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DR DANIEL ESTEBAN PAPARINI (Orcid ID : 0000-0002-3335-4564)

MS FATIMA MERECH (Orcid ID : 0000-0003-4107-9918)

PROFESSOR ROSANNA RAMHORST (Orcid ID : 0000-0003-4222-817X)

Article type : Regular Paper

Decidual factors and vasoactive intestinal peptide guide monocytes to higher migration, efferocytosis and wound healing in term human pregnancy

Daniel E. Paparini[1,*]; Esteban N. Grasso[1,2,*]; Laura del C. Fernandez[1]; Fátima Merech[1]; Rodrigo Weingrill-Barbano[2]; Simone Correa-Silva[2]; Gustavo Izbizky[3]; José I. Abasolo[3]; Vanesa Hauk[1]; Rosanna Ramhorst[1]; Estela Bevilaqcua[2], Claudia Pérez Leirós[1]

* Equal contribution

1 Department of Biological Chemistry, School of Sciences, University of Buenos Aires. IQUIBICEN-CONICET. Ciudad Universitaria, Pab. 2, (1428) Buenos Aires, Argentina.

2 Institute of Biomedical Sciences, Department of Cell and Developmental Biology, University of São Paulo, São Paulo, SP, Brazil

3 Obstetric Service, Hospital Italiano, Buenos Aires, Argentina

Corresponding author:

Prof. Claudia Pérez Leirós

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/apha.13579

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Laboratory of Immunopharmacology. Department of Biological Chemistry, School of Sciences, University of Buenos Aires. IQUIBICEN-CONICET, Ciudad Universitaria, Pab. 2, (1428) Buenos Aires, Argentina.
Tel/fax: 5411-4576-3342
Email: cpleiros@qb.fcen.uba.ar

Running Title: Monocyte shaping at term human pregnancy

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Abstract

Aim: To explore the functional profile of circulating monocytes and decidual macrophages at term human pregnancy and their contribution to tissue repair upon stimulation ex vivo with decidual factors and the vasoactive intestinal peptide (VIP).

Methods: Peripheral blood monocytes were isolated from pregnant and non-pregnant volunteers and tested *in vitro* with decidual explants from term placenta and VIP. The effect of VIP on decidual explants and the effect of its conditioned media on monocytes or decidual macrophages isolated by magnetic beads was carried out by RT-qPCR and ELISA for cytokines expression and release. Migration assays were performed in transwell systems. Efferocytosis was assessed in monocytes or decidual macrophages with CFSE-labelled autologous apoptotic neutrophils and quantified by flow cytometry. Monocyte and decidual macrophages wound healing capacity was evaluated using human endometrial stromal cell monolayers. Immunohistochemistry was performed in serial tissue sections of different placentas.

Results: VIP is expressed in the villi as well as in trophoblast giant cells distributed within the decidua of term placenta. VIP induced the expression of antiinflmammatory markers and monocyte chemoattractant CCL2 and CCL3 in decidual tissues. Monocytes presented higher migration towards decidual explants than CD4 and CD8 cells. VIP-conditioned monocytes displayed an enhanced efferocytosis and wound healing capacity comparable to that of decidual macrophages. Moreover, limited efferocytosis of pregnant women monocytes was restored by VIP-induced decidual factors.

Conclusion: Results show the conditioning of monocytes by decidual factors and VIP to sustain processes required for tissue repair and homeostasis maintenance in term placenta.

Keywords: Macrophages, Repair profile, Pregnancy

Introduction

Decidual macrophages display varying roles throughout pregnancy. They actively participate in vascular transformation at early stages and they restore tissue homeostasis after the acute inflammatory response that characterizes embryo implantation [1–3]. Monocytes are highly recruited towards the maternal-fetal interface from the beginning of pregnancy where they differentiate to decidual macrophages (dMA) under the control of extravillous trophoblast cells [2]. Among several factors including chemokines, hormones and growth factors synthesized by decidual cells that promote monocyte/macrophage recruitment and shaping, the vasoactive intestinal peptide (VIP)

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has been characterized [4,5]. VIP expression in syncyciotrophoblast of first and third trimester placentas was formerly reported[6] and it was shown to induce progesterone release in placental explants and BeWo trophoblast cell line. VIP has been assigned a regulatory role in early human pregnancy through targeting trophoblast, decidual cells and macrophages [4,5], neutrophils [7,8] and Treg cells [9]. VIP modulated trophoblast cell function exhibiting a promigratory and pro-invasive effect that depended on specific glucose uptake and mTOR activation in human cytotrophoblast cell lines in vitro [10,11]. Trophoblastic VIP enhanced apoptotic cell clearance by monocytes with increased production of IL-10 [5,7]. Moreover, VIP silencing in trophoblast cells prevented the conditioning of monocytes which showed reduced phagocytosis of apoptotic cells and IL-10 protein expression [10]. In human first trimester placenta, recent evidence indicates that VIP is expressed and released by invasive extravillous trophoblast cells [12]. VIP localizes in the vascular lumen trophoblast cells in 5-9 weeks human placenta and it favors trophoblast outgrowth. At early stages, VIP shaped the functional profile of NK cells and dMA isolated from first trimester placental explants. Particularly dMA were prompted to a predominant M2 anti-inflammatory profile by VIP treatment with an increased IL-10 production without modifying IL-12 levels [12]. Interestingly, VIP also induced MMP2 expression in dMA from first trimester placenta suggesting a possible contribution to sustaining trophoblast invasion and tissue remodeling at early stages [12]. Although macrophages are found at a constant 20% of immune cells at the decidua until term, little is known about monocyte recruitment and profiling at term human placenta. In fact, increased pro-inflammatory marker expression is observed in the placenta during the last weeks of pregnancy thus preparing the uterus for delivery [13–17]. This response is paralleled by tissue repairing and anti-inflammatory signals that maintain tissue homeostasis and sustain uterus remodelling after delivery [18,19]. Decidual macrophages have a central role in these processes through apoptotic cell clearance and wound healing followed by the release of anti-inflammatory cytokines [20–22]. CD36 is a scavenger receptor required for apoptotic cell clearance by macrophages in cooperation with thrombospondin, phosphatidylserine receptor and the vitronectin receptor (alpha v beta 3)[23,24].

Here we propose that decidual factors and VIP modulate the immune microenvironment in term human placenta. To test this hypothesis, we used human decidual explants and peripheral blood samples in different *in vitro* and *ex vivo* models. Results indicate that VIP is expressed in extravillous trophoblast giant cells fairly distributed in the decidua of term placenta as well as in villous trophoblast cells. VIP induced the expression of anti-inflammatory markers and monocyte chemoattractant CCL2 and CCL3 in decidual tissues. Peripheral blood monocytes presented higher migration towards decidual explants than CD4 and CD8 cells along with an enhanced efferocytosis and wound healing capacity after conditioned with decidual factors and VIP.

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Comparative functional profiles of term pregnant vs. non-pregnant monocytes and decidual macrophages isolated from term placenta are also shown.

Results

VIP localization in human term placenta

Figure 1A shows that VIP is localized to syncytiotrophoblast and cytotrophoblast cells as well as the decidual stroma and fetal vascular tissue within villi (left panel). The right panel microphotographs show VIP expression in the decidua in more detail. The strongest immunostaining for VIP was revealed in cytokeratin-7 ($CK\text{-}7^{+}$) / vimentin (VIM^{-}) cells fairly distributed in the decidual stroma, morphologically consistent with trophoblast giant cells. This result suggest that the trophoblast cells are the main source of locally produced VIP in the placenta. Other stromal cells scarcely localized in the decidua also show a faint labelling.

VIP modulation of immune markers in decidual explants

Next, we investigated the effect of VIP on decidual markers associated with monocyte profiling. We isolated decidua from term placenta explants and incubated the tissue for 20 h with 100 nM VIP, a concentration formerly shown to activate trophoblast cell function in first trimester placental explants [12]. As shown in Figure 1B, decidua of normal gestations at term expresses a relatively higher level of monocyte chemoattractant cytokines CCL2 and CXCL8 than pro-inflammatory cytokines IL-1β and IL-6. The effect of VIP on these markers is shown in Figure 1C. VIP significantly increased CCL2 along with two anti-inflammatory factors IL-10 and IDO whereas it reduced IL-1β. CXCL8, TGF-β and IL-6 did not vary with VIP treatment. It is noteworthy that CCL3, another monocyte recruitment chemokine, was not detected in the decidua in basal conditions, but its expression was induced after VIP treatment (not shown).

Preferential migration of monocytes by decidual factors and VIP

Based on the induction of monocyte chemokine expression in term decidua by VIP, we next analysed the specific migration of peripheral blood mononuclear cells (PBMCs) from non-pregnant volunteers, particularly monocytes ($CD14^{+}$), $CD4^{+}$ and $CD8^{+}$ lymphocytes towards decidual conditioned media. Compared to positive control medium (DMEM:F12 10% FCS), conditioned media of term decidual explants increased the migration of lymphocytes and monocytes. A 3.7-fold increased migration of monocytes towards decidual media vs. basal migration was observed compared to less than 1-fold increase for CD4 and CD8 cells migrated vs. their basal conditions respectively (Figure 2A). Interestingly, monocytes were by far the most recruited population by decidual media, with over 4 times the total amount of CD14+ vs. CD4 and over 10 times CD14+ vs. CD8 cells. Of note, peripheral blood mononuclear samples seeded on the upper side of the Transwell had a 1:9 CD14:T lymphocyte ratio pointing to the specific monocyte *vs.* lymphocyte chemoattraction. The migration of monocytes towards decidual factors

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was further induced in decidua pre-treated with VIP (Figure 2B). In contrast, VIP-treated decidual explants significantly inhibited CD8 lymphocyte migration and showed no effect on CD4 cells (Figure 2B).

Efferocytosis in pregnant *vs.* non-pregnant monocytes

Decidual macrophages have a central role in the maintenance of tissue homeostasis and for sustaining uterus remodelling after delivery through apoptotic cell clearance and wound healing, followed by the release of anti-inflammatory cytokines [20–22]. Considering that monocytes were highly recruited to term decidua and the synergistic effect of VIP, we next explored whether VIP and decidual factors induce apoptotic cell phagocytosis in peripheral blood monocytes from either non-pregnant or pregnant women. First, a significantly lower efferocytosis rate of monocytes from pregnant (P) women compared to non-pregnant (NON-P) donors was observed (Figure 3A). When monocytes were exposed to VIP alone no-changes in efferocytosis were found either in pregnant or non-pregnant donors. However, an enhanced efferocytosis was evident in monocytes that had been conditioned with decidual explants (D) in the presence of VIP. This effect was more pronounced in the pregnant donors (Figure 3B and 3C). Since CD36 is a membrane recognition molecule involved in apoptotic cell clearance, its potential role in pregnant and non-pregnant monocytes' efferocytosis was next investigated. In line with the results obtained in efferocytosis, a higher frequency of CD36 positive cells was induced by decidual factors in non-pregnant monocytes whereas in the pregnant women monocytes with lower apoptotic clearance capacity this effect was not evident (Figure 3D).

Wound healing of stromal cells by pregnant vs. non-pregnant monocytes

Monocytes from pregnant and non-pregnant donors were tested for their wound healing capacity. Stromal endometrial human cell line (HESC) monolayers were used to assess the wound healing effect of monocytes conditioned with VIP, decidual explants' factors or decidual factors from explants pre-treated with VIP in an experimental design similar to assays described above. As shown in Figure 4B, monocytes from pregnant women have a higher wound healing capacity compared to non-pregnant. The limited wound healing observed with non-pregnant monocytes could be boosted by all the three stimuli: VIP, decidual explants (D) and decidual explants pre-treated with VIP (Figure 4C). The wound healing capacity of P monocytes remained elevated without significant changes except with D (VIP) stimulation that increased even further the wound healing capacity (Figure 4D). It should be noted that VIP by itself does not increase wound healing of endometrial stromal cells and in fact, it is reduced. (Supplementary Figure 1).

VIP on decidual macrophages profile and function

Monocytes recruited to the term placenta differentiate to decidual macrophages. We have reported that VIP-conditioning of dMA isolated from 5-9 weeks' placenta induces anti-

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inflammatory and pro-invasive markers [12], both signals required at the onset of placentation. Tissue repair and homeostasis control are required at delivery, so we next characterized the functional profile of dMA isolated from term placenta with special focus on cytokines released, efferocytosis and wound healing and the effect of VIP. As shown in Figure 5A, dMA produced and secreted TGF-β and low levels of TNF-α and IL-1β. VIP (100 nM) increased TGF-β release but did not affect the production of TNF-α nor IL-1β (Figure 5A). Then, we decided to pair monocytes from peripheral blood and dMA of the same patient for efferocytosis and wound healing assays. Thus, in autologous sampling, dMA presented a higher rate of apoptotic cell phagocytosis compared with monocytes from the same pregnant women (Figure 5B). VIP did further increase efferocytosis in dMA. As shown above in Figure 4C, the limited extent of efferocytosis capacity of monocytes from pregnant women could be restored by decidual explants conditioned with VIP reaching efferocytosis rates like dMA (Figure 5B). Wound healing assays revealed that dMA display a high tissue repair capacity, comparable to the high capacity shown for monocytes from the same pregnant woman. VIP was ineffective in boosting this response on dMA but when P-Mo were pre-treated with D (VIP) conditioned media, there was an increment in the wound healing capacity.

Discussion

Functional plasticity characterizes monocyte-derived macrophages in the pregnant uterus from implantation to term. In normal pregnancies, they actively participate in trophoblast invasion and vascular transformation at early stages, whereas they sustain wound repair and restore tissue homeostasis during placentation and until term [25]. Due to their critical role in normal and complicated pregnancies and their potential as therapeutic targets, the mechanisms involved in monocytes recruitment from the periphery to the decidua and their local differentiation and conditioning is the matter of continuous study. Here we demonstrated that VIP in a decidual context, as well as by itself, contributes to the selective migration and profiling of peripheral monocytes from term-pregnant and non-pregnant donors. Monocytes acquire a functional phenotype comparable to dMA at this stage. VIP and decidual factors promote apoptotic cell clearance, tissue repairing, and anti-inflammatory mediator synthesis in term placenta, consistent with the endometrium remodeling events required after placental delivery. Evidence is summarized as follows: First, VIP induces monocyte chemoattractant CCL2 and CCL3 in term decidual explants and preferentially promotes the migration of peripheral monocytes to decidual explants compared to CD4 or CD8 lymphocytes even though lymphocytes count is 9:1 higher in

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peripheral samples tested. Second, critical repairing mechanisms of monocytes and macrophages such as efferocytosis, wound healing and anti-inflammatory cytokines synthesis are enhanced by VIP-induced decidual factors. Also, limited efferocytosis displayed by peripheral monocytes from pregnant women was restored upon stimulation with decidual explants pretreated with VIP to levels similar to those in decidual macrophages.

VIP was formerly described in syncytiotrophoblast, cytotrophoblast cells and vessels of the first trimester and term human placenta [6,26]. It was also detected in the endometrium, where it plays a role in the decidualization process and in the regulation of the immune microenvironment during the peri-implantation period [27–30]. VIP was recently described in 5-9 weeks decidual explants with a strong immunolocalization in invasive extravillous trophoblasts and trophoblast cells in the lumen of transforming vessels suggesting a prominent role in vascular remodelling [12]. An anti-inflammatory milieu with increased IL-10 was induced by VIP in decidual macrophages isolated from first trimester decidua. Metalloproteinase 2 was also induced by VIP in dMA which could assist the trophoblast invasiveness characteristic of this early stage [12]. VIP levels in plasma are higher in pregnant vs. non-pregnant women and they increase in cord blood and plasma by the end of pregnancy [31]. Taken together, these observations support the paracrine and systemic effects of VIP occurring concomitantly to fit the requirements of the different stages until delivery. Our results are in line with a synergistic effect of systemic and decidual VIP. Serum VIP concentration range reported for pregnant women (50-150 pg/ml) is comparable to other species such as the pregnant rat and mouse [32,33]. However, since VIP has a short half-life in serum its effects on different tissues are mostly dependent on VIP released by neuronal endings or locally synthesized in those tissues: in the pregnant uterus the glandular epithelial cells, trophoblast, stromal and vascular cells, all express and release VIP under basal and stimulated conditions [12,27]. A concentration range 1-100 nM was effective to increase trophoblast outgrowth in human first trimester placental explants as well as IL-10 release by dMA isolated from same placental explants [12]. Thus, increasing levels of VIP in plasma and sustained expression of VIP in decidual cells would underlie the constant recruitment and profiling of monocytes to differentiate and attend the varying demands of the uterus to maintain decidual homeostasis. One of those key demands is the constant clearance of apoptotic bodies and cell debris. Differential programming of monocytes by systemic and decidual factors to exert a phagocytic function at term is supported by the higher efferocytosis of monocytes from non-pregnant compared to pregnant donors shown here. The increase of the membrane CD36 phagocytic receptor expression in non-pregnant monocytes *vs.* the lack of CD36 induction in pregnant monocytes is consistent with the poor phagocytic capacity of these cells. The relevance of phagocytosis at the maternal-fetal interface is of such magnitude that not only monocytes are permanently recruited and differentiated to

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phagocytic macrophages to cope with this demand but also the trophoblast cells display phagocytosis and efferocytosis as it was previously shown in murine and human cell designs [34–36].

Consistent with our present observations, circulating leukocytes from pregnant women presented reduced migration index and reduced H_2O_2 production compared with non-pregnant controls [37]. Regarding monocyte efferocytosis capacity during pregnancy, pregnant women monocytes are less efficient than monocytes from non-pregnant controls and the reduced efferocytic capacity was even more pronounced in patients with preeclampsia [38] or systemic lupus erythematosus [39].

Apoptotic cell clearance is deeply associated with tissue repair mechanisms which are central to homeostasis maintenance as well [20,22,40]. VIP conditioning of monocytes favored these events in the decidua. In fact, monocytes from pregnant women presented higher wound healing capacity than monocytes from non-pregnant donors and it was even higher if they were preconditioned with decidual factors and VIP. Tissue repair mechanisms are upregulated after delivery in other species [18,19,41]. Our results support the profiling of circulating monocytes to assist that process before delivery thus contributing to mitigate the decidual disruption by repairing uterine structures. Monocytes potentiated the wound healing promoted by VIP on stromal cells since HESC monolayers treated with VIP in the absence of monocytes not only failed to close the wound but also VIP displayed an inhibitory effect. This negative regulation of VIP in endometrial stromal cell migration is consistent with the promoting effect of VIP on HESC decidualization previously demonstrated [27,42] although this would be at a minimum in term placenta.

Identifying circulatory molecules and/or particles responsible for initiating or exacerbating maternal and fetal sickness is crucial to understanding maternal–fetal communication in pregnancy pathologies[43]. The possibility that VIP emerges as a drug candidate for treating term pregnancy complications is supported by preclinical studies in murine pregnancy models [28,44–46]. A single injection of VIP was enough to restore anti-inflammatory mediators synthesis and apoptotic cell phagocytosis in abortion prone murine models [44,45]. On the other hand, VIP restored reduced fetal weight at later stages of pregnancy in a VIP-deficient mouse model [46] and it modulated Treg cell outflow to the implantation sites related to VIP local and systemic levels [28]..

In the present work we present evidence to demonstrate that in term placenta, decidual macrophages and circulating monocytes are conditioned to sustain key processes required for tissue repair and homeostasis maintenance. VIP guides monocytes to display a higher migration towards term decidua and enhances their efferocytosis and wound healing capacity.

Material and Methods

Placenta samples

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Term placenta tissue (N=23 in Buenos Aires and N=14 in Sao Paulo) were collected from women undergoing programmed caesarean birth. Women participating in the study were 21 to 42 years old and all of them had pre-pregnancy BMI 18.5 to 25. They had negative serology for HIV, HBV, Chagas disease and Syphilis and no clinical records of gestational diseases or more than one pregnancy loss. Samples from women with serious complications during pregnancy such as infections that compromised pregnancy or fetal death were excluded. Approval was obtained from Hospital Italiano of Buenos Aires Research Ethics Committee (HIBA 1681) and the Institute of Biomedical Sciences from University of Sao Paulo (0943/2010). Written informed consent was obtained from all women undergoing elective caesarean birth. Placental with decidual *basalis* tissues were dissected as described previously [47] and processed for cell and tissue culture or immunohistochemistry assays. Briefly, small decidual explants of 5 x 5 x 1 mm were excised from term placenta's cotyledons, washed with cold PBS and then cultured in 24-well polystyrene plates in 500 µl DMEM:F12 complete, with 10% FCS and 100 µg/ml penicillin/ 100 U/ml streptomycin (Life Technologies, Buenos Aires, Argentina) for 20 h without or in the presence of 100 nM VIP as reported previously [12]. The explants were collected for RNA assays in TRizol and the supernatants were centrifuged and conserved at -80°C until use. For immune cell isolation, the decidual explants were washed with PBS and digested with collagenase II (1mg/ml) and DNAse I (0.1mg/ml) followed by Ficoll-Hypaque centrifugation. Then cells were either used for flow cytometry or CD14 cell isolation with magnetic beads coupled to antibodies in a two-step design: non-monocyte immune cells were removed using a negative selection CD14 cell isolation kit (Miltenyi). Then, monocytes were separated from the recovered cells using a CD45-positive selection isolation kit (Miltenyi). In some experiments, cells used for flow cytometry were purified directly using a positive selection CD14 cell isolation kit with magnetic beads coupled antibodies (Miltenyi).

Blood samples

Blood samples were processed from non-pregnant healthy volunteers (N=13) and pregnant (N=12) women at the moment of cesarean birth. Neither donor was under pharmacological treatment for at least 10 days before the day of sampling. Blood was obtained by puncture of the forearm vein, and it was drawn directly into heparin containing sterile plastic tubes. Studies were approved by the Argentine Society of Clinical Investigation Board and Ethical Committee (SAIC 03/17) and the Hospital Italiano de Buenos Aires Research Ethics Committee and Research Committee (HIBA 1681). Samples were collected after corresponding written informed consent was signed by the participants.

Monocyte isolation and conditioning with VIP or decidual media

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Peripheral blood mononuclear cells (PBMC) were isolated from individual subjects by Ficoll-Hypaque and CD14 positive cells separated by Percoll gradient as the manufacturer's protocol. Cell population purity (>80%) was checked by flow cytometry analysis with CD14 labeling as previously described [48]. Monocytes were cultured in 24-well polystyrene plates ($3x10^5$ cells/well) in DMEM:F12 complete in the presence or not of 100 nM VIP, or conditioned media of decidua explants not treated (D or Decidua) or pre-incubated for 20 h with 100 nM VIP and identified as D (VIP) or Decidua VIP; or with first trimester trophoblast cell line, Swan 71 (CT+) for efferocytosis assay. Briefly, Swan 71 cells were cultured for 20 h in 24-well flat-bottom polystyrene plates with DMEM:F12 10% FCS as reported [5]. Cell viability was not affected by the treatments during the incubation times used.

Immunohistochemistry

Paraffin-embedded human term placenta with decidua basalis was stained using colorimetric detection as described previously. Serial tissue sections (5 µm) were immunostained with anti-Vimentin-1/100, anti-Cytokeratin-1/100 and anti-VIP-1/500 Abs. Goat anti-mouse HRP-conjugated (Thermo Fisher Scientific Cat# 62-6520, RRID:AB_2533947) and Donkey anti-rabbit HRP-conjugated antibodies (Thermo Fisher Scientific Cat# PA1-86177, RRID:AB_933717) Abs were obtained from Thermo Fischer (1/200) and immunoreactions performed as previously reported [12]. Microphotographs were acquired using an FV 300® Olympus and Fluoview Software FV-ASW 4.1 Viewer.

Leukocyte migration

Migration assays were performed in Transwell 24-plates across 5 µm polycarbonate membranes (Costar, Corning Incorporated, NY, USA) as previously described [49]. PBMCs ($2x10^5$ cells) were re-suspended in DMEM containing 10% FCS and placed on the upper chamber. The lower chamber contained 300 µl of DMEM 10% FCS (basal condition, —), or conditioned media of decidua explants non-treated (D or Decidua) or that had been pre-treated with 100 nM VIP [D (VIP) or Decidua VIP] as indicated. After incubation for 2 h at 37°C, cells were collected, stained with anti-CD4+, -CD8+ and -CD14+ antibodies and counted with a FACS Aria II cytometer® (Becton Dickinson, San José, CA, USA). The results are expressed as the number of cells migrated in 2 h.

Efferocytosis assays

Phagocytosis of apoptotic neutrophils by autologous CD14+ cells (efferocytosis) from non-pregnant (NON-P) or pregnant (P) blood samples was carried out as previously reported [5,7]. Neutrophils were obtained after the Ficoll-Hypaque gradient and subsequent Dextran purification [7,8]. Apoptotic neutrophils were obtained after 20 h incubation in RPMI 1640 (spontaneous apoptosis) and stained with 3 µM/10^6 cells of CFSE (Life Technologies, Buenos Aires) for 10 min

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in RPMI without FCS. Excess of CFSE was eliminated by serial washes with RPMI 10 % FCS. The percentage of neutrophil apoptosis was higher than 50% as determined by annexin-propidium iodide staining and flow cytometry [5,7]. In parallel, monocytes (3×10^5) were conditioned for 20 h with 100 nM VIP or decidua explants' conditioned media without stimuli (D) or pre-incubated with 100 nM VIP [D (VIP)] or conditioned media from first trimester trophoblast cell line Swan 71 as a positive control (CT+) [5] or DMEM:F12 10% FCS as a positive control (BASAL).

Monocytes were incubated with apoptotic neutrophils in a 1:10 ratio. After 40 min of phagocytosis monocytes were collected, immunostained for CD14 and the percentage of CD14/CFSE double-positive cells was analyzed by flow cytometry as described [5,48].

Cell migration assays

Wound healing assays were carried out using the Human Endometrial Stromal Cell Line (HESC). Briefly, 2×10^4 cells were plated in 96-well polystyrene plates with DMEM-F12 10% FCS and incubated in humidified chamber with 5% CO2 at 37°C. When cells reached confluence, a wound was made with a sterile tip and the monolayer was washed to eliminate unattached cells. First, we analyzed the effect of VIP in HESC treated with 100 nM VIP, or 100 nM VIP antagonist (Synthetic H-Lys-Pro-Arg-Arg-Pro-Tyr-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH2, BACHEM, USA) or with both for 8 h. Later, we evaluated the wound healing-associated factors released by monocytes from either pregnant or non-pregnant donors. For this, monocytes were incubated with DMEM F-12 10% FCS, or 100 nM VIP or conditioned media of decidual explants pretreated with 100 nM VIP [D (VIP)] or not (D) as described for efferocytosis assays above. Photographs were taken at different times in the beginning and after 8h and the images were analyzed using the ImageJ program. Results were expressed as the percentage of wound healing analyzing the whole wound area.

RT-qPCR

Decidual gene expression of cytokines [Interleukin (IL) -1β, -6, -10; Indoleamine 2,3-dioxygenase (IDO), Transforming growth factor (TGF-) β1 and the chemokines (CCL2, CCL3 and CXCL8) was determined by RT-qPCR as previously described [4,5]. Briefly, total RNA was isolated following manufacturer recommendations with Trizol reagent (Life Technologies, Grand Island, NY, USA), cDNAs generated from 1 ug of RNA using a MMLV reverse transcriptase, RNAsin RNAse inhibitor and oligodT kit (Promega Corporation, Madison, WI, USA). Samples were stored at -20 ºC for batch analysis.

RT-qPCR master mix (Biodynamics, Buenos Aires, Argentina) was used according to the manufacturer`s recommendation. Real-time PCR was performed on a Bio-Rad iQ5 Real-time PCR system. The relative gene expression levels were determined using the threshold cycle (CT)

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method ($2^{-\Delta\Delta}$ CT method) with reference to the endogenous GAPDH control. The primer sequences are described in Table I.

Flow cytometry analysis and ELISA

Monocytes or decidual macrophages were cultured in DMEM: F-12 10% FCS without or with 100 nM VIP, or condition media from decidual explants incubated in DMEM:F12 complete (D) or treated with 100 nM VIP [D (VIP)] for 20 hours. Cells were recovered by TrypLE treatment (Invitrogen Life Technologies, Grand Island, NY, USA) and stained with APC or FITC conjugated mAbs directed to CD14 and CD36, respectively (BD Pharmingen, San Diego, CA, USA) or CD40 (Immunotools, AP Biotech, Argentina).

Twenty thousand events were acquired in a FACS Aria II cytometer® (Becton Dickinson, San José, CA, USA) and results were analyzed using FlowJo software (http://www.flowjo.com/). Results were expressed as the percentage of the respective population and the quadrant was set using irrelevant isotype-specific Ab. It was expressed as MFI or double positive cell frequencies by flow cytometry. Particularly for monocyte profile, positive cells were determined inside the electronically gated CD14 positive cell population previously selected in FSC vs. SSC. ELISA was performed to assess cytokine levels in dMA supernatants in the presence or not of 100 nM VIP in duplicates as previously described (Paparini et al., 2015).

Statistical analysis

The significance of the results was analyzed by Wilcoxon matched-pairs or Mann-Whitney U-test for two nonparametric samples. When multiple comparisons were necessary, we used One- or two-way ANOVA test followed by Holm-Sidak or Tukey's multiple comparisson tests. Normality was checked with Shapiro-Wilk test before testing. Differences between groups were considered significant at $P<0.05$ using the GraphPad Prism7 software (GraphPad, San Diego, CA, USA).

Acknowledgements

This work was funded by grants from the ANPCyT (PICT 2014-0657 and 2017-1536), the University of Buenos Aires (UBACyT 20020170100317BA) and São Paulo Research Foundation-FAPESP (08/65780-6). We also acknowledge the Plisser fellowship 2012 to Esteban Grasso.

Conflict of interest disclosure

No commercial or financial conflict of interest.

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Tables

Table I: primers sequences

Gene	Primer	Sequence (5' → 3')
CCL2	Fw	CAGCAGCAAGTGTCCCAAAG
	Rv	GAGTGAGTGTTCAAGTCTTCGG
CCL3	Fw	TTCAGACTTCAGAAGGACAC
	Rv	TGAGCAGGTGACGGAATG
CXCL8	Fw	CCAACACAGAAATTATTGTAAAGC
	Rv	CACTGGCATCTTCACTGATTC
GAPDH	Fw	TGATGACATCAAGAAGGTGGTGAAG
	Rv	TCCTTGGAGGCCATGTAGGCCAT
IDO1	Fw	GGCTGGAAAGGCAACCCCCA
	Rv	GCAGCATGTCCTCCACCAGCA
IL-1β	Fw	TGATGGCTTATTACAGTGGCAATG

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	Rv	GTAGTGGTGGTCGGAGATTCG
IL-6	Fw	CAGATTTGAGAGTAGTGAGGAAC
	Rv	CGCAGAATGAGATGAGTTGTC
IL-10	Fw	TTGCTGGAGGACTTTAAGGGTTAC
	Rv	CTTGATGTCTGGGTCTTGGTTCTC
TGF-β	Fw	GGACACCAACTATTGCTTCAG
	Rv	CCAGGCTCCAAATGTAGGG

Figure legends:

Figure 1: VIP expression and immunomodulatory effect in term human placenta: (A) Serial placenta sections were stained with anti-cytokeratin (CK) 7 and anti-vimentin (VIM) (1/200), or anti-VIP (1/500) Abs and hematoxylin. Microphotographs were taken with an Olympus Microscope in 10X and 40X magnification (squared in the left panel in villi and decidua). Arrows are highlighting the same giant trophoblast cells through different staining sections. A representative of six different placentas at term gestation is shown. (B) The gene relative expression of IL-1β, IL-6, IDO, TGF-β, IL-10, CXCL2 and CXCL8 normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analysed by RT–qPCR as indicated in Materials and Methods. Values represent arbitrary units (A.U.) as mean±S.E.M. of eight term placentas in basal conditions. (C) Placental explants were isolated and cultured in DMEM 10% FCS for 20 h in the absence (-) or presence of VIP (100 nM). Double gradient heat map with the effect of VIP on placenta explants: green (down-regulated), red (up-regulated), and black (unchanged). Results are expressed as mean ± S.E.M. of fold change. Confidence intervals for VIP treated explants *vs*. non-treated (-) were compared. *P<0.05 of eight different placentas.

Fig 2: Leukocyte selective migration towards the decidua. 2×10^5 PBMC from healthy non-pregnant volunteers in DMEM 10% FCS were placed on top of transwell systems and 300 µl of (A) DMEM 10% FCS (-) or conditioned media of decidua explants (D) were placed in the lower

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chamber; or (B) 300 µl of conditioned media of decidua explants cultured without (-) or with 100 nM VIP. Cells in the lower chamber were collected after two hours of migration at 37°C and analysed by flow cytometry. Results are mean ± S.E.M. of at least six experiments. *P: 0.05, †P<0.01, ‡P: 0.001. Two-way ANOVA. Sidak's multiple comparison test when there were more than 3 conditions and Wilcoxon test when there were 2 conditions.

Fig 3: Efferocytosis of peripheral blood monocytes from pregnant and non-pregnant donors. (A) CD14 positive monocytes (3×10^5) from non-pregnant (NON-P) or pregnant (P) donors were plated 20 h in DMEM: F12 10% FCS and then challenged with CFSE-labelled apoptotic neutrophils (aNeu) (10:1) for 40 min. CD14/CFSE double positive cells were determined by flow cytometry. Results are mean±S.E.M. of at least seven independent experiments. *P< 0.05 Mann-Whitney test. Representative dot plot is shown. (B) NONP or (C) P CD14 positive cells were incubated in DMEM:F12 10% FCS (BASAL), with 100 nM VIP (VIP), or decidua conditioned media in DMEM:F12 10% FCS (D), or conditioned media from decidua pretreated with 100 nM VIP [D (VIP)] or conditioned media from trophoblast cell line as a positive control (CT+) for 20 h. Each point represents an independent experiment. Values are median±interquartile range of the number of experiments indicated. *P< 0.05, †P< 0.01 and ‡P<0.001, Friedman test, Dunn's multiple comparisons test. (D) The expression of CD36 on CD14 positive cells was determined as % of double positive cells from NON-P or P by flow cytometry. Results are represented as mean±S.E.M. of at least seven independent experiments. *P<0.05 and †P<0.01. One-way ANOVA, Sidak's multiple comparisons test.

Fig 4: Effect of pregnant and non-pregnant monocytes from peripheral blood on wound healing. (A) The diagram shows the experimental design used. (B) A wound was done in a HESC monolayer and incubated for 8 h with monocyte conditioned media from NON-P or P PBMCs donors. Microphotographs were taken at 0 and 8 h. The wound area was quantified with the ImageJ software. Results are expressed as mean±S.E.M. % of wound healing. †P<0.01, Mann-Whitney U-test of seven independent experiments. HESC were incubated for 8 h with conditioned media from (C) NON-P or (D) P monocytes pre-treated as indicated with 100 nM VIP (VIP), decidual explants (D), decidua pretreated with VIP (D VIP) for 20 h or untreated (Basal). Results are expressed as mean±S.E.M. % of wound healing. *P<0.05, †P<0.01. One-way ANOVA, Sidak's multiple comparisons test of seven experiments.

Fig 5: Functional characterization of dMA compared to autologous Mo. (A) $3x10^5$ dMA were incubated in RPMI 10% FCS in the absence (—) or presence of 100 nM VIP during 20 h and supernatants were used for ELISA of TGF-β, TNF-α and IL-1β. Results are expressed as

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mean±S.E.M. pg/ml. *P<0.05, Mann-Whitney U-test (n=5). (B) and (C) $3x10^5$ dMA were incubated in RPMI 10% FCS with 100 nM VIP or not (-). In parallel, $3x\ 10^5$ peripheral blood Mo from the same donor were incubated with RPMI 10% FCS or D (VIP) conditioned media. Then, dMA or Mo were used for efferocytosis of apoptotic neutrophils (B) or wound healing assays (C). Values are expressed as mean±S.E.M. of double positive CD14/CFSE cells. *P < 0.05; RM-One-way ANOVA, Sidak's multiple comparisons test. Representative dot plots are shown. The wound area was quantified with the ImageJ software. Values are mean±S.E.M. % wound healing. *P<0.05; RM-One-way ANOVA, Tukey's multiple comparisons test. Representative microphotographs are shown at 0 and 8 h.

Supplementary figure 1: VIP decreased wound healing in HESC. Human endometrial stromal cells (HESC) were grown in DMEM:F12 10% FCS until they reached confluence and then wounded and incubated in DMEM 10% FCS (—) or 100 nM VIP (VIP), 100 nM of VIP antagonist (aVIP) or both (VIP-aVIP) for 8 h. Microphotograph were taken at 0 and 8 h. The wound area was quantified with the ImageJ software. (A) Values given are expressed as mean±S.E.M. of the percentage of wound healing. *P<0.05; RM-One-way ANOVA, Sidak's multiple comparisons test from 4. (B) A representative experiment is shown.

Physiological relevance:

The recruitment of monocytes and differentiation at the decidua are critical events that determine the outcome of pregnancy. Their functional plasticity limits the extent of injury and promotes the return to tissue homeostasis from implantation to term. VIP is an immunoregulatory peptide synthesized by trophoblast and decidual cells that promotes trophoblast invasion, vascular remodeling, and functional shaping of decidual macrophages in first trimester placenta. In the present work we provide evidence that in term placenta decidual macrophages and circulating monocytes are conditioned to sustain key processes required for tissue repair and homeostasis maintenance. Our results also show that VIP guides monocytes to display a higher migration towards term decidua and enhances their wound healing capacity.

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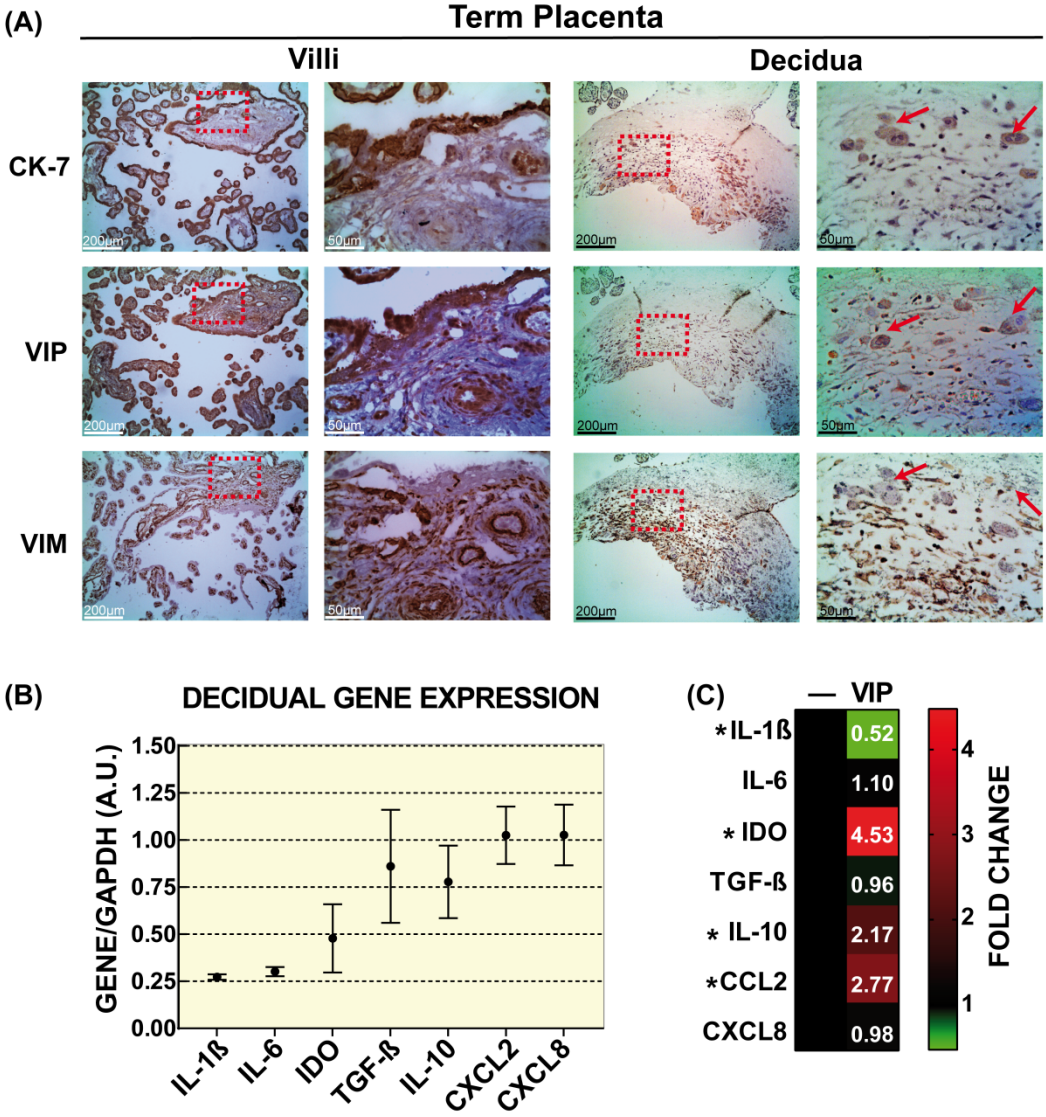

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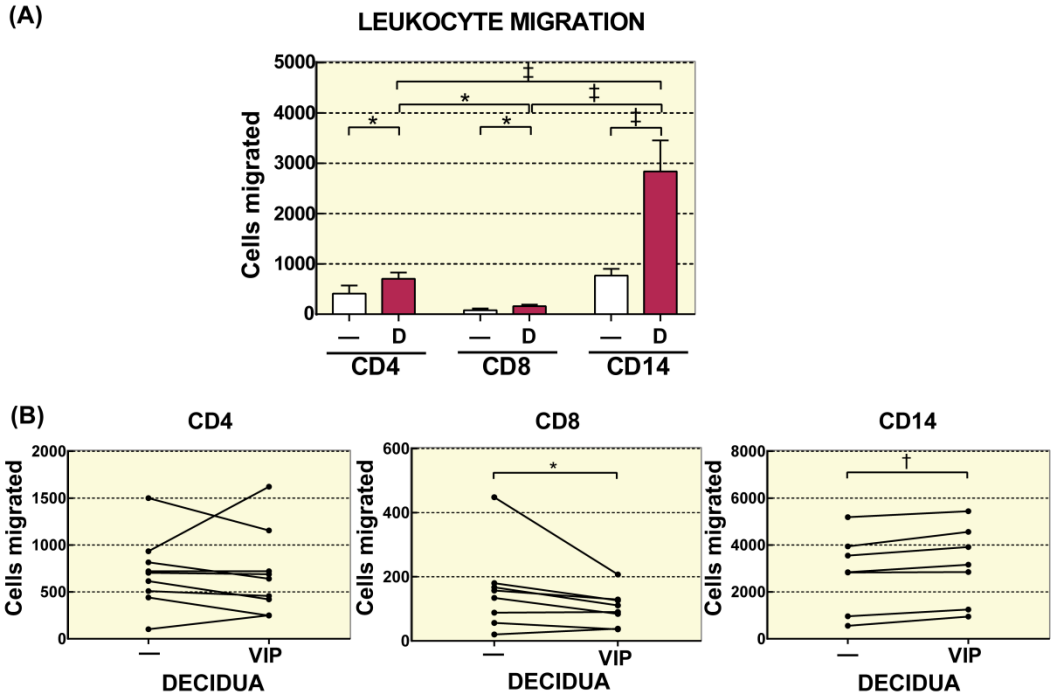

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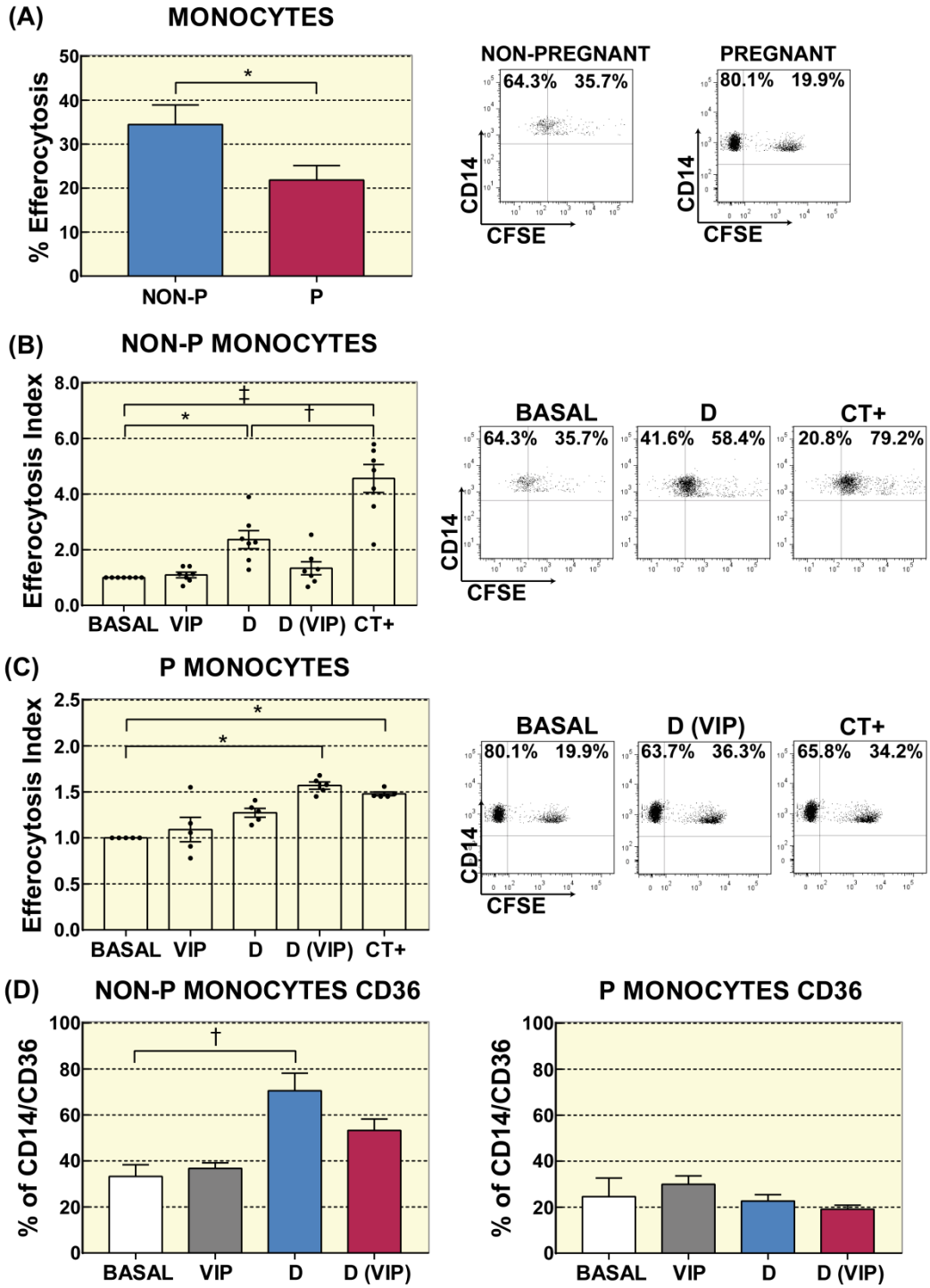

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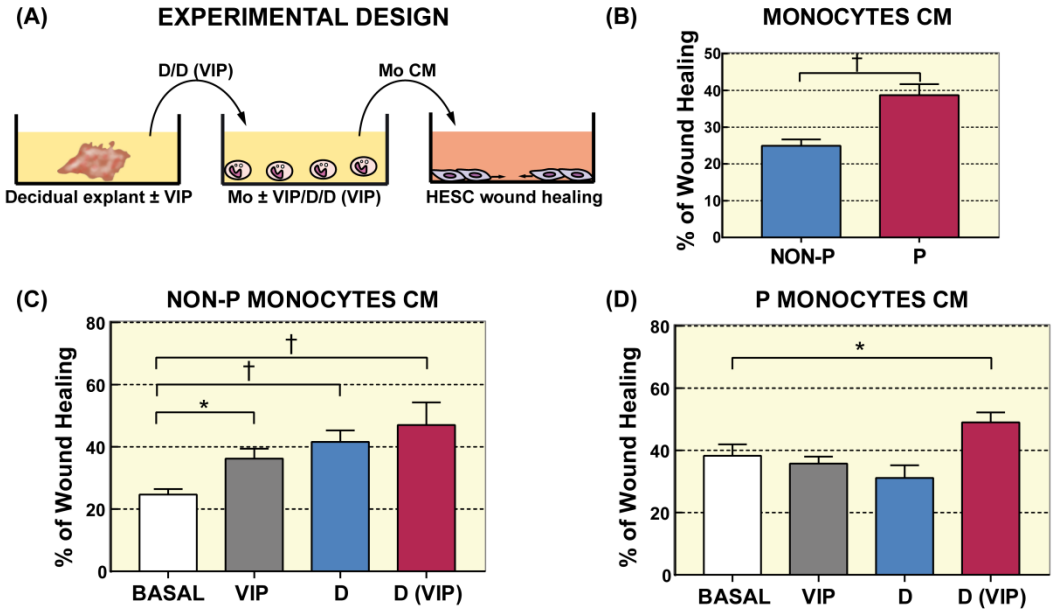

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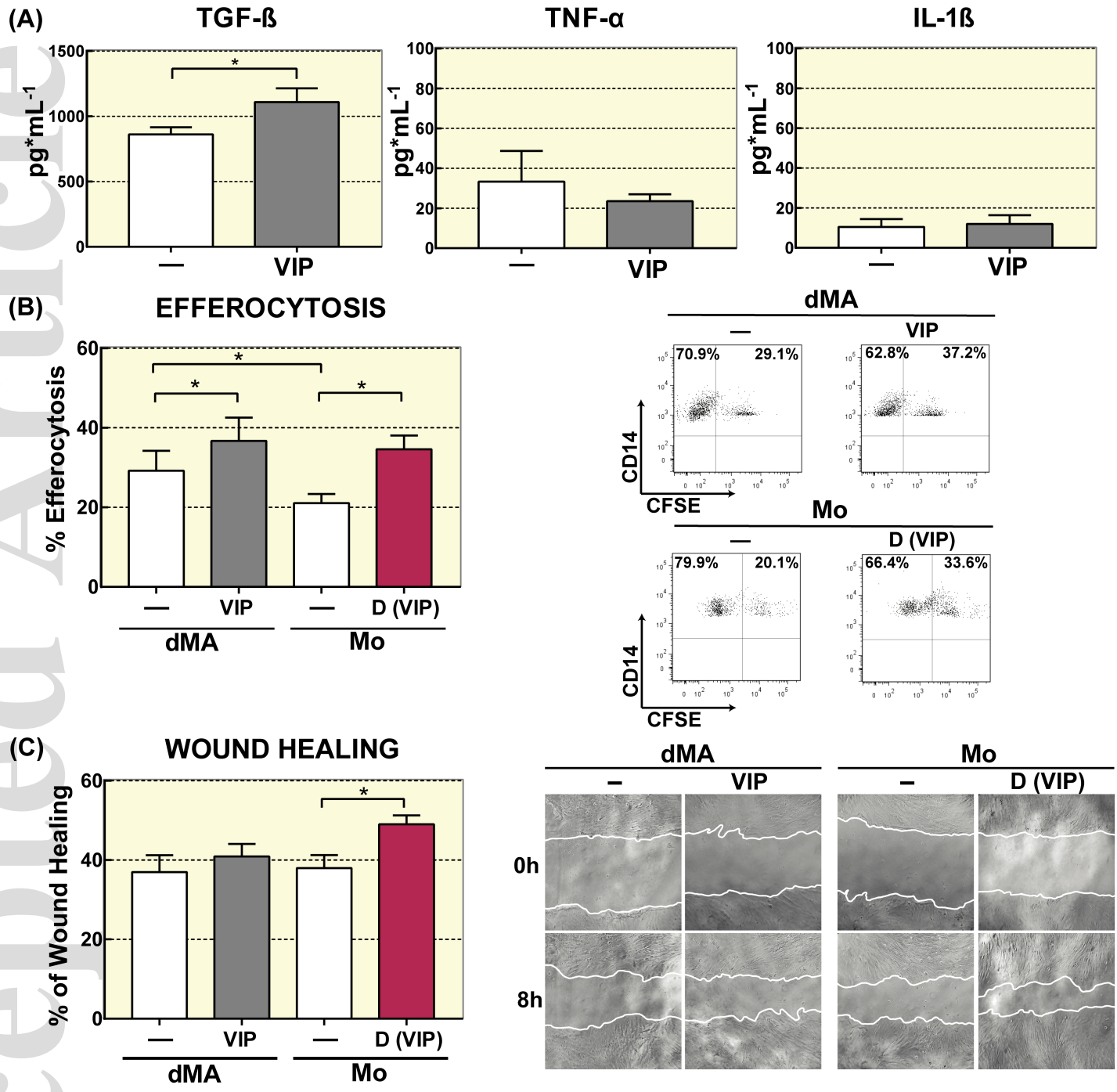

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