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VPAC1 and VPAC2 receptor deficiencies negatively influence pregnancy outcome through distinct and overlapping modulations of immune, trophoblast and vascular functions

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Abstract

Pregnancy outcome relies on the maintenance of immune and metabolic homeostasis at the maternal fetal interface. Maternal and perinatal morbidity and mortality is associated with impaired placental development. Multiple regulatory effects of the endogenous-produced vasoactive intestinal peptide (VIP) on vascular, metabolic and immune functions at the maternal-fetal interface have been reported. Here we studied the involvement of the two primary high affinity receptors for VIP (VPAC1 and VPAC2) on maternal immune response, placental homeostasis and pregnancy outcome. Targeted disruption of each receptor gene led to altered placental structure, vascular and trophoblast functional markers and shaped the functional profiles of macrophages and neutrophils towards a proinflammatory state. Several changes in pregnant mice were receptor specific: ROS production elicited by VIP on neutrophils was selectively dependent on the presence of VPAC1 whereas apoptosis rate was associated with the VPAC2 deletion. In peritoneal macrophages from pregnant mice, levels of MHC-II, TLR2, and IL-10 were selectively altered in VPAC2 receptor-deficient mice, whereas IL-6 gene expression was reduced only in mice lacking VPAC1 receptors. Additionally, MMP9 mRNA in isolated TGCs was

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Author contributions

G. Calo, performed all the *in vivo* and *ex vivo* experiments. V. Hauk helped in RT-qPCR assays and laser capture microdissection readings and interpretation. C. Van collaborated with ELISA measurements. M. Condro helped with mice colonies, mating's and registers. L. Gallino and D. Vota helped with phagocytosis experimental design and evaluation. J. Waschek provided VPAC1 and VPAC2 KO mouse colony. G. Calo, R. Ramhorst, J. Waschek and C. Pérez Leirós designed the whole research, analyzed raw data, discussed the results, and wrote the manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

reduced in VPAC2 receptor deleted mice, while the percentage of IL-12 cells in post-phagocytosis macrophage cultures was selectively reduced in VPAC2 receptor deficient mice. The results indicate that manipulation of VPAC1 and VPAC2 receptor affects immune, vascular and metabolic environment at the maternal fetal interface. These mouse models offer new approaches to study pregnancy complications adding new perspectives to the development of VPAC receptor-selective drugs.

Keywords

VPAC1 / VPAC2 knockout mice; adverse pregnancy outcome models; placental homeostasis disruption; innate response at early pregnancy

1. INTRODUCTION

An altered immune, vascular and metabolic environment at the maternal-placental interface leads to placental homeostasis disruption that associates with severe complications such as maternal and perinatal morbidity, fetal growth restriction and long-term adverse consequences to the offspring [1, 2]. The vasoactive intestinal peptide (VIP) is a 28 amino acid peptide that holds vascular, metabolic and immune properties consistent with its prominent regulatory role at the maternal-fetal interface [3]. VIP displays anti-inflammatory and tolerogenic effects at placentation in mice [4, 5] and induces a dramatic trophic effect on post-implantation mouse embryo *in vitro* [6]. VIP increases myometrial blood flow and inhibits uterine muscle contractility [7]. It is also critical for circadian rhythms synchrony in mice [8, 9]. In line with immune and growth effects of endogenous VIP, VIP knockout (KO) females are sub-fertile and produce only half of the offspring of their WT sisters [10] whereas VIP-deficient fetoplacentas displayed impaired placentation and reduced fetal weight [11]. In this regard, VIP-deficient implantation sites from wild type females crossed to VIP knockout males exhibited decreased frequency of decidual Treg cells, disrupted trophoblast giant cell (TGC) monolayer, vascular defects and lower expression of VEGF-A and angiopoietin 1 [11]. In rats, VIP levels peak in serum at day 11 of pregnancy [12], whereas in mice the maternal VIP expression precedes embryonic VIP, which is expressed in the developing neural tube as early as E10 (embryonic day 10) [13, 14]. Moreover, VIP deficiency in either maternal or embryo/placenta compartments resulted in placental metabolic adaptations with higher placental glucose uptake and transport to the fetus [15]. An abundance of data indicate that VIP effects on embryo growth, immune and vascular modulation involve both VPAC1 and VPAC2 receptors (gene names VIPR1 and VIPR2, respectively, reviewed in [3, 14, 16, 17]). For example, VPAC2 KO mice exhibited greater weight loss, intestinal histopathology and higher levels of proinflammatory mediators (IL-6, IL-1β, and MMP-9) than wild type mice in a model of induced colitis, supporting an immunosuppressive role for this receptor subtype in innate-driven inflammation [17]. Curiously, VPAC1 KO mice exhibited a resistant phenotype to autoimmune encephalitis [18] and colitis [19], suggesting an earlier permissive role for immune responsiveness mediated by this receptor [17]. So far, no studies have been conducted regarding the maternal immune response and pregnancy outcome in VPAC1 and VPAC2 KO mice. Here we present evidence on the differential impact of VPAC1 and VPAC2 knock-down on pregnancy parameters with

special focus on placental homeostasis and the functional shaping of maternal macrophages and neutrophils.

2. MATERIALS AND METHODS

2.1. Mice

8 to 12-week-old C57BL/6 (wild-type (WT)), VPAC1 KO [20] and VPAC2 KO [21] mice were used. VPAC2 colony was kindly provided by M. Sue O'Dorisio. Mice were maintained in specific pathogen-free barrier facilities at the University of California, Los Angeles (UCLA). All husbandry and experimental procedures were performed in compliance with the USDA Animal Welfare Act regulations section 2.31(d)(5), Institutional Policies and Guidelines, and adhered to all principles stated in the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the UCLA Animal Resource Committee (ARC# 1993-302). WT, VPAC1 KO and VPAC2 KO females were mated homozygously to WT, VPAC1 KO or VPAC2 KO males, respectively. Thus, mothers and embryos had the same genotype, either WT, VPAC1$^{-/-}$ or VPAC2$^{-/-}$. Genotyping was performed on all mice in this study. Animals were checked daily for vaginal plugs and separated once mated. The day of the vaginal plug was considered as day 0.5 of pregnancy. Pregnant mice were euthanized on day 7.5 or 8.5 of gestation or followed up until delivery for pregnancy outcome parameter assessment.

2.2. Histologic studies

Implantation sites at gestational day 7.5 and 8.5, from WT, VPAC1 and VPAC2 KO mice were fixed in 4% paraformaldehyde overnight at 4°C. The tissues were embedded in paraffin and 4 mm sections were cut and placed on silanized glass slides. Hematoxylin and eosin (H&E) staining was performed at the Translational Pathology Core Laboratory at UCLA as previously described [11]. Analysis of the photos was performed using a Carl Zeiss binocular microscope.

2.3. Laser Capture microdissection

Laser Capture microdissection of fresh frozen tissue cryosections previously stained with Histogene kit was directed to selected trophoblast giant cells that were then captured on an RNase-free cap with a transfer film (CapSure LCMCap; Arcturus; Molecular Devices, San Jose, CA, USA). Total RNA was extracted from TGCs captured by the LCM transfer film using a PicoPure RNA Isolation Kit (ArcturusXT). This was performed using Leica LMD7000 Laser Microdissection System at the California Nanosystems Institute at UCLA. Total RNA was then treated with DNAase I according to the manufacturer's instructions (Millipore Sigma, Burlington, MA, USA) to avoid DNA contamination, and then samples were reverse transcribed using a MMLV reverse transcriptase, RNAse inhibitor, and oligodT kit (Promega) as previously described [11, 22]

2.4. Neutrophil and macrophage isolation

Neutrophils from pregnant WT or KO mice were isolated from bone marrow as in [23]. Briefly, bone marrow cells were collected, washed and neutrophils isolated by density gradient centrifugation on a two phase Ficoll-Histopaque gradient (Histopaque 1077 and

1119, Sigma Aldrich). Neutrophils recovered at the interface of both layers were washed and double stained with Ly6G and CD11b for purity check (>90%). VPAC1 and VPAC2 expression was assessed in this neutrophil enriched population in the WT by RTqPCR. Both receptors were expressed with a lower Ct for the VPAC1. Macrophages were obtained from pregnant mice at day 7.5–8.5. The peritoneal cavity was washed with ice-cold HANKS as reported and cells re-suspended in culture media and seeded in 24-well plates (Corning Glass, Corning, NY) at 5×10^5 cells/well. After incubation at 37 °C for 2 h, monolayers (> 95% macrophages assessed by F4/80 flow cytometry staining) were used for assays.

2.5. Neutrophil ROS and apoptosis assessment

Purified neutrophils were incubated for 60 min at 37°C with 200 ng/ml PMA in the presence of 10–100 nM VIP (Bachem). To assess ROS production, 5 μM 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) (Sigma-Aldrich) was added for another 15 min and cells analyzed by flow cytometry. On a different set of experiments, neutrophils were incubated or not with 10 nM VIP and apoptosis rate was assessed by flow cytometry staining with Annexin V FITC/PI (Thermofisher).

2.6. Phagocytosis assays

Peritoneal macrophages recovered from pregnant mice at 7.5 or 8.5 day of gestation were cultured with FITC-conjugated latex beads (1 μm, Sigma-Aldrich) at 1:100 ratio (macrophage:beads) as previously [5]. Incubations were performed at 37°C with 5% CO_2 in 24-well plates and after 90 min macrophages were recovered and the percentage of phagocytosis was quantified. Results are expressed as % of FITC+ cells. Negative control of phagocytosis was performed at 4°C. Cytokines were evaluated before and after 120 min of phagocytosis. Supernatants were collected for TNF-α, IL-6 and IL-10 determination by ELISA (BD Biosciences, USA) according to the manufacturer's protocols.

2.7. RT-qPCR

The expression of implantation site and macrophage markers was assessed by quantitative reverse transcription–polymerase chain reaction (RT-qPCR) as previously described [11, 22, 24, 25]. Total RNA (1 μg), routinely checked for integrity, was reverse transcribed using a MMLV reverse transcriptase, RNAse inhibitor and oligo dT kit (Promega Corporation, Madison, WI, USA) and stored at − 20 °C for batch analysis. RNA was quantified and its purity was assessed in all samples using a NanoDrop™ Spectrophotometer. Only RNA with a 260/280 ratio of ~2.0 and a 260/230 ratio of 2.0–2.2 were used. Samples were incubated with SYBR Green PCR Master Mix (Roche, Basel, Switzerland) and the forward and reverse primers (Table 1). RT-qPCR was performed on a QuantStudio 3 PCR system. The relative gene expression levels were determined using the threshold cycle (CT) method (2−ΔCT method) normalized to the endogenous GAPDH or S29 gene control and are expressed as arbitrary units (A.U.). All primers used present an efficiency between 90–100%.

2.8. Flow cytometry

Macrophages were stained with monoclonal antibodies (CD80, CD86, MHCII, TLR2, TLR4) following manufacturer's instructions and isotypes were used as control. IL-12 production was quantified in macrophages of pregnant females before and after phagocytosis of FITC-beads. In the last 4 h of the assay, Stop Golgi (BD, Pharmigen, San José, CA, USA) was added to the culture to promote IL-12 intracellular accumulation. Then macrophages were recovered and stained with anti-F4/80-FITC or F4/80- PerCP mAb depending on the assay, for 30 min, then fixed with Cytofix/cytoperm (BD, Pharmigen, San José, CA, USA) at room temperature and incubated for 30 min with anti-IL12 PerCP/Cy5.5 mAb (eBioscience, USA). Flow cytometry assays were performed at the UCLA Flow Cytometry Core.

Ten thousand events were acquired in a FACSAria cytometer and results were analyzed using the BD software. Negative control samples were incubated in parallel with an irrelevant isotype-matched antibody. Results for positive cells are expressed as a percentage of F4/80 + IL-12+ cells, relative to IL-12 before phagocytosis.

2.9. Cytokine quantification by ELISA

After the phagocytosis assay, macrophages from pregnant females were washed and remained in culture for additional 24 h and then supernatants were collected for cytokine determination. Cytokines were also evaluated before the phagocytosis assay. ELISA for IL-10, IL-6 and TNF-α (BD Biosciences, USA) were performed according to the manufacturer's protocols. Results are expressed in pg/ml.

2.10. Statistical analysis

The significance of the results was analyzed by the Mann–Whitney test or the Wilcoxon test for nonparametric samples. When multiple comparisons were necessary, the Kruskal–Wallis test was used and Dunn's post test or Friedman test when matched or repeated measures were considered. Differences between groups were considered significant at $p < 0.05$ using the GraphPad Prism 9 software (GraphPad, San Diego, CA, USA).

3. RESULTS

3.1. VPAC1 or VPAC2 receptor knockout leads to reproductive failures and adverse pregnancy outcome

Based on previously reported pharmacological effects of VIP and receptor analogs on VPAC1 and VPAC2 receptors at early pregnancy, we studied the impact of VIPR1 and VIPR2 gene disruption on reproductive and pregnancy parameters and litter size. Wild type (WT), VPAC1 KO and VPAC2 KO females were mated homozygously to WT, VPAC1 KO or VPAC2 KO males respectively, thus resulting in control WT, VPAC1 KO or VPAC2 KO mothers and embryo-placental units. These pregnancies were followed daily beginning with vaginal plug observation until delivery. We found that both VPAC1 KO and VPAC2 KO mothers presented a statistically significant reduction in the number of newborn pups (Figure 1 A). When we examined the number of implantation sites at days 7.5–8.5 of pregnancy, we did not find any differences between those from receptor deficient vs control mice (not shown). Curiously, implantation sites were asymmetrically distributed along

uterine horns. Implantation sites in both VPAC1 and VPAC2 KO tended to be implanted on the right horn, compared to the left horn (Figure 1 B–D). No preferences of sides were found in WT mice, as expected.

Moreover, VPAC1 and VPAC2 deficient pregnancies presented a lower pregnancy rate measured as number of pregnant females at time of euthanasia /number of females with a vaginal plug. In our breeding conditions, the fertility rate assessed as births by every 100 mice was reduced for the VPAC KO mice colonies: average 43.5 births per 100 VPAC KO breeding females vs. 58.7 births per 100 WT breeding females, chi-square statistic, $p < 0.05$.

3.2. VPAC1 and VPAC2 KO pregnancies present placental structural disruption

We next performed histological studies to evaluate the structure of implantation sites at gestational days (gd) 7.5 and 8.5. Trophoblast giant cells (TGC), the murine phenotype resembling extravillous invasive human trophoblast, characteristically appear as a continuous organized monolayer in normal pregnancy. Compared to control mice at gd 8.5, in both VPAC1 and VPAC2 deficient implantation sites, a wider and disorganized TGC layer was evident (Figure 2 A). An increased number of TGC layers accompanied by an altered heterogeneous distribution of these cells was seen. Because polymorphonuclear infiltration is associated with deficient placentation and poor pregnancy outcome in mice [26, 27], we then analyzed polymorphonuclear infiltrates in VPAC deficient pregnancies. A substantial neutrophil infiltration along with small areas of necrosis at gd 7.5 was only found in VPAC1 and VPAC2 deficient pregnancies (Figure 2 B). In contrast to control crossings, abnormal ectasia and marked dilation of decidual vessels at implantation sites as well as multivacuolated decidual cells were found in both VPAC1 and VPAC2 KO pregnancies (Figure 2 C).

3.3. Altered angiogenic markers in VPAC deficient pregnancies

Given that VEGF signaling plays a crucial role in maintaining the homeostasis of the maternal vascular space in the mouse placenta, and that modulation of TGC development and differentiation is one of the mechanisms involved [28], we further investigated the expression of this marker in implantation sites. Consistent with the structural vascular alterations observed, a sustained decrease in the expression of VEGF-A was especially evident in VPAC1 KO implantation sites (Figure 3 A).

Hypoxia-inducible factors (HIF) −1 and −2 are oxygen-sensitive basic helix-loop-helix proangiogenic transcription factors that regulate the expression of numerous target genes, including VEGF and other cytoprotective proteins. Since the expression of VEGF-A was lower only in the VPAC1 KO, we next evaluated the expression of VEGF-A related genes only in the implantation sites of the VPAC1 KO model. Figure 3 B shows a marked reduction of both HIF-1α and HIF-2α expression. Interestingly, a lower expression of the anti-inflammatory and invasion regulatory cytokine TGF-β was also evident in VPAC1 KO implantation sites (Figure 3 B). BAX expression was unchanged suggesting that the morphological effects of VPAC1 deficiency does not involve apoptotic events (Figure 3B).

We next studied further the phenotype of TGCs owing to their crucial role in implantation and subsequent placental function in mice. By means of laser-capture microdissection

at gestational day 8.5, single TGCs were isolated as seen in the images (Figure 3 C). Expression of angiogenic related molecules was evaluated in TGCs. Figure 3 C shows a decrease in VEGF-A and Angiopoietin 1 (ANGPT1) corroborating the observed disruption of vascular development in VPAC deficient pregnancies. A reduced expression of metalloproteinase 9 (MMP9), also related to invasive and proangiogenic phenotypes, was seen in VPAC2 KO (WT vs VPAC2 KO, *$p < 0.05$ Mann Whitney test) and a trend decrease in VPAC1 KO.

3.4. VPAC deficiency favors proinflammatory signals in the implantation sites

We have previously shown that VIP is an immunomodulatory peptide in murine pregnancy promoting anti-inflammatory and tolerogenic responses [11, 29–32]. On this basis, we next analyzed if VPAC deficiency could modulate the immune microenvironment at the implantation sites, now focusing on decidual macrophages, a cell type well known to be essential for the establishment of early pregnancy [33, 34]. Figure 4 A shows a lower mRNA expression of F4/80, a widely used unique marker of murine macrophages. Moreover, the expression of CCL2 and CXCL4, that regulate migration and infiltration of monocytes/macrophages (CCL2) and chemotaxis of fibroblasts and monocytes (CXCL4), was also reduced. Particularly in the VPAC1 KO this scenario was accompanied by increased IL-6 and decreased IL-10 production pointing to a proinflammatory bias (Figure 4 B).

3.5. Differential VPAC1 and VPAC2 receptor involvement in neutrophil functional phenotype during pregnancy

The role of neutrophils in early pregnancy has been poorly studied. They contribute to vascular transformation for proper placental development and fetal tolerance but depending on their activation profile and number they are also implicated in pregnancy complications [26, 35]. To address the involvement of VPAC1 and VPAC2 receptors on neutrophil function during pregnancy, we first evaluated reactive oxygen species (ROS) release in the bone marrow neutrophil enriched population isolated from each model. As shown in figure 5 A, 10 and 50 nM VIP can diminish PMA-induced ROS release in WT neutrophils, similarly to what is described for human neutrophils [24]. VPAC1 gene knockout abrogated the effect of VIP on ROS, even at the highest tested concentration of VIP (100 nM) not shown. Conversely, the effects of VIP were not diminished in VPAC2 KO neutrophils. We then examined the previously reported ability of VIP to accelerate spontaneous neutrophil apoptosis [24]. Interestingly, VIP increased apoptosis rate in WT and VPAC1 KO neutrophils but the effect was lost in neutrophils from VPAC2 KO pregnancies (Figure 5 B) Thus, VPAC1 receptors appear to mediate the effects of VIP on neutrophil ROS production during early pregnancy, whereas VPAC2 receptors mediate the corresponding pro-apoptotic action of VIP on neutrophil apoptosis.

3.6. Macrophages from VPAC deficient pregnancies display a proinflammatory profile.

Not only the number but also the phenotype of macrophages in a predominant M2 alternative activation profile conditions normal pregnancy outcome [36]. Macrophages from pregnant VPAC1 and VPAC2 KO females were thus analyzed. Figure 6 shows increased CD80, CD86 and MHC-II expression in macrophages from VPAC1 and VPAC2 KO mice, consistent with a shift to more inflammatory profiles. Interestingly, the increase of

proinflammatory markers appears more evident in VPAC2 KO macrophages. TLR2 and TLR4, two receptors associated with a proinflammatory background appeared upregulated as well (Figure 6).

In order to explore if these molecular phenotypes had functional correlates and reveal a key role for macrophages in the removal of apoptotic cells in a silent anti-inflammatory manner with inhibition of proinflammatory cytokines such as IL-12 and IL-6, we next studied macrophage phagocytosis and post phagocytosis cytokine release. We did not find any difference in the phagocytosis rate of macrophages from VPAC1 or VPAC2 deficient crossings vs. the control (Figure 7 A). However, a consistent proinflammatory profile in macrophages after phagocytosis of fluorescent beads in VPAC1 KO pregnant females compared to WT and VPAC2 KO was evident (Figure 7 B and C). The percentage of proinflammatory cytokine IL-12 producing cells was significantly increased after phagocytosis in VPAC1, but not VPAC2 KO macrophages, compared to the WT (Figure 7 B). Moreover, after phagocytosis there was a marked increase in IL-6 release from VPAC1 KO macrophages that was not seen in the other crossings. (Figure 7 C). As expected for WT macrophages, the anti-inflammatory cytokine IL-10 was increased after phagocytosis but was undetectable in VPAC1 and VPAC2 KO models (Figure 7 D). IL-6 and IL-10 levels mirrored the proinflammatory trend observed in Figure 6. Accordingly, the proinflammatory TNF-α was found increased post phagocytosis in VPAC1 KO pregnancy (pre-phagocytosis: 81.97 pg/ml; post phagocytosis 160,2 N=3; $P<0.05$). Using the same experimental settings, in the WT the levels of TNF-α were below 150 and did not change with the phagocytic stimulus (not shown) whereas in the VPAC2 KO macrophages it was not detected.

4. DISCUSSION

Homeostasis maintenance and vascular transformation at placentation is the result of multiple cell-cell communication loops that involve different populations of leucocytes controlled by trophoblast cells. By means of VPAC1 and VPAC2 KO pregnant mice, here we provide experimental evidence that both receptors are involved in murine placentation through modulating trophoblast cell function, vascular remodeling and immune homeostasis maintenance. Moreover, evidence is presented on the selective effect of VIP on VPAC1 or VPAC2 receptors to shape maternal macrophages and neutrophils.

These conclusions are supported by the following observations. First, reduced litter size accompanied by an asymmetry of implantation sites along the uterine horns was a hallmark of VPAC deficient pregnancies. Second, a disrupted placental structure with expanded layers of trophoblast giant cells, vascular defects and leukocytes infiltration were observed in VPAC KO implantation sites. A deep reduction of vascular marker expression VEGF-A, an altered phenotype of TGC and a predominant proinflammatory microenvironment confirmed this feature. Third, VIP elicited different effects on neutrophils from VPAC1 and VPAC2 KO pregnant models compared to WT pregnancies. ROS production appeared more dependent on the presence of VPAC1 receptors on neutrophils whereas apoptosis rate seems associated preferentially to the VPAC2. Lastly, macrophages from both VPAC KO models displayed an altered phagocytic capacity switched to a proinflammatory profile with increased expression

of CD86, higher release of TNF-α, IL-6, IL-12 and failure to increase the anti-inflammatory cytokine IL-10.

In a normal maternal background, we have previously reported that VIP produced by trophoblast cells is necessary for optimal placentation and sustains fetal growth in mice [11, 15]. Decreased frequency of decidual Treg cells, disrupted trophoblast invasion of decidua and vascular defects were reported in mice in which embryo/placentas were deficient in VIP [11]. Also, VIP deficiency in either maternal or embryo/placenta compartments was associated with placental metabolic maladaptations [15]. It is noteworthy that even with normal maternal VIP levels, evidence presented here supports that either VPAC1 or VPAC2 knockdown affects placentation through altering immune, trophoblast and vascular functions of the placenta. The fact that particular biases were found for certain effects in the VPAC1 and the VPAC2 pregnancies is consistent with previous observations in these gene knockout models [17]. In this sense, it is relevant to point out that the VPAC1 and VPAC2 KO early pregnancy models studied here are subjected to a mild physiological inflammation related to placentation rather to an antigen or tissue damage-driven potent inflammation as in previous reports [18, 37, 38]. In our scenario, characterized by a milder, controlled activation of maternal leukocytes in the placentation period, VPAC1 and VPAC2 KO models share many altered pregnancy parameters. Placental structure, vascular disruption and trophoblast dysfunction underlie adverse pregnancy outcome. Regarding the trophoblast-immune interaction, the lower levels of the macrophage marker F4/80 in both VPAC1 and VPAC2 KO implantation sites could be reflecting an abnormal recruitment capacity of the trophoblast cells consistent with the parallel observed reduction in CCL2 and CXCL4.

VPAC1 and VPAC2 receptors are known to regulate diverse key physiologic functions like vascular homeostasis, feeding behavior, nutrient absorption, sleep and general metabolism [39–42]. Also, a leaner body phenotype with altered metabolic hormones was reported in VIP KO mice [39] whereas VPAC1 and VPAC2 KO mice present altered metabolism and insulin intolerance with changes in their glucose/energy homeostasis [42].

As for neutrophils' profile in these pregnancy models, we can observe some differences that reflect that both receptors are implicated in the proinflammatory phenotype of this bone-marrow derived neutrophil population, although by different mechanisms. While VIP increased the apoptosis rate in WT and VPAC1 KO neutrophils, no effect was found in neutrophils from VPAC2 KO pregnancies. At the same time, VIP selectively inhibited ROS production through VPAC1 receptors on neutrophils from pregnant mice but did not exert a specific effect on VPAC2 receptors. These observations strongly support that VPAC1 and VPAC2 receptors act as a dual complementary anti-inflammatory control mechanism on early pregnancy neutrophils. It is tempting to speculate that in normal pregnancies, neutrophils expressing both VPAC receptors would have redundant complementary mechanisms to ensure the rapid clearance of blood and placental pathogens minimizing a deleterious exacerbated inflammatory response.

Macrophages bearing a predominant M2 phenotype, able to synthesize anti-inflammatory mediators and to silently remove apoptotic cells and cell debris, characteristically control

immune homeostasis during placentation [1]. VIP has emerged as a prominent mediator of this regulatory effect on macrophages in both human and mouse pregnancies [25, 30, 43, 44]. The fact that VPAC1 and VPAC2 KO pregnancies displayed similar proinflammatory functional phenotypes indicate that both receptors can play a role in the required anti-inflammatory removal of apoptotic cells as well as to provide an anti-inflammatory microenvironment for adequate placentation. This observation acquires special relevance when comparing the levels of IL-6 at implantation sites. In fact, although the VPAC1 KO presents a remarkable reduction of macrophages in implantation sites (as evaluated by F4/80), a significant increase in the proinflammatory IL-6 was found.

VPAC1 and VPAC2 receptor diverged more than 300 million years ago and their presence had been maintained in all examined species beyond teleostei. Now, due to differences in promotor/enhancers and coding sequences between the two receptors, they have developed their own cell-specific expression patterns, regulatory mechanisms and, in some cases, signaling pathways. For example, macrophages constitutively express VPAC1 whereas VPAC2 expression is very low in resting cells. This may account for some of the differences found between both KO models. VPAC1 receptor appears to be more necessary to control the inflammatory response in macrophages. Our studies and work from others suggest that the two receptors have many non-reductant functions. For example the EAE phenotype in VPAC1 and VPAC2 KO mice is opposite [18, 37]; VPAC1 and VPAC2 receptors have opposing actions on GABA release from hippocampal nerve terminals through activation of different transduction pathways [45]; VPAC1 receptors but not VPAC2 play a dominant role in PACAP-induced vasorelaxation in female mice [46]; VPAC1 mainly mediates Vasoactive Intestinal Peptide-dependent functional homeostasis of the gut microbiota [47]. However, the present state of knowledge makes it difficult to explain many of these differential findings.

Finally, the pituitary adenylyl cyclase-activating polypeptide (PACAP) is another secretin-family neuropeptide similar to VIP in amino acid sequence and activity on VPAC1 and VPAC2 receptors. Interestingly, VPAC2 KO mice and PACAP KO mice present a similar pattern of immune response to EAE induction, suggesting that endogenous PACAP displays some of its anti-inflammatory actions through VPAC2 receptors [17]. On the other hand, studies in PAC1 KO mice strongly support a role of endogenous PACAP in reproductive function [48] that had been primarily demonstrated with PACAP in fertility rates, at the gonads and in embryo implantation in mice [49, 50]. Taken together, we cannot rule out that the placental defects observed here in the VPAC1 but especially in the VPAC2 KO mice might be not only mediated by VIP but also by PACAP. Similarly, it cannot be ruled out that the effects observed in macrophages and neutrophils of pregnant mice in both KO models are related to extrinsic factors or indirect mechanisms associated to the VPAC deficient background. Extrinsic pathways such as VIP-mediated differential leukocyte trafficking have been proposed in the intestine of postnatal VPAC KO mice [51].

Cumulative evidence supports the potential of VIP for preventing or reducing the impact of pregnancy complications associated with defective placentation. Here we focused on the impact of VIP receptor deficiency in pregnancy outcome by means of VPAC1 or VPAC2 KO models. Notwithstanding the limitations of this kind of approach and the fact that a few

experiments were only carried out in one of the models, our results add new perspectives to receptor-selective drug development since both receptors appeared involved in placental homeostasis and trophoblast functions, but differential responses were observed on innate immune cells.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Nonstandard abbreviations

VIP	vasoactive intestinal peptide
FGR	fetal growth restriction
gd	gestational day
LCM	laser capture microdissection
ROS	reactive oxygen species
ANGPT	angiopoietin
VEGF	vascular endothelial growth factor
TGC	trophoblast giant cell
WT	wild type
KO	knock out
TLR	Toll-like receptors
MMP	matrix metallopeptidase
DCFH-DA	dichlorofluorescin diacetate
PACAP	pituitary adenylyl cyclase-activating polypeptide

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Highlights

- VPAC1 and VPAC2 are involved in murine placentation.
- Targeted disruption of VIP receptors alters trophoblast cell function and placental structure
- VPACs KO shape macrophages and neutrophils functional profiles at early pregnancy

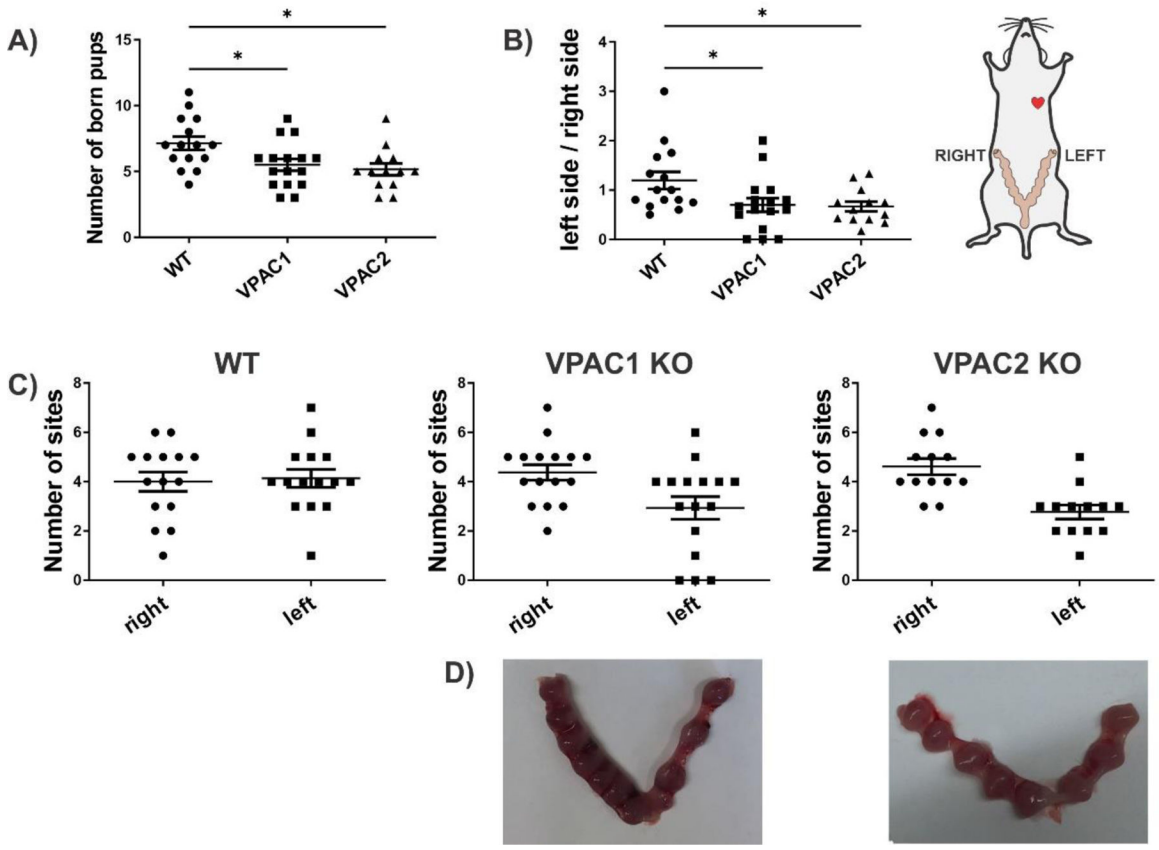

FIGURE 1. Pregnancy alterations in implantation sites of VPAC deficient mice.

A) Number of born pups from each strain. Each dot represents one litter. B) Asymmetry: Number of sites found on left side/number of sites on right side. Scheme showing both sides of the mice, being the left the heart side. Values represent mean ± SEM of at least 13 pregnant mice of each strain, *$p < 0.05$. C) Number of implantation sites found on each side of the three strains. D) Representative images of each strain. Number of mothers WT: 15, VPAC1 KO: 16, VPAC2 KO: 13. All of them analyzed on gestational day 7.5 or 8.5. SEM error bars are shown.

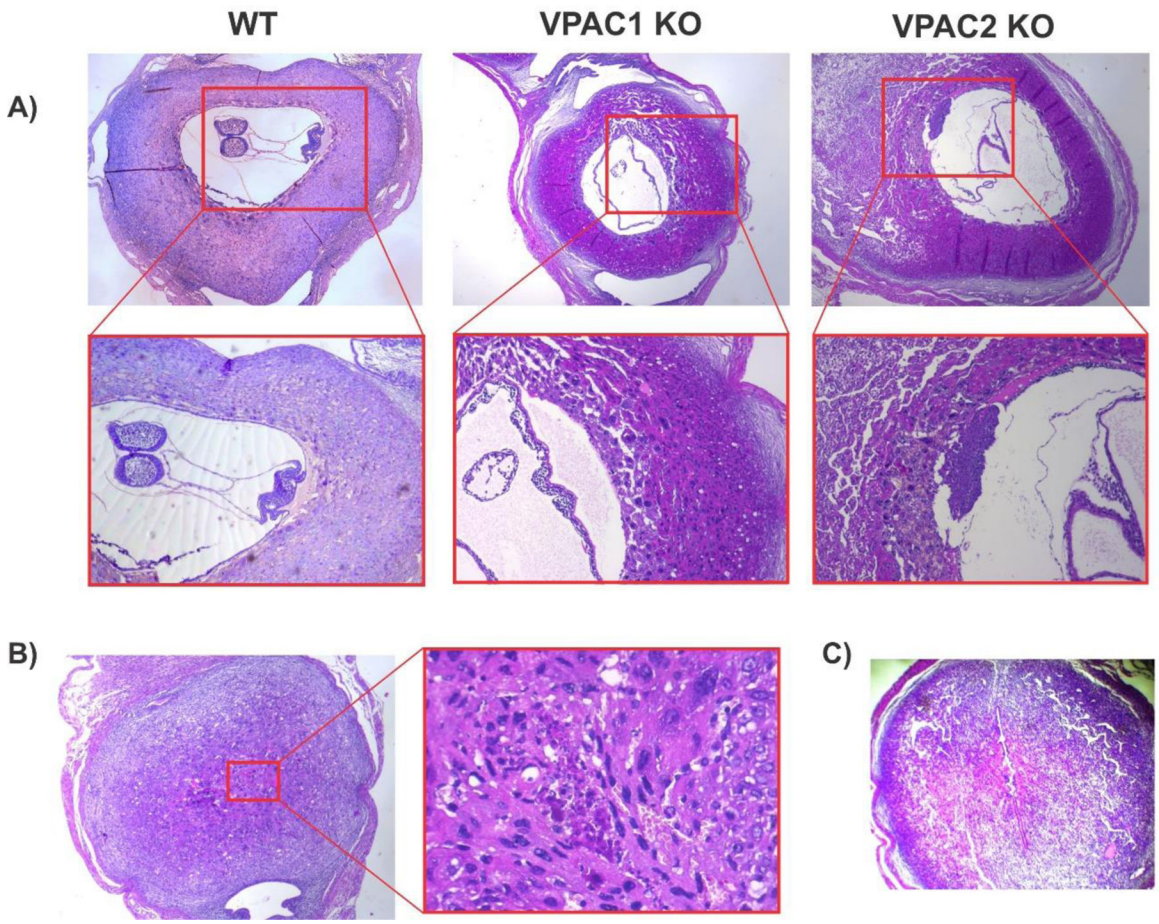

FIGURE 2. Histologic alterations in implantation sites of VPAC1 and VPAC2 KO mice.
A) representative images of WT (left), VPAC1 KO (center) and VPAC2 KO (right) implantation sites at gd 8.5. Higher-magnification images of boxed regions are shown below. B) Representative images showing inflammatory infiltrate of polymorphonuclear cells. Higher-magnification images of boxed regions are shown right. C) Representative image depicting marked vascular dilation and decidual ectatic vessels.

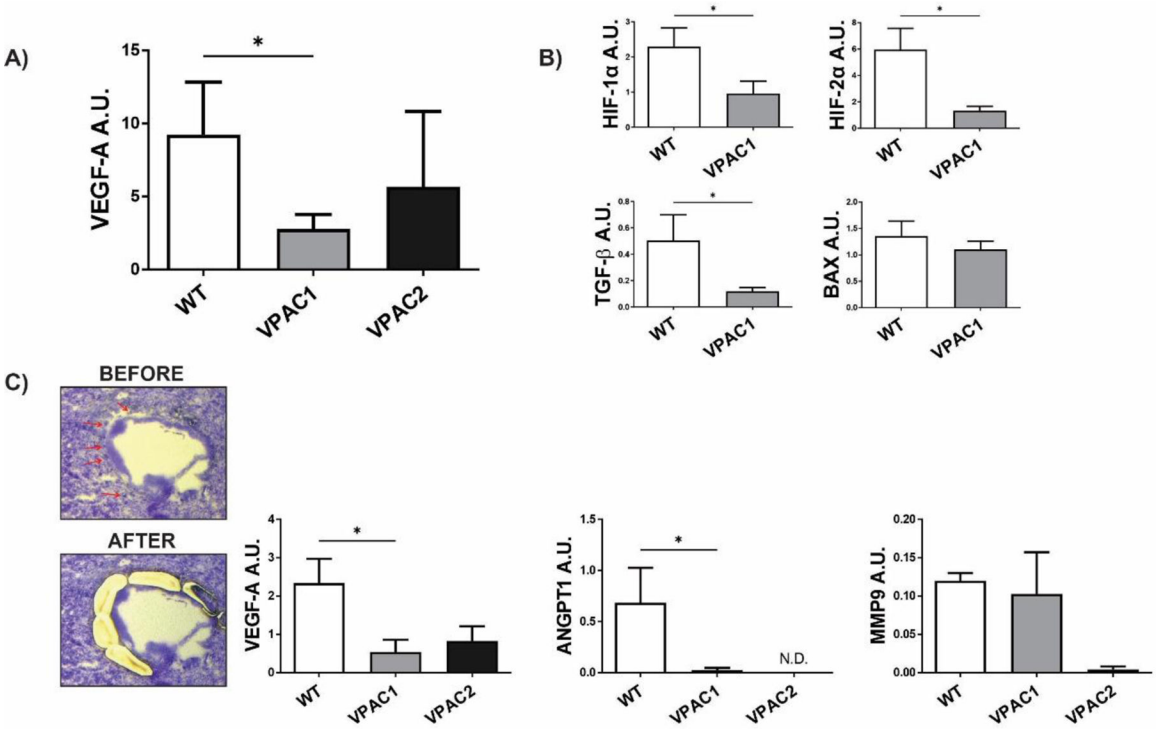

FIGURE 3. Altered vascular related markers in VPAC1 implantation sites.

mRNA expression of (A) VEGF-A and (B) HIF-1α, HIF-2α, TGF-β and BAX were evaluated by quantitative RT-PCR in implantation sites using S29 as a housekeeping gene. Values represent mean ± SEM of at least five pregnant mice of each strain, *$p < 0.05$. C) Photographs show sections of implantation sites before and after TGC isolation using laser-capture microdissection. Red arrows indicate TGCs layer harvested for mRNA analysis. Shown on the right panels of (C) are gene expression values of VEGF-A, ANGPT1 and MMP9 evaluated by quantitative RT-PCR in TGCs. Values represent mean ± SEM of at least three pregnant mice of each strain, *$p < 0.05$.

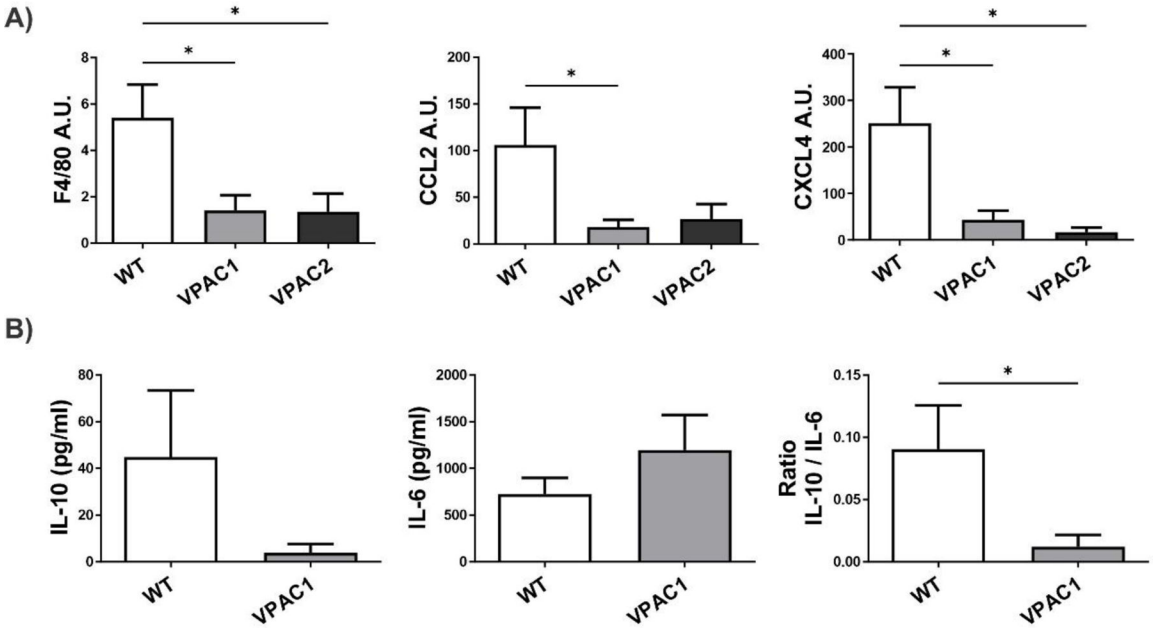

FIGURE 4. Proinflammatory microenvironment in VPAC1 and VPAC2 KO implantation sites.

A) Expression of F4/80, CCL2 and CXCL4 mRNA were evaluated by quantitative RT-PCR in implantation sites. Values represent mean ± SEM of at least five pregnant mice of each strain, *p < 0.05. B) Implantation sites from VPAC1 KO mice were cultured for 8 hours, and supernatants collected. IL-6 and IL-10 were assessed by ELISA. Values represent mean ± SEM of at least five implantation sites from different pregnant mice of each strain, *p < 0.05.

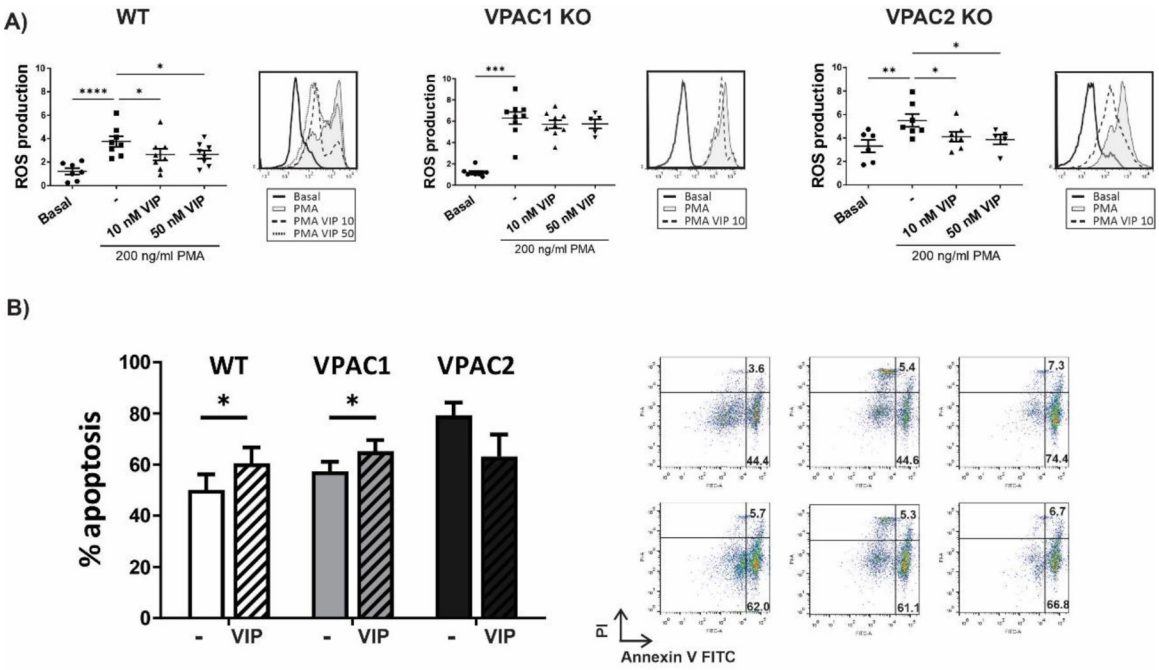

FIGURE 5. Neutrophils from pregnant VPAC KO mice present different functional phenotype compared to WT mice.

A) Isolated neutrophils were stimulated with 200 ng/ml PMA for 45 min to induce the production of ROS which was quantified using the cell permeable fluorogenic probe 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) and detected by flow cytometry. PMA stimulation was carried out in the presence or absence of VIP (10–100 nM). B) Neutrophils were treated or not with 10nM VIP and apoptosis was evaluated after 20 h using Annexin V-FITC / Propidium Iodide staining and flow cytometry. Values represent mean ± S.E.M. of at least 4 mice of each strain. Representative histograms (A) and dot plots (B) are shown.

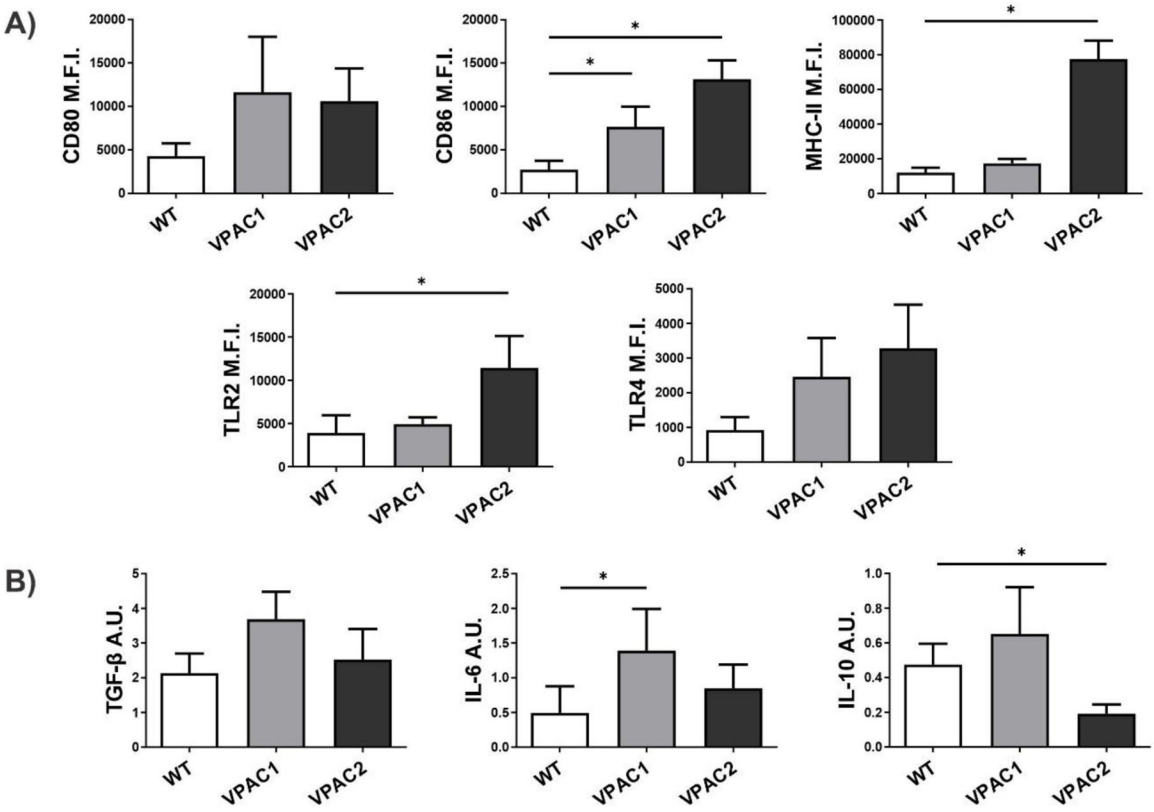

FIGURE 6. VPAC deficient peritoneal macrophages present a proinflammatory phenotype.
Maternal peritoneal macrophages were recovered at day 7.5 or 8.5 and flow cytometry (A) or PCR (B) was performed. Values represent mean ± SEM of at least 3 different pregnant mice of each strain, *$p < 0.05$.

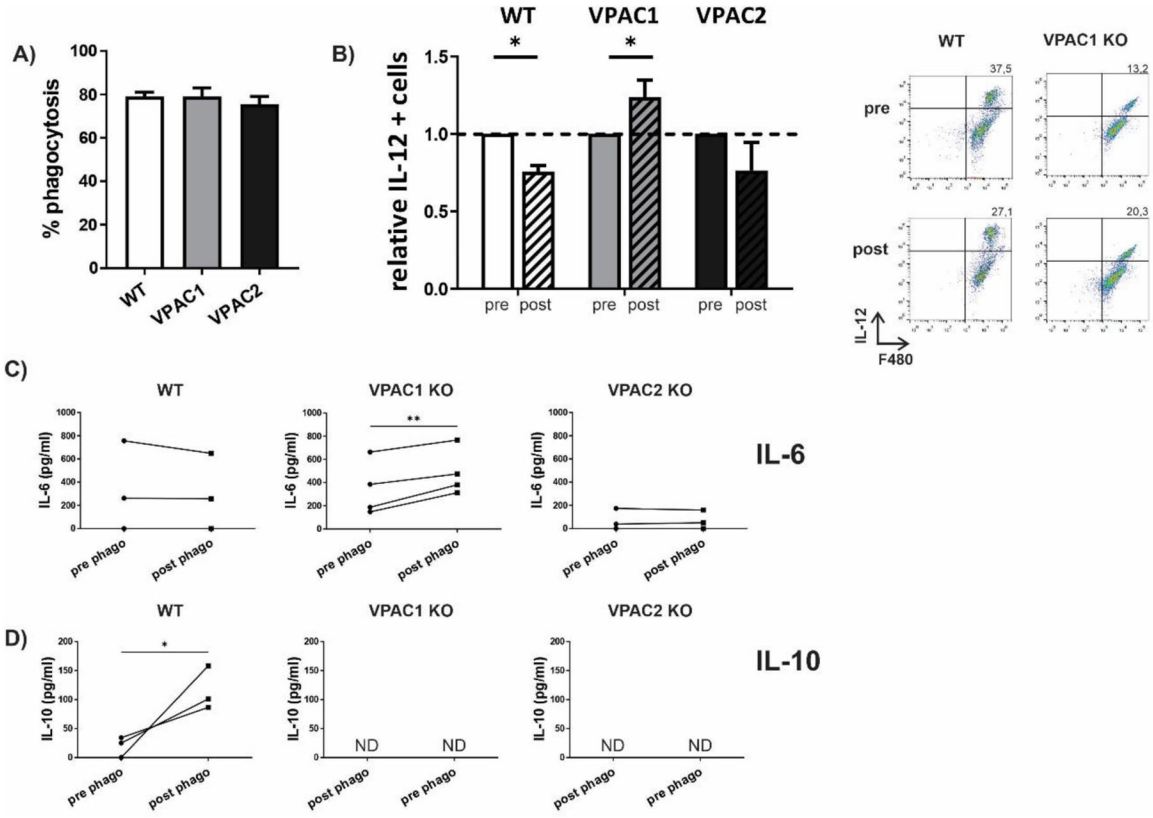

FIGURE 7. Proinflammatory phagocytosis in VPAC deficient peritoneal macrophages.

A) Macrophages were cultured with latex-beads-FITC conjugated for 90 min. Percentage of phagocytosis was evaluated by double staining with F4/80 PerCP. B) Intracellular staining of IL-12 was performed and evaluated by flow cytometry. C and D) Supernatants were collected before and after phagocytosis to perform ELISA according to manufacturer's instructions.

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD80	Biolegend	104713
CD86	Biolegend	105011
MHC-II	ThermoFisher	14-5321-82
TLR2	Biolegend	148603
TLR4	Biolegend	145403
F4/80	ThermoFisher	17-4801-82, 45-4801-82
Ly6G	ThermoFisher	45-5931-80
CD11b	ThermoFisher	53-0112-82
IL-12	ThermoFisher	53-7123-82
ELISA		
IL-10	BD Biosciences	555252
IL-6	BD Biosciences	550950
TNF	BD Biosciences	560478
Reagents		
Phorbol 12-myristate 13-acetate	Sigma Aldrich	P1585
Vasoactive Intestinal Peptide	BACHEM	4011611
2′,7′-Dichlorofluorescin diacetate	Sigma Aldrich	D6883
Annexin V Apoptosis Detection Kit	BD Biosciences	556547
Latex beads, fluorescent yellow-green	Sigma Aldrich	L1030
Culture reagents		
RPMI 1640 Medium	ThermoFisher	12633020
Fetal Bovine Serum	ThermoFisher	16140089
Histopaque-1077	ThermoFisher	10771
Histopaque-1119	ThermoFisher	11191
PCR		
PicoPure RNA Isolation Kit	ThermoFisher	KIT0214
DNAase I	ThermoFisher	EN0521
M-MLV Reverse Transcriptase	ThermoFisher	M1302
FastStart SYBR Green Master	Sigma Aldrich	4673484001

TABLE 1.

Primers used in RT-PCR assays.

Primer sequence, 5′ – 3′.

Gene	Forward	Reverse
S29	GGAGTCACCCACGGAAGTTCGG	GGAAGCACTGGCGGCACATG
GAPDH	TGATGACATCAAGAAGGTGGTGAAG	TCCTTGGAGGCCATGTAGGCCAT
VEGF-A	CACGACAGAAGGAGAGCAGAAG	CTCAATCGGACGGCAGTAGC
HIF-1α	ACCTTCATCGGAAACTCCAAAG	CTGTTAGGCTGGGAAAAGTTAGG
HIF-2α	CTGAGGAAGGAGAAATCCCGT	TGTGTCCGAAGGAAGCTGATG
TGF-β	GACTCTCCACCTGCAAGACCA	TTGGGGGACTGGCGAGCCTT
BAX	GCCTTGGACTGTGTCTTTTCTTC	CCAAAGTGGACCTGAGGTTTATTG
ANGPT1	GGGGGAGGTTGGACAGTAA	CATCAGCTCAATCCTCAGC
MMP9	CCATGCACTGGGCTTAGATCA	GGCCTTGGGTCAGGCTTAGA
CXCL4	GCGTCGCTGCGGTGTTTC	ATCCCAGAGGAGATGGTCTTCC
CCL2	TCTCTTCCTCCACCACCA	GCTCTCCAGCCTACTCATT
F4/80	CTCCAGCACATCCAGCCAAAG	CCAGAAGCCATGATGATAGCCAAG
IL-10	GTTGCCAAGCCTTATCGGAAATG	CACTCTTCACCTGCTCCACTG
IL-6	ACCGCTATGAAGTTCCTCTCTGC	AGTGGTATCCTCTCTGTGAAGTCTCC