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RESEARCH ARTICLE

The activating effect of IFN-γ on monocytes/macrophages is regulated by the LIF–trophoblast–IL-10 axis *via* Stat1 inhibition and Stat3 activation

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Interferon gamma (IFN-γ) and leukemia inhibitory factor (LIF) are key gestational factors that may differentially affect leukocyte function during gestation. Because IFN-γ induces a pro-inflammatory phenotype in macrophages and because trophoblast cells are principal targets of LIF in the placenta, we investigated whether and how soluble factors from trophoblast cells regulate the effects of IFN-γ on macrophage activation. IFN-γ reduces macrophage motility, but enhances Stat1 activation, pro-inflammatory gene expression and cytotoxic functions. Soluble factors from villous cytotrophoblasts (vCT+LIF cells) and BeWo cells (BW/ST+LIF cells) that were differentiated in the presence of LIF inhibit macrophage Stat1 activation but inversely sustain Stat3 activation in response to IFN-γ. vCT+LIF cells produce soluble factors that induce Stat3 activation; this effect is partially abrogated in the presence of neutralizing anti-interleukin 10 (IL-10) antibodies. Moreover, soluble factors from BW/ST+LIF cells reduce cell proliferation but enhance the migratory responses of monocytes. In addition, these factors reverse the inhibitory effect of IFN-γ on monocyte/macrophage motility. BW/ST+LIF cells also generate IFN-γ-activated macrophages with enhanced IL-10 expression, but reduced tumor-necrosis factor alpha (TNF-α), CD14 and CD40 expression as well as impaired cytotoxic function. Additional assays performed in the presence of neutralizing anti-IL-10 antibodies and exogenous IL-10 demonstrate that reduced macrophage cytotoxicity and proliferation, but increased cell motility result from the ability of trophoblast IL-10 to sustain Stat3 activation and suppress IFN-γ-induced Stat1 activation. These *in vitro* studies are the first to describe the regulatory role of the LIF–trophoblast–IL-10 axis in the process of macrophage activation in response to pro-inflammatory cytokines.
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INTRODUCTION

Tissue macrophages (Mϕs) are of interest not only to the field of immunology as part of the innate immune system, but also to other fields because they perform various homeostatic functions that are associated with tissue organization during organogenesis and in mature tissues.[1,2] During the inflammatory response, tissue Mϕs are expected to sequentially develop into three different subpopulations: type-1 (Mϕ-1), type-2 (Mϕ-2) and regulatory (Reg-Mϕs).[3–6] During the initiation phase of inflammation, the pro-inflammatory Mϕ-1 subtype is generated in response to two signals (i.e., Interferon gamma (IFN-γ) and tumor-necrosis factor alpha (TNF-α)) or a Toll-like receptor (TLR) ligand, such as lipopolysaccharide (LPS). During the resolution phase of inflammation, the presence of the type-2 cytokines interleukin 4 (IL-4) and IL-13 leads to the generation of anti-inflammatory Mϕ-2, which are associated with the coordination of tissue repair. The third subtype (i.e., Reg-Mϕs) is fundamentally different from Mϕ-1 and Mϕ-2, and the generation of this subtype requires two signals.[3–6] The first signal may include IL-10, prostaglandins, immune complexes, adenine nucleotides, glucocorticoids and

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apoptotic cells, and the second signal is TLR activation. Reg-Mϕs overproduce IL-10 but not IL-12 and exhibit potent immunosuppressive activity for modulating the acute inflammatory response and limiting tissue damage.[3–6]

Tissue Mϕs constitute one of the most abundant populations of inflammatory cells in the uterus, and the numbers of these cells remain relatively constant in the decidua throughout gestation.[7,8] In normal pregnancies, the phenotype of uterine Mϕs is locally modified to acquire a type-2 phenotype,[9–11] which may contribute to trophoblast differentiation, support embryo implantation and supply growth factors to the placenta.[7,8] However, although Mϕs play important roles at the maternal−fetal interface, aberrant activation of inflammatory pathways in Mϕs can affect trophoblast survival and function,[12] potentially leading to pregnancy complications, such as recurrent spontaneous abortion, pre-eclampsia, fetal growth restriction and intrauterine infection-associated preterm labor in humans.[7,8]

Whereas gestation has long been recognized as an anti-inflammatory Th2 condition, recent studies demonstrated that the process of blastocyst implantation requires a pro-inflammatory Th1 environment.[13,14] The transitory maintenance of this Th1 environment appears to be essential for the growth and survival of trophoblast cells, which in turn produce a balance of pro- and anti-inflammatory cytokines that are linked to the control of leukocyte recruitment, to the regulation of inflammatory activity, and to the promotion of Th2 immune responses.[14] Among the numerous cytokines produced at the implantation site, IFN-γ and TNF-α are potent inducers of the pro-inflammatory type-1 phenotype in tissue Mϕs;[15] in the context of the developing embryo, these cytokines have both beneficial and harmful attributes.[16] Through the activation of the transcription factor signal transducer and activator of transcription 1 (Stat1), IFN-γ is known to induce the expression of several pro-inflammatory genes in Mϕs, such as the cell death-inducing cytokine TNF-α and the cell surface receptor clusters of differentiation 14 and 40 (CD14 and CD40).[15] During early pregnancy, uterine natural killer (uNK) cells secrete IFN-γ, which plays critical roles in the initiation of endometrial vasculature remodeling, in the process of angiogenesis at implantation sites, and in the maintenance of decidual and placental tissues.[16] On the other hand, inflammatory stress in pregnant mice induces higher levels of IFN-γ and TNF-α synthesis by uNK cells and Mϕs, respectively; these cytokines in turn target uterine endothelial cells to provoke vascular damage and placental ischemia.[17,18] For instance, gestational complications have been linked to elevated IFN-γ and/or TNF-α expression in the immune-mediated, early abortion mouse model CBA/J×DBA/2J[17,18] and in the preterm labor and delivery model that employs LPS-treated IL-10 knockout (KO) mice.[19,20] Moreover, as observed during recurrent spontaneous abortion and intrauterine infection-associated preterm labor in humans,[7,8] aberrant inflammatory behavior in Mϕs is associated with spontaneous and endotoxin-mediated abortion in rodents.[21]

Thus, even if a pro-inflammatory Th1 environment appears to be required for successful blastocyst implantation,[13,14] the regulation of pro-inflammatory Mϕ activation within the pregnant uterus may be vital to the avoidance of detrimental Mϕ functions, which could ultimately lead to placental and fetal demise. Embryonic trophoblast cells have been proposed to participate intensively in this process by producing factors that modulate leukocyte function.[22] Recently, Mor *et al.*[14] proposed the theory that trophoblast/immune cell interaction involves three stages. During the attraction step, trophoblast cells secrete chemokines that can recruit immune cells to the implantation site; during the education step, trophoblast cells produce regulatory cytokines that modulate the differentiation process of immune cells; and finally, in the response step, immune cells acquire a trophoblast-supporting phenotype in response to signals from the local microenvironment.[14] However, the molecular mechanism by which uterine Mϕs are programmed to adopt a Th2 phenotype in response to trophoblast-derived factors remains elusive.

Many potential Mϕ programming factors have been described in the pregnant human uterus.[23,24] One factor of interest is the pleiotropic cytokine leukemia inhibitory factor (LIF). LIF plays an important role in the establishment of pregnancy by supporting decidual and placental differentiation and by influencing reproductive tract cells, such as leukocytes and luminal/glandular epithelial cells.[25] The best evidence that LIF may play a role in the regulation of Mϕs comes from data obtained from LIF KO mice, which present a more than half reduced percentage of uterine Mϕs by days 3–5 of pregnancy.[25] The LIF KO mouse model also revealed the central regulatory role of LIF in endotoxic shock and host defense. Essentially, LIF expression following endotoxic shock enhances the expression of hepatic acute-phase proteins and IL-10, which downregulates TNF-α synthesis and release in the liver, conferring protection against endotoxemia.[26] In the uterus of pregnant mice, LIF likely plays a similar but local role in the protective effect of progesterone against endotoxin-induced fetal demise by reducing nitric oxide levels *in utero*.[27]

Another important gestational factor is IL-10, which is a type-2 cytokine that is expected to play a key role in pregnancy immunotolerance through the establishment of a Th2 immune response at the maternal−fetal interface.[28,29] IL-10 inhibits the production of several pro-inflammatory cytokines, including TNF-α in Mϕs and IFN-γ in NK cells, thereby preventing the development of type-1 immune reactions that are deleterious for both the establishment and maintenance of pregnancy.[30–32] Thus, using the IL-10 KO mice model to study LPS-induced preterm parturition, Murphy *et al.*[19,20] demonstrated that in the absence of IL-10, IFN-γ-producing uNK cells may exert cytotoxic functions through TNF-α production and invasiveness into the placental zone, leading to fetal demise or intrauterine fetal growth restriction.[19,20] Moreover, IL-10 is noted for its abilities to inhibit Mϕ proliferation by activating Stat3[33] and to suppress IFN-γ-mediated functions of Mϕs by blocking Stat1 activation.[34]

In the pregnant human uterus, IL-10 is produced in large amounts by both the syncytiotrophoblast and the decidual

Mϕs,[9,10,28,29] while LIF is highly expressed by decidual Mϕs and uNK cells.[35] On the other hand, the IL-10 receptor is constitutively expressed on placental trophoblasts[28,29] and decidual Mϕs are known to be highly responsive to IL-10.[9,10] In contrast, the expression level of LIF receptors is significantly higher in both villous and extravillous trophoblasts than in the decidua,[35] suggesting that all cells of the trophoblast lineage are major targets for the action of LIF in the placenta. However, the possible interplay between LIF, trophoblast cells and IL-10 in the modulation of Mϕ functional phenotypes has never been investigated. Using *in vitro* Mϕ and trophoblast differentiation models that were previously established in our lab,[36–41] we investigate whether and how syncytiotrophoblast-derived factors in general and IL-10 in particular regulate the effects of IFN-γ on the behavior of Mϕs in response to LIF stimulation and describe the mechanism of action of these factors at the molecular level.

MATERIALS AND METHODS

Reagents and chemicals

All cell culture media, serum and reagents were purchased from Wisent (St-Bruno, PQ, CAN). Boyden chambers, cell culture plates and flasks were from Corning (Corning, NY, USA). The cytokines LIF, IL-10 and IFN-γ were purchased from Peprotech (Rocky Hill, NJ, USA). The neutralizing polyclonal anti-human IL-10 antibody (#IC2172F) was purchased from R&D Systems (Minneapolis, MN, USA). The chemicals methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), forskolin (FK), propidium iodide (PI) dye and all electrophoresis grade chemicals were purchased from Sigma Chemical Company (Oakville, ON, CAN). Trizol reagent and PCR primers were purchased from Invitrogen (Carlsbad, CA, USA). Taq DNA polymerase and M-MLV reverse transcriptase were purchased from New England Biolabs (Whitby, ON, USA). Cocktails of proteases and phosphatase inhibitors were purchased from Roche Applied Science (Quebec, PQ, Canada). The specific inhibitors of Stat1 (i.e., JAK inhibitor I) and Stat3 (i.e., Stat3 inhibitor V) were purchased from Calbiochem (San Diego, CA, USA). The rabbit polyclonal antibodies (Abs) against phospho (p) Stat1 (pY701), Stat1, pStat3 (pY705) and Stat3 were purchased from Cell Signaling Technologies (Pickering, ON, CAN). The monoclonal peroxidase-conjugated mouse anti-β-actin antibody was purchased from Sigma Chemical Company, and the horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Bio-Rad Laboratories (Mississauga, ON, CAN).

Isolation and purification of term villous cytotrophoblasts

This study was approved by the ethical committee of CHUM-St-Luc Hospital (Montreal, QC, Canada). Human term placentas (i.e., 37–41 weeks) were obtained immediately after spontaneous vaginal deliveries from uncomplicated pregnancies. Immediately after delivery, the placentas were immersed in DMEM-HG containing antibiotics (i.e., 5 μg/ml of amphotericin, 50 μg/ml of gentamycin and 0.12 mg/ml of penicillin), maintained at 4 °C and processed within 1 h. Villous cytotrophoblasts (vCTs) were isolated and purified as previously described by our group, using the trypsin-DNase/Percoll method.[39–41] Mononuclear vCTs were purified *via* immunomagnetic labelling using an autoMACS Myltenyi Biotec (Auburn, CA, USA) and an anti-HLA-ABC antibody, as described previously.[39] The purity of the immunopurified vCTs was determined *via* flow cytometry using a FITC-conjugated monoclonal antibody against cytokeratin-7. All vCT preparations used in this study were at least 98% pure after cell sorting. The vCTs were centrifuged, suspended in 37 °C culture medium, which consisted of DMEM-High Glucose medium supplemented with 2 mM *L*-glutamine, 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 5% fetal bovine serum (FBS) and a 1× penicillin-streptomycin-neomycin antibiotic mixture, and seeded at a density of 4.5×10^6 cells/well in CellBIND six-well microplates. After seeding, the cells were cultured for 12 h at 37 °C in a humidified atmosphere with 5% CO_2, the medium was removed gently, and the cells were washed twice with pre-warmed medium. Twenty-four hours later, the cells were treated with pre-warmed medium containing 50 ng/ml of human LIF and subsequently cultured for a maximum of 48 h.[39–41]

Villous cytotrophoblast-like BeWo cell differentiation

The BeWo human placental choriocarcinoma cell line (ATCC number CCL-98) was cultured in RPMI-1640 cell culture medium supplemented with 10% heat-inactivated FBS, 1 mM sodium pyruvate, 10 mM HEPES and 50 μg/ml of gentamicin (referred to as 10% FBS-RPMI medium). The human BeWo cell line is the most extensively used *in vitro* cellular model for studying villous cytotrophoblast fusion and function.[42] Undifferentiated BeWo cells are morphologically similar to primary cultures of mononuclear cytotrophoblast cells,[43] with a low spontaneous fusion rate that can be boosted upon treatment with forskolin (FK).[44] As we described previously, FK-stimulated BeWo cells also express markers of typical syncytiotrophoblast cells, such as human chorionic gonadotrophin and markedly reduced E-cadherin expression.[38] Moreover, BeWo cells express IL-10 at the mRNA and the protein levels[45,46] and a recent study demonstrated that this cell line responds very well to exogenous IL-10.[47] Thus, these similarities to trophoblast cells support the use of BeWo cells as a valid and suitable model for studying different aspects of villous cytotrophoblast differentiation and function. In this study, to induce cytotrophoblast differentiation into syncytiotrophoblasts (ST), 10 μM FK was added to BeWo cell cultures (BW/ST), as previously described.[38]

Trophoblast conditioned medium collection

To obtain control medium (CTLm), fresh 10% FBS-RPMI medium was placed in the cell incubator for 48 h. Conditioned medium (CM) was prepared from primary cultures of villous cytotrophoblasts (vCTs) and BW/ST cells that were cultured for 48 h in 5% FBS-RPMI medium in the absence (i.e., CM vCT−LIF and CM BW/ST−LIF) or the presence of 50 ng/ml of LIF (i.e., CM vCT+LIF and CM BW/ST+LIF). CTLm and CM (5 ml) were centrifuged once at 250*g* for 5 min to eliminate cells and cell debris and were then centrifuged in a centrifugal device with a molecular weight cutoff of 3 kDa (Pall Life Science; Port Washington, NY, USA) at 3200*g* for 45 min to separate proteins from low molecular weight molecules, including

residual FK and LIF. The concentrated proteins were diluted with an equal volume of 10% FBS-RPMI medium. Sterile cell-free CTLm and CM were snap-frozen, stored at −80 °C, and thawed on ice when required.

Monocyte and macrophage differentiation

Human subjects provided written informed consent for blood donation at the hemato-oncologic service of the Centre Hospitalier Régional de Trois-Rivières (Trois-Rivières, PQ, Canada), as approved by the Institutional Review Board of Centre Hospitalier Régional de Trois-Rivières. Over a period of 1 year, 35 voluntary blood donors were enrolled in this study. The mean age was 41.5±7.8 years, and the number of male donors (69%) was much higher than the number of female donors (31%). Most of the blood donors in the current study (91%) were both non-smokers and non-alcoholics. The exclusion criteria included a history of chronic immunosuppression, anti-inflammatory use and a history of hepatitis B infection. Blood monocytes were isolated as previously described.[36] Adherent mononuclear cells were recovered using Lymphocyte Separation Medium (Wisent; St-Bruno, PQ, CAN) and cultured for 5 days with 10 ng/ml of GM-CSF to induce Mϕ differentiation. On day 1, the purity of the monocyte-derived Mϕs (MDMϕ) was approximately 98%, as assessed *via* the detection of CD14 by flow cytometry.[36] The THP1 human monocytic leukemia cell line (ATCC number TIB-202) was cultured in 10% FBS-RPMI medium. The THP1 cell line is one of the most widely used cell lines for investigations of the function and differentiation of monocytes and Mϕs in response to various inflammatory mediators, such as IFN-γ and bacterial LPS.[48–50] Undifferentiated THP1 cells resemble primary monocytes/Mϕs isolated from healthy donors or donors with inflammatory diseases, such as diabetes mellitus and atherosclerosis.[50] After treatment with phorbol esters, THP1 cells differentiate into macrophage-like cells, which mimic native monocyte-derived Mϕs in several respects.[49,51] Because of these characteristics, the THP-1 cell line was broadly proposed as a valuable model for studying the regulation of Mϕ-specific genes,[51] the mechanisms involved in Mϕ differentiation[48] and the molecular mechanisms in monocytes and Mϕs that are associated with the physiology and pathophysiology of inflammatory responses.[50] In this study, green fluorescent protein (GFP)-expressing-THP1 cells were cultured for 18 h in 50 nM phorbol 12-myristate 13-acetate to induce monocyte-to-Mϕ differentiation. To investigate whether trophoblast-derived factors regulate the effects of IFN-γ on the behavior of Mϕs, MDMϕs and GFP-THP1-derived Mϕs (TDMϕs) were cultured in the absence of cytokines to obtain control cells (Mϕs) or in the presence of 50 U/ml of IFN-γ to induce a pro-inflammatory type-1 phenotype (Mϕ+IFN-γ).

Migratory/chemotactic assay

Undifferentiated GFP-THP1 cells were used to investigate whether trophoblast cells are able to regulate the chemotactic responsiveness of monocytes (Mo) to trophoblast-derived factors. The transwell migratory assay was conducted in a modified Boyden chamber with membrane inserts with an 8-μm pore size, as described previously.[36,37] Briefly, GFP-THP1 cells were cultured for 24 h in the presence or absence of 50 U/ml of IFN-γ and subsequently washed, counted, and seeded into the upper well of the chamber at a density of 50×10^3 cells/100 μl. Meanwhile, 50×10^3 BW/ST cells that were previously stimulated with a phosphate-buffered saline solution (BW/ST−LIF) or LIF (BW/ST+LIF) were seeded in the lower well. The GFP-THP1 cells that migrated down to the lower well in response to trophoblast-derived chemotactic factors were visualized using fluorescence microscopy and counted from images captured at $t=0$ h and $t=48$ h. All observations were made at ×5 magnification using cell monolayers. Five fields were selected randomly for each treatment.

Motility assay

The *in vitro* scratch wound healing assay was performed to study the effects of trophoblast-derived factors on Mϕ cell migration.[52] Briefly, MDMϕs (300×10^3 cells/ml) and TDMϕs (750×10^3 cells/ml) were seeded into 24-well tissue culture plates to achieve a monolayer with about 70%–80% confluence. The cell monolayers were scraped with a p200 pipet tip in a straight line in one direction to create a 'scratch'. To obtain the same field during image acquisition, another straight line was scratched perpendicular to the first wound line to create a cross in each well. The debris was removed, and the edge of the scratch was smoothed by washing the cells once with 1 ml of Hank's buffer. The cells were then cultured in CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence or absence of 50 U/ml of IFN-γ for the *in vitro* scratch assay. To assess the effects of exogenous IL-10, TDMϕs were pre-treated for 3 h with 25 ng/ml of IL-10 and subsequently washed and activated with 50 U/ml of IFN-γ. Using the cross as a reference point, the plate was placed under an inverted fluorescence microscope and images of the scratch were acquired at $t=0$ h and $t=48$ h. The number of motile cells was determined using the Java-based image processing program ImageJ (National Institutes of Health; Bethesda, MD, USA), and the relative cell motility was expressed as a ratio of motile cells at $t=48$ h/$t=0$ h within the initial wound. To study the impact of inhibiting IFN-γ-induced signaling pathway activation during the healing of the wound, Mϕs were plated as described above. After the scratch cross line was created, the cell monolayers were treated for 60 min with either vehicle (i.e., 0.1% DMSO) or an optimal dose of the chemical inhibitors JAK inhibitor I (JAK Inh I) (i.e., 10 μM) and Stat3 inhibitor V (Stat3 Inh V) (i.e., 5 μM) before incubation with CTLm, CM DMSO, CM FK or CM FK+LIF in the presence or absence of 50 U/ml of IFN-γ. To determine the influence of JAK Inh I and Stat3 Inh V on IFN-γ-induced signaling pathway activation, pre-treated Mϕs were stimulated for 15 min and 30 min and cell lysates were prepared and analyzed *via* immunoblotting, as described previously.[36–38]

Cell viability/proliferation assays

To assess the effects of trophoblast-derived factors on the cell viability of monocytes and Mϕs, undifferentiated GFP-THP1

cells and TDMϕ were cultured in CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence or absence of 50 U/ml of IFN-γ. Cell viability was assessed at $t=0$ h and 48 h using MTT assays, as previously described.[36–38] To evaluate the number of dead cells, the cells were washed and stained for 15 min with 2 μg/ml of PI solution prior to analysis *via* flow cytometry. To estimate the influence of BW/ST cell-derived factors on the cytotoxic activity of Mϕs, TDMϕ were cultured for 24 h with CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence or absence of 50 U/ml of IFN-γ and subsequently washed and cultured for 24 h in serum-free RPMI-1640 culture medium. Undifferentiated BeWo cells were incubated with conditioned medium from these cell cultures. MTT assays and PI staining were performed at $t=0$ h, 24 h and 48 h to evaluate the number of viable and dead cells, respectively. Each assay was performed in quadruplicate and represents three independent experiments.

Protein immunodetection

TDMϕs (750×103 cells/ml) were pre-treated for 24 h with CTLm, CM BW/ST−LIF or CM BW/ST+LIF or pre-treated for 3 h with exogenous IL-10 at concentrations of 5 and 25 ng/ml. The cells were then washed and activated with 50 U/ml of IFN-γ at t=0 min, 15 min and 30 min. Cell lysates were prepared and analyzed by immunoblotting, as described previously.[36–38] Briefly, protein samples were resolved by SDS–PAGE under reducing conditions and transferred onto a polyvinylidene fluoride (PVDF) membrane. The blots were first probed with rabbit polyclonal antibodies against pStat1 and pStat3 (both at dilutions of 1 : 2000) overnight at 4 °C. The blots were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG Ab at a dilution of 1 : 3000 for 1 h at room temperature. The same blots were stripped and then probed with anti-Stat1 and anti-Stat3 Abs, which were both used at dilutions of 1 : 1000. In both cases, the probed molecules were visualized using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific; Waltham, MA, USA).

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR) analyses

Total RNA extraction, preparation of first-strand cDNA by RT PCR were performed as previously described by our group.[36,37] The PCR reaction conditions were chosen to ensure that the amplification of mRNA occurred in the middle of the exponential amplification phase to avoid mRNA amplification close to plateau and saturation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression was used as an internal standard. The sequences of the primers used for amplification were 5′-GTCAGTGGTGGACCTGACCT-3′ (sense (S)) and 5′-TGAGCTTGACAAAGTGGTCG-3′ (antisense (AS)) for GAPDH; 5′-CAGAGGGAAGAGTTCCCCAG-3′ (S) and 5′-CCTTGGTCTGGTAGGAGACG-3′ (AS) for TNF-α; and 5′-TGAGAACCAAGACCCAGACA-3′ (S) and 5′-TCATGGCTTTGTAGATGCCT-3′ (AS) for IL-10.

Surface antigen expression analysis

To study membrane receptor expression, Mϕs were pre-treated for 24 h with CM from BW/ST cell cultures and collected after a 48-hour stimulation period in the presence or absence of 50 U/ml of IFN-γ. The expression levels of CD14 and CD40 were evaluated by flow cytometry, as described previously.[36,37]

IL-10 neutralization assays

To determine the potential involvement of trophoblast-derived IL-10 as a deactivating mediator of Mϕ behavior, we used a polyclonal antibody directed against IL-10 (#IC2172F; R&D System; Minneapolis, MN, USA) to neutralize IL-10 in trophoblast CM. Western blot analysis, MTT assays and *in vitro* scratch wound healing assays were performed as described above. Neutralizing anti-IL-10 or isotype control antibodies, which were both used at concentrations of 2 μg/ml, were added daily to Mϕ monolayers. A representative result from three independent experiments is shown.

Statistical analyses

For all experiments, the values were presented as the mean±s.d. from three independent experiments. The data were analyzed using one-way analysis of variance (ANOVA) followed by the Bonferonni post-test using Prism software, version 3.03 (GraphPad, San Diego, CA, USA). P values $\leqslant 0.05$ were considered to indicate statistical significance.

RESULTS

Villous cytotrophoblast-derived factors repress Stat1 activation but inversely sustain Stat3 activation in IFN-γ-activated Mϕs

This set of experiments was planned to investigate whether primary cultures of vCTs are responsive to LIF and whether LIF-differentiated vCTs have the ability to regulate the effects of IFN-γ on the behavior of MDMϕs. As shown in Figure 1a, the activation of the Stat3 signaling pathway was efficiently induced in vCTs; this effect was maintained at similar levels during 15 min and 30 min of LIF stimulation. Figure 1b shows the representative activation status of Stat1 and Stat3 following stimulation with IFN-γ in MDMϕs preconditioned for 24 h with CTLm, CM vCT−LIF, CM vCT+LIF and recombinant human LIF (rLIF). MDMϕs express low basal levels of pStat1 and pStat3 ($t=0$ min) when preconditioned in CTLm, in CM vCT−LIF, in CM vCT+LIF and in the presence of rLIF (Figure 1b). Very little or no variation in total Stat1 and Stat3 expression was observed, irrespective of treatment. As expected, IFN-γ activates Stat1, with pStat1 levels increasing by 4.3-fold in the presence of CTLm at $t=15$ min and by 7.6-fold at $t=30$ min (Figure 1b). These increases in pStat1 levels were efficiently reduced when MDMϕs were preconditioned with both CM vCT+LIF (1.9-fold at $t=15$ min and 2.8-fold at $t=30$ min) and rLIF (no change at $t=15$ min and 2.1-fold at $t=30$ min) but not with CM vCT−LIF (Figure 1b). In contrast to pStat1, the induction of pStat3 was significantly enhanced by IFN-γ only at $t=15$ min when MDMϕs were preconditioned with CM vCT−LIF (4-fold), CM vCT+LIF (4.5-fold) and rLIF (2.8-fold).

Villous cytotrophoblast-derived IL-10 sustains Stat3 activation in Mϕs

In this set of experiments, we investigated the direct effects of vCTs-derived factors on the induction of pStat3 in MDMϕs and

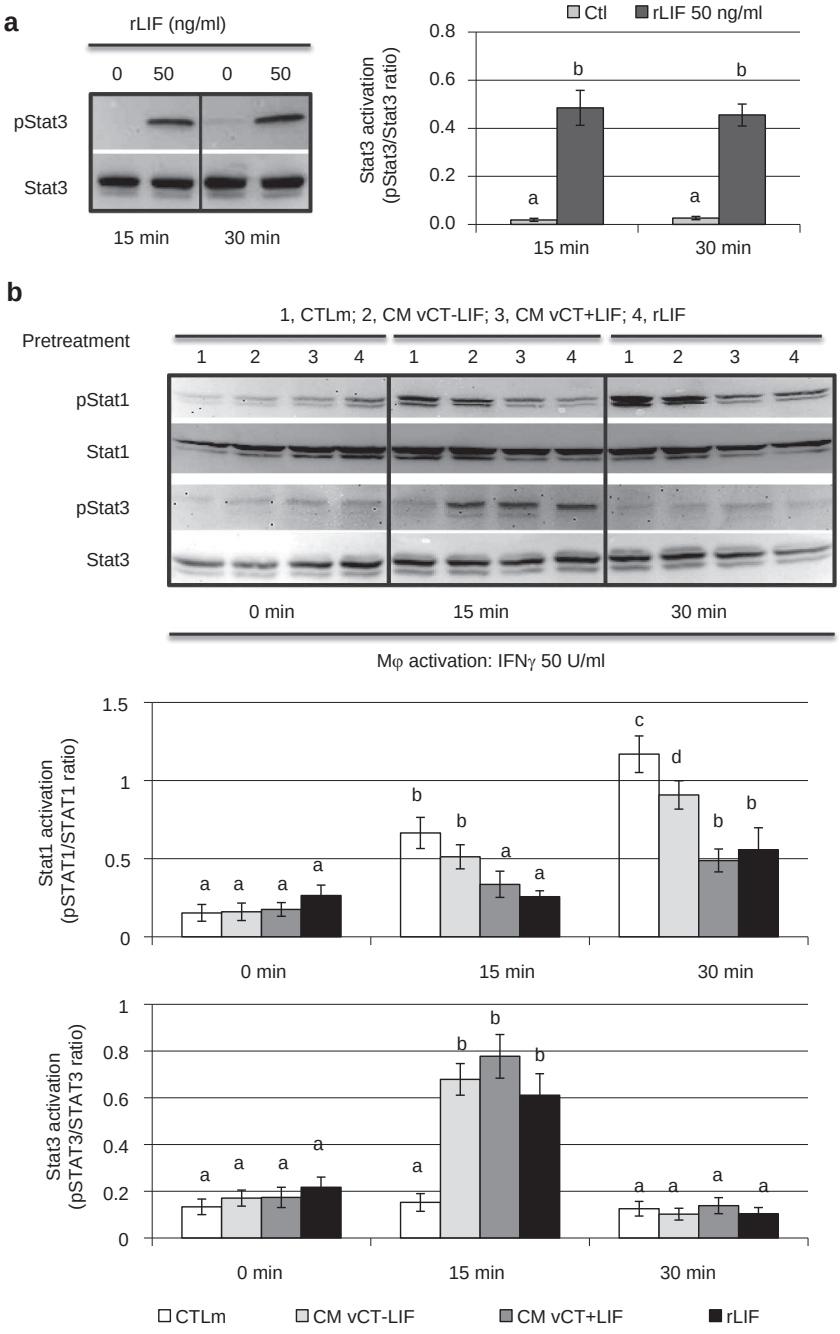

Figure 1 (**a**) Representative images and graphical analysis showing the immunodetection of phosphorylated Stat3 in primary cultures of vCTs stimulated with 50 rLIFs for 15 min and 30 min. (**b**) Representative images and graphical analysis showing the immunodetection of phosphorylated Stat1 and Stat3 in Mϕs pre-treated for 24 h with CTLm, CM vCT−LIF, CM vCT+LIF or rLIF and subsequently activated with 50 U/ml of IFN-γ for 0 min, 15 min and 30 min. (**a**, **b**) The ratio of phosphorylated/unphosphorylated proteins was calculated using densitometric analysis of each sample to evaluate the relative activation of pStat1 or pStat3. Different superscripts denote significant differences between treatments ($P<0.05$). CM, conditioned medium; CTLm, control medium; IFN-γ, interferon gamma; LIF, leukemia inhibitory factor; vCT, villous cytotrophoblast; rLIF, recombinant human LIF; Stat1, signal transducer and activator of transcription 1.

the potential involvement of IL-10 in this process. As shown in Figure 2a, Stat3 is significantly induced in MDMϕs by soluble factors present in CM vCT−LIF and to a greater extent by soluble factors present in CM vCT+LIF and exogenous IL-10. Cell signaling studies performed in the presence of neutralizing anti-IL-10 antibodies demonstrate that IL-10 is required for vCT+LIF cells to trigger enhanced Stat3 activation in MDMϕs (Figure 2a).

For the remaining studies, we opted to use a BeWo cell model due to its relevance and particularly due to its availability and

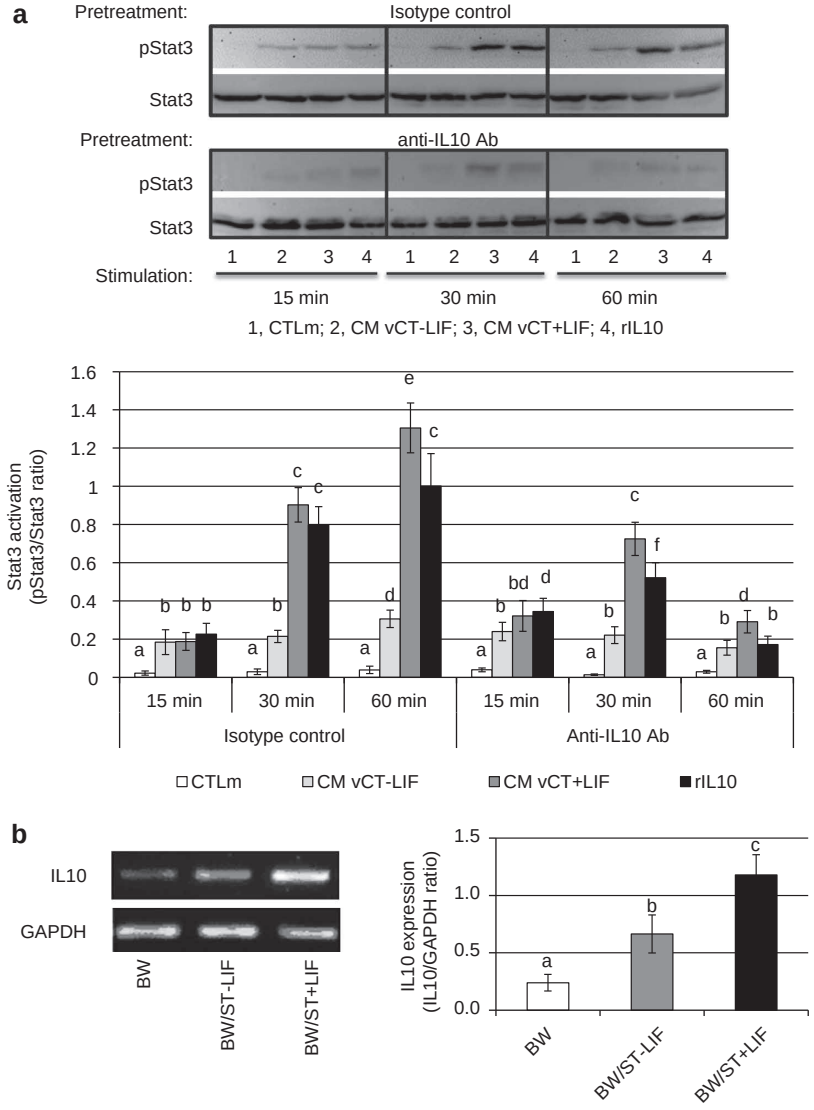

Figure 2 (**a**) Representative images and graphical analysis showing the immunodetection of phosphorylated Stat3 in Mϕs pre-treated for 15 min, 30 min and 60 min with CTLm, CM vCT−LIF, CM vCT+LIF or rIL-10 in the presence of isotype control and anti-IL-10 Abs. The ratio of phosphorylated/unphosphorylated proteins was calculated using densitometric analysis of each sample to evaluate the relative activation of pStat1 or pStat3. (**b**) Representative images and graphical analysis showing the transcription level of IL-10 mRNA in resting (BW) and differentiated BW/ST cells (±LIF), as assessed using RT-PCR. Different superscripts denote significant differences between treatments ($P<0.05$). CM, conditioned medium; CTLm, control medium; Ab, antibody; LIF, leukemia inhibitory factor; Mϕ, macrophage; RT-PCR, reverse transcriptase polymerase chain reaction; rIL-10, recombinant human IL-10; Stat1, signal transducer and activator of transcription 1.

reproducibility when used at early cell culture passages.[38] IL-10 mRNA expression was studied using RT-PCR to validate the use of BeWo cells as a pertinent model for studying the influence of villous cytotrophoblast-derived factors, such as IL-10, on the functional phenotype of pro-inflammatory Mϕs. As expected,[28,29] undifferentiated BeWo cells express basal levels of IL-10 mRNA. However, we found that the IL-10/GAPDH ratio substantially increased from 0.2±0.1 to 0.7±0.2 in BW/ST−LIF cells and to 1.2±0.2 in BW/ST+LIF cells (Figure 2b). Furthermore, cell signaling studies demonstrated that similar to soluble factors from vCT+LIF cells, soluble factors from BW/ST+LIF cells inhibit Mϕ Stat1 activation but inversely sustain Stat3 activation in response to IFN-γ (Supplementary Figure 1).

Syncytiotrophoblast-derived factors affect cell proliferation but enhance the migratory response of IFN-γ-activated monocytes/Mϕs

To determine the influence of ST-derived factors on cell proliferation, monocytes and Mϕs were incubated for 48 h in CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence of 50 U/ml IFN-γ and MTT viability/proliferation assays were then performed. As seen in Figure 3a, the rates of proliferation are considerably higher in IFN-γ-activated monocytes (closed square; relative cell viability=$0.0982t+0.05$; $R^2=1$) than in MDMϕs (closed square; relative cell viability=$0.064t+0.0383$; $R^2=1$). However, strikingly reduced rates of cellular proliferation were observed when IFN-γ-activated monocytes were cultured in

the presence of CM BW/ST−LIF (closed triangle; relative cell viability=0.0476t+0.0924; R^2=1) and to a greater extent in the presence of CM BW/ST+LIF (closed circle; relative cell viability=0.0159t+0.1276; R^2=1). Similar results were observed when IFN-γ-activated MDMϕ were cultured in the presence of CM BW/ST−LIF (closed triangle; relative cell viability=0.0341t+0.061; R^2=1) and CM BW/ST+LIF (closed circle; relative cell viability=0.0103t+0.0863; R^2=1). To explore the possibility that the observed reduction in the number of viable cells occurred as a result of cell death, monocytes and Mϕ were recovered at the end of treatment and stained with PI DNA dye and the number of dead cells was evaluated by flow cytometry. The results indicated that the effect was most likely cytostatic rather than cytotoxic because very low and similar

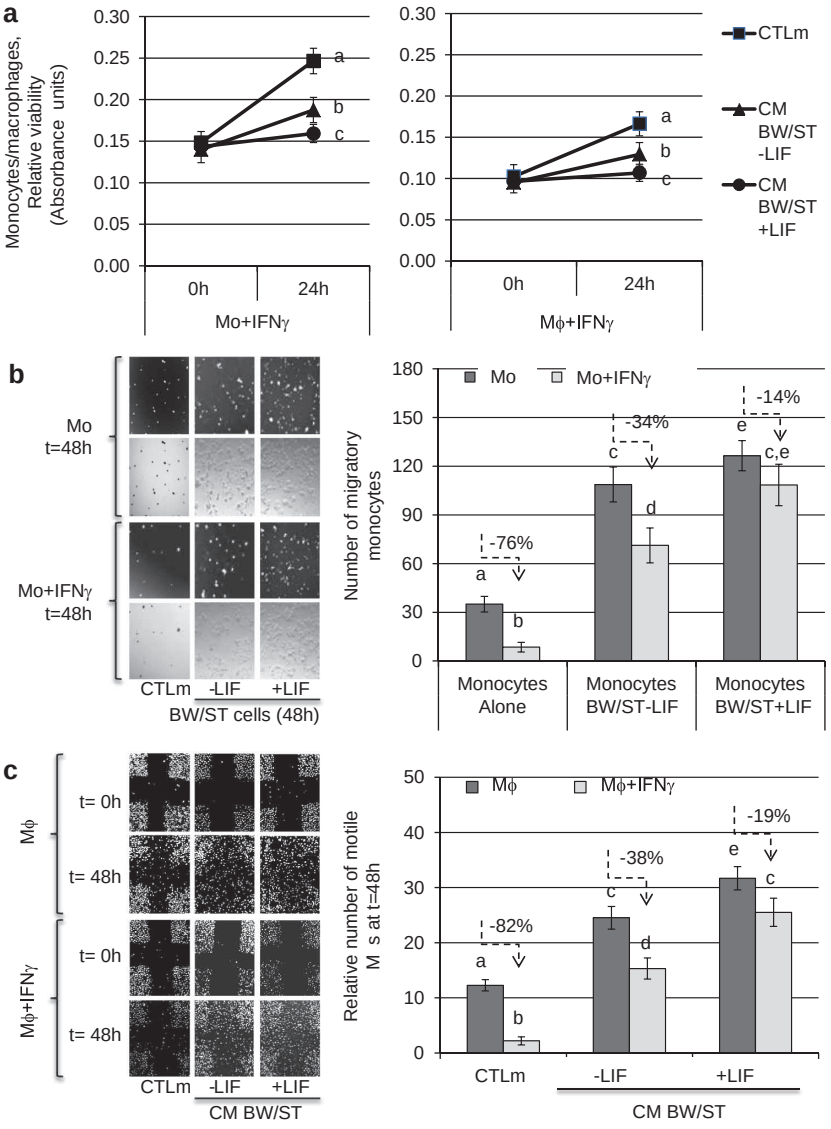

Figure 3 (**a**) The effect of soluble factors from BW/ST cells on the cell proliferation of activated monocyte/Mϕs was assessed using MTT assays. IFN-γ-activated monocytes (Mo+IFN-γ) and Mϕs (Mϕ+IFN-γ) were cultured for 48 h with CTLm, CM BW/ST−LIF or CM BW/ST+LIF. Different superscripts denote significant differences between treatments (P<0.05). CM, conditioned medium; CTLm, control medium; GFP, green fluorescent protein; IFN-γ, interferon gamma; LIF, leukemia inhibitory factor; MTT, methylthiazolyldiphenyl-tetrazolium bromide; Mϕ, macrophage. (**b**) Transwell migratory assays were performed in a modified Boyden chamber with membrane inserts. Monocytes stimulated in the absence (Mo) or presence of 50 U/ml of IFN-γ (Mo+IFN-γ) were seeded into the upper well of the chamber, and previously differentiated BW/ST cells (±LIF) were seeded into the lower well. Monocytes that migrated down to the lower well in response to trophoblast-derived chemotactic factors were visualized using fluorescence microscopy at t=0 h and t=48 h. GFP-expressing monocytes in the lower well were counted from the captured images. (**c**) Representative images and graphical analysis showing scratch wound healing assays performed in MDMϕ monolayers cultured in CTLm and in CM from BW/ST cells (±LIF). A total of 50 U/ml of IFN-γ was added to induce a pro-inflammatory type-1 phenotype (Mϕ+IFN-γ). Images of the scratch were acquired at t=0 h and t=48 h using fluorescence microscopy. (**b**, **c**) Five fields were chosen randomly for each treatment. All observations were made at ×5 magnification. The relative number of motile monocytes and Mϕs was determined and expressed as the ratio of motile cells at t=48 h/t=0 h.

levels of dead cells were found in monocytes and Mϕs cultured with CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence or absence of 50 U/ml of IFN-γ (data not shown).

In this set of migratory/chemotactic assays, undifferentiated GFP-THP1 cells were used to investigate whether BW/ST cells effectively regulate the chemotactic responsiveness of monocytes to LIF. To achieve this aim, monocytes were first cultured for 48 h in the presence and absence of IFN-γ and subsequently placed in the upper well of transwell migratory chambers. As shown in Figure 3b, the number of migratory monocytes passing to the lower well at $t=48$ h in the absence of trophoblast cells was 35±5; this number increased substantially when the cells were cultured in the presence of BW/ST−LIF cells (109±11) and increased to a greater extent when the cells were cultured with BW/ST+LIF cells (127±13). In the absence of BW/ST cells, the number of migratory monocytes decreased from 35±5 to 9±3, which represents a decrease to 24% of the control, when the cells were activated with IFN-γ (Figure 3b). In the presence of BW/ST cells in the lower well, IFN-γ inhibits monocyte migration less effectively (Figure 3b). The proportion of migratory cells activated by IFN-γ gradually increases to 66% when the cells are cocultured with BW/ST−LIF cells (71±13) and to 86% when the cells are cocultured with BW/ST+LIF cells (109±13).

A set of wound healing assays was designed to investigate whether BW/ST factors also change the relative numbers of motile cells in Mϕ cultures pre-treated with CTLm, CM BW/ST−LIF or CM BW/ST+LIF in the presence or absence of 50 U/ml of IFN-γ. IFN-γ stimulation is known to be associated with reduced chemotactic responsiveness and motility in human monocytes/Mϕs *via* the selective inhibition of the expression of CCR2, which is the receptor for the chemoattractant CCL2, which was formerly known as macrophage chemoattractant protein 1 or MCP1.[53] As expected, MDMϕs are motile in the absence of IFN-γ. However, after a 48-hour cell culture period, the cell motility of MDMϕs gradually increased in response to CM BW/ST−LIF and CM BW/ST+LIF (Figure 3c). The relative number of motile cells, which was expressed as a ratio of motile cells at $t=48$ h/$t=0$ h within the initial wound, was determined to be 12±1 with CTLm, 25±2 with CM BW/ST−LIF and 32±2 with CM BW/ST+LIF (Figure 3c). MDMϕs exhibit low motility in the presence of IFN-γ (i.e., a reduction to 18% of the control). However, the percentage of motile cells within the wound gradually increases to 62% and to 81% when the cells are cultured in the presence of factors from BW/ST−LIF and BW/ST+LIF cells, respectively (Figure 3c). Similar results were obtained with TDMϕ cultures, for which factors from BW/ST+LIF cells reversed the effects of IFN-γ on Mϕ motility most efficiently (Supplementary Figure 2).

Inhibition of Mϕ motility is mediated by IFN-γ-induced Stat1 activation

To determine whether Stat1 and Stat3 mediate the inhibitory effects of IFN-γ on Mϕ motility, TDMϕs were separately preconditioned with CTLm and CM BW/ST+LIF for 24 h and subsequently pre-treated with 0.1% DMSO, 10 μM JAK Inh I, or 5 μM Stat3 Inh V for 30 min prior to activation with 50 U/ml of IFN-γ. As shown in Figure 4a, the relative number of motile cells at $t=48$ h in CTLm was determined to be 1.1±0.3 when the cells were pre-treated with DMSO, 4.9±0.8 when the cells were pre-treated with Jak Inh I and 1.1±0.4 when the cells were pre-treated with Stat3 Inh V (Figure 4a). The results obtained using CM BW/ST+LIF demonstrated that as expected, factors from BW/ST+LIF cells promote the motility of Mϕs immobilized by IFN-γ; the relative number of motile cells at $t=48$ h was 7.6±0.9 after pre-treatment with Jak Inh I. It is worth noting that this promoting effect was significantly increased in Mϕs pre-treated with JAK Inh I but not with Stat3 Inh V, with respective ratios of motile cells of 10.7±0.5 and 2.9±0.6 (Figure 4a).

Cell signaling studies indicated that the IFN-γ-mediated arrest of cell motility is mediated by Stat1 activation, as the induction of pStat1 but not that of pStat3 was efficiently impeded by JAK Inh I in response to IFN-γ (Figure 4b). Moreover, as shown in Figure 4c, Stat3 activation is required for the maintenance of cell motility in IFN-γ-immobilized Mϕs by BW/ST+LIF factors; the Mϕs exhibited poor motility after pre-treatment with Stat3 Inh V (Figure 4a).

Syncytiotrophoblast-derived factors modulate Mϕ pro-inflammatory marker expression

This set of experiments was designed to investigate Mϕ TNF-α and IL-10 gene expression using RT-PCR and the expression of CD14 and CD40 using flow cytometry. Consistent with the pro-inflammatory role of IFN-γ, the transcript level of TNF-α was gradually upregulated in Mϕs stimulated with IFN-γ; these levels increased from 0.59±0.06 to 1.39±0.08 after 24 h of stimulation (Figure 5a, left panel). The levels of TNF-α mRNA were downregulated when the Mϕs were pre-treated with CM BW/ST+LIF and stimulated with IFN-γ (0.56±0.06 *versus* 0.39±0.04) (Figure 5a, left panel). In contrast to TNF-α, the levels of IL-10 mRNA were upregulated when the Mϕs were pre-treated with CM BW/ST+LIF; the IL-10/GAPDH ratio increased from 0.21±0.06 to 1.17±0.12 (Figure 5a, right panel). Notably, this rise in IL-10 mRNA expression was maintained even after Mϕ activation with IFN-γ, when the IL-10/GAPDH ratio increased from 0.22±0.08 to 1.00±0.10 (Figure 5a, right panel).

In response to INF-γ, Mϕ CD14 and CD40 expression were enhanced by 2-fold and 3.5-fold, respectively (Figure 5b). However, this rise in CD14 and CD40 expression in IFN-γ-activated Mϕs was blocked when the cells were preconditioned with soluble factors from BW/ST+LIF cells (Figure 5b). Thus, in response to LIF, BW/ST cells have the ability to generate IFN-γ-activated Mϕs with enhanced expression of anti-inflammatory IL-10, but reduced expression of pro-inflammatory TNF-α, CD14 and CD40.

Syncytiotrophoblast-derived IL-10 regulates pro-inflammatory Mϕ function

This set of experiments was designed to investigate the involvement of ST-derived IL-10 in the regulation of Mϕ proliferation, cytotoxicity and motility. The results from MTT assays demonstrated that the antiproliferative effect of BW/ST+LIF cells on

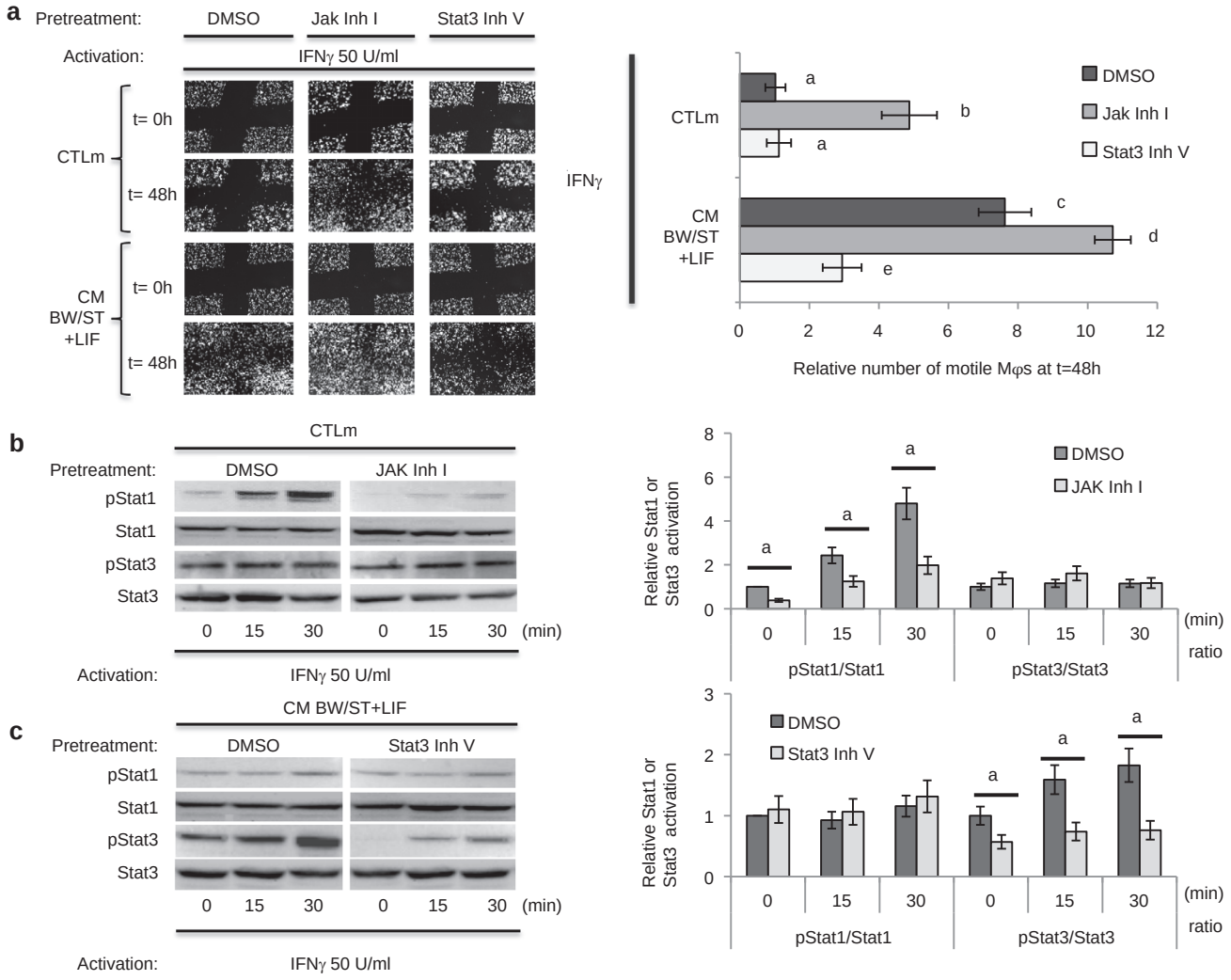

Figure 4 (**a**) Mϕs were separately preconditioned for 24 h with CTLm and CM BW/ST+LIF and subsequently pre-treated for 30 min with 0.1% DMSO, 10 μM JAK Inh I or 5 μM Stat3 Inh V prior to activation with 50 U/ml of IFN-γ. The motility of the Mϕs was evaluated using an *in vitro* scratch assay and visualized using a fluorescence microscope at $t=0$ h and $t=48$ h. Five fields were chosen randomly for each treatment. All observations were made at ×5 magnification. The results are presented as the mean±s.d. from three independent experiments. Different superscripts denote significant differences between treatments ($P<0.05$). (**b**, **c**) Mϕs were preconditioned for 24 h with CTLm or CM BW/ST+LIF and subsequently pre-treated with 0.1% DMSO, 10 μM Jak Inh I or 5 μM Stat3 Inh prior to incubation with 0 and 50 U/ml of IFN-γ for the indicated times. The activation status of Stat1 and Stat3 was assessed using immunoblotting studies. The superscript '*a*' denotes significant differences between DMSO and inhibitor treatments ($P<0.05$). CM, conditioned medium; CTLm, control medium; IFN-γ, interferon gamma; LIF, leukemia inhibitory factor; Mϕ, macrophage; Stat1, signal transducer and activator of transcription 1.

Mϕs was reduced when the cells were preconditioned with neutralizing anti-IL-10 Abs. The number of IFN-γ-activated Mϕs was reduced by 38% in the presence of isotype Abs and by 15% in the presence of anti-IL-10 Abs (Figure 6a). To further confirm that LIF acts on ST/BW cells to produce IL-10 and counteract the activating effects of IFN-γ, Mϕ-mediated cytotoxicity against undifferentiated BeWo cells was evaluated in the presence of neutralizing anti-IL-10 Abs. As shown in Figure 6b, soluble factors from IFN-γ-activated Mϕs preconditioned with CTLm completely blocked the proliferation of BeWo cells. However, the cytotoxic activity of IFN-γ-activated Mϕs against BeWo cells was efficiently neutralized when the Mϕs were preconditioned with CM BW/ST+LIF. Further, Mϕ-mediated cytotoxicity was restored in the presence of neutralizing anti-IL-10 Abs (Figure 6b). Moreover, we found that IL-10 is required for BW/ST+LIF cells to trigger sustained motility in IFN-γ-activated Mϕs (Figure 6c).

Exogenous IL-10 regulates cell motility in IFN-γ-activated Mϕs

Scratch assays were performed to further demonstrate the deactivating effects of IL-10 on IFN-γ-activated Mϕs. We found that pre-treatment for 3 h with exogenous IL-10 significantly enhanced Mϕ motility; the ratio of motile cells increased

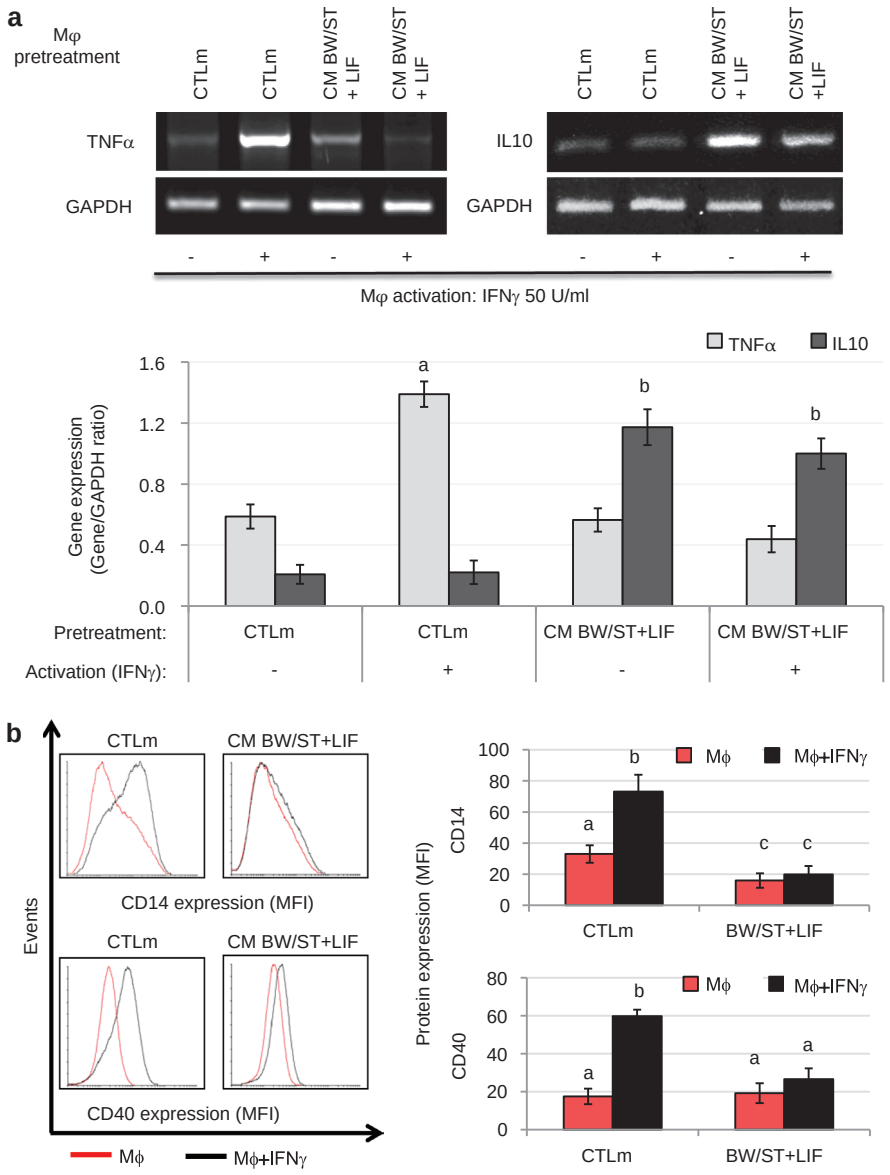

Figure 5 Representative images and graphical analysis showing the expression levels of TNF-α and IL-10 (**a**) and CD14 and CD40 (**b**) in resting and IFN-γ-activated Mϕs pre-treated with CTLm and CM BW/ST+LIF. The Mϕs were pre-incubated with CTLm and CM for 24 h and then activated for 16 h with 50 U/ml of IFN-γ to evaluate mRNA and protein expression using RT-PCR and flow cytometry, respectively. (**a**) The superscript '*a*' denotes significant differences in TNF-α mRNA expression between CTLm−IFN-γ and CTLm+IFN-γ ($P<0.05$). The superscript '*b*' denotes significant differences in IL-10 mRNA expression between CM BW/ST+LIF and CTLm ($P<0.05$). (**b**) Different superscripts denote significant differences between treatments ($P<0.05$). CM, conditioned medium; CTLm, control medium; IFN-γ, interferon gamma; LIF, leukemia inhibitory factor; Mϕ, macrophage; RT-PCR, reverse transcriptase polymerase chain reaction; TNF, tumor-necrosis factor.

by up to 38% compared to phosphate-buffered saline pre-treatment (Figure 7a). However, as observed with BW/ST+LIF factors (Figure 3c), the inhibitory effect of IFN-γ on Mϕ motility was significantly blocked when the Mϕs were pre-treated with IL-10; the ratio of motile cells increased by up to 78% compared to phosphate-buffered saline pre-treatment (Figure 7a). The differential influence of IL-10 on Mϕ proliferation and motility may result from the ability of IL-10 to suppress IFN-γ-induced Stat1 activation while Stat3 activation is maintained (Figure 7b).

DISCUSSION

Although IFN-γ has been defined as an important gestational factor and a potent inducer of the pro-inflammatory type-1 phenotype in Mϕs,[15] this cytokine exhibits both beneficial and harmful attributes in the context of the developing embryo.[54] The present study demonstrates that multinucleated trophoblast cells efficiently deactivate pro-inflammatory Mϕ-1. For instance, Mϕ proliferation, inflammatory gene/protein expression, and cytotoxicity were significantly reduced when IFN-γ-activated Mϕs were preconditioned with soluble factors

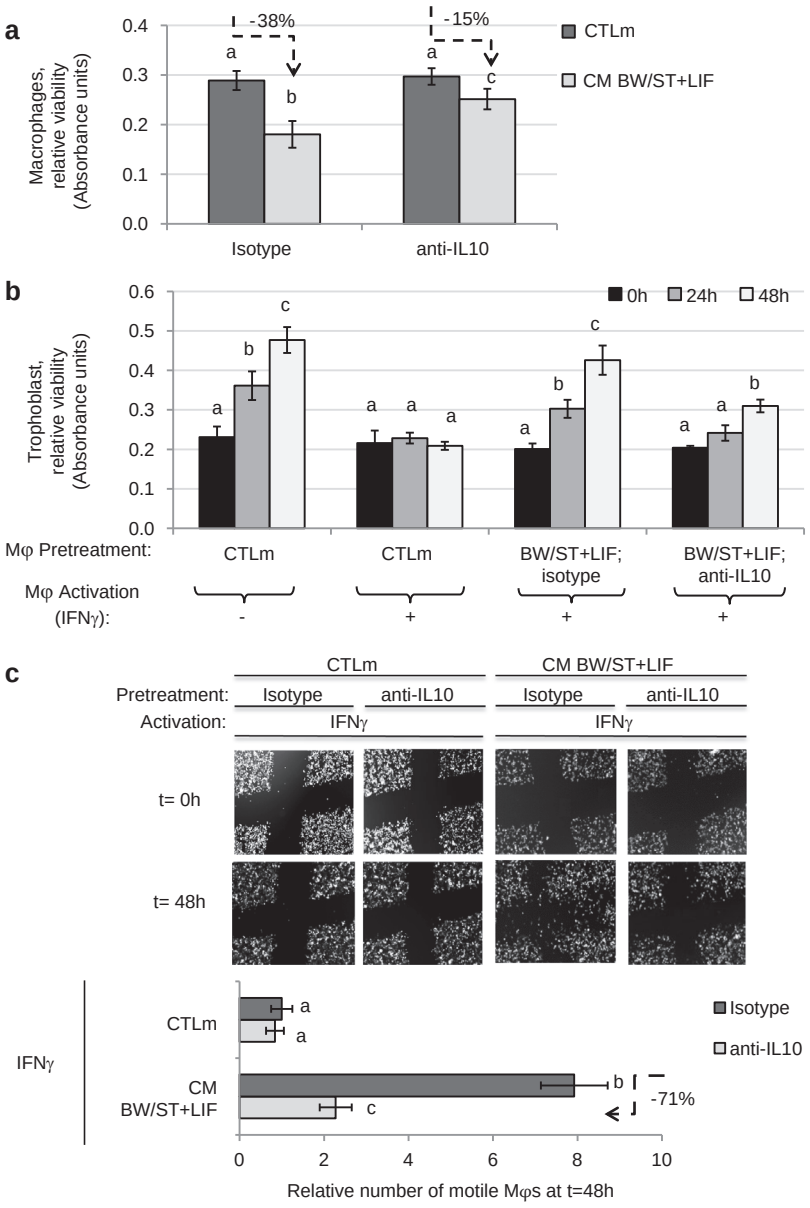

Figure 6 Representative images and graphical analysis showing the involvement of LIF-differentiated BW/ST cells and trophoblast-derived IL-10 in the regulation of Mϕ proliferation (**a**), cytotoxicity (**b**) and motility (**c**). (**a**) MTT assays were performed to determine the cell survival/proliferation of IFN-γ-activated Mϕs incubated for 48 h with CTLm and CM BW/ST+LIF in the presence of isotype control and anti-IL-10 antibodies. (**b**) MTT assays were performed to assess the cell survival/proliferation of undifferentiated BeWo cells incubated with CM from IFN-γ-activated Mϕs that were pre-treated for 24 h with CTLm and CM BW/ST+LIF in the presence of isotype control and anti-IL-10 antibodies. (**c**) Scratch wound healing assays were performed to evaluate the cell motility of IFN-γ-activated Mϕs that were incubated for 24 h with CTLm and CM BW/ST+LIF in the presence of isotype control and anti-IL-10 antibodies. Cell motility was visualized using a fluorescence microscope at t=0 h and t=48 h. Different superscripts denote significant differences between treatments (P<0.05). CM, conditioned medium; CTLm, control medium; IFN-γ, interferon gamma; LIF, leukemia inhibitory factor; MTT, methylthiazolyldiphenyl-tetrazolium bromide; Mϕ, macrophage.

from syncytiotrophoblast cells. In line with our results suggesting the involvement of IL-10 in this process, our previous studies demonstrated that IL-10 and IL-10-producing Mϕ-2 cells inhibit Mϕ-1 cytotoxic activity,[36] likely by blocking the endogenous production of TNF-α, which is the most important microbicidal and tumoricidal mediator produced by IFN-γ-activated Mϕs.[55] Several lines of evidence suggest that IL-10 is an important suppressor of active maternal immunity that may be involved in the process of acceptance of the fetal allograft.[19,20,28,29] IL-10 levels increase markedly in women during early pregnancy and remain elevated up to the third trimester immediately prior to the onset of labor. Of note, compared to syncytiotrophoblasts, extravillous cytotrophoblasts are intrinsically poor in IL-10 production,[28,29] indicating that the syncytium might indeed contribute locally to counteract the deleterious effects of pro-inflammatory cytokines during early pregnancy.

Uterine CD14$^+$ Mϕs are the most important leukocyte population in the pregnant uterus of humans and mice. These cells have potential inflammatory and antigen-presenting cell functions that could be detrimental to the fetus if the cells were over-activated in response to infection.[7,8,13,14,17,22] CD14 is part of a cognate receptor complex, which includes TLR4 and myeloid differentiation factor 2 and is involved in Mϕ detection of Gram-negative bacteria *via* binding to the outer-wall component LPS.[56] IFN-γ-primed Mϕs produced higher levels of pro-inflammatory cytokines and chemokines after stimulation with LPS *via* the upregulation of CD14, TLR4 and myeloid differentiation factor 2 expression.[57] The physiological importance of the downregulation of the expression of the LPS receptor CD14 by syncytiotrophoblast cells becomes apparent in the context of recent studies that demonstrated that LPS may provide the danger signal through which IFN-γ-mediated stress triggers maternal immune activation. This activation would subsequently cause fetal rejection in the abortion-prone CBA/J×DBA/2J mouse model. This interpretation is consistent with results demonstrating that blocking receptors for LPS or neutralizing LPS abrogates the loss of embryos in these pregnant mice.[58]

CD40 is a costimulatory immune receptor that is a member of the TNF receptor superfamily.[59,60] CD40 expression and activation on antigen-presenting cells, including Mϕs and DCs, is crucial for Th1-cell mediated immune responses.[61]

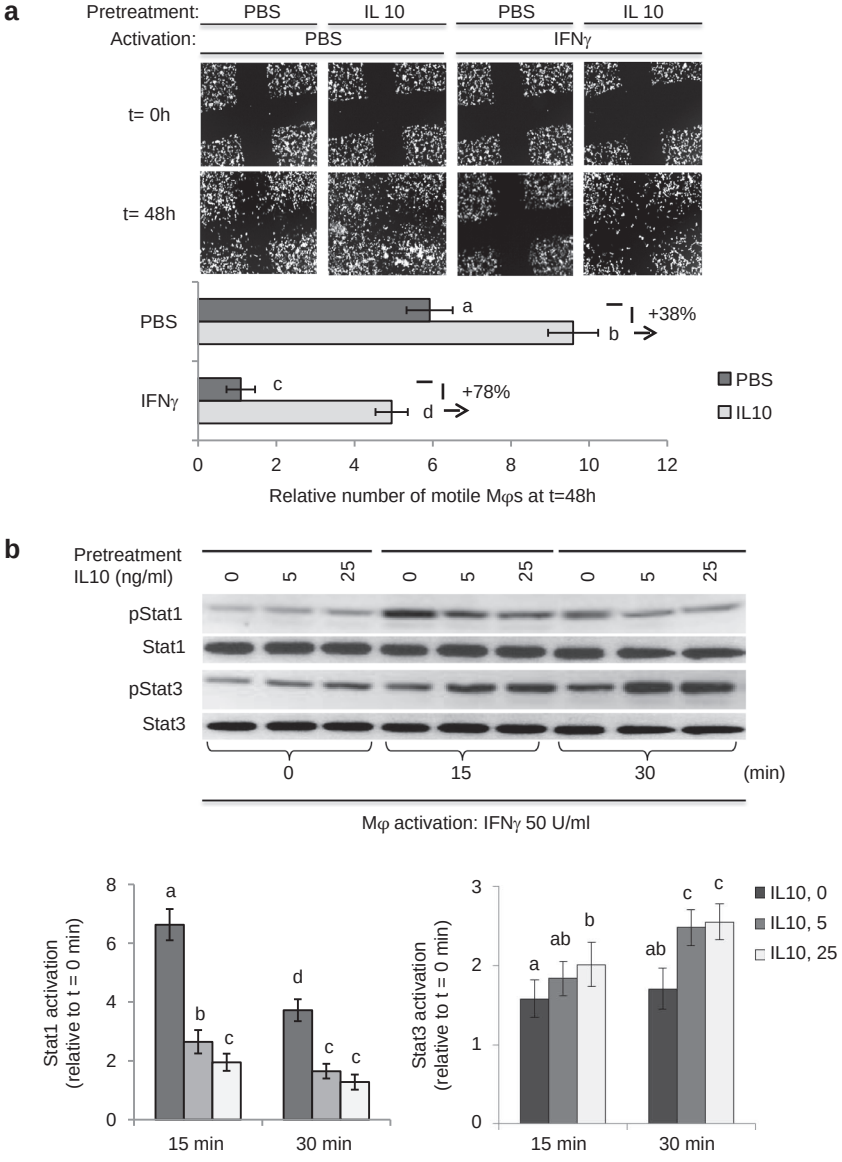

Figure 7 (**a**) Representative images and graphical analysis of scratch wound healing assays showing the involvement of IL-10 in the regulation of cell motility in resting and IFN-γ-activated Mϕs. Images of the scratch were acquired at t=0 h and t=48 h *via* fluorescence microscopy. (**b**) Representative images showing the activation status of Stat1 and Stat3. The Mϕs were pre-treated for 3 h with 5 ng/ml and 25 ng/ml of IL-10 and subsequently activated with 50 U/ml of IFN-γ for 0 min, 15 min or 30 min. Different superscripts denote significant differences between treatments (P<0.05). IFN-γ, interferon gamma; Mϕ, macrophage; Stat1, signal transducer and activator of transcription 1.

IFN-γ is the most potent inducer of CD40 expression in Mϕs,[62] and as we established previously, this induction is associated with enhanced cytotoxic function that triggers robust Mϕ-mediated destruction of tumor cells.[37] Thus, as we suggested for TNF-α and CD14, the repression of Mϕ CD40 expression by syncytiotrophoblast cells could be viewed as a mechanism that leads to reduced cytotoxic and costimulatory functions of IFN-γ-activated Mϕs.

Indeed, recent studies suggested that human decidual Mϕs could represent a regulatory or suppressive type of tissue Mϕs that contribute to the development of appropriate maternal immune responses to the fetus during early pregnancy. Decidual Mϕs spontaneously produced high levels of IL-10.[9–11] IL-10-producing decidual Mϕs expressed CD14 and HLA-DR, but these cells exhibited lower levels of the costimulatory molecules CD40 and CD86 than peripheral blood monocytes from pregnant and non-pregnant women.[11] Furthermore, decidual Mϕs fail to differentiate into dendritic cells under the influence of IL-4 and GM-CSF.[11] Sayama *et al.*[63] recently demonstrated that decidual Mϕs are able to suppress T-cell IFN-γ production. This suppression occurs *via* B7-H1, which is a costimulatory ligand in the B7 family that negatively modulates T-cell activity by binding to the corresponding receptor PD-1. The Fisher's group proposed that decidual Mϕs can also protect fetal cells (i.e., trophoblast cells) against the death-inducing activity of IFN-γ-producing uterine NK cells through a transforming growth factor beta 1-dependent mechanism.[64]

IFN-γ-mediated activation of gene transcription occurs primarily through the transcription factor Stat1.[15] Once Stat1 is phosphorylated by Jak1 and Jak2, Stat1 homodimerizes, translocates to the nucleus, and activates the transcription of multiple genes that contain an IFN-γ-activating sequence in their promoters.[65] As reported here, IFN-γ signaling in Mϕs involves Stat1 activation, and syncytiotrophoblast-derived factors, such as IL-10, have the ability to sustain Stat3 activation but inversely repress Stat1 activation in response to IFN-γ stimulation. Our results strongly suggest that these inverse effects on Stat1 and Stat3 represent a molecular mechanism that could antagonize the inhibitory effects of IFN-γ on Mϕ motility *via* syncytiotrophoblast-derived factors and IL-10. In accordance with our premise, previous studies demonstrated that Stat1 and Stat1-induced gene products mediate the inhibitory effects of IFN-γ on Mϕ migration and that this inhibitory effect is completely abrogated in Stat1-deficient Mϕs.[66] Moreover, these studies demonstrated that Stat3 is required to sustain Mϕ motility. However, Stat3 does not oppose the effects of IFN-γ because this cytokine can still suppress THP1 cell migration, even if the THP1 cells express high levels of activated Stat3.[66] IL-10 can directly inhibit Stat-dependent gene expression induced by IFN-γ in Mϕs by suppressing the tyrosine phosphorylation of Stat1.[34] Furthermore, similar to IL-10, other immunosuppressive cytokines and growth factors, such as transforming growth factor beta 1 and peroxisome proliferator-activated receptor gamma ligands, are also known to promote Mϕ deactivation *via* the suppression of IFN-γ-induced Stat1 phosphorylation and activation.[67,68]

In addition to its effect on Mϕ motility, we also demonstrated that IL-10 mediates the inhibitory action of syncytiotrophoblast cells on Mϕ proliferation. These data are consistent with a previous study that demonstrated that IL-10 inhibits Mϕ proliferation and that this antiproliferative signal was not associated with enhanced apoptosis.[33] Notably, based on the fact that Stat3 is the primary mediator of the effects of IL-10, the authors also demonstrated that the suppression of Mϕ proliferation is a Stat3-dependent event; in contrast, Mϕ deactivation could be mediated *via* a Stat3-independent pathway.[33]

The finding that monocytes and Mϕs are poorly motile in the presence of IFN-γ is in accordance with previous studies that demonstrated that IFN-γ stimulation is associated with reduced chemotactic responsiveness and motility in human monocytes/Mϕs *via* the selective and rapid inhibition of CCR2 expression.[53] For instance, CCR2 is the receptor for CCL2, which is a chemotactic chemokine that is essential for the trafficking and recruitment of monocytes/Mϕs to the decidua.[8,69] The inhibition of CCR2 expression by pro-inflammatory agents such as IFN-γ has been proposed to retain Mϕs at sites of inflammation and to act as a negative feedback loop for monocyte recruitment from the blood.[70] However, because the number of uterine Mϕs remains relatively constant throughout gestation, circulating blood monocytes are expected to be continuously recruited to the decidua by both stromal and trophoblast cells in response to diverse chemotactic factors.[8,69] Moreover, Mϕs are expected to be motile within both the decidua and the fetal membranes, as they express higher levels of genes that promote the activation of migration.[71] One of these genes encodes the nuclear repressor protein glucocorticoid receptor DNA binding factor-1 that antagonizes the inhibitory effect of transforming growth factor beta 1 on monocyte migration.[72] In this context, our results suggest that trophoblast cells could favor the movement of monocytes and Mϕs *via* a similar mechanism, antagonizing the potential blocking effects of IFN-γ on monocyte/Mϕ motility.

In summary, we demonstrate that trophoblast-derived factors, such as IL-10, can induce the differentiation of Mϕs into a trophoblast-supporting phenotype by controlling Mϕ migration/motility and the magnitude of the Mϕ-mediated immune responses that are induced by pro-inflammatory cytokines, particularly IFN-γ and TNF-α. Overall, our study supports the theory that immune-modulating molecules expressed by trophoblast cells are key players in the regulation of Mϕ inflammatory activity and in the promotion of a regulatory/suppressive phenotype in Mϕs.[14]

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Supplementary Information accompanies the paper on *Cellular & Molecular Immunology*'s website. (http://www.nature.com/cmi).

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