From the Department of Clinical Science, Intervention and Technology

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EXPLORING THE ROLE OF MESENCHYMAL STROMAL CELLS IN ENDOMETRIOSIS

Fawaz Abomaray

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Exploring the Role of Mesenchymal Stromal Cells in Endometriosis

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By

Fawaz Abomaray

Main Supervisor:
Associate Professor Cecilia Götherström
Karolinska Institutet
Department of Clinical Science, Intervention and Technology
Division of Obstetrics and Gynecology

Co-supervisor:
Associate Professor Sebastian Gidlöf
Karolinska Institutet
Department of Clinical Science, Intervention and Technology
Division of Obstetrics and Gynecology

Opponent:
Professor Ornella Parolini
Catholic University of the Sacred Heart, Rome, Italy
Institute of Human Anatomy and Cell Biology

Examination Board:
Associate Professor Karl-Henrik Grinnemo
Uppsala University
Department of Clinical Sciences
and Thoracic Surgery, and
Karolinska Institutet
Department of Molecular Medicine
and Surgery

Associate Professor Michael Uhlin
Karolinska Institutet
Department of Clinical Science,
Intervention and Technology

Associate Professor Johan Bengzon
Lund University
Faculty of Medicine, Stem Cell Center

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ABSTRACT

Endometriosis is a chronic inflammatory disease where there is growth of endometrial tissue in ectopic sites, most commonly within the pelvic cavity. The major symptoms of this disease are chronic pelvic pain and infertility. There are both medical and surgical options to treat endometriosis, however, there is a high recurrence rate of ectopic lesions and symptoms, and hence a need for more effective therapies. Although the exact etiology is unclear, the most widely accepted theory describing the origin of endometriosis is Sampson's theory of retrograde menstruation. This theory states that during menstruation there is retrograde movement of endometrial tissue back into the pelvic cavity that grows to become ectopic endometrial tissue through its increased ability to survive, proliferate, migrate, adhere, invade, and promote angiogenesis to support its growth. However, only approximately 10% of women of reproductive age develop endometriosis, while almost all women exhibit retrograde menstruation. Therefore, other mechanisms have been suggested to be involved in the development of endometriosis such as reduced immunosurveillance in the pelvic cavity through modulation or aberrant responses of immune cells such as macrophages and natural killer (NK) cells, and involvement of stem/stromal cells such as mesenchymal stromal cells (MSC).

In endometriosis it is known that there is a predominance of immunosuppressive M2 macrophages, and inhibition of function of NK cells in the pelvic cavity. Both immune cell types have been suggested to contribute to the reduced immunosurvelliance of ectopic endometrial tissue. However, the exact cause of this reduced immunosurveillance is currently unknown. MSC are multipotent cells found in various tissue sources as well as in ectopic endometrial and eutopic endometrial tissue. Their immunomodulatory abilities have been utilized as a treatment in immune mediated diseases through their immunosuppressive effects on immune cells such as macrophages and NK cells. Interestingly, MSC have been suggested to exist as MSC1 (an immunostimulatory phenotype), or as MSC2 (an immunosuppressive phenotype), with the latter phenotype being the most commonly investigated in the literature (see Table 1).

Therefore, herein it was hypothesized that MSC derived from endometriotic ovarian cysts could be involved in the reduced immunosurveillance of ectopic endometrial tissue in the pelvic cavity through their immunosuppressive effects on macrophages and NK cells. Moreover, if autologous MSC are involved in the pathology, then it was hypothesized that the immunomodulatory capabilities of allogeneic MSC could be a potential therapeutic strategy to target the inflammatory component of endometriosis.

Study 1 was an *in vitro* study that found that stromal cells isolated from endometriotic ovarian cysts (ESC_{cyst}) of women with endometriosis are more immunosuppressive than stromal cells isolated from the endometrium (ESC_{endo}) of women with endometriosis. The ESC were also confirmed to be MSC; they expressed MSC markers such as CD73, CD90, and CD105, formed colonies when seeded at low densities, and differentiated into osteoblasts and adipocytes. ESC_{cyst} expressed significantly higher levels of the immunosuppressive enzymes indoleamine 2,3-dioxygenase 1, cyclooxygenase 2, and hemeoxygnase 1, and promoted differentiation of the more immunosuppressive M2 macrophages. Based on the results of this study we used allogeneic adipose tissue-derived MSC (Ad-MSC) in **Study 2** to examine their effects on ESC_{cyst} and ESC_{endo} *in vitro*, as a first step of a long-term goal of developing a potential therapy for endometriosis. We found that culture with allogeneic Ad-MSC either maintained or promoted the proliferation, survival, adhesion, migration and invasion of ESC_{cyst}. Moreover, conditioned medium (CM) derived from Ad-MSC and ESC_{cyst} co-cultures promoted tube formation of human umbilical vein endothelial cells; tube formation is an *in vitro* model of angiogenesis. In **Study 3**, the interactions between ESC_{cyst} and NK cells *in vitro* were examined. It was found that following culture in the CM of ESC_{cyst} or in direct co-culture with ESC_{cyst}, the phenotype, degranulation, and cytotoxic functions of NK cells were similar to the NK cells cultured with ESC_{endo}; the expression of activation, inhibitory, adhesion, and maturation receptors was similar, and the level of the degranulation marker CD107a and expression of the immunostimulatory cytokine interferon gamma was similar.

In conclusion, the findings presented in this thesis suggest that immunosuppressive ESC_{cyst} may indirectly promote the growth of ectopic endometrial tissue through their inhibitory effects on the immunosurveillance in the pelvic cavity through their effects on macrophages but not on NK cells. In addition, autologous ectopic MSC such as ESC_{cyst} may be involved in the pathogenesis of endometriosis through their direct growth promoting effects on ectopic endometrial tissue.

LIST OF SCIENTIFIC PAPERS

I. ***Abomaray F***, *Gidlöf S, Götherström C*
Mesenchymal Stromal Cells Are More Immunosuppressive In vitro If They Are Derived from Endometriotic Lesions than from Eutopic Endometrium
Stem Cells Int. 2017 Nov 5; 2017:3215962: 13 pages.

II. ***Abomaray F***, *Gidlöf S, Bezubik B, Engman M, Götherström C*
Mesenchymal Stromal Cells Support Endometriotic Stromal Cells In Vitro
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III. ***Abomaray F***, *Wagner A, Chrobok M, Gidlöf S, Alici E & Götherström C*
Effect of Mesenchymal Stromal Cells Derived from Endometriotic Lesions on Natural Killer Cell Phenotype and Function
Manuscript.

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LIST OF ABBREVIATIONS

7-AAD	7-aminoactinomycin D
Ad-MSC	Adipose tissue-derived MSC
^{51}Cr	Chromium 51
CFSE	Carboxyfluorescein succinimidyl ester
CFU-F	Colony forming units-fibroblasts
CM	Conditioned medium
COCs	Combined oral contraceptives
COX2	Cyclooxygenase 2
CXCL	C-X-C motif chemokine
DC	Dendritic cells
ESC_{cyst}	Stromal cells derived from endometriotic ovarian cysts
ESC_{endo}	Stromal cells derived from eutopic endometrium
ESC_{endohv}	ESC_{endo} derived from healthy female volunteers
FCS	Fetal calf serum
GnRH	Gonadotropin-releasing hormone
GvHD	Graft versus host disease
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HO-1	Hemeoxygenase 1
HUVEC	Human umbilical vein endothelial cells
IDO	Indoleamine 2,3- dioxygenase
IFN-γ	Interferon gamma
IL	Interleukin
KIRs	Killer immunoglobulin-like receptors
M-CSF	Macrophage-colony stimulating factor

MFI	Median fluorescence intensity
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinases
MSC	Mesenchymal stromal cells
MTT	(d-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
NK	Natural killer
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBMCs	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
qPCR	Quantitative polymerase chain reaction
RANTES	C-C chemokine, regulated on activation, normal T cell expressed and secreted
TGF-β1	Transforming growth factor-beta 1
TH	T helper
TNF-α	Tumor necrosis factor-α
Tregs	T regulatory cells
TSLP	Thymic stromal lymphopoietin
TUNEL	Terminal deoxy transferase-mediated dUTP nick end labelling

1 INTRODUCTION

1.1 ENDOMETRIOSIS

1.1.1 Background

Endometriosis is an estrogen dependent and chronic inflammatory disease defined by the presence of ectopic endometrial tissue, with the pelvic cavity being the most common site (1). Occasionally ectopic lesions may be found in other regions of the body such as the lungs, kidney, and brain (1). Although endometriosis may have systemic effects (2), it is largely seen as a local disease within the pelvic cavity. Laparoscopy is the gold standard for conclusive diagnosis of endometriosis, preferably along with histological confirmation of endometrial glands and stroma in the ectopic lesions (3, 4). Globally, approximately 10% of women of reproductive age suffer from endometriosis, which represents a great number (1). Moreover, around 35-50% of women with chronic pelvic pain and/or infertility have endometriosis (4) The symptoms of endometriosis can be devastating and include dysmenorrhea and dyspareunia, with the two major symptoms being chronic pelvic pain and infertility (1). The total annual healthcare costs of endometriosis are considerable. In the United States alone, endometriosis related spending was estimated to be around $69.4 billion in 2010 following a multicenter prospective study, with these costs being mainly due to a loss of productivity of affected individuals (5).

1.1.2 Etiology

The etiology of endometriosis remains unknown, with Sampson's theory of retrograde menstruation being the most widely accepted for describing the origin of endometriosis (6). This theory states that retrograde flow of endometrial tissue via the fallopian tubes into the pelvic cavity during menstruation leads to the occurrence of endometriosis (6). However, this theory alone is insufficient in explaining the pathology of endometriosis since almost all women of reproductive age exhibit retrograde menstruation, but not all develop the disease (6). Therefore, other factors appear to be important in the development and progression of endometriosis, and other theories may further explain the pathogenesis of endometriosis. There is a theory that suggests that ectopic lesions originate from epithelial-like and/or stromal like stem cells, which are refluxed back into the pelvic cavity through retrograde menstruation supporting a stem cell theory for the pathogenesis of endometriosis (7). For example, several studies have demonstrated that ectopic lesions are monoclonal in origin, and mesenchymal stromal cells (MSC) have been found to exist in menstrual blood, which may carry them to the pelvic cavity during retrograde menstruation (8-11). Additionally, the stem cells may also

originate from the bone marrow, with this being supported by findings that show stem cells engraft in the endometrium via an estradiol regulated C-X-C motif chemokine (CXCL) 12 - C-X-C chemokine receptor type 4 axis. (12-14). Another theory suggests that the epithelial lining of the ovaries and peritoneum may undergo metaplasia, and then transform into endometrium leading to endometriosis (15). Yet another theory suggests that the remaining cells from the mullerian ducts that migrate during embryo development retain the ability to form ectopic lesions when exposed to estrogen during adolescents (1). Autoimmune disease also tends to be more common in patients with endometriosis. This supports the concept that endometriosis may develop due to a defective immune system, preventing the removal of ectopic lesions and consequently allowing the growth of ectopic endometrial tissue (15). Natural killer (NK) cells are one of the immune cells that are recruited to the pelvic cavity, however, their functions have been found to be inhibited (16, 17). Another component related to a defective immune system, and that may partly explain the etiology of endometriosis are the macrophages, which are abundant in ectopic lesions in endometriosis (18).

1.2 MACROPHAGES AND ENDOMETRIOSIS

1.2.1 Background

Macrophages are the major leukocyte recruited to ectopic endometriotic lesions (19), where they undergo polarization into M2 macrophages (18). Moreover, the pelvic cavity contains resident macrophage that may also be involved in the pathology of endometriosis (20). Macrophages are innate immune cells derived from monocytes that play a crucial role in the innate immune response (21). Depending on the signals in their microenvironment, monocytes have been found to be able to differentiate into the M1 macrophages or the M2 macrophages (21) and Table 1. For example, interferon gamma (IFN-γ), tumor necrosis factor-α (TNF-α), and granulocyte macrophage colony stimulating factor induce development into M1 macrophages, while interleukin 4 (IL-4) and IL-13 induce development into M2 macrophages (22, 23). M1 and M2 macrophages have distinctly different properties. M1 macrophages are associated with expression of high levels of pro-inflammatory cytokines such as IL12 and IL13, and promotion of T helper (TH) 1 responses (21) (**Table 1**). Moreover, phenotypically M1 macrophages display low expression of CD14, CD163, CD204, CD206, and B7H4 and increased expression of CD11b (24) (**Table 1**). In contrast, M2 macrophages are associated with a higher expression of CD163, CD204, CD206, CD209, and B7H4, and decreased expression of human leukocyte antigen (HLA)-DR and CD11c (19, 24) (**Table 1**). M2 macrophages are involved in tissue repair and remodeling, promotion of TH2 responses, and secretion of high levels of anti-inflammatory cytokines such as IL-10 and transforming growth

factor-beta 1 (TGF-β1) that allow them to inhibit the responses of other immune cells (19, 21) (**Table 1**). It has been suggested that M2 macrophages have subtypes: M2a, M2b, and M2c, however, their exact phenotypes and functions are still not completely known (25).

Activation of macrophages plays a key role in the pathology of various inflammatory diseases such as atherosclerosis, inflammatory bowel disease, and rheumatoid arthritis (26, 27). Likewise, in endometriosis various studies show the presence of M2 macrophages in the local environment of the pelvic cavity, and that these cells may play a crucial role in the initiation and progression of endometriosis (28-30).

1.2.2 Macrophages in Endometriosis

In endometriosis, macrophages have been found to be recruited to ectopic lesions where they have been suggested to be involved in meditating their growth (31-34). Initially, recruited macrophages secrete factors like cytokines, and phagocytose red blood cells, and other cellular and tissue fragments to help prevent or control the growth of ectopic lesions, and to shape impending adaptive immune responses (35). However, it seems that as the disease progresses, their functions are altered and they become involved in the progression of the disease (36, 37). The signals that lead to their recruitment and activation are still unknown, however, it may be due to local hypoxia in the pelvic cavity, the iron overload as a consequence of dying erythrocytes derived from retrograde menstruation, or from unknown signals (38-40). The following is a discussion regarding current knowledge about macrophages in endometriosis.

Studies in both mice and rat models of endometriosis found that when macrophages were depleted this hindered the initiation and development of endometriosis (28, 41). It has been found that macrophages are needed to deliver trophic and anti-apoptotic signals that induce angiogenesis, survival, and growth of ectopic cells in the hostile hypoxic environment of the pelvic cavity (42-45). This hypothesis is supported by another study that found that the ectopic endometrium expresses the macrophage-colony stimulating factor receptor (M-CSFR), which allows the endometrium to survive and proliferate when stimulated by M-CSF secreted by peritoneal macrophages (46). Moreover, macrophages in mice and also in women with endometriosis have been found to express high levels of CD163 and CD206, an indication of M2 macrophage polarization (28). They suggested that the signals that promoted the differentiation of monocytes into the reparative M2 macrophage phenotype likely existed in the local microenvironment (28). One such signal could be M-CSF, which is known to be able to induce monocyte differentiation into M2 macrophages, and has been found to be at a higher concentration in women with endometriosis than in women without endometriosis (47, 48).

These findings complement what is known about macrophages derived from endometriotic lesions, in that they are involved in clearing dying erythrocytes and endometrial cell debris via phagocytosis, and secreting trophic and angiogenic molecules (40, 49). To test the hypothesis that M2 macrophages play a crucial role in endometriosis development, M1 or M2 macrophages were adoptively transferred into mice following disease establishment (28). Interestingly, M1 macrophages disrupted, while M2 macrophages promoted endometriotic lesion development, respectively (28). The results of these studies demonstrate that it may be possible to treat endometriosis by inhibiting the functions of M2 macrophages in the pelvic cavity.

RANTES (C-C chemokine, regulated on activation, normal T cell expressed and secreted), a pro-inflammatory cytokine present in the ectopic milieu of endometriosis, was found to induce macrophage recruitment and tolerance (50). These recruited tolerant macrophages displayed characteristics of M2 macrophage with increased expression of CD14 and IL-10, but decreased expression of HLA-DR (50). In turn, these macrophages were found to inhibit apoptosis of stromal cells derived from the eutopic endometrium of women with endometriosis (ESC_{endo}) (50). Therefore, the authors concluded that the high levels of RANTES in the peritoneal cavity induces tolerance of macrophages, which promote the survival and growth of ectopic lesions (50). Matrix metalloproteinases (MMP) are endopeptidases that possess the capacity to cleave all components of the extracellular matrix when activated (29). Additionally, they are capable of regulating cell proliferation, survival, migration and angiogenesis, and therefore, their overexpression can contribute to the pathogenesis of endometriosis (29). A study found that MMP27 was specifically expressed in M2 macrophages from endometriotic lesions, and this may contribute to disease progression by favoring survival and growth of ectopic lesions (29). Another study indirectly demonstrated that M2 macrophages are induced in the pelvic cavity by finding that IL1β, an inflammatory cytokine associated with endometriosis, upregulated the expression of thymic stromal lymphopoietin (TSLP) by stromal cells derived from the endometriotic ovarian cysts of women with endometriosis (ESC_{cyst}), which is a master cytokine that drives the TH2 response (51). This complemented their findings that TSLP is found at high levels in the peritoneal fluid of women with endometriosis, and so they suggested that TSLP secretion from ESC_{cyst} may promote the disease via the TH2 response and subsequent induction of M2 macrophages (51). Another study by Nie *et al*. found that ESC_{endo}-derived conditioned medium (CM) induced by IL-6 promoted more polarization of M2 macrophages derived from THP-1 cells (human monocyte leukemia cell line) than ESC_{endo}-derived CM not induced by IL-6 (52). They suggested that IL-6 present in the pelvic cavity of women with endometriosis

may be involved in M2 macrophage induction (52). The aforementioned studies are indirect evidence that stromal cells may be promoting M2 macrophages in endometriosis.

It is generally known that TH2 responses induce polarization of macrophages towards the M2 phenotype (53, 54). For example, cytokines such as IL-4 and IL13 that are associated with a TH2 response are known to drive monocytes towards the M2 phenotype (55). Interestingly, several studies have found that endometriosis is associated with a reduced TH1 immune response, and an increased TH2 response that may allow the survival, implantation, and proliferation of ectopic lesions through activation of M2 macrophages (51, 56-59). One study found that administration of TH1 but not TH2 cytokines inhibited the growth of ectopic lesions in a mouse model of endometriosis. This was suggested to be due to the activation of M1 macrophages, and the cytotoxic effector functions of CD8+ T cells and NK cells (59).

Therefore, the pelvic cavity in endometriosis seems to contain various factors along with stromal cells that are synergistically pushing macrophages towards the M2 phenotype, which seems to be promoting endometriosis development. However, the exact mechanisms by which M2 macrophages are induced in pelvic cavity in endometriosis are unknown. Studies on the interactions of stromal cells and macrophages are discussed later.

1.3 NATURAL KILLER CELLS AND ENDOMETRIOSIS

1.3.1 Background

Just like macrophages, NK cells are also part of the innate immune system, and have also been found to be activated and recruited to ectopic endometriotic lesions early on during the disease (60). Several chemokines are found at high levels in the pelvic cavity, including CXCL12 which is involved in NK cell recruitment; this is not surprising since the levels of chemokines are regulated by estrogen that is known to be increased in endometriosis (61). The normal function of NK cells is to secrete cytokines and to target cells for destruction, both of which help them carry out their cytotoxic activities, and to determine the type of impending responses produced from the adaptive immune system (60). NK cells are a type of lymphocyte, however, unlike the other lymphocytes T cells and B cells of the adaptive immune system, NK cells have the innate ability to distinguish abnormal cells from normal cells, and then to target them for destruction without any requirement for antigen presentation by antigen presenting cells (61).

Although they are not completely clear, the mechanisms by which NK cells carry out their cytotoxic functions are thought to be through a detection system involving a wide range of cell surface activating receptors such as NKG2D and inhibitory receptors such as NKG2A (61). In addition, killer immunoglobulin-like receptors (KIRs) found on the surface of NK cells are

involved in the recognition of HLA molecules on target cells, and the regulation of NK cell cytotoxicity (2). Whether activating or inhibitory receptors predominate on the surface of NK cells depends on the corresponding induced NK cell stress ligands on target cells, presence of HLA proteins on target cells, and the presence of cytokines in their microenvironment, such as IL-2 that is known to activate NK cells (61). Transformed cells may lose their expression of HLA proteins leading to missing-self and the subsequent activation of the NK cells (61). Moreover, stressed cells may upregulate stress ligands such as HLA-E that may engage and activate activation receptors on the surface of the NK cells (61). Activation of NK cells leads to their degranulation and release of granules containing molecules such as the cytolytic protein perforin and the serine protease granzyme b that perforate the membranes of target cells and induce their apoptosis, respectively (62).

Two types of NK cells are able to be distinguished based on their expression of the cell surface markers CD56 and CD16 (63). $CD56^{dim}$ NK cells are more cytotoxic due to their high level of expression of CD16, while $CD56^{bright}$ NK cells are less cytotoxic due to their low level of expression of CD16, but secrete high levels of cytokines such as IFN-γ, and hence are involved in immune regulation (63). Most of the NK cells present in the peripheral blood are of the former type, while most of the NK cells in the endometrium are of the latter type (61). More recently, resident NK cells in the pelvic cavity have been characterized (64), which along with the recruited NK cells, may be involved in the pathogenesis of endometriosis.

1.3.2 Natural Killer Cells in Endometriosis

It is known that NK cells are recruited to ectopic lesions in endometriosis, but it has been found that the number of cytotoxic NK cells is reduced, the number of immature NK cells is increased, and their phenotype, and hence function is inhibited (2, 16, 17, 65-68). The levels of various molecules important in the cytotoxicity of NK cells such as granzyme b, perforin, TNF-related apoptosis-inducing ligand, and the degranulation marker CD107a are all reduced in the peritoneal fluid, and the levels of receptors important in NK cell activation such as NKp46, NKp44, NKG2D, CD16, and CD69 on peritoneal NK cells are also reduced in women with endometriosis; indicating that NK cell function is inhibited in endometriosis (2). The following is a discussion regarding current knowledge about NK cells in endometriosis.

Interestingly, a study examining the phenotype of ectopic endometrial cells found that their expression of HLA-G, which is a ligand of the KIR2DL4, prevented their detection by NK cells and allowed their survival and growth (69). Liu *et al.* found that NK cells from the peritoneal fluid of women with endometriosis that expressed the immunosuppressive enzyme

indoleamine 2,3-dioxygenase (IDO) 1 expressed lower levels of the activation receptors NKp46 and NKG2D and secreted more of the immunosuppressive cytokine IL-10, and hence were more inhibited compared to IDO1 negative NK cells (68). Oosterlynck *et al.* found that NK cells from women with endometriosis have reduced cytotoxicity against the K562 cell line and autologous ESC_{endo} compared to NK cells from women without endometriosis in chromium 51 (^{51}Cr) release assays (16, 17). A similar study found that NK cells from women with endometriosis have reduced cytotoxicity against the K562 cell line in ^{51}Cr release assays compared to women without endometriosis, and suggested that it could be due to their increased expression of KIRs (70). KIRs have also been found to be more highly expressed on NK cells from women with endometriosis compared to women without endometriosis in several other studies (71-73). The NKG2D receptor is known to be a potent activator of NK cells that it has the potential to override inhibitory signals mediated by inhibitory receptors (74). Interestingly, a study found that TGF-β1 derived from platelets isolated from the peritoneal fluid of women with endometriosis can reduce the expression of the NKG2D receptor, and may be involved in the reduced cytotoxicity of NK cells from women with endometriosis (74). Another study related to the NKG2D receptor, found that soluble ligands for the NKG2D receptor were increased in the pelvic cavity in women with endometriosis, which act as decoy receptors and may allow evasion of ectopic cells from NK cells (75). It was also found that peritoneal fluid-derived NK cells expressing the activating NKp46 receptor were reduced in number in women with endometriosis compared to women without endometriosis (76). Furthermore, a study found that CD94/NKG2A+ peritoneal NK cells were increased in women with endometriosis compared to women without endometriosis (77); this is an inhibitory NK cell receptor that interacts with the nonclassical molecule HLA-E that has also been found to be abundant on ectopic and eutopic cells from women with endometriosis (78).

Similar to a pervious study (79), Kang *et al.* found that the peritoneal fluid from women with endometriosis reduced the differentiation and cytotoxicity of NK cells compared to the peritoneal fluid from women without endometriosis (65). They attributed this finding to the increased levels of IL-6 in the peritoneal fluid of women with endometriosis, which they found to be negatively correlated with the cytotoxicity of NK cells (65). IL-6 is a pleiotropic molecule that is able to modulate various aspects of NK cell cytotoxicity (2). Furthermore, it was found that the levels of the Fas ligand (80, 81) are increased in the peritoneal fluid of women with endometriosis, and peritoneal NK cells in women with endometriosis expressed elevated levels of the Fas receptor (82), suggesting that NK cells may actually undergo Fas ligand induced apoptosis allowing immune escape of ectopic cells.

It is clear that there are various mechanisms at play leading to the eventual dysfunction of NK cells in women with endometriosis. However, the exact mechanisms by which the functions of the NK cells are inhibited are not currently known, but the aforementioned studies suggest that certain factors present in the pelvic cavity due to inflammatory changes in the microenvironment, and epithelial and stromal cells may be involved. Studies on the interactions of stromal cells and NK cells are discussed later.

1.4 THERAPIES FOR ENDOMETRIOSIS

There are various medical and surgical treatments for women with endometriosis but treatment failure is frequent and no definite cure is available (83). The initial treatment of a patient with endometriosis depends on the severity of her symptoms and her desire for fertility (83). Pain, the most common symptom of endometriosis, is thought to be due to a consequence of pro-inflammatory cytokines in the peritoneal cavity, bleeding from ectopic lesions, and invasion and irritation of pelvic floor nerves by the ectopic lesions (83). Around 25-50% of infertile women suffer from endometriosis, suggesting that endometriosis causes infertility, even though a clear causal relationship remains to be demonstrated (83, 84). Disturbed ovum transport may be due to distorted pelvic anatomy and chronic inflammation, which may also cause infertility due to reduced receptivity of the uterus, reduced folliculogenesis of the ovaries, and abnormal function of the uterine tubes (83). In infertile women with endometriosis, surgery and assisted reproductive technologies such as superovulation and intrauterine insemination, and *in vitro* fertilization may be considered. There is no evidence that medical therapies can help to improve fertility (83). In fact, medical therapies can delay the ability of a woman to conceive, because they can minimize the effects of the biological processes that are required to allow and maintain a pregnancy (7). However, if fertility is not desired and containment of pain is the only goal, then both medical and surgical treatment options are utilized (83).

Based on the retrograde menstruation theory, and the fact that ectopic lesions behave in a similar fashion to the eutopic endometrium, medical therapy has focused on inducing amenorrhea and reducing growth of the ectopic lesions (85, 86). Therefore, medical therapies are used to inhibit ovulation and menses to reduce inflammation, and the effect of estradiol, and other hormones from the ovaries (87). Medical therapies are predominantly symptomatic and not cytoreductive, and include combined oral contraceptives (COCs), progestogens only contraceptives, and gonadotropin-releasing hormone (GnRH) agonists and antagonists (88). Other medical therapies such as aromatase inhibitors, immunomodulators, selective estrogen and progesterone receptor modulators, histone deacetylase inhibitors, peroxisome proliferator activated receptors-γ ligands, antiangiogenic agents, and melatonin show promise in preclinical

studies, but there is not enough evidence to support their clinical use or they are yet to be tested clinically (88).

The first line of treatment for the pain associated with endometriosis is normally COCs due to their low cost and their long term usability (83). Moreover, another first line treatment are the nonsteroidal anti-inflammatory drugs (NSAIDs), however, evidence demonstrating their effectiveness in reducing the pain associated with endometriosis remains inconclusive (89). Cyclooxygenase 2 (COX2) and prostaglandins are highly expressed in endometriosis and seem to mediate the endometriosis-associated pain, and both are blocked by NSAIDs (88). In contrast, COCs, which are either used cyclically or continuously, function by inhibiting the endogenous production of estrogen by a negative feedback mechanism, leading to a reduction in prostaglandins and inflammation (88). Moreover, COCs may be inducing a pseudopregnancy state by causing decidualization and atrophy of the endometrium (90).

COCs are contraindicated in women with certain co-existing medical disorders. In this case, most women can instead be treated with progestogens only, which can be considered as a second line of treatment for endometriosis-associated pain (83). Progestogens mediate their effects by suppressing endometrial estrogen receptors, leading to decidualization, and eventual atrophy of endometrial tissue (90). Moreover, the suppression of MMP in the endometrium by progesterone may also be contributing to their beneficial effects (90). The side effects of progestogens that include irregular bleeding, weight gain, bloating, fluid retention, breast tenderness, headaches, nausea, and mood changes can limit their use, nevertheless, the reduced bone mineral density loss compared GnRH agonists, make them an attractive option for certain women requiring treatment for their endometriosis-associated pain (91, 92).

When COCs and progestogens cannot be used or when they are contraindicated, or when there is a failure of therapy, GnRH agonists may be considered as a third line of treatment (88). GnRH agonists are modified versions of the native GnRH, which allow them to bind to their receptors for a longer period of time leading to nonpulsatile signaling (83). This causes a hypogonadotrophic and hypoestrogenic state, which leads to amenorrhea and endometrial atrophy (90, 93). However, this hypoestrogenic state leads to bone mineral depletion and vasomotor symptoms, limiting their long term use (93). Due to this, an ‘add back’ therapy utilizing estrogen and progestogen, or estrogen alone is used, which has demonstrated an effectiveness in counteracting the side effects as well as simultaneously controlling the endometriosis-associated pain (90, 93-95).

Not all women respond favorably to medical treatments for endometriosis pain and may require surgical treatment (83). The goals of surgery are to help reduce endometriosis-associated pain by removing ectopic lesions, to restore normal anatomy, and reduce the risk of disease recurrence (83). Surgery may involve ablation, excision or drainage of endometriotic cysts, lysis of adhesions, and destruction of peritoneal implants (87). However, there is a high recurrence rate of ectopic lesions due to microscopic endometriosis lesions that are difficult to remove surgically, and whose removal depend highly on the surgical skills of the surgeon (7).

Although there are various treatment options for endometriosis, there is a high recurrence rate of ectopic lesions and pain symptoms. Moreover, current treatments only target the symptoms of endometriosis and are usually not curative. Therefore, there is an urgent need for new and more effective therapies that not only target the symptoms caused by endometriosis, but also the disease progression. Due to the notion that immunosuppressive MSC may be useful as a potential therapy for inflammatory diseases like endometriosis, we carried out **Study 2**. However, along with our findings in **Study 2**, later in this section it will be discussed how an immunosuppressive subtype of MSC may actually instead be involved in the pathogenesis of endometriosis.

1.5 MESENCHYMAL STROMAL CELLS AND ENDOMETRIOSIS

1.5.1 Background

MSC are multipotent stromal cells that can be isolated from various tissues such as the bone marrow, placenta, endometrium, and umbilical cord, among others (96). The terms mesenchymal stromal cells and mesenchymal stem cells are interchangeable and refer to the same type of cells. MSC were first described as spindle shaped and fibroblast-like cells derived from the bone marrow that were able to form colonies when seeded at low densities (97). The huge surge in research involving MSC lead to the establishment of a set of criteria that these cells need to fulfil in order to be classified as MSC (98). According to these criteria the cells have to be plastic adherent, express a certain repertoire of surface markers such as CD73, CD105, and CD90 and be negative for CD45, CD31, CD34, CD14, and HLA-DR, and be able to undergo multipotent differentiation into the three mesenchymal lineages of adipocytes, osteoblasts, and chondroblasts (98).

MSC are known to be immunomodulatory in that they are able to sense their microenvironment and respond to what they detect by either releasing pro- or anti-inflammatory molecules (99, 100). It is thought that they are able to do this because they contain a machinery for synthesizing and secreting a wide range of cytokines, growth factors, and extracellular vesicles (99, 101). In

essence, MSC are a double edge sword that are able to sense and fine tune their surrounding microenvironment by secreting their repertoire of molecules to maintain homeostasis (99). Due to this ability, MSC have been studied as a therapeutic for a wide range of immune mediated diseases such as multiple sclerosis, graft versus host disease (GvHD), and type 1 diabetes, among others (99).

Recent research suggests that MSC display plasticity in a similar way as macrophages and may exist as different subtypes. Depending on their microenvironment they have been suggested to either develop into the MSC1 or MSC2 subtypes (102). When their surroundings are inflammatory (high levels of IFN-γ/TNF-α) they develop to become immunosuppressive MSC2, which express and secrete high levels of IDO1, COX2, prostaglandin E2 (PGE2), nitric oxide (NO), hepatocyte growth factor (HGF), hemoxygenase 1 (HO-1) and TGF-β1 (103) (**Table 1**). MSC2 induce T regulatory cells (Tregs), suppress T cell responses, and promote an immune response towards M2 macrophage polarization (103) (**Table 1**). Meanwhile, in the absence of inflammatory surroundings (low levels of IFN-γ/TNF-α) they develop to become a pro-inflammatory MSC1, which express and secrete high levels of macrophage migration inhibitory factor (MIF) 1α and 1β, CXCL9, and CXCL10 (**Table 1**) (104, 105). MSC1 induce T cell responses, and promote an immune response towards M1 macrophage polarization (**Table 1**) (104, 105).

Table 1. Summary of phenotypic and functional characteristics of M1 and M2 macrophages, and MSC1 and MSC2.

	Immunostimulatory		Immunosuppressive	
	M1 macrophages	MSC1	M2 macrophages	MSC2
Phenotypic characteristics	High levels of IL-12, IL-13, CD11b	High levels of MIF-1α, MIF-1β, CXCL9, CXCL10	High levels of CD163, CD204, CD206, CD209, B7-H4, IL-10, TGF-β1	High levels of IDO1, COX2, PGE2, NO, HGF, HO, TGF-β1
Functional characteristics	Promote TH1 responses	Promote M1 macrophages, & T cell responses	Promote TH2 responses	Promote M2 macrophages, Tregs & suppress T cell responses

1.5.2 Mesenchymal Stromal Cells in Endometriosis

Indeed, MSC seem to play a role in endometriosis since they are found in ectopic lesions; as mentioned earlier they would be expected to be recruited to ectopic lesions via retrograde menstruation. However, the role MSC play in endometriosis is not completely known, and therefore it was investigated in this PhD project. MSC have been isolated from menstrual blood, eutopic endometrium, and ectopic endometriotic lesions from women with endometriosis (11, 106-109). Some studies have confirmed they are MSC (106, 107), while other studies have not confirmed they are MSC (19, 110), however, herein all the relevant studies of stromal cells derived from women with endometriosis will be mentioned. Moreover, some studies only report that ectopic stromal cells were used, and it is unclear whether the ectopic stromal cells were from endometriotic ovarian cysts, peritoneal endometriotic tissue, or deep infiltrating

endometriosis tissue, which are the three types of endometriosis lesions that can form in endometriosis (111).

The gene expression of COX2 and HO-1 were found to be higher in ESC_{cyst} and stromal cells from peritoneal endometriotic tissue compared to ESC_{endo} (112, 113). Moreover, ectopic stromal cells were found to have a greater protein expression of COX2 and production of PGE2 compared to ESC_{endo} derived from healthy female volunteers (ESC_{endohv}) (114). In another differently designed study, the gene expression of IDO1 and COX2 was found to be higher in menstrual blood-derived stromal cells from women with endometriosis compared to menstrual blood-derived stromal cells from women without endometriosis after culture in a transwell system with peripheral blood mononuclear cells (PBMCs) (11). These studies suggest that immunosuppressive MSC2 may be present in endometriosis. Additionally, peritoneal M2 macrophages from women with endometriosis are known to overexpress both COX2 and have increased production of PGE2 (115). Therefore, both immunosuppressive MSC and M2 macrophages may explain the high levels of PGE2 in the peritoneal cavity, which has been found to have significant roles in the survival and growth of ectopic lesions, and unsurprisingly has been a pharmacological target in several studies (116-120). Moreover, the high levels of PGE2 found in endometriosis has been found to reduce the ability of the peritoneal M2 macrophages to mediate phagocytosis by inhibiting the expression of annexin A2, MMP9, and CD36 (121, 122). Several studies mimicked the *in vivo* inflammatory environment of the pelvic cavity by exposing ESC_{cyst} to pro-inflammatory cytokines such as IL1β, IL17F, and TNF-α *in vitro*, and found that they displayed a significant increase in the gene expression of COX2 and production of PGE2 (112, 123, 124). Moreover, exposure of ectopic stromal cells to IL1β *in vitro* induced the gene and protein expression of COX2 and production of PGE2 (114). Furthermore, a study generated three-dimensional spheroids using ESC_{cyst}, and found that their gene expression of COX2 and production of PGE2 were high, which were then inhibited when treated with the progestin dienogest (125).

As discussed earlier, M2 macrophages seem to play a critical role in the growth of ectopic lesions through direct and indirect mechanisms, however, it is not exactly known what is promoting this phenotype of macrophages in the pelvic cavity in endometriosis. Several studies indirectly suggest that MSC may be promoting M2 macrophages in endometriosis. A study found that ESC_{endo} polarized U937 monocytes stimulated with lipopolysaccharide into M2 macrophages, which showed an increased expression of the immunosuppressive cytokine IL-10 and a decreased expression of the costimulatory molecule CD86 (30). It was suggested that fractaline (FKN) derived from ESC_{endo} drove the M2 polarization of the macrophages (30).

Furthermore, another study found that ectopic stromal cells induced M2 polarization of macrophages (19). The M2 macrophages showed increased expression of CD163 and CD209, and their intracellular expression and extracellular secretion of TGF-β1 and IL-10 were increased compared to macrophages treated with ESC_{endohv} (19). They suggested that IDO1 via IL-33 secreted by the ectopic stromal cells was driving the M2 macrophage polarization. Moreover, the M2 polarized macrophages significantly increased the viability and proliferation, and decreased apoptosis of ESC (126), and enhanced the invasiveness of ESC_{endo} (30). Endometriotic tissue is known to be highly fibrotic and TGF-β1 is widely known to be a key mediator in fibrosis, which has been found to be secreted by both ectopic stromal cells (127) and peritoneal macrophages (128). Further supporting the role of MSC in fibrosis, a recent study found that MSC in ectopic lesions may be involved in fibrosis development in endometriosis via their secretion of TGF-β1 (129). Interestingly, a recent study suggested that that M2a macrophages may play an important role in fibrosis in mice with endometriosis (130). Individual to individual variations in terms of the immunological microenvironment and endocrine factors may occur with the use of ESC_{endohv} as a control in some of the studies mentioned. Moreover, stimulating stromal cells with pro-inflammatory cytokines *in vitro* may not reflect the stromal cells in the *in vivo* microenvironment closely. However, taken together the aforementioned studies indirectly suggest that MSC2 may exist in ectopic lesions, which is known to induce the M2 macrophage phenotype that seems to play a significant role in the initiation and progression of ectopic lesions in endometriosis; this has been directly confirmed by our findings in **Study 1**.

As mentioned earlier, NK cells also seem to be playing a role in the growth of ectopic lesions through indirect mechanisms, however, it is not exactly known why the function of NK cells is inhibited, but stromal cells have been implicated. Liu *et al.* cultured NK cells from the peripheral blood of healthy female volunteers with ESC_{endohv} and ectopic ESC; they found that the NK cells enhanced their expression of IDO1, especially following culture with ectopic ESC, and the effect was partly due to ESC-derived TGF-β1 (68). Moreover, when the NK cells were in turn cultured with ectopic ESC, their cytotoxicity was inhibited, and this was attributed to their expression of IDO1 (68). However, as mentioned earlier comparing the effects of ectopic ESC to ESC_{endohv} on NK cells is not appropriate, because ESC_{endohv} may introduce individual to individual variations in terms of the immunological microenvironment, and endocrine factors. Somigliana *et al.* carried out a study where they observed the effect of supernatant released from ESC_{endohv} on NK cell cytotoxicity using ESC_{endohv} as target cells in ^{51}Cr release assays (131). They found that NK cell cytotoxicity was inhibited, and attributed this finding to soluble intercellular adhesion molecule 1 that may allow the escape of target cells from NK

cell killing (131). Moreover, the same group compared the effects of supernatants released from ESC_{endo} and ESC_{endohv} on NK cell cytotoxicity using ESC_{endohv} as target cells in ^{51}Cr release assays, and found that NK cell cytotoxicity was significantly inhibited by supernatants released from ESC_{endo} compared to ESC_{endohv} (132). Yu *et al.* directly co-cultured NK cells from the peripheral blood of healthy female volunteers with ESC_{cyst} and ESC from other types of ectopic tissue in the presence of IL-15, and compared the results to NK cells cultured alone (133). They found that IFN-γ, NKG2D, and NKp44 expression by the NK cells were reduced, and concluded that ESC suppress the cytotoxic functions of NK cells to allow their immune escape and subsequent growth in endometriosis (133). The same group carried out a study using a system that had ESC_{cyst} and macrophages directly co-cultured with NK cells isolated from the peripheral blood of healthy female volunteers, and compared the results to NK cells cultured alone (60). They found that NK cell cytotoxicity was reduced, and by using blocking studies they determined that it may be due to the secretion of both of the immunosuppressive molecules TGF-β1 and IL-10 from both ESC_{cyst} and the macrophages (60). However, it seems that the effect on the NK cells is mostly due to the macrophages and not ESC_{cyst} (60). Moreover, a comparison to ESC_{endo} cultured with the NK cells would be more relevant to the pathology of endometriosis. Therefore, various factors, including stromal cells in the pelvic cavity in endometriosis seem to be involved in the inhibition of NK function. However, it is not completely clear how NK cells are inhibited in the pelvic cavity, therefore, in **Study 3** we investigated if immunosuppressive ectopic MSC are involved.

Since endometriosis is widely known to be an inflammatory disease with high levels of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α, IFN-γ, and IL1β in the peritoneal cavity, the findings of the aforementioned studies may not be surprising (103, 134). One could speculate that immunosuppressive MSC2 may be induced in order to oppose the high levels of inflammation in their surroundings and attempt to return it to homeostasis by inducing various immunosuppressive pathways. However, this may be the trigger leading to the initiation and progression of this disease by suppressing the immune system from clearing the ectopic lesions.

1.6 MSC2 AND M2 MACROPHAGES IN ENDOMETRIOSIS

The Sampson theory of retrograde menstruation is the most widely accepted theory for explaining the pathogenesis of endometriosis. In addition, the aforementioned studies support the stem cell theory for the pathogenesis of endometriosis (135). The stem cell theory postulates that a putative stem/stromal cell population such as MSC are refluxed back into the peritoneal cavity via retrograde menstruation, which then give rise to and support ectopic lesions (135, 136).

Consequently, one can speculate that endometriosis is caused by a cascade of events involving MSC2 and M2 macrophages as important players initiating and supporting ectopic tissue growth (**Figure 1**). Menstruation is essentially an inflammatory process, so when the menstrual fluid containing MSC is refluxed back into the peritoneal cavity during retrograde menstruation it leads to an inflammatory environment in the peritoneal cavity (6). This inflammatory environment, containing inflammatory cytokines such as IFN-γ and TNF-α, may induce MSC2 that attempts to oppose the high levels of inflammation in the environment by inducing immunosuppressive pathways (137). Then, when innate immune cells such as monocytes are recruited, they are modulated by factors such as IDO1, PGE2, and TGF-β1 secreted by MSC2 to differentiate into M2 macrophages. The M2 macrophages perceive the ectopic endometrial tissue as a wound, and release various anti-apoptotic, trophic, and angiogenic factors that support the survival and growth of MSC2 and the ectopic endometrial tissue. In addition, based on our findings in **Study 2**, MSC2 may directly support the survival and growth of ectopic endometrial tissue. Therefore, although MSC2-induced M2 differentiation of macrophages may allow disease amelioration for other conditions, in the case of endometriosis it may instead be promoting disease progression (137).

If retrograde menstruation occurs in almost all women, then why do only approximately 10% develop endometriosis? It is well documented that the eutopic endometrial tissue from women with endometriosis differs from women without endometriosis in that it has resistance to apoptosis, increased migration, increased adherence to the methothelium lining of the peritoneal cavity, invasion, and induction of angiogenesis to help it receive nutrients that allows its growth (138). It could be speculated that in women not suffering from endometriosis, the endometrial tissue may be dying by apoptosis and not persisting. In contrast, the endometrial tissue from women with endometriosis may persist and is supported by the M2 macrophages to aggressively expand. Moreover, factors released by M2 macrophages and MSC2 such as IDO1, PGE2, M-CSF, IL-10, and TGF-β1 may induce TH2 responses, and continue inducing differentiation towards M2 macrophages, and subsequently inhibit the functions of downstream immune cells such as NK cells, dendritic cells (DC), cytotoxic CD8+ T cells but induce the functions of Tregs. There are reports that DC maturation is reduced in endometriosis with components of the process of antigen capture and presentation altered; there are increased numbers of immature CD1+ DC and reduced numbers of mature CD83+ DC (139, 140). Moreover, it has been shown that peripheral, and peritoneal NK cells have impaired cytotoxic functions in endometriosis, which may promote the implantation and growth of ectopic lesions in endometriosis (65, 69, 77). The cytotoxic functions of CD8+ T cells are also reduced in endometriosis (141). Moreover, several studies show that there is an upregulation in the number

and functions of Tregs in the ectopic milieu, which may contribute to endometriotic tissue immunotolerance and progression of endometriosis (142, 143).

Further supporting the aforementioned are the wide range of conventional studies that demonstrate that MSC are able to induce M2 macrophages, inhibit maturation of DC, reduce proliferation and cytotoxic functions of NK cells, and of CD8+ T cells but instead induce functions of Tregs (24, 144-148). These functions of MSC may be useful in the treatment of inflammatory diseases such as multiple sclerosis, type 1 diabetes, and GvHD, among others (99). However, in the case of endometriosis it may be the complete opposite, in that MSC leads to a disturbance in the surveillance activities of the immune system in the pelvic cavity, and hence allow the survival and growth of ectopic lesions. Therefore, it is unsurprising that a recent study suggested that a disruption of the stem cell recruitment to ectopic lesions may allow their long-term regression and potentially treat endometriosis (149).

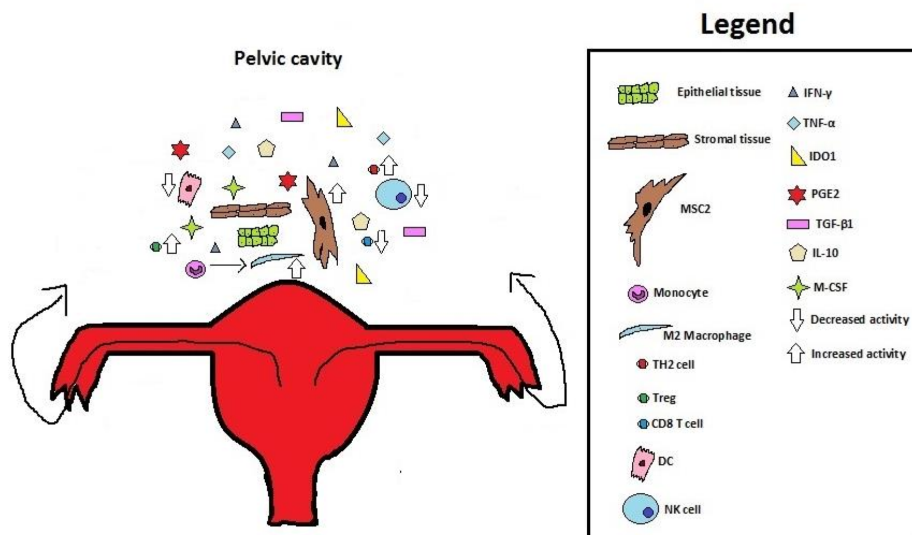

Figure 1. Potential role of MSC2 and M2 macrophages in the pathogenesis of endometriosis.

An illustration of the possible cascade of events involving MSC2 and M2 macrophages in the initiation and support of ectopic (epithelial and stromal) tissue growth causing endometriosis. Immunosuppressive MSC2 is induced by the inflammatory environment created by retrograde menstruation back into the pelvic cavity (e.g. IFN-γ, TNF-α), which then induces recruited monocytes to become M2 macrophages. MSC2 and M2 macrophages cause the levels of IDO1, PGE2, TGF-β1, IL-10, and M-CSF to increase in the pelvic cavity, inducing TH2 responses including Tregs, and decreased functions of DC, NK cells and CD8 T cells. The net effect is promotion of ectopic tissue growth and development of endometriosis.

MSC2: mesenchymal stromal cell type 2, TH2: T helper 2 cell, Treg: T regulatory cell, CD8 T cell: cytotoxic CD8 T cell, DC: dendritic cell, NK cell: natural killer cell, IFN-γ: interferon-gamma, TNF-α: tumour necrosis factor-alpha, IDO1: indoleamine 2,3-dioxygenase 1, PGE2: prostaglandin E2, TGF-β1: transforming growth factor-beta 1, IL-10: interleukin-10, M-CSF: macrophage-colony stimulating factor.

Finally, one could speculate that redirecting MSC2 in ectopic lesions towards MSC1 may be useful in endometriosis by promoting M1 macrophages, and hence TH1 responses. The M1 macrophages could favor increased responses of downstream immune cells such as DC to promote antigen presentation, and cytotoxic NK and T cell functions to help clear the ectopic lesions in the peritoneal cavity, and simultaneously inhibit the immunosuppressive functions

of Tregs. Accordingly, clearing the peritoneal cavity from the fibrotic ectopic lesions, and the associated inflammation may help address the two major symptoms of endometriosis; infertility and chronic pelvic pain, and potentially treat this enigmatic disease.

2 HYPOTHESIS

In this PhD project it was hypothesized that MSC derived from endometriotic ovarian cysts could be involved in the reduced immunosurveillance of ectopic endometrial tissue in the pelvic cavity through their immunosuppressive effects on macrophages and NK cells. Moreover, if autologous MSC are involved in the pathology, then it was hypothesized that the immunomodulatory capabilities of allogeneic MSC could be a potential therapeutic strategy to target the inflammatory component of endometriosis.

3 AIMS

Although MSC have been suggested to be involved in the pathogenesis of endometriosis, their role in its pathogenesis is still not completely clear. Moreover, the therapies that are currently available are not effective, and are associated with a high recurrence rate of ectopic lesions and symptoms. Therefore, the overall aim of this thesis was to understand the role of MSC in the pathogenesis of endometriosis, and to examine whether allogeneic MSC could potentially be a therapy for endometriosis. To fulfill these aims, the following studies were conducted with the following specific aims:

Study 1. To examine if ESC_{cyst} and ESC_{endo} differ in terms of their phenotype and immunomodulatory effects *in vitro*.

Study 2. To examine the direct effects of allogenic adipose tissue-derived MSC (Ad-MSC) on ESC_{cyst} and ESC_{endo} *in vitro*.

Study 3. To examine the interactions of allogeneic NK cells and ESC_{cyst} and ESC_{endo} *in vitro*.

4 MATERIALS AND METHODS

The papers and manuscript included in this thesis contain detailed descriptions of the materials, methods, and statistics used. The publications have the exact manufacturers of reagents, consumables, and hardware so please refer to them. Therefore, this section will briefly discuss the materials and methods that were utilized in the studies.

4.1 ETHICAL CONSIDERATIONS

Ethical approval for all the studies presented in this thesis was obtained from the Regional ethics committee in Stockholm, Sweden. This includes: isolation of stromal cells (2013/1094-31/2) from endometriotic ovarian cysts and the endometrium of women with endometriosis, and isolation of stromal cells from adipose tissue biopsies obtained from women that underwent planned caesarian sections (2017/1017-32). All patients gave oral and signed informed consent, and the studies were in accordance with the Declaration of Helsinki.

4.2 MATERIALS FOR STUDIES

4.2.1 Stromal cells from women with endometriosis and healthy pregnant women

The methods utilized to isolate the stromal cells from women with endometriosis (n=4) that were used for **Studies 1**, **2**, and **3**, and from healthy pregnant women (n=2) that were used for **Study 2** are similar to those used previously by various groups (107, 133, 150, 151), which is why we utilized them. Biopsies of endometriotic ovarian cyst tissues (for ESC_{cyst}) and eutopic endometrial tissues (for ESC_{endo}), and adipose tissues (for allogeneic Ad-MSC) from healthy pregnant women undergoing elective caesarian sections were digested using collagenase type I and II, respectively. Allogeneic Ad-MSC were used because autologous MSC from women with endometriosis were founded to be altered by the pathology in **Study 1**. Following digestion we filtered out contaminating cells including epithelial cells and undigested tissue using 100 and 40 µm cell strainers. Eventually, the cells were resuspended in complete growth medium containing Dulbecco modified essential medium low glucose + 10% MSC certified fetal calf serum (FCS) + 1% antibiotic and antimycotic and cultured at 4000 cells/cm^2 in tissue culture flasks at 37 °C with 5% CO_2. When the cells reached 70-90% confluency, they were trypsinized using 0.05% trypsin/EDTA, and cultured as described above and used for the various experiments in **Studies 1, 2, and 3** from passage three until passage seven. This method of isolation takes advantage of the adherent properties of MSC (152); the idea is that the primary stromal cells eventually outgrow all the other contaminating cells by passage three. The MSC were confirmed using the standard procedures of colony forming units- fibroblasts

(CFU-F), flow cytometry, and differentiation into adipocytes and osteoblasts, as described previously (106), which are important methods to verify MSC characteristics (98). However, there are limitations associated with these methods of isolation, which include potential changes to the phenotypic and functional properties of the MSC following digestion by collagenase and due to culture on plastic surfaces, the potential contamination by a small number of other types of cells, and the heterogeneous nature of the MSC populations used in the experiments (153, 154). Due to such limitations, there has been development of other MSC isolation methods; direct isolation of MSC from cell suspensions obtained following digestion of tissues biopsies using specific MSC markers using fluorescence activated cell sorters for immediate use as native MSC in experiments or following culture on plastic surfaces (155, 156), direct isolation of MSC from cell suspensions obtained following digestion of tissue biopsies using specific MSC markers using magnetic beads for immediate use as native MSC in experiments or following culture on plastic surfaces (157), and harvesting of MSC following their migration from the cut ends of tissue explants cultured on plastic surfaces (96). The utilization of primary MSC from women with endometriosis and healthy pregnant women is an advantage, because these cells are minimally altered when cultured *in vitro*, and closely resemble the original cells found *in vivo*, compared to the use of the commonly used endometriosis 12Z (158) and stromal 22B (158) cell lines that are genetically altered (159). However, compared to cell lines, primary cells do not proliferate much *in vitro*, making their use in experiments more limiting (159).

4.2.2 Monocytes from healthy female volunteers

Monocytes that were used in **Study 1** were isolated from buffy coats donated by healthy female volunteers (n=3) using magnetic beads through a process termed negative selection. Essentially, all the non-monocytes are magnetically labelled with a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and Glyocophorin A, and antibiotin microbeads, which are then removed from the cell suspensions leaving the monocytes for later use in experiments. The monocytes were cultured in complete growth medium containing Roswell Park Memorial Institute 1640 growth medium + 10% FCS + 1% L-glutamine + 1% penicillin and streptomycin. An advantage of this method is that the monocytes are untouched and hence are not activated, and a disadvantage of this method is the phenotypes and functions of the monocytes may have been altered by culture on the plastic surfaces. However, compared to an older method that isolated monocytes through a process of adherence following several hours of culture of PBMCs, this method allows a more pure ($\geq$ 95% pure) population of monocytes to be isolated in a shorter period of time (160).

4.2.3 NK cells from healthy female volunteers

Natural killer (NK) cells that were used in **Study 3** were isolated from buffy coats donated by healthy female volunteers (n=26) using magnetic beads through a process termed negative selection. Essentially, all the non-NK cells such as T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells are indirectly magnetically labelled with a cocktail of biotin-conjugated antibodies and a cocktail of microbeads, then they are removed from the cell suspensions leaving the NK cells for later use in experiments. The NK cells were cultured in complete growth medium containing Stem Cell Growth Medium + 20% FCS + 500 U/mL IL-2. An advantage of this method is that the NK cells are untouched and hence are not activated, allowing a more pure (≥ 95% pure) population of NK cells to be isolated in a short period of time compared to an older method of NK cell isolation (161), and a disadvantage of this method is that the phenotypes and functions of the NK cells may have been altered by culture on the plastic surfaces.

4.2.4 Conditioned medium from MSC

The method used to collect CM from MSC for **Studies 1**, **2**, and **3** is similar to what has been previously used by various groups (24, 162, 163). Briefly, MSC are grown up to 70-90% confluency, then the growth medium is removed, and the cells are washed twice with phosphate buffered saline. Then fresh growth medium is added. Following three days of culture, the growth medium is obtained from the tissue culture flasks, centrifuged at 500x*g* for 10 min, filtered using a 0.2 µm filter to remove contaminating cellular debris, and then stored at -80 °C for later use in experiments. For the CM experiments in **Study** 2, a similar procedure was used to collect CM from Ad-MSC/ESC_{cyst}, and Ad-MSC/ESC_{endo} co-cultures from the *in vitro* cell culture experiments. Obviously, the advantage of using CM is that experiments are simple to prepare compared to experiments employing transwells or direct co-culture between cells, and although they are not entirely representative of the *in vivo* microenvironment, they still offer a snapshot of what may occur *in vivo*.

4.2.5 Other cells and cell lines used

Human umbilical vein endothelial cells (HUVEC) were kindly provided by Dr. Nina Heldring (Karolinska Institutet) and used in **Study 2**. They were isolated (n=2) as previously described (164) and grown on 0.1% gelatin coated surfaces in complete HUVEC growth medium containing human endothelial serum-free basal medium + 10% FCS + 1% penicillin and streptomycin.

The solitary cell line used in this PhD project was in **Study 3**, which was the K562 cell line, chronic myelogenous leukemia cells that were used as target cells in the ^{51}Cr release assay (see below). This cell line is commonly used in this assay because they are suspension cells that are easy to monitor *in vitro*, and their genetic makeup (lack of HLA class I) makes them sensitive to killing by NK cells (165).

4.3 METHODS FOR STUDIES

4.3.1 Cell culture methods

This PhD project utilized the commonly used method of *in vitro* cell culture for **Studies 1**, **2**, and **3**. We did not use *in vivo* animal models for any of the studies, because we first wanted to examine the MSC *in vitro* before examining them further *in vivo* to validate the *in vitro* findings. Nevertheless, the most relevant *in vivo* animal model for endometriosis is primates such as Baboons (166), and not the commonly used rodent (28, 106, 167) and rabbit models (168), because they have a menstrual cycle similar to female humans. The advantages of *in vitro* cell culture studies is that they are less expensive, take a shorter time and hence can be carried out more often than *in vivo* studies, since there is access to a sufficient amount of cells that are easier to maintain than animal models (169). In contrast, the disadvantage of *in vitro* cell culture is that they don't represent the *in vivo* microenvironment as well as *in vivo* animal models (169). Also, it must be mentioned that we used 2D cell culture, which is easier to carry out than 3D cell culture, however, they are less representative of the *in vivo* microenvironment (159). Furthermore, we utilized various cell culture methods such as cell proliferation, apoptosis, adhesion, angiogenesis, migration, and invasion assays. We also used various systems in *in vitro* cell culture including CM, transwell, and direct co-culture systems. The following sections will discuss the various assays and systems we used in *in vitro* cell culture in this PhD project.

4.3.1.1 Cell proliferation assays

In **Study 2**, following the *in vitro* cell cultures employed, several cell proliferation assays were used including the manual cell count, carboxyfluorescein succinimidyl ester (CFSE), and MTT (d-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays to examine the effects of Ad-MSC on the proliferation of ESC_{cyst} and ESC_{endo}. The purpose of using several cell proliferation assays was to validate the findings through more than one method. The manual cell count assay is a routinely used method in *in vitro* cell culture, where in this case the cells are stained with a dye like eosin. Eosin only stains dead cells red due to their disrupted cell membrane, while viable cells remain colorless under the light microscope, giving information

on cell number increases and viability. The advantage is it is a routine simple method, however, since it is prone to human error, we then employed the CFSE and the MTT assays. The CFSE and MTT assays have been previously used by others to measure cell proliferation (170, 171).

Briefly, for the CFSE assay, the CFSE stained ESC_{cyst} and ESC_{endo} were assayed on a flow cytometer following the experiments to measure their level of expression or median fluorescence intensity (MFI) of the CFSE dye; essentially the CFSE dye is diluted when cells proliferate leading to a reduced fluorescence and hence MFI with time, the greater the cell proliferation then the lower MFI (172). The CFSE assay is easy to use to quickly label target cells, the dye is also retained for a while in cells, and it is non-toxic with minimal effects on the proliferation ability of the stained cells (173). In addition, it is a more quantitative method than the other assays we employed for cell proliferation since it uses flow cytometry. However, a limitation is that it is prone to bleaching and may lead to irreproducibility, and therefore care must be taken to avoid exposure to light (173). However, CFSE is a common method used to measure cellular proliferation, which is why it was utilized (173).

Briefly, for the colorimetric MTT assay, ESC_{cyst} and ESC_{endo} were stained with the MTT dye following the experiments to measure their optical density using a spectrophotometer. Essentially, the MTT dye is converted into purple formazan crystals that are then solubilized using DMSO in viable proliferating cells, hence giving information regarding cell number and proliferation (174). This assay is not expensive, and it is a simple and quick alternative to derive cell proliferation data before validation using more expensive methods (174). Compared to fluorescent methods such as the CFSE assay, this assay is less sensitive, which is why we also used the CFSE assay (174). Moreover, the number of washes and steps in the procedure has led to the development of assays such has the MTS assay that does not require any washing steps (175). Nevertheless, the MTT assay is a commonly used assay that provides valuable information on cell proliferation (176).

4.3.1.2 Cell apoptosis assay

In **Study 2**, the annexin V assay was utilized to examine the apoptosis of ESC_{cyst} and ESC_{endo} following their *in vitro* cell culture with Ad-MSC. Briefly, following the experiments ESC_{cyst} and ESC_{endo} were stained with the annexin V antibody and 7-AAD. Annexin V binds to phosphatidyl serine that becomes exposed on a cell's surface during apoptosis, and 7-AAD binds to intracellular DNA in membrane compromised cells undergoing necrotic death (177). Then the cells are assayed on a flow cytometer and scatterplots are created with annexin V versus 7-AAD giving data on the percentage of cells undergoing early apoptosis (only annexin

positive), late apoptosis (annexin and 7-AAD positive), or necrosis (only 7-AAD positive) (178). The advantage of this assay is that it gives information on both cell viability and the various types of cell death. In addition, it gives more quantitative data than methods such as the comet assay, since it makes use of flow cytometry (179). The disadvantages of this assay is that only live cells can be used during staining compared to the terminal deoxy transferase-mediated dUTP nick end labelling (TUNEL) assay that can be done with fixed cells, and unlike the TUNEL assay it does not give information regarding the phase of the cell cycle apoptosis is occurring (179). However, the annexin V assay has been previously used in the field of endometriosis to measure apoptosis, which is why we utilized it (180, 181).

4.3.1.3 Cell adhesion assay

In **Study 2**, the fibronectin adhesion assay was utilized to examine the adhesion of ESC_{cyst} and ESC_{endo} following their *in vitro* cell culture with Ad-MSC. Briefly, following the experiments ESC_{cyst} and ESC_{endo} were incubated on fibronectin coated wells of 96 well plates for two hours. Then the cells were washed so that only the adherent cells were measured in the MTT assay as described above. Fibronectin is an extracellular matrix protein normally found in the pelvic cavity in women with endometriosis, which is why we used it (182). As stated above, the MTT assay is a cheap, easy and quick method to employ, however, compared to fluorescent methods it is less sensitive (174). Nonetheless, this assay is similar to what has been previously used in the field of endometriosis to measure cell adhesion (182, 183).

4.3.1.4 Tube formation assay

In **Study 2**, the tube formation assay was utilized to examine the tube formation of HUVEC on matrigel coated wells in 96 well plates while they were being cultured in the CM derived from co-cultures of Ad-MSC/ESC_{cyst} and Ad-MSC/ESC_{endo}. This method is an *in vitro* model of angiogenesis that has been established previously (184). Briefly, HUVEC were allowed to grow on matrigel coated wells in the aforementioned CM for 17-18 hours, then images were taken of the tubes the HUVEC formed at 4x magnification that displayed the whole well, and the number of tubes formed per well were quantified using the angiogenesis analyzer plugin on ImageJ as previously described (185). The limitations of this assay is that there may be a lack of consistent lumen formation in the rings, which may affect reproducibility of experiments, and its correlation to the *in vivo* situation is unclear (186). However, an advantage of this assay is that it is a good representation of all the processes that endothelial cells must undergo *in vivo* during angiogenesis in order to form rings, such as migration, proliferation,

and matrix degradation (186). This procedure is similar to what has been used previously in the field of endometriosis to measure tube formation (187, 188).

4.3.1.5 Cell migration and invasion assays

In **Study 2**, migration and invasion transwell assays were utilized to examine the migration and invasion of ESC_{cyst} and ESC_{endo}, respectively, following their *in vitro* cell culture with Ad-MSC. These assays have been previously used in the field of endometriosis to measure migration and invasion of ESC (189, 190). Briefly, following culture with Ad-MSC, ESC_{cyst} and ESC_{endo} were harvested and seeded on 8 µm pore transwell inserts, with FBS placed in the bottom to allow a chemotactic gradient to induce migration or invasion of the ESC through the pores and then to attach to the other side of the inserts. For the invasion assay, matrigel was used to coat the upper surface of the transwell inserts, so that the ESC also have to degrade and invade the extracellular matrix before migrating to the bottom side of the inserts. The non-migrated or non-invaded cells were removed using cotton swabs, then the migrated or invaded cells were stained with eosin. Finally, images were taken of the inserts in five randomly chosen areas at 10x magnification, and the number of cells migrated or invaded were counted using ImageJ. A disadvantage of these assays is that they are endpoint assays that do not provide much information about the actual processes involved in migration and invasion (191). However, the availability of different inserts and sizes, and the ease at which the experimental setup is prepared are advantages (191).

4.3.1.6 Conditioned medium, transwell and direct co-culture systems

In this PhD project, the CM, transwell, and direct co-culture systems were employed during *in vitro* cell culture; **Figure 2** shows an example of the systems in **Study 2**. These systems were carried out, because they are normally used in the field of *in vitro* cell culture, and have been used previously by others (24, 60, 144). Each of the systems provides a different snapshot of the *in vivo* microenvironment, so that together they help to form a more complete picture of what might potentially occur. In the CM system the factors that are present are examined for their effect on the target cells. In contrast, in the transwell system the target cells in the bottom wells influence the type of factors that are secreted by the cells in the upper inserts, so it is mimicking paracrine signaling. Finally, in the direct co-culture system both cell types are cultured together in the same well to examine their effects on each other in terms of a certain parameter, for example in **Study 2**, we examined the effect of culture of Ad-MSC with ESC on the apoptosis and survival of ESC.

Figure 2. Showing the various systems that were employed during *in vitro* cell culture in **Study 2**.

An illustration showing the various systems that were employed during in vitro cell culture in ***Study 2****. The CM system contained already secreted factors from Ad-MSC, which were examined for their effect on ESC. Meanwhile, the transwell system had ESC in the bottom wells that controlled the type of factors that were secreted by Ad-MSC in the upper inserts. Finally, in the direct co-culture system both Ad-MSC and ESC were cultured together in the same well to examine their effects on each other in terms of a certain parameter, for example, apoptosis and survival of ESC.*

4.3.2 Flow cytometry

Flow cytometry is a commonly used method to carry out phenotypical and functional studies involving cells of interest (192). In this PhD project we used this method in **Studies 1**, **2**, and **3**. The main use of this method was to characterize various cell types in the studies such as MSC, macrophages, and NK cells. Flow cytometry can be used to stain cells extracellularly on the cell surface or intracellularly within the cells (192), with both techniques being used in this PhD project. For the former, cells are incubated with the appropriate antibodies targeting certain antigens on the cell surface (192). However, for the latter the cells need to be fixed and permeabilized in order to prepare them for incubation with antibodies that will enter and stain antigens within the cells (192). Eventually, the cell suspensions are assayed on a flow cytometer, and parameters such as side scatter (cell granularity) and forward scatter (cell size) on scatterplots, are used to locate and further analyze certain cell populations for the percentage of cells that express a certain antigen using histograms and scatterplots (192). In addition, the intensity of expression of a certain antigen on the cells of a population of interest is measured via a parameter termed the MFI (192).

The advantages of flow cytometry is that it is quiet easy to employ, can be carried out quite fast, and is much more quantitative compared to other protein measurement methods such as western blotting, immunofluorescence microscopy, immunocytochemistry, and immunohistochemistry, which require several days to carry out. However, it certainly would be more complete to have quantitative data regarding a protein using flow cytometry, and fluorescence or colorimetric imaging using immunofluorescence microscopy or immunohistochemistry of the same protein in the target tissue. Moreover, the quantitative nature of the method makes it possible to have access to a multitude of parameters that can be used to understand the cell populations of interest due to the potential use of various antibodies conjugated with various fluorophores against various cellular antigens (192). A disadvantage of this method is that surface molecules on cells may be cleaved off following trypsinization, and the cells must be in suspension so information regarding tissue architecture or cell-cell interactions of adherent cells is lost (193). Moreover, it can be costly due to the price of antibodies, and the fact that the actual flow cytometers are expensive to purchase and to maintain. Also, sometimes a large number of cells are required for analysis, which may not be possible to obtain if slowly growing primary cells are used. The fluorophores that are conjugated to the antibodies are used to quantify the various antigens of interest via their emission of fluorescence of various wavelengths, however, there can be overlap between the emission wavelengths of different fluorophores, which makes them difficult to distinguish from each other especially when a large number of fluorophores are used in an experiment, even if strict compensation and fluorescence minus one controls are employed to remove fluorescence spillage (193). Therefore, recently there has been development of mass cytometry that overcomes the spectral overlap associated with flow cytometry, by utilizing probes linked to unique stable, heavy-metal isotopes that can be distinguished with high accuracy (194). Nevertheless, flow cytometry is still a widely used method that offers the possibility of extracting a large amount of data from experiments, hence why it was used in this PhD project.

4.3.3 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was used in **Study 1**, because it is a common method to quantify gene expression, and because only several genes were examined. Briefly, RNA was extracted from the MSC, then it was reverse transcribed into cDNA, which was eventually quantified for various genes using customized primers. The advantages of this method compared to conventional PCR and the older method of Northern blot is that it is highly sensitive since it allows the detection of low-copy targets, it has a wide dynamic range, it is more accurate, is more quick to carry out due to less steps, is less expensive, is highly

quantitative as absolute copy numbers can be determined, and there is elimination of cross-contamination due to the closed-tube format (195, 196). However, more recent methods such as digital PCR, microarrays, serial analysis of gene expression, and RNA sequencing have the ability to examine the expression of many genes (was not our aim) so they are less time consuming, are highly reproducible, and require less starting amounts of RNA compared to qPCR (197). Moreover, we utilized SYBR Green as a probe, which has disadvantages compared to other probes like TaqMan, of being less accurate and specific, and it is not possible to do multiplex PCR or to determine the expression of several genes simultaneously (198). However, compared to the TaqMan method, SYBR Green is less expensive, provides relative and not absolute quantitation (what we wanted), and it is easier to use, which is why we utilized it in the PhD project (198).

4.3.4 **Chromium 51 release assay**

The ^{51}Cr release assay is a standard assay that has been widely utilized in the study of NK cell cytotoxicity, hence why we used it in **Study 3** (199). Briefly, we labelled the target cells in **Study 3** with ^{51}Cr for 1 hour which were the K562 cell line or the ESC, then we cultured them with the effector NK cells in V-bottom 96 well plates for four hours. Then the supernatants that contained the ^{51}Cr released following the lysis of either the K562 cell line or the ESC were collected and assayed on an automated gamma counter. Negative control wells containing only complete growth medium for spontaneous release of ^{51}Cr, and positive control wells containing 2M hydrochloric acid for maximum lysis of ^{51}Cr labelled target cells and maximum release of ^{51}Cr were included. Finally, the counts per minute were used to calculate the percentage killing of the target cells carried out by the effector NK cells using the standard formula (199):

% cytotoxicity = [(experimental - spontaneous release)/(maximum release - spontaneous release) x 100]

The advantages of this assay is that as previously mentioned, it uses the K562 cell line that is easy to maintain, and that is sensitive to NK cell killing due to the lack of expression of HLA-I (165). Moreover, the assay only requires a small number of cells. However, the disadvantages of this assay are the costs and the handling of radioactivity involved, as well as the fact that only dead cells are measured, and there is no information on both live and dead cells or cells undergoing various stages of cell death (200). Various other assays have been developed to overcome the disadvantages such as the real-time digital bio-imaging cytotoxicity assay (200), a bioluminescence imaging assay (201), and the antibody-dependent cell-mediated cytotoxicity and complement dependent cytotoxicity assays (202), among others. However, the ^{51}Cr release

assay is still widely used, and remains the most sensitive assay to examine NK cell cytotoxicity (200), hence why we used it in this PhD project. We also carried out the degranulation assay in **Study 3**, which examines NK cell cytotoxicity, but by measuring the expression of the degranulation marker CD107a in NK cells (203). Degranulation is a process where there is a release of granules from NK cells that are involved in NK cell killing of target cells (203).

5 RESULTS AND DISCUSSION

The papers and the manuscript included in this thesis contain the detailed description and discussion of the results. Therefore, this section will only briefly describe and discuss the results of the studies carried out in this PhD project.

5.1 STUDY 1

MSC have been found in the pelvic cavity of women with endometriosis (106), however, it is not completely known what the involvement of MSC may be in endometriosis. Therefore, we examined the phenotype and function of MSC in women with endometriosis to gain more insight regarding their potential role in endometriosis.

Initially, we confirmed that both ESC_{cyst} and ESC_{endo} were MSC following the use of the commonly used standard assays of CFU-F, flow cytometry for MSC markers, and osteoblast and adipocyte differentiation, as shown before (106). Then ESC_{cyst} and ESC_{endo} were compared phenotypically and functionally. Phenotypically, we examined the expression of IDO1, COX2, and HO-1, which are immunosuppressive enzymes that have been suggested to be highly expressed by immunosuppressive MSC2 (103). Moreover, MSC2 has been suggested to express lower levels of immunostimulatory molecules such as CXCL12, so we also examined its expression in the ESC (103). ESC_{cyst} expressed IDO1, COX2, and HO-1 significantly more than ESC_{endo} using both qPCR for gene expression, and flow cytometry for protein expression. These results are similar to some of the studies carried out previously (112, 113), however, some of the other studies (110, 150) have had conflicting results to ours. In addition, sometimes the comparisons have been made to ESC_{endohv}, however, these control cells may introduce individual to individual variations in terms of the immunological microenvironment and endocrine factors (11, 108). Also, in some cases it was not clear if the ectopic stromal cells were from peritoneal endometriotic tissue, endometriotic ovarian cysts, or deep infiltrating endometriosis tissue, which are different types of endometriosis lesions (108). Moreover, herein we did not stimulate our ESC with immune cells or cytokines, in order to reflect the *in vivo* microenvironment more closely, in contrast to a previous study (11).

Interestingly, ESC_{cyst} expressed significantly higher and lower levels of the protein and gene of CXCL12 compared to ESC_{endo}, respectively. This finding is similar to a study by another group (204), but they stimulated their cells with estrogen or progesterone, and unlike our study it does not reflect the *in vivo* microenvironment closely. The discrepancy of the protein and gene results may be due to posttranscriptional and posttranslational processes, since it is known that there are weak correlations between mRNA and protein expression (205). However,

proteins are more important in cellular function (205), and so therefore ESC_{cyst} may also be more immunostimulatory than ESC_{endo}: this may due to their dynamic responses to fluctuations in the levels of pathological inflammation in the pelvic cavity.

IDO1, COX2 via secretion of PGE2, and HO-1 have been suggested to induce immunosuppressive M2 macrophages. Interestingly, M2 macrophages are predominantly found in ectopic lesions in women with endometriosis, however, the mechanisms of their induction are not known (18, 28). Therefore, we carried out a functional study, where we found that ESC_{cyst} induced more monocytes to differentiate into M2 macrophages than ESC_{cyst}; ESC_{cyst} treated monocytes expressed CD14 and CD163 more significantly than ESC_{endo} treated monocytes, and appeared to be more spindle shaped when they differentiated into macrophages. Accordingly, immunosuppressive ectopic MSC may be inducing immunosuppressive M2 macrophages in the pelvic cavity in endometriosis. Previously, several differently designed studies have suggested that M2 macrophages are induced in endometriosis due to ESC (19, 30). However, as mentioned earlier we believe the use of ESC_{endohv} as control cells is not appropriate, and it is important to know the type of endometriosis lesions from which the ectopic stromal cells are isolated. Moreover, we compared the effects of ESC_{cyst} and ESC_{endo} on unstimulated human primary monocytes, and to our knowledge this is the first time that this has been performed.

The data shows that ESC_{cyst} is more immunosuppressive than ESC_{endo} both phenotypically and functionally. The pelvic cavity in women with endometriosis displays high levels of inflammation (206), and MSC are known to respond to their immediate microenvironment to attempt to maintain homeostasis (103). Therefore, the inflamed pelvic cavity may induce ESC_{cyst} to become more immunosuppressive to allow them to reduce inflammation and promote tissue homeostasis. It is well known that the pelvic cavity in women of endometriosis has reduced immunosurveillance leading to immune escape of ectopic lesions, but it is not currently known why this occurs (35). However, it may be associated with the immunosuppressive effects of ESC_{cyst}, which may be mediated partly via their promotion of recruited monocytes into immunosuppressive M2 macrophages. Finally, the M2 macrophages may directly (18) and indirectly (207) through their immunosuppressive effects on other immune cells, support the growth of ectopic lesions in endometriosis.

5.2 STUDY 2

Endometriosis is known to be an inflammatory disease (208), and MSC are known to be immunosuppressive; so MSC may be potentially useful as a therapy for endometriosis (209). Moreover, based on the findings in **Study 1**, it seemed that autologous MSC are altered by the pathology of endometriosis. Therefore, in this study we examined the effects of allogeneic MSC on ESC *in vitro* in various cell co-culture assays to examine if MSC could potentially be a therapy for endometriosis. Specifically, cell proliferation, apoptosis, adhesion, migration, invasion, and tube formation assays were carried out to examine the effects of Ad-MSC on ESC_{cyst} and ESC_{endo} *in vitro*.

Initially, the effects of Ad-MSC on the proliferation of ESC_{cyst} and ESC_{endo} was carried out. The three methods of manual cell count, CFSE, and MTT assays were used in order to validate the findings through the use of more than one method. The CM system induced cell proliferation of ESC_{cyst} and ESC_{endo} significantly compared to untreated controls, while the transwell system had no effect on cell proliferation, using all three methods. Some of these findings were similar to a previous study (129), and conflicting with other studies (210, 211), which may be due to the use of different sources of MSC, as has been previously described (212). There is a paracrine effect in the transwell system that is absent in the CM system, which may lead ESC_{cyst} and ESC_{endo} to secrete factors that may inhibit the Ad-MSC in the top inserts from secreting proliferation-promoting factors (213). Meanwhile, the direct co-culture system had in some cases no effect or in some cases caused a decrease in the proliferation of ESC_{cyst} and ESC_{endo}. The decrease in cell proliferation may be due to the contacted mediated inhibitory effects by Ad-MSC.

It was found that the Ad-MSC promoted the survival and reduced the apoptosis of ESC_{cyst} and ESC_{endo}. These findings are similar to other studies (214-216) carried out in other cell types, and they differ from a study that used MSC from another source (211). Moreover, the findings suggest that MSC present in the pelvic cavity in women with endometriosis could potentially promote the survival and reduce apoptosis of stromal cells.

The transwell system caused a significant increase in the adhesion of ESC_{endo}, but had no effect on ESC_{cyst}. Meanwhile, the CM system caused a significant decrease in the adhesion of ESC_{cyst}, but it had no effect on the adhesion of ESC_{endo}. These findings go in line with the cell proliferation data, since cells that divide rapidly are less likely to be adherent (217). In addition, the differences between the transwell and CM systems is due to a paracrine effect present in the former and absent in the latter. There have been no other previous studies that examined the effects of MSC on the adhesion of ESC_{cyst} and ESC_{endo}.

The CM collected from Ad-MSC/ESC_{cyst} and Ad-MSC/ESC_{endo} co-cultures significantly promoted the tube formation of HUVEC compared to untreated controls. There have been no similar previous studies conducted. Moreover, these findings are in corroboration with others who found that MSC have the ability to promote tube formation (218-220).

The transwell system significantly increased the migration of ESC_{cyst} but had no effect on ESC_{endo}, and it had no effect on the invasion of either cell type, compared to the untreated controls. The CM system significantly reduced the migration and invasion of both cell types. This data is in accordance with the cell adhesion data, since migration and invasion require initial adhesion of cells (221). Also, the differences between the transwell and CM systems are due to the presence of a paracrine effect in the former. A similar study found conflicting data to ours (129), which could be explained by their different source of MSC.

We in **Study 1** and others (106) have found that MSC are recruited to ectopic lesion in women with endometriosis. Therefore, based on the findings in this study it seems that ectopic MSC may be involved in the promotion of cell proliferation, survival, and migration, as well as supporting endothelial cells in angiogenesis, to allow the direct growth of ectopic lesions. Finally, it must be remembered that these findings may not hold true for MSC from other sources, which should still be investigated as a potential therapy for endometriosis.

5.3 STUDY 3

As mentioned earlier, various immune cells such as NK cells are recruited to the pelvic cavity in women with endometriosis, however, their functions have been found to be reduced (16, 17, 67). Although it is not completely known why there is immune inhibition in the pelvic cavity of women with endometriosis, in **Study 1** we found that ESC_{cyst} from women with endometriosis may be involved in part through their promotion of immunosuppressive M2 macrophages. Therefore, herein we wanted to examine if ESC_{cyst} also had similar inhibitory effects on the phenotype and function of allogeneic NK cells.

NK cells were cultured in the CM of ESC_{cyst} and ESC_{endo}, and their cytotoxic function was examined. We found that there were no differences in NK cell cytotoxicity between ESC_{cyst} and ESC_{endo} using the ^{51}Cr release assay. Similarly, the phenotype of the NK cells treated with the CM of ESC_{cyst} was similar to the NK cells treated with the CM of ESC_{endo}, with no significant differences in the expression of activating, inhibitory, maturation or adhesion

markers. In addition, there were no significant differences in the NK cell degranulation marker CD107a, or in the expression of the proinflammatory cytokine IFN-γ from the NK cells. Furthermore, the percentage of NK cells expressing IFN-γ and CD107a was similar for both ESC_{cyst} and ESC_{endo}. Secreted factors may not have been present at a significant concentration in the CM of ESC_{cyst} to have an evident effect on NK cell cytotoxicity since they may have been short-lived as many cytokines are (222). To the best of our knowledge, no previous studies comparing the CM of ESC_{cyst} and ESC_{endo} on the phenotype of NK cells have been carried out. However, there have been differently designed studies that have generally found that NK cell cytotoxicity is significantly inhibited in women with endometriosis compared to women without endometriosis, and this may be due to the effects of ESC_{endo} compared to ESC_{endohv} (16, 17, 132). However, a comparison to ESC_{endohv} may not be appropriate since ESC_{endohv} may introduce inter-individual variations in terms of the immunological microenvironment and endocrine factors. Furthermore, healthy eutopic endometrium serves as an inappropriate model for studying NK cell responses in endometriosis as ESC from ectopic endometriotic lesions may have significantly different behavior.

Direct co-culture experiments with ESC_{cyst} and ESC_{endo} were carried out to examine their effects on NK cell cytotoxicity. The cytotoxicity of the NK cells in direct contact with ESC_{cyst} was similar to NK cells that had been in direct contact with ESC_{endo}. Therefore, the cell-cell interactions that occur in direct co-culture with ESC_{cyst} compared to ESC_{endo} may have a similar effect on NK cell cytotoxicity. According to the literature, there have been no similar previous studies comparing the effects of ESC_{cyst} and ESC_{endo} on the cytotoxicity of NK cells following direct co-culture. Instead, Oosterlynck *et al.* found that NK cells from the peripheral blood and peritoneal fluid of women with endometriosis have significantly reduced cytotoxicity against the K562 cell line and autologous ESC_{endo} compared to NK cells from women without endometriosis (16, 17). Moreover, a similar study found that NK cells derived from the peripheral blood and peritoneal fluid of women with endometriosis had significantly reduced cytotoxicity against the K562 cell line compared to women without endometriosis (70).

In contrast to the aforementioned NK cell cytotoxicity studies, we used NK cells from healthy female volunteers similar to a number of previous studies (60, 68, 133), which could explain the conflicting results. The use of NK cells from healthy female volunteers may not be appropriate for the investigation of NK cell immunomodulation in endometriosis. In conclusion, the reduced function of NK cells in the pelvic cavity in women with endometriosis may be due to other factors besides ESC_{cyst}. However, NK cells from women with

endometriosis should be utilized in a future study in a similar co-culture system as ours, in order to confirm our findings.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 CONCLUSIONS

Several conclusions can be derived from this thesis:

- Firstly, ESC_{cyst} are more immunosuppressive than ESC_{endo}. This could be due to ESC_{cyst} responding to and opposing the high levels of inflammation in the pelvic cavity in order to attempt to maintain homeostasis. ESC_{cyst} may be one of the mechanisms by which reduced immunosurveillance of ectopic endometrial tissue in the pelvic cavity occurs. They may be inducing recruited monocytes to differentiate into immunosuppressive M2 macrophages, which may directly support the growth of ectopic endometrial tissue, and inhibit the functions of other immune cells. The net effect might be an indirect promotion of the growth of ectopic endometrial tissue by ESC_{cyst}.
- Secondly, although MSC such as Ad-MSC are known to be immunosuppressive and endometriosis is an inflammatory disease, Ad-MSC should not be investigated further as a potential therapy for endometriosis, because they may directly support the growth of ectopic endometrial tissue and worsen the pathology. This also suggests that ectopic MSC such as ESC_{cyst} may directly support the growth of ectopic endometrial tissue.
- Thirdly, ESC_{cyst} may not be involved in the known inhibition of NK cells in the pelvic cavity of women with endometriosis, suggesting that other factors may be involved.

Taken together, ESC_{cyst} may directly and indirectly through immunosuppressive M2 macrophages support the growth of ectopic endometrial tissue in women with endometriosis.

6.2 FUTURE PERSPECTIVES

The involvement of ESC_{cyst} and immunosuppressive M2 macrophages in the pathogenesis of endometriosis could be further investigated by carrying out mechanistic studies to examine the mechanisms by which ESC_{cyst} may be promoting immunosuppressive M2 macrophages. The subtypes of macrophages promoted could also be examined. Moreover, a similar co-culture system should be utilized in a future study but with NK cells from women with endometriosis to confirm our NK cell findings. To have a more complete story showing the effects of ESC_{cyst} on the immune system in endometriosis, other immune cells such as DC, B cells, and T cells could also be investigated.

Furthermore, although allogeneic Ad-MSC may directly support the growth of ectopic endometrial tissue in endometriosis, this may not be true for other sources of MSC, which should also be investigated. ESC_{cyst} were examined in this thesis, however, MSC from other ectopic lesions such as early stage superficial peritoneal lesions and deep infiltrating endometriosis lesions should also be investigated as they may provide a different snapshot of the pathology.

Finally, the findings of such *in vitro* studies could be further confirmed through the use of a relevant *in vivo* model such as the Baboon to conclude if MSC are in fact one of the major players in the pathology of endometriosis through their direct and indirect effects on the growth of ectopic endometrial tissue in the pelvic cavity. An improved understanding of the role of MSC in the pathology of endometriosis will help future research to devise ways to target them during the development of novel therapeutics.

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9 ORIGINAL PAPERS I-III