Biopsy-induced inflammatory conditions improve endometrial receptivity: the mechanism of action

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Abstract

A decade ago, we first reported that endometrial biopsy significantly improves the success of pregnancy in IVF patients with recurrent implantation failure, an observation that was later confirmed by others. Recently, we have demonstrated that this treatment elevated the levels of endometrial pro-inflammatory cytokines and increased the abundance of macrophages (Mac) and dendritic cells (DCs). We therefore hypothesised that the biopsy-related successful pregnancy is secondary to an inflammatory response, and aimed at deciphering its mechanism of action. Supporting our hypothesis, we found that the pro-inflammatory TNFα stimulated primary endometrial stromal cells to express cytokines that attracted monocytes and induced their differentiation into DCs. These monocyte-derived DCs stimulated endometrial epithelial cells to express the adhesive molecule SPP1 (osteopontin (OPN)) and its receptors ITGB3 and CD44, whereas MUC16, which interferes with adhesion, was downregulated. Other implantation-associated genes, such as CHST2, CCL4 (MIP1B) and GROA, were upregulated by monocyte-derived Mac. These findings suggest that uterine receptivity is mediated by the expression of molecules associated with inflammation. Such an inflammatory milieu is not generated in some IVF patients with recurrent implantation failure in the absence of local injury provoked by the biopsy treatment.

Introduction

Human embryo implantation is a three-stage process that includes apposition, adhesion and invasion. During apposition, chemokines and cytokines, produced by the endometrial cells, guide the blastocyst to the site of implantation and the embryonic l-selectin binds to its ligands on the luminal epithelium, enabling the initial contact of the blastocyst with the uterus. During adhesion, the interaction between the blastocyst and the uterus is further established by binding of the epithelial adhesion molecules, such as intergrins, to their ligands on the trophoblast cells. Invasion of the trophoblast is regulated by its crosstalk with the different endometrial cell components (Lessey 2002, van Mourik et al. 2009).

Successful implantation is dependent on both, the development of high-quality embryo and the acquisition of endometrial receptivity. Although fertilisation rate is relatively high and embryo culture conditions are constantly being improved, implantation remains a rate-limiting step for the success of IVF treatments. A major reason for implantation failure of the in vitro grown embryo is still an inadequate uterine function (Edwards 1994, Simon et al. 1998, Fatemi & Popovic-Todorovic 2013). In humans, the uterus becomes receptive during the mid-secretory phase (days 19–23) of the menstrual cycle, known as the window of implantation (WOI). This period is characterised by morphological and transcriptional changes in the endometrium such as, cell proliferation and their differentiation; accumulation of specific immune cells, macrophages (Mac), dendritic cells (DCs) and natural killer (NK) cells and expression of different cytokines/chemokines and growth factors as well as adhesion molecules. All these changes facilitate the embryo–endometrium interaction enabling implantation (van Mourik et al. 2009, Granot et al. 2012).

It was a decade ago that we first demonstrated that endometrial biopsies taken during the spontaneous cycle preceding the IVF treatment substantially increase the rates of implantation, clinical pregnancies and live births (Barash et al. 2003). Such favourable effect of local endometrial injury was later confirmed by other IVF clinics worldwide (Raziel et al. 2007, Zhou et al. 2008, Karimzadeh et al. 2009, Almog et al. 2010, Narvekar et al. 2010, Tiboni et al. 2011, Nastri et al. 2013).
Moreover, two meta-analyses of the results of the above-mentioned studies as well as a randomised clinical trial recently published strongly support the notion that local endometrial injury significantly improves clinical pregnancy and live birth rates in the subsequent IVF cycle (El-Toukhby et al. 2012, Potdar et al. 2012, Gibreel et al. 2013). Furthermore, it has been recently shown that biopsy treatment has a positive effect on spontaneous pregnancies as well (Brinsden et al. 2009).

Investigating the mechanism by which the biopsy treatment increases endometrial receptivity, we previously demonstrated that the local injury induces an inflammatory response that is characterised by elevated levels of pro-inflammatory cytokines/chemokines, such as macrophage inflammatory protein (MIP)1B, tumour necrosis factor alpha (TNFα), growth-regulated oncogene alpha (GROα), SPP1 osteopontin (OPN) and IL15 (Gnainsky et al. 2010). In this study, we also demonstrated an increased abundance of specific CD11c+HLA-DR+ immune cells, representing Mac and DCS. Most importantly, we showed a positive correlation between the biopsy-induced pro-inflammatory cytokines and successful pregnancy, suggesting that these molecules may serve as possible biomarkers for implantation competence (Gnainsky et al. 2010). Taking the above-mentioned findings into consideration, we hypothesise that endometrial biopsy triggers an inflammatory response that induces the transition of the endometrium from its non-receptive to its receptive state (Dekel et al. 2014).

The present study was designed to test this hypothesis. Due to the obvious ethical and technical limitations of investigating implantation in human in vivo, we employed an in vitro experimental model using primary human endometrial cells isolated from the biopsies taken from IVF patients as well as an endometrial cell line. We demonstrate that injury-induced pro-inflammatory cytokines secreted by the endometrial stromal cells (ESCs) attract monocytes and induce their differentiation into DC-like cells. Furthermore, monocyte-derived DCs directly affect ESCs and endometrial epithelial cells (EECs) to express a specific repertoire of chemokines and adhesion molecules that are involved in the uterine–blastocyst interaction. We suggest that mechanical intervention by endometrial biopsy may be necessary in IVF patients with recurrent implantation failures for provoking the inflammatory response required for acquisition of endometrial receptivity.

Materials and Methods

Patients and endometrial sample collection

IVF patients, 22–39 years of age with menstrual cycles of 28–30 days and are good responders to the hormonal stimulation were selected. Patients with endometriosis and/or hydrosalpinx were excluded. Endometrial samples of the functional layer of the endometrium were retrieved using a biopsy catheter (Pipelle de Cornier, Prodimed, Neuilly-en-Thelle, France) (Barash et al. 2003, Gnainsky et al. 2010). In each biopsy, the endometrial sample was taken from several spots. Biopsies were taken during the spontaneous menstrual cycle preceding the IVF cycle, on day 12, the non-receptive, and/or day 21 (WOI), the receptive phase of the cycle. The samples were immediately transferred into culture medium for the isolation of endometrial cells.

The protocol of this study has been approved by the Kaplan Medical Center Committee on the use of Human Subjects in Medical Research in accordance with the Helsinki declaration. Informed consent was received from all participants.

Isolation of endometrial stromal and epithelial cells

Endometrial cells were isolated as described previously (Gnainsky et al. 2010). In brief, fresh endometrial samples were minced into 1–2 mm fragments and subjected to enzymatic digestion using 2.5 mg/ml collagenase (Sigma–Aldrich, St. Louis, MO, USA) and DNase (Roche Applied Science, Penzberg, Germany) for 1.5 h. The cells were then transferred through a 100 μm cell strainer. ESCs and ESCs were separated by their filtration though a 40 μm cell strainer (BD Biosciences, San Jose, CA, USA) according to their size difference. The stromal cells were seeded and were used for further experiments on their first passage. Epithelial sheets were plated onto 30 mm plate and incubated for 30 min to allow the contaminating stromal cells to adhere. The media containing the non-adherent EECs were transferred to a 24-well plate for further experiments.

Cell culture

Freshly-isolated human ESCs and EECs were cultured in DMEM/F12 (Biological Industries, Kibbutz Beit Haemek, Israel). Human monocyte cell line (THP-1), blood-derived monocytes, Mac and DCS were cultured in complete RPMI-1640 (Biological Industries). All culture media were supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco-Invitrogen, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin (Biological Industries).

To induce an injury-like environment in vitro, ESCs were incubated with 25 nM TNFα (Peprotech, Rocky Hill, NJ, USA), a pro-inflammatory cytokine that is known to initiate inflammatory response. To confirm its inflammatory effect, the conditioned medium (CM) was collected after 24 h of incubation and analysed for cytokine secretion profile using Multiplex analysis (see below).

Migration assay

The capacity of the TNFα-treated ESC to attract monocytes (monocyte cell line, THP-1) was tested using transwell migration assay. For this purpose, THP-1 cells (2×10⁶) were resuspended in RPMI medium and placed in transwell inserts with 5 μm pore size (Millipore, Billerica, MA, USA). CM collected from either TNFα-treated or untreated ESC was placed in the bottom of the well. After 2 h of incubation, inserts were removed and the number of THP-1 cells migrated to the bottom of the well was assessed using haemacytometer.
Isolation of monocytes from peripheral blood

Mononuclear cells were isolated from blood samples using Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, UK), according to manufacturer’s instructions. Leukocyte-enriched blood samples were purchased from the Blood Bank in Rabin Medical Center. The protocol of isolating cells from blood samples for research was approved by the Kaplan Medical Center Committee on the use of Human Subjects in Medical Research in accordance with the Helsinki declaration. The viability of the isolated mononuclear cells was assessed by staining with Trypan blue. The cells were then seeded in 10 cm cell culture plates. After 1 h of incubation, non-adhesive mononuclear cells were removed; attached monocytes were washed and further used for differentiation experiments. The purity of the isolated monocytes was tested by FACS as described below.

Generation of Mac and DCs

Human Mac and DCs were generated either from THP-1 monocyte cell line or from peripheral blood-derived monocytes using conventional in vitro differentiation methods as previously described (Berges et al. 2005, Hanlon et al. 2011). Briefly, to induce differentiation to DCs, monocytes were incubated with 100 ng/ml of IL4 and GM-CSF (Peprotech). To induce differentiation to Mac, monocytes were incubated with either 100 ng/ml M-CSF (Peprotech) or 20 nM TPA (Sigma–Aldrich). Following 3 and 6 days of incubation, cell phenotype was assessed by analysing the expression of specific surface molecules of Mac and DCs using FACS (see below). The CM was used for further experiments and was analysed for cytokine production using Multiplex Technology, as described below.

To study the effect of TNFα-induced pro-inflammatory cytokines on monocyte differentiation, freshly isolated monocytes were incubated with CM from TNFα-treated and untreated day 21-endometrial stromal cells. Following 3 days of culture, cell phenotype was assessed by FACS analysis. Monocyte-derived DCs and Mac served as reference for differentiation status.

Multiplex analysis

The levels of cytokines and chemokines secreted by the stromal and the immune cells to their culture medium were determined by multiplex analysis using the BioPlex assay (Bio-Rad, Hercules, CA, USA), in which Luminex 100 IS system (Luminex, Austin, TX, USA) was used for detection and analysis (Aldo et al. 2013).

Analysis of expression of implantation-associated genes in endometrial cells

Human ESCs and EECs isolated from day 12 endometrial samples representing non-receptive endometrium were treated with or without CM collected from different types of the immune cells (monocytes, Mac and DCs). After 24 h of culture, RNA was extracted using the PerfectPure RNA Cell kit (5 Prime, Gaithersburg, MD, USA). The expression of implantation associated genes was analysed by quantitative PCR. For the analysis of protein expression, epithelial cells were grown on round coverslips placed in 24-well plates with or without immune cell CM. After 48 h of incubation, the cells were fixed with 4% paraformaldehyde for 10 min and immunofluorescent staining was performed as described below.

Quantitative real-time PCR

Primers for quantitative real-time PCR (qPCR) were designed by Primer Express software (Applied Biosystems, Foster City, CA, USA) and analysed using the BLAT–UCSC Genome Browser for their specificity. Relative quantification of the mRNA was carried out using SYBR Green PCR Master Mix (Applied Biosystems) in the StepOne system v2.1 (Applied Biosystems). As internal control, hypoxanthine phosphor-ribosyltransferase 1 (HPRT1) was amplified in parallel for each sample and used for normalisation. The results of qPCR are presented as a fold change (mean ± S.E.M.) normalised to control samples.

FACS analysis

Immune cells were incubated with human immunoglobulin for 10 min and stained with fluorescent antibodies against specific markers of monocytes and monocyte-derived Mac and DCs, such as anti-CD14 (Milenynt, Bergisch Gladbach, Germany), anti-CD11c (Becton-Dickinson, San Jose, CA, USA), anti-HLA-DR and anti-CD1a (Biolegend, San Diego, CA, USA). After 30 min of incubation at 4°C, the cells were washed and analysed by FACS sort flow cytomter (Becton-Dickinson). Unlabelled cells and the respective isotype antibodies were used as control. The results were analysed using FCS express, version 3 software.

Immunofluorescent staining

For immunofluorescence staining, epithelial cells were fixed with 4% PFA for 10 min, blocked with 10% serum (Vector, Burlingame, CA, USA), and incubated overnight with mouse anti-human CD44 antibody (1:50; Novus Biologicals, Littleton, CO, USA) or mouse anti-human MUC16 (1:50; Abcam, Cambridge, MA, USA). The next day, the coverslips were washed and cy3–labelled goat anti-mouse secondary antibody was applied for 1 h (1:100; Jackson, West Grove, PA, USA). After washing, the nucleus were stained with Hoechst (1:2000; Sigma–Aldrich) for 1 min and then coverslips were mounted with Vectashield medium (Vector).

Statistical analysis

Statistical analysis was performed using computerised software GraphPad Prism (San Diego, CA, USA) and Statistica version 10 (Tulsa, OK, USA). The results have been analysed using non-parametric tests. Mann–Whitney U test was used for comparison between two groups and Kruskal–Wallis test followed by Tukey’s test for multiple comparisons. P<0.05 was considered statistically significant.

Results

TNFα-treated endometrial cells secret chemokines that attract monocytes

We have previously demonstrated that in addition to its effect on cytokine production, endometrial injury
induces a local increase in monocyte-derived immune cells, such as DCs and Mac (Gnainsky et al. 2010). We therefore hypothesised that the endometrial biopsy provokes an inflammatory response that stimulates the cells to secrete chemokines, which in turn recruit immune cells from the circulation to the site of injury. To test this hypothesis, we assessed the ability of endometrial cells to recruit monocytes in vitro. For this purpose, we isolated stromal cells from endometrial samples recovered from IVF patients on days 12 and 21.

![Figure 1](image-url)

**Figure 1** TNFα stimulates endometrial stromal cells to produce pro-inflammatory cytokines that attract monocytes. (A) Quantitative analysis of cytokine secretion by TNFα-treated (T) and untreated (C) stromal cells isolated from endometrial samples recovered on days 12 and 21 of the menstrual cycle. The box plot horizontal lines represent the median and the 25th to 75th percentile (n=4–5 independent experiments). *P<0.05, Kruskal–Wallis test, followed by Tukey’s test. (B) Migration of THP-1 cells in response to 20 ng/ml TNFα, conditioned medium of TNFα-treated and untreated stromal cells isolated from day-12 (12T and 12C respectively) and conditioned medium of TNFα-treated and untreated stromal cells isolated from 21 endometrial samples (21T and 21C respectively). Chemotaxis index represents the number of migrating cells under treatment with conditioned medium, divided by the number of migrating cells in the presence of cell-free medium (DMEM). Columns represent the average of three independent experiments ± S.E.M. Small cap letters indicate significant differences based on Kruskal-Wallis test followed by Tukey’s test (P<0.05). (C) Migration of THP-1 cells in response to conditioned medium of TNFα-treated stromal cells (CM TNFα) following neutralization of selected cytokines. (n=5 independent experiments). Small cap letters indicate significant differences based on Kruskal-Wallis test followed by Tukey’s test (P<0.05). (D) PCR analysis for the relevant chemokine receptors (CXCR2, CCR1, CCR2, and CCR5) expressed by THP-1 cells. Hypoxanthine phosphor-ribosyltransferase 1 (HPRT1) was used as a housekeeping gene.
of their spontaneous menstrual cycle. Inflammation was induced by incubating these cells for 24 h with 25 ng/ml TNFα (one of the cytokines that initiate an inflammatory response). CM was then collected and tested for cytokine profile and its ability to trigger monocyte migration.

Analysis of the cytokine profile of the CM collected from TNFα-treated stromal cell revealed increased levels of RANTES, GROα, IL8, IL6, monocyte chemotactic protein 1 (MCP1), GM-CSF, interferon γ-induced protein (IP-10) and G-CSF, whereas the levels of VEGF were decreased by this treatment (Fig. 1A). This response of stromal cells to TNFα was similar in cells isolated from endometrial samples taken on both days 12 and 21. Analysis of the cytokine RNA levels showed compatible results (data not shown).

The ability of CM collected from TNFα-treated stromal cells to attract monocytes was tested by the transwell migration assay. We found a higher number of monocytes (THP-1 cells) recruited by CM from TNFα-treated cells as compared with CM from untreated cells (Fig. 1B). To identify which of the chemokines in the TNFα-treated stromal-cell CM attract monocytes, another set of migration assays was performed with the addition of relevant antibodies to neutralise specific cytokines. We found that anti-MCP1 and anti-GROα antibodies inhibited the THP-1 cell migration (Fig. 1C), suggesting that these chemokines could be directly involved in monocyte recruitment in response to the endometrial biopsy-induced inflammation. Complimentary experiments demonstrated that THP-1 cells do express the receptors for these cytokines (Fig. 1D).

**TNFα-treated endometrial cells secrete chemokines that induce monocyte differentiation**

Monocyte differentiation into Mac and DCs is determined by the specific tissue microenvironment (Geissmann et al. 2010). We therefore assumed that differentiation of the recruited monocyte into uterine DCs or Mac is mediated by the cytokines secreted by the TNFα-treated endometrial stromal cells. In our next experiments, we studied the effect of CM collected from TNFα-stimulated stromal cells on monocyte differentiation. For this purpose, we evaluated the expression of the commonly used, Mac and DC surface markers (HLA-DR, CD14, CD11c, and CD1a) in response to TNFα-treated and untreated (control) stromal cell CM. For reference, monocyte-derived DCs and Mac that underwent *in vitro* differentiation induced by GM-CSF and IL4 or by M-CSF, respectively, were employed. FACS analysis revealed that monocytes incubated with TNFα-treated stromal cell CM exhibited a DC-like cell phenotype (Fig. 2). Specifically, CD14 expression was reduced (Fig. 2A and C), CD1a expression was elevated (Fig. 2B and D), and HLA-DR expression was slightly decreased (Fig. 2E) with no change observed in CD11c expression.

![Figure 2](https://www.reproduction-online.org)Conditioned medium (CM) collected from TNFα-treated stromal cells induces differentiation of blood-derived monocytes into DC-like cells. (A and B) A representative FACS analysis of monocyte differentiation using the specific cell surface markers for Mac and DCs: HLA-DR, CD14, CD1a, and CD11c. Freshly isolated monocytes (control) were cultured for 3 days with CM from TNFα-treated (CM T) and untreated (CM C) stromal cells. As a positive control, cells were incubated with GM-CSF and IL4 for DC differentiation and with M-CSF for Mac differentiation. (C, D, E and F) Quantification of the cell surface marker expression determined by FACS as shown in A and B. The results represent three independent experiments each of which conducted using CM from endometrial stromal cells of a different patient. Small cap letters indicate significant differences based on Kruskal-Wallis test followed by Tukey’s test (P<0.05).
expression (Fig. 2F). The phenotype of monocytes that were incubated with CM of untreated cell was characterised by HLA-DRlow CD11c+ CD14− CD14+CD1a+ (Fig. 2), a phenotype that is associated with tissue Mac.

To further characterise this effect and remove any potential contaminant immune cell present in the primary cultures, we carried out experiments as described above using the well-characterised endometrial stromal cell line HESC (Krikun et al. 2004). The CM collected from TNFα-treated HESC induced differentiation that was similar to that described above for the primary cultures (data not shown), indicating that the TNFα-treated stromal cells are directly involved in monocyte differentiation into DCs.

**Monocyte-derived DCs directly affect endometrial cell receptivity**

We assumed that the recruited immune cells are involved in the acquisition of endometrial receptivity. We therefore studied their direct effect on endometrial cells *in vitro*. For this purpose, freshly isolated endometrial stromal and epithelial cells were cultured with CM collected from either Mac or DCs derived from either THP-1 or peripheral blood monocytes. Specifically, we tested the effect of the immune cell CM on endometrial cell expression of implantation-associated genes. We found that CM collected from both THP-derived DCs and Mac affect the expression of genes that are involved in the embryo–endometrium interaction and that this effect is cell and gene specific (Fig. 3). Mac CM significantly increased the expression of CHST2 that is responsible for the production of the functional ε-selectin ligands in the epithelial cells. This CM also elevated *CCL4* (*MIP1B*) and *GROα* mRNA in the stromal cells. CM collected from DCs regulated the expression of genes that are involved in the adhesion of the blastocyst to the luminal uterine surface. Specifically, mRNA expression of *SPP1* and, its receptors, *ITGB3* and *CD44*, were upregulated, whereas the expression of the adhesion inhibitory molecule *MUC16* was down-regulated. A similar effect on the adhesion molecule expression was exerted by CM collected from the blood monocyte-derived DCs (Fig. 4A). Complementary immunofluorescent staining confirmed these findings at the protein level (Fig. 4B).

Multiplex analysis of the cytokine profile of DC-CM detected a variety of cytokines such as RANTES, CCL4, GROα, IL6, MCP1, GM-CSF, IP10, and SPP1 that may be potential mediators of the effect of DCs described above.

**Discussion**

This study unravels the role of pro-inflammatory cytokines and immune cells in preparing the endometrium for implantation. Specifically, we demonstrate herein that inflammatory conditions achieved by exposure to TNFα stimulate the endometrial stromal cells to produce a set of cytokines capable of stimulating monocyte migration and their differentiation into DCs. These immune cells in turn modify the expression of specific adhesion molecules by the endometrial epithelium.

Accumulating evidence that endometrial injury improves the rate of implantation generated by us and

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Figure 3 Regulation of the expression of implantation-associated genes in endometrial stromal and epithelial cells by THP-1 cell-derived Mac and DCs. Freshly isolated, either stromal or epithelial cells, from day-12 endometrial samples were treated with either RPMI-culture medium (control) or with conditioned medium recovered from either monocytes (THP-1), Mac or DCs. The effect of these immune cells on the expression of the implantation-associated genes was tested by qPCR. *CCL4* and *GROα* were analysed in both epithelial and stromal cells (white and black columns respectively), while *CHST2*, *SPP1*, integrins αv and β3 and *MUC16* were analysed only in the epithelial cells. The results of qPCR are normalized to hypoxanthine phosphor-ribosyltransferase 1 and represent the mean ± s.e.m. of five independent experiments, each of which was conducted on cells of a different patient. *P* < 0.05, Kruskal–Wallis test.
others endorsed a role of inflammation in acquisition of uterine receptivity (Barash et al. 2003, Raziel et al. 2007, Zhou et al. 2008, Karimzadeh et al. 2009, Dekel et al. 2010, Narvekar et al. 2010, Tiboni et al. 2011, Granot et al. 2012, Nastri et al. 2013). Furthermore, the strong correlation between successful implantation and increased levels of endometrial pro-inflammatory cytokines, as well as elevated abundance of immune cells, following endometrial local injury (Gnainsky et al. 2010) reinforces this notion. The results of this study indicate that the pro-inflammatory TNFα, which is upregulated upon endometrial biopsy (Gnainsky et al. 2010), could contribute to the injury-induced rise in endometrial pro-inflammatory cytokines. Previous studies demonstrated that some of these cytokines are directly involved in implantation. For instance, IL6 and CCL4 were found as the effective chemoattractants for trophoblast cells in human and IP10 has been shown to take part in the regulation of blastocyst migration, apposition and initial adhesion (Hannan et al. 2006, Dominguez et al. 2008, Sela et al. 2013). High levels of CCL4 and IP10 were positively associated with elevated rates of implantation in IVF patients (Boomsma et al. 2009, Gnainsky et al. 2010). Another cytokine, GM-CSF, was shown to be necessary for the growth and survival of the foetus (Seymour et al. 1997). Its addition to human embryo culture medium significantly increased pregnancy and live birth rates (Ziebe et al. 2013). The production of the injury-induced cytokines IP10, MCP1, and CCL4 was shown to be regulated by estrogen (Boucher et al. 2000, Kitaya et al. 2003, Sentman et al. 2004, Carlino et al. 2008). Local injury of the endometrium during the proliferative phase of the menstrual cycle substantially increases endometrial expression of estrogen receptors in the following secretory phase (Li & Hao 2009). Therefore, this reciprocal regulation of estrogen and pro-inflammatory cytokines may indicate that the estrogen-induced proliferation in the endometrium is mediated by cytokines. Taken together, these findings could possibly suggest that in IVF patients with recurrent implantation failures, estrogen is insufficient for provoking endometrial receptivity. In these patients, mechanical intervention by endometrial biopsy is necessary to elicit the inflammatory response required for successful implantation.

We show herein that under a TNFα-provoked inflammatory state, endometrial stromal cells secrete cytokines that stimulate monocyte migration specifically identifying MCP1 and GROα as the cytokines that elicit this response. These findings are in line with our previous demonstrations that the endometrial biopsy-induced increase in pro-inflammatory cytokines is associated with accumulation of HLA-DR+CD11c+CD14− (DCs) and HLA-DR+CD11c+CD14+ (Mac) cells in the endometrium (Gnainsky et al. 2010). They also agree with previous findings that pro-inflammatory cytokines act as chemokines that induce the recruitment of monocytes to injured tissues (Shober & Weber 2005, Shi & Pamer 2011). We further found that the CM recovered from TNFα-treated endometrial stromal cells provides pro-inflammatory environment that stimulates the differentiation of monocytes into DC-like cells. Differentiation of the recruited monocytes into specific immune cells, such as Mac and DCs, that is dependent on environmental signals has been described also in previous reports (Chapuis et al. 1997, Iwamoto et al. 2007).

It has been reported that the abundance of DCs and Mac in human endometrium reaches its highest levels during the WOI (Rieger et al. 2004, Kämmerer 2005, Laskarin et al. 2007). These reports go in line with our previous demonstration that an increased abundance of these cells in the endometrium following biopsy treatment significantly associated with improved pregnancy rates (Gnainsky et al. 2010). In support of these reports, studies carried out in mice provided strong evidence for the indispensability of DCs in implantation (Blois et al. 2004, Plaks et al. 2008). Accordingly, it has been shown that therapy by DCs administration significantly decreased the rate of spontaneous resorption of mouse embryos (Blois et al. 2004). These studies further demonstrate that DCs and Mac secrete an array of cytokines/chemokines and enzymes that may play a
role in tissue remodeling and angiogenesis, regulating trophoblast invasion and decidualisation (Goetzl et al. 1996, Plaks et al. 2008, David Dong et al. 2009, Blois et al. 2011). However, the specific role of DCs and Mac in preparation of the human uterus for implantation remained unclear. Our attempts to decipher the mechanism by which monocyte-derived cells affect the capacity of endometrial epithelium to interact with the embryo revealed that CM collected from Mac elevated mRNA levels of CHST2 and CCL4 in epithelial and stromal endometrial cells respectively. These genes were proposed to be associated with the initial contact of the blastocyst with the endometrial epithelium. It was shown that CHST2 is responsible for the production of the functional endometrial l-selectin ligands that interact with embryonic l-selectins (Genbacev et al. 2003). In human endometrium, elevated level of l-selectin ligands has been associated with improved implantation (Margarit et al. 2009). In this context, decreased expression of CHST2 was associated with infertility (Margarit et al. 2009). Another molecule apparently involved in embryo–uterine interaction is CCL4. Among its other functions, this cytokine has the ability to attract blastocyst to the site of implantation (Hannan et al. 2006). We previously found a strong correlation between CCL4 levels in the endometrium and successful pregnancy in biopsy-treated IVF patients, suggesting that CCL4 could potentially serve as a biomarker for the prediction of implantation competence (Gnainsky et al. 2010).

Other molecules that could be possibly involved in the attachment of the embryo to the uterine wall and are directly modified by monocyte-derived cells are the anti-adhesive MUC16 and the adhesion molecules SPP1 and its receptors, ITGb3, and CD44. The membrane glycoprotein MUC16 is expressed in the non-receptive area of the luminal uterine surface, preventing undesirable embryo–uterine interaction and its misplaced implantation (Gipson et al. 2008). Removal of MUC16 from epithelial cells in vitro increased the adhesion of trophoblast cells (Gipson et al. 2008). Our results suggest that DCs positively affect the adhesive capacity of EECs by downregulating the MUC16 along with upregulation of the SPP1, ITGb3 and CD44. These findings agree with the previous reports of the association between an increase of

Figure 5 The postulated model of the injury-induced events in the endometrium that lead to an increase in uterine receptivity. (A) Endometrial biopsy, performed during the proliferative phase of the spontaneous menstrual cycle, triggers the secretion of a repertoire of pro-inflammatory cytokines by the stromal cells. These cytokines recruit monocytes from the circulation and further induce their differentiation into DCs and Mac. These immune cells, in turn, secrete a set of cytokines that trigger the stromal and epithelial cells to express specific implantation-associated genes. Specifically, Mac induces the epithelial cells to express CHST2 and the subsequent production of functional l-selectin ligands whereas DCs upregulate the epithelial cell production of the adhesion molecule SPP1 and its receptors ITGb3 and CD44 with a concomitant downregulation of the anti-adhesive molecule MUC16. (B) The recruited monocytes reside in the endometrial tissue and elicit the effects mentioned above on endometrial receptivity facilitating implantation at the following IVF cycle.
these adhesive molecules and the endometrial receptive phase (Lessey et al. 1992, Apparao et al. 2001, Afify et al. 2006, Horcajadas et al. 2007). Since in addition to the EECs, SPP1 receptors are expressed on the trophoblast cells, it was suggested that SPP1 serves as a bridge that links the luminal uterine cells with the blastocyst (Apparao et al. 2001, Johnson et al. 2003), thus playing a crucial role in establishing the embryo–endometrium interaction. Studies carried out in mice and rabbits indeed showed that using specific antibodies for functional blocking of endometrial, either ITGαvβ3 or SPP1 in vivo significantly reduced the number of implantation sites (Illera et al. 2000, 2003, Liu et al. 2013). Further in vitro experiments demonstrated that blastocysts failed to attach to EECs pre-treated with ITGβ3 siRNA (Kaneko et al. 2011).

It is important to note that the favourable effect of the biopsy treatment on endometrial receptivity was observed in the IVF treatment performed in the following cycle (Barash et al. 2003). This delayed response is dependent on 'tissue memory' apparently contributed by the recruited monocytes that reside in the tissue for several months, during which time they can constitute distinct-resident tissue Mac and DC populations (Chomarat et al. 2003, Luster et al. 2005, McIntire et al. 2008). In this context, it should be noted that during menstruation the endometrial thickness is reduced due to the loss of fluid and shrinkage of the spongy layer, whereas most of the stroma and apparently the embedded immune cells remain intact (Brenner & Slayden 1994). Under these conditions, the modified properties of endometrial stromal and epithelial cells, provoked by the biopsy treatment could be preserved for the following IVF cycle.

In summary, our findings support the idea that the injury-induced improved endometrial receptivity in IVF patients with repeated implantation failure is mediated by an inflammatory response that comprises the cooperation between the two endometrial compartments, stromal and epithelial cells, orchestrated by the recruited immune cells Mac and DCs. The following is our proposed model for the series of events that occur in response to the endometrial injury (Fig. 5). Endometrial biopsy upregulates the expression of pro-inflammatory cytokines that recruit monocytes/Mac to the site of injury further inducing their differentiation into Mac and DCs that in turn trigger the stromal and epithelial cells to express some particular implantation-associated genes, which are involved in the apposition and adhesion of the blastocyst. Specifically, Mac-induced CHST2 together with CCL4, produced by the stromal cells, guide the blastocyst to the desired implantation site, whereas DCs-induced upregulation of epithelial cell adhesion molecules SPP1, ITGαvβ3, and CD44 in combination with downregulation of the anti-adhesive MUC16 enable the attachment of the blastocyst to the uterine epithelial surface, facilitating implantation. These events probably do not take place in patients with repeated implantation failures in the absence of the endometrial biopsy treatment.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**Author contribution statement**

Y Gnainsky designed and conducted the experiments, analysed and interpreted the data, wrote and revised the manuscript. I Granot collected the samples, designed the experiments, analysed and interpreted the data, wrote and revised the manuscript. P Aldo conducted the experiments, analysed the data and revised the article. A Barash and Y Or recruited patients for the study, performed clinical procedures and sample collection and revised the manuscript. N Dekel and G Mor interpreted the data, contributed to discussion, and revised the manuscript. All authors gave their final approval of the version to be published.

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