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Understanding the natural selection of human embryos: blastocyst quality modulates the inflammatory response during the peri-implantation period

Running title: Immunoregulation by Blastocyst conditioned-media

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Abstract

Problem: Decidualized cells display an active role during embryo implantation sensing blastocyst quality, allowing the implantation of normal developed blastocysts and preventing the invasion of impaired developed ones. Here, we characterized the immune microenvironment generated by decidualized cells in response to soluble factors secreted by blastocysts that shape the receptive milieu.

Method of Study: We used an *in vitro* model of decidualization based on the Human Endometrial Stromal Cells line (HESC) differentiated with medroxiprogesterone and dibutyryl-cAMP, then treated with human blastocysts-conditioned media (BCM) classified according to their quality.

Results: Decidualized cells treated with BCM from impaired developed blastocysts increased IL-1β production. Next, we evaluated the ability of decidualized cells to modulate other mediators associated with menstruation as chemokines. Decidualized cells responded to stimulation with BCM from impaired developed blastocysts increasing CXCL12 expression and CXCL8 secretion. The modulation of these markers was associated with the recruitment and activation of neutrophils, while regulatory T cells recruitment was restrained. These changes were not observed in the presence of BCM from normal developed blastocysts.

Conclusion: Soluble factors released by impaired developed blastocysts induce an exacerbated inflammatory response associated with neutrophils recruitment and activation, providing new clues to understand the molecular basis of the embryo-endometrial dialogue.

Keywords: decidualization – embryo quality – implantation window

Introduction

Decidualized cells display an active role during embryo implantation, which is associated with an initial sterile and physiological inflammatory response (1–3). The decidualization program in humans starts on each menstrual cycle and implies not only phenotypical changes on the endometrial stromal cells, but also in their secretory profile, contributing to implantation. After decidualization, endometrial stromal cells acquire the ability to change their secretome according to the quality of the embryo (4). Brosens et al. described that in co-culture systems between decidualized endometrial cells and morphologically arrested blastocysts, the former significantly decreased the production of pro-implantatory and immunomodulatory factors. However, when these assays were performed in the presence of non decidualized stromal cells, no changes were observed *in vitro* (5).

In this sense, decidualized cells undergo endoplasmic reticulum stress, which triggers the unfolded protein response in order to restore its cellular homeostasis (6). Prolonged or strong reticulum stress conditions are associated with caspase-1 inflammasome activation. This multiprotein complex is functionally active, inducing the maturation of caspase-1 and, through this enzyme, the production of the active form of IL-1β (7). This cytokine is one of the main mediators in placental viviparity and endometrium receptivity (8,9). In fact, the pharmacological inhibition of reticulum stress-pathway prevented not only IL-1β production but also trophoblast invasion evaluated in an *in vitro* model of implantation, suggesting that IL-1β favours a microenvironment for embryo implantation (7). However, IL-1β could act as a "double edge sword": on one hand, it is a pro-implantatory mediator; but, on the other hand, it is a powerful pro-inflammatory factor. In this sense, an exacerbated inflammatory response during the peri-implantation period can compromise the endometrial receptivity and fertility in women (10).

In this inflammatory context, neutrophils are consistently sparse in the late secretory phase of endometrial samples and are dramatically increased at the onset of menstruation (11). The changes in neutrophil abundance across the peri-menstrual phase of the menstrual cycle reveal a tightly temporally regulated influx. In general, neutrophil migration, survival and activation lead up to menstruation (11). A number of histological changes in the endometrium are observed, such as tissue edema, breakdown of the basal lamina supporting endothelial cells, and augmented blood vessel permeability and fragility (12). These histological changes are further accompanied by the activation of matrix metalloproteinases (MMPs) and the production of chemokines, inflammatory cytokines and several mediators such as VEGF-A (11,12). Regarding the latter, pro-angiogenic properties have also been described in a novel resident population of decidual neutrophils at the second trimester (13). However, at earlier stages, neutrophil excess was associated with a negative pregnancy outcome. In this sense, Mizugishi et al. reported in a mouse model that massive neutrophils infiltration into the feto-maternal interface at pregnancy day 7.5 resulted in fetal death (14). Moreover, this effect was reversed by the blockage of neutrophils influx (14). In contrast, since the earliest stages of embryo implantation, regulatory T cells (Tregs CD4+CD25+FoxP3+) contribute

to sustain a local tolerogenic microenvironment (15–17). In this context, Tregs are selectively recruited towards the uterus in waves, being the first one composed of natural Tregs that might control the initial inflammatory response, helping to sustain uterine homeostasis throughout the menstrual cycle (18–22). The importance of Tregs during the peri-implantation period was evaluated in several studies. Using a mouse model, Teles et al. showed that Tregs depletion prior to mating resulted in impaired embryo implantation in both syngeneic and allogeneic matings (23). In humans, while a higher Tregs circulating levels were associated with better *in vitro* fertilization outcome, a lower Tregs frequency has been proposed as a potential biomarker of reproductive failures (24,25).

Therefore, a dynamic and adequate immune response is crucial to control the endometrial receptivity (3,26,27). However, most current data is focused on maternal immune modulation during early pregnancy, but the primary role of the blastocyst-produced mediators in shaping the receptive immunomodulatory milieu is still an open question. Previous reports suggest the existence of a preimplantation embryo-mediated paracrine regulation of decidualized cells that promotes implantation (28). Here, we evaluated whether blastocysts contribute to shaping the receptive immunomodulatory milieu according to their quality. Particularly, we focused on the ability of the decidual cells to respond to soluble factors from impaired developed blastocysts to potentiate an inflammatory microenvironment with the production of specific mediators and the recruitment and activation of neutrophils in a menstruation-like process.

Methods

Human samples

Blastocysts conditioned media: Blastocysts-conditioned (BCM) media was obtained as previously described (29). Briefly, mature oocytes were inseminated (conventional IVF or ICSI procedures were applied according to male evaluation) in drops of 30 µl of G-IVF plus medium (Vitrolife AB, Sweden, Cat. 10136). Fertilization was observed at 16-18 h after insemination and fertilized eggs continued their development in drops of 30 µl of G1 plus medium (Vitrolife, Goteborg, Sweden, Cat. 10128). On day 3, embryos were individually transferred to 30 µl drops of G2 plus medium (Vitrolife, Goteborg, Sweden, Cat. 10132) until day 5. Culture was carried out in dry mini-volume chambers, at 37ºC, 5% O_2, 6% CO_2. Embryos were classified as normal developed or impaired developed blastocysts according to Istanbul consensus (30). After embryos were transferred or cryopreserved, the G2 plus drops were collected and allocated individually in vials. For further analysis, BCM obtained from three normal developing blastocysts or three impaired ones were pooled and used as described below.

Blood samples: Peripheral blood samples were obtained from healthy volunteers who were not under pharmacological treatment for at least 10 days before the day of sampling. Blood was obtained by puncture of the forearm vein and was drawn directly into heparinized plastic tubes. Studies were approved by the Argentine Society of Clinical Investigation Board and Ethical Committee (Ref. SAIC 03/17). All healthy donors provided written

informed consent for sample collection and subsequent analysis. Cells from different donors were used on each assay. Cells from each individual donor were considered independent samples and tested individually. Peripheral blood mononuclear cells (PBMCs) were purified by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden, Cat. GE17-1440-02) density gradient as previously reported (29). Neutrophils were purified by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden, Cat. GE17-1440-02) gradient centrifugation, Dextran (MP Biomedicals, Santa Ana, CA, USA, Cat. 101514) sedimentation and hypotonic lysis as previously reported (31). Cell viability was evaluated by trypan blue exclusion.

***In vitro* decidualization model**

Cell line: Human endometrial stromal cell line (HESC) was used in this study (32). Cells were maintained in DMEM-F12 (Gibco, Life Tech, Cat. 12400-024) supplemented with 10% fetal bovine serum (FBS, Internegocios, Buenos Aires, Argentina, Cat. FBI), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Life Technologies, Cat. 15140122) and 2mM glutamine (Gibco, Life Technologies, Cat. G3126). For the different assays, HESC cells (20.000 cells/well) were cultured in 24 well plates until they reached 70% confluence. This cell line was kindly provided by Dr. Gil Mor, Wayne State University, USA.

Decidualization protocol: HESC cells were cultured in 24 well plates in the presence of medroxyprogesterone (MPA) (10^{-7}M, Sigma, Cat. M1629) and dibutyryl-cAMP (dbcAMP) (5. 10^{-4}M, Sigma, Cat. D0260) for 8 days (Dec), renewing the stimuli every 48 h. In all cases, cells were washed and the differentiation media were replaced by fresh medium (270 µl/well) after decidualization was complete. The decidualization process was confirmed by the evaluation of decidual markers and cell viability, as previously described (29). Non decidualized cells were cultured simultaneously in similar conditions without decidualization stimuli.

Blastocysts-conditioned media stimulation: Decidualized HESC cells were stimulated with 30 µl of pooled human blastocysts-conditioned media (BCM) (final dilution 1/10) obtained from three normal developing blastocysts (ND) or three impaired ones (ID) during 24 for CXCL12 expression by RT-PCR detection, or 48 h for CXCL8 quantification by ELISA and IL-1β by flow cytometry. Considering that leukocytes populations display different kinetic-recruitment towards the generation of the maternal-fetal interface we used different stimulation periods for the migration assays. Since CXCL8 is one of the main chemokines involved in neutrophils recruitment and significantly increases after 48 h of stimulation, we performed neutrophils migration and activation assays at 48 h. On the other hand, since natural Tregs are selectively recruited towards the uterus prior to mating (23), stimulation of decidualized cells with BCM for Tregs migration were performed during 24 h.

Caspase-1 activity Assay

Decidualized cells were stimulated with Blastocysts-conditioned media during 48 h and Fluorochrome Inhibitor of Caspases (FAM-FLICA® Caspase-1 (YVAD) Assay Kit) reagent was added for determination of caspase-1

activation following manufacturer's instructions (Immunochemistry, Technologies, USA, Cat. 97) as we previously described (7). Briefly, the FLICA reagent FAM-YVAD-FMK enters each cell and irreversibly binds to activated caspase-1. Because the FLICA reagent becomes covalently coupled to the active enzyme, it is retained within the cell, while any unbound FLICA reagent diffuses out of the cell and is washed away. The remaining green fluorescent signal is a direct measure of the active caspase-1 enzyme activity present in the cell at the time the reagent was added. Finally, cells were analyzed by flow cytometry and results were expressed as the percentage of positive cells.

Intracellular IL-1β

Decidualized cells were stimulated with blastocysts-conditioned media during 48 h, then cells recovered and IL-1β was intracellularly stained. Therefore, cells were fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences, USA, Cat. 554715) and then stained with dilution 1/30 of PE-conjugated mAb anti IL-1β (eBioscience, Cat. 12-7018-82, clone CRM56) for detection of cytokine active form, or with PE-conjugated mouse IgG1 kappa isotype control (eBioscience, Cat. 12-4714-41, clone P3.6.2.8.1) according to manufacturer's instructions. Finally, cells were analyzed by flow cytometry and results were expressed as the percentage of positive cells.

Real Time PCR

Decidualized cells were stimulated with blastocyst-conditioned media during 24 h and CXCL12 expression was evaluated as previously described (29). Briefly, total RNA was isolated using TRI Reagent (Molecular Research Center Inc., USA, Cat. 12400-024) following manufacturer's recommendations. cDNAs were generated from 1µg of RNA using a MMLV reverse transcriptase(Promega, USA, Cat. M1701), recombinant RNasin(R) RNase inhibitor (Promega, USA. Cat. N2511) and oligo dT kit (Biodynamics, Argentina Cat. B071-40) and stored at -20°C for batch analysis. PCR assays were performed using FastStart universal SYBR green master mix (Roche, Germany, Cat. 4913850001) following manufacturer's recommendations on an iQ5 real time PCR (Bio-Rad, USA). Primers sequences and melting temperatures were previously described (29). Gene expression was quantified relative to the expression of the endogenous reference gene GAPDH by comparative Ct method, using the $2^{-\Delta\Delta}$Ct calculation and expressed as fold change vs unstimulated decidualized cells.

CXCL8 secretion

Decidualized cells were stimulated with blastocysts-conditioned media and after 48 h supernatants were collected. CXCL8 concentration was quantified by ELISA (BD Biosciences, USA, Cat. 555244) according to the manufacturer's instructions. Results are expressed as ng/ml.

Neutrophils migration assay

Neutrophils migration was evaluated using a transwell system with 5µm-pore inserts (BD Falcon cell culture inserts). Therefore, decidualized HESC cells treated with blastocysts-conditioned media during 48 h, then supernatants seeded in the lower compartment, while neutrophils were seeded in the upper one ($2x10^5$ cells/insert). After 30 minutes, neutrophils were recovered from the lower compartment and the total events were assessed by flow cytometry. Results were expressed relative to non decidualized condition for each assay (N= 4 donors).

ROS production

Neutrophils were incubated for 45 min at 37°C with HESC conditioned media from 48 h BCM stimulation. Then 5 µM 2',7'-Dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich, USA Cat.D6883) was added for 15 min and cells analyzed by flow cytometry (n= 4 donors). Results are expressed as Mean Intensity (MIF) Fluorescence from 4 independent experiments.

Tregs migration assay

Tregs migration was evaluated using a transwell system with 5µm-inserts (BD Falcon cell culture inserts). Decidualized HESC cells were treated with blastocysts-conditioned media during 24 h, then supernatants were recovered and seeded in the lower compartment, while PBMCs were seeded in the upper one (3×10^5 cells/insert). After 24 h, PBMCs were recovered from the lower compartment, washed and stained with dilution 1/30 of PE-conjugated anti-CD4 mAb (BD Biosciences, CA, USA, Cat. 555347, clone RPA-T4 or mouse IgG1 kappa isotype control eBioscience, Cat. 12-4714-41, clone P3.6.2.8.1). Cells were then fixed/permeabilized with Human FoxP3 Buffer Set (BD Pharmigen, Cat. 560098, clone 259D/C7) and intranuclear stained with dilution of 1/15 of Alexa 488-conjugated anti-FoxP3 mAb (BD Biosciences, CA, USA, Cat. 560047). Then, cells were washed with a permeabilization buffer and analyzed by flow cytometry. Results were expressed relative to non decidualized condition for each assay (n= 4 donors).

Statistical analysis

Data was analysed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Wilcoxon test was used in case of pairwise comparisons, while Kruskal-Wallis or Friedman with Dunn´s post test was used in case of multiple comparisons, depending on the assay. Results were expressed as Mean ± S.E.M. Statistical significance was defined as $p<0.05$.

Results

Decidualized cells differentially respond to blastocyst quality modulating IL-1β production.

Decidualization of endometrial stromal cells is accompanied by a physiological inflammatory response; however, an exacerbated response is linked with endometrial receptivity failure. Considering that, we tested whether blastocyst-derived factors might contribute to control IL-1β production by decidualized cells. Hence, HESC cells were decidualized and then stimulated with blastocysts-conditioned media (BCM) from normal developed blastocyst (ND) or impaired developed blastocyst (ID) as described in the M&M section. First, we evaluated the inflammasome activation, particularly caspase-1 activation by FLICA-probe. As shown in Figure 1A, there was no significant frequency modulation observed in caspase-1+ cells by blastocysts-conditioned media treatment. However, we observed a significantly increased production of the active form of IL-1β in decidualized cell treated with BCM from impaired developed blastocysts, compared to unstimulated decidualized cells (Figure 1B). On the contrary, BCM from normal developed blastocysts decreased active IL-1β production in decidualized cells (Figure 1B). The right panel shows dot plots of one representative experiment.

Decidualized cells differentially respond to blastocyst quality modulating a pro-inflammatory chemokines microenvironment.

Since decidualized cells displayed the ability to sense blastocyst quality and to modulate IL-1β production accordingly, we next evaluated other known inflammatory mediators such as chemokines production. We focus on the CXCL12 (SDF-1) and CXCL8 (IL-8) expression since are the main chemokines associated with the recruitment of leukocytes towards inflammatory sites. As shown in Figure 2A and 2B, the stimulation of decidualized cells with BCM from impaired developed blastocysts induces a significant increase of CXCL12 expression and CXCL8 secretion, that might be associated with the initial inflammation that accompanies the triggering of menstruation.

Decidualized cells differentially recruit neutrophils and regulatory T cells accordingly to blastocyst quality.

Considering that BCM from impaired developed blastocysts displayed the ability to induce proinflammatory mediators in decidualized cells, first we evaluated neutrophils migration and activation. Therefore, decidualized cells were treated with BCM, then supernatants were recovered and seeded in the lower chamber of a transwell system and tested for neutrophils chemoattraction. As depicted in Figure 3A, neutrophils migration was significantly increased in response to the decidualized cells stimulated with BCM from impaired developed blastocysts, which could be associated with the increased production of chemokines tested previously. In addition, we also observed a higher reactive oxygen species (ROS) production by neutrophils under the same treatment, suggesting an increased activation of them in this condition (Figure 3B). Accordingly, these changes were not observed in decidualized cells stimulated with BCM from normal developed blastocysts. The right panel shows a representative FACS analysis.

As a counterpart, we studied regulatory T cells (Tregs) recruitment, since it is one of the earliest immunoregulatory mechanisms that operate during decidualization. For this, decidualized cells were treated with BCM, and then supernatants were recovered and seeded in the lower chamber of a transwell system. PBMCs were seeded in the upper compartment and the number of CD4+FoxP3+ cells was quantified in the lower compartment by flow cytometry. We observed a significant decrease in the recruitment of CD4+FoxP3+ cells under the stimulation with BCM from impaired developed blastocysts (Figure 4). The right panel shows a representative migration assay.

Discussion

It has been proposed that the ability of the human endometrium to generate an adequate decidual response is based on successive inflammatory events that might contribute to a sensitization of the uterine tissues. Under this hypothesis, a tight immune homeostatic control prior to implantation is required (19,22). This control is mediated by highly complex signaling pathways and several families of soluble factors that activate regulatory circuits. One interlocutor in this dialogue is the embryo, which produces soluble ligands and expresses receptors for different autocrine and paracrine factors. The other interlocutor is the receptive endometrium, which produces mediators to regulate proliferation, differentiation, adhesion and invasiveness of the embryo, among other processes (8,33,34).

Here, we focused on the role of blastocyst-derived factors in shaping the receptive immunomodulatory milieu. In this sense, we observed that the decidualized cells are able to display a differential response according to blastocyst quality. Our conclusions are based on several observations. First, decidualized cells significantly increased the production of the active form of IL-1β in response to soluble factors from impaired developed blastocysts, Second, decidualized cells under BCM from impaired developed blastocysts stimulation significantly increased CXCL12 expression and CXCL8 production, which was accompanied with a higher neutrophils migration and activation. Third, Tregs recruitment was significantly reduced under the same treatment. Finally, BCM from normal developed blastocysts decreased IL-1β, did not modulate CXCL12 expression and CXCL8 production nor the recruitment of the leukocyte subpopulations evaluated. Therefore, our results suggest that developmentally impaired human blastocyst, rather than being biologically silent or inert, are able to actively induce an exacerbated inflammatory response as well as to prevent tolerogenic mechanisms through the secretion of soluble factors. In tis sense, in response to BCM from impaired blastocysts, decidualized cells increased ~2 folds IL-1β and CXCL12 expression in comparison with normal blastocysts stimulation. Moreover, they recruited the double of the neutrophils and ~half of Tregs highlighting whether blastocysts might condition decidual-microenvoirenment. It is important to note that, even though we observed a different behavior, normal developed blastocysts, classified by morphological criteria, are not always genetically normal, able to implant or achieve a successful pregnancy.

It has been proposed that an excessive secretion of pro-inflammatory mediators such as TNFα by the decidual cells, could have embryotoxic effects that limits the implantation (35). It was demonstrated that decidualized cells selectively recognize and respond to the presence of a developmentally impaired embryo (5,20,28). Here, we showed that it is able to increase IL-1β production and to induce a chemokine microenvironment associated with an inflammatory response. Regarding to possible mediators involved, previous reports indicated a wide range of molecules involved in the inflammasome activation, such as PAMPs, DAMPs or alarmins as well as others mediators like potassium efflux, lysosomal rupture, mitochondrial dysfunction, calcium influx and decreased intracellular cAMP levels (36). In this sense, several DAMPs or alarmins are known to trigger inflammatory processes in the amniotic cavity and chorioamniotic membranes such as HMGB1, alarmins S100B and IL-1α (37,38). Further research is needed to determine their possible role in the embryo-endometrium dialogue.

Despite the clinical significance of endometrial embryo selection, little is known about the link between the unsuccessful implantation of a low quality embryo and the posterior menstruation onset. Recently, it was reported that neutrophils represent 1-10% of total cells of the endometrial stroma from days 26-28 of the cycle, which is accompanied by transcriptional increase of proinflammatory chemokines and cytokines such as CCL2, CXCL8, IL-6 and TNFα (39). In this sense, CXCL8 is a potent chemoattractant and activator of neutrophils, with the highest expression in the menstrual phase (39). These events were depicted in this *in vitro* model by the upregulation of CXCL12 and CXCL8 by decidualized cells in response to soluble factors from impaired developed blastocysts, which was accompanied by an increase in neutrophils migration and activation. Particularly, CXCL12 has been associated to neutrophils recruitment to inflammatory sites in other models (40,41). The importance of neutrophils on this stage of the menstrual cycle has been reported by Salamonsen's group. Using a mouse model of endometrial breakdown, they observed an increase in the number of neutrophils during breakdown and early repair. Furthermore, neutrophils depletion affected both processes (42). In turn, this data suggests that soluble factors from impaired blastocysts induce a “menstruation-like” proinflammatory microenvironment. However, further studies are necessary to elucidate whether the mechanisms operate similarly *in vivo* and rule out any factor not contemplated *in vitro*.

In order to sustain homeostasis at the decidua, it was reported that Tregs are recruited previous to the implantation to form an immune tolerant microenvironment. This was assessed not only in humans but also in murine models (16,43). Here we showed that decidualized cells decreased Tregs recruitment in response to soluble factors from impaired blastocysts favouring a proinflammatory microenvironment and highlighting once again the unbalance induced by impaired blastocysts.

Considering that this *in vitro* model does not involve direct contact between the embryo and the endometrium, the presented evidence indicates that a paracrine signaling is able to modulate the immune response during the peri-implantation period. In accordance with this, a previous study in cattle showed that

blastocyst conditioned medium alone was sufficient to alter the abundance of specific transcripts, such as interferon-stimulated genes (44). Besides the paracrine interaction evaluated here, other studies have characterized the immune microenvironment on *in vitro* co-culture systems between stromal cells and human blastocysts. In this sense, Teklenburg et al. obtained different results regarding IL-1β secreted levels, they observed a reduction in IL-1β production (4). This difference might be associated with the *in vitro* experimental design. On one hand, Teklenburg et al. used an *in vitro* model that involved co-culture of decidualized endometrial stromal cells and human blastocysts. That *in vitro* model involved not only paracrine signaling, but also cell-to-cell interaction. The latter are not being contemplated in the present study due to the use of blastocysts-conditioned media. On the other hand, in our *in vitro* model we used blastocysts-conditioned media that contain soluble factors secreted by blastocysts between days 4 to 5; while Teklenburg et al. seeded day 5 blastocysts on the decidualized endometrial stromal cells and evaluated IL-1β secretion 72h later. These experimental differences in the *in vitro* design might explain the different results observed in IL-1β production.

These effects support the notion that local embryo-maternal interaction may occur in early stages, even before the embryo directly contacts the endometrium and may display dynamic changes throughout the implantation process.

In this sense, many data propose a variety of soluble factors produced by the human preimplantation embryo as growth factors, cytokines, chemokines, micro-RNAs, metabolites and hormones (45,46). Human embryo also secretes soluble factors with immunomodulatory properties as the LIF (leukemia inhibitor factor), TGF family, CFS-1 and CFS-2, chemokines as CCL3, CXCL13, CXCL1 among others. Particularly, human leukocyte antigen G (HLA-G) both insoluble and soluble isoforms, is also expressed by the preimplantation embryo and was indeed associated with embryo-derived extracellular vesicles (47,48). HLA-G belongs to major histocompatibility complex (MHC) I class proteins and its embryonic secretion was proposed as a suitable biomarker of embryo viability and competence, positively associated with embryo implantation (49,50). Moreover, soluble HLA-G is believed to represent a major player in the initiation of immunotolerance for the invading blastocyst into the decidual tissues (51,52). Accordingly, recent reports showed the induction of HLA-G^{+} T cells, a novel subset of Tregs $FoxP3^{neg}$, by HLA-G expressing-dendritic cells, both present in human decidua or induced by decidualized cells as we recently demonstrated (53–55).

Considering the study of embryo-endometrial dialogue is key to improving reproductive outcomes and human studies imply ethical and technical challenges, this *in vitro* model provide new clues about how decidualized cells display immunomodulatory effects in response to blastocyst quality, to throw new light on the ‘‘black box’’ of embryo implantation.

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Authors' contributions

CPL and RR designed the study, supervised the experimental work and wrote the manuscript. LF and ES carried out all the experiments using the in vitro model of decidualization, BCM stimulation and FACS analysis.

EG performed Tregs assays. GC performed neutrophils activation assays. VH performed neutrophils migration assays. SG and LG did the RT-PCRs data analysis and interpretation. FS did ELISA assays. GM and MI recruited the patients and provided the BCM. CPL and RR supervised the whole study. All authors read and approved the final manuscript.

Conflict of Interest Disclosure: The authors declare no conflict of interest.

Data Availability statement

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

Figure Legends

Figure 1: Decidualized cells differentially respond to blastocyst quality modulating IL-1β production.

HESC cells were decidualized and then cultured with blastocysts-conditioned media (BCM) obtained from normal developed blastocysts (ND) or impaired developed blastocysts (ID). After 48 h of stimulation we evaluated **A)** the activity of Inflammasome caspase-1 using a fluorescent inhibitor probe, FAM-FLICA, and analyzed by flow cytometry (n=4 independent experiments); and **B)** the production of the active form of IL-1β by intracellular staining and flow cytometry analysis (n=8 independent experiments). The right panel shows dot plots of one representative experiment with the corresponding percentages. Dotted lines indicate the mean levels of non-decidualized cells and (-) represents decidualized cells cultured with G2 plus medium (unstimulated decidualized cells). Results were expressed as the percentage of positive cells obtained from independent experiments. Data are displayed by a box and whiskers plot, showing minimum, lower
quartile, median, upper quartile, and maximum. *p<0.05, Wilcoxon test.

Figure 2: Decidualized cells differentially respond to blastocyst quality modulating a pro-inflammatory chemokines microenvironment.

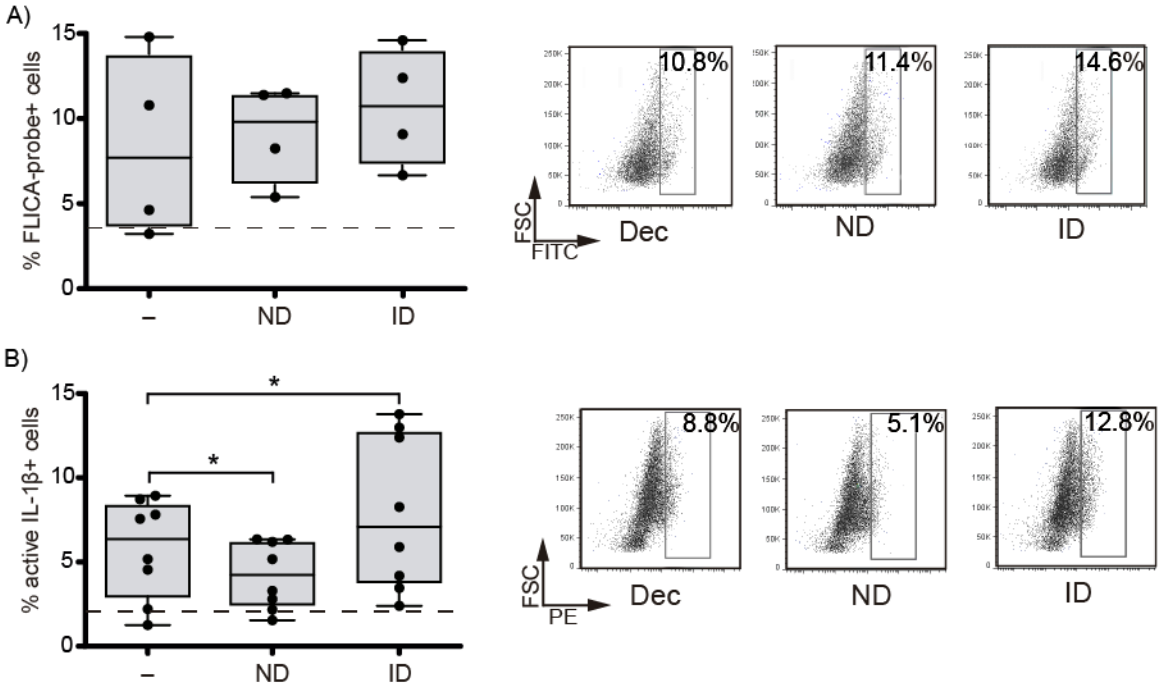
Decidualized cells were cultured in the presence of BCM from normal developed blastocysts (ND) or impaired ones (ID) and then we evaluated the following parameters. **A)** CXCL12 expression by RT-qPCR after 24 h (n=6 independent experiments). Values expressed as fold changes of unstimulated decidualized cells. **B)** CXCL8 production was quantified by ELISA after 48 h of stimulation (n=8 independent experiments). Dotted lines indicate the mean levels of non-decidualized cells and (-) represents decidualized cells cultured with G2 plus medium (unstimulated decidualized cells). Data are displayed by a box and whiskers plot, showing minimum, lower quartile, median, upper quartile, and maximum. *p<0.05, Wilcoxon test.

Figure 3: Decidualized cells differentially modulate neutrophils migration and activation according to blastocyst quality.

Decidualized cells were treated with BCM from normal developed blastocysts (ND) or impaired ones (ID) for 48 h, then supernatants were recovered and neutrophils migration and stimulation assays were performed. **A)** Neutrophils were seeded in the upper compartment of 5µm-inserts and HESC conditioned media was seeded in the lower compartment. Migration was quantified by flow cytometry after 30 min and values were normalized to non decidualized HESC condition media (A. U.= arbitrary units). The right panel shows dot plots of one representative experiment (n=4 donors) with the corresponding events number. **B)** Neutrophils were stimulated with HESC conditioned media and ROS production was determined by DCFH-DA probe staining and flow cytometry analysis. Results are expressed as Mean Intensity Fluorescence (MIF) from 4 independent experiments (n=4 donors). The right panel shows histograms of one representative experiment. Dotted lines indicate the mean levels of non-decidualized cells and (-) represents decidualized cells cultured with G2 plus medium (unstimulated decidualized cells). Data are displayed by a box and whiskers plot, showing minimum, lower quartile, median, upper quartile, and maximum. *p<0.05, Kruskal Wallis followed by Dunn's post test or Friedman followed by Dunn's post test.

Figure 4: Decidualized cells differentially recruit regulatory T cells according to blastocyst quality.

Decidualized cells were treated with BCM from normal developed blastocysts (ND) or impaired ones (ID) for 24 h,, then supernatants were recovered and Tregs migration assay was performed. PBMCs were seeded in the upper compartment of 5µm-inserts and HESC conditioned media was seeded in the lower compartment. Migration of CD4+FoxP3+ cells was quantified by flow cytometry and values normalized to non decidualized HESC condition media (n=4 donors). The right panel shows CD4+lymphocytes gated dot plots of one representative experiment with the corresponding event numbers for CD4+FoxP3+. Dotted lines indicate the mean levels of non-decidualized cells and (-) represents decidualized cells cultured with G2 plus medium (unstimulated decidualized cells). Data are displayed by a box and whiskers plot, showing minimum, lower quartile, median, upper quartile, and maximum. *p<0.05, Kruskal Wallis followed by Dunn's post test.



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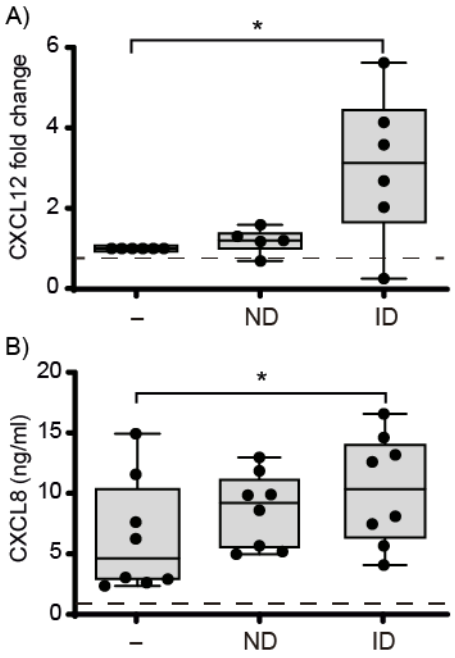

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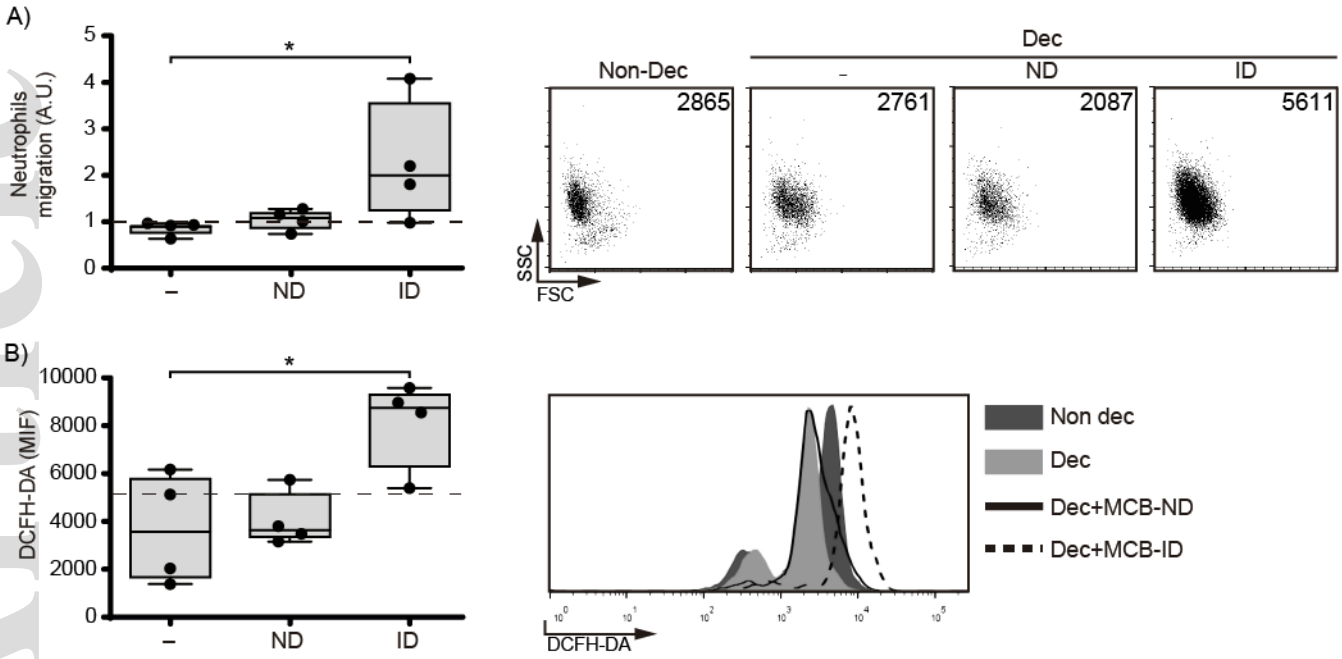

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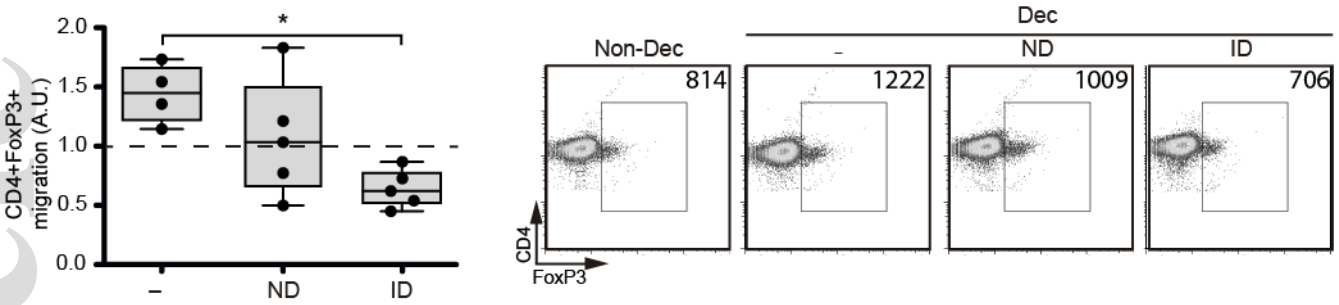

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