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Trophoblast induces monocyte differentiation into CD14⁺/CD16⁺ macrophages

Paulomi B Aldo¹, Karen Racicot¹, Vinicius Craviero¹, Seth Guller¹, Roberto Romero², and Gil Mor¹

¹Department of Obstetrics Gynecology and Reproductive Science, Yale University School of Medicine

²Perinatology Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, DHHS, Detroit

Abstract

Problem—During early pregnancy, macrophages and trophoblast come into close contact during placenta development, and regulated cross talk between these cellular compartments is crucial for maintaining a healthy pregnancy. As trophoblast cells constitutively secrete many chemokines and cytokines, we hypothesize that trophoblast secreted factors can differentiate monocytes into a decidual phenotype. In this study, we describe a unique macrophage phenotype following monocytes' exposure to trophoblast soluble factors.

Method of Study—Peripheral blood monocytes were treated with or without conditioned media (CM) from first trimester trophoblast cells. Phenotypic changes and phagocytic capacity were determined by flow cytometry. Cytokine and chemokine production was determined by multiplex analysis.

Results—Monocytes exposed to trophoblast factors undergo morphologic changes characterized by a gain in size and complexity and acquire a unique phenotype characterized by gain of CD14 surface expression as well as CD16. The presence of CD14⁺/CD16⁺ macrophages was confirmed in normal decidua. These cells secrete higher levels of IL-1b, IL-10, and IP-10 and have increased capacity for phagocytosis.

Conclusion—We demonstrate that trophoblast secreted factors can induce monocyte differentiation into a unique macrophage phenotype. These findings suggest that the microenvironment of the placenta can modulate the phenotype of macrophages present at the decidua.

Introduction

Macrophages are a major component of the innate immune system that function in both the immune response to infection as well as homeostasis of the surrounding environment^{1, 2}. As macrophages are plastic to their environment making them tissue specific the cells

Corresponding Author: Gil Mor, MD, PhD, Department of Obstetrics, Gynecology & Reproductive Sciences, Division of Reproductive Sciences, Yale University School of Medicine, 333 Cedar St., LSOG 305A, New Haven, CT 06520, Tel: 203 785 6294, Fax: 203 785 4883, gil.mor@yale.edu.

surrounding or in contact with macrophages influence their differentiation and therefore their function^{3, 4}. Distinct subsets of monocytes/ macrophages have been found in various tissues in both normal and disease states^{5, 6}. Macrophage differentiation and polarization occur in vivo under the influence of the localized cytokine milieu^{3, 7}. As specificity of function relies heavily on differentiation, the factors regulating differentiation in specific tissue environments become crucial to understanding the role of macrophages within the tissue.

The maternal-fetal interface is made of both placental and decidual tissue and within this tissue are components of the maternal immune system^{8, 9}. One of the most abundant immune cells present throughout pregnancy are macrophages, accounting for 20–25% of the total leukocyte population in the first trimester^{10, 11} and maintaining their presence throughout pregnancy. Gene expression profiling of early human decidua has shown an M2 dominant macrophage subtype characterized by CD14, HLA-DR, CD209, expression of anti-inflammatory genes, and production of IL-10¹². Decidual macrophages belong to a unique phenotype associated with homeostatic and tolerogenic properties and play an important role in tissue repair. Although their function it is still not fully defined, it is thought that decidual macrophages are important for spiral artery remodeling^{7, 13–16}, recruitment of Tregs¹⁷, and clearance of apoptotic cells¹⁸. Additionally, macrophage infiltration into the decidua is an essential event in the induction of labor¹⁹, suggesting that these cells may not only contribute to the immunoregulatory environment at the maternal/fetal interface, but also to the normal development of the pregnancy. The polarization of decidual macrophages to an anti-inflammatory state is critical to protect the fetus and the pregnancy²⁰.

In contrast to anti-inflammatory type 2 (M2) macrophages, cells stimulated with Th1 cytokines polarize toward pro-inflammatory type 1 macrophages (M1 cells)²¹ and primarily defend against infections²¹. As infection can be detrimental to pregnancy^{22, 23}, it is also important that decidual macrophages respond appropriately to invading pathogens or endogenous ligands. Even though decidual macrophages are dominantly M2-like, new evidence shows that macrophages can phenotypically fall into both categories, as macrophages will differentiate depending on what stimulus they are exposed to^{24–26}. Therefore, factors altering the normal phenotype and function of decidual macrophages may predispose to pregnancy complications such as preeclampsia or preterm birth^{11, 18, 20}. Although we have improved in our understanding of the phenotype of decidual macrophages, the factors regulating this differentiation process still remain unclear.

Innate and adaptive immune cells are present at the maternal-fetal interface in close proximity with decidua and trophoblast cells. It is thought that peripheral blood monocytes migrate into the decidualized endometrium and undergo differentiation within the specific microenvironment. Indeed, macrophages are found in close proximity to trophoblast cells of the placenta and decidual cells of the endometrium²⁷. We posit that the interaction between trophoblast and decidual macrophages is essential for the normal development of the placenta and the protection of the fetus against infections.

We and others have shown that trophoblast cells have immune-like functions as they respond to bacterial and viral ligands^{28–32} and, most importantly, constitutively secrete a

host of growth factors, chemokines and cytokines such as IL-8, IL-6, and TGF β ³⁰. Consequently, we hypothesized that trophoblast cells, through this array of factors, can regulate the migration, differentiation and function of decidual macrophages³³; conferring them to the unique phenotype required for the success of pregnancy.

The objective of this study was to develop an in vitro model of trophoblast-monocyte/macrophage interaction in order to evaluate the role of trophoblast cells on macrophage differentiation. We demonstrate that trophoblast-secreted factors contribute to the differentiation and function of macrophages by promoting a unique phenotype that resembles decidual macrophages.

Materials and Methods

Reagents and Antibodies

LPS isolated from *Escherichia coli* (0111:B4) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Multiplex beads for: IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12 (p70), IL-17, G-CSF, GM-CSF, IFN- γ , MCP-1, MIP-1 α /1 β , RANTES, TNF- α , VEGF, IP-10, and GRO- α were purchased from Bio-Rad Laboratories (Hercules, CA, USA). TGF β was purchased from Peprotech (Rocky Hill, NJ, USA) and neutralizing TGF β Ab was purchased from R&D (Minneapolis, MN, USA). For flow cytometry PE-conjugated anti human-CD14 and FITC-conjugated anti human-CD16 Abs purchased from eBioscience (San Diego, CA, USA) were used at a 1:50 dilution.

Cell Culture

The human monocytic cell line, THP-1, was a kind gift from Dr. P. Guyre (Dartmouth Medical School, Lebanon, NH, USA). The human first trimester trophoblast cell line, Sw. 71, was isolated from a normal 7-week placenta and immortalized by telomerase-mediated transformation.¹⁷ All cells were maintained at 37°C in 5% CO₂. Sw.71 cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; Life Technologies, Carlsbad, CA, USA) and THP-1 cells were cultured in RPMI-1640 (Life Technologies). All growth media were supplemented with 10% fetal bovine serum (FBS; Gemini, Sacramento, CA, USA), 10 mM HEPES, 0.1 mM minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, and 100 nM of penicillin and streptomycin (Life Technologies).

Monocyte Isolation

CD14⁺ monocytes were isolated from concentrated leukocytes from normal donors and purified using the EasySep Isolation System (EasySep, Vancouver, BC, CA). In brief, leukocytes were diluted 1:1 in phosphate-buffered saline, layered onto lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH, USA) and centrifuged without brake at 2000rpm for 20 min at room temperature. The mononuclear layer was then resuspended in phosphate-buffered saline and centrifuged at 1200 rpm for 10 min at room temperature. The pellet was then resuspended in Easy Sep buffer (0.5% bovine serum albumin, 2 mM ethylenediaminetetraacetic acid, pH 7.2) and incubated with human CD14 Positive Selection Cocktail and EasySep Magnetic Nanoparticles according to the

manufacture's protocol. The purity of the CD14⁺ cells was >95% as determined by flow cytometry.

Trophoblast CM preparation

Isolation of trophoblast cells from first trimester placentas was performed as previously described³⁴. First trimester trophoblast were plated at 9.0×10^5 cells/100mm dish and allowed to attach overnight. Cells were then changed to 1% serum DMEM/F-12 media and incubated for 48hrs. Cell supernatant was then collected and spun down to remove any cellular debris. Cell free supernatant was aliquotted and stored at -80° until use.

Education Studies

Freshly isolated CD14⁺ cells (PMs) were plated at 2×10^6 cell/well in 1% DMEM/F-12 media. Cells were then treated with or without 50% conditioned media from the first trimester trophoblast cell line, Swan 71 (Sw.71)³⁴ for a total of 6 days (TCM); media was refreshed every 2 days.

For LPS studies, d3 PM or d6-educated macrophages were treated with 10ng/ml LPS for 24hrs after which conditioned media was collected, prepared as described above for cytokine analysis.

For TGF β studies, freshly isolated CD14⁺ were treated with 10ng/ml TGF β for 6 days with media being refreshed every 2 days. Additionally, CD14⁺ cells cultured in trophoblast CM were treated with or without a TGF β neutralizing Ab (clone AB-100-NA) for 6 days with media being refreshed every 2 days. Cells were collected on day 6 for flow cytometry or cytokine analysis.

FACS analysis and sorting

Cells were collected from differentiating cultures and analyzed by FACS (FACSCalibur; Beckton Dickinson, Franklin Lakes, NJ, USA) for detection of various specific cell surface markers. We used mouse monoclonal PE and FITC-conjugated antibodies against the following human antigens: CD14(PE) and CD16 (FITC) (eBioscience). FACS data were analyzed with Cell Quest software (Beckton Dickinson).

Double Staining Immunocytochemistry

Standard IHC protocol was followed to stain normal 3rd trimester placentas using the mouse monoclonal antibody against hCD14 (Novus Biologicals, Littleton, CO, USA) and hCD16 (AbDSerotec, Raleigh, NC, USA); acquisition of human tissue was approved by the Yale University Human Investigation Committee. Briefly, 5 μ m sized paraffin embedded tissue sections were deparaffinized with HistoSolve and tissue sections were dehydrated through graded alcohols and subjected to antigen retrieval using 1x Target Retrieval Solution from Dako (Carpinteria, CA, USA). Sections were washed with Dako Wash Buffer and then blocked with 5% BSA (Bovine Serum Albumin; Gemini Bio) for 30m. Slides were incubated with the mouse monoclonal primary antibody CD14 or IgG control diluted (1:750) with Dako Wash buffer overnight at 4° . Slides were then washed for 5 minutes in wash buffer twice and incubated for 1 hour in the dark with the Alexa546 at 1:500 (Life

Technologies). After washing, mouse monoclonal primary antibody CD16 or IgG control diluted (1:750) with Dako Wash buffer overnight at 4°. Slides were then washed for 5 minutes in wash buffer twice and incubated for 1 hour in the dark with the Alexa488 at 1:500 (Life Technologies). After washing, slides were counterstained with Hoechst (1:10,000) for 5min in dark, and wash repeated. Slides were then mounted and stored in dark at 4°. Slides were then observed under a fluorescent microscope (Axio Observer, Carl Zeiss).

Cytokine/Chemokine Analysis

The effect of trophoblast supernatant on immune cell chemokine and cytokine production was determined using the cytokine multiplex assay from BioRad Laboratories. Briefly, wells of a 96-well filter plate were loaded with either 50 µl of prepared standard solution, or 50µl of cell-free supernatant and incubated with a custom 17plex ProBead mix from BioRad at ± 800 rpm for 30 min in the dark at room temperature. Wells were then vacuum-washed three times with 100µl wash buffer. Samples were then incubated with 25µl of biotinylated detection antibody at ± 800 rpm for 30 min at room temperature in the dark. After three washes, 50µl of streptavidin-phycoerythrin was added to each well and incubated for 10 min at ± 800 rpm at room temperature in the dark. After a final wash, the beads were resuspended in 125µl of sheath buffer for measurement with the LUMINEX 200 (LUMINEX, Austin, TX, USA).

Wound/repair assay

Human endometrial stromal cells were plated in a 24-well Image Lock Plate (Essen Bioscience, Ann Arbor, MI). After reaching confluency cells were wounded using a semi-manual wound maker tool. Cells were washed and then incubated for up to 48hrs with TEM CM, PM CM, or HESC media. Wound width was calculated by imaging plates using the Incucyte system (Essen Instruments), during around-the-clock kinetic imaging using Incucyte Imaging Software.

Phagocytosis Assay

Peripheral CD14+ cells and TEMs were incubated with 1.0µm FluoSphere FITC labeled carboxylated latex beads purchased from Life Technologies. Beads were added at a 1:1000 dilution and incubated for 4hrs in a 5% CO₂ incubator at 37°. Cells were then collected and directly analyzed by FACS analysis. An identical plate was incubated at 4° for control.

Results

Trophoblast CM induces morphological change in monocytes

Decidual macrophages represent a unique cell population, which differ from other tissue macrophages in terms of markers and function^{9, 35}. As first trimester trophoblast are in close proximity to CD14+ cells (Fig. 1), our first objective was to determine whether the placenta and, more specifically, the trophoblast contributes to the establishment of this unique macrophage population. In order to evaluate this premise we established an in vitro model for trophoblast-immune interaction using peripheral blood monocytes or the pre-myeloid THP-1 cell line exposed to condition media (CM) from first trimester trophoblast cells. A

first set of experiments utilized the pre-myeloid THP-1 cell line, which has the capacity to differentiate into macrophages according to the stimuli that is used. THP-1 cells were incubated with trophoblast CM for up to five days and morphological changes monitored. As shown in Figure 2, the starting culture is characterized by round floating cells (Fig. 2A), and after 2 days in the presence of trophoblast CM, a high number of monocytes attached to the plate and underwent morphological changes associated with bigger cellular size and increased number of membrane projections (Fig. 2B). This was further enhanced at day five (Fig. 2C). To confirm the morphological changes observed by microscopy we analyzed the monocyte population by flow cytometry. While the original THP-1 population is highly homogeneous in terms of their size and density (Fig. 2Di), after exposure to trophoblast CM we observed a significant number of cells undergoing a shift in size and density, further confirming the differentiation process induced by trophoblast CM (Fig. 2Dii).

Characterization of trophoblast-educated macrophage gene expression

To further characterize the phenotype of trophoblast-educated macrophages, we performed a gene array on monocytes differentiated with condition media (CM) obtained from first trimester trophoblast cells. Gene expression data showed that 270 genes were differentially regulated in trophoblast-educated macrophages (TEMs) as compared to CD14⁺ peripheral monocytes. As shown in Table I, genes previously reported in decidual macrophages^{36, 37} are up regulated in TEMs, including TGF β , MMP9, GAS6, and SPP1. Additionally, CD14 and CD163, known decidual macrophage markers, are also significantly increased. Table II shows those genes that are down regulated in trophoblast-educated macrophages; the role of these genes in decidual macrophage function needs to be further elucidated. Our data suggest that trophoblast soluble factors mediate, in part, macrophage differentiation into an M2 phenotype associated with immune regulation and repair.

Next we validated the findings from the THP-1 array using CD14⁺ peripheral blood monocytes (PM). We exposed CD14⁺ monocytes to trophoblast CM and evaluated their morphology and expression levels of CD14 by flow cytometry and production of TGF β 1 by ELISA. As shown in Figure 3, CD14⁺ PM exposed to trophoblast CM undergo similar morphologic differentiation as shown with THP-1 cells, including change in size and density (Fig. 3A). Furthermore, these macrophages express high levels of membranous CD14 (Fig. 3B) and secrete significantly higher levels of TGF β 1 ($p < 0.001$) (Fig. 3C). CD163 and IL-8 protein expression levels are also increased in TEMs, therefore confirming the findings of the array (data not shown).

Trophoblast CM induces a unique population of macrophages with a distinct phenotype

Circulating monocytes are characterized by the expression of CD14; however, once they migrate to a tissue and undergo tissue macrophage differentiation they usually lose CD14 expression. That is not the case for decidual macrophages, which maintain CD14 expression³⁷. As indicated above, CD14 is one of the genes identified in the gene array that was significantly induced by trophoblast CM. Therefore, we evaluated whether the increase in CD14 expression was associated with the cells undergoing differentiation. PMs were exposed to trophoblast CM for 6 days and CD14⁺ cells were evaluated in the undifferentiated (R1) and differentiated (R2) regions determined by their morphologic

characteristics (Fig. 4). As shown in Figure 4Ai, 97.3% of CD14⁺ PM are clustered in R1, corresponding with a pure and homogeneous CD14⁺ monocyte population. However, after exposure to trophoblast CM, 83.3% of CD14⁺ cells are observed in R2 (Fig. 4Aii) corresponding to trophoblast-educated macrophages. These findings suggest that PM, in the presence of trophoblast CM, maintain CD14 expression.

Trophoblast-educated macrophages are CD14⁺/CD16⁺ cells

CD16 is a common marker for identifying subsets of monocytes; therefore, we evaluated the percentage of CD16⁺ cells in PMs and TEMs. While PM CD16⁺ cells are less than 5%, a significant number (> 50%) of TEMs acquired CD16⁺ expression (Fig. 4Bii). Interestingly, all the CD16⁺ cells are also CD14⁺ (Fig. 4C). This suggests that trophoblast soluble factors shift the population of monocytes to a macrophage-like cell that maintain and even increase their CD14⁺ expression while concurrently gaining CD16. Our next objective was to determine whether this phenotype could also be found at the implantation site of normal pregnancies. Thus, we double stained third trimester placentas for CD14 and CD16 using fluorescence labeled antibodies. We observed a high number of CD14⁺ cells as well as CD16⁺ cells in the decidua of normal pregnancies in close proximity to trophoblast cells. Double staining for CD14 and CD16 revealed that a high percentage of CD14⁺ macrophages are also CD16⁺, this suggests that macrophages present in the decidua are CD14⁺^{high} and CD16⁺^{high} (Fig. 5).

Trophoblast-educated macrophage parallel M2 function

To determine what functional changes are associated with this unique population of macrophages we first looked at the cytokine profile of CD14⁺ PM versus trophoblast-educated macrophages. As shown in Table III, TEMs revealed a distinct cytokine profile characterized by high production of IL1 β , IL-10, IL-8, IL-6, IP-10 and GRO- α . Furthermore, contrary to PM, trophoblast-educated macrophages do not produce VEGF and RANTES (Table III).

The cytokine profile of trophoblast-educated macrophages is suggestive of an M2 macrophage. As M2 macrophages are associated with enhanced capacity for tissue repair and phagocytosis, we next evaluated these functional outcomes in TEMs. As shown in Figure 6A, PM differentiation into trophoblast-educated macrophages revealed a significant increase in their phagocytic capacity as demonstrated by their ability to uptake FITC labeled beads ($p < 0.001$); control conditions described in material and methods were used as baseline (data not shown). Furthermore we evaluated their capacity to promote wound repair by using a wound/healing in vitro model³⁸. A mechanical “wound” was performed in endometrial stromal cell cultures followed by incubation with CM from TEMs or PM and rate of wound repair was monitored by Live Cell imaging³⁸. We observed a 50% decrease in the repair time on the cultures treated with CM from trophoblast-educated macrophages (12h vs 24 and 32h), suggesting that the cytokines/chemokines secreted by these cells have the capacity to promote cell migration/proliferation and enhance tissue repair (Fig. 6B and 6C).

The role of TGF β in the differentiation of monocytes

Our next objective was to elucidate the potential factor(s) produced by trophoblast cells responsible for the expression of CD16 in trophoblast-educated macrophages. Although trophoblast constitutively secrete many cytokines, growth factors, and chemokines, TGF β is one of the main regulatory factors found in trophoblast CM ³⁹.

To first determine the direct effect of TGF β on monocyte differentiation we treated CD14+ PM with 10ng/ml TGF β 1 for six days and evaluated CD16 expression by flow cytometry. As shown in Fig. 7A, TGF β treatment increased the expression of CD16 in CD14+PM, similar to what was observed with trophoblast CM. To define if trophoblast secreted TGF β is responsible for the induction of CD16 expression, we neutralized TGF β 1 in the CM of trophoblast using a specific TGF β neutralizing antibody. Our data showed that by inhibiting TGF β we decreased the induction of CD16 expression as well as the production of IL-10, a marker of M2 macrophages (Figure 7B and C). Our data suggests that TGF β , produced locally by the trophoblast, contributes to maintaining the phenotype of macrophages found in the decidua.

Response of educated monocytes to LPS

It is well known that monocytes are highly responsive to TLR ligands, especially LPS. However, having a high inflammatory response at the maternal fetal interface can be very detrimental to pregnancy. We have previously shown that trophoblast could alter the response of CD14+ cells to LPS by dampening the inflammatory response ³³ as compared to PMs. We confirmed this profile in our model, showing that after PM differentiation with trophoblast CM the characteristic pro-inflammatory response to LPS (TNF α , IL-6, GCSF) is inhibited and, moreover, there is a shift in response as characterized by the production of high levels of RANTES, and IP-10 (Table IV).

Discussion

In the current study, we demonstrate that trophoblast cells contribute to the differentiation and function of decidual macrophages by promoting a unique phenotype that supports pregnancy. Soluble trophoblast factors induce monocyte differentiation into a macrophage that is CD14^{high}/CD16^{high} and expresses immunoregulatory genes representative of an M2-like macrophage. These cells also produce factors that support homeostasis, such as IL-10, TGF-beta, and IL-6 as well as have a dampened response to endotoxins such as LPS.

Macrophages are one of the main leukocyte populations present throughout pregnancy, they exhibit a high degree of plasticity and participate in very diverse physiologic processes¹³. The functional differentiation of macrophages has been defined in a manner analogous to the well-described concept of Th1/Th2 effector T cells. Macrophages activated under the influence of pro-inflammatory cytokines and LPS are termed M1, secrete TNF- α and IL-12, and participate in the progression of inflammation ^{13, 14, 40}. M2 polarization of macrophages is characterized by the expression of anti-inflammatory cytokines, IL-10, TGF β , IL13, enhanced expression of scavenger and mannose receptors, as well as enhanced expression of innate immunity receptors ⁴¹. Additionally, M2-type macrophages are involved in tissue

repair and the control of inflammation^{21, 42}. There is strong evidence suggesting that majority of macrophages present at the decidua and in contact with trophoblast cells have the characteristics of a M2-type macrophage¹²; however, what regulates their polarization and resulting function is still unclear. As macrophage polarization is specifically regulated and heavily influenced by their environment, we proposed that the surrounding trophoblast cells might play a role in their differentiation and unique phenotype. Indeed, when we exposed peripheral monocytes to trophoblast secreted factors we observed: 1) morphological differentiation; 2) acquisition of specific markers of differentiation; 3) change in their cytokine profile; 4) differential response to LPS; and 5) increased phagocytic and tissue repair characteristics.

Trophoblast cells are a well-established source of regulation at the maternal-fetal interface^{43, 44}. Previously we have shown that trophoblast cells have an immunomodulatory role via their constitutive secretion of cytokines, chemokines and growth factors^{30, 44}. They express a variety of HLA class I molecules that signal with decidual NK cells to prevent recognition of paternal antigens^{14, 45}. Trophoblast cells also contribute to the Treg population found in early pregnancy⁴⁶ as well as increase migration of CD14+ monocytes³³. In this study, we have demonstrated that trophoblast derived factors differentiate monocytes into a macrophage population that maintains the expression of CD14 and increase the expression of CD16, and this double positive population is found in human decidual/placenta tissue. To our knowledge, this is the first report of this double positive population at the implantation site.

Circulating CD14+ monocytes are now categorized based on their concomitant CD16 expression, CD14++/CD16- (classical), CD14++/cd16+ (intermediate), and CD14+/CD16++ (non-classical). Interestingly, we observed that monocytes differentiated under trophoblast-derived factors maintain their CD14 expression, which correlated with previous reports of CD14+ decidual macrophages; however, they acquire CD16+ expression. When we evaluate the expression of CD16 on decidual samples we found the co-expression of CD14 and CD16 on decidual macrophages in proximity to trophoblast cells. This data suggests that in normal pregnancy, the macrophages, which might be associated with the support of trophoblast invasion and placental growth, are CD14^{high}/CD16^{high}. Interestingly, an increase of non-classical CD14^{high}/CD16^{high} circulating monocytes in peripheral blood of third trimester pregnant women⁴⁷ was recently reported.

The expression of these markers might allow us to determine the normal macrophage phenotype and their interaction with the invading trophoblast. Indeed, in the past we have reported the differential localization of decidual macrophages in samples from normal pregnancy and from patients complicated with preeclampsia¹⁸. Samples from normal pregnancies localize macrophages behind invading trophoblast cells that are migrating towards the spiral arteries, while in samples from preeclamptic patients, the macrophages had a different morphology and were surrounding the spiral arteries forming a barrier-like structure to the trophoblast⁴⁸. These differences further support the plasticity of decidual macrophages and the importance of the signals responsible for the specific microenvironment where these cells undergo differentiation.

CD14⁺ monocytes differentiated by trophoblast factors express similar genes as reported for decidual macrophages, such as IFI30 and TGF β , and appear to be similar in function by having enhanced phagocytosis and high capacity of tissue repair^{49,50}. Additionally, gene array analysis showed that trophoblast educated macrophages express a number of immunoregulatory genes associated with decidual macrophages¹², such as APOE, TREM2 and GAS6, known negative regulators of inflammation. Other similarities include MMP9 and PAD14, both important regulators of tissue remodeling⁴⁴.

As decidual macrophages are a source of homeostasis as well as inflammatory responses to infection, we determined what effect trophoblast conditioned media had on phagocytosis, wound repair as well as macrophage capacity to respond to LPS. We show that after education, phagocytosis as well as endometrial stromal repair, two established functions of M2 macrophages, was significantly enhanced in TEMs as compared to peripheral monocytes and MCSF-derived macrophages (data not shown). Tissue integrity and abnormal clearance of apoptotic debris have contributed to complications such as preeclampsia and IUGR^{8, 47}, which are both associated with trophoblast dysfunction. This further drives the importance of trophoblast cross talk as contributing to the normal function of local macrophages. Furthermore, after treatment with LPS, TEMs showed a dampened classical response to LPS as shown by decreased secretion of TNF α , IL-6 and GCSF. This suggests that although functional the NF- κ B mediated pathway is altered. Moreover, the dominant factors being produced, RANTES and IP-10, after stimulation with LPS are downstream of TRIF, suggest education with trophoblast soluble factors promotes activation of this pathway. Ongoing studies are being done to provide the mechanism of TLR4 signaling in these cells.

As trophoblast are known to secrete a variety of factors, we then asked the question: What factor(s) could be a contributing to the enhancement of this double positive population with M2 like properties? As TGF β has been a widely studied immunoregulatory factor and produced locally by cytotrophoblast as well as decidual macrophages⁵¹, we examined its role in monocyte differentiation and function. We demonstrated that neutralizing TGF β directly in trophoblast CM decreased CD16 expression and downregulated M2 associated factors, such as IL-10. Inhibition of CD16 expression did not affect cells' phagocytic capacity, showing the specificity of the effect on function.

We demonstrate that trophoblast-educated CD14⁺high/CD16⁺high macrophages have enhanced capacity to phagocytize particles, repair stroma, and modulate responses to bacteria. Macrophages with phagocytic activity were found to infiltrate remodeling decidual vessels suggesting they are involved in the degradation of the extracellular matrix and clearance of apoptotic cells⁵²; functions that are necessary for normal placentation.

Together our data ascertains a role of trophoblast-mediated factors promoting the differentiation of monocytes to an M2-like macrophage associated with high expression of CD14 and an increase in CD16 along with a phenotype similar to decidual macrophages. Additionally, we suggest that TGF β produced by the trophoblast regulates some aspects of this phenotype, as inhibiting TGF β decreases CD16 expression while altering their constitutive production of cytokines and chemokines. Pregnancy complications are closely

linked to alterations in function and phenotype of decidual macrophages so understanding what can regulate their dysfunction may allow for targeted therapies.

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References

1. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. *Immunity*. 2005; 23:344–346. [PubMed: 16226499]
2. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leuk Biol*. 2004; 76:509–513.
3. Bruck W, Porada P, Poser S, Rieckmann P, Hanefeld F, Kretschmar HA, Lassmann H. Monocyte/macrophage differentiation in early multiple sclerosis lesions. *Ann Neurol*. 1995; 38:788–796. [PubMed: 7486871]
4. Bornstein SR, Rutkowski H, Vrezas I. Cytokines and steroidogenesis. *Mol Cell Endocrinol*. 2004; 215:135–141. [PubMed: 15026186]
5. Alvero AB, Montagna MK, Craveiro V, Liu L, Mor G. Distinct Subpopulations of Epithelial Ovarian Cancer Cells Can Differentially Induce Macrophages and T Regulatory Cells Toward a Pro-Tumor Phenotype. *Am J Reprod Immunol*. 2011; 67:256–265. [PubMed: 21917055]
6. Barua A, Yoshimura Y, Tamura T. The effects of age and sex steroids on the macrophage population in the ovary of the chicken, *Gallus domesticus*. *J Reprod Fertil*. 1998; 114:253–258. [PubMed: 10070355]
7. Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. *Clin Sci (Lond)*. 2003; 104:27–38. [PubMed: 12519085]
8. Svensson-Arvelund J, Ernerudh J, Buse E, Cline JM, Haeger JD, Dixon D, Markert UR, Pfarrer C, De Vos P, Faas MM. The placenta in toxicology. Part II: Systemic and local immune adaptations in pregnancy. *Toxicologic pathology*. 2014; 42:327–338. [PubMed: 23531796]
9. Mor G, Cardenas I. The immune system in pregnancy: A unique complexity. *Am J Reprod Immunol*. 2010:63.
10. Bulmer JN, Pace D, Ritson A. Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function. *Reprod Nutr Dev*. 1988; 28:1599–1613. [PubMed: 3073448]
11. Houser BL, Tilburgs T, Hill J, Nicotra ML, Strominger JL. Two unique human decidual macrophage populations. *J Immunol*. 2011; 186:2633–2642. [PubMed: 21257965]
12. Gustafsson C, Mjosberg J, Matussek A, Geffers R, Matthiesen L, Berg G, Sharma S, Buer J, Ernerudh J. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. *PLoS One*. 2008; 3:e2078. [PubMed: 18446208]
13. Nagamatsu T, Schust DJ. The contribution of macrophages to normal and pathological pregnancies. *Am J Reprod Immunol*. 2010; 63:460–471. [PubMed: 20163399]
14. Nagamatsu T, Schust DJ. The immunomodulatory roles of macrophages at the maternal-fetal interface. *Reprod Sci*. 2010; 17:209–218. [PubMed: 20065301]
15. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nature reviews Immunology*. 2005; 5:953–964.
16. Haimovici F, Hill JA, Anderson DJ. The effects of soluble products of activated lymphocytes and macrophages on blastocyst implantation events in vitro. *Biol Reprod*. 1991; 44:69–75. [PubMed: 2015353]
17. Vacca P, Cantoni C, Vitale M, Prato C, Canegallo F, Fenoglio D, Ragni N, Moretta L, Mingari MC. Crosstalk between decidual NK and CD14⁺ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci U S A*. 2010; 107:11918–11923. [PubMed: 20547831]

18. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and apoptotic cell clearance during pregnancy. *Am J Reprod Immunol.* 2004; 51:275–282. [PubMed: 15212680]
19. Jaiswal MK, Agrawal V, Mallers T, Gilman-Sachs A, Hirsch E, Beaman KD. Regulation of apoptosis and innate immune stimuli in inflammation-induced preterm labor. *J Immunol.* 2013; 191:5702–5713. [PubMed: 24163412]
20. Ben Amara A, Gorvel L, Baulan K, Derain-Court J, Buffat C, Verollet C, Textoris J, Ghigo E, Bretelle F, Maridonneau-Parini I, Mege JL. Placental macrophages are impaired in chorioamnionitis, an infectious pathology of the placenta. *J Immunol.* 2013; 191:5501–5514. [PubMed: 24163411]
21. Gordon S. Targeting a monocyte subset to reduce inflammation. *Circulation research.* 2012; 110:1546–1548. [PubMed: 22679136]
22. Chaiworapongsa T, Romero R, Espinoza J, Kim YM, Edwin S, Bujold E, Gomez R, Kuivaniemi H. Macrophage migration inhibitory factor in patients with preterm parturition and microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med.* 2005; 18:405–416. [PubMed: 16390807]
23. Madsen-Bouterse SA, Romero R, Tarca AL, Kusanovic JP, Espinoza J, Kim CJ, Kim JS, Edwin SS, Gomez R, Draghici S. The transcriptome of the fetal inflammatory response syndrome. *Am J Reprod Immunol.* 2010; 63:73–92. [PubMed: 20059468]
24. Kim YM, Romero R, Chaiworapongsa T, Kim GJ, Kim MR, Kuivaniemi H, Tromp G, Espinoza J, Bujold E, Abrahams VM, Mor G. Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *Am J Obstet Gynecol.* 2004; 191:1346–1355. [PubMed: 15507964]
25. Kim JS, Romero R, Cushenberry E, Kim YM, Erez O, Nien JK, Yoon BH, Espinoza J, Kim CJ. Distribution of CD14+ and CD68+ macrophages in the placental bed and basal plate of women with preeclampsia and preterm labor. *Placenta.* 2007; 28:571–576. [PubMed: 17052752]
26. Schatz F, Soderland C, Hendricks-Munoz KD, Gerrets RP, Lockwood CJ. Human endometrial endothelial cells: isolation, characterization, and inflammatory-mediated expression of tissue factor and type 1 plasminogen activator inhibitor. *Biol Reprod.* 2000; 62:691–697. [PubMed: 10684811]
27. Mor G, Cardenas I, Abrahams V, Guller S. Inflammation and pregnancy: the role of the immune system at the implantation site. *Annals of the New York Academy of Sciences.* 2011; 1221:80–87. [PubMed: 21401634]
28. Abrahams VM, Romero R, Mor G. TLR-3 and TLR-4 mediate differential chemokine production and immune cell recruitment by first trimester trophoblast cells. *Am J Reprod Immunol.* 2005; 53:279. ASRI205-202.
29. Abrahams VM, Aldo PB, Murphy SP, Visintin I, Koga K, Wilson G, Romero R, Sharma S, Mor G. TLR6 modulates first trimester trophoblast responses to peptidoglycan. *J Immunol.* 2008; 180:6035–6043. [PubMed: 18424724]
30. Abrahams VM, Bole-Aldo P, Kim YM, Straszewski-Chavez SL, Chaiworapongsa T, Romero R, Mor G. Divergent trophoblast responses to bacterial products mediated by TLRs. *J Immunol.* 2004; 173:4286–4296. [PubMed: 15383557]
31. Gonzalez JM, Xu H, Ofori E, Elovitz MA. Toll-like receptors in the uterus, cervix, and placenta: is pregnancy an immunosuppressed state? *Am J Obstet Gynecol.* 2007; 197:296. e291–296. [PubMed: 17826427]
32. Hirschfeld AF, Jiang R, Robinson WP, McFadden DE, Turvey SE. Toll-like receptor 4 polymorphisms and idiopathic chromosomally normal miscarriage. *Hum Reprod.* 2007; 22:440–443. [PubMed: 16982657]
33. Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, Chen R, Chavez SL, Romero R, Mor G. Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. *Am J Reprod Immunol.* 2007; 57:55–66. [PubMed: 17156192]
34. Straszewski-Chavez SL, Abrahams VM, Aldo PB, Mor G. Isolation and characterization of a novel telomerase-immortalized human first trimester trophoblast cell line. *Placenta.* 2005; 26:A.62.

35. Cardenas I, Means RE, Aldo P, Koga K, Lang SM, Booth C, Manzur A, Oyarzun E, Romero R, Mor G. Viral Infection of the Placenta Leads to Fetal Inflammation and Sensitization to Bacterial Products Predisposing to Preterm Labor. *J Immunol.* 2010;185.
36. Mattsson N, Tabatabaei S, Johansson P, Hansson O, Andreasson U, Mansson JE, Johansson JO, Olsson B, Wallin A, Svensson J, Blennow K, Zetterberg H. Cerebrospinal fluid microglial markers in Alzheimer's disease: elevated chitotriosidase activity but lack of diagnostic utility. *Neuromolecular medicine.* 2011; 13:151–159. [PubMed: 21567187]
37. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. *J Immunol.* 2011; 187:3671–3682. [PubMed: 21890660]
38. Chefetz I, Alvero AB, Holmberg JC, Lebowitz N, Craveiro V, Yang-Hartwich Y, Yin G, Squillace L, Gurra Soteras M, Aldo P, Mor G. TLR2 enhances ovarian cancer stem cell self-renewal and promotes tumor repair and recurrence. *Cell Cycle.* 2013; 12:511–521. [PubMed: 23324344]
39. Ramhorst R, Fraccaroli L, Aldo P, Alvero AB, Cardenas I, Leiros CP, Mor G. Modulation and Recruitment of Inducible Regulatory T Cells by First Trimester Trophoblast Cells. *Am J Reprod Immunol.* 2011
40. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol.* 2006; 177:7303–7311. [PubMed: 17082649]
41. Taylor PR, Gordon S. Monocyte heterogeneity and innate immunity. *Immunity.* 2003; 19:2–4. [PubMed: 12871633]
42. Gordon S. Alternative activation of macrophages. *Nature reviews Immunology.* 2003; 3:23–35.
43. Heikkinen J, Mottonen M, Komi J, Alanen A, Lassila O. Phenotypic characterization of human decidual macrophages. *Clinical and experimental immunology.* 2003; 131:498–505. [PubMed: 12605704]
44. McIntire R, Ganacias K, Hunt J. Programming of Human Monocytes by the Uteroplacental Environment. *Reproductive Sciences.* 2008
45. Hunt J. HLA and maternal-fetal recognition. *FASEB J.* 1992; 6:2344–2348. [PubMed: 1544544]
46. Ramhorst R, Fraccaroli L, Aldo P, Alvero AB, Cardenas I, Leiros CP, Mor G. Modulation and recruitment of inducible regulatory T cells by first trimester trophoblast cells. *Am J Reprod Immunol.* 2012; 67:17–27. [PubMed: 21819477]
47. Melgert BN, Spaans F, Borghuis T, Klok PA, Groen B, Bolt A, de Vos P, van Pampus MG, Wong TY, van Goor H, Bakker WW, Faas MM. Pregnancy and preeclampsia affect monocyte subsets in humans and rats. *PLoS One.* 2012; 7:e45229. [PubMed: 23028864]
48. Mor, G.; Romero, R.; Abrahams, V. *Macrophages and Pregnancy.* New York, NY: Springer/Landes BioScience; 2006.
49. Oliveira LJ, McClellan S, Hansen PJ. Differentiation of the endometrial macrophage during pregnancy in the cow. *PLoS One.* 2010; 5:e13213. [PubMed: 20949061]
50. Zawada AM, Rogacev KS, Rotter B, Winter P, Marell RR, Fliser D, Heine GH. SuperSAGE evidence for CD14⁺⁺CD16⁺ monocytes as a third monocyte subset. *Blood.* 2011; 118:e50–e61. [PubMed: 21803849]
51. Reed D, Shen Y, Shelat AA, Arnold LA, Ferreira AM, Zhu F, Mills N, Smithson DC, Regni CA, Bashford D, Cicero SA, Schulman BA, Jochemsen AG, Guy RK, Dyer MA. Identification and characterization of the first small molecule inhibitor of MDMX. *J Biol Chem.* 2010; 285:10786–10796. [PubMed: 20080970]
52. Hazan AD, Smith SD, Jones RL, Whittle W, Lye SJ, Dunk CE. Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro. *Am J Pathol.* 2010; 177:1017–1030. [PubMed: 20558572]

Fig 1.
First trimester placenta samples from normal tissue show CD14+ cells (—●) are in close proximity to trophoblast cells (—→). Images are representative of 3 patients.

Fig 2.

Monocytes become macrophage-like when exposed to trophoblast soluble factors. THP-1 cells were exposed to 1st trimester trophoblast CM for 5 days after which cells became adherent and formed podia (**A–C**). When analyzed for size (FSC) and complexity (SSC) cells educated with trophoblast CM increased in size and granularity (**D**).

Fig. 3. CD14⁺ peripheral monocytes differentiate into macrophages after education with trophoblast CM. Freshly isolated CD14⁺ cells were exposed to 1st trimester trophoblast CM for 6 days, after which cells were analyzed by flow cytometry for morphology and CD14

expression and supernatant analyzed for TGF β 1 by ELISA. CD14+ educated cells increased in both size and granularity (**A**), express higher CD14 (**B**) and secrete TGF β 1 (**C**) validating the array.

Fig 4.

Education with trophoblast CM induces a unique macrophage that maintains CD14 expression while gaining CD16. CD14⁺ monocytes were isolated and cultured in trophoblast CM for 6 days. Cells were then stained for CD14 and CD16 by flow cytometry. Both CD14 and CD16 expression were analyzed after isolation (**Ai and Bi**) and after education (**Aii, Bii, and C**). As seen in panel Aii, cells that have shifted into R2 maintain levels of CD14 as seen in R1 of monocytes (**Ai**); additionally, the cells in R2 gain CD16 (**Bii**). Double staining FACS analysis shows that the population gaining CD16 are the CD14⁺ cells (**C**). Data is representative of four independent experiments; data shown is % of positive cells gated on live CD14⁺ cells.

Fig 5. Presence of CD14+/CD16+ macrophages in first trimester placenta. Representative immunohistochemistry of double positive cells in normal first trimester placenta. (A) and (B) show single staining of CD14 (red) and CD16 (green) respectively; (C) shows a merged imaged. (D) Close interaction between trophoblast cells and CD14+/CD16+ cells. Images are representative of 3 patients.

A

Fig 6. Trophoblast educated monocytes (TEM) function in an M2-like manner with enhanced capacity for phagocytosis and repair. After 6 days of education, TEMs uptake a significantly higher percentage of FITC labeled beads compared to peripheral monocytes. Time = 4 hours after exposure to beads; n=2; ***p<0.001(A). Additionally, trophoblast educated macrophages (TEMs) are able to enhance endometrial stromal wound repair. **B)** 12hr real-time images of wound repair **C)** Quantitative analysis of rate of wound repair over time. n=2; ***p<0.001

A **B**

C **Fig 7.**

TGFb regulates expression of CD16. Treating CD14+ cells directly with TGFb for 6 days increased CD16 expression similar to TEMs (**A**). Furthermore, when a neutralizing TGFb Ab was added to trophoblast CM, CD16 expression was inhibited (**B**) as was IL-10 production (**C**). Data is representative of two independent experiments.

Table I

Selection of genes upregulated in trophoblast-educated macrophages compared with peripheral blood CD14+ cells

Gene Name	Fold Difference	Name
C3	4.79	Complement component 3
SPP1	3.46	Secreted phosphoprotein 1 (OPN)
CD14	3.4	Cluster of differentiation 14
APOE	3.4	Apolipoprotein E
IFI30	3.25	Interferon-inducible gene 30
MMP9	3.13	Matrix metalloproteinase 9
CD163	2.83	Macrophage scavenger receptor
TREM2	2.81	Triggering receptor expressed on myeloid cells 2
IL1B	2.67	Interleukin 1, beta
GAS6	2.34	Growth arrest specific 6
TGFB1	2.24	Transforming growth factor beta 1
IL-8	2.18	Interleukin-8

Data shown as fold change with only genes included that were upregulated by a factor of at least 2.

Table II

Selection of genes downregulated in trophoblast-educated macrophages compared with peripheral blood CD14+ cells

Gene Name	Fold Difference	Name
CTSG	-2.94	Cathepsin G
IL18RAP	-2.57	Interleukin-18 receptor accessory protein
DAPK3	-2.57	Death-associated protein kinase 3
NCF1	-2.52	Neutrophil cytosolic factor 1
IL18R1	-2.41	Interleukin 18 receptor
LRRFIP1	-2.32	Leucine-rich repeat (in FLII) interacting protein 1
CCR7	-2.3	Chemokine (C-C motif) receptor 7
MCM4	-2.5	Mini chromosome maintenance deficient 2
STMN3	-2.1	Stathmin 1/oncoprotein 3
NOG	-2.01	Noggin

Data shown as fold change with only genes included that were downregulated by a factor of at least 2.

Table IIICytokine profile of peripheral monocytes versus TEMs^a

Peripheral monocytes	Trophoblast educated
IL-1 β ^{lo}	IL-1 β ^{hi}
IL-10 ^{lo}	IL-10 ^{hi}
IL-8 ^{lo}	IL-8 ^{hi}
IL-6 ^{lo}	IL-6 ^{hi}
GRO- α ^{lo}	GRO- α ^{hi}
IP-10 ^{lo}	IP-10 ^{hi}
GSCF ^{lo}	GSCF ^{lo}
VEGF ^{hi}	VEGF
RANTES ^{hi}	RANTES

^a CD14⁺ cells were cultured for either 3 days in media or 6 days with first trimester trophoblast CM after which cell supernatant was analyzed using a human multiplex kit.

Table IVCytokine production by peripheral monocytes and TEMs following ligation of TLR-4^a

Cytokine	PM	TEMs
IL-1 β	75.1	178.9
IL-10	118.2	5.8
TNF- α	1036	132.4
IL-6	818.9	1.9
GSCF	360.13	0.2
VEGF	0.56	0.29
RANTES	238.6	832.4
IP-10	1.74	5

^aCD14+ cells were cultured for either 3 days in media or 6 days with first trimester trophoblast CM and then were incubated for 24 hr with either no treatment or LPS (10 ng/mL) after which cell supernatant was analyzed for cytokine expression using a human multiplex (Bio-Rad). Data are shown as fold change compared with the untreated control.