNCTIONS

BY

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DISSERTATION

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Abstract

Endometriosis is a debilitating disease that is diagnosed in 0.5-5% fertile and 25-40% of infertile women, but the underlying mechanisms involved in progression of the disease as well as the associated infertility are yet unclear. Research now shows evidence that the immune system has a pivotal role in endometriosis development as well as the related infertility. Among other immune cells, the T-helper lymphocytes accumulate in the normal endometrium during the mid- to late-secretory phase of the menstrual cycle and are considered essential for endometrial receptivity. The goal of this project was to 1) study the relationship between anti-inflammatory T-regulatory (Treg) and pro-inflammatory T-helper-17 (Th17) lymphocytes in the eutopic endometrium of patients with the primary complaint of infertility; 2) explore the involvement of interleukin-17 (IL-17) in the promotion of a pro-inflammatory environment and the consequences of this altered microenvironment on eutopic endometrial and ectopic endometriotic cells; and 3) characterize EMMPRIN expression in the eutopic endometrium in women with endometriosis-related infertility and its correlation to elevated IL-17 expression.

Endometrial biopsy samples collected from patients during the mid- to late-secretory phase of their menstrual cycles were evaluated for Treg and Th17 lymphocyte subsets and the Th17 specific cytokine, IL-17 expression. These data were compared to the fertility status in these patients. Overall, Treg cell counts were higher and Th17 cell counts were lower in patients who conceived compared to those that did not get pregnant. Conversely, patients who maintained their infertile status had a lower Treg cell count and higher Th17 cell count in their eutopic endometrium. The ratio of Treg:Th17 cell counts was significantly correlated to their fertility status. Patients with a ratio less than 3 failed to conceive in spite of medical or surgical intervention. Laparoscopic intervention for ectopic lesion excision had a boosting effect on the

endometrial Treg cell population which was in turn correlated to a positive pregnancy outcome. The IL-17 expression was elevated in both the glandular and stromal compartments of the endometrium in patients with a low Treg:high Th17 cell ratio.

IL-17 is associated with various inflammatory conditions including endometriosis. IL-17 treatment did not have any effect on cell proliferation in endometrial or endometriotic cell type. But IL-17 did positively affect cell migration and invasion in the endometriotic cells, though not in the endometrial cells. To understand this differential effect we assessed IL-17 receptor (IL-17R) expression in both cell types and observed that the receptor expression was fairly similar in both cell types. Thus we concluded that the differential effect was probably due to specific interaction processes of IL-17 with different cell types or another co-receptor expressed specifically on the endometriotic cells could mediate this effect. The inflammatory NFκB pathway is the hallmark of IL-17 activity, but our results showed that in our uterine cell lines, IL-17 did not induce the NFκB pathway. Instead IL-17 activated an alternate MAPK signal transduction pathway, but only in the endometriotic 12Z cells.

Because it is known that cell motility requires MMP induction, we also evaluated changes in EMMPRIN expression in these cells when treated with IL-17. Endometriotic cells showed a transient increase in EMMPRIN expression post treatment which could partially explain their enhanced motility in the presence of IL-17. We also assessed the correlation between IL-17 and EMMPRIN expression in the eutopic endometrial samples from patients with endometriosis. We found that IL-17 expression positively correlated with EMMPRIN expression in about 90% of the samples tested.

Our experiments using IL-17 may reveal the mechanisms involved with the creation of a pro-inflammatory environment and its consequence on eutopic endometrial cells as well as

processes involved in the establishment of ectopic endometriotic lesions. We believe this project addresses questions that will greatly increase our understanding of how a specific immune cell niche may regulate endometrial receptivity. Moreover, a better understanding of the mechanisms of Epithelial to Mesenchymal Transition will aid research in the field of endometriosis as well as cancer.

DEDICATION

This dissertation is dedicated to my best friend, my husband, Swanand Sathe, for always standing by me, in the good times and bad, as I made my way through this research program.

This dissertation is also dedicated to my mother, Sudha Adur, and father, Kishore Adur, who have always put their children before themselves, and given me and my siblings everything possible to succeed in our personal and professional lives.

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"It takes a village to raise a child"

- African proverb

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CHAPTER 1

INTRODUCTION

Endometrial receptivity is a complex phenomenon dependent upon the interaction and precise synchronization of multiple factors that control structural changes in the endometrial lining in response to changes in circulating steroid hormones(1). Previous studies have suggested that a delicate and stage-specific leukocyte and cytokine equilibrium is also involved in the control of uterine receptivity. It is therefore important to understand the immunological factors involved in implantation and early pregnancy, particularly in reproductive pathologies that may result in infertility. One such example is the establishment of endometriosis and subsequent infertility.

Endometriosis is a debilitating inflammatory disease that is diagnosed in 5-10% of women of reproductive age. In addition to causing chronic pelvic pain, up to 30-50% of women with endometriosis are infertile (2). The pathogenesis of endometriosis is considered to be multifactorial, but the actual underlying mechanisms are as yet unclear. Research now shows evidence that the immune system plays a pivotal role in the development of endometriosis as well as the related subfertility. In patients with endometriosis, immune responses are altered in favor of the survival and establishment of endometriotic tissue in extra-uterine locations (3). A key feature of endometriosis is inflammation, which involves an overproduction of cytokines, chemokines, prostaglandins and metalloproteinases. Peritoneal immune cells such as T-helper cells secrete IL-17, one such inflammatory factor, which possibly facilitates the adhesion, invasion, and proliferation of endometriotic cells and the progression of endometriosis.

Evidence suggests that the increased concentrations of certain growth factors and/or cytokines in the peritoneal fluid of patients with endometriosis induce establishment and proliferation of the ectopic endometriotic implants, and also may inhibit early reproductive events. It has been proposed that both pro-inflammatory and immune tolerance factors co-exist at the maternal-fetal interface in a normal pregnancy and an imbalance between these anti- and pro-inflammatory factors leading to immune dysregulation in the reproductive tract of women with endometriosis may have a profound negative impact on the outcome of implantation and pregnancy. Hence our hypothesis was that the presence of endometriotic lesions promotes a pro-inflammatory environment leading to shifts in the T-helper cell profiles and related cytokine milieu in the eutopic endometrium during the window of implantation (WOI). To test this hypothesis, our objectives were:

1. to characterize alterations from an anti-inflammatory lymphocyte phenotype toward a pro-inflammatory phenotype in the eutopic endometrium of patients with infertility.
2. to explore the mechanisms involved in the promotion of a pro-inflammatory environment and the consequences of this altered microenvironment on eutopic endometrial cells and on the processes involved in the establishment of ectopic endometriotic lesions. We conducted *in vitro* experiments using recombinant human IL-17 (recHuIL-17) to evaluate effects on cell migration, epithelial to mesenchymal transition, and cell signaling.
3. to characterize EMMPRIN expression in the eutopic endometrium in women with endometriosis-related infertility. We also evaluated the effect of recHuIL-17 on EMMPRIN expression in endometrial and endometriotic cells and the signaling pathway involved.

Hence the overall goal of this project was to gain a better understanding of how a specific immune cell niche might regulate eutopic endometrial and endometriotic cell function. In chapter 2, an overview of endometrial receptivity, immune factors involved in its pathophysiology, endometriosis and its pathogenesis, various factors and signaling pathways involved in its establishment and its effect on endometrial receptivity are presented. Chapter 3 presents *ex vivo* data demonstrating an alteration in the T-cell profiles of the eutopic endometrium in women with endometriosis and its correlation to subfertility in these women. T-regulatory cells accumulate in the normal endometrium during the mid- to late-secretory phase of the menstrual cycle and are considered essential for uterine receptivity. To this end we have characterized and quantified the eutopic endometrial expression of T-regulatory cells, T-helper 17 cells, ratio of Treg to Th17 cells and expression of the cytokine Interleukin 17 in fertile and infertile women with endometriosis. These data were then compared to patient fertility history to evaluate these endpoints as possible uterine receptivity biomarkers. Chapter 4 presents our findings on the effects of recHuIL-17 on endometrial and endometriotic cell function *in vitro*. Elevated levels of IL-17 are known to trigger signaling pathways that augment the pro-inflammatory milieu systemically as well as in the local environment. We have evaluated the effect of recHuIL-17 treatment on immortalized eutopic endometrial and ectopic endometriotic cell lines to assess changes in cell proliferation, migration and invasion, and activation of signaling pathways. An IL-17 enriched local environment is known to support peritoneal inflammation and adhesions. EMMPRIN is another factor known to be involved in matrix remodeling, metalloproteinase production, inflammation and EMT, processes known to affect embryo implantation and establishment of metastatic implants. We evaluated the correlation between IL-17 and EMMPRIN protein expression in the eutopic endometrium of women with endometriosis related

subfertility and the effect of recHuIL-17 treatment on EMMPRIN expression in endometrial and endometriotic cell lines to determine changes in EMMPRIN expression in these cells. Chapter 5 presents a summary of our findings and future directions in this area of research. Chapter 6 includes detailed information on the materials and techniques used in our studies.

FIGURE

Overall Model for Specific Aims

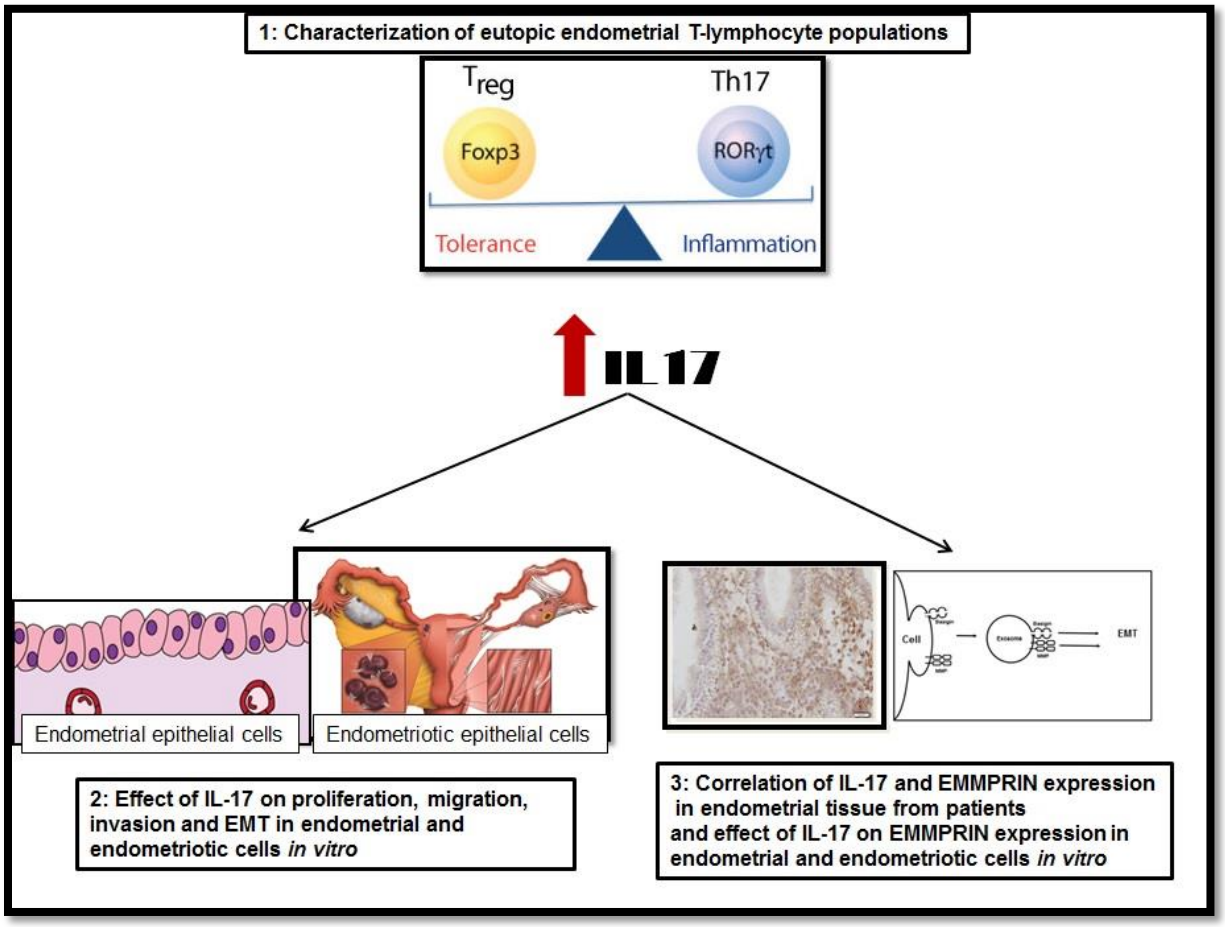

Figure 1.1: Overall model presents the three objectives of this study which aim to evaluate the altered endometrial immune cell status and its effects on endometrial and endometriotic cell function *in vitro* as well as its relation to EMMPRIN activity.

CHAPTER 2

LITERATURE REVIEW

ENDOMETRIAL RECEPTIVITY

The two main components of the uterus, the endometrium and the myometrium, work in perfect coordination to allow an embryo to attach, develop during the course of the pregnancy into a mature fetus and be expulsed during parturition. Our focus is on the endometrial tissue which is composed of glandular and luminal epithelium, stromal fibroblasts, cells associated with blood vessels and subpopulations of leukocytes, whose numbers and phenotypes vary with menstrual cycle stage (4). As is commonly known, the endometrium undergoes specific changes during the menstrual cycle under the influence of sex steroid hormones and other key factors that are especially evident during the mid- to late-secretory phase of the cycle when the endometrium prepares for the advent of the embryo. Major changes occur in the endometrial epithelium associated with the window of implantation. These include increases in secretory activity of the glands while the luminal epithelium undergoes synchronized modifications including the loss of specific inhibitory components that could prevent blastocyst attachment, the simultaneous acquisition of adhesion ligands and microvilli known as pinopods as well as alterations in its junctional integrity to allow blastocyst attachment and invasion. The stromal cells undergo a transformation commonly known as the predecidual response, which uniquely in women, occurs independently of the presence of the embryo. A substantial increase in the numbers of leukocytes and the development of long, coiled spiral arteries are also critically important as these subsequently form the decidual compartment of the placenta (152,153).

Involvement of Sex Steroids:

Sex steroid hormones are the most prominent players in this dynamic process making the uterus capable of supporting pregnancy when exposed to a suitable balance and appropriate sequence of estrogen and progesterone during the menstrual cycle. Progesterone is the hormone known to be absolutely essential for establishment and maintenance of pregnancy by virtue of its genomic activity via the progesterone receptors PRA and PRB. Some of the effects of progesterone include an anti-estrogen effect through down-regulation of the estrogen receptor (5), controlling the expression of cell cycle progression and growth factors as well as their receptors, and simultaneously repressing cell cycle arrest proteins (6), induction of specific marker expression such as pinopods on the endometrial surface (7) and osteopontin (ligand believed to bind integrin receptors (8)) on the trophoblast side of the maternal-fetal interface. Progesterone also acts via non-genomic pathways but the physiological and/or pathological relevance of this action in the uterus is yet largely unknown (6). Several studies have also established the critical role played by progesterone in the process of decidualization of endometrial stromal cells.

The Decidua:

The crosstalk between the sex steroid hormones, estrogen and progesterone, and cAMP (9) regulates morphological and biochemical changes in the stromal compartment of the endometrium during this key phenotypic transition. The stromal cells are transformed during this process from elongated fibroblast-like cells to enlarged epithelioid-like cells with polyploid nuclei. They also synthesize new cellular products of the extracellular matrix, hormones and peptides, cytokines and chemokines, growth factors and matrix metalloproteinases that all aid in

this remodeling. Additional changes in the decidua include accumulation of glycogen, lipids and proteins that serve as nutrient medium to support embryo growth until the placenta is completely developed and takes over in early pregnancy (10). The decidua thus plays an important role in the remodeling of the materno-fetal interface, wherein it is proposed to "encapsulate" the developing embryo thus keeping trophoblast invasion in check and protecting the maternal tissues from excessive invasion (11). In addition to this complex interaction with embryonic trophoblast cells, human decidual cells secrete cytokines and chemokines that attract peripheral immune cells to the maternal decidua thus orchestrating local immunomodulation that supports placental as well as embryonic development. Progesterone and its receptor also play a key role in the localized suppression of the maternal immune system and control of cytokine cascades that are the prime mediators of the dialogue at the maternal-fetal interface.

Thus the endometrium may be viewed as a guardian, allowing the embryo to attach and invade under a specific set of precisely controlled molecular and cellular events, coordinated by autocrine, paracrine and endocrine factors that are vital to endometrial receptivity during the putative window of implantation (WOI). The overall data in the literature strongly suggest that misregulation of these WOI factors leads to an inhospitable environment for the embryo and one in which implantation often fails.

UTERINE MUCOSAL IMMUNITY

The uterine mucosa needs to be prepared to respond to antigenic challenges, just as the other mucosal surfaces in our body such as the intestinal, bronchial, salivary, mammary, nasal- and ocular-associated glandular tissues. Although these mucosal immune systems share structural and functional similarities, the human uterus is unique, as it undergoes cyclical

proliferation and shedding under the control of sex steroid hormones (9, 154). These hormones along with a number of other factors have considerable effect on both the afferent and efferent immune events in the gravid as well as non-gravid uterus (13). As a result, the leukocyte sub-populations within the endometrium vary considerably across the menstrual cycle, with maximal numbers associated with the processes of implantation and also menstruation (152).

Uterine Mucosal Leukocytes:

In the normal physiological setting the populations of dendritic cells, uNK and specific subsets of T-lymphocyte cells increase at the implantation site. These cells maintain a local immunosuppressive environment in the uterus so that the semi-allogeneic embryo is protected from the maternal immune system and allowed to attach. Moreover, they also interact with the invading trophoblast cells to direct as well as limit their invasion into the endometrium (14). During this receptive phase, these specific populations of leukocytes constitute approximately 20-40% of all endometrial cells, and hence it has been proposed that the sub-types of cells trafficking to the endometrium are likely to be critical for endometrial receptivity (14).

T-helper Lymphocytes:

Among these key immune cells are the T-lymphocytes, identified on the basis of their expression of surface CD4 and T-cell receptor (TCR) molecules. These cells account for a significant 5-10% of the endometrial leukocyte population in both the cyclic and pregnant endometrium of all species investigated so far (154). These T-lymphocytes, also known as T-helper (Th) cells can be classified into Th1 cells that are involved in cellular immunity, Th2 cells that are involved in humoral immunity, Th17 cells that produce pro-inflammatory cytokines and

are responsible for induction of inflammation and Treg cells which play central roles in immunoregulation and induction of tolerance (15). The Th1 / Th2 hypothesis originally provided the rationale to explain immune regulation during pregnancy, but was found incomplete when Th2-dominant immunity, observed to support pregnancy, was also reported in women with recurrent abortion as well as in implantation failure. Thus the pregnancy paradigm was expanded to include the Th1/Th2/Th17 and Treg cell model (Figure 2.1).

Balance between Treg and Th17 lymphocytes:

The Treg cells are especially critical for localized immunosuppression at the implantation site as they are capable of suppressing other immune responses in vitro and in vivo. There are two main distinct lineages of Treg lymphocytes: nTregs that originate in the thymus and iTregs that are induced in the peripheral circulation by activation of naïve T cells in the presence of TGFβ. Both these types of Treg cells have T-cell receptors specific for self-antigens and are distinguished from other CD4 cells by their surface expression of CD25 and the unique use and expression of a transcription factor known as Forkhead Box P3 or FoxP3 (155). FoxP3 is now an established marker for Treg cells in mice and humans and is shown to be present in the endometrium of pregnancy (16). Various studies and reviews have confirmed that Treg lymphocyte populations are increased in the peripheral circulation (16,17) as well as the decidua (18–20) during early pregnancy, showing a stable and highly suppressive phenotype and hence are believed to be critical for embryo tolerance, invasion and establishment of pregnancy (21,22).

However, it has also been proposed that the balance between Treg cells and their reciprocal cell types should be considered when evaluating immune status, especially since the presence of Treg cells and their reciprocal subset known as Th17 cells are inversely related to

each other in the decidua (18). These Th17 lymphocytes express the retinoid-related orphan receptor-γt (RORγt), secrete large quantities of the cytokine IL-17 and are directly involved in neutrophil-mediated inflammatory processes (155). Because the Th17 cells are involved in inflammatory processes, it would be best that the proportion of Th17 cells should be reduced during pregnancy to prevent embryonic rejection. However, Nakashima et.al, (23) showed that the Th17 cell population remains stable in the peripheral circulation as well as decidua of pregnant women as compared to non-pregnant women, throughout the entire duration of pregnancy. This continuing presence can be explained by the fact that these cells are required to prevent in-utero infections and so are not removed from the uterus completely.

Treg and Th17 cells share a common lineage with their relative abundance determined by the cytokine environment in which naïve T-cell differentiation occurs. The key to preferential differentiation of naïve T cells to the Th17 phenotype rather than the Treg phenotype is the presence of the pro-inflammatory cytokine IL-6 in addition to TGFβ (which on its own induces Treg differentiation). It has therefore been proposed that the Treg and Th17 cell subsets, with their common developmental pathways but opposite effects, may have evolved to regulate tissue inflammation (24). The net result is that Th17-dominated tissues undergo further immune-mediated tissue damage, precipitating towards a chronic pro-inflammatory milieu. This delicate balance between the differing T-cell populations further substantiates the functional significance of T-lymphocytes in the uterine environment and their very probable association with pathologies of pregnancy.

Role of T-helper lymphocytes in uterine pathology:

Extensive research in the field of reproductive immunology has now linked these aberrant T-cell population phenotypes with the occurrence of various conditions such as recurrent pregnancy loss (RPL), miscarriage (RM) or spontaneous abortions (RSA) as well as preeclampsia. Women who suffer from RPL/RM/RSA have been shown to have reduced peripheral levels of CD4+CD25+Tregs (20,24–26) and significantly increased levels of Th17 cells (20,24,25,27,28) as well as increased ratios of Th17/Treg cells (24,25) when compared to normal fertile controls. Similar phenotypes were also observed in the decidual tissues of these patients (20,27). Women with preeclampsia were also observed to have decreased numbers of peripheral as well as decidual Treg cells (29). Decreases in the endometrial Treg phenotype have also been implicated as a cause for unexplained implantation failure (30).

All this evidence supports the hypothesis that the absolute numbers as well as relative proportions of T-lymphocytes found in the uterus and peripheral circulation are one of the major determining factors for embryo acceptance or rejection.

CYTOKINES

Cytokines are small glycoproteins critical for the normal functioning of the immune system. They are involved in the growth, development and activation of immune cells and also mediate crucial inflammatory responses via specific cell-surface receptors. Every cell in the body is exposed to specific combinations of cytokines at a given time, sometimes with similar functions, resulting in a synergistic or antagonistic interaction, the overall consequence of which influences the ultimate effect on the cell. These potent molecules induce a cascade of biochemical events within the cell leading to various outcomes such as alteration in cellular

proliferation, apoptosis, differentiation, motility, extracellular matrix deposition or breakdown and/or release of other cytokines. By virtue of these multiple effects, these molecules are receiving increasing attention in regards to their role in the uterus, particularly during embryonic implantation.

Uterine cytokines:

Cytokines are among the first and most abundant molecules produced locally and secreted by the endometrium during the receptive phase of the menstrual cycle. A major source of cytokines is the subsets of leukocytes present in the uterus at this time. Cytokines are often grouped based on the type of leukocytes they are produced by. Since the different types of leukocytes produce unique profiles of cytokines that induce distinct responses, the cytokines are often grouped depending on their ability to promote or inhibit an inflammatory response. These proteins, due to their small radius of activity, dictate cellular functions by autocrine or paracrine effects, thus promoting a unique immune environment essential to successful implantation. Not only do cytokines play a part in the selective recruitment of specific leukocytes to the endometrium, but also act as potent intercellular signals regulating adhesive properties of the endometrial epithelium and trophoblast, spiral arteriole remodeling, blastocyst attachment and invasion as well as trophoblast proliferation and differentiation (28–31,152). Several cytokines known to influence leukocyte maturation, function and consequent inflammation, are also expressed by non-immune cells in the endometrial tissue such as endometrial stromal, epithelial, and decidual cells and trophoblast cells under the regulation of ovarian steroid hormones (31,35).

Cytokines and endometrial receptivity:

The role of inflammation in the endometrium, during the window of implantation, emphasizes the critical function of cytokines in regulating maternal receptivity as well as embryo implantation. Studies have compared the production and/or presence of these cytokines in the peripheral circulation as well as endometrium of fertile and infertile women, thus defining the specific role of cytokines in this process. Research also shows that cytokines, though necessary for implantation, also have the capacity to skew local immune environment through their influence on the recruitment, differentiation and expansion of specific lymphocyte populations at the implantation site. This function could possibly account for reproductive anomalies such as recurrent miscarriages, habitual abortions and/or implantation failure. The exact mechanism by which these abnormal immune factors cause these conditions is unknown, but there is strong evidence that it may involve modulations or an imbalance between the various cells of the immune system, especially the T-lymphocyte subgroups (as stated earlier in this review), as also by the cytokine secretion profile (36–38). Moreover, it has also been shown that patients who underwent ovarian stimulation during IVF cycles (versus natural cycles) had significantly higher pro-inflammatory cytokine profiles in their endometrial secretions and this was hypothesized to be the reason for an unreceptive intrauterine milieu (39).

INTERLEUKIN 17:

One such pro-inflammatory cytokine is Interleukin 17 (IL-17) which is mainly secreted by activated CD4+ Th17 cells but is also known to be produced by other cells of the innate and adaptive immune systems (40). IL-17 belongs to the IL-17 family of cytokines, which includes IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F.

Comparison of the human IL-17s with other species suggests that this family of cytokines is highly conserved across vertebrates (41). Although the cellular sources and expression patterns of the IL-17 cytokines is different across species, they all have pro-inflammatory functions. All the members of this family are similarly sized secreted proteins that are expressed as homo- or heterodimers. Although IL-17 has an important role in maintaining mucosal barrier integrity during homeostasis, its dysregulation mediated inflammation can be destructive to tissues (42). This cytokine has been classified as a pro-inflammatory cytokine because of its striking ability to act on a broad range of cell types to induce expression of many inflammatory mediators such as cytokines, chemokines and metalloproteinases (40). IL-17 induces production of these pro-inflammatory cytokines which then either forms a positive feedback loop (as seen with IL-6 that is needed for Th17 differentiation) or synergizes with them (as seen with TNFα and IL-1β) to induce a larger amount of inflammatory factors (43). IL-17A/IL-17 in particular has a more important role in driving autoimmunity than the other family members. Extensive reviews by Moseley et.al, (44) and Witowski et.al (45) among others, discuss the association of increased levels of IL-17 with various conditions including rheumatoid arthritis, airway inflammation, inflammatory bowel disease, intraperitoneal abscesses and adhesions as well as allograft rejections.

The IL-17 Receptor:

The IL-17 receptor family comprises five receptor subunits, IL-17RA – IL-17RE (46). The receptor for IL-17A/IL-17 (IL-17RA/IL-17R) is a single-pass transmembrane protein of approximately 130kDa (44). Though the IL-17 cytokine is expressed by activated T-cells, its receptor is expressed ubiquitously on all nucleated cells. Fibroblasts, epithelial cells and

endothelial cells express both IL-17RA and IL-17RC, whereas T cells express IL-17RA but not the other receptors (42). The IL-17 receptor is not related to any of the other known cytokine receptors and in spite of its relatively large size it does not possess similarity to any other known protein (41). The receptor is a long transmembrane protein with an N-terminal signal peptide followed by a fibronectin III-like extracellular domain, a short transmembrane domain and an unusually long cytoplasmic SEF/IL-17R (SEFIR) domain (45,46). Early studies have shown that the affinity of IL-17R for its ligand, IL-17, is lower than that needed to mediate responses, which indicates the need for an additional subunit for binding the ligand and/or eliciting signaling. Since it is used by multiple members of the IL-17 family, IL-17R could also have a shared cytokine receptor subunit, such as the gp130, which is a common signal transducer for the IL-6 family of cytokines (46). The binding of its ligand to the IL-17R activates the NFκB, a hallmark transcription factor associated with a highly pro-inflammatory program of gene expression. It is also known to activate other common signaling pathways including MAPKs, JNK, p38 and ERK, PI3K and JAK/STATs (43).

SIGNALING PATHWAYS

Emerging knowledge about IL-17 and its effects on different organ systems has provided insight into the possible signaling pathways involved. The IL-17 family of receptors has unique structural features and mediates signaling events that are distinct from those induced by other adaptive immunity related cytokines. Studies show that IL-17 activates a highly pro-inflammatory program of gene expression via activation of NFκB and/or MAPK signal transduction pathways.

Nuclear Factor-κB Pathway:

NFκB is a hallmark transcription factor associated with induction of inflammation as it can modulate production of chemokines, adhesion molecules, and matrix metalloproteinases and can also induce prostaglandin synthesis enzymes such as COX-2, thus controlling the expression of many downstream pro-inflammatory molecules. IL-17 induces the NFκB signal transduction pathway by engagement of a heteromeric complex of receptors and subsequent recruitment of an essential adaptor known as Act1. This adaptor ubiquitinates the TRAF6 ligase which promotes activation of the inhibitor of NFκB kinase (IKK) complex and thus induces the classical/canonical NFκB signaling pathway via degradation of IκB and activation of the p50 and p65 subunits of this pathway (46–48). The involvement of this pathway in IL-17 signaling effects was conclusively proven by both in vitro (RNA-interference knockdown of Act1) as well as in vivo (Act1, IKK or TRAF6 deficient mice) studies. NFκB activity and hence its inflammatory consequences can be inhibited by stimulating synthesis of IκB, the molecule that restrains NFκB in the cytosol or by competitive nuclear receptor binding brought about by progesterone (49). However it is uncertain whether specific inhibition of only NFκB or its signaling factors would suffice for treating the inflammatory condition produced as a result of this pathway. Moreover, NFκB has some vital roles in normal immune responses and so it would be prudent to target post-transcriptional expression of specific pro-inflammatory molecules without blocking NFκB activity altogether.

Mitogen Activated Protein Kinase Pathway:

Another possible player to consider in the control of this complex interaction is that IL-17 can also induce Act1-dependent, TRAF6-independent signaling events via activation of the

Mitogen-activated protein kinase (MAPK) transduction pathway. MAPK pathways are among the most common signal transduction pathways and are involved in many physiological processes that regulate gene expression, mitosis, motility, cell survival or apoptosis, and differentiation(50). Though not completely understood, the effect produced by IL-17 is thought to be mediated through rapid and strong phosphorylation of extracellular signal-regulated kinases (ERK) which aids in controlling the stability of mRNA transcripts. MAPKs can thus increase mRNA transcript half-life, in turn increasing their concentration(46,47). Since many IL-17 targets are genes related to chemokine and cytokine synthesis, stabilization of such molecules can promote the inflammatory response induced by IL-17.

Role of signaling pathways in the uterus:

Both these signaling pathways (NFκB and MAPK) play key roles during the menstrual cycle too. King et.al, (51) have shown that premenstrual progesterone withdrawal induces the NFκB signaling pathway via upregulation of IKKβ mRNA which is involved with the inflammatory effects seen during the process of menstruation. They also showed that the IKKα mRNA, which is involved with morphogenesis signaling to NFκB pathway, is upregulated in the decidua under continually maintained progesterone levels thus supporting the differentiation taking place in the decidua during the pre-implantation stage. This differential regulation of the IKK subunits was shown to be due to their preferential phosphorylation by MAPK factors. Similarly, Murk et.al, (52) showed a menstrual cycle dependent involvement of the MAPK pathway in the normal menstrual cycle, with sex steroid hormone regulated ERK phosphorylation thus implying its possible role in decidualization, glandular differentiation and cell survival. Due to this very specific and tightly defined involvement of these signaling

pathways in the normal menstrual cycle as well as the preimplantation priming of the endometrium, it would be reasonable to suppose that any dysregulation in these pathways would possibly lead to an aberrant and unresponsive endometrium and consequent pregnancy failure as has been observed in patients with elevated IL-17 levels.

ENDOMETRIOSIS

One of the most commonly encountered gynecological conditions in women of reproductive age; endometriosis is an estrogen-dependent disorder characterized by the presence of endometrial glandular and stromal cells outside the uterus, primarily on the pelvic peritoneum, ovaries, recto-vaginal pouch and in some cases in extra-pelvic regions such as the chest wall, lungs, bone and brain. Endometriosis is a very poorly understood and extremely debilitating benign gynecological condition, causing chronic pelvic pain, dysmenorrhea with or without abnormal bleeding and infertility (53–55), and accounts for up to 40% of infertility cases (56). Though the pathogenesis of this condition is not yet definitive, many theories have been proposed to explain the establishment of the disease. Among the most popular is the "retrograde menstruation theory" proposed by Sampson (1927) that states the sloughing of eutopic endometrium through patent fallopian ducts into the peritoneal cavity during menstruation is the source of endometriotic tissue in ectopic regions. This sequence of events has been shown to be influenced by factors such as impaired antegrade menstruation due to congenital or postnatally developed lower tract obstruction, alteration in the eutopic endometrial as well as peritoneal environment to support establishment of lesions and possible genetic changes of the cells at the implantation site to promote survival. It has been proposed that a defective immunosurveillance in these women may support attachment and persistence of these ectopic lesions (57). Hence

multiple factors work together to increase the propensity of the endometrial tissue to attach and implant in the ectopic locations in these women.

Role of Inflammation in Endometriosis:

Although a benign disorder, the process by which endometrial cells attach and invade ectopic surfaces is similar to malignancies. Studies show that endometrial cells will implant only on surfaces that have some amount of mesothelial cell injury. Though it is yet unknown what could be the cause for this damage, it is accepted that inflammation is a key feature of endometriotic tissue. This is seen as an overproduction of pro-inflammatory factors such as matrix metalloproteinases, prostaglandins, cytokines and chemokines by these ectopic lesions as well as the peritoneal lining which supports their survival and progress. Pro-inflammatory leukocytes and associated cytokines and growth factor levels are elevated in the peritoneal fluid (58–61) as well as serum (62) in women with endometriosis. Moreover, Gonzalez-Ramos et.al, (63) showed constitutive NFκB activation in peritoneal endometriosis in women and further discuss the significant role played by this inflammatory signaling pathway in the initiation and progression of endometriosis (64). This is also supported by previous studies in which inhibitors of the NFκB pathway caused a significant reduction in xenograft development in the nude mouse model (65) as well as the rat model (66) of endometriosis.

Role of the "Epithelial to Mesenchymal Transition" Process in Endometriosis:

The presence of these inflammatory factors is thought to enhance the establishment of ectopic lesions via Epithelial to Mesenchymal Transition (EMT). During this process an epithelial cell undergoes multiple biochemical changes that then help it assume a mesenchymal

cell phenotype, which gives the cell an enhanced migratory capacity, invasiveness, and increased resistance to apoptosis (67). This process of endometrial cell attachment and transmesothelial invasion is observed to be rapid and dynamic in vitro (68) and hence difficult to be witnessed under in vivo conditions. But studies, with primary tissue samples from women with endometriosis, show a distinct difference in the expression of key EMT markers between endometrial and endometriotic tissue, thus confirming the role played by EMT in lesion establishment. Matsuzaki and Darcha,(69) observed that samples from initial stages of endometriotic lesions such as red peritoneal endometriosis as well as ovarian endometrioma expressed lower epithelial and higher mesenchymal marker levels as compared to menstrual endometrium. They further showed that chronic lesions such as black peritoneal and deep infiltrating endometriosis expressed higher epithelial marker levels than menstrual endometrium, red lesions or ovarian endometrioma, but also retained some mesenchymal marker expression. This is supported by Bartley et.al,(70) who showed an increased mesenchymal marker expression in endometriotic tissue as compared to endometrium with respect to both the gene and protein expression.

EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER:

Extracellular Matrix Metalloproteinase Inducer (EMMPRIN), also known as Basic immunoglobulin superfamily (Basigin) or Cluster of Differentiation147 (CD147) is a widely expressed transmembrane glycoprotein that belongs to the immunoglobulin superfamily (71). It was first described as a factor made by tumor cells that stimulated production of matrix metalloproteinases (72) but is now known to be expressed on normal cells as well, including hematopoietic, endothelial, epithelial cells and leukocytes. EMMPRIN is a highly conserved

protein in several species and is expressed in both embryonic and adult tissues (73,74). Four different isoforms of EMMPRIN have been identified. Isoform 1 is a retina specific molecule (75) and has three Ig-like domains. The more ubiquitous isoform 2 is expressed in most tumor and fibroblast cells (76) and is also the only secreted form (77). Isoforms 3 and 4 are less abundant and were identified in human endometrial stromal cells and cervical carcinoma cells. The human EMMPRIN protein is 269 amino acid-long with a predicted molecular mass of 28kDa and the intact glycoprotein has a molecular weight that ranges from 43kDa to 66kDa (72,74). This wide molecular weight range is explained by the differentially glycosylated extracellular N-terminal which consists of two immunoglobulin-like domains (Fig. 4) (71,78). The MMP-inducing function of EMMPRIN depends on the level of glycosylation of this N-terminal Ig domain. Furthermore, the highly glycosylated form of EMMPRIN is increased on exposure to inflammatory signals (79,80). This MMP-inducing property of EMMPRIN plays a significant role in various processes such as wound healing, tumor invasion and metastasis (73).

Role of EMMPRIN in Reproductive Physiology and Pathology:

EMMPRIN also plays an important role in reproductive processes such as menstruation and embryo implantation among others. EMMPRIN is expressed in the mouse uterus during the peri-implantation period (81) and plays an important role in reproduction, as it is needed for normal uterine stromal cell proliferation and decidualization in mice (82), embryo implantation (83) and null mice (both male and female) are sterile (84). EMMPRIN is also important for human reproduction as it is known to be expressed in the human endometrium during the menstrual cycle (85) and is involved with endometrial remodeling (86). The mechanisms of EMMPRIN regulation are not completely understood but studies have shown that ovarian steroid

hormones regulate EMMPRIN expression in the endometrium (86,87). EMMPRIN expression is highest in the epithelial cells throughout the proliferative phase of the menstrual cycle when estradiol levels are high. During the secretory phase, EMMPRIN expression is stronger in the stromal compartment of the endometrium but weaker in the glandular and luminal epithelium and thought to be under the influence of the elevated progesterone levels (86,88). This increase in EMMPRIN expression is correlated to the increase in MMPs that play a significant role in endometrial tissue breakdown during menses. Besides being regulated by steroid hormones, EMMPRIN expression is also regulated by certain cytokines (89) and in turn can induce expression of cytokines and chemokines (90). Expression of EMMPRIN is also seen to be elevated in ectopic endometriotic tissue throughout the menstrual cycle (86). It is hypothesized that this could be involved in stimulating MMP production at the site of endometriotic tissue invasion into the peritoneal wall or any other abdominal organ. The baboon endometriosis model has also shown an increased EMMPRIN expression in the eutopic as well as ectopic endometrial tissue especially in the animals with earlier stages of endometriosis (88). Elevated EMMPRIN expression in ectopic lesions is also correlated with cell survival and migration (91).

ROLE OF IMMUNE FACTORS IN ENDOMETRIOSIS RELATED INFERTILITY:

The invasive process of endometriotic lesion establishment is supported by pro-inflammatory leukocytes and their associated cytokines, which contribute to the development of endometriosis and also maintain a subclinical inflammatory environment in the peritoneal cavity (as mentioned earlier in this review). This further exerts a pathological effect on the eutopic endometrium especially during the window of implantation (92), leading to alterations in normal function such as impaired decidualization (93–95), which could account for the unexplained

infertility and recurrent pregnancy loss seen in women with minimal and mild endometriosis (14). This subclinical peritoneal inflammation can also influence the population of leukocytes in the eutopic endometrium especially the sub-populations of T-helper lymphocytes. As discussed earlier in this review, an imbalance in the T-lymphocytes towards a pro-inflammatory phenotype with an increase in Th17 cells and decrease in Treg cells is associated with pregnancy failure. A similar phenotype has been shown in the peritoneal environment of women with endometriosis, but has not been studied in the eutopic endometrium of women with endometriosis related infertility.

Moreover, the peritoneal IL-17 profiles are elevated in women with endometriosis (96), but the eutopic endometrial expression has not been characterized in women with endometriosis, and neither its effect on eutopic endometrial function. Though Pongcharoen et.al, (97) have proposed that the IL-17 cytokine plays an important role in angiogenesis and hence for establishment of pregnancy, it has also been shown that decreased ratios of serum Treg/Th17-related-cytokines may play a role in the pathogenesis of defects resulting in implantation failure (98). This was supported by Wang et.al. (28), who detected significantly elevated IL-17 cytokine levels in both peripheral blood and decidua of unexplained RSA patients. Soluble IL-17 levels from cell culture supernatants were significantly higher in patients with unexplained recurrent spontaneous abortions as compared to normal early pregnancy levels (24). Furthermore, Liu et.al, (24) suggested that cells other than the lymphocytes at the maternal-fetal interface, specifically decidualized stromal cells and glandular epithelial cells could also express and secrete IL-17 leading to a localized pro-inflammatory environment in the endometrium. Thus an overproduction of IL-17 may be involved in aggravation of inflammatory responses in the

endometrium and the consequent unreceptive milieu, making it an important factor for further exploration and a potential target for therapeutic intervention in cases of failed pregnancy.

FIGURES

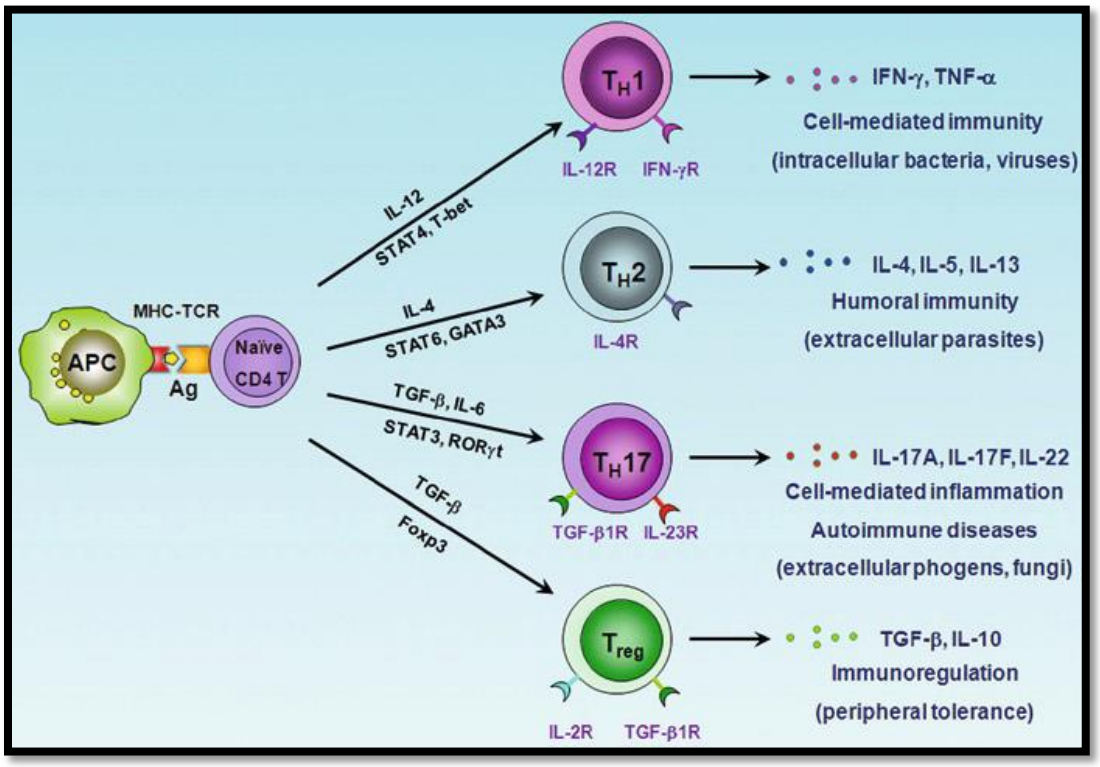

Figure 2.1: Th1/Th2/Th17/Treg Model

The T-lymphocytes, also known as T-helper (Th) cells are classified into Th1 cells that are involved in cellular immunity, Th2 cells that are involved in humoral immunity, Th17 cells that produce pro-inflammatory cytokines and are responsible for induction of inflammation and Treg cells which play central roles in immunoregulation and induction of tolerance. Adapted from review article (99).

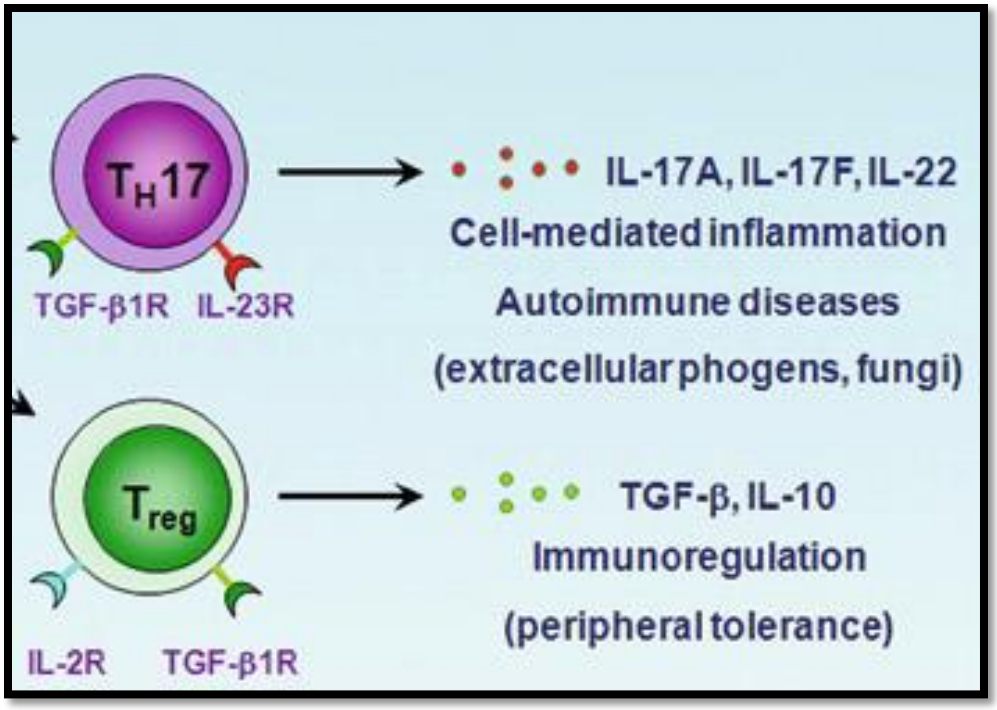

Figure 2.2: Cytokine secretion profiles for T-lymphocyte sub-populations

Th17 cells produce pro-inflammatory cytokines that in turn induce production of other inflammatory factors that play a role in host defense mechanism (physiological effect) and autoimmunity (pathological effect). Treg cells produce anti-inflammatory cytokines that play important roles in peripheral and local immune tolerance. Adapted from review article (99).

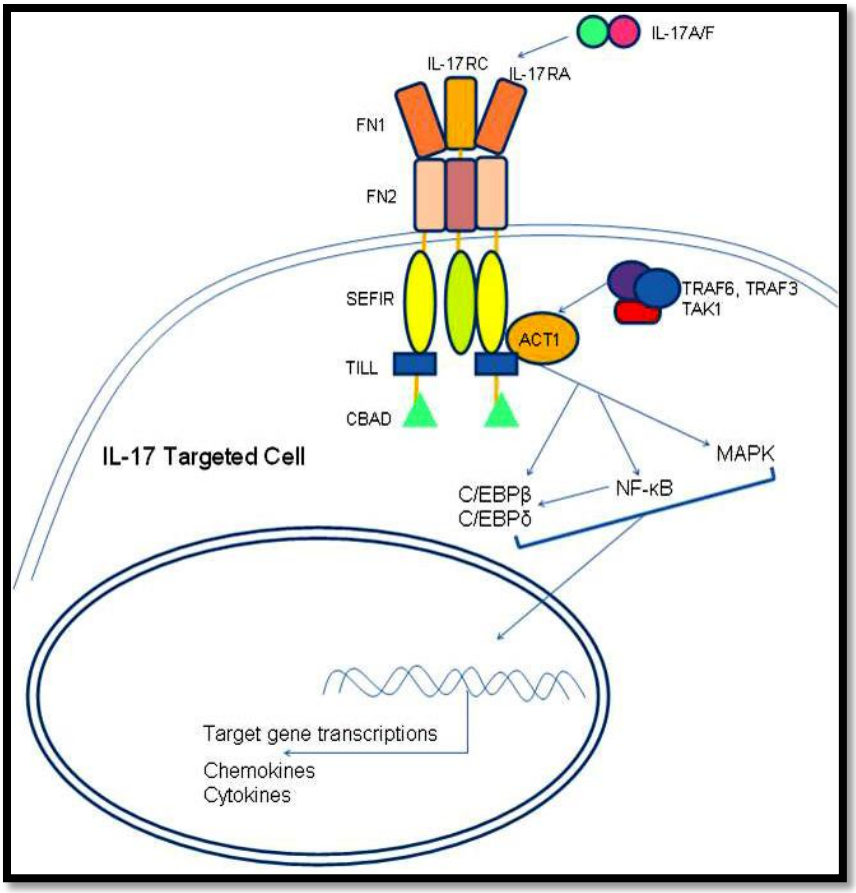

Figure 2.3: IL-17 signaling pathway

IL-17 activates a highly pro-inflammatory program of gene expression via activation of NFκB and/or MAPK signal transduction pathways.

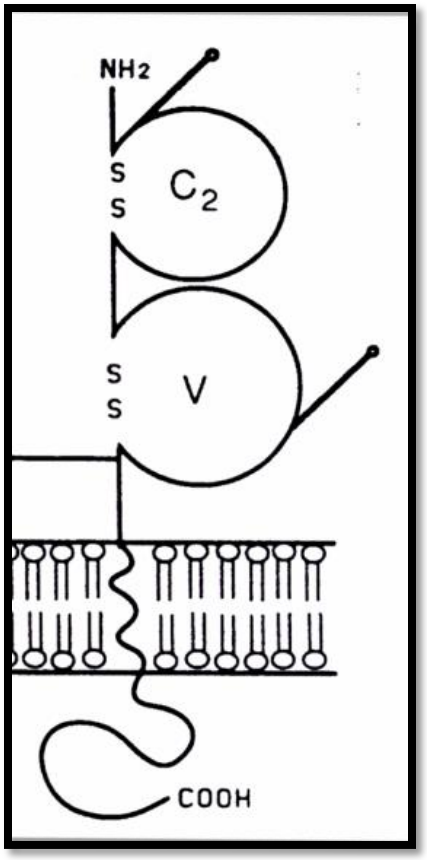

Figure 2.4: Structure of EMMPRIN

EMMPRIN is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. It has a wide molecular weight range which is explained by the differentially glycosylated extracellular N-terminal which consists of two immunoglobulin-like domains. (Image courtesy of Dr. Braundmeier).

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CHAPTER 3

THE EUTOPIC ENDOMETRIAL T-HELPER CELL PROFILE IN WOMEN WITH ENDOMETRIOSIS RELATED INFERTILITY IS PRO-INFLAMMATORY RATHER THAN THE CHARACTERISTIC ANTI-INFLAMMATORY PROFILE OBSERVED NORMALLY DURING THE WINDOW OF IMPLANTATION

ABSTRACT

The endometrium, under the influence of specific set of precisely controlled molecular and cellular events, allows the semi-allogeneic embryo to attach and invade during the putative window of implantation (WOI). Of the many factors controlling endometrial receptivity, T-regulatory cells are one subset of T-helper lymphocytes that accumulate in the normal endometrium during the mid- to late-secretory phase of the cycle and are considered essential for receptivity. The goal of this project was to study the relationship between Treg and Th17 cells in the eutopic endometrium of women with the primary complaint of infertility. The endometrial biopsy samples had been collected prospectively from patients during the mid- to late-secretory period of their menstrual cycles. Tissue samples were evaluated immunohistochemically for Treg and Th17 lymphocyte subsets and the Th17 specific cytokine, IL-17 expression. These data were compared to the fertility status in these patients. Overall, Treg cell counts were higher and Th17 cell counts were lower in patients who conceived compared to those that did not get pregnant. Conversely, patients who maintained their infertile status, even after laparoscopic intervention for ectopic lesion removal, had a lower Treg cell count and higher Th17 cell count in their eutopic endometrium. The ratio of Treg:Th17 cell counts was significantly correlated to their fertility status. Patients with a ratio less than 3 failed to conceive in spite of medical or surgical intervention. Laparoscopic intervention seemed to have a boosting effect on the endometrial Treg cell population which was in turn correlated to a positive pregnancy outcome. The IL-17

expression was elevated in both the glandular and stromal compartments of the endometrium in patients with a low Treg:high Th17 cell ratio. Hence, it can be concluded that patients with a high Treg:Low Th17 cell count ratio in their eutopic endometrium during the secretory phase of their menstrual cycle are more likely to conceive, especially after laparoscopic removal of endometriotic lesions.

INTRODUCTION

Recurrent Pregnancy Loss (RPL) also referred to as recurrent miscarriage (RM) or habitual abortion (HAB) is defined as two or more consecutive pregnancy losses prior to 20 weeks from the last menstrual period. Currently a number of etiologies are accepted for such pregnancy losses such as parental chromosomal abnormalities, certain uterine anatomic abnormalities, endocrine disorders, infections, and possibly immunologic abnormalities (100). However when none of these factors are evident, they are classified as idiopathic or unexplained pregnancy losses. Coincidental findings of concurrent disease conditions such as endometriosis may be considered a reason for these recurrent losses.

Endometriosis is a poorly understood and extremely debilitating benign gynecological condition commonly encountered in women of reproductive age. It is an estrogen dependent disorder characterized by the presence of endometrial glandular and stromal cells outside the uterus, primarily on the pelvic and/or abdominal organs (101) and in some cases in extra-pelvic regions such as chest wall, lungs, brain and bone (102). Chronic pelvic pain, dysmenorrhea with or without abnormal bleeding are common symptoms of this condition (53,54). Endometriosis also accounts for 30-50% of infertility in women (56). Endometriosis related infertility has numerous causes but in this chapter we are focusing on the role of T-helper lymphocyte subsets.

In a normal physiological setting specific populations of immune cells increase at the implantation site to help maintain a local immunosuppressive environment in the uterus for the semi-allogeneic embryo to attach (14). Among these key immune cells are the T-helper lymphocytes, which account for a significant 5-10% of the endometrial leukocyte population. The T-regulatory (Treg) cells are especially critical for localized immunosuppression at the implantation site and are seen to increase in the peripheral circulation (17,103) as well as the decidua (18,19) during early pregnancy, showing a stable and highly suppressive phenotype and hence are believed to be critical for embryo tolerance, invasion and establishment of pregnancy (21,22). However, it has also been proposed that the balance between Treg cells and their reciprocal cell types should be considered when evaluating immune status, especially since the presence of Treg cells and their reciprocal pro-inflammatory subset known as T-helper 17 (Th17) cells are inversely related to each other in the decidua (18). Hua et.al (98) have also shown that decreased ratios of serum Treg/Th17-related-cytokines may play a role in the pathogenesis of defects resulting in implantation failure, which was supported by Wang et.al, (104), who detected significantly elevated Interleukin 17 (IL-17) cytokine levels in both peripheral blood and decidua of patients with unexplained spontaneous abortion.

Our goal in this first aim was to evaluate whether women with endometriosis have a pro-inflammatory rather than an anti-inflammatory T-cell and related cytokine milieu in their eutopic endometrium during the receptive phase of their menstrual cycle. Eutopic endometrial biopsies were collected from women presented with complaints of unexplained infertility or unexplained recurrent pregnancy loss. These women were between 25-40 years of age and had not received any medical treatment for their fertility issues prior to undergoing the endometrial biopsy. A subset of patients had undergone a laparoscopy for removal of endometriotic lesions. The

endometrial biopsies were collected during the mid- to late-secretory phase (specifically 7 days post LH surge) from the middle front or back region of the uterine body (not close to the cornua). The biopsy tissue sections were assessed for Treg and Th17 cell profiles as well as for the cytokine Interleukin 17 (IL-17). The results were correlated with the fertility status of these women to ascertain potential markers for uterine receptivity and positive pregnancy outcome. To keep the study completely unbiased, I was blind to the patient history and/or categorization.

RESULTS

Characterization of the T-helper lymphocyte subsets in the eutopic endometrium of patients with UI/uRPL and/or confirmed endometriosis

Treg cells are known to accumulate in the normal endometrium during the mid- to late-secretory phase and are considered essential for uterine receptivity. On the other hand Th17 cells play a critical role in autoimmunity, which suggests that Th17 cells may hinder the mechanisms mediating maternal tolerance towards the conceptus and hence impair maintenance of pregnancy. Thus we first evaluated the Treg and Th17 lymphocyte subset populations in the eutopic endometrium of patients, with the primary complaint of recurrent pregnancy loss, using established immunohistochemistry techniques. The Treg cell count per unit area of eutopic endometrium analyzed was not different in "UI/uRPL Fertile" patients (27.50±3.97 cells/100mm^2 tissue area) as compared to the UI/uRPL Infertile" patients (23.42± 2.86 cells/10mm^2 tissue area) (Figure 3.1.C). But when patients with confirmed endometriosis were evaluated, it was observed that "Fertile with endometriosis" patients had a significantly higher Treg cell number (27±4.07 cells/10mm^2 tissue area) in their eutopic endometrium as compared to "Infertile with endometriosis" patients (17±2.10 cells/10mm^2 tissue area) (Figure 3.1.D). These

findings concur with data shown by Sasaki et.al. (20) that Treg cells are found in higher numbers in the decidua of normal early pregnancy as compared to spontaneous abortion cases. Conversely, we also observed that the pro-inflammatory Th17 cell numbers in the eutopic endometrium of "UI/uRPL Fertile" patients (10.10±2.09 cells/10mm^2 tissue area) was not different than that observed in "UI/uRPL Infertile" patients (16.27±1.98 cells/10mm^2 tissue area) (Figure 3.2.C). But again, when the patients with confirmed endometriosis were evaluated, it was observed that "Fertile with endometriosis" patients had a significantly lower Th17 cell number (6±1.45 cells/10mm^2 tissue area) in their eutopic endometrium as compared to the "Infertile with endometriosis" patients (17±2.07 cells/10mm^2 tissue area) (Figure 3.2.D). Liu et.al. (24) have shown that the proportion of Th17 cells was significantly higher in the decidua of patients with unexplained recurrent spontaneous abortions. These results indicate that the immunosuppressive FoxP3+ Treg lymphocytes might indeed play an important role in maintenance of a receptive endometrium. And a converse increase in inflammatory RORγt+ Th17 cell population in the endometrium might interfere with this receptivity.

Characterization of the T-helper lymphocyte subsets in the eutopic endometrium of patients with different stages of endometriosis

We then concentrated on the patients with endometriosis and compared between patients who had different stages of endometriosis as confirmed by laparoscopy. Our results showed that the Treg population in the eutopic endometrium was highest when the patients had early stage of the disease (36.92±13.23 cells/10mm^2 tissue area) as compared to stage II endometriosis (18.64±3.19 cells/10mm^2 tissue area) or stage III endometriosis (29.11±9.83 cells/10mm^2 tissue area) when the ectopic lesions are well established (Figure 3.3.A). The Th17 cell population was highest in

the stage III patients (14.98±3.61 cells/10mm^2 tissue area) than the stage I (12.33±4.86 cells/10mm^2 tissue area) or stage II (10.95±2.82 cells/10mm^2 tissue area) (Figure 3.3.B), indicating a consistent pro-inflammatory environment in the endometrium once the disease is established and also during the initial stages of ectopic lesion invasion. These data were unfortunately not statistically significant probably due to small sample size in each comparison.

Characterization of the Treg and Th17 lymphocyte subsets in the eutopic endometrium of patients who underwent laparoscopy for removal of ectopic lesions

Due to the wide range in the Treg and Th17 cell populations mentioned above, we decided to evaluate the T-cell populations in patients who underwent the endometrial biopsy before laparoscopic intervention for ectopic lesion removal as compared to those patients who had the biopsy after the laparoscopy. Our results showed that patients who had the biopsy before the laparoscopic intervention and who conceived successfully had a higher Treg population (26.16±11.15 cells/10mm^2 tissue area) in their eutopic endometrium as compared to patients who did not conceive (14.56±3.29 cells/10mm^2 tissue area). Moreover, it was interesting to see that post laparoscopic intervention, a larger number of patients conceived and had a much higher Treg cell population (37.18±10.53 cells/10mm^2 tissue area) than patients who did not get pregnant in spite of the laparoscopy (8.39±3.80 cells/10mm^2 tissue area) (Figure 3.4.A). Conversely, patients who had the biopsy before the laparoscopy and who conceived had a significantly lower Th17 cell population to begin with (3.22±1.62 cells/10mm^2 tissue area) as compared to those who did not get pregnant (13.99±3.17 cells/10mm^2 tissue area). And the laparoscopic intervention did seem to have a significant effect on the Th17 cell population in patients who conceived post laparoscopy (4.43±0.94 cells/10mm^2 tissue area) as compared to

patients who continued to be infertile (18.41±2.29 cells/10mm^2 tissue area) (Figure 3.4.B). This finding could suggest a boost in the "helpful" Treg immune status as a result of the laparoscopy which was also observed in the baboon model of endometriosis (105).

Evaluation of the Ratio of Treg to Th17 cell populations in the eutopic endometrium of patients

As mentioned earlier, a receptive uterus usually has an anti-inflammatory environment in preparation for the advent of the semi-allogeneic embryo. It has been previously proposed that the balance between specific T-cell subsets and/or their related cytokines better reflects the immune status in the endometrium than Treg cell frequency alone(18,106). Our initial evaluation of the Treg:Th17 cell ratios in patients with a history of UI/uRPL indicates that the ratio of Treg:Th17 cells was highly significant and correlated with pregnancy outcomes (Figure 3.5.A). UI/uRPL patients with a higher Treg:Th17 cell ratio (5.59±1.23) had a better chance at conceiving than patients with a lower ratio (2.97±0.72). Moreover, "Fertile with endometriosis" patients had a significantly higher Treg:Th17 ratio (7.29±1.82) as compared to the "Infertile with endometriosis" patients (1.85±0.45) (Figure 3.5.B). It was consistently observed that few patients conceived successfully when this Treg:Th17 cell ratio was less than 3. We confirmed this finding on further evaluation of patients who underwent the biopsy before the laparoscopic intervention as compared to those after. Patients who underwent the biopsy after the laparoscopic intervention had significantly better Treg:Th17 ratios and a better chance of conceiving (8.53±1.75 in fertile group v/s 0.50±0.25 in infertile group) than the patients who underwent the biopsy before laparoscopy (9.84±5.51 in fertile group v/s 1.76±0.87 in infertile group) (Figure 3.5.C). Thus again confirming our previous findings that the laparoscopic intervention might indeed be

helping the immune system shift towards a more normal anti-inflammatory environment in the endometrium.

Evaluation of Th-17 cell specific cytokine - Interleukin-17 expression in eutopic endometrial samples of patients and correlation with fertility status

Cytokines with the capacity to alter immune responses are abundant in and around the implantation site. Hence we decided to evaluate the expression of a Th-17 cell specific cytokine, IL-17, in the eutopic endometrial biopsy samples to determine whether elevated IL-17 levels play a part in promoting the pro-inflammatory environment in the eutopic endometrium in patients with endometriosis related infertility as judged by the Treg:Th17 cell ratio data. Endometrial sections stained for IL-17 expression were analyzed using a semi-quantitative H-Score technique. Five independent fields each were evaluated for glandular and stromal expression of the cytokine in each patient's sample. A significant inverse relationship between this Treg:Th17 cell ratio and the IL-17 expression H-Score analyses (Figure 3.6.i) was observed. Patients with a low IL-17 H-Score had a high Treg:Th17 cell ratio whereas patients with a high IL-17 H-Score had a low Treg:Th17 cell ratio. This trend was observed to be consistent in both the glandular as well as the stromal compartments of the eutopic endometrium (Figure 3.6.A-D). Furthermore, these elevated levels stayed consistent in the patients who did not conceive in spite of the laparoscopic intervention (2.67±0.33) as compared to the other groups mentioned earlier who had H-scores less than 2.08±0.05 on an average, irrespective of pregnancy outcome. Liu et.al.(24) have shown a similar increase in IL-17 expression in decidualized stromal cells and decidual glands of patients with unexplained recurrent spontaneous abortions as compared to normal fertile women.

DISCUSSION

It is known that the endometrium undergoes specific changes during the mid- to late-secretory phase of the menstrual cycle wherein it acquires a "receptive" phenotype thus preparing for the advent of the semi-allogeneic embryo. Compromised receptivity as seen in unexplained infertility or the various forms of pregnancy loss has been associated with various cellular and molecular defects in the endometrium (107). Extensive research in the field of reproductive immunology has now linked aberrant immune responses with the occurrence of recurrent pregnancy loss, and atypical T-cell subset populations are considered at least partly responsible for implantation failure. Our hypothesis was that the absolute numbers as well as relative proportions of T-helper lymphocytes, specifically the Treg and Th17 cell populations, found in the secretory phase endometrium, are strongly associated with the patients' fertility status and hence are some of the major determining factors for uterine receptivity and embryo acceptance or rejection. We determined that a relationship does exist between these cell sub-populations and endometrial receptivity in women with endometriosis related infertility. We evaluated the presence and number of Treg and Th17 cells in the eutopic endometrium of fertile and infertile patients, during the mid- to late-secretory phase of their menstrual cycles. We observed that patients with high Treg numbers had a better chance at conceiving than patients with low Treg numbers in their endometrium. Conversely, patients with a higher pro-inflammatory Th17 cell count in their endometrium had a significantly lower chance of conceiving. Interestingly, we observed that the ratio of the Treg to Th17 cell counts was a better and more reliable correlation to patient fertility status than the individual cell counts by themselves. Moreover, our results suggest that the correlation between the relative numbers of

these cell types (Treg:Th17 ratios) and the pro-inflammatory cytokine IL-17 was a good indicator of a patients' ability to conceive. A subset of these patients underwent laparoscopic intervention for excision of ectopic endometriotic lesions before the endometrial biopsy was conducted for the above mentioned assessments. We compared all the above mentioned parameters in the patients who underwent the biopsy before laparoscopic intervention to those who underwent the biopsy after the laparoscopic intervention to assess whether this surgical procedure had any significant effect on restoring the imbalanced endometrial immune cell milieu and in turn helping endometrial receptivity. We found that the laparoscopic intervention did indeed have a boosting effect on the patients' endometrial immune response which increased their chance of conceiving.

An expansion in the CD4+CD25+FoxP3+Treg cell pool occurs around the time of implantation in mice (108) but it is not known whether these cells are preferentially recruited to the endometrium or undergo local expansion at the materno-fetal interface. Treg cells appear to play a similar role during establishment of human pregnancy. Decreases in the endometrial expression of the Treg transcription factor FoxP3 mRNA, have been implicated as a cause for unexplained infertility (30). Our results show a similar trend with regards to T-lymphocyte cell counts. We observed that women with endometriosis who had a higher anti-inflammatory Treg cell population in their eutopic endometrium (during the window of implantation) had a better chance of conceiving than the women who had a lower Treg cell population to begin with. Previous research has shown that the peripheral blood (17,103) and decidual (17) CD4+CD25+ Treg cell population were highest during the first trimester of pregnancy. In fact it was also shown that the CD4+CD25+Foxp3+ Treg cell population was enriched in the early normal pregnancy decidua as compared to the peripheral blood of the same pregnant woman(18,19).

This phenotype of increased Treg cell population is hence thought to contribute to the immunosuppressive environment in the endometrium and allows tolerance to the fetal semi-allogeneic cells. Its dysregulation in reproductive pathologies is often thought to contribute to the associated infertility. Circulating Treg cells were shown to be reduced in women with unexplained recurrent spontaneous abortion(24) and recurrent pregnancy loss (25).

Concurrently, pro-inflammatory Th17 cell numbers were shown to be significantly increased in the peripheral circulation in patients with unexplained recurrent spontaneous abortion(24) and recurrent miscarriage(104). A similar increase in Th17 cells was shown in the decidua of patients with recurrent pregnancy loss (109) and recurrent or inevitable spontaneous abortion (27,28). Our results showed that the patients with a higher pro-inflammatory Th17 cell population in their eutopic endometrium had a significantly lower chance of conceiving as compared to patients with a lower Th17 cell population. Th17 cells play an important role in host defense mechanisms. But an over production of these Th17 cells and a concurrent decrease in Treg cells is also associated with autoimmune and inflammatory disease conditions. This effect may be due to the reciprocal developmental pathways for both cell types and the opposing effects of these cells on immune processes.

And so it has been proposed that a fine balance between these two cell types is crucial to maintaining peripheral and/or local immune equilibrium. Various studies of Hashimoto thyroiditis (110), acute respiratory distress syndrome (111), coronary atherosclerosis (112) to list a few have shown the importance of this balance. It has also been shown that peripheral circulation levels of Th17 to Treg cell ratios are increased in patients with recurrent pregnancy loss (25) and unexplained recurrent spontaneous abortion (24) as compared to normal fertile controls. We observed that the ratio of Treg cell to Th17 cell populations (Treg:Th17) was a

better indicator of fertility status than the absolute individual values of the T-cell subsets. Patients with a consistently low Treg:Th17 ratio (less than 3) failed to conceive. Though it has been shown that the decidual populations of both T-cell types are significantly inversely correlated in recurrent spontaneous abortion cases (28), nothing specific has been studied with regards to this ratio and it's correlation to fertility. Hence our result is a novel finding and may be used as a reliable marker for endometrial receptivity. This novel marker would be especially helpful when assessing endometrial receptivity in patients who are undergoing assisted reproduction treatments.

Because the patients we were assessing for the above mentioned parameters had a confirmed diagnosis of endometriosis, we next decided to evaluate whether the stage of disease had an impact on these T-cell phenotypes. Studies have proposed that a defective T-cell response is associated with endometriosis establishment. Peritoneal T-cell characterization has shown that the CD4+ Treg cells are decreased in stage I and II of the disease (113), but they did not evaluate the endometrial T-cell populations. Very few studies have focused on the effect of endometriosis on the eutopic endometrial T-cell population. One study showed that the total CD4+ T cell number in the eutopic endometrium was lower than that seen in the ectopic lesions (114), but they did not characterize the individual T-cell subsets nor did they compare these parameters to fertility. We observed that the stage of endometriosis did not have a statistically significant effect on the T-cell populations; though the Treg cells did seem to decline as the disease progressed. The baboon model of endometriosis showed a similar phenotype. The systemic and eutopic endometrial Treg lymphocytes in the animals with endometriosis were reduced at 1 month post inoculation of ectopic lesions and this reduction was maintained through the 15 months of the study as compared to the disease free animals (105). We also observed that the Th17 cells

gradually increased (though not significantly) as the disease progressed, indicative of a consistent pro-inflammatory environment once the disease was established. This supports our earlier findings that the low Treg and concurrent high Th17 cell phenotype is responsible for the endometriosis related infertility. Though Th17 cells have not been characterized previously for stage of disease, the cell specific cytokine Interleukin-17 (IL-17) has been shown to be significantly higher in the peritoneal fluid of patients with minimal/mild stage endometriosis as compared to those with moderate/severe stage of disease (115,116).

Other studies have also shown abnormal serum and peritoneal fluid T-cell related cytokine levels in patients with endometriosis. Peritoneal fluid IL-17 levels were shown to be significantly higher in patients with endometriosis related infertility as compared to patients who were fertile (115). On the other hand, decreased ratios of Th17/Treg-related-cytokines in the serum have also been implicated in the pathogenesis of implantation failure (98). Our data shows that elevated expression of IL17, in both the glandular and stromal compartments of the eutopic endometrium, is inversely related to the T-cell ratios. Even though the R^2 value is low (0.105), we observed that patients with a higher IL17 H-score consistently had a lower Treg:Th17 cell ratio. These data in turn suggest that the pro-inflammatory environment produced by the elevated IL-17 levels may be one of the reasons for the unreceptive endometrium during the secretory phase in these infertile patients. This result concurs with previous data that have shown significantly elevated expression of IL-17 mRNA and protein in the decidua of patients with recurrent spontaneous abortion (28). IL-17 acts through the induction and expression of other pro-inflammatory cytokines and chemokines, which mediate immune cell infiltration and destruction of tissue. Various treatment modalities are under investigation currently for autoimmune and pro-inflammatory conditions that focus on targeting the IL-17 cytokine or its

receptor. IL-17 cytokine and receptor inhibitors are being tested for treatment of psoriasis, psoriatic arthritis and ankylosing spondylitis (117), lung damage in bleomycin-treated mice (118), attenuation of pro-inflammatory molecules in astrocytes (119), amelioration of experimental autoimmune encephalomyelitis (120) and collagen-induced arthritis in rats (121) to name a few among many. Current medical treatments for endometriosis associated symptoms usually target hormonal pathways (122,123). Newer drugs targeting non-hormonal pathways that affect inflammation, angiogenesis, tissue adhesion and invasion are being considered for endometriosis treatment (123,124). But all these treatment modalities have significant side effects as their targets also play important roles in physiological processes.

Currently, surgical excision of the ectopic lesions is the only known treatment that has true benefits without the side effects associated with hormonal therapy. Laparoscopic excision of endometriotic lesions significantly reduced the associated symptoms such as dysmenorrhea, pelvic pain, dyspareunia and improved quality of life for patients (125–127). Moreover, laparoscopic excision of lesions has also been shown to improve subsequent conception and live birth rates irrespective of whether they underwent IVF/ICSI cycles or not (125,128,129). But speculations on why the fertility index improves post excision are yet unclear. One explanation could be the immunobiological effect of surgery. The baboon model of endometriosis showed that early laparoscopic intervention, whether excisional or sham, resulted in a recovery of Foxp3 mRNA and Foxp3-positive cells (105). Our results showed a similar trend. Patients who underwent laparoscopic intervention for removal of lesions had a higher endometrial Treg count, significantly lower endometrial Th17 count, significantly better Treg to Th17 ratio and had a much higher conception rate, as compared to patients who did not undergo the excisional surgery before the endometrial biopsy were collected. These findings could be suggestive of the

immunobiological boosting effect mentioned earlier, which helps the body transition back towards an anti-inflammatory milieu from the pro-inflammatory one due to endometriosis. Other studies have shown a similar improvement in endometrial receptivity brought on by the effect of endometrial biopsy-induced inflammation (130) and lipiodol hysterosalpingogram (131) and base their inference on an immunomodulatory effect of these treatments.

Tomaccetti et.al, (92) proposed in an extensive review that altered humoral and cell-mediated immunological function may possibly alter endometrial characteristics during the crucial implantation window and thus affect endometrial receptivity in women with endometriosis. Overall, our experiments characterizing the eutopic endometrial T-cell populations in specific groups of patients have yielded promising results that support our hypothesis that an anti-inflammatory T-lymphocyte phenotype (high Treg-low Th17) in the eutopic endometrium is associated with a positive pregnancy outcome, whereas a pro-inflammatory T-lymphocyte phenotype (high Th17-low Treg) in the eutopic endometrium is associated with infertility and pregnancy loss (Figure 3.7). Hence our data strongly indicates using the ratio of the Treg:Th17 cell populations in the endometrium as a novel and reliable marker of uterine receptivity and predictor of successful pregnancy. The specific role of excisional laparoscopy in boosting the T-cell phenotypes and its positive fertility outcome is also a novel finding which could be further explored with larger sample size. The IL-17 cytokine expression data further add strength to this hypothesis and support its use as a better target for treatment in patients with endometriosis related fertility problems. These data can further be used to assess exactly how the elevated IL-17 levels affect specific endometrial cell functions to add to the pro-inflammatory environment induced in the eutopic endometrium by the presence of ectopic endometriotic lesions.

FIGURES

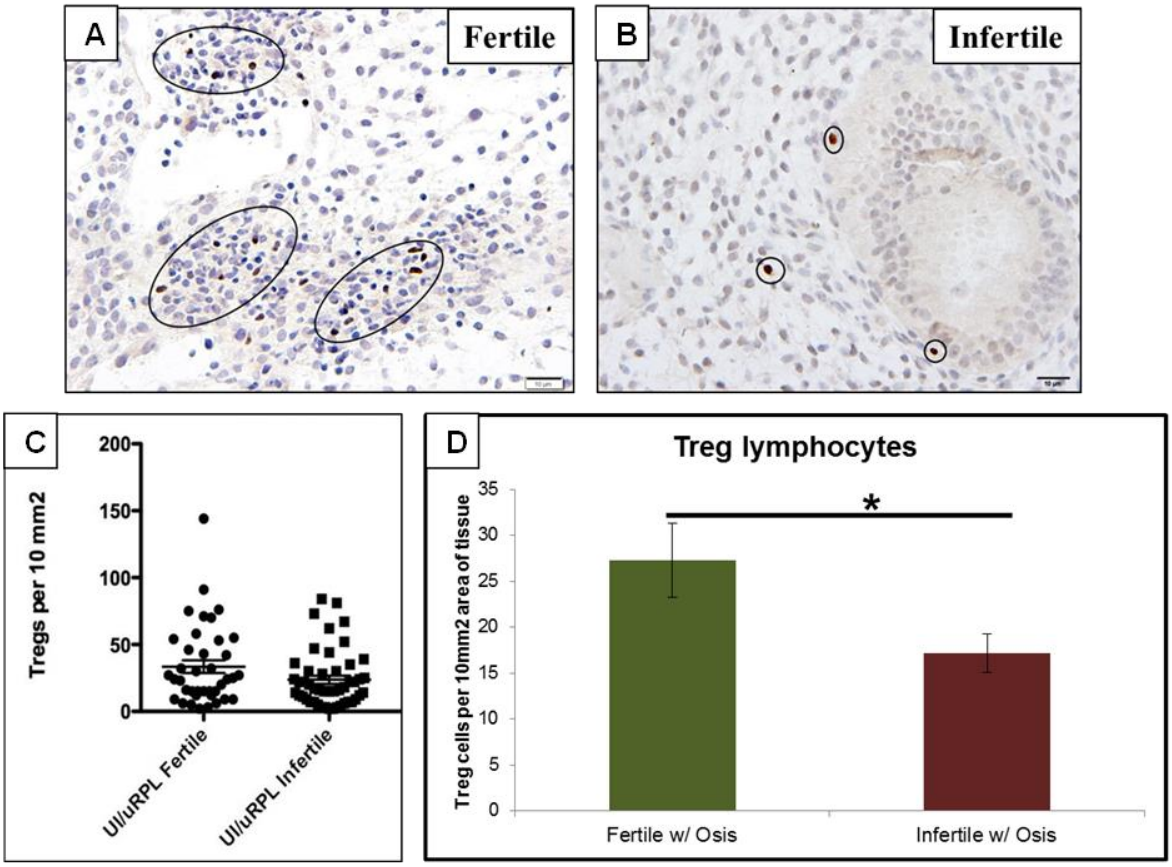

Figure 3.1. Characterization of Treg lymphocyte numbers in the eutopic endometrium during the receptive phase of the menstrual cycle.

Fertile women (A) had a higher number of endometrial Treg cells than infertile women (B). The Treg cell numbers per unit area of tissue analyzed in the "UI/uRPL Fertile" patients did not differ from that observed in the "UI/uRPL Infertile" patients (C). The Treg cell numbers per unit area of tissue analyzed in the "Fertile with endometriosis" patients was significantly higher than in the "Infertile with endometriosis" patients (D). (n=31 UI/uRPL Fertile, n=34 UI/uRPL Infertile, n=19 Fertile w/ Osis, n=17 Infertile w/ Osis; $p<0.05$)

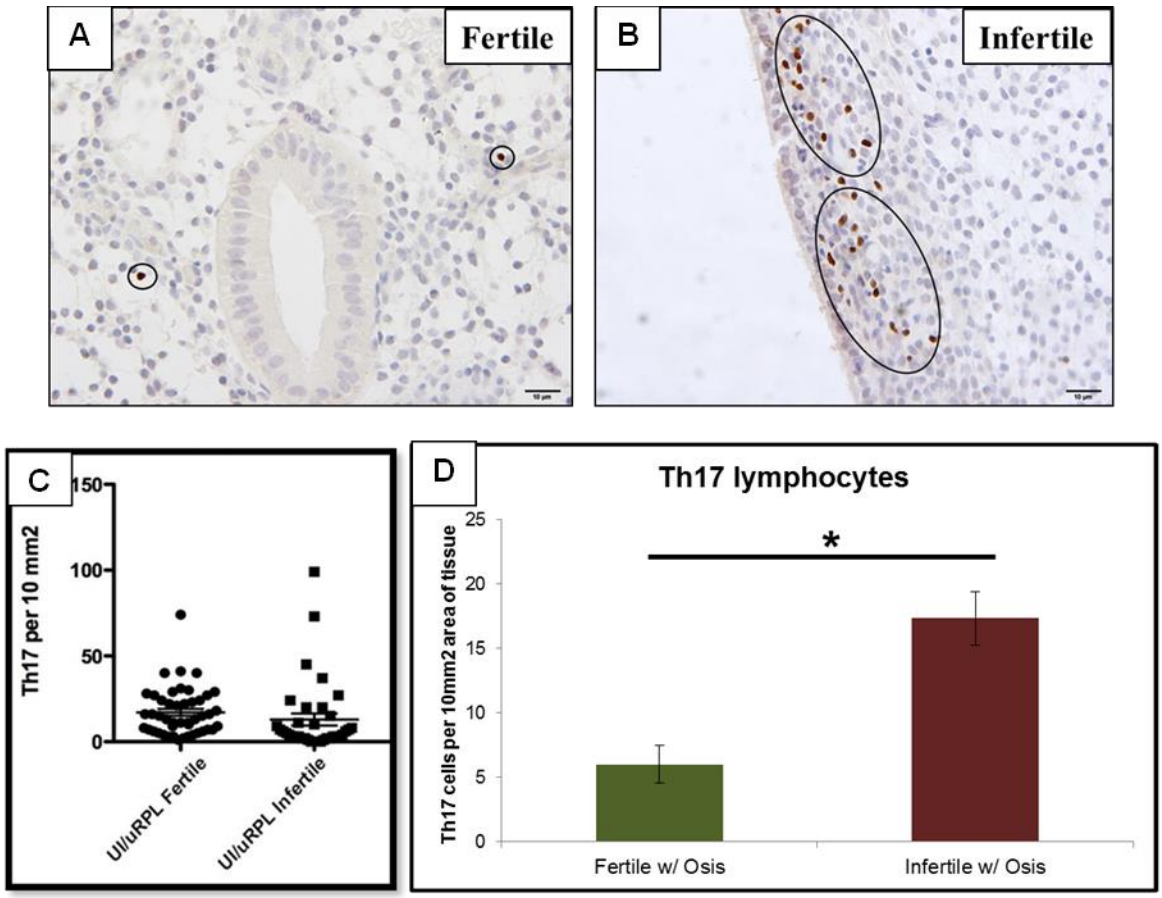

Figure 3.2. Characterization of Th17 lymphocyte numbers in the eutopic endometrium during the receptive phase of the menstrual cycle.

Fertile women (A) had a lower number of endometrial Th17 cells than infertile women (B). The Th17 cell numbers per unit area of tissue analyzed in the "UI/uRPL Fertile" patients did not differ from that observed in the "UI/uRPL Infertile" patients (C). The Th17 cell numbers per unit area of tissue analyzed in the "Fertile with endometriosis" patients was significantly lower than in the "Infertile with endometriosis" patients (D). (n=31 UI/uRPL Fertile, n=34 UI/uRPL Infertile, n=19 Fertile w/ Osis, n=17 Infertile w/ Osis; $p<0.05$)

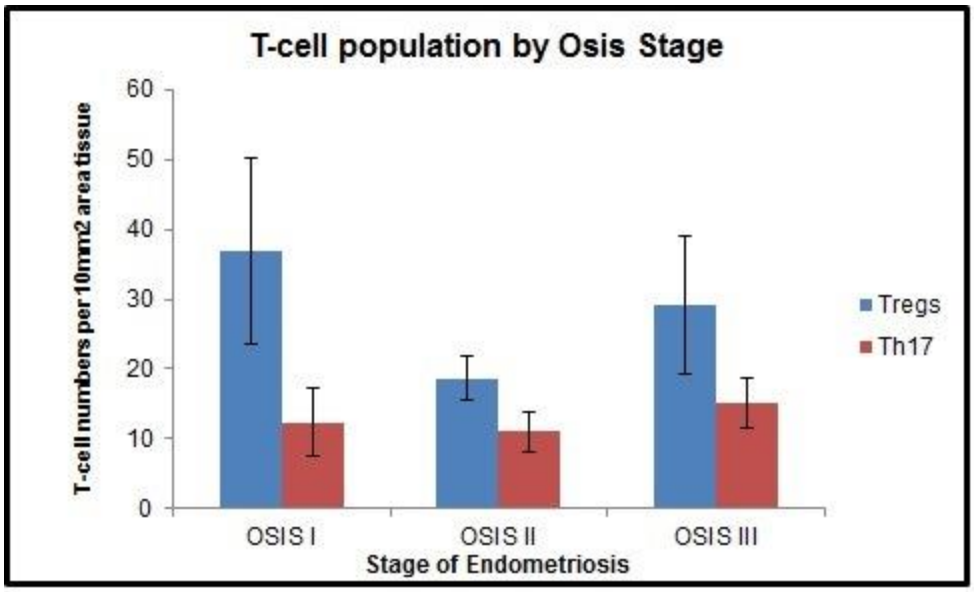

Figure 3.3. Characterization of the T-helper lymphocyte subsets in the eutopic endometrium of patients with different stages of endometriosis.

Patients with Stage I endometriosis had the highest Treg counts as compared to Stage II and Stage III patients (as indicated with blue bars). Patients with Stage III endometriosis had the highest Th17 numbers than women with Stage I or Stage II (as indicated with red bars) indicative of consistent pro-inflammatory milieu once the disease is established. Differences were not statistically significant probably due to small sample size. (n=7 Osis I, n=17 Osis II, n=5 Osis III; $p>0.05$)

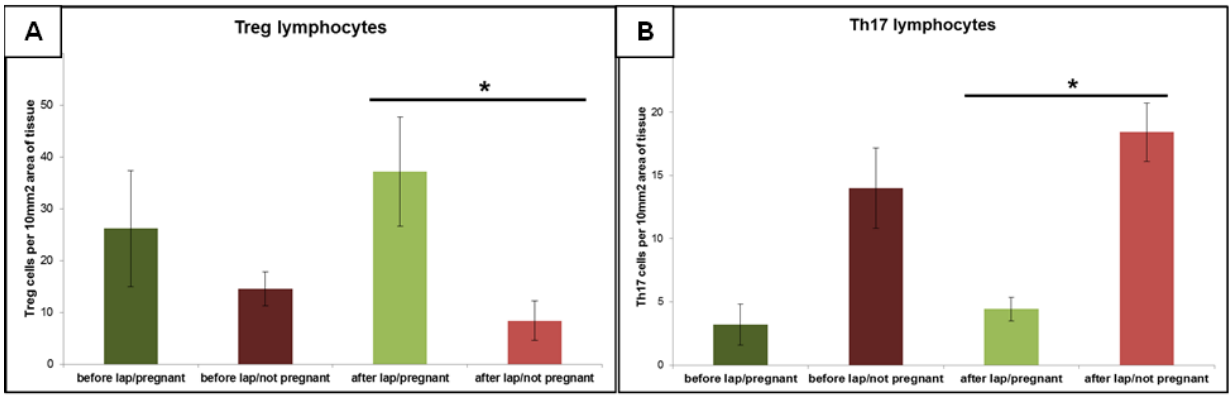

Figure 3.4. Characterization of the Treg and Th17 lymphocyte subsets in the eutopic endometrium of patients who underwent laparoscopy for removal of ectopic endometriotic lesions.

Patients who had the laparoscopic removal of ectopic lesions showed an increase in the Treg cell population and had a better chance at conceiving whereas patients who showed no increase in Treg numbers post laparoscopy were consistently infertile (A). Concurrently, patients who had laparoscopic removal of lesions but maintained their significantly high Th17 cell profile continued to be infertile (B). (n=3 biopsy before laparoscopy+pregnant, n=6 biopsy before laparoscopy+not pregnant, n=8 biopsy after laparoscopy+pregnant, n=3 biopsy after laparoscopy+not pregnant; $p<0.05$)

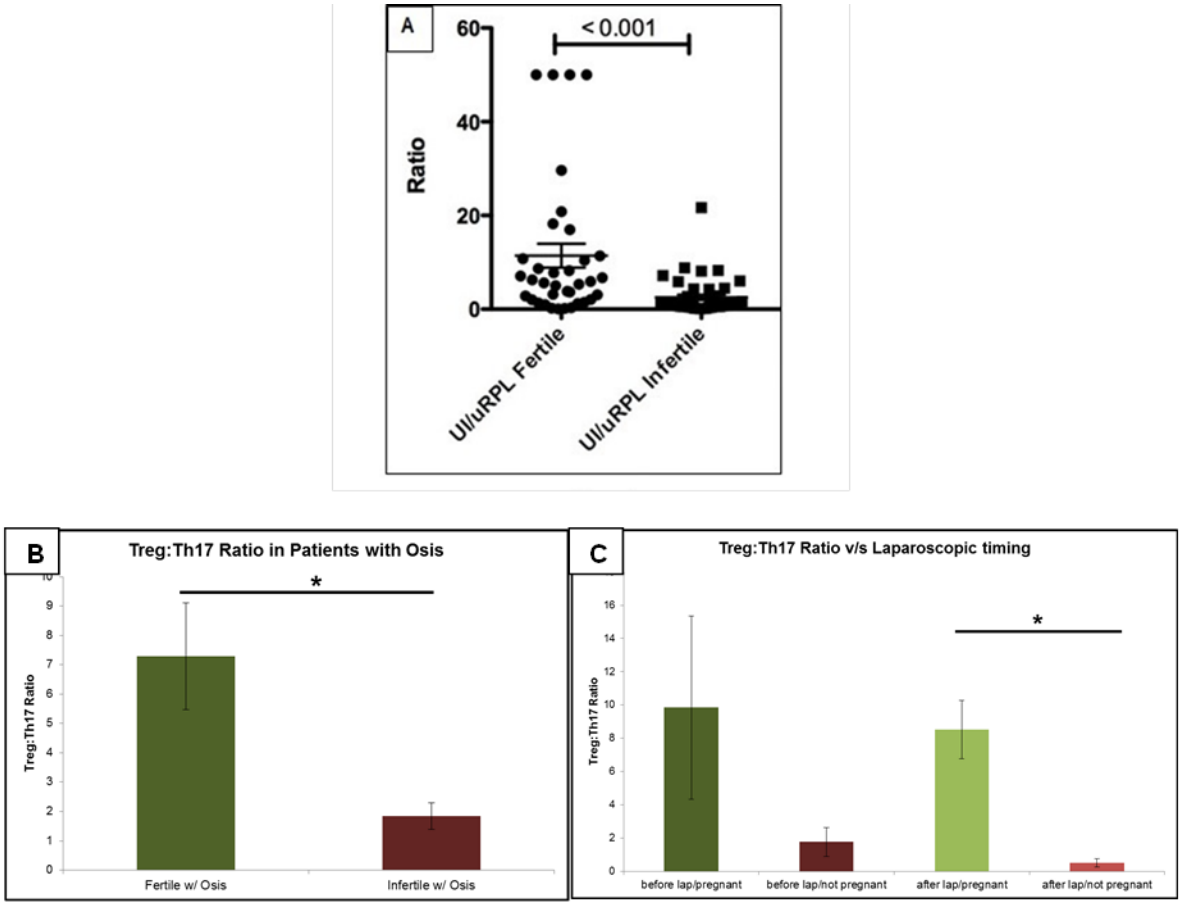

Figure 3.5. Evaluation of the Ratio of Treg to Th17 cell populations in the eutopic endometrium of patients.

Treg:Th17 cell ratio was highly significant and correlated with pregnancy outcomes. UI/uRPL patients with a higher Treg:Th17 cell ratio had a better chance at conceiving than patients with a lower ratio (A). "Fertile with endometriosis" patients had significantly higher Treg/Th17cell ratios than the "Infertile with endometriosis" patients (B). Patients who had a ratio of less than 3 even after laparoscopic removal of ectopic lesions continued to be infertile (C). (n=31 UI/uRPL Fertile, n=34 UI/uRPL Infertile, n=19 Fertile w/ Osis, n=17 Infertile w/ Osis, n=3 biopsy before laparoscopy+pregnant, n=6 biopsy before laparoscopy+not pregnant, n=8 biopsy after laparoscopy+pregnant, n=3 biopsy after laparoscopy+not pregnant; $p<0.05$)

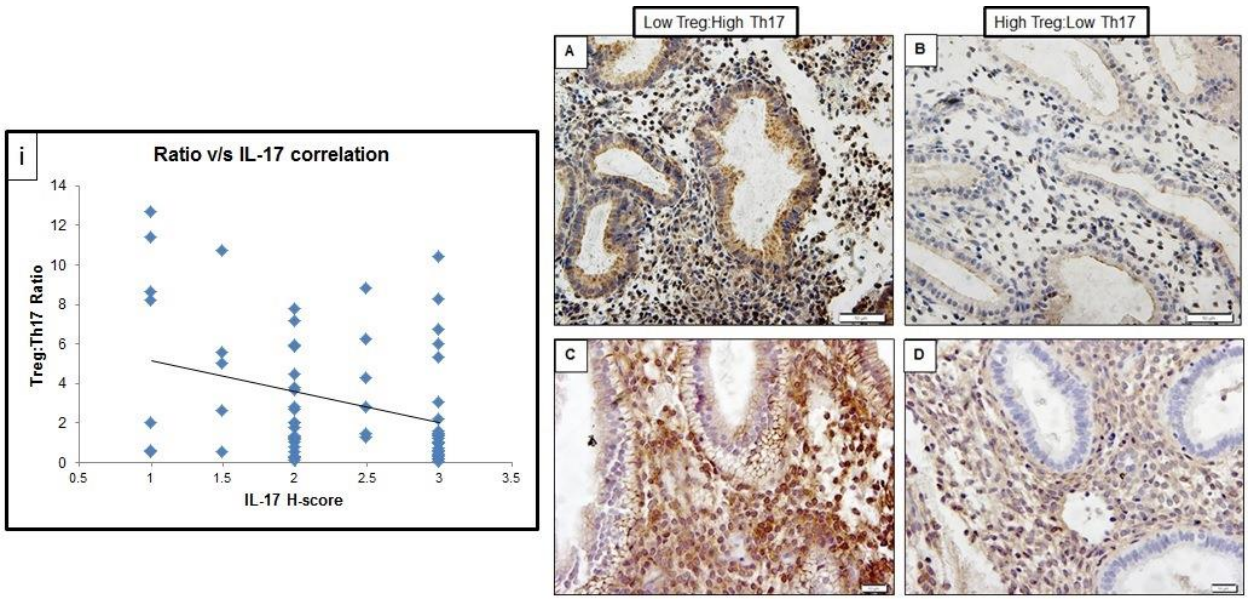

Figure 3.6. Evaluation of Th-17 cell specific cytokine - Interleukin-17 expression in eutopic endometrial samples of patients and correlation with fertility status.

A significant inverse relationship ($p<0.0001$) between Treg:Th17 cell ratio and the IL-17 expression H-Score was observed (i). Patients with a low Treg to high Th17 ratio showed increased expression of IL-17 in both glandular (A) and stromal (C) compartments of the endometrium. Conversely, patients with a high Treg to low Th17 ratio showed a lower expression level for IL-17 in both the glandular (B) and stromal (D) compartments of the endometrium. ($n=66$, $p<0.05$)

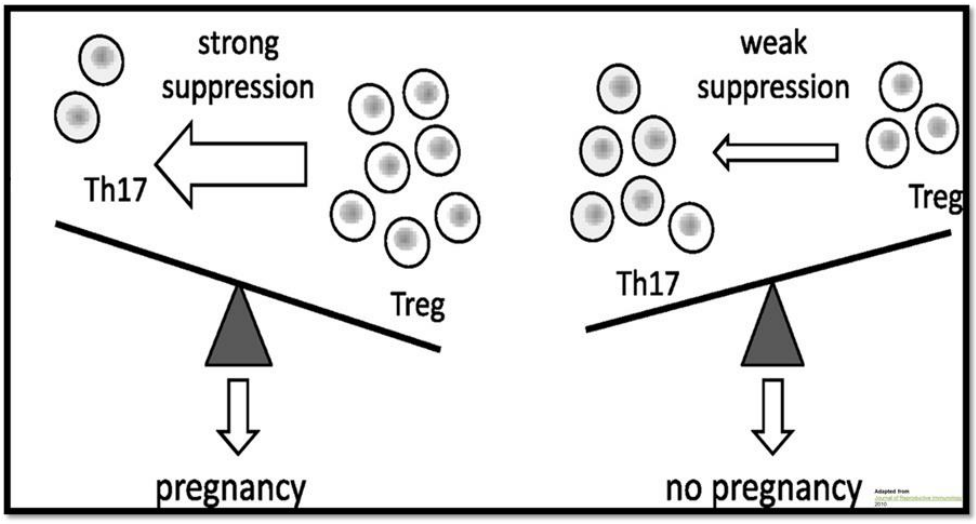

Figure 3.7. Model for overall hypothesis.

The anti-inflammatory T-lymphocyte phenotype (high Treg-low Th17) in the eutopic endometrium is associated with a positive pregnancy outcome, whereas a pro-inflammatory T-lymphocyte phenotype (high Th17-low Treg) is associated with infertility and pregnancy loss. (Image adapted from Journal of Reproductive Immunology 2010)

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CHAPTER 4

EFFECTS OF INTERLEUKIN-17 ON ENDOMETRIAL AND ENDOMETRIOTIC CELL MIGRATION, INVASION AND ACTIVATION OF SIGNALING PATHWAYS

ABSTRACT

The pathophysiology of endometriosis is yet unclear but is known to involve complex interactions between ectopic endometriotic cells and the peritoneal mesothelium. Endometriotic lesion establishment and growth are supported by various factors that regulate cell growth and survival, migration and invasion. Overall, a pro-inflammatory microenvironment in the vicinity of an ectopic endometriotic lesion is thought to aid its survival, attachment and invasion into extra-uterine surfaces. One such cytokine is interleukin-17 (IL-17) which is associated with various inflammatory conditions including endometriosis. The goal of this study was to evaluate the effect of IL-17 treatment on endometrial (Ishikawa) cells and endometriotic (12Z) cells with respect to cell proliferation, motility and induction of signaling pathways. IL-17 treatment did not have any effect on cell proliferation in either cell type. IL-17 positively affected cell migration and invasion in the endometriotic cells but not in the endometrial cells. To understand this differential effect we assessed IL-17 receptor (IL-17R) expression in both cell types and observed that the receptor expression was fairly similar in both cell types. Thus we concluded that the differential effect observed was probably due to factors other than the IL-17R expression in these endometrial cell types. We investigated which signaling pathway was utilized by IL-17 in these uterine cell types. The inflammatory NFκB pathway is the hallmark of IL-17 activity, but our results showed that in our uterine cell lines, IL-17 did not induce the NFκB pathway. Instead IL-17 activated an alternate MAPK signal transduction pathway, but only in the endometriotic 12Z cells. Since it is known that cell motility requires MMP induction, we also

evaluated changes in EMMPRIN expression in these cells when treated with IL-17. Endometriotic cells showed an increase in EMMPRIN expression post treatment which could partially explain their enhanced motility in the presence of IL-17. We then assessed the correlation between IL-17 and EMMPRIN expression in eutopic endometrial samples from patients with endometriosis. We found that IL-17 expression positively correlated with EMMPRIN expression in about 90% of the samples tested. All these data together support a role for the pro-inflammatory cytokine IL-17 in cell migration and invasion through its effects on EMMPRIN expression and the induction of MAPK signaling pathway.

INTRODUCTION

Endometriosis is one of the most common gynecological disease conditions in women of reproductive age characterized by the presence of endometrial cells outside the uterus (102). The most popular hypothesis for the pathogenesis of this condition is the "retrograde menstruation theory" proposed by Sampson (1927), which suggests that eutopic endometrial fragments pass through patent fallopian ducts into the peritoneal cavity during menstruation and invade and establish as ectopic lesions on extra-uterine surfaces. This induces an inflammatory micro-environment in that area and is thought to be responsible for the symptoms associated with endometriosis, such as chronic pelvic pain, dysmenorrhea and infertility (53).

Although a benign condition, the endometrial cell attachment and invasion into the ectopic surfaces, is thought to occur in a manner similar to cancer metastasis. This process not only involves chronic inflammation but also the process of epithelial to mesenchymal transition. Epithelial to mesenchymal transition (EMT) defines a process by which stationary, polarized epithelial cells undergo a transformation into motile mesenchymal cells (132). This

transformation helps the cells acquire a phenotype that increases their migratory and invasive capabilities, which in turn helps in the establishment of endometriotic ectopic lesions. The endometrial cells that undergo this transition acquire mesenchymal markers especially in the initial stages of lesion establishment (69). The inflammatory microenvironment is also thought to be supported by pro-inflammatory leukocytes and their associated cytokines. One such cytokine is interleukin-17 (IL-17), a pro-inflammatory cytokine produced primarily by Th17 cells , that is found to be elevated in the peritoneal fluid of women with endometriosis (115).

Our goal in this second aim was to assess the effect of IL-17 on eutopic and ectopic endometrial cells in culture and evaluate effects on processes that affect endometriotic lesion establishment. Due to the unavailability of primary cells from patients with endometriosis, we decided to conduct our study using immortalized/transformed endometrial (Ishikawa) cells and endometriotic (12Z) cells that have been characterized and are representative of these cell types. Both cell types were treated with doses of recombinant human IL-17 (recHuIL-17) ranging from 10 pg/ml to 100 ng/ml. Both cell types were also treated with positive and negative control recombinant proteins as needed for each experiment. Several parameters important to the process of EMT were assessed such as cell proliferation, migration and invasion, expression of EMT marker proteins and activation of the NFκB signaling pathway.

RESULTS

Effect of IL-17 on endometrial and endometriotic cell proliferation

Cell proliferation assays were used to evaluate the effect of the pro-inflammatory cytokine IL-17 on endometrial (Ishikawa) and endometriotic (12Z) cell growth. Proliferation of both cell types was assessed 48 hours after treatment with different doses of recHuIL-17 by

conducting cell counts and tritiated thymidine incorporation experiments. Results showed that the cell proliferation rate was similar between cells treated with and without the recHuIL-17 (Figure 4.1).

Effect of IL-17 on endometrial and endometriotic cell motility

The effect of IL-17 on cell motility was evaluated using the Radius^{TM} 24-well cell migration assay and the CytoSelect^{TM} 24-well cell invasion assay. The migration assay showed that the highest dose of recHuIL17 (100 ng/ml) promoted cell migration when compared to the vehicle control treated cells (8.16±1.50% v/s 3.54±0.74%) in the endometriotic (12Z) cells at 24 hours post treatment. However, IL-17 had no effect on the endometrial (Ishikawa) cells at any of the treatment doses or time-points (Figure 4.2).

The invasion assay showed a similar pattern. The highest dose of recHuIL-17 (100 ng/ml) increased invasiveness of the endometriotic (12Z) cells at 24 hours post treatment as compared to the vehicle control treated cells, but had no effect on the endometrial (Ishikawa) cells (Figure 4.3). These data suggest that IL-17 enhances the cell migration and invasive ability in the endometriotic cells but not in the endometrial cells.

IL-17 Receptor expression in endometrial and endometriotic cells

Due to the differential effect of IL-17 on endometrial and endometriotic cell migration and invasion, immunoblots were conducted to confirm and assess IL-17 receptor (IL-17R) expression in these cells. The THP-1 monocyte cell line was used as a positive control for IL-17R expression. Interestingly both the endometrial (Ishikawa) and endometriotic (12Z) cells showed near equal expression of the IL-17R when normalized to GAPDH (Figure 4.4). This

suggested that IL-17 may exert differential effects that are dependent on cell type or other co-receptor expression levels.

Effect of IL-17 on Epithelial to Mesenchymal Transition markers

To investigate whether recHuIL-17 induced alterations in the EMT phenotype of endometrial and endometriotic cells in culture, we treated both cell types with different doses (100 ng/ml, 10 ng/ml, 1 ng/ml and 10 pg/ml) of IL-17 and assessed morphological and EMT-related markers at 0, 15 min, 60 min and 24 hours post treatment.

Endometriotic (12Z) cells acquired an elongated, fusiform appearance with dendritic processes when treated with the 10 ng/ml and 100 ng/ml doses of IL-17. None of the other doses had any effect on morphology of the endometriotic cells. Moreover, the treatments had no effect on the morphology of the endometrial (Ishikawa) cells (Figure 4.5). RecHuTNFα treatment was used as a positive control to induce EMT and had a positive effect on both cell types.

As EMT is an important mechanism for cell migration and invasion, we further evaluated the effect of recHuIL-17 on EMT protein marker end-points including cytokeratin (epithelial marker) and vimentin (mesenchymal marker). Both cell types expressed the mesenchymal marker vimentin, at all time-points irrespective of treatment, though the endometriotic (12Z) cells had a higher expression of vimentin as compared to the endometrial (Ishikawa) cells (Figure 4.7). This can be explained by the fact that these immortalized cells may have undergone EMT already and probably retain some of the mesenchymal markers. Conversely, the epithelial marker, cytokeratin expression was not consistent between the cell types. Only the endometrial (Ishikawa) cells expressed this epithelial marker at all time-points irrespective of treatment (Figure 4.6).

Activation of the NFκB signaling pathway

NFκB is a transcription factor often associated with inflammatory conditions and has been shown to act as a downstream target of IL-17 signaling (133). We next tested the effect of recHuIL-17 on activation of this signaling pathway by evaluating phosphorylation of its inhibitor molecule IκBα and nuclear translocation of the p65 subunit of this pathway, as both are processes involved in NFκB pathway induction (46).

Phosphorylation of IκBα and its subsequent degradation is considered the most common proximal step of the signaling pathway activation. We treated both endometrial and endometriotic cells with recHuIL-17 (100 ng/ml, 10 ng/ml, 1 ng/ml and 10 pg/ml) and assessed cell lysates at 0, 15 min, 60 min and 24 hours post treatment for pIκBα and total IκBα protein expression. Unfortunately the phosphorylated and total protein forms of the IκBα molecule were very difficult to assess on immunoblots and the results were inconsistent. However, from some of the better immunoblots showing total IκBα protein estimation, it appeared that IL-17 treatment at higher concentrations (100 ng/ml and 10 ng/ml) did reduce the total IκBα protein levels in the endometriotic (12Z) cells at 24 hours post treatment, though this effect was not statistically significant (Figure 4.8.A and 4.8.B). This reduction in total IκBα protein expression could be interpreted as loss due to phosphorylation and degradation of the molecule.

Due to the difficulty in assessing phosphorylation of the IκBα protein expression, we decided to evaluate the next step in the signaling pathway activation, which was nuclear translocation of the p65 subunit of the NFκB molecule. Both endometrial (Ishikawa) and endometriotic (12Z) cells were treated as described above and observed for localization of p65 using immunofluorescence. Interestingly we observed that the highest dose of recHuIL-17 (100

ng/ml) increased the cytoplasmic expression of p65 in the endometriotic (12Z) cells at 24 hours post treatment, but nuclear translocation of the protein was not observed (Figure 4.9). None of the other concentrations had an effect on p65 protein localization. The IL-17 had no effect on the endometrial (Ishikawa) cells at any of the time-points irrespective of treatment.

Activation of the MAPK signaling pathway

Given the variable results obtained with the IκBα phosphorylation and p65 nuclear translocation assessment, we then decided to evaluate whether IL-17 was acting on the endometrial and endometriotic cells through an alternate signaling pathway. The mitogen activated protein kinase (MAPK) is one such signal transduction pathway that is commonly associated with processes that regulate mitosis, cell motility, survival or apoptosis and differentiation. IL-17 is thought to activate this pathway through phosphorylation of extracellular signal-regulated kinases (ERK 1/2), the major signaling molecule in the MAPK pathway. We tested this by treating both endometrial (Ishikawa) and endometriotic (12Z) cells with different doses of recHuIL-17 (100 ng/ml, 10 ng/ml, 1 ng/ml and 10 pg/ml) and assessed ERK 1/2 phosphorylation at 0, 15 min, 60 min and 24 hours. We observed that the higher concentrations of IL-17 (100 ng/ml and 10 ng/ml) strongly phosphorylated ERK1/2 in the endometriotic (12Z) cells but did not have a definitive effect on the endometrial (Ishikawa) cells (Figure 4.10.A and 4.10.B). This effect was seen to peak at 60 minutes post treatment in the endometriotic (12Z) cells and seemed to wane by 24 hours post treatment.

EMMPRIN Expression

Because elevated EMMPRIN expression in ectopic lesions is associated with cell survival and migration (91), we wanted to determine whether the heightened cell motility observed in the earlier experiments could be due to increased EMMPRIN expression as a result of IL-17 signaling. We treated both cell types with recHuIL-17 (100 ng/ml, 10 ng/ml, 1 ng/ml and 10 pg/ml) and evaluated EMMPRIN protein expression in cell lysates at 0, 15 min, 60 min and 24 hours. Our results showed that IL-17 treatment at all doses increased EMMPRIN expression starting at 15 min and peaking at 60 minutes post treatment. This effect was more apparent in the endometriotic (12Z) cells and was not very consistent in the endometrial (Ishikawa) cells (Figure 4.11.A and 4.11.B).

IL-17 and EMMPRIN expression in *ex vivo* patient samples

Since EMMPRIN is involved in a number of pro-inflammatory conditions, we decided to assess the correlation between IL-17 and EMMPRIN expression in endometrial biopsy samples collected from patients with endometriosis. H-Score analyses of immunohistochemistry samples revealed that for a majority of patients, IL-17 and EMMPRIN protein expression were positively correlated. Patients with a high IL-17 expression H-Score showed elevated EMMPRIN expression in both the glandular and stromal compartments of the endometrium. Patients with a low IL-17 H-Score showed a physiological stromal expression, which was expected as the samples were collected during the secretory phase of the patients' menstrual cycles (Figure 4.12). From these results we can propose that uterine epithelial cell (glandular) expression of EMMPRIN may possibly be regulated by IL-17 action.

DISCUSSION

Endometriosis is an estrogen-dependent disorder defined by the presence of endometrial cells outside the uterus, which induces a chronic inflammatory microenvironment. This condition is associated with chronic pelvic pain, dysmenorrhea, ovulation pain, abnormal bleeding and infertility (134). Despite significant steps in endometriosis research, the pathogenesis of endometriosis is still unclear. Sampson's retrograde menstruation theory explains how the endometrial tissue reaches the extra-uterine regions but it is yet not completely understood how these tissue fragments form the ectopic lesions. But it is accepted that inflammation is a key feature associated with endometriosis. Pro-inflammatory factors such as cytokines, chemokines and matrix metalloproteinases are produced by these ectopic lesions and found in the peritoneal fluid of patients (58,59) and in turn are thought to aid in the survival of these ectopic lesions. One such cytokine is Interleukin 17 (IL-17), which is known to drive inflammation (46), is elevated in the peritoneal fluid of patients with endometriosis (115) and in turn induces the production of other pro-inflammatory factors (135) that affect endometriotic cells. But it is not known whether this pro-inflammatory cytokine plays a role in the initial stages of endometriotic lesion establishment. Our results show that the pro-inflammatory cytokine interleukin-17 (IL-17) positively impacts endometriotic cell migration and invasion, and induces epithelial to mesenchymal transition in these ectopic cells but has no effect on the endometrial epithelial cells.

Cell proliferation and motility are key properties epithelial cells acquire as they transition into a mesenchymal phenotype. It is known that endometrial cells from different sources i.e. from eutopic endometrium and ectopic endometriotic lesions have different proliferative capabilities (136). And it is also known that cytokines affect cell proliferation and motility (39).

Our proliferation assay experiments showed that neither of the doses had any significant effect on endometrial or endometriotic cell growth. A previous study has shown that higher ng/ml IL-17 doses (similar to our 100ng/ml and 10ng/ml doses) had a positive effect on the proliferation of endometriotic stromal cells (137) after 48 hours of treatment. But on the other hand it has also been shown that IL-17 treatment has no effect on the proliferation of JEG-3 human choriocarcinoma cells in culture (138) or hepatocellular carcinoma cells in culture (133). These results could indicate that IL-17 has a differential effect on cell proliferation depending on the cell type it interacts with.

We then tested the effect of IL-17 treatments on cell motility parameters of migration and invasion. The 100ng/ml treatment had a significant positive effect on the endometriotic (12Z) cell migration at 24 hours post treatment as compared to the vehicle treated controls. IL-17 has a similar effect on rheumatoid arthritis synoviocyte migration (139) and hepatocellular carcinoma cells in culture (133). None of the other doses tested (10ng/ml, 1ng/ml or 10pg/ml) had an effect on the endometriotic cells. Moreover, the IL-17 treatment had no effect on the endometrial (Ishikawa) cells at any of the treatment levels or time-points. Another property of cell motility is the invasive capacity of the cell. IL-17 has been shown to enhance the invasive ability of hepatocellular carcinoma cells in culture (133) and JEG-3 choriocarcinoma cells in culture (138). Our cell invasion assay also showed us a similar result. The two high doses of 100ng/ml and 10ng/ml increased endometriotic (12Z) cell invasion through a basement membrane-like membrane at 24 hours post treatment, but had no effect on the endometrial (Ishikawa) cells.

This differential effect of IL-17 on endometrial and endometriotic cells in culture was a very interesting finding. Then again, eutopic and ectopic stromal cells from patients have been shown to exhibit differential properties with regards to their proliferative and motility parameters

(136). Hence we decided to assess whether the IL-17 receptor (IL-17R) expression levels were contributing to this differential effect observed. The IL-17R family comprises of five subunits, IL-17RA-IL-17RE, that act as homo- or heterodimers depending on the IL-17 ligand interaction. We concentrated on the IL-17RA subunit as this is a common receptor for most IL-17 ligand subunits and is ubiquitously expressed. Moreover, this receptor subunit is involved in the main response to IL-17A that occurs in epithelial and fibroblast cells (46). Our results showed that the IL-17R had a near equal expression level in both our cell types. This was even more intriguing as it confirmed our previous hypothesis that the cytokine had a differential effect depending on the cell type it interacted with. This differential effect could be explained by the involvement of another IL-17R subunit or a shared receptor subunit (such as the gp130 for IL-6R) in this interaction. It could be further explored by evaluating the expression of the other known IL-17R subunits in both cell types.

This differential effect is not confined to cell motility. It has been shown that the process of EMT, which is important for cell migration and invasion, is inherently differently regulated in endometrial and endometriotic cells (140). We wanted to evaluate whether IL-17 could add to this differential effect on EMT in our cell types. Upon treatment with the 100ng/ml dose of IL-17 for 24 hours, the endometriotic (12Z) cells showed an elongated fusiform appearance with the presence of dendritic processes. This is a common morphology acquired by cells that are actively undergoing the EMT process (109,139). The endometrial (Ishikawa) cells showed this change only with the TNFα (positive control) treated group. Moreover, we did not observe any differences in the typical EMT protein marker expression. The cytokeratin (epithelial marker) expression was seen only in the endometrial (Ishikawa) cells and not in the endometriotic (12Z) cells, even though both cell types are established epithelial cell lines. On the other hand, both cell

types expressed vimentin (mesenchymal marker), irrespective of the treatment dose or time-point. Earlier studies have also shown similar results wherein they observed functional effects but none on EMT markers (133,139). Our findings on the EMT marker expression could also be explained by the fact that we used immortalized cell lines that have undergone the EMT process and hence possibly retain some of their mesenchymal markers acquired during that transformation (141). This also indicates that we need to evaluate some other protein markers that play a role in EMT related cell motility, such as Snail, Slug and Twist, among others.

With our results thus far, we observed that IL-17 had a functional effect on cell motility but did not alter the typical EMT marker expression. Thus we wanted to assess the signaling pathway of choice for the functional effect seen. The Nuclear Factor κB (NFκB) pathway is considered the classical pro-inflammatory signaling pathway as it is induced by inflammatory cytokines and in turn enhances the expression of pro-inflammatory genes and factors (142). IL-17 is known to activate a highly pro-inflammatory program of gene expression via activation of the NFκB pathway. It does this by causing phosphorylation of the IκB molecule (an inhibitor of the NFκB molecule) which then activates the canonical NFκB pathway (46). Our immunoblot experiments to assess the phosphorylated form of IκBα (pIκBα) were inconsistent with regards to acquiring the band of choice. On further discussion with other labs that had had a similar problem visualizing the pIκBα protein, we decided to see if the total IκBα protein showed any reduction in its expression as compared to GAPDH (as loading control). In spite of our failure with visualizing the pIκBα protein, we were able to observe a consistent and sustained effect of IL-17 on the total protein expression. In the endometriotic (12Z) cells, all the IL-17 doses seemed to increase the total IκBα protein expression at 15 minutes post treatment and this expression then dropped over time to its lowest at 24 hours post treatment. In the endometrial

(Ishikawa) cells, the total IκBα protein expression seemed to drop at 15 minutes post treatment but came back to the original levels by 60 minutes and stayed elevated at 24 hours post treatment. Unfortunately, none of these changes were statistically significant.

Due to our problems with visualizing the pIκBα protein and the not so significant results with the total IκBα protein immunoblots, we decided to assess the nuclear translocation of p50 and p65 subunits, which are components of the NFκB pathway. Previous research has shown that treatment with IL-17 causes these subunits to move into the nucleus and induce the downstream signaling that eventually leads to cell motility and EMT effects (133,143). Our results with the detection of the p65 subunit revealed that treatment with IL-17 at the highest dose of 100ng/ml caused an enhanced cytoplasmic expression of p65 as detected by the fluorophore in the endometriotic (12Z) cells, but we did not observe a significant nuclear translocation of the protein. The endometrial (Ishikawa) cells did not show any effect on the p65 expression post IL-17 treatment. A different treatment time-point, either a 12 hour or 36 hour time-point, might help us better understand whether the cytoplasmic increase in p65 was in fact preceding the nuclear translocation of the protein or was a remnant of its activity. Overall our data did not give us significant proof that the IL-17 treatment was causing activation of the NFκB pathway in our uterine cell types. This could be explained by the fact that IL-17 is considered a weak NFκB activator and usually has a stronger pro-inflammatory effect secondary to TNFα activation of the NFκB pathway (144).

It could also be indicative that IL-17 could be acting via a different signaling pathway in our uterine cells. It has been suggested that IL-17 activates extracellular signal-regulated protein kinase (ERK) which in turn up-regulates other pro-inflammatory factors (41,44). MAPK pathways are some of the most common signaling pathways associated with cell motility,

survival and differentiation (50). In fact treatment of endometriotic stromal cells with IL-17 caused an immediate increase in the phosphorylation of P42/44 MAPK (ERK1/2) (137). Our results showed an increase in the ERK 1/2 phosphorylation in the endometriotic (12Z) cells when they were treated with the two higher doses of IL-17 (100ng/ml and 1ng/ml) but not with the lower doses (1ng/ml and 10pg/ml). This increase in pERK 1/2 peaked at 60 minutes post treatment and decreased to the original levels by 24 hours post treatment. The endometrial (Ishikawa) cells showed an increase in pERK 1/2 but this effect was variable at each treatment x time-point interaction and hence not consistent. This enhanced pERK 1/2 and MAPK activation could explain the increased migration and invasion capabilities of the endometriotic (12Z) cells that we have observed with our cells. As it has been shown earlier that IL-17 induced ERK 1/2 activation is responsible for enhanced migration and invasion of cervical cancer cells (143) and p38 MAPK activation is responsible for enhanced migration and invasion of nasopharyngeal cells (145). In fact in the baboon model of endometriosis, they assessed the gene expression of different signaling pathways and their components and found that the ERK/MAPK pathway was dysregulated which was thought to be responsible for the increased MMP activity that helped in lesion establishment and invasion (146). The ERK 1/2 pathway activation is also linked to enhanced EMMPRIN and MMP expression in ovarian carcinomas (147).

And because it is well known that enhanced MMP activity is needed for cell migration and invasion, we decided to focus on the upstream factors that regulate MMP activity. One such pro-inflammatory factor that upregulates MMPs which in turn degrade basement membrane proteins thus allowing cells to invade into surfaces is the Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) (89). EMMPRIN plays an important role in reproductive processes as it plays a role physiological endometrial remodeling and is also thought to aid in ectopic lesion

establishment (86). Moreover, it has been proposed that EMMPRIN interacts with other proteins to affect tumor cell invasion and metastasis (148). Hence we wanted to see if IL-17 had an effect on the EMMPRIN expression in our cell types and if this could be correlated to the functional effects observed. Interestingly, we observed that all the doses of IL-17 (100ng/ml, 10ng/ml, 1ng/ml and 10pg/ml) enhanced the EMMPRIN expression at 15 minutes, which peaked at 60 minutes and decreased by 24 hours post treatment in the endometriotic (12Z) cells. This concurred with earlier findings from our lab that pro-inflammatory cytokines enhance the EMMPRIN expression in endometrial cells and in turn increase the MMP expression (149). The MMP-inducing function of EMMPRIN is thought to depend on the level of glycosylation of the extracellular N-terminal Ig domain, which is increased on exposure to inflammatory signals (79,80). We could suppose that is how IL-17 acts on enhancing the EMMPRIN expression in the endometriotic cells in culture.

The endometrial (Ishikawa) cells did not show similar response to IL-17 treatment. But it has been shown that eutopic endometrium from baboons that have endometriotic lesions express increased EMMPRIN expression in their eutopic endometrium (88) as compared to disease free controls. Hence we decided to assess the relation between IL-17 expression and EMMPRIN expression in eutopic endometrial biopsy sections taken from patients with endometriosis. Overall there was a positive correlation in about 90% of the patient samples tested. Patients with a high IL-17 expression showed an increased expression of EMMPRIN in their stromal as well as glandular compartments whereas patients with moderate IL-17 expression had an enhanced stromal expression but EMMPRIN was not expressed in their glands. This could suggest that elevated IL-17 levels increase the epithelial cell expression of EMMPRIN, which we saw with our cell culture experiments as well (though our endometrial cells did not respond to IL-17

treatment). In the patients who showed a negative correlation, we could assume other factors are activating MMP production for establishment of endometriotic lesions. Cytokines are known to enhance MMP activity in human endometrial fibroblasts through processes that do not involve increases in EMMPRIN (150). Moreover, IL-17 has been shown to increase MMP expression in human periodontal ligament fibroblasts but not have any effect on EMMPRIN expression in these cells (151).

Overall our data suggests that the pro-inflammatory environment produced by elevated IL-17 levels activates EMMPRIN expression and both these factors either in coordination or on their own act on induction of the MAPK pathway, which in turn regulates the functional effects we observed with our cell culture experiments. As research in the area of IL-17 regulated EMMPRIN expression is scarce, our data could be one of the first to show a positive correlation between the two pro-inflammatory factors. It would be interesting to assess the actual process by which IL-17 regulates EMMPRIN expression. Cytokines are known to enhance the sequestration of EMMPRIN in microvesicles released by human uterine fibroblast cells (149). Moreover, it is known that protease laden exosomes, released by tumor cells, aid in tumor invasion and metastasis (152). Hence it would be interesting to evaluate the exosome production and EMMPRIN sequestration therein as an effect of IL-17 on endometrial and endometriotic cells.

FIGURES and TABLES

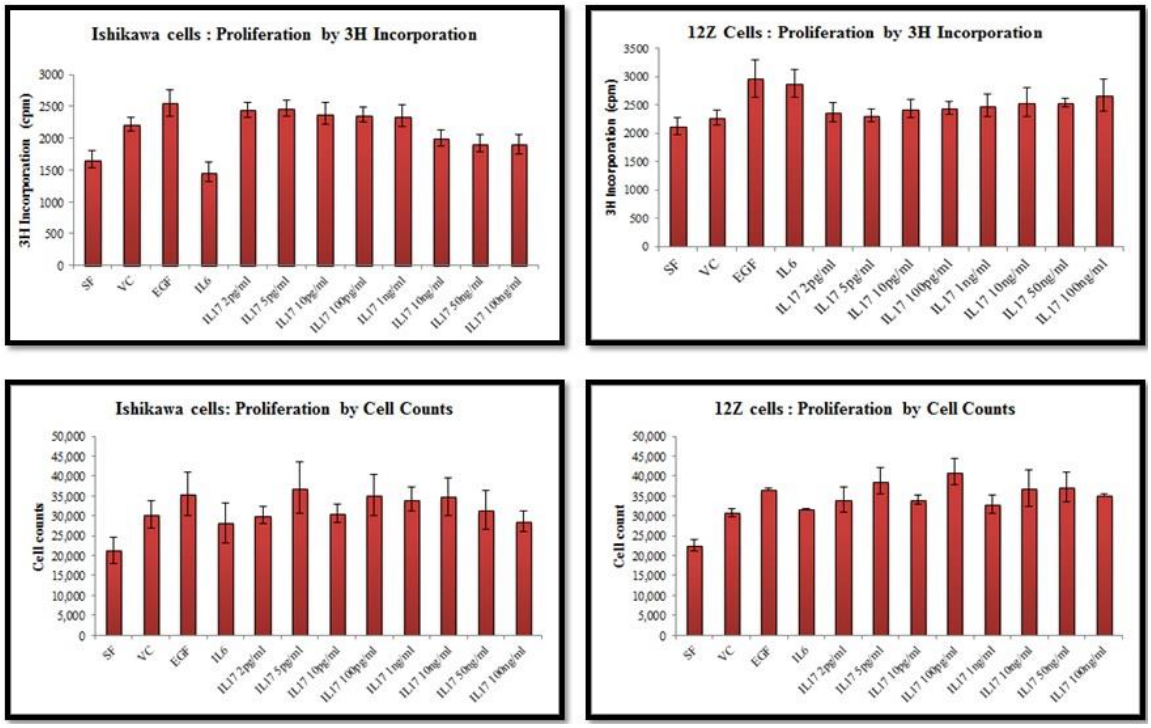


Figure 4.1. IL-17 has no effect on the proliferation of endometrial (Ishikawa) and endometriotic (12Z) cells in culture.

Endometrial and endometriotic cells were treated with recHuIL-17 doses ranging from 2 pg/ml to 100 ng/ml. Effect on cell proliferation was assessed by cell tritiated thymidine incorporation and cell count assays. (n=3, $p>0.05$).

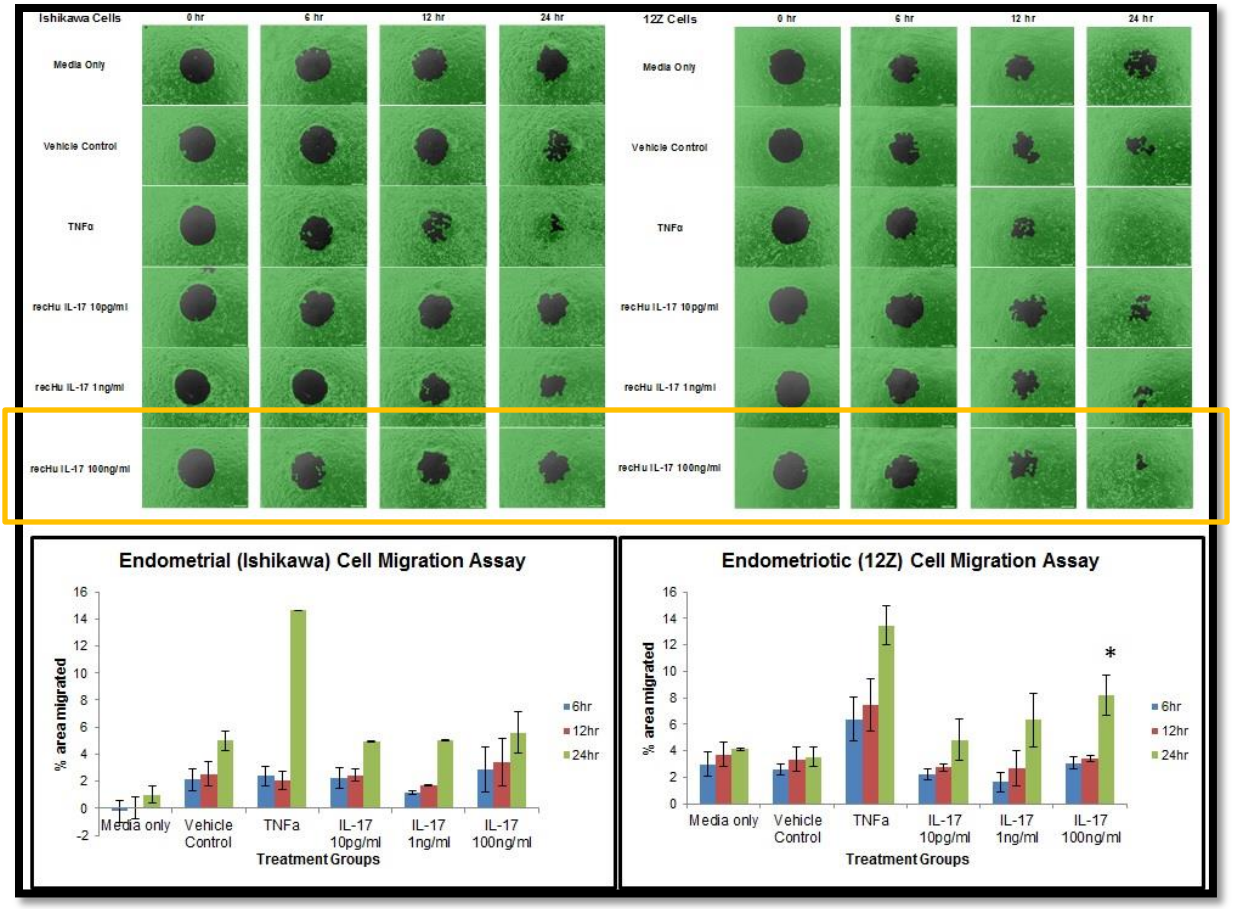


Figure 4.2. IL-17 positively affects migration of endometriotic (12Z) cells but has no effect on the migration of endometrial (Ishikawa) cells in culture.

Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, and 100 ng/ml. Cell migration was assessed at 0, 6, 12 and 24 hours post treatment. Graphs represent actual percent area the cells migrated as compared to the 0 hour starting point. (n=3, $p<0.05$)

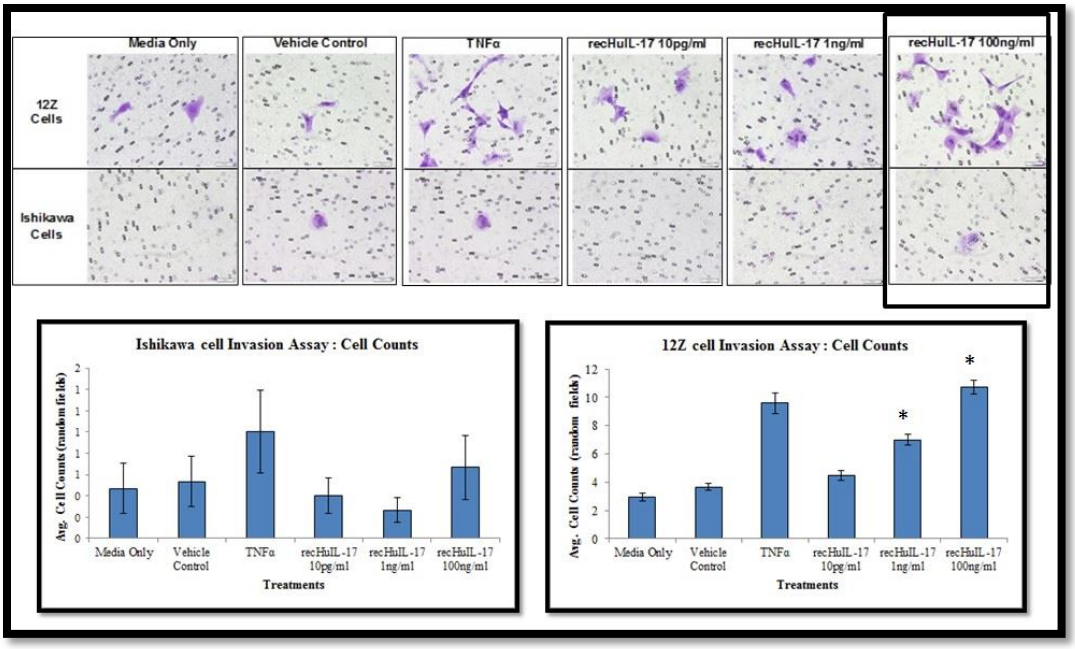


Figure 4.3. IL-17 has a positive effect on the invasive capability of endometriotic (12Z) cells but has no effect on the endometrial (Ishikawa) cells in culture.

Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, and 100 ng/ml. Cell invasion through a basement membrane-like membrane was assessed at 24 hours post treatment. Graphs represent the cell counts for the invading cells evaluated from five separate random fields for each treatment. ($n=3$, $p<0.05$)

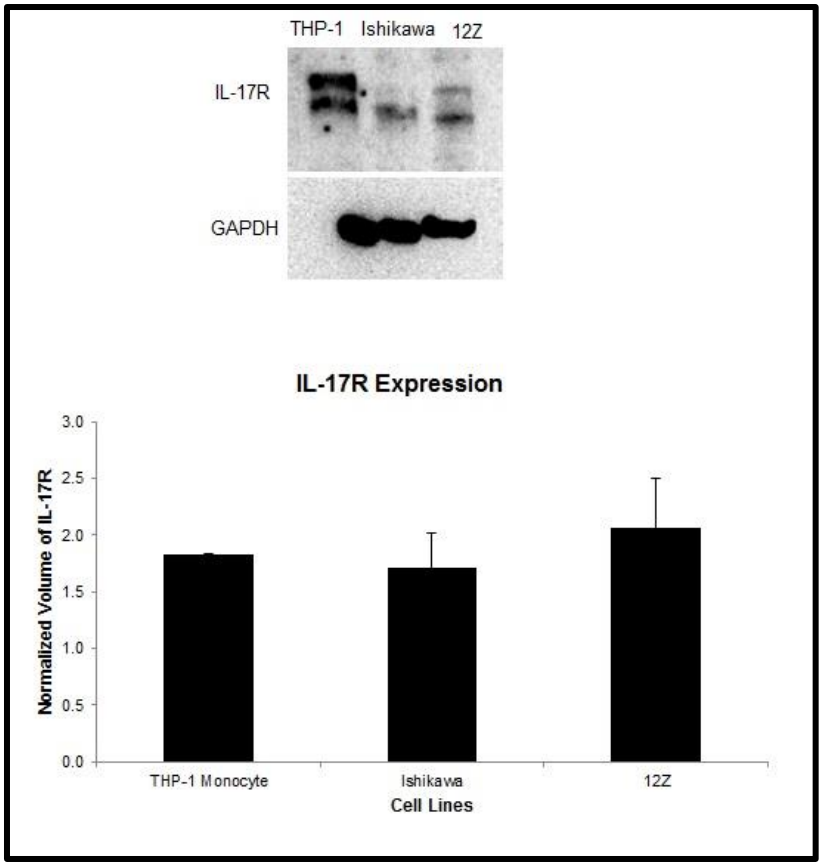


Figure 4.4. IL-17-Receptor expression in both endometrial (Ishikawa) cells and endometriotic (12Z) cells is similar.

Cell lysates collected from endometrial and endometriotic cells grown in 2% FBS containing medium were used for immunoblot estimation of the IL-17 receptor. THP-1 monocyte cell line was used as a positive control for receptor expression. Receptor expression on the immunoblot was normalized to GAPDH expression in the respective samples. (n=3, $p>0.05$)

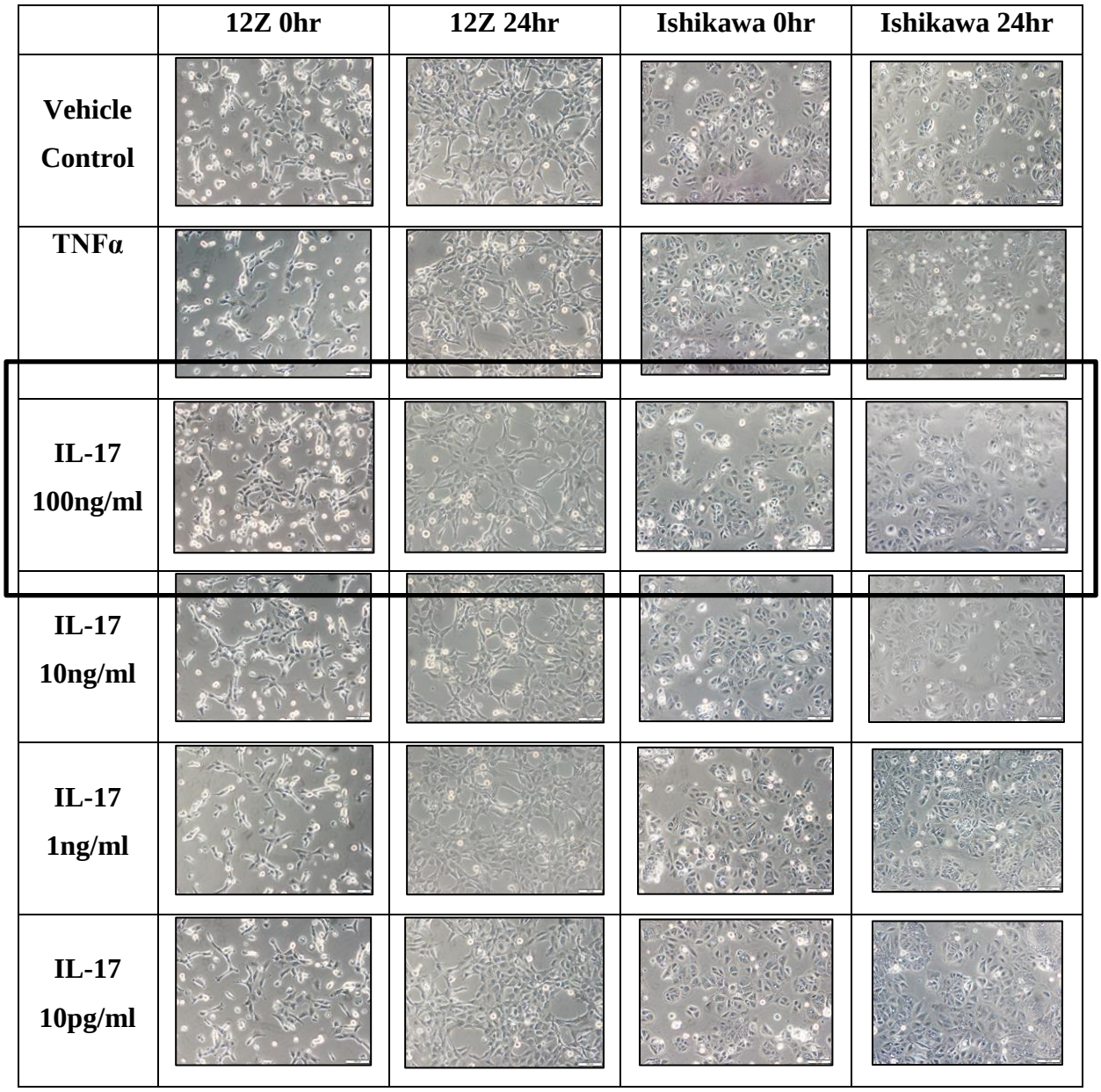


Figure 4.5. Morphological evaluation of EMT: IL-17 treatment shows a fusiform morphology with dendritic processes in the endometriotic (12Z) cells but has no effect on the endometrial (Ishikawa) cells.

Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell morphology was assessed at 0, 6, 12 and 24 hours post treatment. Only the 0 hour and 24 hour images are shown here as the 6 and 12 hour treatments did not show any change in morphology. All images collected at 10X magnification. (n=3)

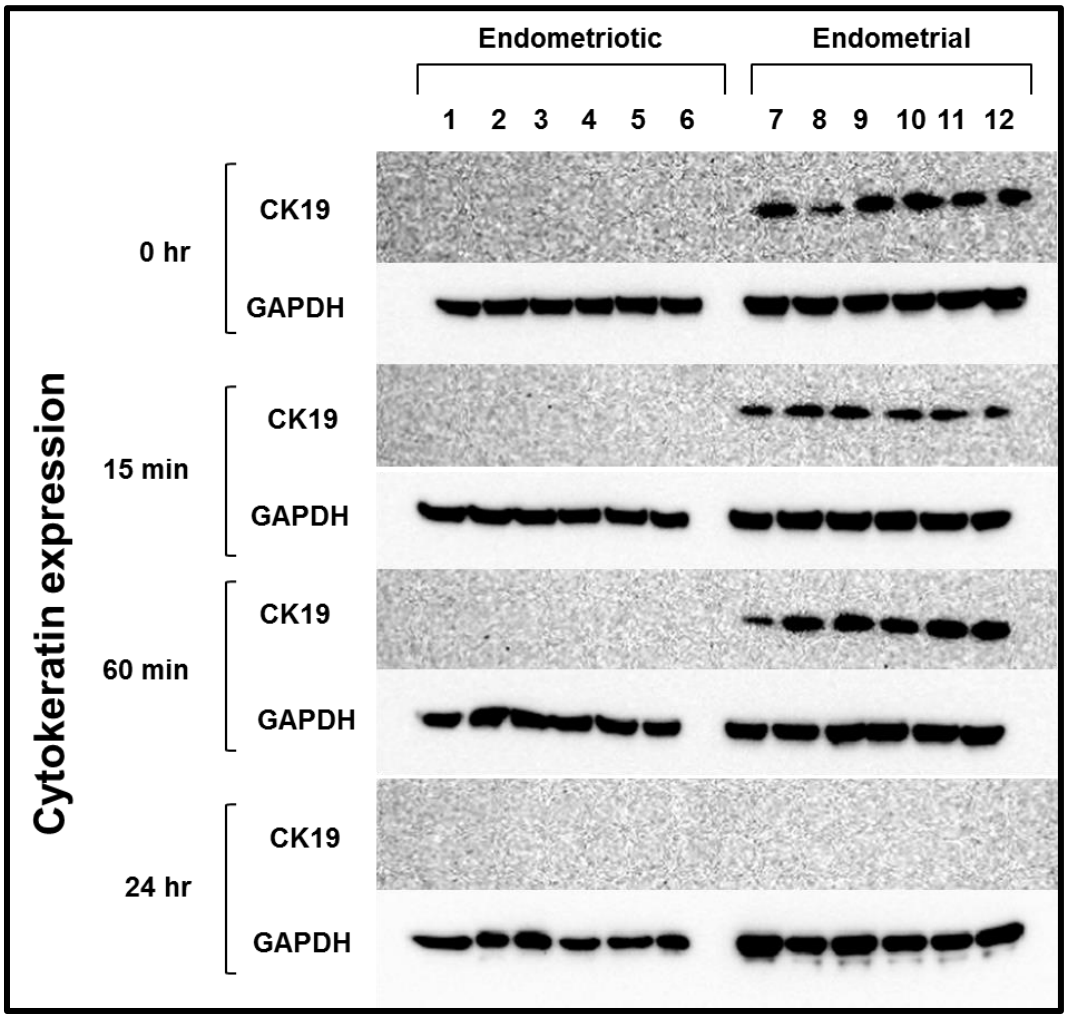


Figure 4.6. IL-17 had no significant effect on the cytokeratin protein expression.
Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell lysates collected at 0, 15 min, 60 min and 24 hours post treatment were processed by immunoblotting techniques and probed for cytokeratin protein expression. Bands obtained for each treatment x time-point combination were normalized to GAPDH expression for respective treatment x time-point combination. Lanes 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Lanes 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. ($n=3$, $p>0.05$)

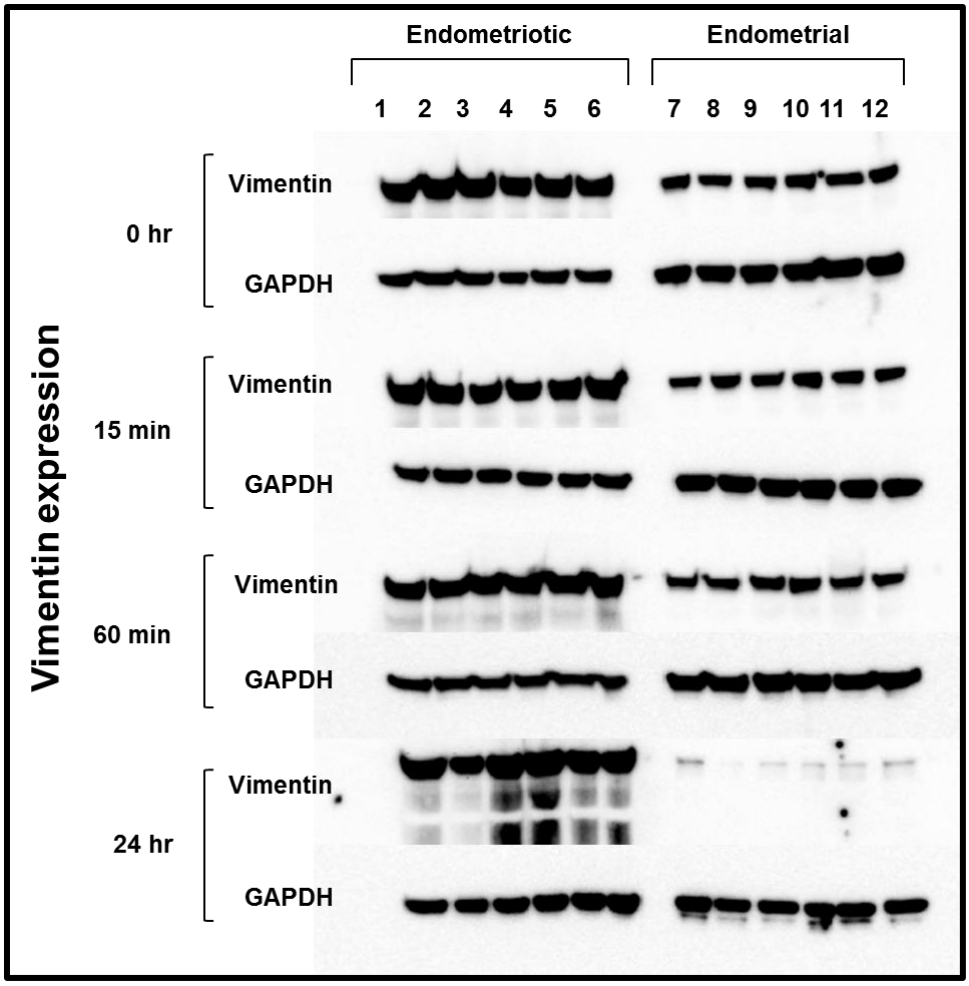


Figure 4.7. IL-17 had no significant effect on the vimentin protein expression.
Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell lysates collected at 0, 15 min, 60 min and 24 hours post treatment were processed by immunoblotting techniques and probed for vimentin protein expression. Bands obtained for each treatment x time-point combination were normalized to GAPDH expression for respective treatment x time-point combination. Lanes 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Lanes 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. (n=3, $p>0.05$)

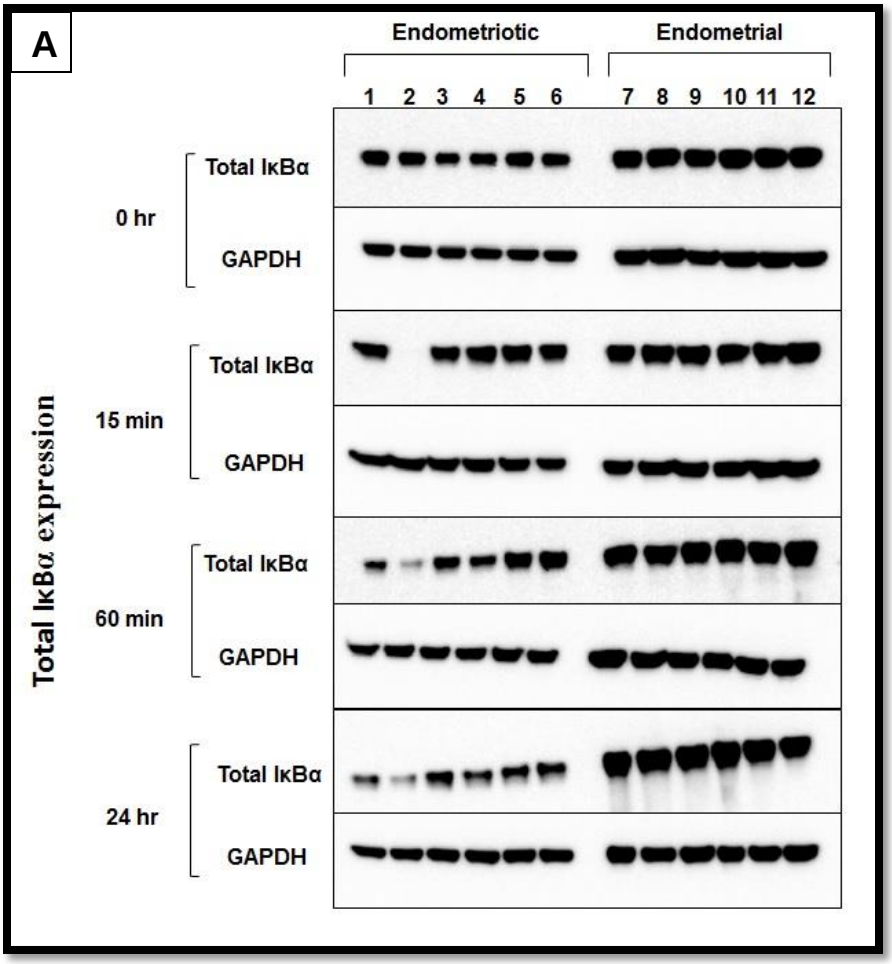


Figure 4.8.A. IL-17 had no significant effect on the total IκBα protein expression.
Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell lysates collected at 0, 15 min, 60 min and 24 hours post treatment were processed by immunoblotting techniques and probed for total IκBα protein expression. Bands obtained for each treatment x time-point combination were normalized to GAPDH expression for respective treatment x time-point combination. Lanes 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Lanes 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. (n=3, $p>0.05$)

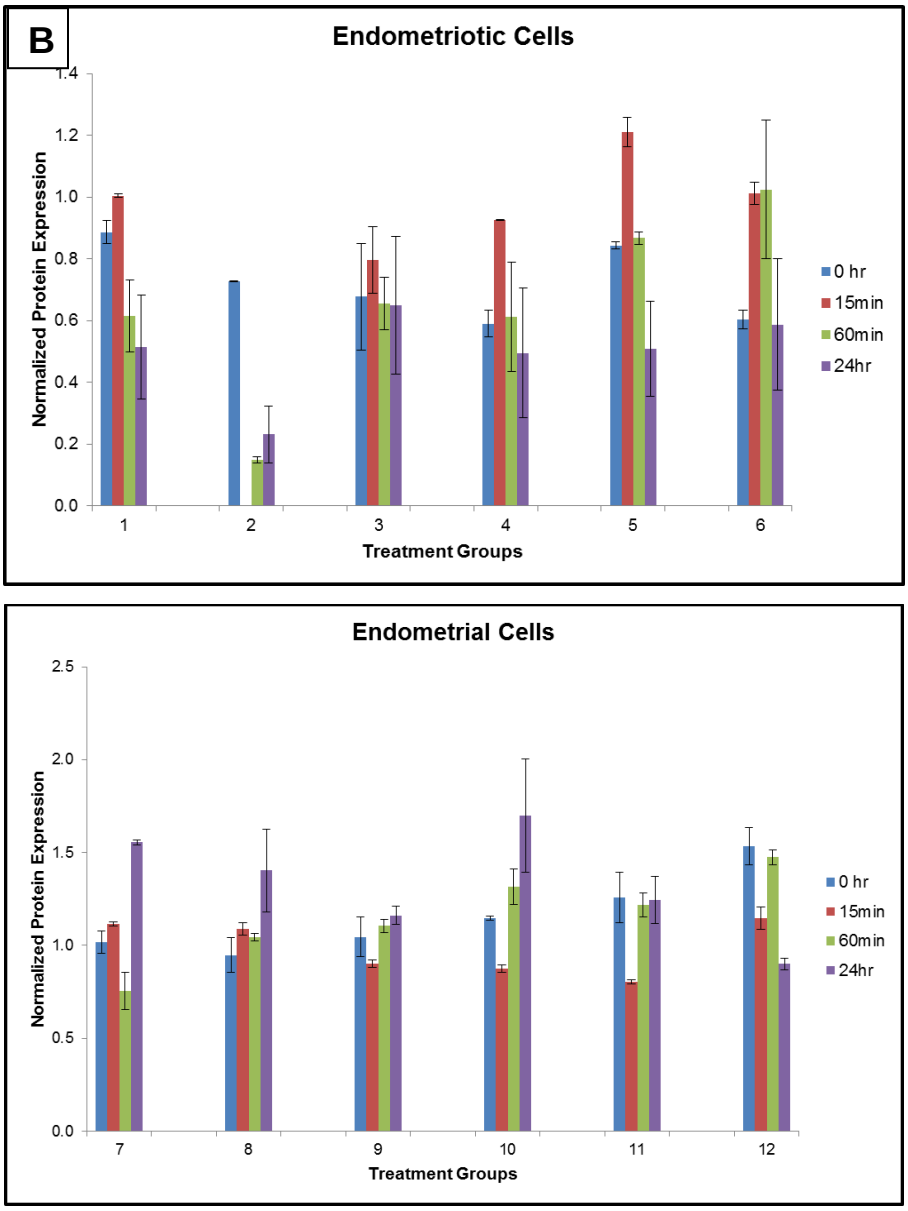


Figure 4.8.B. IL-17 had no significant effect on the total IκBα protein expression.
Densitometric analysis of immunoblot shown in figure 4.6.A. Immunoblot bands obtained for each treatment x time-point combination were normalized to GAPDH expression for respective treatment x time-point combination. Treatment groups 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Treatment groups 7–12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. (n=3, $p>0.05$)

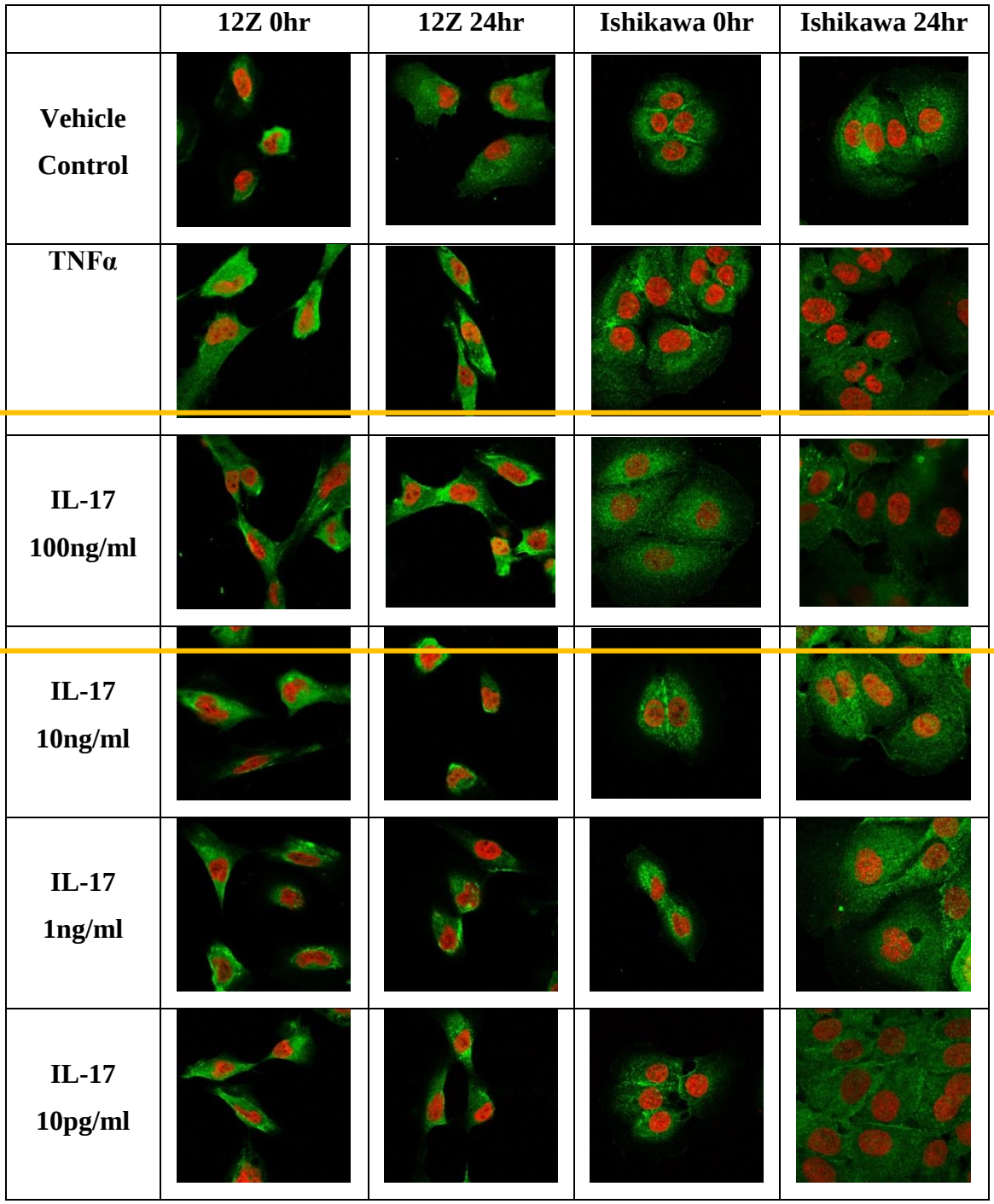


Figure 4.9. IL-17 enhanced the cytoplasmic expression of p65 subunit in the 100ng/ml IL-17 treated endometriotic (12Z) cells but had no effect on the endometrial (Ishikawa) cells. Endometrial and endometriotic cells were treated with recHuIL-17 at 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Immunofluorescence techniques were utilized to visualize p65 subunit movement within the cells. All images were obtained at 60x magnification. (n=3)

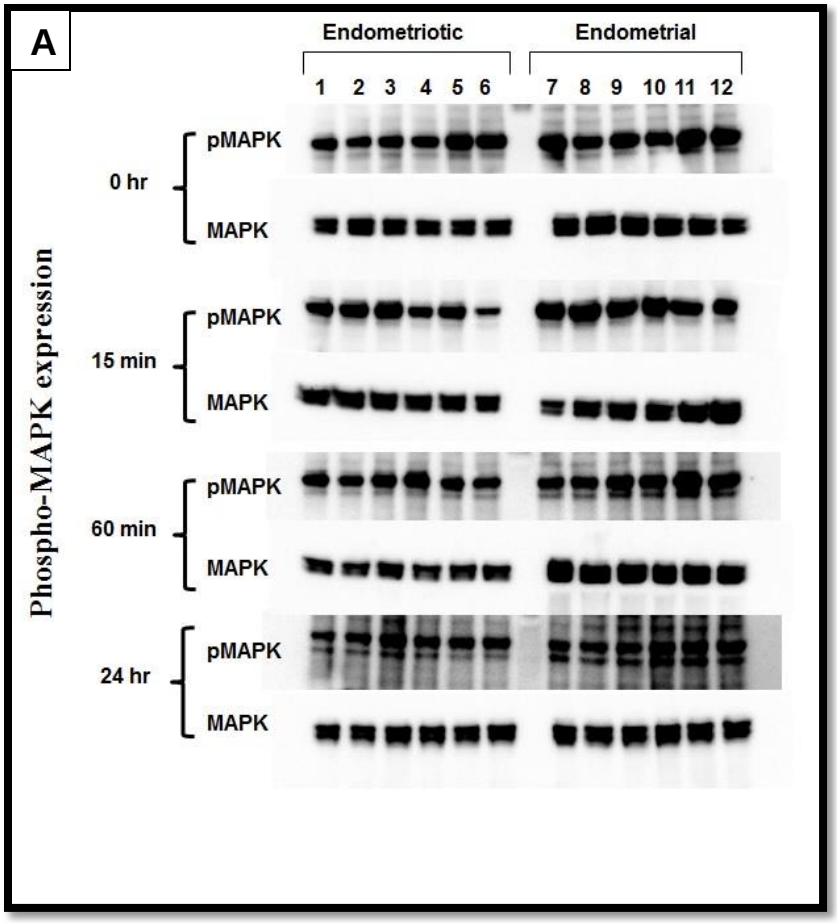


Figure 4.10.A. IL-17 increased phosphorylation of MAPK (ERK 1/2) in endometriotic (12Z) cells but had no specific effect in the endometrial (Ishikawa) cells.

Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell lysates collected at 0, 15 min, 60 min and 24 hours post treatment were analyzed by immunoblotting technique and probed for pMAPK and total MAPK protein expression. pMAPK bands for each treatment x time-point combination was normalized to its respective total MAPK band. Lanes 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Lanes 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml ($n=3$, $p<0.05$)

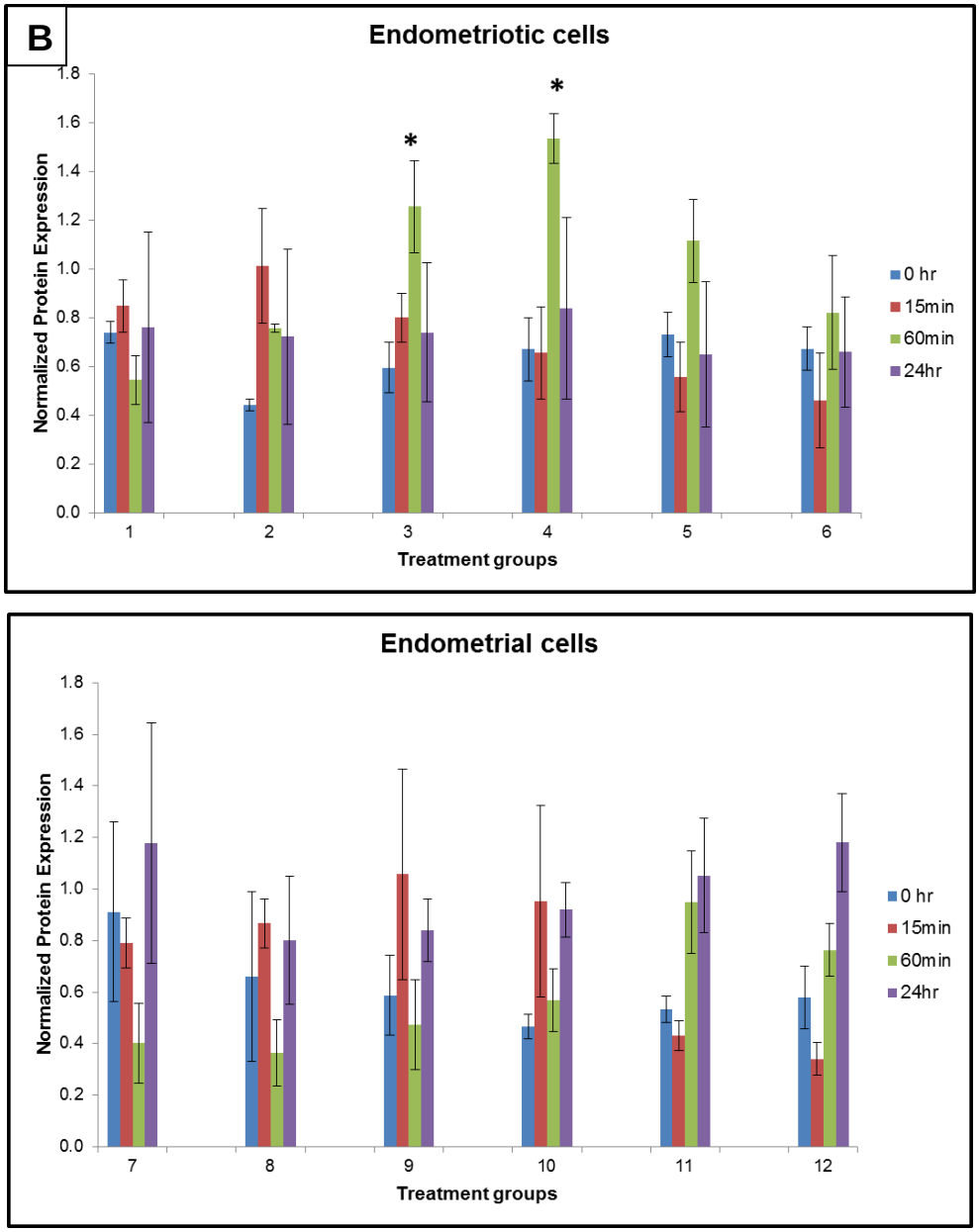


Figure 4.10.B. IL-17 increased phosphorylation of MAPK (ERK 1/2) in endometriotic (12Z) cells but had no specific effect in the endometrial (Ishikawa) cells.

Densitometric analysis of immunoblot shown in figure 4.8.A. phospho-MAPK bands obtained for each treatment x time-point combination were normalized to total-MAPK expression for respective treatment x time-point combination. Treatment groups 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Treatment groups 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml (n=3, $p<0.05$)

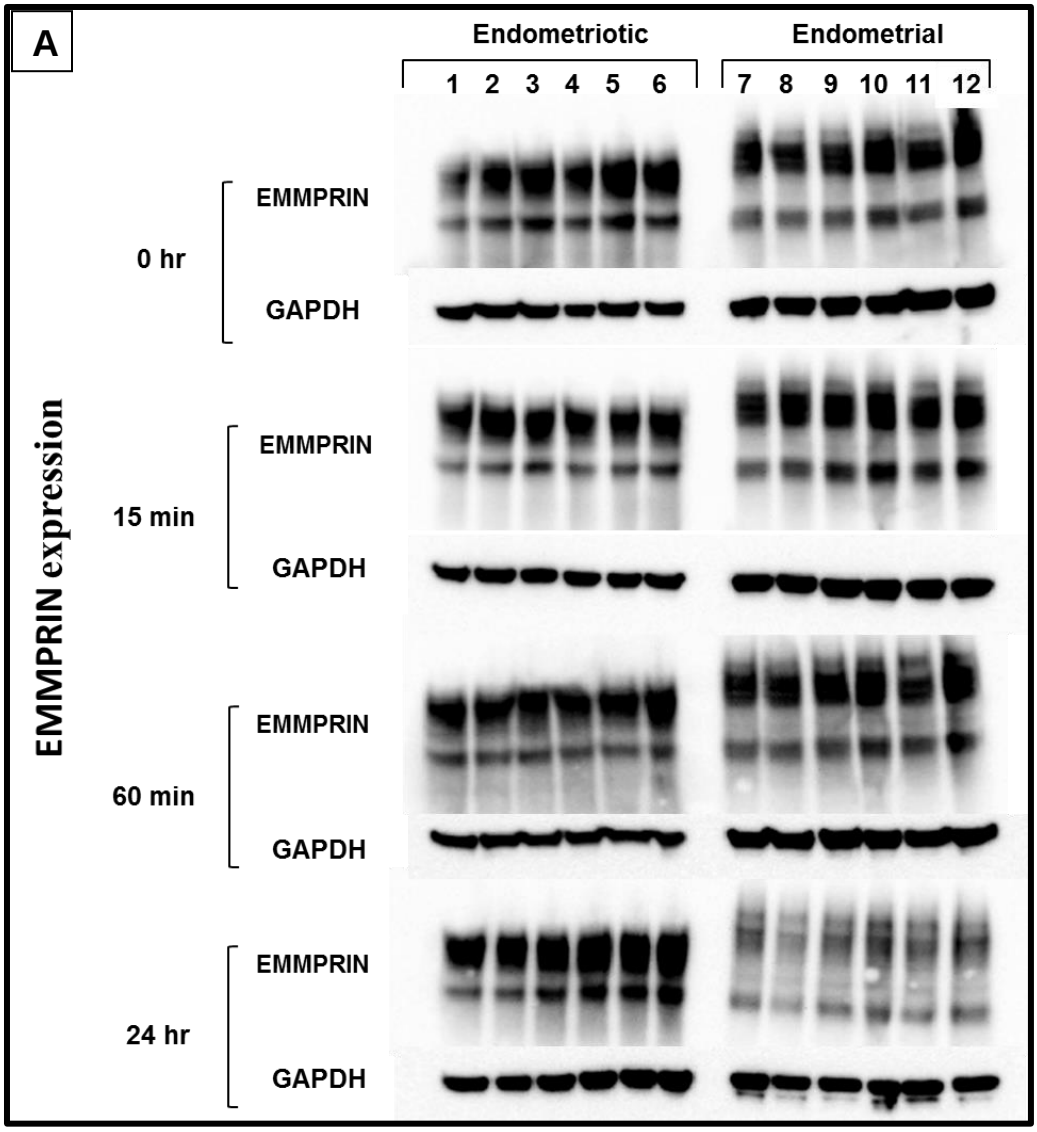


Figure 4.11.A. IL-17 increased EMMPRIN expression in endometriotic (12Z) cells but had no specific effect in the endometrial (Ishikawa) cells.

Endometrial and endometriotic cells were treated with recHuIL-17 doses of 10 pg/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml. Cell lysates collected at 0, 15 min, 60 min and 24 hours post treatment were analyzed by immunoblotting technique and probed for EMMPRIN protein expression. EMMPRIN bands for each treatment x time-point combination were normalized to their respective GAPDH band. Lanes 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Lanes 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml ($n=3$, $p<0.05$)

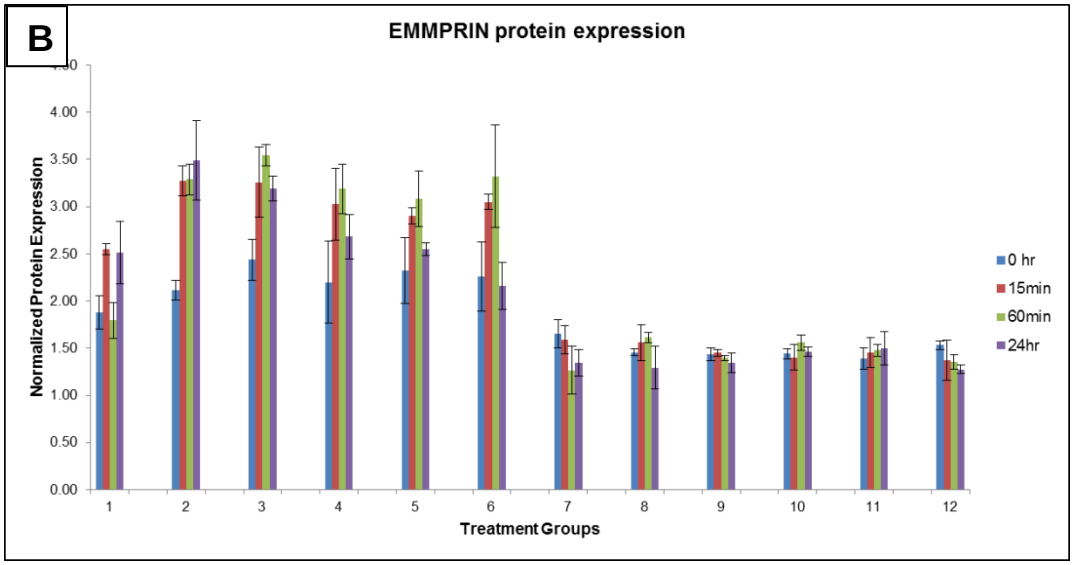
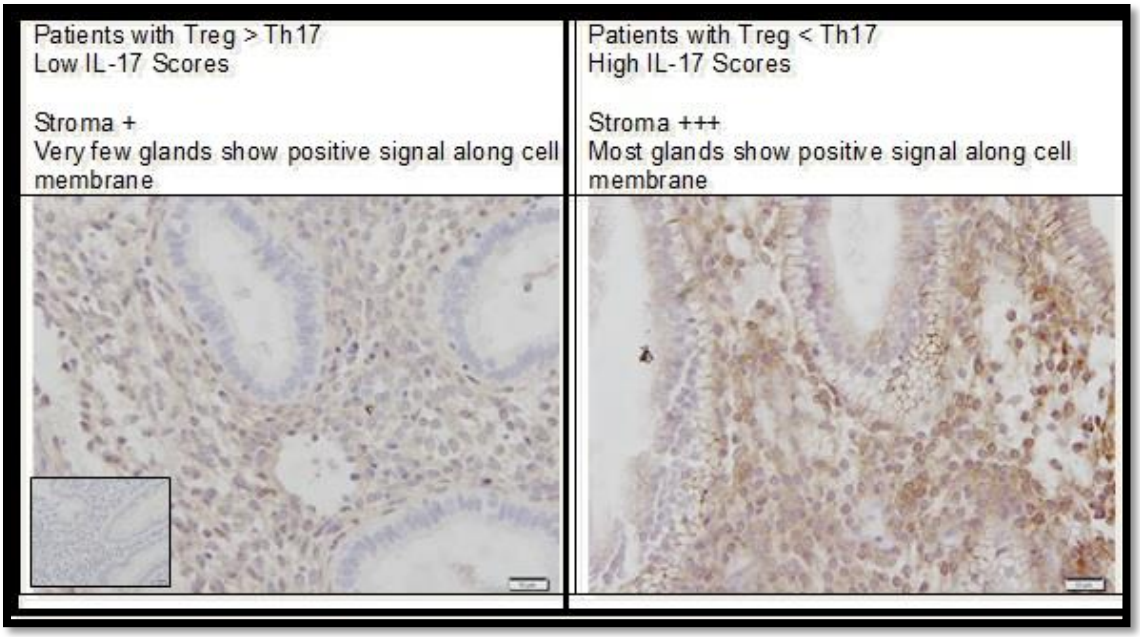


Figure 4.11.B. IL-17 increased EMMPRIN expression in endometriotic (12Z) cells but had no specific effect in the endometrial (Ishikawa) cells.

Densitometric analysis of immunoblot shown in figure 4.9.A. EMMPRIN bands obtained for each treatment x time-point combination were normalized to GAPDH expression for respective treatment x time-point combination. Treatment groups 1-6 are samples from endometriotic 12Z cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml. Treatment groups 7-12 are samples from endometrial Ishikawa cells treated with vehicle control, recHuTNFα, IL-17 100 ng/ml, IL-17 10 ng/ml, IL-17 1 ng/ml and IL-17 10 pg/ml ($n=3$, $p<0.05$)



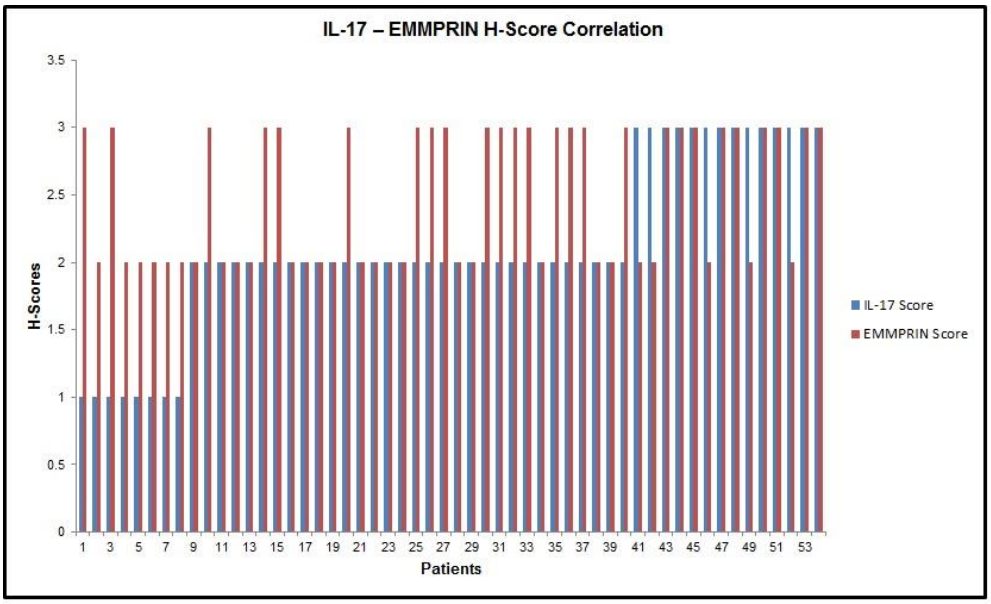


Figure 4.12. Elevated IL-17 expression is positively correlated to elevated EMMPRIN expression in eutopic endometrial biopsy specimens collected form patients with endometriosis.

Endometrial biopsies collected from patients during the secretory phase of their menstrual cycles were evaluated using immunohistochemistry for IL-17 and EMMPRIN expression. (n=54)

Experimental Endpoints	Endometrial cells (Ishikawa cells)	Endometriotic cells (12Z cells)
Cell Proliferation	No Effect	No Effect
Cell Migration	No Effect	Increased
Cell Invasion	No Effect	Increased
IL-17 Receptor Expression	Similar	Similar
EMT Morphology	No Effect	Fusiform with dendritic processes (100 ng/ml, $p<0.05$)
Cytokeratin Expression	No Effect	Not Expressed
Vimentin Expression	No Effect	No Effect
Total IκBα Expression	No Effect	No Effect
p65 Subunit Nuclear Sequestration	No Effect	Increased cytoplasmic expression at 24 hours (100 ng/ml)
pMAPK Expression	No Effect	Increased at 60 min (100 ng/ml and 10 ng/ml; $p<0.05$)
EMMPRIN Expression	No Effect	Increased at 60 min (100 ng/ml and 10 ng/ml; $p<0.05$)

Table 4.1. Functional and protein expression end points in endometrial and endometriotic cells.

A differential effect of IL-17 treatment was observed with respect to migration and invasion in endometrial and endometriotic cells in culture. This effect was not due to a differential IL-17R expression as receptor expression was similar in both cell types. IL-17 treatment did not induce the hallmark NFκB signaling pathway but instead caused activation of the alternative MAPK signaling pathway in the endometriotic cells. Overall, IL-17 treatment did not have any effect on the endometrial cells.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTION

Endometrial receptivity is a complex phenomenon dependent on the precise synchronization of multiple factors that control structural changes in the endometrium in response to changes in circulating steroid hormones and a delicate leukocyte and cytokine equilibrium. It is therefore important to understand the immunological factors involved in implantation and early pregnancy, particularly in reproductive pathologies that may result in infertility. Endometriosis is one such condition which not only has a debilitating and inflammatory effect but is also estimated to account for up to 30-50% of otherwise unexplained infertility. Evidence suggests that the increased concentrations of certain growth factors and/or cytokines in the peritoneal fluid of patients with endometriosis induce establishment and proliferation of the endometrial implants, and also may inhibit early reproductive events. It has been shown that both pro-inflammatory and immune tolerance factors co-exist at the maternal-fetal interface in a normal pregnancy. An imbalance between these factors leading to immune dysregulation in the reproductive tract of women with endometriosis may have a profound impact on the outcome of implantation and pregnancy. A key feature of endometriosis is inflammation, which involves an overproduction of cytokines, chemokines, prostaglandins and metalloproteinases. Hence our hypothesis was that presence of endometriotic lesions promotes a pro-inflammatory environment leading to shifts in the T-helper cell profiles and related cytokine milieu in the eutopic endometrium during the window of implantation (WOI).

Our summarized findings and future directions are as follows:

High Treg and low Th17 cell ratio in the eutopic endometrium is predictive of receptivity

We characterized alterations in lymphocyte cell populations in the endometrium and assessed whether the shift from an anti-inflammatory to a pro-inflammatory phenotype in the eutopic endometrium was associated with infertility. We found that patients with a high Treg and low Th17 cell population had a better chance at conceiving. In fact patients with a consistently and significantly high pro-inflammatory Th17 cell count maintained their infertile status. The ratio of the Treg to Th17 cells was a better indicator of fertility status as compared to the individual cell counts. Patients with a ratio less than 3 did not get pregnant. We also showed that laparoscopic intervention for ectopic lesion excision provided a boost to the anti-inflammatory cells and increased the chance of pregnancy in subsequent cycles. Moreover, the IL-17 expression was elevated in both the glandular and stromal compartments of the endometrium and had a strong inverse correlation to the Treg:Th17 ratio.

IL-17 promotes migration and invasion of endometriotic cells via the induction of EMT

We studied the effect of IL-17 on endometrial and endometriotic cells in culture to explore the consequences of an altered pro-inflammatory microenvironment on eutopic endometrial cells and on the processes involved in the establishment of ectopic endometriotic lesions. Though IL-17 had no effect on the proliferation of these cell types, it did have a significantly positive effect on the migration and invasion of the endometriotic cells but not the endometrial cells. This differential effect could be explained by specific interaction of this ligand with different cell types or the involvement of shared receptors, because the IL-17R was expressed at similar levels

in both cell types. Furthermore, the endometriotic cells underwent EMT-like morphological changes with fusiform shape and dendritic filaments when they were treated with IL-17. But the cytokine had no such effect on the endometrial cells. Unfortunately, we did not see any marked differences in the traditional EMT protein marker end-points. But it was interesting that IL-17 triggered the MAPK signaling pathway instead of its traditional NFκB pathway in our cell types.

IL-17 possibly regulates and/or synergizes with EMMPRIN expression in endometriotic cells but not in the endometrial cells

EMMPRIN is known to be involved in inflammatory processes and also plays an important role in matrix breakdown and cell motility. We characterized the EMMPRIN expression in the eutopic endometrium collected from the patients with endometriosis-related infertility. Our results show a positive direct correlation between the IL-17 and EMMPRIN expression in the eutopic endometrium. Our in vitro experiments with IL-17 showed an increase in EMMPRIN expression in the endometriotic cells but not in the endometrial cells. This interaction could also explain the induction of the MAPK pathway observed as both IL-17 and EMMPRIN are known to induce pro-inflammatory events via this signaling pathway.

We conclude that this project has increased our understanding of how a specific immune cell niche regulates endometrial receptivity and also plays a role in ectopic lesion establishment. Future experiments should focus on further elucidation of this effect on eutopic and ectopic cell function.

1. Our novel finding with the boosting effect of laparoscopic intervention should be pursued by increasing the sample size for patients with endometriosis related infertility in each group. Other studies have also shown promising results with

endometrial biopsy related boosting effects, which we did not observe in our patient set. This could be used as a therapeutic modality in patients undergoing IVF treatment.

2. Because laparoscopy is invasive (though minimally), alternate methods to induce the boosting effect could be explored. Use of a fallopian tube contrast agent has shown a similar effect possibly by leakage into the peritoneal cavity. Use of a less inflammatory flushing solution in a similar manner could possibly provide the desired effect.
3. MUC proteins are important during the implantation process. Loss of mucins in a controlled manner on the luminal epithelium is necessary for embryo implantation. Elevated IL-17 is known to increase mucin expression in lung epithelium. It would be interesting to see if the mucin expression in the eutopic endometrium of patients with elevated IL-17 expression is altered and whether this is associated with the infertility seen.
4. The IL-17 family of receptors has multiple subunits that act as homo- or heterodimers. As our in vitro experiments showed a differential effect with regards to cell motility, it would be beneficial to assess the role of a shared receptor in bringing about this effect.
5. The experimental techniques utilized to assess NFκB pathway involvement were not very conclusive in our study. It would be beneficial to confirm these findings with alternate and possibly more accurate techniques such as the Luciferase assay.

6. Alternatively, this could also be tested by conducting microarray analyses of IL-17 treated cells to evaluate changes in expression of genes involved in the pro-inflammatory NFκB pathway.
7. As our data show the involvement of MAPK signaling pathway, it would be prudent to assess how exactly this pathway is involved with the effects seen on cell motility. In addition, MAPK is also involved with cell survival. Endometriotic cells undergo changes that make them "resistant" to apoptosis. It would be interesting to see whether IL-17 treatment via the MAPK pathway confers this "resistance" to apoptosis.
8. Endometriotic lesion establishment involves attachment before invasion of cells takes place. Co-culture experiments with endometriotic and mesothelial cells would help us evaluate whether IL-17 could enhance the attachment and invasion of endometriotic cells into a mesothelial monolayer. This experiment could then be conducted in a 3-D co-culture system and true invasion of cells into the matrix could be evaluated.
9. Our experiments showed an enhancement of EMMPRIN expression in IL-17 treated cells. It would be good to evaluate whether downstream factors such as MMPs are also induced as a result of this. Moreover, IL-17 treatment following knock-down of EMMPRIN in cells could help elucidate whether the effect seen in the presence of EMMPRIN is also seen with it absent.
10. IL-17 is known to increase the expression of other inflammatory factors such as IL-6 which then has a positive feedback loop effect as IL-6 is needed for Th17 cell differentiation. IL-6 activity is enhanced manifold when its trans-signaling pathway is induced. This can occur due to shedding of the membrane bound receptor into a

soluble receptor. One interesting experiment could be to assess whether EMMPRIN can cause shedding of this receptor thus enhancing the pro-inflammatory effect overall.

11. Prospective inhibitors of factors involved in the inflammatory signaling pathways discussed, could be tested in the future, for clinical use in patients with endometriosis related inflammation, pain and/or infertility.

All of these and many more such ideas could help elucidate the role of IL-17 in producing the pro-inflammatory microenvironment in the uterine cells. This knowledge would help us better understand endometriosis related immune dysregulation.

CHAPTER 6

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco's Modified Eagle's Medium (DMEM F/12) (Cat#11039-047; Invitrogen), penicillin-streptomycin (Cat#30002CI; Corning), and L-glutamine (Cat#25005CI; Corning) were purchased from Life Technologies/ThermoFisher Brand. Fetal Bovine Serum (FBS Premium) (Cat#S11150) was purchased from Atlanta Biologicals. Recombinant human IL-17, recombinant human TNFα, recombinant EGF, recombinant IL-6 were purchased from R&D Systems (Refer Appendix B for details). Tritiated thymidine ([^{3}H] thymidine) was purchased from PerkinElmer. Precise Protein Gels (Cat#PI25244), BupH Tris/Hepes/SDS Running buffer (Cat#PI28398), Restore Western Blot Stripping Buffer (Cat#PI21059), SuperSignal West Pico Chemiluminescent Substrate (Cat#PI34080), Pierce ® BCA protein assay kit (Cat#23225), Immobilon-P PVDF membrane (Cat#IPVH00010; Millipore), hydrogen peroxide (Cat#H325-100), xylene (Cat#X5-4), were purchased from ThermoScientific Fisher Brand. Mayer's Hematoxylin solution (Cat#MHS32), Triton X-100 (Cat#X100), Ponceau S solution (Cat#P7170) were purchased from Sigma. Fluorophore antibodies (refer Appendix B for details), Image-IT Signal FX Enhancer (Cat#I36933), DAPI (Cat#D3571), Prolong Gold (P36930) were purchased from Invitrogen. DAKO Target Retrieval Solution pH 9.0 (Cat#S236784) was obtained from DAKO. Ibidi dishes (Cat#80826) were purchased from Ibidi, paraformaldehyde (Cat#15710) from Electron Microscopy Sciences, Precision Plus Protein All Blue Standards (Cat#1610373) from Biorad and antibodies were obtained from Abcam, Cell Signaling Technology and Santa Cruz Biotechnology Inc (refer Appendix B for details).

Cell culture

Endometrial and endometriotic epithelial immortalized/transformed cell lines were used for all cell culture experiments. Ishikawa endometrial epithelial cells were used as representative eutopic endometrial epithelial cells and were obtained from Dr. Bruce Lessey (South Carolina). The 12Z endometriotic epithelial cells were used as representative ectopic endometriotic epithelial cells and were obtained from Dr. Asgi Fazleabas (Michigan State University). These cells were cultured in DMEM/F-12 medium containing 2% heat inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin. Cells were cultured on plastic culture dishes at 37^{o}C in a humidified atmosphere of 5% $CO_{2.}$ All experimental replicates were restricted to passage number 52-54 for 12Z cells and split number 9-11 for Ishikawa cells.

Cell Proliferation Assay: Cell counts

Cells were cultured in flasks, washed once with 1x PBS to remove dead cells before dissociation with 0.25% Trypsin in 0.53mM EDTA for 5 minutes for the 12Z cells and 12-15 minutes for the Ishikawa cells at 37^{o}C. Dishes were checked for cell detachment, then 2% heat inactivated FBS containing medium was added for enzyme inactivation. Cell suspensions were centrifuged at a 100 RCF for 5 minutes, supernatant was removed, and cell pellets were resuspended in 2% heat inactivated FBS containing medium. A 10ul aliquot of this cell suspension was placed on two separate chambers of a hemocytometer for cell counts under an inverted bright field microscope. To evaluate proliferation, 10000 cells of each cell type were plated separately in each well of a 96 well plate and allowed to grow for 24 hours. Cells were then treated with serum free medium, vehicle control (0.1% BSA/PBS), recombinant EGF (100 pg/ml), IL-6 (200 pg/ml) and IL-17

(100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml, 5 pg/ml, and 2 pg/ml). Cells were trypsinized and counted 48 hours post treatments. Three experimental replicates were conducted for each cell line.

Cell Proliferation Assay: Thymidine Incorporation Assay

Thymidine incorporation assays were also used to evaluate proliferation of endometrial and endometriotic epithelial cells in response to different treatments. Cells were plated in 96 well plates as described above and allowed to grow for 24 hours. Cells were then treated with serum free medium, vehicle control (0.1% BSA/PBS), recombinant EGF (100 pg/ml), IL-6 (200 pg/ml) and IL-17 (100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml, 5 pg/ml and 2 pg/ml). Twenty four hours after treatments were added, cells were labeled with 0.01uCi/ul tritiated thymidine in 20 ul medium. After additional 24 hour incubation, cells were trypsinized in 100ul of 0.25% Trypsin-EDTA and transferred into 96 well polyethylene terephthalate sample plates (Cat#1450-401; Perkin Elmer). One hundred microliters of scintillation fluid were then added to each well and mixed well with the cell suspension. Plates were sealed using sealing tape and quantified in a Wallac Microbeta liquid scintillation counter. Three experimental replicates were carried out per cell line.

Cell Migration Assay

Migration of endometrial and endometriotic epithelial cells was evaluated using the Radius^{TM} 24-well cell migration assay kit (Cat#CBA-125; Cell Biolabs, Inc.). Both cell types were plated in the culture wells provided in the kit at a density of 75,000 cells per well in five hundred microliters of medium and allowed to proliferate for 24 hours, when they reached 80%

confluency. The gel insert in the center was then removed using the gel removal solution provided in the kit. Cells were then washed and treated with medium only, vehicle control (0.1% BSA/PBS), recombinant TNFα (15 ng/ml) or recombinant IL-17 (100 ng/ml, 1 ng/ml and 10 pg/ml). Cell migration over time was evaluated by imaging wells at 0, 6, 12, and 24 hours post treatment. Images thus obtained were analyzed by Wimasis Image Analysis, Germany for area covered by cells.

Cell Invasion Assay

Invasion of endometrial and endometriotic epithelial cells through a basement membrane-like structure was evaluated using the CytoSelect ^{TM} 24-well Cell Invasion Assay (Basement Membrane, Colorimetric Format, Cat#CBA-110; Cell Biolabs Inc.). Cell suspensions of both cell types were prepared with 250,000 cells in 500ul of medium. Treatments prepared and administered were medium only, vehicle control (0.1% BSA/PBS), recombinant TNFα (15 ng/ml) and recombinant IL-17 (100 ng/ml, 1 ng/ml and 10 pg/ml). Cell suspensions with added treatments were plated onto the inserts in each well provided in the kit. Five hundred microliters of 10% FBS-containing medium were added to the lower well of each invasion plate. Cells were incubated for 24 hours at 37^{o}C in 5% CO_2 atmosphere. The medium from the inserts was then carefully aspirated. The insides of the inserts were carefully wiped with the cotton swabs provided in the kit. Inserts were then transferred to a clean well in the plate and incubated in the Cell Stain Solution provided for 10 minutes at room temperature. The inserts were then gently washed in MiliQ purified water and allowed to dry. Cells on the underside of the insert from five random fields were counted under high magnification for each treatment group. The inserts were then transferred to a clean empty well in the plate and treated with Extraction solution provided

in the kit for 10 minutes on an orbital shaker. One hundred microliters of each sample were transferred to a 96-well microtiter plate and optical density was measured at 560nm in a plate reader.

Patient Endometrial Biopsy Samples

Patients who came with the primary complaint of Unexplained Infertility (UI) or unexplained Recurrent Pregnancy Loss (uRPL), to Dr. Bruce Lessey's clinic were included in this study. These women were between 18-40 years of age and had not received any treatment for their fertility issues prior to undergoing the biopsy. Endometrial biopsies were collected during the mid- to late-secretory phase (specifically 7 days post LH surge) from the middle front or back region of the uterine body (not close to the cornua). A subset of these patients underwent laparoscopic excision of endometriotic lesions before the endometrial biopsy sample was collected. The samples were processed in Dr. Lessey's laboratory for histological analysis and we received tissue sections on coated glass slides. These tissue sections were assessed for Treg and Th17 cell numbers as well as for the cytokine Interleukin 17 expression. The results were correlated with the fertility status of these women to ascertain potential markers for uterine receptivity and positive pregnancy outcome. In order to keep the study completely unbiased, I was blind to the patient history and/or categorization. The results of the immunohistochemistry analyses were sent to Dr. Lessey and he conducted the statistical analysis.

Immunohistochemistry

Immunohistochemistry was performed on paraffin embedded human eutopic endometrial tissue sections to detect Treg and Th17 cells, IL-17 cytokine and EMMPRIN expression. Antibodies

against FoxP3 (Treg cell transcription factor), RORγt (Th17 cell transcription factor), IL-17 (Th17 specific cytokine) and anti-human EMMPRIN were used for this purpose. After deparaffinization and rehydration of sections, antigen retrieval was performed by incubating the sections in 1X DAKO Target Retrieval solution (pH 9.0) at 97-99^{o}C for 20 minutes and then cooled to room temperature. Quenching of endogenous peroxidase activity was performed (for IL-17 and EMMPRIN only) by incubating the slides in 0.3% hydrogen peroxide solution in methanol for 15 minutes at room temperature. Non-specific binding was blocked using 5% normal horse serum (for FoxP3), 5% normal rabbit serum (for RORγt), 5% normal goat serum (for IL-17) or 5% normal rabbit serum (for EMMPRIN) in 1% BSA/PBST at room temperature for 30 minutes. The sections were then incubated with 10ug/ml of mouse anti-human FoxP3, 5ug/ml of rat anti-human RORγt, 2ug/ml of rabbit anti-human IL-17 or 1ug/ml goat anti-human EMMPRIN in 1% BSA/PBST at 4^{o}C overnight. The sections were washed 3 times for 5 minutes each with PBST and then incubated with biotinylated horse anti-mouse IgG, biotinylated rabbit anti-rat IgG, biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-goat IgG diluted 1:200 with 1% BSA/PBST for 45 min at room temperature for FoxP3, RORγt, IL-17 and EMMPRIN respectively. Indirect detection was performed by incubation of sections with avidin-biotinylated peroxidase complex (Reagents A and B in Vectastain Elite ABC Kit), reacted with 0.6 mg/ml 3, 3'-Diaminobenzidine (DAB; Cat#AC11209; Fisher) in Tris-HCl buffer and finally counterstained with Mayer's hematoxylin nuclear stain. The slides were then dehydrated, mounted with cover-slip and kept overnight for drying. Negative control sections were treated with non-specific IgG at similar concentration for each primary antibody. Stained sections were scanned using a Nanozoomer 2.0HT (Hamamatsu) and these digital images were used for quantification of FoxP3 and RORγt positive cells in the endometrial sections and H-Score

analysis of IL-17 and EMMPRIN expression. Quantitation and scoring were carried out in a blinded fashion to avoid any bias. The results thus obtained were then compared to the fertility history for each patient to determine potential correlation.

Immunofluorescence Microscopy

Cells were grown in 8-chamber Ibidi dishes and allowed to proliferate for 48 hours. Cells were then treated with vehicle control (0.1% BSA/PBS), recombinant TNFα (15 ng/ml) or recombinant IL-17 (100 ng/ml, 10 ng/ml, 1 ng/ml and 10 pg/ml) for 0, 60 minutes and 24 hours. Cells were washed with 1x PBS between each step in the entire procedure. Cells were fixed at the respective time-points with 4% paraformaldehyde for 30 minutes at room temperature, and then permeabilized in 0.5% Triton X-100 for 15 minutes. Cells were then incubated in ultra-cold methanol for 15 minutes at -20^{o}C. Blocking of non-specific activity was carried out with Image-IT^{TM} FX Signal Enhancer for 30 minutes. Cells were then incubated with 1 ug/ml of anti-human p65 antibody for 2 hours at 37^{o}C. After further PBS washes, cells were incubated in a 1:200 dilution of anti-rabbit Alexa Fluor 568 conjugated secondary antibody for 1 hour at 37^{o}C. Cells were then incubated in 10ug/ml DAPI for 15 minutes at room temperature. Prolong Gold was added to each well and allowed to cure in the wells for 24 hours at room temperature. Dishes were kept at 4^{o}C in the dark till imaging was conducted with Zeiss LSM700 microscope.

Collecting Cell Lysates for Immunoblotting

Total cell lysates from endometrial and endometriotic epithelial cells that had received various treatments in specific experiments were collected in 1x RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulphate) with

cOmplete Mini EDTA-free Protease Inhibitor Cocktail Tablets (Roche). Cells were kept in 1x RIPA buffer on ice for 30 minutes, lysates were then collected into Eppendorf tubes and centrifuged at 12,000 RPM at 4^{o}C for 15 minutes to remove nuclear and cell debris. Supernatants were then transferred to fresh Eppendorf tubes for further processing. Lysates were kept on ice throughout the entire harvest procedure.

Immunoblotting

BCA protein assays were performed on cell lysates to determine total protein concentrations for all samples. Ten micrograms of total protein for each sample were loaded onto 4-20% gradient Tris-HEPES-SDS Precast Polyacrylamide Mini gels and run for 1 hour at 90V. Proteins thus separated were transferred to Polyvinylidene Fluoride membranes. Membranes were stained for 5 minutes with Ponceau S stain to check for proper transfer of proteins onto the membranes. Membranes were then incubated in 5% non-fat dry milk in TBST (20 mM Tris base, 150 mM NaCl, 0.1% Tween-20, pH 8.0) for 1 hour at room temperature to block non-specific activity. Membranes were then incubated in the respective primary antibody at 4^{o}C overnight according to specific conditions for each primary antibody. Membranes were probed for phospho-IκBα, total- IκBα, EMMPRIN, cytokeratin, vimentin, phopho-MAPK, and MAPK (refer Appendix B for details). After 3 washes with TBST, membranes were incubated in a 1:10,000 dilution of respective HRP-conjugated secondary antibody (refer Appendix B for details) for 60 minutes at room temperature. After further washes with TBST, the membranes were incubated in the SuperSignal West Pico Chemiluminescent Substrate for 6 minutes. Membranes were then visualized for protein bands using ImageQuant LAS4000. Membranes were first probed with phosphorylated protein antibodies, then stripped using Restore western blotting stripping buffer

for 15 minutes at room temperature and reprobed with antibody against the total protein. GAPDH was used as a loading control for all other proteins probed.

Densitometric Analysis of Immunoblots

To quantify changes in protein expression in response to treatment over time, images obtained using the ImageQuant LAS4000 were analyzed using the image analysis software. Proteins of interest were measured by drawing a rectangle around the bands and obtaining numerical values for optical density of the band thus selected. Measurement of bands for the phosphorylated protein was normalized to measurements for total protein bands. Other protein bands were normalized to GAPDH bands.

Statistical Analysis

The n value for each experiment is mentioned in the figure legend. Results are expressed as Mean ± SEM. Experimental variability between treatments and time-points was determined by conducting the Shapiro-Wilk test for normality on obtained data. Parametric ANOVA, Student's *t*-test or linear regression analyses were conducted (as needed) using SAS (9.0). Significant differences between groups were detected by post hoc analyses using Tukey's or Bonferroni's test (as appropriate). Statistical significance was set as $P<0.05$.

APPENDIX A

LIST OF ABBREVIATIONS

Cell lines

12Z :- Immortalized Human Epithelial Endometriotic cell line

22B :- Immortalized Human Stromal Endometriotic cell line

JEG-3 :- Human Placenta Choriocarcinoma cell line

THP-1 :- Human Monocytic cell line

Reagents

DMEM/F12 :- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

FBS :- Fetal Bovine Serum

DAB :- 3, 3`- Diaminobenzidine

H_2O_2 :- Hydrogen Peroxide

PBST :- Phosphate Buffered Saline with Tween 20

recHuEGF :- recombinant Human Epidermal Growth Factor

recHuIL-17 :- recombinant Human Interleukin 17

recHuIL6 :- recombinant Human Interleukin 6

recHuTNFα :- recombinant Human Tumor Necrosis Factor alpha

Pathological conditions

EMT :- Epithelial to Mesenchymal Transition →is a biologic process that allows a polarized epithelial cell, which normally interacts with the basement membrane to undergo multiple biochemical changes that enable it to lose cell polarity and cell-cell adhesion, and gain migratory and invasive properties.

MET :- Mesenchymal to Epithelial Transition →is a reversible biological process that involves the transition from motile, multipolar or spindle-shaped mesenchymal cells to planar arrays of polarized epithelial cells.

RPL, RM or RSA is defined as 3 consecutive pregnancy losses prior to 20 weeks from the last menstrual period.

RPL :- Recurrent Pregnancy Loss

iRPL :- idiopathic Recurrent Pregnancy Loss

uRPL :- unexplained Recurrent Pregnancy Loss

RSA :- Recurrent Spontaneous Abortion

URSA :- unexplained Recurrent Spontaneous Abortion

RM :- Recurrent Miscarriage

Factors involved with pathways

Act1:- NFκB Activator 1 → NFκB activating protein; Act1 associates with and activates IκB kinase (IKK), leading to the liberation of NF-κB from its complex with IκB.

cAMP :- cyclic adenosine monophosphate → a second messenger important in many biological processes.

CAPN7 :- Calpain 7 → Calpains are ubiquitous, well-conserved family of calcium-dependent, cysteine proteases.

CCL2 :- Chemokine (C-C motif) ligand 2 → CCL2 is a small cytokine that belongs to the CC chemokine family. CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection.

COX2 :- Cyclooxygenase 2 → an enzyme that is responsible for formation of important biological mediators such as prostaglandins, prostacyclin and thromboxane.

ERK :- Extracellular-Signal-Regulated Kinases → widely expressed protein kinase intracellular signaling molecules that are involved in functions including the regulation of meiosis, mitosis and post-mitotic functions in differentiated cells.

IGFBP1 :- Insulin-like Growth Factor-binding Protein 1 → serves as a carrier protein for IGF.

IgG :- Immunoglobulin isotype G →Most abundant antibody isotype found in blood circulation; synthesized and secreted by plasma B cells.

IκB :- Inhibitor of NFκB → a protein that inactivates the NF-κB transcription factors and keeps them sequestered in the cytoplasm.

IKK :- IκB kinase → The IκB kinase enzyme complex is part of the upstream NFκB signal transduction cascade.

IKKα :- IκB kinase alpha →catalytic subunit of IκB kinase.

IKKβ :- IκB kinase beta → catalytic subunit of IκB kinase.

LH :- Luteinizing Hormone → is a hormone produced by gonadotrophs in the anterior pituitary; it triggers ovulation in females and stimulates Leydig cell production of testosterone in males.

MAPK :- Mitogen-activated Protein Kinases → specific protein kinases that are involved in cellular responses to stimuli and hence regulate proliferation, differentiation, cell survival etc.

MMP :- Matrix Metalloproteinase → are zinc-dependent proteases that are capable of degrading extracellular matrix proteins, and are also thought to play a major role in cell behaviors such as proliferation, migration, differentiation, and apoptosis etc.

mRNA :- messenger Ribonucleic acid → a large family of ribonucleic acid molecules that convey genetic information from DNA to the ribosome, where they specify the amino acid sequence of the protein products of gene expression.

NFκB :- Nuclear Factor Kappa-light-chain-enhancer of activated B cells → is a protein complex that controls transcription of DNA, is involved in cellular responses to stimuli, plays a key role in regulating the immune response to infection by regulating cytokine production and cell survival.

p50 :- Protein subunit of NFκB transcription factor complex

p65 :- Protein subunit of NFκB transcription factor complex

PDGF :- Platelet Derived Growth Factor → growth factor or protein that regulates cell growth and plays important role in angiogenesis.

PI3K :- Phosphoinositide 3-Kinase → a family of related intracellular signal transducer enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer.

PRA :- Progesterone Receptor A

PRB :- Progesterone Receptor B

PRL :- Prolactin → is a protein secreted from the pituitary gland that is best known for its role in milk production in the female; but also plays an essential role in metabolism and regulation of the immune system.

TRAF6 :- Tumor Necrosis Factor Receptor Associated Factor → a signal transducing protein that is involved in regulation of NFκB signaling and activation of MAP Kinases.

TIMP-2 :- Tissue Inhibitor of Metalloproteinases 2 → encoded proteins that are natural inhibitors of matrix metalloproteinases, and can also directly suppress proliferation of endothelial cells.

Raf1 :- Rapidly Accelerated Fibrosarcoma → is an enzyme that is a "gatekeeper" of the ERK pathway.

Immune cells and related factors

CD4 :- Cluster of Differentiation 4 → a glycoprotein found on the surface of immune cells such as T helper cells, monocytes, macrophages, and dendritic cells

CD25 :- Cluster of differentiation 25 → a type I transmembrane protein present on activated T cells, activated B cells, some thymocytes, myeloid precursors, and oligodendrocytes that associates with CD122 to form a heterodimer that can act as a high-affinity receptor for IL-2.

FoxP3 :- Forkhead Box P 3 → a protein involved in immune system responses, it appears to function as a transcription factor in the development and function of Treg cells.

IL-17 :- Interleukin 17 → a cytokine that acts as a potent mediator of inflammation and attracts monocytes and neutrophils to the site of inflammation.

RORγt :- RAR-related orphan receptor gamma t → is a transcription factor with essential functionality in the immune system for lymphoid organogenesis, and promoting thymocyte differentiation into pro-inflammatory T helper 17 (Th17) cells.

TCR :- T cell receptor → is a molecule found on the surface of T lymphocytes that recognizes antigens bound to major histocompatibility complex molecules.

TGFβ :- Transforming Growth Factor beta →is a protein that plays an important role in proliferation, cellular differentiation and other functions in most cells.

Th1 :- T helper 1 → T_h1 helper cells are the host immunity effectors against intracellular bacteria and protozoa. The main effector cells of T_h1 immunity are macrophages as well as CD8 T cells, IgG B cells, and IFN-γ CD4 T cells.

Th2 :- T helper 2 → T_h2 helper cells are the host immunity effectors against multicellular helminths. The main effector cells are eosinophils, basophils, and mast cells as well as IgE B cells, and IL-4/IL-5 CD4 T cells.

Th-17 :- T-helper 17 → Th-17 helper cells mediate host immunity against extracellular bacteria and fungi. Its main effector cytokines are IL-17a, IL-21, and IL-22. The main T_h17 effector cells are neutrophils as well as IgM/IgA B cells, and IL-17 CD4 T cells. The key T_h17 transcription factors are STAT3 and RORgt.

Treg :- T-regulatory → The regulatory T cells, formerly known as suppressor T cells, are a subpopulation of T cells which modulate the immune system, maintain tolerance to self-antigens, and abrogate autoimmune disease.

iTregs :- inducible Treg → also known as adaptive regulatory T cells. They develop outside the thymus under a variety of conditions. iTregs are non-redundant and essential for tolerance at mucosal surfaces, yet their mechanisms of suppression and stability are unknown.

nTregs :- natural /naïve Treg → Natural regulatory T cells develop in the thymus and express the transcription factor Foxp3. They are required for systemic immunological tolerance.

uNK :- uterine Natural Killer → a type of cytotoxic lymphocyte critical to the innate immune system. Since pregnancies involve two individuals who are not tissue matched, successful pregnancy requires the mother's immune system to be suppressed. Uterine NK cells are thought to be important cells in this process. They are in the NK cell subset, potent at cytokine secretion, but with low cytotoxic ability and relatively similar to peripheral NK cells.

Miscellaneous abbreviations

WOI :- Window of Implantation → The receptive phase of the endometrium of the uterus is usually termed the "implantation window" and lasts about 4 days. The implantation window follows around 6-7 days after the peak in LH levels. The implantation window is characterized by changes to the endometrium cells, both structurally and in the composition of its secretions.

ANOVA :- Analysis of Variance →is a collection of statistical models used to analyze the differences between group means and their associated procedures (such as "variation" among and between groups) and are useful in comparing three or more variables for statistical significance.

QRT-PCR :- Real Time Polymerase Chain Reaction → is a laboratory technique of molecular biology based on PCR technology, which enables amplification, detection and simultaneous measurement of targeted DNA products generated during each cycle of PCR process.

APPENDIX B

LIST OF ANTIBODIES AND RECOMBINANT PROTEINS USED

Recombinant Proteins	Catalog number	Final Concentration	Company
Recombinant EGF	236-EG-200	100pg/ml	R & D Systems
Recombinant TNFα	210-TA-010	15 ng/ml	R & D Systems
Recombinant IL-17	7955-IL-025	100 ng/ml to 10 pg/ml	R & D Systems

Primary Antibody [Clone]	Catalog number	Dilution [Application]	Company
Mouse Anti-Human FOXP3 [236A/E7]	ab20034	10 ug/ml [IHC]	abcam
Rat Anti- Human/Mouse RORγt [AFKJS-9]	14-6988	5 ug/ml [IHC]	eBioscience
Rabbit Anti- Human IL-17 [H-132]	sc-7927	2 ug/ml [IHC]	Santa Cruz Biotechnology
Goat Anti-Human EMMPRIN	sc-9754	1 ug/ml [IHC]	Santa Cruz Biotechnology
Rabbit Anti-Human IL-17RA	5503	1:1000 [WB]	Cell Signaling
Rabbit Anti-Human p65	ab16502	1 ug/ml [IF]	abcam
Mouse Anti-Human phospho-IκBα [5A5]	9246	1:1000 [WB]	Cell Signaling
Rabbit Anti-Human IκBα (total)	9242	1:1000 [WB]	Cell Signaling
Mouse Anti-Human EMMPRIN [HIM6]	555961	1:2000 [WB]	BD Biosciences
Rabbit Anti-Human/Mouse Cytokeratin-19 [EP1580Y]	ab52625	1:1000 [WB]	abcam
Goat Anti-Human Vimentin	AF2105	1:200 [WB]	R & D Systems
Rabbit Anti- Human phospho-MAPK [D13.14.4E]	4370	1:1000 [WB]	Cell Signaling
Rabbit Anti-Human MAPK	9102	1:1000 [WB]	Cell Signaling
Mouse Anti-Human GAPDH [1D4]	NB300-221	1:1000 [WB]	Novus Bio
Rabbit Anti-Human MMP2 [H-76]	sc-10736	1:500 [WB]	Santa Cruz Biotechnology

Secondary Antibodies	Catalog number [Kit]	Dilution [Application]	Company
Biotinylated Horse Anti-Mouse IgG	PK-6102 [Vectastain Elite ABC Kit Mouse IgG]	1:200 [IHC]	Vector Labs
Biotinylated Rabbit Anti-Rat IgG	PK-6104 [Vectastain Elite ABC Kit Rat IgG]	1:200 [IHC]	Vector Labs
Biotinylated Goat Anti-Rabbit IgG	PK-6101 [Vectastain Elite ABC Kit Rabbit IgG]	1:200 [IHC]	Vector Labs
Biotinylated Rabbit Anti-Goat IgG	PK-6105 [Vectastain Elite ABC Kit Goat IgG]	1:200 [IHC]	Vector Labs
Anti-Rabbit HRP-conjugated IgG	7074S	1:10,000 [WB]	Cell Signaling
Anti-Mouse HRP-conjugated IgG	7076S	1:10,000 [WB]	Cell Signaling
Anti-Goat HRP-conjugated IgG	ab6885	1:10,000 [WB]	abcam