Dissertation

Hofbauer cells across preeclampsia subtypes: immune programming, effector functions, and TGF-β signaling

submitted by

Monika HORVAT MERČNIK, Mag. Mikrobiol. dipl. mikrobiol.

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Department of Obstetrics and Gynecology

under the supervision of

Prof. Dr. Christian WADSACK

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STATUTORY DECLARATION

I hereby declare that this thesis is my original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Furthermore, I hereby declare that if artificial intelligence (AI) tools were used for the generation and/or correction of certain text passages in the creation of this work, such employment was conducted in compliance with ethical principles, academic integrity, and the regulations of my university. Additionally, it was ensured that this usage was transparently disclosed and appropriately attributed.

Graz, 13.04.2026 Monika Horvat Merčnik

Disclosures

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Co-authors contribution:

Fluhr H [1] contributed to data interpretation and edited the manuscript.

Sanchez-Duffhues G [2] contributed to conceptualization of the manuscript writing and editing

Schliefsteiner C [1] supported in designing concepts, supervised experimental performance of all projects, contributed to manuscript writing and editing

Wadsack C. [1,3] supported in designing concepts, supervised experimental performance of all projects, contributed to manuscript writing and editing

1 Perinatal Research Laboratory, Department of Obstetrics and Gynecology, Medical

University of Graz, Graz, Austria

2 Nanomaterials and Nanotechnology Research Center (CINN-CSIC), Tissue-specific BMP signalling ISPA-HUCA, Avda. de Roma, s/n. 33011-Oviedo, Spain

3 BioTechMed-Graz, Graz, Austria

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Table of content

Abbreviations

ACTA2 – smooth muscle actin alpha 2

ACVR – activin A receptor

AKT – protein kinase B

ALK – activin receptor-like kinase

ARG1 – arginase 1

BMI – body mass index

BMPR – bone morphogenetic protein receptor

BMP – bone morphogenetic protein

CAT – catalase

CD – cluster of differentiation

CK7 – cytokeratin 7

CT – cytotrophoblast

DC-SIGN – dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DNase I – deoxyribonuclease I

ECM – extracellular matrix

ECA – fetoplacental arterial endothelial cells

ECV – fetoplacental venous endothelial cells

EGFR – epidermal growth factor receptor

EMT – epithelial-to-mesenchymal transition

ENG – endoglin

EndoMT – endothelial-to-mesenchymal transition

EO-PE – early-onset preeclampsia

EVT – extravillous trophoblast

Fc – fragment crystallizable region of immunoglobulin

FACS – fluorescence-activated cell sorting

FOLR2 – folate receptor beta

FSC – forward scatter

hCG – human chorionic gonadotropin

hPL – human placental lactogen

HBC – Hofbauer cell

HLA – human leukocyte antigen

HSP70 – heat shock protein 70

IFN – interferon

IL – interleukin

iNOS – inducible nitric oxide synthase,

IRF – interferon regulatory factor

JAK – Janus kinase

KDR – kinase insert domain receptor

LO-PE – late-onset preeclampsia

LPS – lipopolysaccharide

MAPK – mitogen-activated protein kinase

MHC II – major histocompatibility complex class II

MMP – matrix metalloproteinase

NAD(P)H – nicotinamide adenine dinucleotide (phosphate),

NF-κB – nuclear factor kappa B

NK – natural killer

NOS2 – nitric oxide synthase 2

PAMM – placenta-associated maternal macrophage

PE – preeclampsia

PI3K – phosphoinositide 3-kinase

PlGF – placental growth factor

PPAR – peroxisome proliferator-activated receptor

ROS – reactive oxygen species

ST – syncytiotrophoblast

sFlt-1 – soluble fms-like tyrosine kinase 1,

SMA – smooth muscle actin

SOD – superoxide dismutase

SSC – side scatter

STAT – signal transducer and activator of transcription

TGF – transforming growth factor

Th17 – T helper 17 cell

TIMP – tissue inhibitor of metalloproteinases

TLR – Toll-like receptor

TNF – tumor necrosis factor

Treg – regulatory T cell

VEGF – vascular endothelial growth factor

VWF – von Willebrand factor

Zusammenfassung

Hofbauer Zellen (HBCs) sind fetoplazentare Makrophagen im villösen Stroma des Plazentagewebes. Durch ihre hohe Plastizität und ihre Fähigkeit auf lokale, mikroumgebungsabhängige Signale zu reagieren, unterstützen HBCs die Gewebehomöostase, einschließlich der Beseitigung von Zelldebris und des Gewebeumbaus. Präeklampsie (PE) bezeichnet man als das Auftreten einer Hypertonie in der Schwangerschaft verbunden mit mindestens einer weiteren Organmanifestation in der Mutter. PE geht mit einer schlechten Durchblutung der Plazentazotten, einem Ungleichgewicht in der Bildung und dem Umbau von Blutgefäßen, sowie einer Aktivierung von Entzündungssignalen einher, welche auch die Plazentafunktion beeinträchtigen. Basierend auf dem Gestationsalter zum Zeitpunkt der Diagnose wird PE üblicherweise in früh auftretende (engl. early onset, EO) und spät auftretende (engl. late onset, LO) PE unterteilt. Da sich diese Subtypen in Bezug auf die Plazentapathologie, den klinischen Verlauf und dem Einfluss auf den wachsenden Fetus unterscheiden, analysieren wir sie in der vorliegenden Studie getrennt. Die Hypothese lautet, dass sich die Polarisation und die Effektorfunktionen der HBCs zwischen EO- und LO-PE unterscheiden. Die Ursache dieser Veränderungen da könnten mit einer veränderten TGF-β abhängigen Signalübertragung an HBCs in Zusammenhang stehen.

Zunächst wurde ein optimierter Arbeitsablauf für die Isolierung und Qualitätskontrolle von HBCs aus termingerecht geborenen Plazenten entwickelt, um eine zuverlässige -Profilierung zu ermöglichen. Das vorliegende Protokoll umfasst eine präzise Dissektion der Villi, die Entfernung der Dezidua, eine kontrollierte enzymatische Dissoziation, eine Percoll-basierte Anreicherung sowie eine Durchflusszytometrie nach der Isolierung. Die Qualitätskontrolle ist als Kernkomponente des Arbeitsablaufs integriert und umfasst die Überprüfung der Reinheit, Identität und Eigenschaften der Zellen, sowie den Ausschluss wichtiger Kontaminanten.

Der hier beschriebene Arbeitsablauf demonstriert, dass PE die Polarisierung und Funktion von HBCs beeinflusst. Die Ergebnisse zeigen, dass es nicht einen einzigen, einheitlichen Phänotyp gibt, sondern, dass EO- und LO-PE sich unterschiedlich auswirken. EO-PE HBCs

zeigten ein gewebeumgestaltendes M2-Profil mit einer verstärkten Expression von entzündlichen Komponenten. Diese Beobachtung wurde durch höhere Werte von CD86, TLR-4, HLA-DR, IRF5 und *NOS2* gestützt, wobei regulatorische Merkmale wie *ARG1* und eine erhöhte TGF-β1-Sekretion beibehalten wurden. LO-PE zeigte ein eher phagozytotisches, CD209-armes M2-Profil mit einer weniger ausgeprägten Entzündungsaktivierung, einer stärkeren IRF4- und *ARG1*-Expression, einer erhöhten TGF-β1-Sekretion und einer verstärkten Phagozytose. Bei beiden Subtypen blieben die Kernfunktionen der Phagozytose und der Matrixumgestaltung aktiv, was für die aufrechterhaltene Plastizität mit krankheitsassoziierter Modifikation spricht.

Im weiteren Verlauf wurde wurde die Signalübertragung der TGF-β-Superfamilie in gesunden Plazenten sowie in Plazenten mit PE untersucht und dabei Trophoblasten, Endothelzellen und Immunzellen analysiert. Im Anschluss daran erfolgte eine Interpretation der Ergebnisse in Bezug auf diese Muster. Im Rahmen einer physiologischen Schwangerschaft unterstützt die TGF-β-Signalübertragung verschiedene Prozesse, darunter die Differenzierung und Invasion von Trophoblasten, das angiogene Gleichgewicht sowie die Endothelfunktion. Daüber hinaus fördert sie die Immuntoleranz, indem sie die Auswirkungen auf regulatorische T-Zellen (Tregs), deziduale natürliche Killerzellen (dNK-Zellen) und die Makrophagenpolarisation koordiniert. Im Rahmen dieser Studie wurde durch Verfügbarkeit von Liganden und der Rezeptorzusammensetzung eine Störung der TGF-β-Signalübertragung bei PE beobachtet. Diese Beobachtungen stehen im Zusammenhang mit einer beeinträchtigten Immunregulation und vaskulären Dysfunktion bei PE und verbindet die veränderte Signalübertragung der TGF-β-Superfamilie mit den in dieser Studie beobachteten HBC-Phänotypen der humanen Plazenta.

Zusammenfassend wurde ein zuverlässiges Protokoll zur Isolierung und Qualitätskontrolle von HBCs entwickelt und angewendet, um unterschiedliche, vom Zeitpunkt der klinischen Manifestation abhängige phänotypische und funktionelle HBC-Profile bei PE zu identifizieren, die mit einer erhöhten TGF-β1-Sekretion verbunden sind. Die vorliegende Arbeit zeigt eine signifikante Lücke in der Signalübertragung, die durch die Integration der Ergebnisse mit der berichteten Störung der TGF-β-Superfamilien-Signalübertragung bei PE entsteht. Die Rolle der TGF-β-Superfamilien-Signalübertragung in HBCs bedarf weiterer Klärung. Es sind weitere Untersuchungen erforderlich, um die Bioverfügbarkeit von

Liganden und das Rezeptorgleichgewicht in Bezug auf Makrophagen-Effektorfunktionen bei nach ihrem Auftreten stratifizierter PE zu untersuchen.

Abstract

Hofbauer cells (HBCs), are resident macrophages in the villous stroma, that support tissue homeostasis, including debris clearance and tissue remodeling, through high plasticity and responsiveness to the microenvironmental cues. Preeclampsia (PE) is a heterogeneous placental disorder that is characterized by new onset of hypertension during pregnancy, accompanied by at least one additional maternal organ manifestation. It is associated with villous malperfusion, angiogenic imbalance, and inflammatory activation, all of which impair placental function. Based on gestational age at diagnosis, PE is commonly subdivided into early-onset (EO-) and late-onset (LO-) PE. These two subtypes differ in terms of placental pathology, clinical course, and fetal outcome. We hypothesized that the HBCs polarization and effector functions differ between EO- and LO-PE and that these onset-dependent patterns are linked, at least in part, to altered TGF-β superfamily signaling within the villous niche.

First, we developed an optimized HBCs isolation and quality control workflow for term placentas to enable reliable profiling. This protocol incorporated strict villous dissection, decidua removal, controlled enzymatic dissociation, Percoll-based enrichment, and flow cytometry following isolation. Quality control was embedded as a core component of the workflow and included viability assessment, purity and identity verification, and exclusion of major contaminants. We demonstrate that PE is associated with onset-dependent changes in HBC polarization and function rather than the development of a single, uniform phenotype. EO-PE exhibited a tissue-remodeling M2 program with a stronger inflammatory component, as evidenced by higher levels of CD86, TLR-4, HLA-DR, IRF5, and *NOS2*, while maintaining regulatory features, such as *ARG1*, and increased TGF-β1 secretion. LO-PE exhibited a more phagocytic, CD209-low M2 profile, demonstrating less pronounced inflammatory activation, stronger IRF4 and *ARG1* expression, together with increased TGF-β1 secretion and enhanced phagocytosis. Across both subtypes, the core functions of phagocytosis and matrix remodeling remained active which supports maintained plasticity with disease-associated skewing.

Next, we reviewed TGF-β superfamily signaling in healthy and PE placentas, examining the trophoblast, endothelial, and immune compartments. Then, we interpreted our findings in relation to these TGF-β patterns. In a healthy pregnancy, TGF-β signaling supports trophoblast differentiation and invasion, angiogenic balance, and the endothelial phenotype. It also promotes immune tolerance by coordinating the effects on Tregs, decidual NK cells, and macrophage polarization. In PE, disruption of TGF-β superfamily signaling is reported at the levels of ligand availability and receptor composition. This is consistent with impaired immune regulation and vascular dysfunction in PE, linking altered TGF-β superfamily signaling to the HBCs phenotypes observed in our study.

In conclusion, we developed a reliable HBCs isolation and quality control protocol and applied it to identify distinct, onset-dependent phenotypic and functional HBCs profiles in PE, accompanied by increased TGF-β1 secretion. Integrating these results with the reported disruption of TGF-β superfamily signaling in PE reveals a significant signaling gap. The role of TGF-β superfamily signaling in HBCs remains unclear and further research is needed to investigate ligand bioavailability and receptor balance in relation to macrophage effector programs in onset-stratified PE.

Introduction

The villous placenta - an immune-vascular organ

The placenta is a unique and essential organ that connects the mother and the developing fetus (8). It supports fetal development by enabling the exchange of gases and nutrients, the removal of waste products, and endocrine signaling (9). Development of the human placenta begins shortly after implantation from the trophectoderm, and involves the fusion of trophoblasts to establish the maternal-fetal interface (10). During early gestation, trophoblast differentiation, villous development, and maternal uterine adaptation occur simultaneously in order to form an organ that facilitates highly efficient exchange (9,11). Before full placental perfusion is established, extraembryonic tissues such as the yolk sac contribute to early embryonic support and hematopoiesis (12).

Trophoblasts differentiate into specialized subtypes with distinct roles (10). Some cytotrophoblasts (CTs) proliferate and fuse to form the multinucleated syncytiotrophoblast (STs), which cover the villous surface and form the primary exchange barrier (10). STs secrete pregnancy hormones such as human chorionic gonadotropin beta (hCGβ) and placental lactogen (hPL) (13,14). In anchoring villi, CTs form columns of trophoblasts that generate extravillous trophoblasts (EVTs) (10). The differentiation of EVTs includes an epithelial-to-mesenchymal transition (EMT)-like program that supports their migration into the decidua and contributes to spiral artery remodeling (15). Coordinated trophoblast invasion and vascular remodeling are central to normal placentation (16).

In parallel, the chorionic villi branch and mature, thereby increasing the surface area available for exchange (17). Fetal vessels develop within the villous core, extending into the terminal villi (18). In this area, the process of dense capillarization brings fetal blood close to the surface of trophoblasts (17). The villous stroma contains fetal immune cells, including Hofbauer cells (HBCs), a type of placental macrophages (19). HBCs have been observed to be present throughout gestation and located in close proximity to the trophoblast and fetal vessels (20). This positioning enables HBCs to contribute to stromal homeostasis, debris clearance, and immune regulation within the villous core (21).

Pregnancy requires tightly regulated immune adaptation given the semi-allogeneic nature of fetal tissue (22). Maternal immune cells in the decidua, including NK cells, decidual macrophages, and T cell subsets, interact with EVTs and contribute to tolerance, tissue remodeling, and host defense (23–25). In parallel, fetal HBCs have been demonstrated to facilitate villous homeostasis within the chorionic villi (26). The development of the placenta is depending on the coordinated regulation of the immune system across the decidua and the villous stroma. The functional integration between these compartments is mediated by direct cell-to-cell interactions and the secretion of soluble factors, including cytokines and growth factors (22,24).

By term, the placenta has a well-defined villous structure. The side of the tissue towards the fetus consists of the chorionic plate, which is covered by the amnion and connected to the fetus by the umbilical cord. The side attached to the maternal uterus is formed by the basal plate (18). The intervening compartment, known as the intervillous space, is the primary site of exchange between the mother and the developing fetus. It contains branching villous trees arranged into distinct cotyledons that are connected to the fetal circulation through chorionic vessels (17). Together, this organization enables efficient maternal-fetal exchange, defining the villous placenta as a highly vascularized organ (9,27). Importantly, placental exchange occurs across two anatomically distinct circulations: the maternal uteroplacental circulation and the fetal fetoplacental circulation (28). Although maternal and fetal blood come into close proximity at the villous surface, they remain strictly separated. Maternal blood perfuses the intervillous space, while fetal blood remains within the placental vasculature. Consequently, exchange occurs across the placental barrier (29,30). To support this function, uteroplacental vascular remodeling establishes a low-resistance, high-capacitance perfusion system, and the fetoplacental circulation adapts to meet the growing demands of the fetus. Deoxygenated blood reaches the placenta through the umbilical arteries and oxygenated blood returns to the fetus through the umbilical vein (28,31). Throughout gestation, these structural and hemodynamic adaptations are accompanied by local immune and microenvironmental signals that continuously shape cellular phenotype and function (9,32).

Polarization of macrophages as a local milieu-dependent process

Macrophages play a key role in regulating inflammation, host defense, tissue repair, and homeostasis. A major feature of macrophages is their plasticity, evident in their capacity to alter their polarization and functionality in response to local stimuli (33). Although the activation of macrophages is often described along the M1 to M2 polarization axis, tissue macrophages *in vivo* rarely fit into fixed condition (34). Instead, they exhibit overlapping and intermediate phenotypes, which are influenced by factors such as cytokines, metabolites, oxygen availability, extracellular matrix signals, and tissue injury (35).

M1-associated polarization is typically induced by inflammatory stimuli, such as lipopolysaccharide (LPS) and interferon-gamma (IFN-γ), and is associated with inflammatory cytokine production, antigen presentation, and antimicrobial activity (36,37). In contrast, M2-associated polarization is promoted by factors such as interleukin-4 (IL-4), IL-13, IL-10, transforming growth factor-β (TGF-β), and glucocorticoids (35,38–40). It is associated with immune regulation, tissue remodeling, angiogenesis, and repair. In tissues, including the placenta, macrophage phenotypes are more accurately defined by the combination of polarization and functional readouts than by single markers alone (41,42). These readouts include secretome of cytokines and chemokines, phagocytosis and efferocytosis, metabolic programs, and sustained transcriptional regulation (Figure 1).

A key biochemical axis linked to macrophage polarization involves arginine metabolism through inducible nitric oxide synthase (iNOS) and arginase-1 (ARG1). iNOS is associated with M1-like polarization, where it converts arginine into nitric oxide and citrulline and thereby supports inflammatory and antimicrobial responses (43–45). In contrast, ARG1 is typically associated with M2-like polarization, where it converts arginine into ornithine and urea, thereby supporting tissue remodeling and repair (46,47). *In vivo*, macrophages engage in both polarization pathways to varying degrees, and changes in their relative activity are accompanied by changes in inflammatory versus homeostatic function (42).

Macrophage functionality and polarization states are further regulated by complex signaling pathways and transcriptional networks. Key pathways include Toll-like receptor (TLR-), Janus kinase (JAK), and signal transducer and activator of transcription (STAT), TGF-β and SMAD, peroxisome proliferator-activated receptor gamma (PPARγ)-linked lipid sensing; Notch, and non-coding RNA networks (48–51). Transcription factors such as interferon regulatory factor 5 (IRF5) and IRF4 have been associated with inflammatory and regulatory macrophage programs, respectively (42,51–54). Metabolic and redox regulation further shape macrophage behavior, including the production of reactive oxygen species (ROS) and the regulation of antioxidant defenses by enzymes such as superoxide dismutase and catalase (55–58). Epigenetic mechanisms provide an additional layer of control over polarization associated gene expression through DNA methylation and histone modifications (59,60). Collectively, these regulatory mechanisms support macrophage polarization as a dynamic and reversible spectrum of phenotypes, that adapts to change in cytokine signals, metabolic stress, oxygen availability, and tissue injury, thereby maintaining host defense, tissue repair, and homeostasis (58).

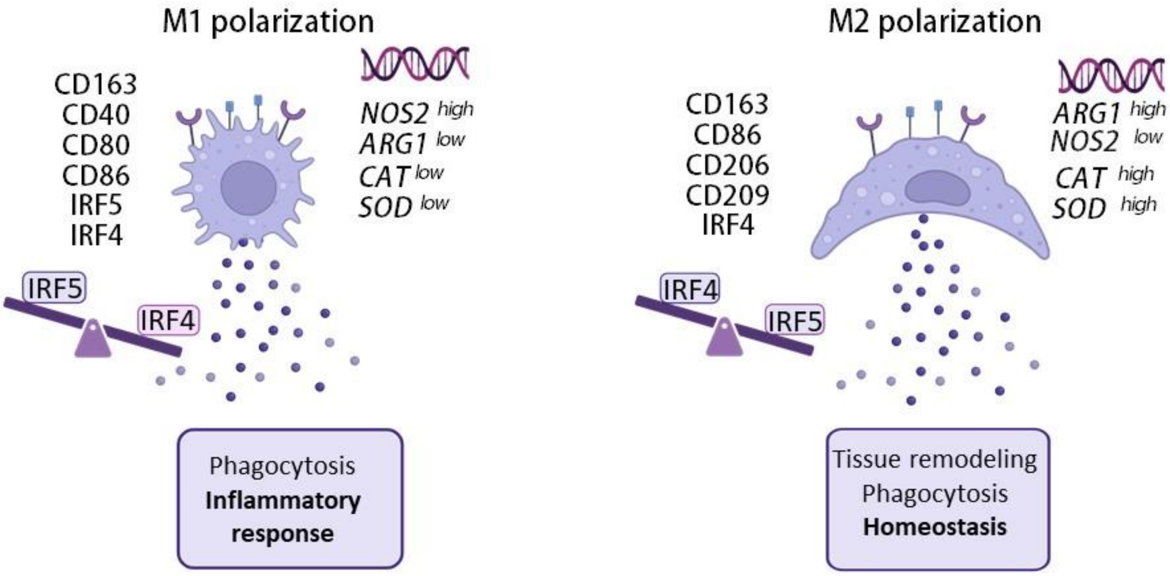

Figure 1: Overview of the general M1 and M2 macrophage polarization concept. Marker profiles are shown next to the macrophages on the left, and representative expressed genes are shown on the right. The bottom section summarizes the main functions and secreted molecules associated with each polarization state. Figure was created with BioRender.

Hofbauer cells – tissue resident macrophages of the villous stroma

Hofbauer cells (HBCs), are fetoplacental macrophages of the villous stroma that appear early in gestation, around day 18 after conception (61). Current evidence suggests that HBCs origin from early primitive hematopoiesis. The characteristics of HBCs resembles yolk sac-derived macrophages rather than macrophages derived from circulating monocytes (62–64). Morphologically, HBCs are large cells, typically ranging from 10 to 30 µm in diameter (26,65). They have abundant vacuoles and granular cytoplasm and can appear round, pleomorphic, or stellate (66,67). This pleomorphism is consistent with the concept of macrophage plasticity and reflects local microenvironmental cues within the villous niche (63,68).

HBCs have been observed in proximity to trophoblasts and fetal villous vessels (67). In first trimester villi, ultrastructural studies have shown that they are located within channel-like stromal spaces (17,69). This finding is consistent with their motility within the stroma during villous expansion and reorganization (65). This localization supports their roles in clearing debris, limiting danger signal amplification, and supporting stromal and vascular development (21,26,67). This is also consistent with their reported association with early vasculogenic and angiogenic structures (21).

Phenotypically, HBCs are widely described as M2-like macrophages with dominant anti-inflammatory and tissue-remodeling properties and limited M1 specifics (68,70). In addition, HBCs have been found to express macrophage markers, including CD68 and Fc gamma receptors (Fcγ), with CD64 being a notable example (60,71). They also express scavenger and lectin-associated receptors, which are involved in clearance and tissue support (72). The following receptors have been identified: CD163, CD206/MRC1, and frequently, CD209/DC-SIGN (68,70,72,73). In the early stages of gestation, HBCs are frequently characterized by high-affinity binding sites for folate receptor β (FOLR2) and low or absent expression of the human leucocyte antigen-DR (HLA-DR) (71). This distinction facilitates the identification of fetal HBCs and maternal macrophages in placental digests. As gestation progresses, HBCs become increasingly heterogeneous, with the emergence of subsets exhibiting elevated HLA-DR expression. This is consistent with their augmented range of immune functions (64,66,68,71).

In healthy placentas, HBCs exhibit an ARG1-linked polarization pattern and produce mediators such as IL-10 and TGF-β (59,66,68,72). Furthermore, HBCs have been demonstrated to produce factors associated with villous vascular development and remodeling. These factors under discussion include vascular endothelial growth factor-A (VEGF-A), osteopontin, metalloproteinase-9 (MMP9), and Sprouty family proteins (21,71,74)**.** This phenotype supports efficient phagocytosis and efferocytosis, accompanied by a recurrent reduction in inflammatory output, a process that is critical for maintaining villous homeostasis. In this context, continuous clearance and repair of trophoblast renewal, cell death-associated debris, and extracellular matrix remodeling are imperative (58,71,75,76).

During pregnancy, HBCs have been demonstrated to support villous homeostasis, vascular maintenance, and immune defense (58,68,77,78). These cells maintain a predominantly regulatory phenotype, whilst concurrently expressing functional TLR-receptors, including TLR-2, TLR-3, TLR-4, TLR-7, and TLR-8. This capacity enables the cells to elicit antimicrobial responses and to sense viruses (71,79). This combination of regulatory and host-defense functions is a key feature of HBCs biology across gestation (78).

HBCs represent a significant immune cell population within the placenta. Several maternal immune myeloid populations are also present in placental tissue, which must be distinguished from fetal HBCs (71). Maternal immune cells, including decidual macrophages, populate the decidua and interact with EVTs (64,71). These macrophages have been shown to contribute to tissue remodeling, vascular adaptation, and immune tolerance. Additionally, placenta-associated maternal macrophages (PAMMs) have been observed to adhere to the STs on the villous surface. PAMM populations include monocyte-like cells and a macrophage-like subset, PAMM1a, which exhibits a repair-oriented program (71). These cells have been shown to localize to sites of syncytial damage and fibrin deposition, suggesting a role in preserving the syncytial surface (64,71,80,81). Since these maternal populations contribute substantially to CD14-positive cells in placental digests, rigorous fetal–maternal separation is crucial to prevent the misattribution of maternal macrophages to HBCs, particularly during the early stages of gestation (71,80).

Hofbauer cell isolation and its quality control

The biological composition of HBCs has been predominantly studied by the utilization of histological techniques and related *in situ* approaches. These methodologies are essential for localization and morphological assessment, yet they provide only limited insight into functional behavior and signaling outputs (65). Consequently, the utilization of *ex vivo* isolation methodologies have emerged for the purpose of conducting phenotypic profiling and subsequent downstream assays. A central challenge is that isolation itself can alter the phenotype of macrophages (81). Therefore, a robust isolation methodology is employed in order to ensure the preservation of viability, the achievement of adequate cell yield, and rigorously control of contamination by other placental cell populations and maternal macrophages.

The majority of isolation workflows include the following steps: careful villous dissection, removal of decidual tissue, dissociation of the tissue to generate a single-cell suspension, density-based mononuclear enrichment, and flow cytometry-based phenotyping or purification (70,80,82–84). The disparities observed in the protocols employed for first-trimester and term placentas are primarily attributed to the influence gestational age on tissue mass and composition, in addition to variations in validation of fetal origin (71). Additional variation arises from dissociation conditions, density gradient media, magnetic bead selection strategy (positive or negative) and the stringency used to exclude maternal cells (70,80,82,84).

It is important to acknowledge that the processing of tissue samples during the isolation procedure has the potential to induce substantial alterations in the HBC activation and phenotype. Consequently, the implementation of rigorous quality control measures constitutes a pivotal component within the study design (80). Key elements include viability assessment, reporting of activation-associated markers, fetal origin validation using HLA allotypes or sex chromosome genotyping, and exclusion of major non-myeloid contaminants (80,84). Common orthogonal quality control procedures include the use of markers for the exclusion of trophoblasts, stromal, and vascular contaminants, together with immunocytochemistry for confirmatory purposes (68,80). These steps are followed in order to guarantee that the downstream phenotype and functional readouts accurately reflect the biology of HBCs, as opposed to bias that may be induced by isolation.

Preeclampsia - a heterogeneous placental disorder with immune and microenvironmental imbalance

Preeclampsia (PE) is a heterogeneous disorder that develops after 20 weeks of gestation and is defined by new-onset hypertension (systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg) (85). The clinical manifestations include proteinuria, thrombocytopenia, renal impairment, liver dysfunction, pulmonary edema, and neurological complications (86). PE has been observed to affect approximately 5 - 10 % of pregnancies worldwide (87). It has been identified as a leading cause of preterm delivery, fetal growth restriction, and is associated with an increased long-term cardiovascular risk for both the mother and her offspring (88–90). The only definitive treatment is delivery, while all other management efforts aim to prevent maternal and fetal complications and optimize the timing of delivery (85,86).

It is widely accepted that placental dysfunction and the maternal response to placental stress are considered key factors in the pathogenesis of PE (91). In PE, shallow EVT invasion and incomplete spiral artery remodeling occurring in the first trimester lead to reduced uteroplacental perfusion. This, in turn, has been shown to promote a villous microenvironment characterized by intermittent hypoxia, oxidative stress, and endoplasmic reticulum stress (91,92). These cues have been demonstrated to activate inflammatory signaling and alter cell-to-cell communication across the trophoblast, stromal, and fetoplacental vascular compartments (93,94). A significant systemic consequence of this condition is angiogenic dysfunction, which is driven by the placental release of anti-angiogenic mediators, such as soluble sFlt-1 and soluble endoglin (sENG) (95,96). These mediators have been shown to reduce VEGF and PlGF activity, contributing to endothelial dysfunction (95,97).

Immune dysregulation constitutes a central element the pathophysiology in PE (98). The maintenance of a healthy pregnancy is contingent upon the regulation of innate and adaptive immune responses, thereby facilitating tolerance, controlled remodeling, and host defense (22–25). However, in the context of PE, this equilibrium is shifted towards an inflammatory response, as evidenced by the altered regulation of macrophages and lymphocytes, reduced tolerogenic control, and increased inflammatory mediators (99,100). These factors contribute to the exacerbation of trophoblast stress and vascular injury (101–103). In line, molecular profiling indicates that PE is mechanistically heterogeneous, with variable contributions from malperfusion-related stress, angiogenic dysfunction, immune activation, and maternal susceptibility (92,104–107).

Clinically, the condition is commonly subdivided into two distinct categories: early-onset PE (EO-PE, diagnosed before 34 weeks) and late-onset PE (LO-PE, diagnosed from 34 weeks onwards) (108). The evidence suggests that EO-PE is more consistently associated with primary placental pathology, including, but not limited to, impaired placentation, uteroplacental malperfusion, fetal growth restriction, and preterm delivery (109,110). The majority of cases are attributable to LO-PE, which is more frequently associated with maternal susceptibility and manifests later in gestation (109). Despite the varying initiating mechanisms differ, both subtypes ultimately results in endothelial dysfunction and inflammation, accompanied by subtype-specific differences in immune programming (111,112). It has been demonstrated that in cases of EO-PE, there is a greater risk for the manifestation of a more extensive placental immune activation, which has been linked to conditions of hypoxia and oxidative stress. Conversely, in instances of LO-PE, the inflammatory profile that is exhibited is more selective in nature, characterized by a distinct equilibrium of maternal and placental signals (113). In both subtypes, anti-angiogenic mediators, including sFlt-1 and sENG, together with a shift in cytokines toward TNFα, IL-6, IL-1β, IFN-γ, and IL-17 and away from IL-10, shape a villous environment likely sensed by HBCs (72,95,114,115). It has been hypothesized that these local cues may promote HBCs polarization shifts, which in turn may further reinforce inflammation and vascular dysfunction.

Hofbauer cells in preeclampsia

During pregnancy in PE, HBCs are exposed to stress signals and inflammatory mediators in the placenta, including those associated with hypoxia, oxidative stress and cytokine imbalance (116,117). Several *in situ* and *ex vivo* studies reported altered abundance and phenotype of HBCs compared with those in normotensive placentas (73,118). However, the results vary depending on the sampling region, gestational age at delivery, and marker strategy used. Commonly used markers include CD68, CD163, and CD14 (39,68,70,73,118). Studies using additional scavenger and lectin-associated markers have described shifts in specific regulatory feature subsets (72,73).

Reduced CD209/DC-SIGN- and CD163-associated readouts as well as lower IL-10 levels, are consistent findings across studies of PE (72). A smaller number of studies also report altered cytokine secretion and tissue remodeling programs (114,115,119). IL-10 and TGF-β have been identified as integral components of a homeostatic HBC phenotype, which appears to be attenuated in PE (72,120). In conjunction with the altered expression of immune-inhibitory regulators, this supports a shift towards inflammation-associated phenotypes (72,73).

The stratification of PE into the categories of EO-PE or LO-PE available datasets has been shown to suggest the presence of immune and metabolic differences, which are dependent on the specific subtype. In addition, further subtype variation observed is contingent on fetal sex, as evidenced by existing datasets (60,121). EO-PE has been found to be more frequently associated with broader changes in HBC immune programs, including stronger interferon and inflammatory cytokine signaling, increased antigen presentation and phagocytic activity, and altered extracellular matrix and tissue remodeling pathways (113,114). Conversely, LO-PE

HBCs exhibit a retention of anti-inflammatory characteristics, accompanied by selective alterations in cytokine and chemokine secretion (114).

TGF-β superfamily signaling in placental biology in health and preeclampsia

The TGF-β superfamily has been described to play a central role in intercellular signaling in the placenta, linking trophoblast biology, endothelial phenotype, and immune regulation (122). Canonical signaling is mediated through Smad pathways. TGF-β, activins, and nodal typically signal through Smad2/3, while BMP ligands predominantly signal through Smad1/5/8 depending on receptor composition (123). Non-canonical signaling through MAPK, PI3K-AKT, and Rho GTPase pathways further modulates cellular responses, particularly under inflammatory or stressful conditions (124). The final cellular output depends on the availability of ligands, the composition of receptors and co-receptors, and the balance between Smad-dependent and Smad-independent signaling (125).

During early placentation, the TGF-β signaling pathway regulates trophoblast differentiation and the acquisition of the invasive EVT phenotype (126). This contributes to spiral artery remodeling by affecting motility, matrix interactions, and protease-linked remodeling (127–129). Concurrently, TGF-β, activins, and BMPs promote immune adaptation at the maternal-placental interface (122). This process is facilitated by the promotion of regulatory T cell differentiation, the modulation of the phenotype of uterine NK cells, and the regulation of the functions of decidual macrophages involved in clearance, matrix remodeling, and inflammatory control (22–24). The immune and trophoblast processes are tightly linked, as excessive inflammation has been shown to impair trophoblast invasion and vascular remodeling (101,130,131).

As gestation progresses, the TGF-β superfamily continues to coordinate the biology of trophoblasts, endothelial cells, stromal cells, and HBCs (122). In the context of placental endothelial cells, the signaling of TGF-β and BMP plays a regulatory role in angiogenic remodeling, the properties of the barrier, and the maintenance of the endothelial phenotype (132). Within the villous microenvironment, trophoblasts, stromal cells, endothelial cells, and HBCs interact through paracrine signaling to limit excessive inflammation and support tissue repair and remodeling (67,133,134). HBCs are central to this immune-vascular unit, given that, as resident fetal macrophages, they are located in close proximity to villous vessels and stromal structures (67), thereby directly contributing to local ligand availability (119).

HBCs have been found to express components of the TGF-β pathway and secrete active TGF-β1, TGF-β2, and TGF-β3 (119). Furthermore, these cells have also been reported to express BMP2, suggesting that they provide local BMP cues involved in stromal remodeling and vascular development (134). TGF-β is a significant regulator of the macrophage phenotype in both decidual macrophages and HBCs, thereby supporting anti-inflammatory and homeostatic functions (20). Notably, term HBCs have been observed to secrete TGF-β1 during inflammatory activation, thereby indicating that inflammatory polarization does not results in the elimination of TGF-β-mediated immunoregulatory activity (66,68).

In the field of PE, the signaling of the TGF-β superfamily is observed to be compromised across the various placental compartments at multiple levels, including the bioavailability of ligands, the composition of receptors and co-receptors, and the downstream signaling output (122). Altered TGF-β and activin signaling in trophoblasts has been linked to the impaired acquisition of an invasive extravillous trophoblast phenotype and defective spiral artery remodeling (135–139). In endothelial cells, dysregulated TGF-β superfamily signaling contributes to impaired vascular homeostasis (95). ENG, a member of the TGF-β superfamily, is expressed by endothelial cells and trophoblasts and has been identified is a key modifier (95,140,141). Increased ENG shedding in PE has been shown to generate sEng, which antagonizes TGF-β family signaling and contribute to endothelial dysfunction (95).

Within the immune compartment, altered TGF-β signaling has been linked to impaired tolerance, a reduced frequency or suppressive capacity of regulatory T cells, and an increased Th17-to-Treg ratio (142). The phenotype of decidual NK cells is also shaped by local TGF-β signaling (143). Altered levels of TGF-β in PE are consistent with disrupted tuning of NK cell activity (120,143). Macrophage phenotypes are similarly affected. Decidual macrophages are dependent on TGF-β-dependent signals to restrain inflammation and remodel tissue within the spiral artery niche (23,144). Together, these findings suggest that TGF-β superfamily signaling could represent a biologically plausible axis linking placental stress, immune dysregulation, and endothelial pathology in PE.

However, significant knowledge gaps regarding HBCs remain. This observation underscores a broader limitation in placental cell biology as cell- and subtype-resolved analyses of TGF-β superfamily signaling within villous compartments are still insufficient (Figure 2). In particular, evidence on TGF-β superfamily signaling activity in HBCs, and its relationship to polarization and functional outputs in EO-PE and LO-PE, is limited. Addressing this gap is important for our understanding of the underlying mechanisms through which villous immune dysregulation and fetoplacental vascular dysfunction contribute to the development of preeclampsia.

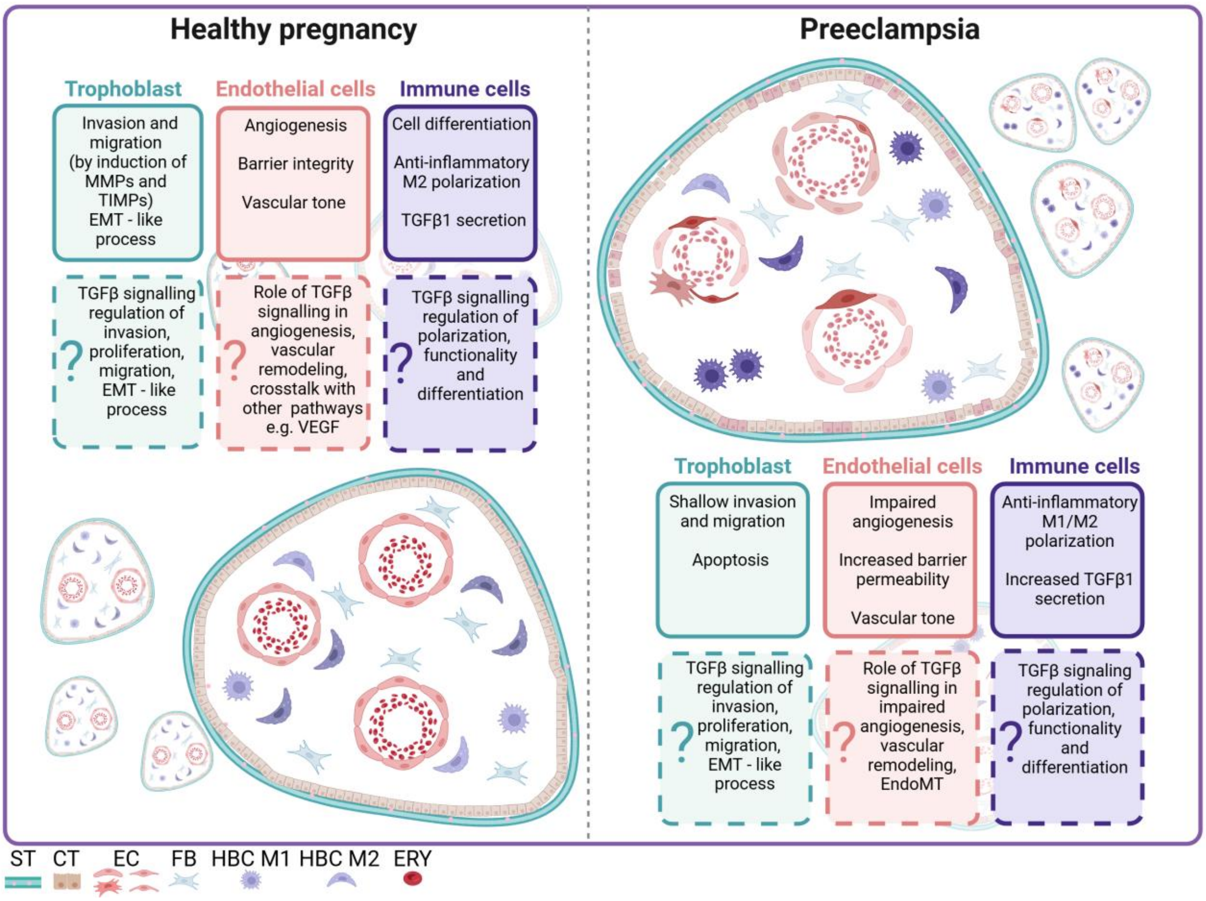

Figure 2: An overview of TGF-β superfamily signaling across placental villous cell types in PE. The illustration shows a cross-sectional view of a healthy and a PE placenta. Trophoblast, endothelial, and immune cell populations are depicted alongside their established functions. Question marks indicate areas where TGF-β-related signaling is not well understood in PE. The highlighted gaps include trophoblast biology, endothelial remodeling and angiogenesis, and immune cell phenotype shifts. FB: fibroblasts; HBC M1: M1-polarized Hofbauer cells; M2: M2-polarized Hofbauer cells; ERY: erythrocytes. Adapted from Horvat Mercnik et al.(2), with permission of Human Reproduction Update, under Creative Commons Attributions Licence.

Hypothesis and objectives:

We hypothesized that the polarization patterns of HBCs differ between early- and late-onset preeclampsia, reflecting distinct phenotypes within the placenta. Furthermore, we hypothesized that these differences, which dependent on disease onset are linked, at least in part, to disrupted TGF-β superfamily signaling in the local placental microenvironment.

To address this hypothesis, we pursued the following objectives:

1. Optimize and validate the isolation workflow of HBCs by improving the preparation of the tissue, enzymatic digestion, and enrichment steps in order to increase the yield, viability, and purity of the cells while preserving their suitability for downstream functional analyses.
2. Establish and validate a quality control protocol for HBCs isolation that enables the assessment of cell purity and the identification of contaminating cell populations.
3. Define Hofbauer cell polarization patterns in preeclampsia and determine how they differ between early- and late-onset cases
4. Assess differences in HBC phenotype features between early- and late-onset preeclampsia.
5. Integrating HBCs phenotype findings within TGF-β superfamily signaling across placental cell types in health and preeclampsia

Results

This section offers a synopsis of the findings presented in the following publications.

1) Mercnik MH, Schliefsteiner C, Fluhr H, Wadsack C. Placental macrophages present distinct polarization pattern and effector functions depending on clinical onset of preeclampsia. Front Immunol. 2023 Jan 12;13:1095879. doi:10.3389/fimmu.2022.1095879
2) Mercnik MH, Schliefsteiner C, Sanchez-Duffhues G, Wadsack C. TGFβ signalling: a nexus between inflammation, placental health and preeclampsia throughout pregnancy. Hum Reprod Update. 2024 Jul 1;30(4):442–71. doi:10.1093/humupd/dmae007
3) Mercnik MH, Wadsack C, Schliefsteiner C. Unlocking the secrets of Hofbauer cells in placental (patho-) physiology: Isolation and quality assessment in human term placenta. Placenta. 2025 Jun 13;166:41–53. doi:10.1016/j.placenta.2024.07.004

Hofbauer cells have been demonstrated to play a pivotal role in the regulation of immune homeostasis, tissue remodeling, and vascular development in the placental villous stroma (26). Despite their importance, their functional biology remains incompletely defined because they have primarily been studied using immunohistochemistry and other *in situ* approaches (39,73). While these methodologies are imperative for cell localization and marker-based characterization, they do not permit the direct evaluation of HBC function or signaling. To enable downstream phenotypic and functional analyses, we developed and optimized an isolation protocol for HBCs (3). The protocol has been developed in accordance with the method established by Tang et al. (70), incorporating targeted improvements in tissue processing and quality assessment. The procedure combines villous tissue dissection with decidua removal, enzymatic digestion, Percoll-based density enrichment and immune selection, followed by flow cytometry-based quality control after isolation (3). The refinement of tissue preparation, digestion, and enrichment techniques has been demonstrated to enhance cell yield, viability, and purity, thereby facilitating reliable phenotypic profiling. It is important to note that the isolation workflow itself did not induce detectable activation or polarization shifts (3,68). The implementation of flow cytometry-based quality control procedures was essential, as it facilitated the evaluation of purity and the exclusion of non-target placental cells, such as smooth muscle cells and fibroblasts. The effects of cryopreservation were also determined; however, it was found that freezing and thawing introduced clear limitations, with the result that freshly isolated cells were used wherever possible (3).

Subsequently, we implemented an optimized isolation protocol to evaluate HBC polarization in PE, with a separate analysis of EO-PE and LO-PE. The onset-stratified analysis revealed that PE is not associated with a single HBC phenotype but rather with distinct phenotype patterns in EO-PE and LO-PE. In this study, the presence of an M2 phenotype in EO-PE HBCs was observed, accompanied by a notable shift towards M1-associated features, as indicated by elevated levels of CD86, TLR-4, HLA-DR, IRF5, and *NOS2*. However, in the same time these HBCS maintained their M2 phenotype with higher TGF-β1 secretion and *ARG1* expression. In contrast, LO-PE HBCs exhibited a more phagocytic, CD209^{low} M2 phenotype with a less pronounced pro-inflammatory pattern. The enhanced phagocytosis and MMP9 secretion, in conjunction with anti-inflammatory cytokine secretion, supported persistent HBC plasticity in both groups. These findings suggest that HBC polarization in PE is better described as an onset- and local milieu-dependent phenotype spectrum than by a binary M1/M2 classification (1).

In the following paper, a study of TGF-β superfamily signaling in healthy and PE placentas was conducted, with the trophoblast, endothelial, and immune compartments being the focus of this analysis. In healthy placenta, the TGF-β superfamily coordinates signaling in trophoblasts, where it is associated with differentiation, invasion, and remodeling; in endothelial cells, where it promotes angiogenic balance and the endothelial phenotype; and in immune cells, where it contributes to controlled inflammation and immune regulation. In field of PE, we have identified recurring disturbance in this signaling axis across various compartments. This disruption encompasses alterations in ligand availability, receptor and co-receptor composition, and downstream signaling output (2). These changes are linked to impaired trophoblast function, endothelial dysfunction, and inflammatory skewing (2,122). Endoglin, particularly the soluble form, has been identified as a key modifier of this imbalance, with the strongest evidence supporting this finding in endothelial cells (2,95).

The review emphasized that HBCs remain understudied within this signaling axis despite their localization near trophoblasts and fetal vessels and their potential involvement in villous immune-vascular signaling (2). This is directly relevant to our experimental findings, as we demonstrated HBC polarization patterns dependent on the onset of PE and identified HBC-associated endpoints, including MMP9 and TGF-β1 (1). However, direct, cell-resolved analyses of TGF-β superfamily signaling in HBCs remain largely unexplored, especially in studies that stratify EO-PE and LO-PE (2,113).

In summary, we established an optimized and validated protocol for isolation of HBCs, a defined onset-dependent HBC polarization pattern in PE has been established. The review article aimed to identify major mechanistic gaps for future work: e.g., direct analysis of signaling pathways in stratified PE cohorts.

Discussion

In my thesis, the inflammatory environment of PE was examined in relation to the polarization and functional properties of HBCs. First, the protocol for isolating HBCs was optimized and validated with rapid contamination control and streamlined polarization readout (3). Subsequently, the protocol was applied to placentas from patients with EO- and LO- PE, with the objective of isolating the cells. In order to define subtype-specific polarization patterns, an analysis of the cells was conducted at the mRNA, protein, secretory, and functional levels, with a focus on tissue remodeling and phagocytosis (1). In consideration of the pivotal role of TGF-β signaling in PE, a comprehensive review was put together across placental cell populations. The findings of this publication were incorporated into the broader pathobiology of PE (2).

Hofbauer cell isolation in term placenta: methodological challenges and limitations

HBCs represent the predominant immune cell population in placental villi, contributing to tissue homeostasis, host defense, and inflammation resolution (19). Despite their importance in the context of placental development in the early stages of pregnancy, advancement has been constrained by the absence of a cohesive isolation protocol (3,19,70,80). Although immunohistochemistry and other *in situ* approaches are essential for defining HBC localization and marker expression (73,79,145); they provide limited insight into functional behavior and signaling activity.

In vitro studies are essential; however, current protocols differ at every key step, including tissue dissection, enzymatic digestion, enrichment by density centrifugation, purification, and phenotyping (19). In the case of term placentas, the majority of isolation protocols start with rigorous trimming of the decidual membrane, with the objective of minimizing maternal macrophage contamination (3,70,84). Subsequent to this, villous dissection and dissociation are undertaken, employing trypsin, collagenase V, and DNase I (70). Next, density enrichment is performed using Percoll or Ficoll-type media (70,80,84). These methodological differences directly affect viability, marker detectability, and downstream functional readouts.

In the present study, the villous tissue was subjected to three sequential trypsin and DNase I digestions with intermediate washing, followed by a final collagenase V and DNase I digestion (3). This step is particularly important in term placentas, where the villi are more compact and releasing the stromal cells is more difficult than in first trimester tissue (80,81). The protocol was optimized by shortening the early enzymatic incubations while maintaining adequate dissociation. Furthermore, the inclusion of DNase I throughout all digestion steps was found to reduce clumping, lower viscosity, and improve filtration (3,80). Additionally, the potential of cryopreservation as component of the optimization process was assessed. However, reduced post-thaw viability and adherence limited its suitability for reproducible downstream functional assays. Consequently, we advocate the use of fresh isolates whenever feasible (3).

The process of digestion in isolation has been identified as a significant factor in the recovery of HBCs and the induction of phenotype shifts during isolation. Trypsin has been observed to reduce trophoblast-rich fractions, while collagenase has been shown to release stromal-embedded HBCs, particularly at term (80,83). However, prolonged enzymatic exposure has been associated with reduced viability and altered marker detectability (146). In some cases, older protocols include dispase to loosen the matrix; however, this can introduce variability in epitope preservation (83,146). Of note, the application of warm enzymatic digestion increased TNFα, IL1β, IL10, and HSP70 while reducing CD163 in placental macrophage preparations compared with cold mechanical dissociation (82). These shifts should be interpreted with caution as they may reflect digestion-induced effects and early culture adaptation (70,82). However, the present study found that HBC polarization remained stable and aligned with *in situ* marker expression in placental tissue (66,68).

Density enrichment is another key source of variability. Percoll has been demonstrated to be particularly effective in the complex term placental digest where layered gradients enable effective separation of debris, trophoblast-rich fractions, stromal cells, and immune cells (70). Ficoll-type media, including Histopaque and Pancoll, enrich the mononuclear fraction by density; however, this separation is less selective in term villous digests and typically require stricter downstream purification (82–84). Following density enrichment, purification can be achieved through either magnetic immunopurification or FACS sorting. It has been demonstrated that magnetic bead-based approaches, including CD14 enrichment or EGFR/CD10 negative depletion, are more straightforward to scale up large term digests and generally provide enhanced absolute macrophage recovery (70,147). In the context of functional studies, negative depletion was selected as the preferred approach, given the potential of positive magnetic selection to alter the activation-sensitive profile of macrophages through receptor ligation (147,148). In contrast, omission of immune selection may lead to a higher number of undesired placental cells, (e.g., fibroblasts) (84). Alternatively, cell sorting by FACS offers stronger phenotypic resolution, stricter contaminant exclusion, and potential maternal-fetal discrimination (80). However, final recovery rate for the cells of interest is lower due to pre-enrichment losses, strict gating, viability and doublet exclusion, and longer processing times in large, debris-rich term digests (71,80).

Another significant source of protocol inconsistency is the selection of markers for either FACS sorting or immunopurification. While CD68 has been considered as a useful in immunohistochemistry and immunocytochemistry, it has been demonstrated to be insufficient for flow cytometric purity claims in placental digests when used alone, since it can be detected in CT and placental fibroblasts, too (58,70,84). In term HBC, CD163 is widely recognized as the most informative marker (84). However, interpretation of the staining requires caution since surface staining measures only membrane bound CD163, whereas intracellular staining reflects total CD163. Of note, the enzymatic digestion process during HBC isolation may temporarily reduce CD163 surface detectability without necessarily reducing overall expression (70). Studies often use CD45 and CD14; although, these markers are only effective for the enrichment of leukocytes and myeloid cells (80,147). Importantly to note that neither marker is HBC-specific, which limits the use of CD14-based term protocols when the fetal origin cannot be verified independently (71,81). This is particularly pronounced in first trimester placental digests, where maternal macrophages can constitute a substantial proportion of CD14-positive cells (80). Consequently, studies conducted during the first trimester frequently employ a combination of FACS sorting of CD45$^+$CD14$^+$FOLR2$^+$ cells with fetal origin detection based on HLA class I allotypes and the HLA-DR phenotype of HBCs (64,71,80). In the event of a maternal-fetal HLA mismatch, the HLA allotypes facilitates the direct separation of the fetal and maternal cells (80). In the absence of an informative mismatch, the presence of HLA-DR negativity has been demonstrated to support the identification of HBCs (64). However, at term, this approach is less reliable because some HBC subsets express HLA-DR (64,149). Importantly, it is only possible to undertake a HLA-based separation if the mother and fetus exhibit different HLA allotypes (80). Term protocols therefore primarily rely on rigorous trimming and removal of the decidual membrane before digestion to minimize contamination from decidual macrophages (3,70). Independent confirmation of fetal origin is preferable for term HBCs, for example, through sex chromosome assays or genotype-based approaches (58,63,70,150).

A further critical step in the isolation protocol is the identification and exclusion of other not desired placental cell populations. CK7 has been shown to facilitate the exclusion of trophoblasts, while αSMA and CD90 have been demonstrated to enhance the detection of stromal, perivascular, and fibroblast-like contaminants, a process that is particularly significant in term villous digests (3,58,70,84). In the present study, we implemented a rapid flow cytometry quality-control panel including vimentin, CD163, αSMA, and CD90 was implemented to assess contamination by mesenchymal cells, smooth muscle cells, and fibroblasts (3). CK7 was not included in the initial panel, which is a limitation; however, trophoblast exclusion was assessed by immunocytochemistry as shown in our earlier work (67). Orthogonal immunocytochemistry remains a valuable method since it independently confirms cell composition.

In addition to isolation and contamination control, we introduced a rapid polarization readout as an early screening step. Although full polarization assessment requires integrated mRNA, protein, secretory, and functional analyses, intracellular regulatory markers such as IRF4 and IRF5 may provide a useful early indication of polarization status (35). This is particularly relevant for HBCs, which often display mixed polarization phenotypes with concurrent pro-inflammatory and anti-inflammatory characteristics (68). For example, IRF5 has been linked to an inflammatory macrophage programming, whereas IRF4 is associated with alternative polarization (52,54,151,152). Intracellular flow cytometry is recommended to apply in cultured HBCs, as surface-marker interpretation is influenced by enzyme exposure and short-term adaptation (70). In this protocol, the authors identified CD163-positive HBCs, subsequently assessing IRF4 and IRF5 alongside pro-inflammatory markers (CD80 and CD40) and alternative polarization markers (CD206 and CD209) (3). This approach enhances the resolution of the dominant polarization program within each sample in a timely manner.

Differential Hofbauer cell polarization in early- and late-onset preeclampsia

There is already good evidence that HBCs contribute to the maintenance of placental physiology by ensuring homeostasis, sustaining a tolerogenic immune environment, and promoting the resolution of inflammation (26). The functional diversity of these cells relies on their high plasticity, which enables them to rapidly adopt to a variety of polarization patterns in response to microenvironmental cues (33,40)**.** In PE, the villous niche is exposed to a complex series of alterations, including immune activation, trophoblast stress and shedding, endothelial dysfunction, and stromal remodeling associated with hypoxia and oxidative stress (116,153–155). PE cases were stratified into two categories namely, early- (EO-) and late-onset (LO-) PE, due to the evidence that dominant placental cues vary across subtypes, (156,157). The prevailing hypothesis suggests that EO-PE is more closely associated with impaired placentation, malperfusion, hypoxia-linked stress, oxidative injury, and placenta-centered inflammatory signaling (157). In contrast, LO-PE is more strongly influenced by maternal cardiovascular and metabolic susceptibility, as well as systemic endothelial dysfunction (156). Onset stratification was mechanistically necessary, since subtype-specific alterations in the local villous milieu may redirect HBC polarization and thereby contribute to placental dysfunction (157,158). Consistent with this, it has been shown that EO-PE placentas more frequently exhibit reduced placental size, impaired villous vascular development, and reduced perfusion. These characteristics may bias isolation-based *in vitro* readouts (158,159).

Accordingly, the first part of this thesis focused on establishing a robust HBC isolation protocol for PE placental tissue that facilitates regulated phenotypic and functional profiling (3). This study confirmed that the workflow yielded PE HBC preparations with sufficient purity and recovery as well (1,3). This approach ensures that any downstream differences can be interpreted as being of biological origin, rather than being driven factors such as recovery bias, contamination, or variability in tissue quality.

Due to the altered inflammatory environment of the villous stroma in PE, the initial assessment focused on determining whether the abundance of HBCs in villous tissue was altered. The accuracy of quantification depends on the selection of appropriate markers, as inflammation and tissue disruption may alter cellular composition and reduce the reliability of some markers (35). CD68, CD14, and CD163 are commonly used to identify HBCs; however, CD68 and CD14 lack of specificity for HBCs (160). Inconsistent reports are likely to reflect differences in marker panels, which capture distinct macrophage populations and therefore yield divergent readouts. CD163 has been stated as a reliable and informative marker of HBCs under inflammatory placental pathology (1,3,68), consistent with the findings of our isolation data, which showed a predominant CD163-positive HBC fraction (3). Accordingly, HBC abundance was quantified using CD163 immunohistochemistry and directly correlated with isolation yield (1). The results indicate a decline in both measures within the EO-PE and LO-PE groups, suggesting a reduction in HBC abundance rather than an isolation effect (3). In line, Tang et al. reported reduced CD163-positive HBC numbers in PE (70,73), while Broekhuizen et al., using combined CD68 and CD163 staining, observed reduced abundance in EO-PE but no change in LO-PE (118). Notably, transcriptomic-based approaches including methylation-based deconvolution and single-cell or spatial datasets also support the observation that reduced HBC abundance and compositional shifts in PE are observed (113,161–165). A reduction in HBC abundance has been described to have functional consequences like the promotion of inflammation, the limitation of efferocytosis and debris clearance, as well as the reduction of macrophage-derived regulation of cytokine expression and matrix remodeling (160). Since HBCs are involved in the role of established paracrine signaling with trophoblasts and fetoplacental endothelial cells, a decrease in HBC content could potentially disrupt these intercellular cues, thereby influencing trophoblast behavior and endothelial phenotype (67,133).

Transcriptomic profiling has shown that HBC polarization is heterogeneous and cannot be adequately described by a single marker or an oversimplified M1/M2 dichotomy (58,162). Therefore, rather than employing a single marker, an array of readouts was utilized to assess polarization, integrating polarization markers and cytokine secretion with measures of arginine metabolism, inflammatory versus regulatory transcriptional programs, antioxidant capacity, and redox control (1,35).

EO-PE HBCs exhibited concurrent inflammatory activation while maintaining anti-inflammatory and tissue remodeling programs. Surface profiling revealed increased pro-inflammatory activation, evidenced by higher levels of CD86, TLR-4, and HLA-DR. This finding was supported by trends indicating increased levels of CD11b, CD11c, CD40, CD80, and TLR-1. However, the activation profile was not uniform across the TLR- axis. Despite increased expression of TLR-4 and TLR-1, TLR-2 levels were decreased, indicating modulation of TLR activation rather than a global increase in TLR expression (1). IRF5 increased with reduced IRF4, consistent with pro-inflammatory transcriptional programming (1,166,167). Furthermore, the increased expression of *NFKB1* and *TNFA*, together with *NOS2* induction, supports a shift toward an M1-like inflammatory program (1,35). This finding is consistent with the activation of a TLR-4–NF-κB axis (168).

Nevertheless, a complete transition to an M1-like state was not achieved by EO-PE HBCs. The levels of *ARG1* and *TGFB1* increased, accompanied by elevated TGF-β secretion, and upward trends in IL-4 and IL-10. These results are consistent with the persistence of regulatory and repair-associated programs (1). The equilibrium between M1-like activation and M2-like regulation in EO-PE HBCs is further influenced by two molecular switches. The first is the IRF5–IRF4 axis, which has been demonstrated to affect transcriptional programming and in EO-PE HBCs has shown to favour inflammatory IRF5 (1,166,167). The second is the iNOS–ARG1 axis, which reflects competing arginine metabolic pathways that support inflammatory activity versus metabolism associated with repair (46,47). In classical alternative activation, IRF4 and ARG1 have been shown to dominate and suppress TLR-4-driven inflammation (46,169). In this particular situation, however, inflammatory and regulatory modules coexist, suggesting partial counter-regulation rather than polarization to one extreme (1). The co-induction of *NOS2* and *ARG1* points to the presence of competition within the L-arginine axis (43). Meanwhile, reduced *CAT* and *SOD* levels signify diminished antioxidant activity, which may sustain ROS-mediated signaling and limit effective resolution of inflammation (170).

This EO-PE pattern is consistent with reports linking severe or early disease to reduced HBC abundance or attenuation of M2-associated features, in conjunction with stronger pro-inflammatory polarization, including reduced CD163, FR-β, CD209/DC-SIGN, and IL-10, as observed in multiple studies (72,73,171). However, a significant number of these analyses fail to stratify EO-PE and LO-PE, and they also do not include gestational age-matched controls. This is likely to be a contribution factor to the inconsistent findings across studies (72). Single-cell and spatial transcriptomic studies have also reported a shift in EO-PE HBC composition. This is characterized by a decrease of in the number of HBC populations, which are enriched for oxygen-stress response genes and programs linked to stromal and vascular interaction (113).

Overall, EO-PE HBCs have been shown to activate an inflammatory program associated with TLR-4-NF-κB, yet they also retain regulatory and repair-associated outputs that prevent a complete M1-like shift (1). Furthermore, transcriptomics supports the hypothesis of a compositional shift with reduced representation of HBC populations linked to oxygen-stress responses and stromal or vascular interaction programs. This finding is consistent with the concept of impaired adaptation under conditions of hypoxia and oxidative stress, as well as a bias towards inflammatory activation over resolution (1,99,113,172,173).

The present study demonstrated that LO-PE HBC polarization differed from both controls and EO-PE, and that it was most accurately described as M2-like with a defined pro-inflammatory activation. This result was corroborated by elevated levels IRF4 and *ARG1*, as along with lower levels of *NOS2* and IRF5 (1). Together with increased secretion of TGF-β, IL-4, and IL-13, these results argue against an M1 switch (1,35). The increased TNF-α, reduced CD209/DC-SIGN, and reduced *CAT* and *SOD* were evident, consistent with oxidative stress that can sustain inflammatory signaling within an immunoregulatory program (1). This pattern is consistent with observations in term HBCs, in which TNF-α can increase alongside IL-10-associated regulation. This supports a balanced pro-inflammatory activation state rather than a M1 switch (20,60). In accordance with this, spatial transcriptomics has reported the enrichment of TNF-α and NF-κB-linked pathways in LO-PE-associated macrophages, which is consistent with the increased TNF-α detected in our secretome (113,162). Integrated spatial and transcriptomic analyses further differentiate LO-PE from EO-PE by associating LO-PE signatures with interferon-related signaling and mitochondrial stress, whereas EO-PE signatures are more strongly linked to hypoxia-associated programs and broader phenotype shifts (113). Together, these data suggest that LO-PE and EO-PE drive distinct HBC activation patterns rather than a single, shared PE macrophage phenotype.

Decoding the link between Hofbauer cell polarization and TGF-β superfamily signaling

Macrophage plasticity facilitates the coordinated transition from inflammatory control to resolution (37,174,175). This transition includes the downregulation of inflammatory signaling, the clearance of apoptotic and necrotic material, and subsequent tissue repair through regulated extracellular matrix turnover and pro-angiogenic support (175,176).

Within the placental villous stroma, phagocytosis is a fundamental HBC function, where both term and first-trimester HBCs demonstrate substantial clearance capacity *in vitro* (1,3,70,71,177). In cases of PE, impaired placental perfusion has been demonstrated to induce oxidative injury and cell death within the villi (178–181). This may lead to an increased local burden of apoptotic material and inflammatory debris, thereby elevating the demand for clearance by macrophages. This in turn, places HBC function at the center of stromal homeostasis (76,182–185). The expression of Fcγ receptors (CD64, CD32, and CD16) by HBC enables Fc-mediated uptake of Ig-opsonized targets and immune complexes, as well as phagocytosis of debris (71,81). In the field of PE, the presence of elevated circulating immune complexes, higher complement activation, and increased placental complement deposition has been reported. These findings provide evidence to support the hypothesis of an increased burden of opsonized material that can enter stromal clearance pathways, (186–189). CD74, a required component in the process of for MHC II antigen processing, has been reported to be reduced in PE HBCs. This reduction has been linked to a more pro-inflammatory phenotype, suggesting that CD74 uptake may favor clearance-associated activation over efficient antigen processing (189). In accordance with an increased clearance demand, it was observed that phagocytic activity increased in both EO- and LO-PE, reaching statistical significance in the latter (1). The most relevant substrates for HBC clearance *in situ* are apoptotic cells and trophoblast-derived debris; thus, enhanced phagocytic capacity is expected to support efferocytosis-associated resolution programs (183). Similarly, the presence of stable IL-10, together with elevated TGF-β has been observed to facilitate immunoregulatory and repair processes, in addition to active clearance (1). The observation that CD209/DC-SIGN levels are reduced indicates that this particular tolerogenic module is attenuated. Increased TGF-β, as well as TNF-α or NF-κB-linked signatures, indicate that inflammatory activation persists within an otherwise immunoregulatory program rather than reflecting a full conversion to an M1-like state (1,113).

In the presented review article, the TGF-β superfamily is summarized in relation to regulation of placental development and function from the first trimester to term, with particular reference to its effects on the trophoblast, feto-placental endothelium, and immune compartments (2). Dysregulation of this pathway has been linked to PE (2,122). TGF-β has been identified as a marker of an immunoregulatory program, and furthermore, it functions as a regulator that modifies the balance between IRF5- and *NOS2*-driven inflammatory activation and IRF4- and *ARG1*-associated regulatory and repair pathways (54,190,191). In various models of macrophages, TGF-β has been observed to induce an M2-like polarization shift through the actions of SNAIL, a process, which has been linked to depend on SMAD2/3 and PI3K/AKT signaling (190,192,193). Conversely, the inhibition of the TGFβ–SNAIL axis has been found to restore pro-inflammatory cytokine production (193). Concurrently, TGFβ-1 limits inflammatory activation by suppressing STAT1 signaling and reducing iNOS protein stability (190). SMAD3 has been demonstrated to contribute to this inhibitory effect and has been associated with reduced expression of M1-related genes, including iNOS and MMP12, in LPS-stimulated macrophages (192). In LO-PE, higher IRF4 and *ARG1*, together with lower IRF5 and *NOS2*, are compatible with stronger TGFβ-driven immune regulation; *NOS2* remains low despite ongoing inflammatory activation (1). In the context of EO-PE, an upregulation of *TGFB1* and TGF-β secretion has been observed corroborated with the induction of IRF5 and *NOS2* induction. These findings indicate that inflammatory signaling persists despite elevated TGF-β availability (1,2). This phenomenon may be attributed to the predominance of inflammatory pathways or a reduction in downstream TGF-β signaling output. However, the study's design did not permit the quantification of the output of the canonical TGF-β pathway in HBCs at the protein and mRNA level. Thus, the relationship between increased TGF-β availability and the regulation of iNOS and polarization programs cannot be assessed directly.

The process of matrix remodeling driven by HBCs is a function that is tightly linked to their polarization phenotype. Pro-inflammatory cytokines, such as TNF-α, IL1β, and IL6, have been shown to induce gelatinase expression and activity (194–196), while programs associated with repair have been observed to converge on TGF-β signaling to coordinate extracellular matrix (ECM) turnover and stromal organization (127,197–199). In this process, a key determinant of proteolytic activity is the inhibition of matrix metalloproteinases (MMPs) by tissue inhibitors of metalloproteinases (TIMPs), particularly TIMP1 and TIMP2 (198,200). By constraining MMP activity, including MMP9-driven gelatinolysis, TIMPs limit the magnitude and distribution of matrix degradation, thereby supporting regulated ECM turnover (198,199,201). Consistent with the findings of our study, numerous reports have been characterized PE as a protease-leaning environment, most commonly reported as increased MMP9 together with reduced TIMP1 and/or TIMP2

(1,202–204). Consequently, an imbalance in the MMP9-to-TIMP ratio has been shown to increase gelatinolytic activity, thereby leading to the destabilization of basement membranes and stromal scaffolds, and consequently affecting villous microarchitecture (194,205,206). In parallel, enhanced proteolysis can increase the release and activation of ECM-sequestered mediators, including latent TGF-β and VEGF family ligands (207–210). Increased MMP9 levels was identified in both PE subtypes, accompanied by elevated gelatinolytic activity that was significant in EO-PE and trending in LO-PE. These results support the active contribution of HBCs to stromal remodeling (1,2,205). This finding is consistent with increased TGF-β in both subtypes, thereby, linking immune programs to matrix remodeling and ECM production (195,196). Mechanistically, MMP9 has been shown to activate latent TGF-β complexes, and TGF-β has been demonstrated to reinforce MMP9 expression in turn, thus supporting a remodeling feedback circuit (195,209,211–213). MMP9 has been found to be associated with programs of macrophage remodeling that extend beyond matrix degradation (42,214,215). By altering the stromal niche and promoting the protease-mediated activation of latent TGFβ, MMP9 increases the local bioactivity of TGFβ, which acts on neighboring trophoblast and endothelial cells (2,130,205,216). This process establishes a correlation between the remodeling activity of macrophages and the organization of stromal tissue, in addition to vascular homeostasis (2). In the context of EO-PE, this finding is consistent with the observation of heightened inflammatory activation and elevated gelatinolysis within a more inflammatory milieu. In LO-PE, this pattern is associated with a more regulatory profile and comparatively less pronounced proteolysis (1).

HBCs have been found to reside in close proximity to trophoblasts and fetoplacental endothelial cells (67). In a healthy placenta, it has been demonstrated that they support villous vascular development by secreting pro-angiogenic mediators, while influencing trophoblast and endothelial function (67,133). During the early stages of gestation, the mechanisms of EVT invasion and spiral artery remodeling are subject to regulation by TGF-β superfamily signaling and matrix remodeling systems. These systems control extracellular matrix turnover and local ligand availability (2). First-trimester HBCs have been shown to contribute to this developmental environment by secreting pro-remodeling mediators, such as MMP9 and osteopontin, as well as immunoregulatory ligands, including TGF-β (64,71). Impaired early placentation and inadequate EVT invasion are the hallmarks of PE, which consequently results in incomplete spiral artery remodeling, thereby reducing uteroplacental perfusion (91,217,218). Altered TGF-β superfamily signaling has been linked to these processes (2,137,219). Interestingly, HBCs have been identified as a significant source of BMP2 in PE, and there has been observed increase in BMP2 abundance and epigenetic signatures consistent with increased BMP2 expression (134,220,221). In terms of functionality, HBC-derived BMP2 has been shown to enhance trophoblast invasion and promote endovascular-like behavior via BMPR1A and downstream SMAD2/3–SMAD4 signaling (134). This finding identifies the BMP2 axis as a potential compensatory response that may reinforce invasion when trophoblast-driven vascular remodeling remains superficial (134,220). At term, HBC-trophoblast paracrine communication shifts towards regulating trophoblast endocrine and vasoactive outputs rather than invasion (26,133). It has been demonstrated that HBC-conditioned medium modulates trophoblast secretion of hCGβ and hPL, and influences the release of vasoactive mediators, including endothelin-1, angiotensin II, and prostanoids (133).

In the field of PE, impaired villous perfusion and endothelial stress have been described to alter local oxygen availability and redox balance, reshape chemokine and growth factor signaling, and increase cellular debris (155,185,222). These cues have been shown to modulate HBC polarization, thereby modifying clearance activity and paracrine endothelial support (1,2,67). Under physiological conditions, the maintenance of the endothelial phenotype is found to be strongly influenced by TGF-β superfamily signaling (140,223) The equilibrium between ALK1- and ALK5-driven programs is pivotal in coordinating the processes of angiogenic activation and vessel maturation, with the interpretation of these cues being modulated by endoglin (223,224). In PE cases, an increase of TGF-β availability has been observed to coincide with an increase in maternal endoglin, including elevated soluble endoglin (95,225,226). It has been demonstrated that this has the capacity to modify the interpretation of TGF-β cues, by endothelial cells, thus contributing to the promotion of endothelial dysfunction (95,140). Within the villous core, increased HBC-derived TGF-β contributes to the activated TGF-β–endoglin axis (2,227). Together with HBC-derived TNF-α and IL-8, it has the capacity to influence endothelial activation and junctional stability (1,154,228–230). TNF-α and IL-8 have been shown to amplify endothelial inflammatory signaling and chemokine circuits, while concomitantly weakening junctional organization (230,231). Conversely, increased TGF-β has been observed to promote remodeling-associated shifts in endothelial phenotype, including altered ECM signaling and reduced homeostatic features (140,232). These alterations in cytokines indicate a potential contribution of PE HBCs to endothelial dysfunction, despite the absence of changes in conditioned-medium readouts, such as proliferation or NAD(P)H-linked metabolic activity, remain unchanged (1,2,233). It is hypothesized that the paracrine effects of HBCs are more likely to involve endothelial activation, barrier integrity, stress responses, and inflammatory signaling than growth (1,233). Concurrently, the reduction of IL-17 in EO-PE and LO-PE, along with decreased VEGFA and KDR, is consistent with the attenuation of an IL-17-associated angiogenic and matrix-interacting program, as well as reduced pro-angiogenic support (1,234,235). Together, these findings suggest that, in PE, the HBC-ECA paracrine axis undergoes a shift towards inflammatory activation and remodeling rather than endothelial homeostasis (1). Increased HBC-derived TGF-β, together with TNF-α and IL-8, has been demonstrated to induce destabilization of junctional organization, thereby amplifying endothelial activation programs, and skewing ECAs towards a dysfunction-associated endothelial-to-mesenchymal transition (EndoMT)-like state (1,2).

Conclusion

In this thesis, a combined concept integrating a robust HBC isolation workflow, onset-resolved polarization profiling, and TGF-β superfamily signaling was followed. Together, these components mechanistically link the villous placental microenvironment to HBC functional states. In particular, a new validated methodological approach for isolating and characterizing term HBCs with strong contaminant control and practical early polarization readouts has been introduced. A key limitation is that a proportion of the phenotyping was performed after short-term culture, which can affect marker detectability and cellular phenotype during post-isolation recovery (3). Notably, the *in vitro* polarization profile was concordant with the *in situ* marker expression (3,66,68), supporting the biological relevance of the readouts. Nevertheless, future studies should include marker assessments immediately after isolation and again ffollowing short-term culture to more precisely delineate isolation-induced changes. In addition, routine verification of fetal origin—ideally via informative HLA allotyping and, when needed, complementary methods—should be implemented to strengthen conclusions specific to term HBCs.

Using this optimized and validated workflow, we demonstrated that EO-PE and LO-PE have distinct HBC polarization and functional profiles. Despite a shared clinical syndrome, these profiles are consistent with different underlying pathophysiological disease origins. EO-PE exhibited a pronounced TLR-4–NF-κB-associated pro-inflammatory program, while immunoregulatory M2-associated features and MMP9-mediated tissue remodeling remained active. In contrast, LO-PE exhibited a profile associated with ARG1 and IRF4, showing selective inflammatory activation and enhanced phagocytosis. Both subtypes exhibited phagocytosis and matrix remodeling associated with MMP9, while cytokine shifts, including increased TGF-β, higher TNFα, and reduced IL-17, were consistent with altered TGF-β pathway signaling and reduced VEGF-axis support (1). This combination may have reduced the paracrine support provided to neighboring trophoblasts and fetoplacental endothelial cells, resulting in local signaling that favors endothelial activation and remodeling rather than homeostasis (67,133). A key strength of the present work is the stratification of PE by clinical onset. Many studies still do not distinguish between EO-PE and LO-PE, which likely contributes to divergent and sometimes conflicting macrophage readouts. This underscores the need to consistently analyze EO-PE and LO-PE separately and to validate onset-dependent signatures in HBCs (39,73,79). Interpreting HBC polarization is challenging since omics studies demonstrate that the HBC population is heterogeneous and comprises multiple transcriptional subtypes (58,60,113,236,237). The relative representation of these subtypes varies with gestational age and is influenced by fetal sex, maternal BMI, delivery and labor methods, medication exposure, and comorbidities (58). These findings highlight the need for prospective recruitment with tighter matching for these covariates, as well as subtype-aware analyses using single-cell or spatial datasets, to distinguish subtype redistribution from true phenotypic change (58,236). Since stratifying by EO-PE and LO-PE reduces group size, sample size must be planned carefully to avoid loss of statistical power. Fetal sex is an important modifier of HBC immunometabolism and efferocytosis (60,238). Female HBCs show increased fatty acid uptake and lipid droplet accumulation, while male HBCs show higher glucose uptake, ROS, and a more glycolysis-linked inflammatory response (60,177,238). Therefore, fetal sex should be predefined as a stratifier in future onset-resolved studies. Finally, future work should combine metabolic phenotyping with functional assays that test how PE reshapes HBCs paracrine signaling within the placental niche. This can be achieved using coculture systems with TNF-α and TGF-β perturbations and endothelial readouts that report on direct dysfunction, including barrier integrity, inflammatory activation, migration, and tube formation.

Nevertheless, significant knowledge gaps remain regarding HBC biology in PE. This reflects a broader limitation across placental cell types, as analyses of TGF-β superfamily signaling in the villous compartment has not been fully resolved at the cellular and subtype levels (2). Specifically, the activity of TGF-β superfamily signaling in HBCs and its relationship to polarization and functional outputs in EO-PE versus LO-PE remain poorly defined. Taken together, these findings suggest that perturbed TGF-β superfamily signaling links placental stress to trophoblast dysfunction, immune dysregulation, and endothelial pathology in PE. Future studies that integrate subtype-resolved profiling with functional validation are necessary to determine the role of HBC-specific TGF-β superfamily signaling in the onset and progression of PE, as well as its contribution to placental adaptation across different disease subtypes.

References

1. Mercnik MH, Schliefsteiner C, Fluhr H, Wadsack C. Placental macrophages present distinct polarization pattern and effector functions depending on clinical onset of preeclampsia. Front Immunol. 2023 Jan 12;13: 1095879. doi:10.3389/FIMMU.2022.1095879 PubMed PMID: 36713449.

2. Mercnik MH, Schliefsteiner C, Sanchez-Duffhues G, Wadsack C. TGFβ signalling: a nexus between inflammation, placental health and preeclampsia throughout pregnancy. Hum Reprod Update. 2024 Jul 1;30(4):442–71. doi:10.1093/HUMUPD/DMAE007 PubMed PMID: 38519450.

3. Mercnik MH, Wadsack C, Schliefsteiner C. Unlocking the secrets of Hofbauer cells in placental (patho-) physiology: Isolation and quality assessment in human term placenta. Placenta. 2025 Jun 13;166:41–53. doi:10.1016/j.placenta.2024.07.004 PubMed PMID: 39004544.

4. Gajić M, Schröder-Heurich B, Horvat Mercnik M, Cervar-Zivkovic M, Wadsack C, von Versen-Höynck F, et al. The Impact of Hydroxychloroquine on Primary Feto-Placental Endothelial Cells from Healthy and Early-Onset Preeclamptic Placentas. Int J Mol Sci. 2023;24(13):10934. doi:10.3390/ijms241310934 PubMed PMID: 37446111.

5. Allerkamp HH, Bondarenko AI, Tawfik I, Kamali-Simsek N, Mercnik MH, Madreiter-Sokolowski CT, et al. In vitro examination of Piezo1-TRPV4 dynamics: implications for placental endothelial function in normal and preeclamptic pregnancies. Am J Physiol Cell Physiol. 2025 Jan 1;328(1):C227–44. doi:10.1152/ajpcell.00794.2024 PubMed PMID: 39652778.

6. Erlandsson L, Hirschmugl B, Hansson E, Mercnik MH, Wadsack C, Hansson SR. Urban air pollution disrupts placental microarchitecture and shifts hofbauer cells towards a pro-inflammatory state. J Environ Sci. 2026 Feb 1;160(7):124–34. doi:10.1016/j.jes.2025.03.043 PubMed PMID: 41177590.

7. Stoiber M, Horvat Mercnik M, Hirschmugl B, Darnhofer B, Pernitsch D, Leopold-Posch B, et al. Proteomic Epithelial-To-Mesenchymal Transition Signature in Fetoplacental

Small Extracellular Vesicles of Early-Onset Preeclampsia. J Extracell Biol. 2026 Mar 1;5(3):e70122. doi:10.1002/JEX2.70122 PubMed PMID: 41788817.

8. Burton GJ, Fowden AL. The placenta: A multifaceted, transient organ. Philos Trans R Soc Lond B Biol Sci. 2015 Mar 5;370(1663):20140066. doi:10.1098/rstb.2014.0066 PubMed PMID: 25602070.

9. Huppertz B. The anatomy of the normal placenta. J Clin Pathol. 2008 Dec;61(12):1296–302. doi:10.1136/JCP.2008.055277 PubMed PMID: 18755720.

10. Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage TKJB, James J. Human placenta and trophoblast development: key molecular mechanisms and model systems. Cell Mol Life Sci. 2019 May 3;76(18):3479–96. doi:10.1007/S00018-019-03104-6 PubMed PMID: 31049600.

11. Gauster M, Moser G, Wernitznig S, Kupper N, Huppertz B. Early human trophoblast development: from morphology to function. Cell Mol Life Sci. 2022 Jun 5;79(6):345. doi:10.1007/S00018-022-04377-0 PubMed PMID: 35661923.

12. Boss AL, Chamley LW, James JL. Placental formation in early pregnancy: how is the centre of the placenta made? Hum Reprod Update. 2018 Nov 1;24(6):750–60. doi:10.1093/humupd/dmy030 PubMed PMID: 30257012.

13. Handschuh K, Guibourdenche J, Tsatsaris V, Guesnon M, Laurendeau I, Evain-Brion D, et al. Human Chorionic Gonadotropin Produced by the Invasive Trophoblast But Not the Villous Trophoblast Promotes Cell Invasion and Is Down-Regulated by Peroxisome Proliferator-Activated Receptor-γ. Endocrinology. 2007 Oct 1;148(10):5011–9. doi:10.1210/en.2007-0286 PubMed PMID: 17628005.

14. Rassie K, Giri R, Joham AE, Teede H, Mousa A. Human Placental Lactogen in Relation to Maternal Metabolic Health and Fetal Outcomes: A Systematic Review and Meta-Analysis. Int J Mol Sci. 2022 Dec 1;23(24):15621. doi:10.3390/ijms232415621 PubMed PMID: 36555258.

15. E. Davies J, Pollheimer J, Yong HEJ, Kokkinos MI, Kalionis B, Knöfler M, et al. Epithelial-mesenchymal transition during extravillous trophoblast differentiation. Cell

Adh Migr. 2016 May 3;10(3):310–21. doi:10.1080/19336918.2016.1170258 PubMed PMID: 27070187.

16. Park JY, Mani S, Clair G, Olson HM, Paurus VL, Ansong CK, et al. A microphysiological model of human trophoblast invasion during implantation. Nat Commun. 2022 Mar 15;13(1):1252. doi:10.1038/s41467-022-28663-4 PubMed PMID: 35292627.

17. Castellucci M, Kosanke G, Verdenelli F, Huppertz B, Kaufmann P. Villous sprouting: Fundamental mechanisms of human placental development. Hum Reprod Update. 2000;6(5):485–94. doi:10.1093/humupd/6.5.485 PubMed PMID: 11045879.

18. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res. 2004;114(5-6):397-407. doi: 10.1016/j.thromres.2004.06.038. PubMed PMID: 15507270.

19. Tang Z, Abrahams VM, Mor G, Guller S. Placental Hofbauer cells and complications of pregnancy. Ann N Y Acad Sci. 2011 Mar 1;1221(1):103–8. doi:10.1111/j.1749-6632.2010.05932.x. PubMed PMID: 21401637.

20. Bezemer RE, Faas MM, van Goor H, Gordijn SJ, Prins JR. Decidual macrophages and Hofbauer cells in fetal growth restriction. Front Immunol. 2024 Jun 28;15:1379537. doi:10.3389/fimmu.2024.1379537 PubMed PMID: 39007150.

21. Seval Y, Korgun ET, Demir R. Hofbauer Cells in Early Human Placenta: Possible Implications in Vasculogenesis and Angiogenesis. Placenta. 2007 Aug 1;28(8–9):841–5. doi:10.1016/j.placenta.2007.01.010 PubMed PMID: 17350092.

22. Svensson-Arvelund J, Mehta RB, Lindau R, Mirrasekhian E, Rodriguez-Martinez H, Berg G, et al. The Human Fetal Placenta Promotes Tolerance against the Semiallogeneic Fetus by Inducing Regulatory T Cells and Homeostatic M2 Macrophages. J Immunol. 2015 Feb 15;194(4):1534–44. doi:10.4049/jimmunol.1401536 PubMed PMID: 25560409.

23. Vondra S, Höbler AL, Lackner AI, Raffetseder J, Mihalic ZN, Vogel A, et al. The human placenta shapes the phenotype of decidual macrophages. Cell Rep. 2023 Jan 31;42(1):111977. doi:10.1016/J.CELREP.2022.111977 PubMed PMID: 36640334.

24. Alijotas-Reig J, Llurba E, Gris JM. Potentiating maternal immune tolerance in pregnancy: A new challenging role for regulatory T cells. Placenta. 2014 Apr 1;35(4):241–8. doi:10.1016/j.placenta.2014.02.004 PubMed PMID: 24581729.

25. Yagel S. The developmental role of natural killer cells at the fetal-maternal interface. Am J Obstet Gynecol. 2009 Oct 1;201(4):344–50. doi:10.1016/j.ajog.2009.02.030 PubMed PMID: 19788966.

26. Reyes L, Golos TG. Hofbauer cells: Their role in healthy and complicated pregnancy. Front Immunol. 2018 Nov 15;9(NOV):2628. doi:10.3389/fimmu.2018.02628 PubMed PMID: 30498493.

27. Lager S, Powell TL. Regulation of Nutrient Transport across the Placenta. J Pregnancy. 2012 Jan 1;2012(1):179827. doi:10.1155/2012/179827 PubMed PMID: 23304511.

28. Kingdom J, Huppertz B, Seaward G, Kaufmann P. Development of the placental villous tree and its consequences for fetal growth. Eur J Obstet Gynecol Reprod Biol. 2000 Sep 1;92(1):35–43. doi:10.1016/S0301-2115(00)00423-1 PubMed PMID: 10986432.

29. Martin L, Higgins L, Westwood M, Brownbill P. Pulsatility effects of flow on vascular tone in the fetoplacental circulation. Placenta. 2020 Nov 1;101:163–8. doi:10.1016/j.placenta.2020.09.003 PubMed PMID: 33002776.

30. Burton GJ, Woods AW, Jauniaux E, Kingdom JCP. Rheological and Physiological Consequences of Conversion of the Maternal Spiral Arteries for Uteroplacental Blood Flow during Human Pregnancy. Placenta. 2009 Jun 1;30(6):473–82. doi:10.1016/J.PLACENTA.2009.02.009 PubMed PMID: 19375795.

31. Li Y, Lorca RA, Su EJ. Molecular and cellular underpinnings of normal and abnormal human placental blood flows. J Mol Endocrinol. 2018 Jan 1;60(1):R9–22. doi:10.1530/JME-17-0139 PubMed PMID: 29097590.

32. Burton GJ, Jauniaux E. What is the placenta? Am J Obstet Gynecol. 2015 Oct 1;213(4):S6.e1-S6.e4. doi:10.1016/j.ajog.2015.07.050 PubMed PMID: 26428504.

33. Sica A, Mantovani A. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest. 2012 Mar 1;122(3):787–95. doi:10.1172/JCI59643 PubMed PMID: 22378047.

34. Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. J Leukoc Biol. 2011 Apr 1;89(4):557–63. doi:10.1189/jlb.0710409 PubMed PMID: 21248152.

35. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. Immunity. 2014 Jul 17;41(1):14–20. doi:10.1016/j.immuni.2014.06.008 PubMed PMID: 25035950.

36. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 2018 Sep;233(9):6425-6440. doi: 10.1002/jcp.26429. PubMed PMID: 29319160.

37. Luo M, Zhao F, Cheng H, Su M, Wang Y. Macrophage polarization: an important role in inflammatory diseases. Front Immunol. 2024 Apr 10;15:1352946. doi:10.3389/fimmu.2024.1352946 PubMed PMID: 38660308.

38. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002 Nov;23(11):549-55. doi: 10.1016/s1471-4906(02)02302-5. PubMed PMID: 12401408.

39. Tang Z, Niven-Fairchild T, Tadesse S, Norwitz ER, Buhimschi CS, Buhimschi IA, et al. Glucocorticoids Enhance CD163 Expression in Placental Hofbauer Cells. Endocrinology. 2013 Jan 1;154(1):471–82. doi:10.1210/EN.2012-1575 PubMed PMID: 23142809.

40. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci. 2008;13(2):453–61. doi:10.2741/2692 PubMed PMID: 17981560.

41. Hoeffel G, Ginhoux F. Fetal monocytes and the origins of tissue-resident macrophages. Cell Immunol. 2018 Aug 1;330:5–15. doi:10.1016/j.cellimm.2018.01.001 PubMed PMID: 29475558.

42. T’Jonck W, Guilliams M, Bonnardel J. Niche signals and transcription factors involved in tissue-resident macrophage development. Cell Immunol. 2018 Aug 1;330:43–53. doi:10.1016/j.cellimm.2018.02.005 PubMed PMID: 29463401.

43. Kang JH, Kim JH, Gim JA, Lee MY. iNOS in Macrophage Polarization: Pharmacological and Regulatory Insights. Int J Mol Sci. 2025 Dec 15;26(24):12056. doi:10.3390/ijms262412056 PubMed PMID: 41465487.

44. Wu KK lok, Xu X, Wu M, Li X, Hoque M, Li GHY, et al. MDM2 induces pro-inflammatory and glycolytic responses in M1 macrophages by integrating iNOS-nitric oxide and HIF-1α pathways in mice. Nat Commun. 2024 Oct 4;15(1):8624. doi:10.1038/s41467-024-53006-w. PubMed PMID: 39366973.

45. Mitani T, Terashima M, Yoshimura H, Nariai Y, Tanigawa Y. TGF-beta1 enhances degradation of IFN-gamma-induced iNOS protein via proteasomes in RAW 264.7 cells. Nitric Oxide. 2005;13(1):78–87. doi:10.1016/j.niox.2005.05.001. PubMed PMID: 15964225.

46. Rath M, Müller I, Kropf P, Closs EI, Munder M. Metabolism via arginase or nitric oxide synthase: Two competing arginine pathways in macrophages. Front Immunol. 2014 Oct 27;5(OCT):532. doi:10.3389/fimmu.2014.00532 PubMed PMID: 25386178.

47. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPβ. Gene. 2005 Jun 20;353(1):98–106. doi:10.1016/J.GENE.2005.04.004. PubMed PMID: 15922518.

48. Sugiyama Y, Kakoi K, Kimura A, Takada I, Kashiwagi I, Wakabayashi Y, et al. Smad2 and Smad3 are redundantly essential for the suppression of iNOS synthesis in macrophages by regulating IRF3 and STAT1 pathways. Int Immunol. 2012 Apr 1;24(4):253–65. doi:10.1093/intimm/dxr126. PubMed PMID: 22331441.

49. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature. 2005 Mar 10;434(7030):243-9. doi: 10.1038/nature03308. PubMed PMID: 15665823.

50. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-Based Network Analysis Reveals a Spectrum Model of Human Macrophage Activation. Immunity. 2014 Feb 20;40(2):274-88. doi:10.1016/J.IMMUNI.2014.01.006 PubMed PMID: 24530056.

51. Lawrence T, Natoli G. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol. 2011 Oct 25;11(11):750-61. doi: 10.1038/nri3088. PubMed PMID: 22025054.

52. Corbin AL, Gomez-Vazquez M, Berthold DL, Attar M, Arnold IC, Powrie FM, et al. IRF5 guides monocytes toward an inflammatory CD11c+ macrophage phenotype and promotes intestinal inflammation. Sci Immunol. 2020 May 22;5(47):eaax6085. doi:10.1126/sciimmunol.aax6085 PubMed PMID: 32444476.

53. Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, et al. Negative regulation of Toll-like-receptor signaling by IRF-4. Proceedings of the National Academy of Sciences. 2005 Nov 1;102(44):15989–94. doi:10.1073/PNAS.0508327102 PubMed PMID: 16236719.

54. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. Nat Immunol. 2011 Jan 16;12(3):231–8. doi:10.1038/ni.1990 PubMed PMID: 21240265.

55. Zhu B, Song L, Li M, Cheng M, Tang W, Miao L. Comparison of the effects between catalase and superoxide dismutase on regulating macrophage inflammatory response and protecting osteogenic function of periodontal ligament cells. Biochem Biophys Res Commun. 2025 Apr 5;756:151523. doi:10.1016/j.bbrc.2025.151523 PubMed PMID: 40058309.

56. Morris G, Gevezova M, Sarafian V, Maes M. Redox regulation of the immune response. Cellular & Molecular Immunology 2022 19:10. 2022 Sep 2;19(10):1079–101. doi:10.1038/s41423-022-00902-0 PubMed PMID: 36056148.

57. He C, Carter AB. The Metabolic Prospective and Redox Regulation of Macrophage Polarization. J Clin Cell Immunol. 2015;6(6):371. doi:10.4172/2155-9899.1000371 PubMed PMID: 26962470.

58. Baráth BR, Bojcsuk D, Bene K, Caballero-Sánchez N, Cseh T, Freitas JCR de, et al. Distinct transcriptional and epigenomic programs define Hofbauer cells in term placenta. JCI Insight. 2026 Feb 9;11(3):e195801. doi:10.1172/jci.insight.195801 PubMed PMID: 41433105.

59. Kim SY, Romero R, Tarca AL, Bhatti G, Kim CJ, Lee J, et al. Methylome of Fetal and Maternal Monocytes and Macrophages at the Feto-Maternal Interface. Am J Reprod Immunol. 2012 Jul 1;68(1):8–27. doi:10.1111/j.1600-0897.2012.01108.x PubMed PMID: 22385097.

60. Paparini DE, Grasso E, Aguilera F, Arslanian MA, Lella V, Lara B, et al. Sex-specific phenotypical, functional and metabolic profiles of human term placenta macrophages. Biol Sex Differ. 2024 Oct 17;15(1):80. doi:10.1186/s13293-024-00652-w PubMed PMID: 39420346.

61. Turco MY, Moffett A. Development of the human placenta. Development. 2019 Nov 27;146(22):dev163428. doi: 10.1242/dev.163428. PubMed PMID: 31776138.

62. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-resident macrophages originate from yolk-sac-derived erythro-myeloid progenitors. Nature. 2015 Feb 26;518(7540):547–51. doi:10.1038/nature13989 PubMed PMID: 25470051.

63. Reyes L, Wolfe B, Golos T. Hofbauer cells: Placental macrophages of fetal origin. In: Results Probl Cell Differ. 2017;62:45-60. doi: 10.1007/978-3-319-54090-0_3. PubMed PMID: 28455705.

64. Thomas JR, Appios A, Calderbank EF, Yoshida N, Zhao X, Hamilton RS, et al. Primitive haematopoiesis in the human placenta gives rise to macrophages with epigenetically silenced HLA-DR. Nat Commun. 2023 Mar 30;14(1):1764. doi:10.1038/s41467-023-37383-2 PubMed PMID: 36997537.

65. Ingman K, Cookson VJKW, Jones CJP, Aplin JD. Characterisation of Hofbauer Cells in First and Second Trimester Placenta: Incidence, Phenotype, Survival in vitro and Motility. Placenta. 2010 Jun 1;31(6):535–44. doi:10.1016/j.placenta.2010.03.003 PubMed PMID: 20347485.

66. Schliefsteiner C, Peinhaupt M, Kopp S, Lögl J, Lang-Olip I, Hiden U, et al. Human placental Hofbauer cells maintain an anti-inflammatory M2 phenotype despite the presence of gestational diabetes mellitus. Front Immunol. 2017 Jul 31;8:888. doi: 10.3389/fimmu.2017.00888.PubMed PMID: 28824621.

67. Loegl J, Hiden U, Nussbaumer E, Schliefsteiner C, Cvitic S, Lang I, et al. Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis. Reproduction. 2016;152(5):447–55. doi:10.1530/REP-16-0159. PubMed PMID: 27534571.

68. Schliefsteiner C, Ibesich S, Wadsack C. Placental Hofbauer Cell Polarization Resists Inflammatory Cues In Vitro. Int J Mol Sci. 2020 Jan 22;21(3):736. doi:10.3390/ijms21030736. PubMed PMID: 31979196.

69. Kaufmann P, Stark J, Stegner HE. The villous stroma of the human placenta. I. The ultrastructure of fixed connective tissue cells. Cell Tissue Res. 1977 Feb;177(1):105–21. doi:10.1007/BF00221122 PubMed PMID: 837398.

70. Tang Z, Tadesse S, Norwitz E, Mor G, Abrahams VM, Guller S. Isolation of Hofbauer cells from human term placentas with high yield and purity. American Journal of Reproductive Immunology. 2011 Oct;66(4):336–48. doi:10.1111/j.1600-0897.2011.01006.x PubMed PMID: 21545365.

71. Thomas JR, Appios A, Zhao X, Dutkiewicz R, Donde M, Lee CYC, et al. Phenotypic and functional characterization of first-trimester human placental macrophages, Hofbauer cells. J Exp Med. 2021 Oct 19;218(1):e20200891. doi:10.1084/JEM.20200891 PubMed PMID: 33075123.

72. Yang SW, Cho EH, Choi SY, Lee YK, Park JH, Kim MK, et al. DC-SIGN expression in Hofbauer cells may play an important role in immune tolerance in fetal chorionic villi during the development of preeclampsia. J Reprod Immunol. 2017 Nov 1;124:30–7. doi:10.1016/j.jri.2017.09.012 PubMed PMID: 29049918.

73. Tang Z, Buhimschi IA, Buhimschi CS, Tadesse S, Norwitz E, Niven-Fairchild T, et al. Decreased Levels of Folate Receptor-β and Reduced Numbers of Fetal Macrophages (Hofbauer Cells) in Placentas from Pregnancies with Severe Pre-Eclampsia. American

Journal of Reproductive Immunology. 2013 Aug 1;70(2):104–15. doi:10.1111/AJI.12112. PubMed PMID: 23480364.

74. Anteby EY, Natanson-Yaron S, Greenfield C, Goldman-Wohl D, Haimov-Kochman R, Holzer H, et al. Human Placental Hofbauer Cells Express Sprouty Proteins: a Possible Modulating Mechanism of Villous Branching. Placenta. 2005 Jul 1;26(6):476–83. doi:10.1016/J.PLACENTA.2004.08.008. PubMed PMID: 15950061.

75. Abumaree MH, Stone PR, Chamley LW. The effects of apoptotic, deported human placental trophoblast on macrophages: Possible consequences for pregnancy. J Reprod Immunol. 2006 Dec 1;72(1–2):33–45. doi:10.1016/j.jri.2006.03.001 PubMed PMID: 16843534.

76. Abumaree MH, Chamley LW, Badri M, El-Muzaini MF. Trophoblast debris modulates the expression of immune proteins in macrophages: a key to maternal tolerance of the fetal allograft? J Reprod Immunol. 2012 Jun 1;94(2):131–41. doi:10.1016/j.jri.2012.03.488 PubMed PMID: 22542910.

77. Fakonti G, Pantazi P, Bokun V, Holder B. Placental Macrophage (Hofbauer Cell) Responses to Infection During Pregnancy: A Systematic Scoping Review. Front Immunol. 2022 Feb 11;12:756035. doi:10.3389/fimmu.2021.756035. PubMed PMID:

78. Yoshida N, Appios A, Li Q, Hutton JP, Wood G, Potts M, et al. Interactions between placental Hofbauer cells and L. monocytogenes change throughout gestation. Sci Immunol. 2025 Jul 18;10(109):eadq3066. doi: 10.1126/sciimmunol.adq3066. PubMed PMID: 40680145.

79. Young OM, Tang Z, Niven-Fairchild T, Tadesse S, Krikun G, Norwitz ER, et al. Toll-like receptor-mediated responses by placental Hofbauer cells (HBCs): a potential pro-inflammatory role for fetal M2 macrophages. Am J Reprod Immunol. 2015 Jan 1;73(1):22–35. doi:10.1111/AJI.12336 PubMed PMID: 25345551.

80. Appios A, Thomas JR, McGovern N. Isolation of First-Trimester and Full-term Human Placental Hofbauer Cells. Bio Protoc. 2021 Jun 5;11(11):e4044. doi:10.21769/BioProtoc.4044. PubMed PMID: 34250210.

81. Thomas JR, Naidu P, Appios A, McGovern N. The Ontogeny and Function of Placental Macrophages. Front Immunol. 2021 Oct 21;12:771054. doi:10.3389/fimmu.2021.771054 PubMed PMID: 34745147.

82. Eskandar S, Bezemer RE, Eggen BJL, Prins JR. Cold Mechanical Isolation of Placental Macrophages as a Method to Limit Procedure-Induced Activation of Macrophages. J Immunol. 2023 Dec 15;211(12):1868–76. doi:10.4049/jimmunol.2300379 PubMed PMID: 37909834.

83. Wetzka B, Clark DE, Charaock-Jones DS, Zahradnik HP, Smith SK. Isolation of macrophages (Hofbauer cells) from human term placenta and their prostaglandin E2 and thromboxane production. Hum Reprod. 1997 Apr 1;12(4):847–52. doi:10.1093/HUMREP/12.4.847 PubMed PMID: 9159455.

84. Szukiewicz D, Lasch M, Sudan K, Paul C, Schulz C, Kolben T, et al. Isolation of Decidual Macrophages and Hofbauer Cells from Term Placenta-Comparison of the Expression of CD163 and CD80. Int J Mol Sci. 2022 May 30;23(11):6113. doi:10.3390/IJMS23116113 PubMed PMID: 35682791.

85. Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. Obstet Gynecol. 2020 Jun 1;135(6):e237–60. doi:10.1097/AOG.0000000000003891 PubMed PMID: 32443079.

86. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics. ACOG Practice Bulletin No. 203: Chronic Hypertension in Pregnancy. Obstet Gynecol. 2019 Jan 1;133(1):e26–50. doi:10.1097/AOG.0000000000003020 PubMed PMID: 30575676.

87. Mustary M, Ansariadi, Syam A, Riskiyani S, Erika KA, Moedjiono AI, et al. Preeclampsia: Etiology, Pathophysiology, Risk Factors, Impact and Prevention: A Narrative Review. Iran J Public Health. 2024 Nov 1;53(11):2392–403. doi:10.18502/ijph.v53i11.16941 PubMed PMID: 39619908.

88. Fox R, Kitt J, Leeson P, Aye CYL, Lewandowski AJ. Preeclampsia: Risk Factors, Diagnosis, Management, and the Cardiovascular Impact on the Offspring. J Clin Med. 2019 Oct 4;8(10):1625. doi:10.3390/JCM8101625 PubMed PMID: 31590294.

89. Palmiero P, Caretto P, Ciccone MM, Maiello M, on behalf of the I.C.I.S.C.U. (Italian Chapter of International Society Cardiovascular Ultrasound). Long-Term Cardiovascular Risk and Maternal History of Pre-Eclampsia. J Clin Med. 2025 Apr 30;14(9):3121. doi:10.3390/JCM14093121 PubMed PMID: 40364153.

90. Mery EE, Hajjar J, Sudade S, Vasam G, Benton S, Gaudet L, et al. Placental anti-angiogenic and inflammatory markers and postpartum cardiovascular risk following preeclampsia. Placenta. 2026 Mar 25;176:43–52. doi:10.1016/j.placenta.2026.01.016 PubMed PMID: 41679190.

91. Huppertz B. Placental origins of preeclampsia: Challenging the current hypothesis. Hypertension. 2008 Apr;51(4):970-5. doi: 10.1161/HYPERTENSIONAHA.107.107607. PubMed PMID: 18259009.

92. Than NG, Posta M, Györffy D, Orosz L, Orosz G, Rossi SW, et al. Early pathways, biomarkers, and four distinct molecular subclasses of preeclampsia: The intersection of clinical, pathological, and high-dimensional biology studies. Placenta. 2022 Jul 1;125:10–19. doi:10.1016/j.placenta.2022.03.009 PubMed PMID: 35428514.

93. Redline RW, Patterson P. Pre-eclampsia is associated with an excess of proliferative immature intermediate trophoblast. Hum Pathol. 1995 Jun 1;26(6):594–600. doi:10.1016/0046-8177(95)90162-0 PubMed PMID: 7774887.

94. Ding J, Zhang Y, Cai X, Diao L, Yang C, Yang J. Crosstalk Between Trophoblast and Macrophage at the Maternal-Fetal Interface: Current Status and Future Perspectives. Front Immunol. 2021 Oct 21;12:758281. doi:10.3389/fimmu.2021.758281 PubMed PMID: 34745133.

95. Venkatesha S, Toporsian M, Lam C, Hanai JI, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med. 2006 Jun 4;12(6):642–9. doi:10.1038/nm1429 PubMed PMID: 16751767.

96. Leaños-Miranda A, Navarro-Romero CS, Sillas-Pardo LJ, Ramírez-Valenzuela KL, Isordia-Salas I, Jiménez-Trejo LM. Soluble Endoglin As a Marker for Preeclampsia, Its Severity, and the Occurrence of Adverse Outcomes. Hypertension. 2019 Oct 1;74(4):991–7. doi:10.1161/HYPERTENSIONAHA.119.13348 PubMed PMID: 31446801.

97. Cudmore MJ, Ahmad S, Sissaoui S, Ramma W, Ma B, Fujisawa T, et al. Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: identification of inter-dependency between Akt-1 and heme oxygenase-1. Eur Heart J. 2012 May 1;33(9):1150–8. doi:10.1093/EURHEARTJ/EHR065 PubMed PMID: 21411816.

98. Ma Y, Ye Y, Zhang J, Ruan CC, Gao PJ. Immune imbalance is associated with the development of preeclampsia. Medicine (Baltimore). 2019;98(14):e15080. doi:10.1097/MD.0000000000015080 PubMed PMID: 30946359.

99. Mannaerts D, Faes E, Cos P, Briedé JJ, Gyselaers W, Cornette J, et al. Oxidative stress in healthy pregnancy and preeclampsia is linked to chronic inflammation, iron status and vascular function. PLoS One. 2018 Sep 1;13(9):e0202919. doi:10.1371/JOURNAL.PONE.0202919 PubMed PMID: 30204759.

100. Murthi P, Pinar AA, Dimitriadis E, Samuel CS. Inflammasomes—A Molecular Link for Altered Immunoregulation and Inflammation Mediated Vascular Dysfunction in Preeclampsia. Int J Mol Sci. 2020 Feb 19;21(4):1406. doi:10.3390/IJMS21041406 PubMed PMID: 32093005.

101. Campbell N, Robbins M, Nembaware H, Deer E, Cornelius D, LaMarca B. Immune Cells in Preeclampsia. Int J Mol Sci. 2025 Dec 21;27(1):74. doi:10.3390/ijms27010074 PubMed PMID: 41515952.

102. Christensen AP, Bert S, Nadkarni S. Immune Mechanisms of Vascular Disease in Preeclampsia. Arterioscler Thromb Vasc Biol. 2025;45:1715–23. doi:10.1161/ATVBAHA.124.321670 PubMed PMID: 40874307.

103. Miller D, Motomura K, Galaz J, Gershater M, Lee ED, Romero R, et al. Cellular immune responses in the pathophysiology of preeclampsia. J Leukoc Biol. 2021 Dec 28;111(1):237–60. doi:10.1002/JLB.5RU1120-787RR PubMed PMID: 33847419.

104. Nelson DB, Ziadie MS, McIntire DD, Rogers BB, Leveno KJ. Placental pathology suggesting that preeclampsia is more than one disease. Am J Obstet Gynecol. 2014 Jan 1;210(1):66.e1-66.e7. doi:10.1016/J.AJOG.2013.09.010. PubMed PMID: 24036400.

105. O'Brien M, Baczyk D, Kingdom JC. Endothelial Dysfunction in Severe Preeclampsia is Mediated by Soluble Factors, Rather than Extracellular Vesicles. Sci Rep. 2017 Jul 19;7(1):5887. doi: 10.1038/s41598-017-06178-z. PubMed PMID: 28725005.

106. Than NG, Romero R, Tarca AL, Kekesi KA, Xu Y, Xu Z, et al. Integrated systems biology approach identifies novel maternal and placental pathways of preeclampsia. Front Immunol. 2018 Aug 8;9(8):1661. doi:10.3389/fimmu.2018.01661 PubMed PMID: 30135684.

107. Than NG, Romero R, Györffy D, Posta M, Bhatti G, Done B, et al. Molecular subclasses of preeclampsia characterized by a longitudinal maternal proteomics study: Distinct biomarkers, disease pathways and options for prevention. J Perinat Med. 2023 Jan 1;51(1):51–68. doi:10.1515/jpm-2022-0433 PubMed PMID: 36253935.

108. Von Dadelszen P, Magee LA, Roberts JM. Subclassification of Preeclampsia. Hypertens Pregnancy. 2003;22(2):143–8. doi:10.1081/PRG-120021060 PubMed PMID: 12908998.

109. Valensise H, Vasapollo B, Gagliardi G, Novelli GP. Early and Late Preeclampsia. Hypertension. 2008 Nov 1;52(5):873–80. doi:10.1161/HYPERTENSIONAHA.108.117358 PubMed PMID: 18824660.

110. Li XL, Chen TT, Dong X, Gou WL, Lau S, Stone P, et al. Early onset preeclampsia in subsequent pregnancies correlates with early onset preeclampsia in first pregnancy. Eur J Obstet Gynecol Reprod Biol. 2014 Jun 1;177:94–9. doi:10.1016/J.EJOGRB.2014.03.043 PubMed PMID: 24784713.

111. Stepan H, Hund M, Andraczek T. Combining Biomarkers to Predict Pregnancy Complications and Redefine Preeclampsia. Hypertension. 2020 Apr 1;75(4):918–26. doi:10.1161/HYPERTENSIONAHA.119.13763 PubMed PMID: 32063058.

112. Wu K, Liu F, Wu W, Chen Y, Zhang W. Bioinformatics approach reveals the critical role of TGF-β signaling pathway in pre-eclampsia development. European Journal of Obstetrics & Gynecology and Reproductive Biology. 2019 Sep 1;240:130–8. doi:10.1016/J.EJOGRB.2019.06.034 PubMed PMID: 31280059.

113. Chen Y, Wu L, Huang D, Wu X, Liu K, Sun B, et al. Subtype specific immune-metabolic reprogramming in preeclampsia revealed by multiomics and serum biomarkers. Hypertens Res. 2025 Dec 19;49(3):641–57. doi:10.1038/s41440-025-02504-5 PubMed PMID: 41419624.

114. Admati I, Skarbianskis N, Hochgerner H, Ophir O, Weiner Z, Yagel S, et al. Two distinct molecular faces of preeclampsia revealed by single-cell transcriptomics. Med. 2023 Oct 13;4(10):687-709.e7. doi:10.1016/j.medj.2023.07.005 PubMed PMID: 37572658.

115. Ren Z, Gao Y, Gao Y, Liang G, Chen Q, Jiang S, et al. Distinct placental molecular processes associated with early-onset and late-onset preeclampsia. Theranostics. 2021 Mar 5;11(10):5028–44. doi:10.7150/THNO.56141 PubMed PMID: 33754042.

116. Fei H, Lu X, Shi Z, Liu X, Yang C, Zhu X, et al. Deciphering the preeclampsia-specific immune microenvironment and the role of pro-inflammatory macrophages at the maternal–fetal interface. Elife. 2025 Mar 28;13:RP100002. doi:10.7554/elife.100002. PubMed PMID: 40152904.

117. Wang Y, Li B, Zhao Y. Inflammation in Preeclampsia: Genetic Biomarkers, Mechanisms, and Therapeutic Strategies. Front Immunol. 2022 Jul 8;13:883404. doi:10.3389/fimmu.2022.883404 PubMed PMID: 35880174.

118. Broekhuizen M, Hitzerd E, van den Bosch TPP, Dumas J, Verdijk RM, van Rijn BB, et al. The Placental Innate Immune System Is Altered in Early-Onset Preeclampsia, but Not in Late-Onset Preeclampsia. Front Immunol. 2021 Dec 21;12:780043. doi:10.3389/fimmu.2021.780043 PubMed PMID: 34992598.

119. Pavlov O V., Selutin A V., Pavlova OM, Selkov SA. Two patterns of cytokine production by placental macrophages. Placenta. 2020 Feb 1;91:1–10. doi:10.1016/J.PLACENTA.2020.01.005 PubMed PMID: 31941612.

120. Yang D, Dai F, Yuan M, Zheng Y, Liu S, Deng Z, et al. Role of Transforming Growth Factor-β1 in Regulating Fetal-Maternal Immune Tolerance in Normal and Pathological Pregnancy. Front Immunol. 2021 Aug 31;12:689181. doi:10.3389/fimmu.2021.689181 PubMed PMID: 34531852.

121. Mittelberger J, Seefried M, Löb S, Kuhn C, Franitza M, Garrido F, et al. The expression of TIM-3 and Gal-9 on macrophages and Hofbauer cells in the placenta of preeclampsia patients. J Reprod Immunol. 2024 Aug 1;164(4):104296. doi:10.1016/j.jri.2024.104296 PubMed PMID: 38972269.

122. Chuva de Sousa Lopes SM, Alexdottir MS, Valdimarsdottir G. The TGFβ Family in Human Placental Development at the Fetal-Maternal Interface. Biomolecules. 2020 Mar 13;10(3):453. doi:10.3390/biom10030453. PubMed PMID: 32183218.

123. Tzavlaki K, Moustakas A. TGF-β Signaling. Biomolecules. 2020 Mar 23;10(3):487. doi:10.3390/biom10030487. PubMed PMID: 32210029.

124. Moustakas A, Heldin CH. Non-Smad TGF-β signals. J Cell Sci. 2005 Aug 15;118(Pt 16):3573-84. doi: 10.1242/jcs.02554. PubMed PMID: 16105881.

125. Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-β signaling in regulation of cell growth and differentiation. Immunol Lett. 2002 Jun 3;82(1–2):85–91. doi:10.1016/S0165-2478(02)00023-8 PubMed PMID: 12008039.

126. Haider S, Lackner AI, Dietrich B, Kunihs V, Haslinger P, Meinhardt G, et al. Transforming growth factor-β signaling governs the differentiation program of extravillous trophoblasts in the developing human placenta. Proc Natl Acad Sci U S A. 2022 Jul 12;119(28):e2120667119. doi:10.1073/pnas.2120667119 PubMed PMID: 35867736.

127. Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of Trophoblast Cell Invasion by TGFB1, 2, and 3 Is Associated with a Decrease in Active Proteases. Biol Reprod. 2005 Aug 1;73(2):374–81. doi:10.1095/BIOLREPROD.105.040337 PubMed PMID: 15858216.

128. Lyall F, Robson SC, Bulmer JN. Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction relationship to clinical outcome. Hypertension. 2013;62(6):1046–54. doi:10.1161/HYPERTENSIONAHA.113.01892 PubMed PMID: 24060885.

129. Tse WK, Whitley GSJ, Cartwright JE. Transforming Growth Factor-β1 Regulates Hepatocyte Growth Factor-induced Trophoblast Motility and Invasion. Placenta. 2002 Nov 1;23(10):699–705. doi:10.1053/PLAC.2002.0866. PubMed PMID: 12398809.

130. Wang Y, Lewis DF, Gu Y, Zhang Y, Alexander JS, Granger DN. Placental Trophoblast-Derived Factors Diminish Endothelial Barrier Function. J Clin Endocrinol Metab. 2004 May 1;89(5):2421–8. doi:10.1210/JC.2003-031707 PubMed PMID: 15126573.

131. Wang K, Lu C, Liu Y, Tao Y. In vitro effects of sEng and TGF-β on human umbilical vein endothelial cells and trophoblasts. J Obstet Gynaecol Res. 2018 Jun 1;44(6):1023–30. doi:10.1111/JOG.13643 PubMed PMID: 29673026.

132. Possomato-Vieira JS, Khalil RA. Mechanisms of Endothelial Dysfunction in Hypertensive Pregnancy and Preeclampsia. Adv Pharmacol. 2016;77:361–431. doi:10.1016/bs.apha.2016.04.008. PubMed PMID: 27451103.

133. Cervar M, Blaschitz A, Dohr G, Desoye G. Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages. Cell Tissue Res. 1999;295(2):297–305. doi:10.1007/S004410051236. PubMed PMID: 9931376

134. Deng J, Zhao HJ, Zhong Y, Hu C, Meng J, Wang C, et al. H3K27me3-modulated Hofbauer cell BMP2 signalling enhancement compensates for shallow trophoblast invasion in preeclampsia. EBioMedicine. 2023 Jul 1;93:104664. doi:10.1016/j.ebiom.2023.104664. PubMed PMID: 37331163.

135. Liu E, Liu Z, Zhou Y, Chen M, Wang L, Li J. MicroRNA-142-3p inhibits trophoblast cell migration and invasion by disrupting the TGF-β1/Smad3 signaling pathway. Mol Med Rep. 2019 May 1;19(5):3775–82. doi:10.3892/mmr.2019.9997 PubMed PMID: 30864732.

136. Caniggia I, Grisaru-Gravnosky S, Kuliszewsky M, Post M, Lye SJ. Inhibition of TGF-β3 restores the invasive capability of extravillous trophoblasts in preeclamptic pregnancies. J Clin Invest. 1999 Jun 15;103(12):1641–50. doi:10.1172/JCI6380 PubMed PMID: 10377170.

137. Xu J, Sivasubramaniyam T, Yinon Y, Tagliaferro A, Ray J, Nevo O, et al. Aberrant TGFβ Signaling Contributes to Altered Trophoblast Differentiation in Preeclampsia.

Endocrinology. 2016 Feb 1;157(2):883–99. doi:10.1210/EN.2015-1696 PubMed PMID: 26653761.

138. Nadeem L, Munir S, Fu G, Dunk C, Baczyk D, Caniggia I, et al. Nodal Signals through Activin Receptor-Like Kinase 7 to Inhibit Trophoblast Migration and Invasion: Implication in the Pathogenesis of Preeclampsia. Am J Pathol. 2011 Mar 1;178(3):1177–89. doi:10.1016/J.AJPATH.2010.11.066. PubMed PMID: 21356369.

139. Caniggia I, Lye SJ, Cross JC. Activin Is a Local Regulator of Human Cytotrophoblast Cell Differentiation. Endocrinology. 1997 Sep 1;138(9):3976–86. doi:10.1210/ENDO.138.9.5403. PubMed PMID: 9275089.

140. Lebrin F, Goumans MJ, Jonker L, Carvalho RLC, Valdimarsdottir G, Thorikay M, et al. Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. EMBO J. 2004 Oct 13;23(20):4018–28. doi:10.1038/SJ.EMBOJ.7600386 PubMed PMID: 15385967.

141. Caniggia I, Taylor C V., Ritchie JWK, Lye SJ, Letarte M. Endoglin Regulates Trophoblast Differentiation along the Invasive Pathway in Human Placental Villous Explants. Endocrinology. 1997 Nov 1;138(11):4977–88. doi:10.1210/ENDO.138.11.5475. PubMed PMID: 9348229.

142. Wu L, Luo LH, Zhang YX, Li Q, Xu B, Zhou GX, et al. Alteration of Th17 and Treg cells in patients with unexplained recurrent spontaneous abortion before and after lymphocyte immunization therapy. Reprod Biol Endocrinol. 2014 Aug 3;12(1):74. doi:10.1186/1477-7827-12-74 PubMed PMID: 25086467.

143. Zhang J, Dunk CE, Shynlova O, Caniggia I, Lye SJ. TGFb1 suppresses the activation of distinct dNK subpopulations in preeclampsia. EBioMedicine. 2019 Jan 1;39:531–9. doi:10.1016/J.EBIOM.2018.12.015 PubMed PMID: 30579870.

144. Ning F, Liu H, Lash GE. The Role of Decidual Macrophages During Normal and Pathological Pregnancy. Am J Reprod Immunol. 2016 Mar;75(3):298-309. doi: 10.1111/aji.12477. PubMed PMID: 26750089.

145. Johnson EL, Chakraborty R. Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of

immunoregulatory cytokines. Retrovirology. 2012 Dec 5;9(1):101. doi:10.1186/1742-4690-9-101 PubMed PMID: 23217137.

146. Shcherbakova A, Utkina M, Valyaeva A, Pachuashvili N, Bondarenko E, Urusova L, et al. Factors Affecting Cell Viability during the Enzymatic Dissociation of Human Endocrine Tumor Tissues. Biology (Basel). 2024 Sep 1;13(9):665. doi:10.3390/biology13090665. PubMed PMID: 39336093

147. Mezouar S, Ben Amara A, Chartier C, Gorvel L, Mege JL. A Fast and Reliable Method to Isolate Human Placental Macrophages. Curr Protoc Immunol. 2019 Jun 1;125(1):e77. doi:10.1002/cpim.77 PubMed PMID: 31125190.

148. Hornschuh M, Haas V, Winkel PP, Gökyildirim MY, Mullins CS, Wrobel IM, et al. Negative Magnetic Sorting Preserves the Functionality of Ex Vivo Cultivated Non-Adherent Human Monocytes. Biology (Basel). 2022 Nov 1;11(11):1583. doi:10.3390/biology11111583. PubMed PMID: 36358284.

149. Sutton L, Mason DY, Redman CW. HLA-DR positive cells in the human placenta. Immunology. 1983 May;49(1):103-112. PubMed PMID: 6220962.

150. Kim JS, Romero R, Kim MR, Kim YM, Friel L, Espinoza J, et al. Involvement of Hofbauer cells and maternal T cells in villitis of unknown aetiology. Histopathology. 2008 Mar 1;52(4):457–64. doi:10.1111/J.1365-2559.2008.02964.X. PubMed PMID: 18315598.

151. Al Mamun A, Chauhan A, Qi S, Ngwa C, Xu Y, Sharmeen R, et al. Microglial IRF5-IRF4 regulatory axis regulates neuroinflammation after cerebral ischemia and impacts stroke outcomes. Proc Natl Acad Sci U S A. 2020 Jan 21;117(3):1742-1752. doi: 10.1073/pnas.1914742117. PubMed PMID: 31892541.

152. Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol. 2010 Oct;11(10):936-44. doi: 10.1038/ni.1920. PubMed PMID: 20729857.

153. Tranquilli AL, Brown MA, Zeeman GG, Dekker G, Sibai BM. The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of

Hypertension in Pregnancy (ISSHP). Pregnancy Hypertens. 2013 Jan 1;3(1):44–7. doi:10.1016/J.PREGHY.2012.11.001. PubMed PMID: 26105740

154. LaMarca B. Endothelial dysfunction. An important mediator in the pathophysiology of hypertension during pre-eclampsia. Minerva Ginecol. 2012 Aug;64(4):309-20. PubMed PMID: 22728575.

155. Sánchez-Aranguren LC, Prada CE, Riaño-Medina CE, Lopez M. Endothelial dysfunction and preeclampsia: Role of oxidative stress. Front Physiol. 2014 Oct 10;5:372. doi: 10.3389/fphys.2014.00372. PubMed PMID:25346691.

156. Aneman I, Pienaar D, Suvakov S, Simic TP, Garovic VD, McClements L. Mechanisms of Key Innate Immune Cells in Early- and Late-Onset Preeclampsia. Front Immunol. 2020 Aug 18;11:1864. doi:10.3389/fimmu.2020.01864 PubMed PMID: 33013837.

157. Kariori M, Katsi V, Tsioufis C. Late vs. Early Preeclampsia. International Journal of Molecular Sciences 2025, Vol 26, Page 11091. 2025 Nov 16;26(22):11091. doi:10.3390/ijms262211091 PubMed PMID: 41303573.

158. Herzog EM, Eggink AJ, Reijnierse A, Kerkhof MAM, de Krijger RR, Roks AJM, et al. Impact of early- and late-onset preeclampsia on features of placental and newborn vascular health. Placenta. 2017 Jan 1;49:72–9. doi:10.1016/j.placenta.2016.11.014 PubMed PMID: 28012458.

159. Li M, Han X, Peng Y, He Y, You Q, Zhang J. Placental vascular remodeling in preeclampsia: A three-dimensional analysis of microvascular alterations across disease severity. Placenta. 2026 Feb 1;174:96–108. doi:10.1016/j.placenta.2025.12.005 PubMed PMID: 41391446.

160. Zulu MZ, Martinez FO, Gordon S, Gray CM. The Elusive Role of Placental Macrophages: The Hofbauer Cell. J Innate Immun. 2019;11(6):447-456. doi: 10.1159/000497416. PubMed PMID:30970346.

161. Derisoud E, Jiang H, Zhao A, Chavatte-Palmer P, Deng Q. Revealing the molecular landscape of human placenta: a systematic review and meta-analysis of single-cell RNA sequencing studies. Hum Reprod Update. 2024 Jul 1;30(4):410–41. doi:10.1093/humupd/dmae006. PubMed PMID: 38478759.

162. Wei X, Li K, Lu S, Li F, Zhang M, Liu Y, et al. Single-cell RNA sequencing reveals systemic and placental immune landscape in preeclampsia. Placenta. 2025 Oct 1;170:26–35. doi:10.1016/j.placenta.2025.08.325 PubMed PMID: 40803063.

163. Campbell KA, Colacino JA, Puttabyatappa M, Dou JF, Elkin ER, Hammoud SS, et al. Placental cell type deconvolution reveals that cell proportions drive preeclampsia gene expression differences. Commun Biol. 2023 Mar 13;6(1):264. doi:10.1038/s42003-023-04623-6 PubMed PMID: 36914823.

164. Zheng X, Wen Y, Zhao X. Adjusting methylation levels with nucleus proportions highlights functional significance of differentially methylated cytosines associated with pre-eclampsia. Mol Hum Reprod. 2024 Oct 12;30(10):gaae032. doi:10.1093/molehr/gaae032 PubMed PMID: 39254642.

165. Chen Z, Yang L, Geng L, Zhang X, Du M, Sun Y, et al. Placental whole transcriptome expression profile in patients with early-onset, late-onset preeclampsia and gestational diabetes mellitus. Sci Rep. 2025 Jun 3;15(1):19476. doi:10.1038/s41598-025-04836-1 PubMed PMID: 40461719.

166. Almuttaqi H, Udalova IA. Advances and challenges in targeting IRF5, a key regulator of inflammation. FEBS J. 2019 May 1;286(9):1624–37. doi:10.1111/FEBS.14654 PubMed PMID: 30199605.

167. Hedl M, Yan J, Abraham C. IRF5 and IRF5 Disease-Risk Variants Increase Glycolysis and Human M1 Macrophage Polarization by Regulating Proximal Signaling and Akt2 Activation. Cell Rep. 2016 Aug 30;16(9):2442–55. doi:10.1016/j.celrep.2016.07.060 PubMed PMID: 27545875.

168. Firmal P, Shah VK, Chattopadhyay S. Insight Into TLR4-Mediated Immunomodulation in Normal Pregnancy and Related Disorders. Front Immunol. 2020 May 19;11:807. doi:10.3389/FIMMU.2020.00807. PubMed PMID: 32508811.

169. Gordon S, Martinez FO. Alternative Activation of Macrophages: Mechanism and Functions. Immunity. 2010 May 28;32(5):593–604. doi:10.1016/J.IMMUNI.2010.05.007. PubMed PMID: 20510870.

170. Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, Liu ZG. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. Cell Res. 2013 Jun 11;23(7):898–914. doi:10.1038/cr.2013.75 PubMed PMID: 23752925.

171. Choi H, Yang SW, Joo JS, Park M, Jin Y, Kim JW, et al. Sialylated IVIg binding to DC-SIGN+ Hofbauer cells induces immune tolerance through the caveolin-1/NF-kB pathway and IL-10 secretion. Clinical Immunology. 2023 Jan 1;246:109215. doi:10.1016/j.clim.2022.109215 PubMed PMID: 36581222.

172. Huppertz B. Oxygenation of the placenta and its role in pre-eclampsia. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health. 2014 Jul 1;4(3):244–5. doi:10.1016/J.PREGHY.2014.04.016. PubMed PMID: 26104644.

173. Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS. Placental Endoplasmic Reticulum Stress and Oxidative Stress in the Pathophysiology of Unexplained Intrauterine Growth Restriction and Early Onset Preeclampsia. Placenta. 2009 Mar;30 Suppl A(Suppl):S43-8. doi: 10.1016/j.placenta.2008.11.003. PubMed PMID: 19081132.

174. Oishi Y, Manabe I. Macrophages in inflammation, repair and regeneration. Int Immunol. 2018 Oct 29;30(11):511–28. doi:10.1093/INTIMM/DXY054 PubMed PMID: 30165385.

175. Cohen HB, Mosser DM. Extrinsic and intrinsic control of macrophage inflammatory responses. J Leukoc Biol. 2013 Nov 1;94(5):913–9. doi:10.1189/jlb.0413236 PubMed PMID: 23964115.

176. Porcheray F, Viaud S, Rimaniol AC, Léone C, Samah B, Dereuddre-Bosquet N, et al. Macrophage activation switching: an asset for the resolution of inflammation. Clin Exp Immunol. 2005 Dec 1;142(3):481–9. doi:10.1111/J.1365-2249.2005.02934.X PubMed PMID: 16297160.

177. Pantazi P, Kaforou M, Tang Z, Abrahams VM, McArdle A, Guller S, et al. Placental macrophage responses to viral and bacterial ligands and the influence of fetal sex. iScience. 2022 Dec 22;25(12):105653. doi:10.1016/j.isci.2022.105653. PubMed PMID: 36505933.

178. Schoots MH, Gordijn SJ, Scherjon SA, van Goor H, Hillebrands JL. Oxidative stress in placental pathology. Placenta. 2018 Sep 1;69(10):153–61. doi:10.1016/j.placenta.2018.03.003 PubMed PMID: 29622278.

179. Tenório MB, Ferreira RC, Moura FA, Bueno NB, Menezes De Oliveira AC, Oliveira M, et al. Cross-Talk between Oxidative Stress and Inflammation in Preeclampsia. Oxid Med Cell Longev. 2019 Nov 4;2019:8238727. doi:10.1155/2019/8238727. PubMed PMID: 31781353.

180. Marín R, Chiarello DI, Abad C, Rojas D, Toledo F, Sobrevia L. Oxidative stress and mitochondrial dysfunction in early-onset and late-onset preeclampsia. Biochim Biophys Acta Mol Basis Dis. 2020 Dec 1;1866(12):165961. doi:10.1016/J.BBADIS.2020.165961 PubMed PMID: 32916282.

181. Abrahams VM, Tang Z, Mor G, Guller S. NLRP3 inflammasome function and pyroptotic cell death in human placental Hofbauer cells. J Reprod Immunol. 2020 Nov 1;142:103214. doi:10.1016/J.JRI.2020.103214 PubMed PMID: 33152658.

182. Bailey LJ, Alahari S, Tagliaferro A, Post M, Caniggia I. Augmented trophoblast cell death in preeclampsia can proceed via ceramide-mediated necroptosis. Cell Death Dis. 2017 Feb 2;8(2):e2590. doi:10.1038/cddis.2016.483 PubMed PMID: 28151467.

183. Abrahams VM, Kim YM, Straszewski SL, Romero R, Mor G. Macrophages and Apoptotic Cell Clearance During Pregnancy. Am J Reprod Immunol. 2004 Apr 1;51(4):275–82. doi:10.1111/j.1600-0897.2004.00156.x PubMed PMID: 15212680.

184. Huppertz B, Kingdom JCP. Apoptosis in the trophoblast--role of apoptosis in placental morphogenesis. J Soc Gynecol Investig. 2004 Sep;11(6):353–62. doi:10.1016/j.jsgi.2004.06.002 PubMed PMID: 15350247.

185. DiFederico E, Genbacev O, Fisher SJ. Preeclampsia Is Associated with Widespread Apoptosis of Placental Cytotrophoblasts within the Uterine Wall. Am J Pathol. 1999;155(1):293-301. doi:10.1016/S0002-9440(10)65123-1 PubMed PMID: 10393861.

186. Blakey H, Sun R, Xie L, Russell R, Sarween N, Hodson J, et al. Pre-eclampsia is associated with complement pathway activation in the maternal and fetal circulation, and

placental tissue. Pregnancy Hypertens. 2023 Jun 1;32(1):43–9. doi:10.1016/j.preghy.2023.04.001 PubMed PMID: 37088032.

187. Burwick RM, Java A, Regal JF. The role of complement in normal pregnancy and preeclampsia. Front Immunol. 2025 Jul 24;16:1643896. doi:10.3389/fimmu.2025.1643896 PubMed PMID: 40777009.

188. Stirrat GM, Redman CWG, Levinsky RJ. Circulating immune complexes in pre-eclampsia. Br Med J. 1978 Jun 3;1(6125):1450–1. doi:10.1136/bmj.1.6125.1450 PubMed PMID: 647331.

189. Przybyl L, Haase N, Golic M, Rugor J, Solano ME, Arck PC, et al. CD74-Downregulation of Placental Macrophage-Trophoblastic Interactions in Preeclampsia. Circ Res. 2016 Jun 24;119(1):55–68. doi:10.1161/CIRCRESAHA.116.308304 PubMed PMID: 27199465.

190. Takaki H, Minoda Y, Koga K, Takaesu G, Yoshimura A, Kobayashi T. TGF-β1 suppresses IFN-γ-induced NO production in macrophages by suppressing STAT1 activation and accelerating iNOS protein degradation. Genes Cells. 2006 Aug 1;11(8):871–82. doi:10.1111/j.1365-2443.2006.00988.x PubMed PMID: 16866871.

191. Gong D, Shi W, Yi S ju, Chen H, Groffen J, Heisterkamp N. BMC Immunol. 2012 Jun 15;13:31. doi: 10.1186/1471-2172-13-31. PubMed PMID: 22703233.

192. Werner F, Jain MK, Feinberg MW, Sibinga NES, Pellacani A, Wiesel P, et al. Transforming Growth Factor-β1 Inhibition of Macrophage Activation Is Mediated via Smad3. J Biol Chem. 2000 Nov 24;275(47):36653–8. doi:10.1074/jbc.M004536200 PubMed PMID: 10973958.

193. Zhang F, Wang H, Wang X, Jiang G, Liu H, Zhang G, et al. TGF-β induces M2-like macrophage polarization via SNAIL-mediated suppression of a pro-inflammatory phenotype. Oncotarget. 2016 Aug 1;7(32):52294–306. doi:10.18632/oncotarget.10561 PubMed PMID: 27418133.

194. Lockwood CJ, Oner C, Uz YH, Kayisli UA, Huang SJ, Buchwalder LF, et al. Matrix Metalloproteinase 9 (MMP9) Expression in Preeclamptic Decidua and MMP9 Induction by Tumor Necrosis Factor Alpha and Interleukin 1 Beta in Human First Trimester

Decidual Cells. Biol Reprod. 2008 Jun;78(6):1064–72. doi:10.1095/biolreprod.107.063743 PubMed PMID: 18276934.

195. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. Circ Res. 2003 May 2;92(8):827–39. doi:10.1161/01.RES.0000070112.80711.3D PubMed PMID: 12730128.

196. Massagué J, Sheppard D. TGF-β signaling in health and disease. Cell. 2023 Sep 14;186(19):4007–37. doi:10.1016/j.cell.2023.07.036 PubMed PMID: 37714133.

197. Lech M, Anders HJ. Macrophages and fibrosis: How resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair. Biochim Biophys Acta. 2013 Jul;1832(7):989-97. doi: 10.1016/j.bbadis.2012.12.001. PubMed PMID: 23246690.

198. Lu P, Takai K, Weaver VM, Werb Z. Extracellular Matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol. 2011 Dec;3(12):a005058. doi:10.1101/cshperspect.a005058 PubMed PMID: 21917992.

199. Wang X, Khalil RA. Matrix Metalloproteinases, Vascular Remodeling, and Vascular Disease. Adv Pharmacol. 2018;81:241–330. doi:10.1016/bs.apha.2017.08.002. PubMed PMID: 29310800.

200. Stergiotou I, Crispi F, Valenzuela-Alcaraz B, Bijnens B, Gratacos E. Patterns of maternal vascular remodeling and responsiveness in early- versus late-onset preeclampsia. Am J Obstet Gynecol. 2013 Dec 1;209(6):558.e1-558.e14. doi:10.1016/J.AJOG.2013.07.030. PubMed PMID: 23911383.

201. Batra J, Soares AS, Mehner C, Radisky ES. Matrix Metalloproteinase-10/TIMP-2 Structure and Analyses Define Conserved Core Interactions and Diverse Exosite Interactions in MMP/TIMP Complexes. PLoS One. 2013 Sep 20;8(9):e75836. doi:10.1371/journal.pone.0075836 PubMed PMID: 24073280.

202. Tency I, Verstraelen H, Kroes I, Holtappels G, Verhasselt B, Vaneechoutte M, et al. Imbalances between Matrix Metalloproteinases (MMPs) and Tissue Inhibitor of

Metalloproteinases (TIMPs) in Maternal Serum during Preterm Labor. PLoS One. 2012 Nov 8;7(11):e49042. doi:10.1371/journal.pone.0049042 PubMed PMID: 23145060.

203. Zhu JY, Zhong M, Pang ZJ, Yu YH. Dysregulated expression of matrix metalloproteinases and their inhibitors may participate in the pathogenesis of pre-eclampsia and fetal growth restriction. Early Hum Dev. 2014 Oct 1;90(10):657–64. doi:10.1016/j.earlhumdev.2014.08.007 PubMed PMID: 25194834.

204. Wang Z, Lu S, Liu C, Zhao B, Pei K, Tian L, et al. Expressional and epigenetic alterations of placental matrix metalloproteinase 9 in preeclampsia. Gynecol Endocrinol. 2010 Feb;26(2):96–102. doi:10.3109/09513590903184100 PubMed PMID: 19724956.

205. Majali-Martinez A, Hiden U, Ghaffari-Tabrizi-Wizsy N, Lang U, Desoye G, Dieber-Rotheneder M. Placental membrane-type metalloproteinases (MT-MMPs): Key players in pregnancy. Cell Adh Migr. 2016 Mar 3;10(1-2):136-46. doi: 10.1080/19336918.2015.1110671. PubMed PMID:26745344.

206. Nacu N, Luzina IG, Highsmith K, Lockatell V, Pochetuhen K, Cooper ZA, et al. Macrophages Produce TGF-β-Induced (β-ig-h3) following Ingestion of Apoptotic Cells and Regulate MMP14 Levels and Collagen Turnover in Fibroblasts. J Immunol. 2008;180(7):5036–44. doi:10.4049/jimmunol.180.7.5036 PubMed PMID: 18354229.

207. Vempati P, Popel AS, Mac Gabhann F. Extracellular regulation of VEGF: Isoforms, proteolysis, and vascular patterning. Cytokine Growth Factor Rev. 2014 Feb 1;25(1):1–19. doi:10.1016/j.cytogfr.2013.11.002 PubMed PMID: 24332926.

208. Hawinkels LJAC, Zuidwijk K, Verspaget HW, de Jonge-Muller ESM, Duijn W van, Ferreira V, et al. VEGF release by MMP-9 mediated heparan sulphate cleavage induces colorectal cancer angiogenesis. Eur J Cancer. 2008 Sep 1;44(13):1904–13. doi:10.1016/j.ejca.2008.06.031 PubMed PMID: 18691882.

209. Dayer C, Stamenkovic I. Recruitment of matrix metalloproteinase-9 (MMP-9) to the fibroblast cell surface by lysyl hydroxylase 3 (LH3) triggers transforming growth factor-β (TGF-β) activation and fibroblast differentiation. J Biol Chem. 2015 May 29;290(22):13763–78. doi:10.1074/jbc.M114.622274 PubMed PMID: 25825495.

210. Bourboulia D, Stetler-Stevenson WG. Matrix MetalloProteinases (MMPs) andTissue Inhibitors of MetalloProteinases (TIMPs): positive and negative regulators intumor cell adhesion. Semin Cancer Biol. 2010 Jun;20(3):161–8. doi:10.1016/j.semcancer.2010.05.002 PubMed PMID: 20470890.

211. Dallas SL, Rosser JL, Mundy GR, Bonewald LF. Proteolysis of Latent Transforming Growth Factor-β (TGF-β)-binding Protein-1 by Osteoclasts. J Biol Chem. 2002 Jun 14;277(24):21352–60. doi:10.1074/jbc.m111663200. PubMed PMID: 11929865.

212. Santibanez JF, Guerrero J, Quintanilla M, Fabra A, Martínez J. Transforming growth factor-β1 modulates matrix metalloproteinase-9 production through the Ras/MAPK signaling pathway in transformed keratinocytes. Biochem Biophys Res Commun. 2002 Aug 16;296(2):267–73. doi:10.1016/S0006-291X(02)00864-1 PubMed PMID: 12163012.

213. Sinpitaksakul SN, Pimkhaokham A, Sanchavanakit N, Pavasant P. TGF-β1 induced MMP-9 expression in HNSCC cell lines via Smad/MLCK pathway. Biochem Biophys Res Commun. 2008 Jul 11;371(4):713–8. doi:10.1016/j.bbrc.2008.04.128 PubMed PMID: 18457660.

214. Guan F, Wang R, Yi Z, Luo P, Liu W, Xie Y, et al. Tissue macrophages: origin, heterogenity, biological functions, diseases and therapeutic targets. Signal Transduct Target Ther. 2025 Mar 7;10(1):93. doi:10.1038/s41392-025-02124-y PubMed PMID: 40055311.

215. Graney PL, Ben-Shaul S, Landau S, Bajpai A, Singh B, Eager J, et al. Macrophages of diverse phenotypes drive vascularization of engineered tissues. Sci Adv. 2020 Apr 1;6(18):eaay6391. doi:10.1126/sciadv.aay6391 PubMed PMID: 32494664.

216. Xuan YH, Choi YL, Shin YK, Ahn GH, Kim KH, Kim WJ, et al. Expression of TGF-beta signaling proteins in normal placenta and gestational trophoblastic disease. Histol Histopathol. 2007 Jan;22(3):227–34. doi:10.14670/HH-22.227 PubMed PMID: 17163397.

217. Ali M, Ahmed M, Memon M, Chandio F, Shaikh Q, Parveen A, et al. Preeclampsia: A comprehensive review. Clin Chim Acta. 2024 Sep 15;563:119922. doi:10.1016/j.cca.2024.119922 PubMed PMID: 39142550.

218. Rana S, Lemoine E, Granger J, Karumanchi SA. Preeclampsia: Pathophysiology, Challenges, and Perspectives. Circ Res. 2019 Mar 29;124(7):1094–112. doi:10.1161/CIRCRESAHA.118.313276 PubMed PMID: 30920918.

219. Martin E, Ray PD, Smeester L, Grace MR, Boggess K, Fry RC. Epigenetics and Preeclampsia: Defining Functional Epimutations in the Preeclamptic Placenta Related to the TGF-β Pathway. PLoS One. 2015 Oct 28;10(10):e0141294. doi:10.1371/JOURNAL.PONE.0141294 PubMed PMID: 26510177.

220. You J, Wang W, Chang HM, Yi Y, Zhao H, Zhu H, et al. The BMP2 Signaling Axis Promotes Invasive Differentiation of Human Trophoblasts. Front Cell Dev Biol. 2021 Feb 4;9:607332. doi:10.3389/fcell.2021.607332. PubMed PMID: 33614644.

221. Yi Y, Zhu H, Klausen C, Chang HM, Inkster AM, Terry J, et al. Dysregulated BMP2 in the Placenta May Contribute to Early-Onset Preeclampsia by Regulating Human Trophoblast Expression of Extracellular Matrix and Adhesion Molecules. Front Cell Dev Biol. 2021 Dec 14;9:768669. doi:10.3389/FCELL.2021.768669. PubMed PMID: 34970543.

222. Raguema N, Moustadraf S, Bertagnolli M. Immune and Apoptosis Mechanisms Regulating Placental Development and Vascularization in Preeclampsia. Front Physiol. 2020 Feb 11;11:98. doi:10.3389/fphys.2020.00098. PubMed PMID: 32116801.

223. Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-β receptor function in the endothelium. Cardiovasc Res. 2005 Feb 15;65(3):599–608. doi:10.1016/j.cardiores.2004.10.036 PubMed PMID: 15664386.

224. Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, et al. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. Mol Cell. 2003 Oct 1;12(4):817–28. doi:10.1016/S1097-2765(03)00386-1 PubMed PMID: 14580334.

225. Perucci LO, Gomes KB, Freitas LG, Godoi LC, Alpoim PN, Pinheiro MB, et al. Soluble Endoglin, Transforming Growth Factor-Beta 1 and Soluble Tumor Necrosis Factor Alpha Receptors in Different Clinical Manifestations of Preeclampsia. PLoS One. 2014 May 22;9(5):e97632. doi:10.1371/JOURNAL.PONE.0097632 PubMed PMID: 24851923.

226. Ten Dijke P, Goumans MJ, Pardali E. Endoglin in angiogenesis and vascular diseases. Angiogenesis. 2008 Mar 19;11(1):79–89. doi:10.1007/s10456-008-9101-9 PubMed PMID: 18283546.

227. Margioula-Siarkou G, Margioula-Siarkou C, Petousis S, Margaritis K, Vavoulidis E, Gullo G, et al. The role of endoglin and its soluble form in pathogenesis of preeclampsia. Mol Cell Biochem. 2022 Feb 1;477(2):479–91. doi:10.1007/S11010-021-04294-Z PubMed PMID: 34783962.

228. Boeldt DS, Bird IM. Vascular adaptation in pregnancy and endothelial dysfunction in preeclampsia. J Endocrinol. 2017 Jan;232(1):R27-R44. doi: 10.1530/JOE-16-0340. PubMed PMID: 27729465.

229. Scioscia M, Ananth Karumanchi S, Goldman-Wohl D, Robillard PY. Endothelial dysfunction and metabolic syndrome in preeclampsia: an alternative viewpoint. J Reprod Immunol. 2015;108:42–7. doi:10.1016/j.jri.2015.01.009. PubMed PMID: 25766966.

230. Cho JG, Lee A, Chang W, Lee MS, Kim J. Endothelial to mesenchymal transition represents a key link in the interaction between inflammation and endothelial dysfunction. Front Immunol. 2018 Feb 20;9:294. doi: 10.3389/fimmu.2018.00294. PubMed PMID: 29515588.

231. Chen LM, Liu B, Zhao HB, Stone P, Chen Q, Chamley L. IL-6, TNFα and TGFβ Promote Nonapoptotic Trophoblast Deportation and Subsequently Causes Endothelial Cell Activation. Placenta. 2010 Jan 1;31(1):75–80. doi:10.1016/J.PLACENTA.2009.11.005 PubMed PMID: 19948358.

232. Sanjabi S, Oh SA, Li MO. Regulation of the immune response by TGF-β: From conception to autoimmunity and infection. Cold Spring Harb Perspect Biol. 2017 Jun 1;9(6):a022236. doi:10.1101/cshperspect.a022236. PubMed PMID: 28108486.

233. Goumans MJ, Liu Z, Ten Dijke P. TGF-β signaling in vascular biology and dysfunction. Cell Res. 2009 Jan;19(1):116-27. doi: 10.1038/cr.2008.326. PubMed PMID: 19114994.

234. Torry DS, Leavenworth J, Chang M, Maheshwari V, Groesch K, Ball ER, et al. Angiogenesis in implantation. J Assist Reprod Genet. 2007 Jul 7;24(7):303–15. doi:10.1007/s10815-007-9152-7 PubMed PMID: 17616801.

235. Camargo L do N, Righetti RF, Aristóteles LR de CRB, dos Santos TM, de Souza FCR, Fukuzaki S, et al. Effects of Anti-IL-17 on Inflammation, Remodeling, and Oxidative Stress in an Experimental Model of Asthma Exacerbated by LPS. Front Immunol. 2018 Jan 5;8:1835. doi:10.3389/fimmu.2017.01835. PubMed PMID: 29379497.

236. Fitzgerald E, Shen M, Yong HEJ, Wang Z, Pokhvisneva I, Patel S, et al. Hofbauer cell function in the term placenta associates with adult cardiovascular and depressive outcomes. Nat Commun. 2023 Nov 14;14(1):7120. doi:10.1038/s41467-023-42300-8 PubMed PMID: 37963865.

237. Liu Y, Fan X, Wang R, Lu X, Dang YL, Wang H, et al. Single-cell RNA-seq reveals the diversity of trophoblast subtypes and patterns of differentiation in the human placenta. Cell Res. 2018 Jul 24;28(8):819–32. doi:10.1038/s41422-018-0066-y PubMed PMID: 30042384.

238. Zhou C, Yan Q, Zou QY, Zhong XQ, Tyler CT, Magness RR, et al. Sexual Dimorphisms of Preeclampsia-Dysregulated Transcriptomic Profiles and Cell Function in Fetal Endothelial Cells. Hypertension. 2019 Jul 1;74(1):154–63. doi:10.1161/HYPERTENSIONAHA.118.12569 PubMed PMID: 31154903.

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EDITED BY
Ruoxi Yuan,
Hospital for Special Surgery, United States

REVIEWED BY
Poonam Dhillon,
University of Pennsylvania, United States
Xinjun Wu,
University of North Carolina at Chapel Hill,
United States

*CORRESPONDENCE
Christian Wadsack
✉ christian.wadsack@medunigraz.at

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Placental macrophages present distinct polarization pattern and effector functions depending on clinical onset of preeclampsia

Monika Horvat Mercnik[1], Carolin Schliefsteiner[1], Herbert Fluhr[1] and Christian Wadsack[1,2]*

[1]Department of Obstetrics and Gynaecology, Medical University of Graz, Graz, Austria, [2]BioTechMed-Graz, Graz, Austria

Hofbauer cells (HBCs) are resident macrophages of the human placenta, regulating immune tolerance and tissue homeostasis. HBCs of a normal placenta (CTR) exhibit mainly an anti-inflammatory M2 phenotype. Under exaggerated chronic inflammation during pregnancy, as in preeclampsia (PE), a phenotypic switch towards M1 polarization has been proposed. PE, defined as maternally derived syndrome can be distinguished into two different entities: early-onset (EO) preeclampsia and late-onset (LO) preeclampsia. Although the clinical presenting characteristics overlap, both can be identified by biochemical markers, heritability, and different maternal and fetal outcomes. To date, no study has specifically investigated polarization and phenotype of EO- and LO-PE HBCs and looked at possible changes in HBC functionality. Primary HBCs were isolated from CTR and PE placentae. First, *in vitro* morphological differences were observed between CTR and PE HBCs, with both PE groups exhibiting features of M1 macrophages alongside M2 forms. Interestingly, a different polarization pattern was observed between EO- and LO-PE HBCs. EO-PE HBCs develop a tissue remodeling M2 phenotype that is strongly shifted toward M1 polarization and showed a significant upregulation of CD86, TLR4, and HLA-DR. Furthermore, this pro-inflammatory signature is corroborated by higher expression of IRF5 and of *NOS2* ($p \leq 0.05$). However, their M2 characteristics is reflected by significant TGF-β secretion and *ARG1* expression. In contrast, LO-PE HBCs developed a phagocytic CD209-low M2 phenotype in which the M1 pattern was not as pronounced as they downregulated the *NOS2* gene, but expressed increased levels of pro-inflammatory CD80 and TLR1 ($p \leq 0.05$). The enhanced phagocytosis and MMP-9 secretion alongside the increased secretion of anti-inflammatory IL -4, IL -13 and TGF-β in both EO- and LO-PE HBCs suggests their adaptive role and plasticity in resolving inflammation and tissue homeostasis.

KEYWORDS

hofbauer cell, preeclampsia, polarization, early onset preeeclampsia, late onset preeclampsia, inflammation, macrophage, human placenta

1 Introduction

Preeclampsia is a maternally derived inflammatory syndrome, affecting 4-5% of pregnancies worldwide. It is a leading cause of preterm delivery and intrauterine growth restriction, mainly due to the insufficient nutrient supply across the placenta and chronic hypoxia exposure of the fetus (1, 2). PE is clinically defined as *de novo* onset of hypertension (≥140/90 mmHg) accompanied by one or more of the following new-onset conditions: proteinuria, thrombocytopenia, renal failure, impaired liver function, pulmonary edema, neurological complications, or uteroplacental dysfunction, occurring after 20 weeks of gestation (1–3). Depending on the time of diagnosis, this syndrome can be divided into two subgroups, namely before (early-onset, EO) or after (late-onset, LO) 34 weeks of gestation (4–6). PE is a complex and heterogeneous disorder whose pathophysiological mechanisms are still not fully understood (7). Of note, different aetiology of EO- and LO-PE has been suggested. Briefly, EO-PE is associated with placental dysfunction and is more likely to affect the fetus (8, 9), whereas LO-PE is mediated by maternal factors, therefore more favourable for successful fetal outcome (10). The placenta in PE is characterized by profound morphological and functional alterations (11), due to poor placentation and placental ischemia (12, 13). In addition, placental dysfunction has been associated with an imbalanced immune function, excessive inflammation accompanied with increased production of pro-inflammatory factors, and simultaneously a decrease in the number of regulatory immune cells and anti-inflammatory cytokines, all together contributing to the development and progress of PE (14–16).

Both the maternal and feto-placental immune system play a crucial role in the development of pregnancy (16). Hence, in contrast to normal pregnancy, where the immune systems contribute to the maintenance of feto-maternal tolerance and placental development, a pro-inflammatory environment leads to excessive activation of innate immune cells and consequently to placental dysfunction and/or poor maternal vascular adaptation (16–19). Macrophages represent a diverse group of innate immune cells, vital for the regulation of inflammation, tissue homeostasis, and defence (20). Due to their remarkable plasticity that allows them fast and direct response to the stimuli and to the adaptive capability of their micro-environmental milieu (21), they are important key players in the progression of pregnancy and could be involved in the development and progression of PE (16, 19). Macrophages are keen to develop a broad spectrum of phenotypes along the M1 and M2 axis, which allows to divide the cells into defined classical M1 and M2 polarized groups (22, 23) The balance between the different polarization states often plays an important role in the resolution or progression of inflammation (24, 25). Their phenotypic heterogeneity is also reflected in their effector functions. In general, M1 macrophages are thought to be pro-inflammatory, while M2 macrophages limit inflammation and promote tissue repair, angiogenesis and homeostasis (26, 27).

Hofbauer cells (HBCs) are placental macrophages residing from day 18 after conception (28) in the chorionic villi of the human placenta (29). In a normal pregnancy HBCs are M2 polarized (26, 30–33) long spindled cells with large vacuoles (34). Due to their phenotypic heterogeneity, HBCs fulfil a variety of functions (35). As placental immune cells, they exhibit micro-biocidal activity (36, 37) and promote maternal tolerance towards the fetus (38). They are known to engage in tight and specific interactions with surrounding placental cells, therefore promoting trophoblast function (39, 40), tissue remodeling (36) and angiogenesis (26, 36, 41, 42). Perturbations in the homeostatic functions of HBCs are often associated with inflammation (43–46) and infection (47). Despite their crucial role in placental tissue, knowledge about the role of HBCs in PE is still lacking. A deeper understanding of HBC function offers the potential for therapeutical immune manipulation during compromised pregnancies in relation to gestational age, which determines both maternal and perinatal outcomes.

This study aimed to investigate polarization and phenotypic differences of primary human HBCs isolated from normal and PE placentae. In addition, we tested whether changes of the HBC phenotype might be linked to altered functionality, specifically to phagocytosis, tissue remodeling and the ability of macrophages to activate feto-placental endothelial cells (fpEC). Further, as gestational age has been identified as the most important clinical variable, we hypothesized that stratification of PE (EO-PE vs LO-PE) may account for the observed functional changes of HBCs within each group. These findings, while somewhat preliminary (due to case numbers), demonstrate that the inflammatory placental environment of EO-PE alters the immunoregulatory phenotype of HBCs with an increased pro-inflammatory M1 signature. Interestingly, LO-PE HBCs remained M2 polarized cells, but with a different polarization pattern as controls.

2 Materials and methods

2.1 Study population

In this study preeclampsia was defined according to the guidelines of the American College of Obstetricians and Gynaecologists as already mentioned above (1, 2). The institutional ethics committee of the Medical University of Graz (29-319 ex 16/17) approved the study. Subjects included in the study signed an informed consent form before participation, the characteristics of which are shown in the Table 1. Included placentae from singleton pregnancies were used within 30 minutes of caesarean section or vaginal delivery. PE was defined as a sustained blood pressure of 140/90 mm Hg or greater (on two occasions at least 4 hours apart) occurring after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by one or more of the following new onset conditions: proteinuria, thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, neurological complications or uteroplacental dysfunction. Onset of PE was defined as early (EO, delivered and detected before 34 weeks of gestation) or late (LO, delivered and detected after 34 weeks of gestation) (1). Placentae from normal pregnancies served as controls.

2.2 Isolation of HBCs

Primary HBCs were isolated according to a modified protocol as described by Tang et al. (48). To avoid contamination with decidual macrophages, the decidual membrane was removed before isolating

TABLE 1 Subject characteristics of women and their offspring included in the study.

	CTR (n=22)	EO-PE (n=8)	LO-PE (n=6)
Age	29.4 ± 3.5	34.4 ± 3.6 **	34.4 ± 4.0 †
BMI before pregnancy (kg/m2)	22.5 ± 2.9	21.0 ± 2.1	23.4 ± 3.2
Week of gestation	38.7 ± 1.5	34.2 ± 1.1 ****	37.7 ± 1.1
Mode of the delivery	SP 10/CS 12	CS 8	SP 3/CS 3
Fetal sex	8♀ 14♂	4♀, 4♂	2♀, 4♂
Umbilical cord blood Arterial, pH	7.30 ± 0.06	7.31 ± 0.03	7.25 ± 0.09
Umbilical cord blood Venous, pH	7.37 ± 0.06	7.36 ± 0.02	7.34 ± 0.05
Placental weight (g)	616.7 ± 123.0	425.7 ± 63.7	506.7 ± 82.8
Birth weight (g)	3345 ± 398	1996 ± 404.7 ****	2955 ± 288.9
Birth weight percentile	49.2 ± 21.2	24.0 ± 18.7 **	34.3 ± 11.3
Systolic blood pressure (mmHg)	115.1 ± 6.9	162 ± 12.1****	153.2 ± 20.9 ††††
Diastolic blood pressure (mmHg)	72.9 ± 10.2	94.3 ± 8***	103 ± 13.3 ††††
sFlt-1 [pg/mL]	/	14472 ± 5083 ‡	8257 ± 2881
PlGF [pg/mL]	/	66.2 ± 24.3	81.6 ± 13.02
sFlt-1/PlGF [pg/mL]	/	248.7 ± 118.1	105.5 ± 51.9
Platelets [109/L]	218.3 ± 63.1	221.9 ± 106.3	191.8 ± 60.8
Uric acid [mg/dL]	/	6.3 ± 1.4	5.9 ± 0.7
AST [U/L]	/	21.0 ± 6.3	21.2 ± 4.2
ALT [U/L]	/	17.4± 5.6	12.7 ± 4.8

BMI, body-mass index; SP, spontaneous delivery; CS, caesarean section; All data are shown as mean ± SD. Statistical significance was assessed by one-way ANOVA with Tukey's *post-hoc* test. If normality testing failed, Kruskal-Wallis test with Dunn's *post-hoc* test was used. When comparing two groups' Students t-test was used. **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001; whereas *CTR vs EO-PE. †p ≤ 0.05 and † † † †p ≤ 0.0001; whereas †CTR vs LO-PE. ‡ represents comparison between EO-PE vs LO-PE; whereas ‡ p ≤ 0.05.

HBCs. The villous tissue was dissected, washed in 0.4% saline solution (Fresenius, Cat #C924228), and finely minced. Between 60 and 100 g of the minced tissue was stored overnight at 4°C in 1 x phosphate buffered saline (PBS, Medicado, Cat #09-9400-100). On the next day, the tissue was digested with trypsin (0.25%, Sigma Aldrich, Cat #T4549) and DNase I (0.08 mg/ml; Roche, Cat #10104159001), followed by digestion with collagenase A (1 mg/ml; Roche, Cat #10103586001) and DNase I (0.08 mg/ml, Roche, Cat #10104159001). The cell suspension containing the HBCs was applied to a Percoll gradient (20-40%, Sigma Aldrich, Cat #P4937) and centrifuged unrestrained at 1000 g for 30 min. At this point, the HBCs appearing as bands between 30 and 35% gradient layers were aspirated and purified by negative selection using Dynabeads (Invitrogen, Cat #11033) coated with antibodies against epithelial growth factor receptor (EGFR, Santa Cruz, Cat #sc-120) and CD10 (Sigma Aldrich, Cat #SAB4700440). After immunopurification, cells were seeded in macrophage medium (MaM, ScienCell, Cat #SC1921) containing 5% FBS (ScienCell, Cat #SC1921), PenStrep (ScienCell, Cat #SC1921) and macrophage growth supplements (ScienCell, Cat #SC1921) at a cell density of $1x10^6$ cells/ml. Cells were cultured at 21% oxygen and 37°C. Quality control of the isolated HBCs was performed on the fixed cells after 6 days by immunocytochemistry for CD163 (Thermo Fischer Scientific, Cat #MA1-82342), CD90 (Dianova, Cat # DIA100), CD80 (Abcam, Cat #ab86473), CD68 (Dako, Cat #GA613), CD86 (Abcam, Cat #ab270719), CD206 (Novus, Cat #H00004360), CD209 (R&D Systems, Cat #MAB1621), and isotype control (Dako, Cat #X0931) as previously described by Schliefsteiner et al. (31). Cell culture images were obtained using brightfield microscope with a SC50 Olympus camera and CellSens software.

2.3 Immunohistochemistry

Tissue sections were taken from four different areas of placenta (reaching from chorionic plate to the decidual side and a central region of the placental disk) and fixed overnight in 4% neutral buffered paraformaldehyde solution. After paraffin embedding, tissue sections with a thickness of 5μm were mounted on glass slides. The paraffin was then removed with xylene and rehydrated in an ethanol dilution series. Antigen retrieval was performed using a citrate buffer (Gatt, Cat #403139070) adjusted to pH 6.0. UltraVision LP detection system (Thermo Fischer Scientific, Cat #TL125HL) was used for histochemical immunostaining. Tissue was incubated with Hydrogen Peroxide Block (Thermo Fischer Scientific, Cat #TL125HL) for 15 minutes and washed in TBE buffer (Gatt, Cat #403211370), followed by a 5-minute incubation with Ultra V protein block (Thermo Fischer Scientific, Cat #TL125HL). The primary antibody isotype control (1:200, Dako, Cat #X0931) and CD163 (1:200, Thermo Fischer Scientific, Cat #MA1-82342) were diluted in antibody diluent (Agilent, Dako, Cat #S0809) and incubated overnight at 4°C in a humidified chamber. After washing step, primary antibody enhancer (Thermo Fischer Scientific, Cat #TL125HL) was applied for 20 minutes. After another washing step samples were incubated with Large HRP Polymer (Thermo Fischer Scientific, Cat #TL125HL) solution for 30 minutes, followed by intensive washing and incubation with AEC Chromogen Solution (Abcam, Cat #64252) for 10 minutes. The tissue was counterstained with Haematoxylin (Gatt-Koller Cat #401296170) for 1 minute and mounted with embedding medium. Images were acquired using CellSens Standard software and an Olympus BX53 light microscope with an Olympus UC90 camera. Per slide, images of 5-10 different areas were taken and quantified with Qupath software (49).

2.4 Fluorescence assisted cell sorting (FACS)

FACS was performed to quantify the cell populations expressing M1 and M2 polarization markers of HBCs. On the fifth day after isolation, cells were harvested using accutase (Thermo Fischer Scientific, Cat #00-4555-56) and gentle scraping. Viability and number of cells after scraping was determined using a CASY cell counter model TT (Innovatis, Bielefeld). At least 1x10^5 viable cells per tube were used for the experiment. Cells were resuspended in 3% FCS - HBSS solution for 10 min at room temperature to block Fc-receptors and reduce non-specific binding. For surface staining, cells were incubated with a fluorochrome-conjugated antibody in the amount indicated in the Supplementary Table 1 for 20 minutes at 4°C in the dark. Cells were washed with staining buffer [PBS containing 0.1% BSA (Sigma Aldrich, Cat #A2153) and 2mM EDTA (Thermo Fischer Scientific, Cat #15575020)], centrifuged at 300 g for 5 minutes and resuspended in 200 µL staining buffer. For detection of surface molecules, a minimum of 10000 live events per sample were counted. In order to identify expression of surface markers, cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination. In the next step, cells were discriminated into live and dead cells using the 7-AAD dye (BD Biosciences, Cat #559925) by plotting it against the SSC area. In the fourth step cells were plotted for the respective marker against the SSC area. For staining of intracellular molecules, cells were fixed and permeabilized with BD Cytofix/Cytoperm kit (BD Biosciences, Cat #554714). Staining was performed according to the manufacturer's instructions. A minimum of 10000 events per sample were counted. To investigate the expression of intracellular polarization markers cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination and gating against SSC-area and respective marker. The same gating strategy was employed on CTR and PE macrophages. Surface molecules were compensated by individual staining on OneComp eBeadsTM Compensation Beads (Thermo Fischer Scientific, Cat #01-1111-42). Isotype controls corresponding to each fluorochrome in the experiment were used to detect non-specific positive signals. Antibodies used for FACS analysis and their corresponding dilutions are listed in the Supplementary Table 1. Cell sorting was performed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) and analysed with FlowJoTM v10.8 software for gate setting and data analysis.

2.5 Multiplex ELISA-on-bead Assay

Inflammation 20-Plex Human ProcartaPlex^{TM} Panel (Invitrogen, Thermo Fischer Scientific, Cat #EPX200-12185-901) was used to quantify the secretion of pro- and anti-inflammatory molecules. Human TIMP Magnetic Luminex Performance Assay 4- Plex Kit (R&D Systems, Cat #LKTM003) was used to analyse TIMP secretion. HBCs were cultured in MaM for 5-6 days before supernatants were collected and centrifuged at 4000 rpm, 4°C for 15 minutes. MaM medium processed under the same conditions as the samples served as a blank. Multiplex assays were performed according to the manufacturer's instructions. Cytokines reaching the detection limits were normalized to total protein content in the supernatant using the Pierce BCA kit (Thermo Fischer Scientific, Cat #23225).

2.6 Enzyme- linked immunosorbent assay (ELISA)

TGF-beta 1 Quantikine ELISA kit for human/mouse/rat/porcine/rabbit (R&D Systems, Cat #DB100B) was used for the detection of TGF-β1. Next, to quantify secretion of IL-8, human IL -8/CXCL8 Quantikine ELISA kit (R&D Systems, Cat #D8000C) was used. Both ELISA kits were performed according to the manufacturers' instructions. To quantify the amount of secreted TGF-β1 and IL-8, cells were cultured in MaM for 5-6 days before collection of the supernatants. MaM medium processed under the same conditions as the samples, but without cells served as a blank. Cytokine levels were normalized to the total protein content in the supernatant measured with the Pierce BCA kit (Thermo Fischer Scientific, Cat #23225), in order to account for deviating volume concentrations.

2.7 Quantitative Real-Time PCR (RT-qPCR)

HBCs were washed twice with ice cold Hanks' salt balanced solution (HBSS, Thermo Fischer Scientific, Cat #14175-053) and harvested in 700 µL QIazol Lysis Reagent (Quiagen, Cat #79306). Total RNA content was isolated using miRNeasy Mini Kit (Quiagen, Cat #217004). Reverse transcription was performed using 1 µg of RNA and Luna Script RT SuperMix Kit (New England BioLabs, Cat #M3010). For qPCR analysis, SYBR Green Luna Universal qPCR Master Mix (New England BioLabs, Cat #M3003) and CFX-384 Touch Real time PCR detection system (Bio-Rad) were used. Expression of target genes was normalized to the following housekeeping genes (*18S*, *RPL30* and *HPRT*) using 2^($^{\Delta\Delta Ct}$) method. Primer sequences used for qPCR analysis are listed in the Supplementary Table 2.

2.8 Phagocytosis assay measured with FACS

Phagocytosis Assay Kit (Abcam, Cat #ab234053) was used according to the manufacturer's instructions. For detection of phagocytic activity 1 x 10^6 cells/ml were used. On the fifth day post isolation HBCs were treated with zymosan slurry and incubated for 3 hours at 21% oxygen and 37°C. After washing steps, cells were detached using accutase (Thermo Fischer Scientific, Cat #00-4555-56) and careful scraping, followed by a washing step with staining buffer containing PBS with 0.1% BSA (Sigma Aldrich, Cat #A2153) and 2mM EDTA (Thermo Fischer Scientific, Cat #15575020). Afterwards measurements were performed in the FITC channel. Untreated cells served as controls. Cells were separated by size using FSC-A and SSC-A, followed by doublet discrimination gating the area and height of FSC. Lastly, the FITC fluorescent signal was determined using histograms. Cell sorting was performed on a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) using FlowJoTM v10.8software for gate setting and data analysis.

2.9 Phagocytosis assay with high-content confocal screening microscope

Phagocytosis Assay Kit (Abcam, Cat #ab234053) was used according to the manufacturer's instructions. HBCs were seeded at the density of 0.5 x 10^6 cells/ml in 24-well black/clear bottom plates. 5 µL of zymosan slurry was added to the cells and incubated at 21% oxygen and 37°C for 3 hours, followed by a washing step. Next, cells were fixed in a plate containing 4% neutral buffered paraformaldehyde solution, followed by an intensive wash step with TBE buffer containing 1x TBE and 0.1% Tween (Thermo Fischer Scientific, Cat #003005). HBCs were incubated with Protein Block (Thermo Fischer Scientific, Cat #TL125HL) for 20 minutes. Cells were then counterstained with CD163 (1:100, Thermo Fischer Scientific, Cat #MA1-82342), diluted to working concentration in Antibody diluent (Agilent, Dako, Cat #S0809) and incubated overnight at 4°C. After serial washing steps, the plate was incubated with the secondary antibody Dylight633 (goat versus mouse 1:200, Thermo Fischer Scientific, Cat #35512) for 2 h at room temperature. To stain the nuclei, the plate was counterstained with DAPI (1:1000, Sigma Aldrich, Cat #D9542) diluted in antibody diluent for 10 min. After intensive washing, 300 µL of PBS was added to each well and stored at 4°C. Image acquisition was performed using a Nikon microscope with the Zyla sCMON camera. All statistical analysis was carried out on 25 different locations per well using 20x magnification. For better visualization of phagocytosis shown images in the Figures 4C–E were taken with 40x magnification. The number of FITC labelled beads was counted within the cells positive for CD163 and DAPI staining using Nis Elements viewer version 5.20.01 software. Cell surface area was measured using the measuring tool provided within the software.

2.10 Gelatin zymography

HBCs supernatants were collected on the fifth day post isolation. The supernatants were centrifuged at 4000 rpm for 15 minutes at 4°C. Total protein concentration was determined using Pierce BCA kit (Thermo Fischer Scientific, Cat #23225) according to the manufacturer's guidelines. A total of 15 µg of protein sample was diluted with Tris-Glycine SDS sample buffer (Thermo Fisher Scientific, Cat # LC2676) and loaded onto 10% Tris-Glycine gels containing 0.1% gelatin (Thermo Fisher Scientific, Cat #ZY00105BOX) and separated for 135 min at 125 V, 35 mA. After electrophoresis, the gels were incubated in 1x Zymogram Renaturating buffer (Thermo Fisher Scientific, Cat #LC2670) at room temperature with gentle agitation. Followed by 30 minutes incubation with 1x Zymogram developing buffer (Thermo Fisher Scientific, Cat #LC2671). Fresh developing buffer was added and the gels were stored overnight at 37°C. Next day, gels were stained with Coomassie Brilliant Blue (Sigma Aldrich, Cat #1.15444) for 50 minutes and decolorized in 50% distilled water (Fresenius, Cat #C920928): 40% methanol (Sigma Aldrich, Cat #322415): 10% acetic acid (Roth, Cat #3738.5) solution for 10 minutes. Protease activity appearing as a clear band on the dark background was visualized with the ChemiDoc^{TM}Touch Imaging System (Bio-Rad). Band densitometry was determined using Image Lab Software Version 6.1 (Bio-Rad).

2.11 Statistical analysis

SPSS (IBM SPSS Statistics version 26) was used for statistical calculations. Next, graphs were generated using Graph Pad Prism 9.3.1 software (GraphPad Software Inc.). To test normal distribution Shapiro-Wilk test was used. Skewed data were transformed using natural logarithm (ln) before applied to statistical analysis and re-transformed for the graphical presentation. To assess statistical significance of the patient characteristics (Table 1) one-way ANOVA with Tukey's *post-hoc* test was used. If normality testing failed, Kruskal-Wallis test with Dunn's *post-hoc* test was performed. Next, to compare differences between three groups (CTR; EO-PE and LO-PE) ANCOVA with adjustment for gestational age and Sidak's *post-hoc* test was used. Equal variances of variables were verified by Levene´s test. When comparing the effect of HBCs conditioned medium on fpECs without adjustment for gestational age (Supplementary Figure 5) two-way ANOVA with Sidak's *post-hoc* test was used. All values are given as mean ± S.E.M. p-values ≤ 0.05 were considered statistically significant.

3 Results

3.1 The number and morphology of HBCs is affected by PE

The characteristics of the study population are shown in Table 1. Women who developed early (EO, n=8) and late onset (LO, n=6) PE were included in this pilot study. Advanced maternal age, high pre-pregnancy BMI, nulliparity, gestational diabetes, chronic hypertension are some of the risk factors for the development of PE (1). Women in PE groups were significantly older as those in CTR group and their pre-pregnancy BMI ranged from 19.6 to 25.4 kg/m2. As expected, systolic and diastolic blood pressure levels differed significantly between the CTR and PE groups. Gestational age of EO-PE group was significantly lower than of CTR and LO-PE group. Consequently, early gestational age of the EO-PE group is directly related to placental- and fetal weight, both of which were significantly lower than in CTRs. Since development of the placenta depends on gestational age (50), we adjusted the (normally distributed) data for that respective factor. We found a significant difference in the levels of sFlt between EO- and LO-PE group, while there were no significant differences between other clinical parameters (PlGF, platelets, uric acid, AST and ALT).

To study polarization, we first examined the distribution and number of HBCs in placental tissue using immunohistochemistry approach. Since HBCs have been shown to be strongly positive for CD163, we stained 5 µm serial sections of CTR (n=5), EO-PE (n=6) and LO-PE (n=6) placental tissue, mouse IgG served as a negative control. CD163 is used as a marker used for placenta resident macrophages, and if combined with other markers (e.g. Folate receptor-β, CD206, CD209) is often associated with M2 polarization (26, 30, 31, 51). HBCs positive for CD163 were found in the villous stroma, moreover in stem, intermediate and terminal villi of CTR, EO- and LO- PE placentae (Figure 1A). Furthermore, quantification of CD163-positive cells revealed a significantly decreased number of stained (Figure 1B) cells per mm^2 in both PE groups, indicating reduced number of HBCs in respective groups. Next, we adjusted the number of isolated HBCs to the wet weight (grams) of placental tissue used for isolation. Consistent with immunohistochemical analysis, we found significantly reduced number of primary LO-PE HBCs. Reduction of HBCs was also observed in the EO-PE group, however, did not reach significance compared to the CTRs (Figure 1C).

In vitro, cell morphology of HBCs isolated from CTR, EO- and LO-PE placentae showed substantial differences (Supplementary Figure S1). Normally, directly after isolation, HBCs are round shaped cells with many vacuoles in the cytosol. Within 48-72 hours after isolation, cells differentiate and develop different shapes. Usually, M2 characterized macrophages exhibit an elongated, spindle-shaped morphology, whereas M1 polarized cells form a round, dendritic cell-like morphology with large filopodia (52, 53). CTR HBCs developed typical M2 features (Supplementary Figure 1A), whereas within isolations of EO- and LO-PE HBCs more of round shaped cells with larger filopodia next to M2 morphologies were found (Supplementary Figures 1B, C).

3.2 EO- and LO-PE HBCs are characterized by different expression of polarization markers

Basal expression of surface and intracellular M1 and M2 markers was determined by FACS (Tables 2 and 3) on primary isolated CTR (n=12), EO-PE (n=6) and LO-PE (n=5) HBCs. The gating strategy is shown in Supplementary Figure 2. FACS analysis revealed distinct expression of M1 and M2 markers in EO- and LO- PE HBCs (Table 2). EO-PE HBCs tend to express higher levels of pro-inflammatory CD11b, CD11c, CD40 (p=0.09) and TLR4 ($p \leq 0.05$), whereas expression of listed markers was similarly distributed

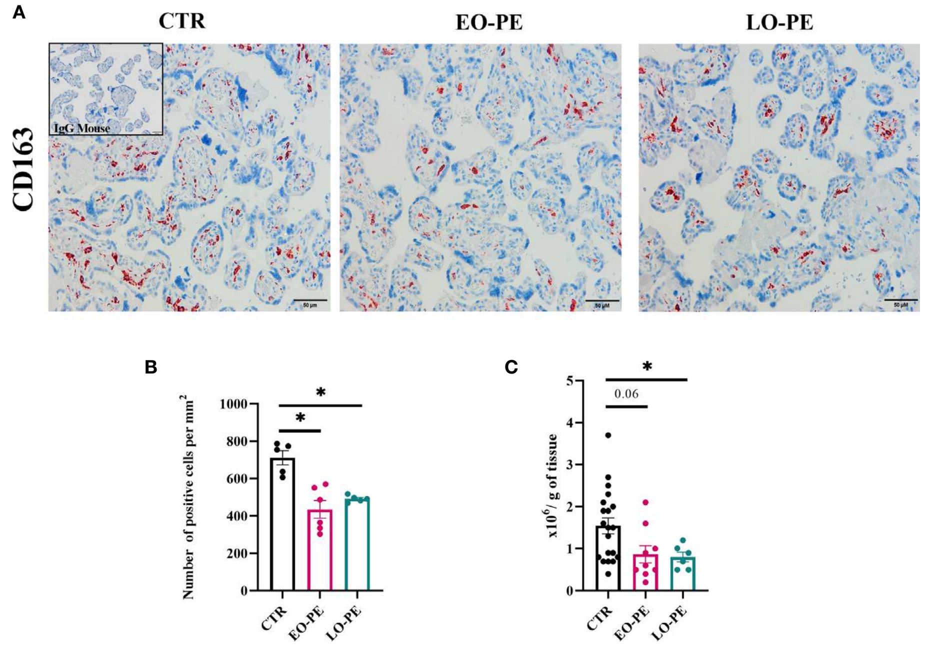

FIGURE 1
Immunohistochemical assessment of CD163 in placental tissue. **(A)** Representative images of serial sections of CTR (n=5), EO- (n=6) and LO-PE (n=6) are shown. Insert in the top right corner of CTR image depicts negative control stainings (blue). Images were taken at 20x magnification, scale bar represents 50µm. **(B)** Quantification of cells positive for CD163 in CTR, EO- and LO-PE group. Cells were quantified by using Quapath software. **(C)** Average yield of HBCs, adjusted to the wet weight (in grams) of minced tissue for each respective isolation, CTR (n=22), EO-PE (n=8), LO-PE (n=6). Data on **(B)** in **(C)** graphs are presented as mean ± S.E.M, ANCOVA with adjustment for gestational age, followed by Sidak's *post-hoc* test to examine statistical significance, *$p \leq 0.05$.

TABLE 2 Percentage of live cells positive for respective surface polarization markers within the CTR (n=12), EO- (n=6) and LO-PE (n=5) groups.

Surface polarization marker	CTR (% of live cells)	EO-PE (% of live cells)	LO-PE (% of live cells)
CD11B	22.2 ± 9.6	38.7 ± 16.5	28.8 ± 17.0
CD11C	45.1 ± 14.3	69.9 ± 17.8	51.9 ± 11.8
CD40	17.1 ± 5.9	38.9 ± 12.6	22.6 ± 10.9
CD80	2.9 ± 1.8	6.5 ± 3.5	10.5 ± 8.9
CD86	25.0 ± 10.3 *	41.7 ± 3.6 ‡	21.6 ± 6.8
CD163	89.4 ± 3.9	82.0 ± 4.5	81.8 ± 6.8
CD206	47.6 ± 9.8	33.5 ± 6.5	38.2 ± 13.3
CD209	53.3 ± 6.9 †††	43.7± 12.1 ‡‡	22.1 ± 5.2
TLR1	5. 9 ± 6.2 †	9.5 ± 6.2	18.8 ± 13.6
TLR2	6.1 ± 3.9 *	1.2 ± 1.5	5.4 ± 4.5
TLR4	37.3 ± 12.3 *	79.7 ± 19.9	54.2 ± 18.8
HLA-DR	33.8 ± 14.1 ***	77.9 ± 12.6 ‡‡	44.3 ± 18.7
FR-β	79.2 ± 10.0	77.6 ± 20.7	68.4 ± 15.3

Data are presented as mean ± SD. Statistical significance is represented with *$p \le 0.05$, ***$p \le 0.001$; whereas *CTR vs EO-PE; †$p \le 0.05$, †††$p \le 0.001$; whereas †CTR vs LO-PE; ‡ $p \le 0.05$, ‡‡$p \le 0.01$; whereas ‡EO-PE vs LO-PE, by ANCOVA with Sidak's post-hoc test and adjustment for gestational age.

between LO-PE and CTR HBCs, except for CD209 ($p \le 0.001$). Furthermore, we found significant increase of the major histocompatibility class (MHC) II molecule HLA-DR within the EO-PE group compared to the CTR group ($p \le 0.001$). Interestingly, the HLA-DR expression was significantly different between EO- and LO-PE HBCs ($p \le 0.01$) as well. Among the M1 markers only expression of CD80 ($p=0.05$) and TLR1 ($p \le 0.05$) were elevated in LO-PE group. Notably, expression of CD80 and TLR1 was elevated in EO-PE group as well, but only by trend. Interestingly, surface expression of TLR2 was reduced in both EO- ($p \le 0.05$) and LO-PE group. Next, we investigated the expression levels of CD86. Since CD86 can serve as M1 or M2b marker (54, 55), the secretion profile and expression of other markers should be taken into account when interpreting its expression. We found significant induction of the expression of the respective marker in EO-PE HBCs ($p \le 0.05$) compared to the CTR ($p \le 0.05$) or LO-PE ($p \le 0.05$) group. In LO-PE HBCs the expression of CD86 did not differ from the control. In contrast to the increase of pro-inflammatory markers in EO-PE HBCs, a slight decrease in the anti-inflammatory markers CD206 and folate receptor β (FR-β) was observed. Expression pattern of CD206 and FR-β was similar in LO-PE HBCs as well. Expression of CD163 on primary isolated HBCs was evenly distributed between all investigated groups, confirming that CD163 can be used rather as a reliable tissue resident marker (Figures 1A, B) than a direct indicator of M2 phenotype. Next, M2 marker CD209 was suppressed in the LO-PE group ($p \le 0.001$). Its expression was only minimally decreased by 10% in EO-PE HBCs group. Interestingly, we found a significant difference in the expression of CD209 between EO- and LO-PE HBCs ($p \le 0.01$).

In addition to the investigation of surface polarization markers, we studied intracellular markers (Table 3). Next to the pan-macrophage marker CD68, two important regulators of TLR-Myd88 signaling (56, 57), IRF4 and IRF5 were investigated. Noteworthy, IRF4/IRF5 axis is involved in the initiation control of a specific M1/M2 polarization program. IRF5 as a positive regulator of Myd88 induces the expression of pro-inflammatory genes and establishment of M1 phenotype (58). Whereas IRF4, as a negative regulator of Myd88, leads to the activation of anti-inflammatory genes and initiation of M2 polarization (56, 59).We found the highest expression of IRF5 in EO-PE group and the lowest in CTRs. Expression of IRF4 the regulator of M2 polarization was evenly distributed between CTR and LO-PE group. Importantly, expression of IRF4 in EO-PE HBCs was reduced. Moreover, balance in favour of IRF5 together with higher expression of other surface M1 markers (Table 2) indicates possible phenotypic switch towards M1 polarization of EO-PE HBCs.

TABLE 3 Percentage of positive CTR (n=12), EO-PE (n=5) and LO-PE (n=4) HBCs for respective intracellular polarization markers.

Intracellular polarization marker	CTR (% of positive cells)	EO-PE (% of positive cells)	LO-PE (% of positive cells)
CD68	84.0 ± 9.8	70.4 ± 9.1	87.5 ± 5.4
IRF4	91.6 ± 8.0	85.7 ± 11.8	96.2 ± 2.0
IRF5	73.1 ± 12.8	91.5 ± 10.8	85.2 ± 12.0

Data are presented as mean ± SD. Statistical significance was tested by ANCOVA with Sidak's post-hoc test and adjustment for gestational age.

3.3 HBC secretion profile of cytokines and adhesion molecules differs in PE

Polarized macrophages are known to secrete specific patterns of cytokines, chemokines, and growth factors, allowing us to characterize polarization states (60). Using multiplex ELISA-on-bead technology, we determined the secretion profile of cytokines and chemokines secreted by CTR (n=8), EO-PE (n=6) and LO-PE (n=4) HBCs (Figure 2, Supplementary Figure 3). Moreover, secretion of IL-8 was determined using ELISA, since its secretion excided the detection limit of the multiplex ELISA (CTR n=10, EO-PE n=5, LO-PE n= 5; Figure 2J). Notably, TGF-β1 was measured with ELISA (CTR n=12, EO-PE n=6, LO-PE n=6, Figure 2K) since the sample preparation requires acidification of the samples for binding of TGF-β epitopes.

Secretion profile of EO- and LO-PE HBCs differs from the CTR group. First, among pro-inflammatory cytokines, we discovered a trend of an increased secretion of IL-6, IL-12p70 and P-Selectin by

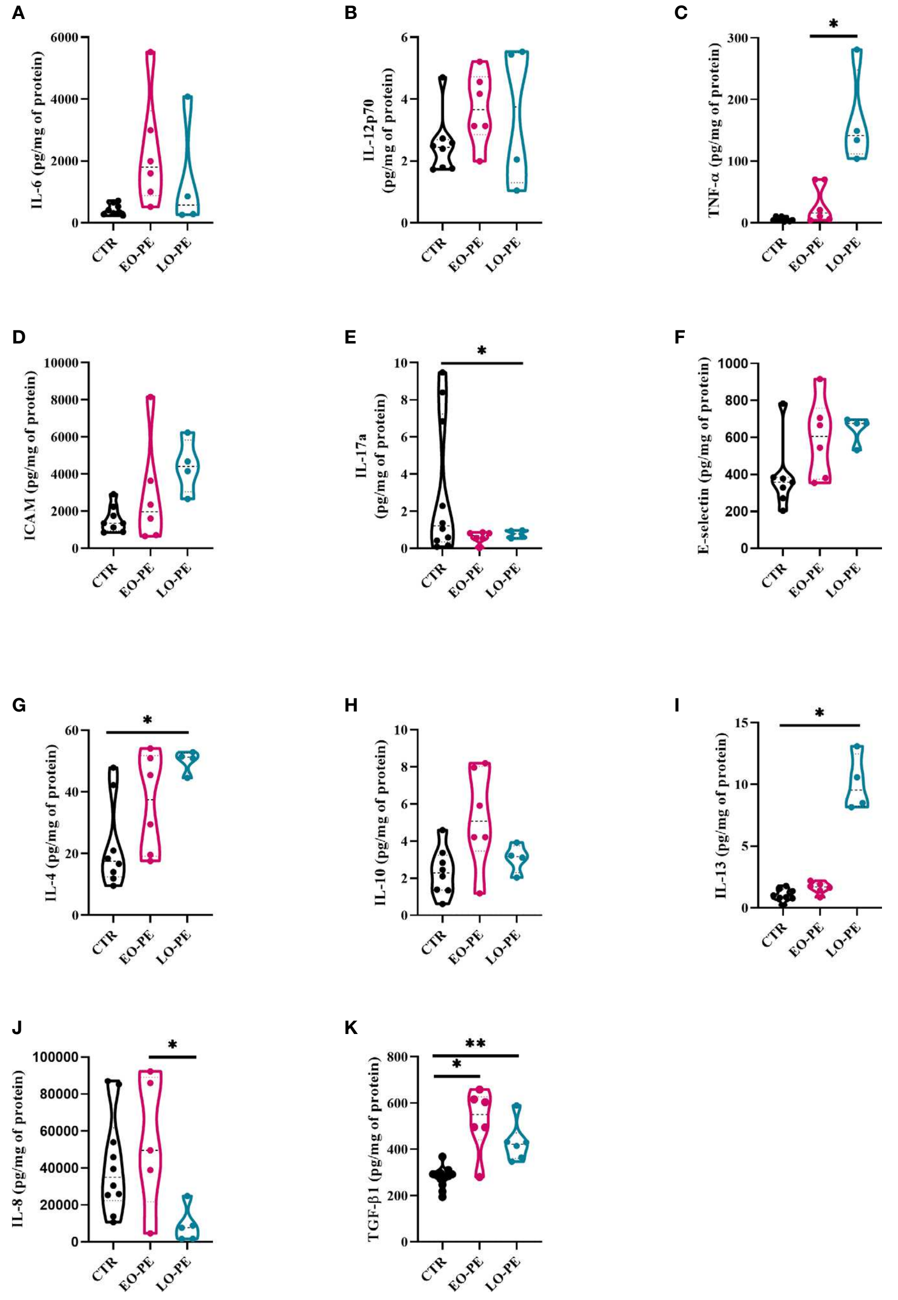

FIGURE 2
Secretion of cytokines and adhesion molecules by CTR (n=8), EO- (n=6) and LO-PE (n=4) HBCs. Multiplex-ELISA-on-beads assay for followed pro-inflammatory and anti-inflammatory cytokines **(A)** IL-6, **(B)** IL-12p-70, **(C)** TNF-α, **(D)** ICAM, **(E)** IL-17a, **(F)** E-selectin, **(G)** IL-4, **(H)** IL-10 and **(I)** IL-13, respectively. Multiplex was performed in duplicates. **(J)** ELISA against IL-8 performed in duplicates (CTR n=10, EO-PE n=5 and LO-PE n=5). **(K)** ELISA assay against TGF-β1 performed in duplicates (CTR n=12, EO-PE n=6 and LO-PE n=6). Secretion of respective cytokines was normalized to the total protein content measured in the cell culture supernatants. Statistical significance was tested using ANCOVA with adjustment for gestational age followed by Sidak's *post-hoc* test. *p ≤ 0.05, **p ≤0.01.

EO-PE HBCs (Figures 2A, B; Supplementary Figure 3A). Contrary, to the EO-PE HBCs where secretion of TNF-α was unchanged, LO-PE released higher amounts of TNF-α as CTRs or EO-PE HBCs ($p \leq 0.05$) (Figure 2C). LO-PE HBCs secreted higher amounts of ICAM, but only by trend (Figure 2D). Moreover, both PE groups secreted less IL-17a compared to CTRs (Figure 2E). Although, release of E-Selectin was higher in both PE groups, we did not find any differences in the production of other pro-inflammatory cytokines, such as: IL-1α, IL-1 β, CCL-3, CCL-4, IFN- α, and IFN-γ between investigated groups (Figure 2F, Supplementary Figures 3B–H). A decrease of IL-8, measured by ELISA was detected in LO-PE HBC group, whereas unchanged between CTR HBCs and EO-PE group. Interestingly, we found a significant difference in the release of IL-8 between EO- and LO-PE HBCs ($p \leq 0.05$) (Figure 2J).

Next, we examined the secretion of anti-inflammatory cytokines namely, IL-4, IL-10, IL-13, and TGF-β1, which serve as important drivers of M2 polarization (53, 61). We did not find any differences in the secretion of IL-13; however, secretion of IL-4 and IL-10 was increased, but only by trend in EO-PE HBCs. LO-PE HBCs secreted significantly higher levels of IL-4 ($p \leq 0.05$) and IL-13 ($p \leq 0.05$) compared to CTR HBCs (Figures 2G–I). Interestingly, both PE groups released significantly higher amounts of anti-inflammatory TGF-β1, which was even more pronounced in the LO-PE group ($p \leq 0.01$) (Figure 2K).

Noteworthy, some of the secreted pro-inflammatory cytokines are not reliable identifiers of a specific phenotype, since they are expressed by both M1 and M2 macrophages. E.g. IL-6 is a pro-inflammatory cytokine produced by both M1- and M2a-polarized macrophages (62). In addition, secretion of ICAM is mediated by NF-κB, but can serve as both an M1 and M2 cytokine due to its pro-angiogenic nature (63). The observed changes in the secretion profile indicate a switch in the phenotype and possible protective mechanisms of PE HBCs in an attempt to reduce the extent of inflammation by increasing the production of anti-inflammatory cytokines.

3.4 Preeclampsia triggers transcriptional changes of HBC-genes involved in inflammation

Macrophages are capable of responding to the local stimuli and acquiring different phenotypes and functions to meet changing physiological needs (64). Next, we examined transcriptional changes in HBCs that may be triggered by PE. Basal gene expression of CTR (n=12), EO- (n=5) and LO-PE (n=5) HBCs was determined on the fifth day after isolation. We analysed selected genes associated with phenotype and functionality of macrophages undergoing inflammation (Figure 3). Dynamic changes in gene expression were observed between the PE subgroups EO and LO. First, we found -as expected - an upregulation of *NFKB1* in both EO- and LO-PE groups ($p \leq 0.05$) (Figure 3A). Among the inflammatory pathways involved in M1 polarization, NF-κB plays an important role and regulates the expression of pro-inflammatory genes such as cytokines, adhesion molecules and growth factors (65). Next, the expression of *HIF1*, another M1-associated gene was upregulated in the LO-PE group ($p \leq 0.05$), whereas its expression was surprisingly decreased in EO-PE group ($p \leq 0.05$) (Supplementary Figure 4). In line with the increased secretion of *ICAM* (Figure 2H), mRNA of the respective gene was upregulated in the both PE groups (Supplementary Figure 4). In contrast to the expression of *ICAM*, *VCAM* was downregulated in EO-PE, whereas in LO-PE group remained on the level of CTRs (Supplementary Figure 4). Consistent with secretion of IL-8 (Figure 2J), higher fold change of *IL8* was detected in CTR group (Supplementary Figure 4). The expression of *TGFB1*, which acts as important M2 inducer (66), was significantly elevated in both, EO- and LO-PE HBCs ($p \leq 0.05$) (Figure 3B). Although, the differences in the secretion of CCL-4 (Supplementary Figure 3H) were not noticeable, higher expression was detected in the CTR group by RT-qPCR. In contrast to the secretion profile of IL -6 and IL -10, which was higher in the PE group (Figures 2A, H), qPCR analysis revealed downregulation of respective cytokines in the EO- and LO-PE groups (Supplementary Figure 4), possibly due to the tight post-transcriptional gene regulation of these cytokines in particular (67). As polarized macrophages metabolise L-arginine differently, M2 *via* arginase-1 and M1 macrophages *via* nitric oxide synthase (iNOS) (68), we looked at expression of *ARG1* gene, encoding arginase-1, and *NOS2*, encoding iNOS (68). Interestingly, *ARG1* expression was increased in both PE groups (Figure 3C). However, expression of *NOS2* was strongly upregulated in EO-PE ($p \leq 0.05$); whereas expression of the respective gene in LO-PE HBCs was even lower as in CTR HBCs (Figure 3D). In addition to the metabolism of L-arginine, regulation of reactive oxygen species (ROS), represents an important link between M1 and M2 polarization (69). M1 macrophages produce higher amounts of ROS and consequently downregulate antioxidant enzymes such as *CAT* encoding catalase, or *SOD* encoding superoxide dismutase (70), whereas, M2 macrophages are thought to produce lower levels of ROS and express higher levels of *CAT* or *SOD* (71). The expression of the genes *CAT* and *SOD*, was significantly attenuated in both, EO- and LO-PE groups (Figure 3E, F). Next in respect to observed TGF-β1 differences, we looked at the expression of genes involved in tissue remodeling and adhesion. Importantly, we found upregulation of *MMP9* in both PE groups (Figure 3G). The expression of other genes involved in tissue remodeling (*MMP2, MMP12, TIMP1, TIMP2*), did not differ between groups (Supplementary Figure 4). Adhesion molecules, such as *CDH2* has been downregulated in both EO-PE ($p \leq 0.05$) and LO-PE HBCs (Figure 3H). Similarly, as *CDH2* we identified reduced expression of *CDH5* in both, EO- and LO- PE HBCs (Figure 3I).

In the healthy placenta, HBCs are often found in close proximity to feto-placental endothelial cells (fpEC), and M2 macrophages have the ability to regulate placental angiogenesis by secretion of pro-angiogenic factors (26). To gain insight into their role in angiogenesis, HBCs were examined for the expression of *FLT, VEGFA, KDR*, and *EGFR*; which were all downregulated in EO- and LO-PE group (Supplementary Figure 4).

Furthermore, EO-PE and CTR fpECs were treated with conditioned medium (CM) collected from CTR and EO-PE HBCs. In order to further investigate the influence of HBCs on the fpEC, metabolic activity of fpECs using the MTS assay (Supplementary Figure 5A) and the proliferation of the fpEC by incorporation of BrDU (Supplementary Figure 5B) were measured. CM of CTR HBCs increased the NAD(P)H dehydrogenase activity of CTR fpEC,

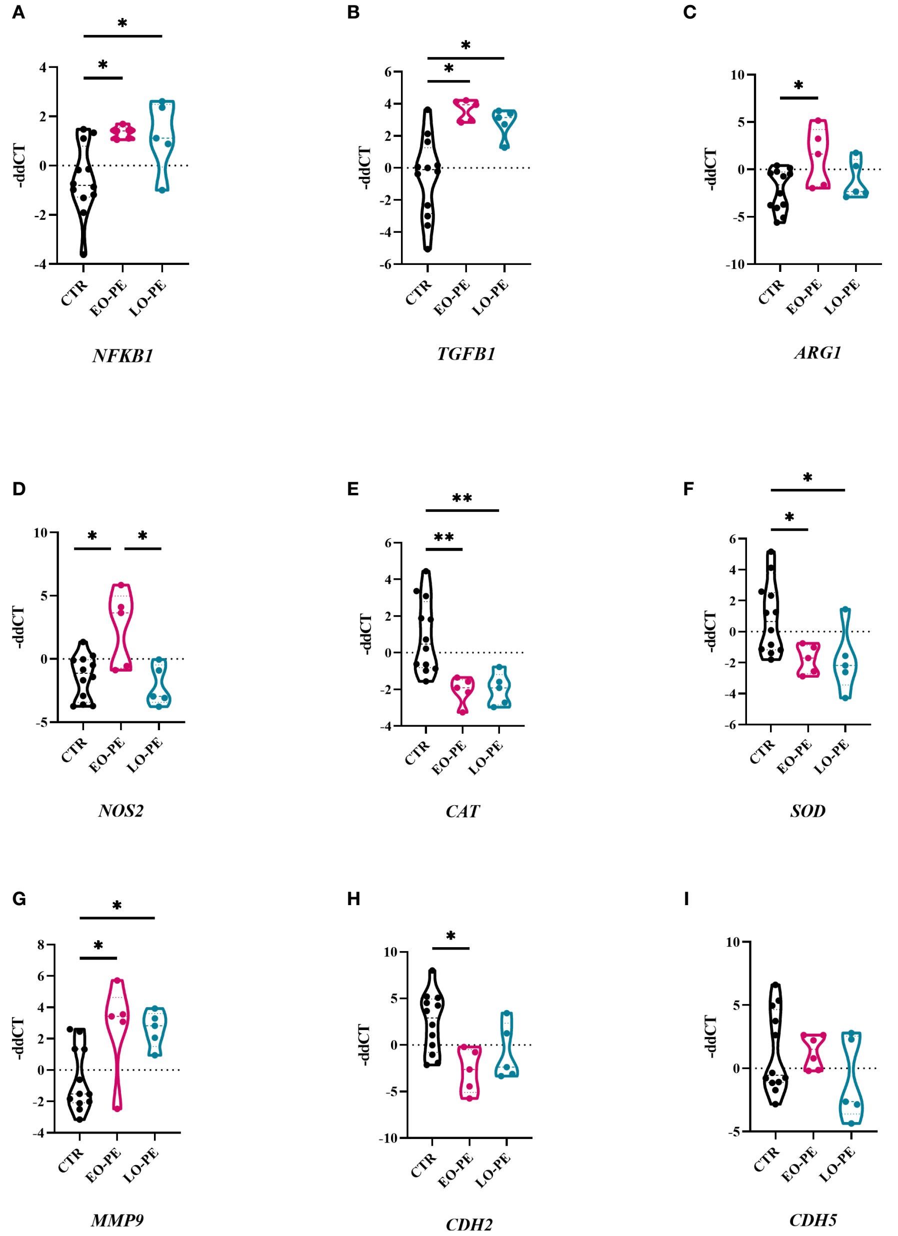

FIGURE 3

Preeclampsia alters inflammatory related gene expression in HBCs. **(A)** Total RNA of cultivated HBCs was harvested on the fifth day post isolation and analysed by RT-qPCR. **(A)** *NFKB1*, **(B)** *TGFB1*, **(C)** *ARG1*, **(D)** *NOS2*, **(E)** *CAT*, **(F)** *SOD*, **(G)** *MMP9*, **(H)** *CDH2*, **(I)** *CDH5*. In total 12 CTR, 5 EO-PE and 5 LO-PE HBCs isolations in three technical replicates were used. Expression of target genes was normalized to the following housekeeping genes (*18S*, *RPL30* and *HPRT1*) using $2^{\Delta\Delta Ct}$ method. Statistical significance was tested using ANCOVA with adjustment for gestational age followed by Sidak's *post-hoc* test. *$p \le 0.05$ and **$p \le 0.01$.

whereas PE CM had no effect on the activity of PE fpEC (Supplementary Figure 5A). A similar effect was observed when the proliferation of CTR fpEC was measured (Supplementary Figure 5B).

3.5 Phagocytosis of HBCs is altered in PE

Macrophages, as professional phagocytes eliminate pathogens and apoptotic cells. The elimination of apoptotic cells plays an important regulatory role regarding the reduction of the inflammatory burden (72). Phagocytosis was measured and visualised using two different approaches. First, it was assessed by FACS (CTR n=10, EO-PE n=5, LO-PE n=5), where median fluorescence intensity (MFI) was used to quantify the phagocytic activity. PE HBC showed significantly higher phagocytic activity ($p \le 0.01$) than CTRs (Figures 4A, B). Second, we analysed phagocytic activity using HCS (Figures 4C–E). For better visualisation cells were stained with HBCs tissue resident marker CD163. Analysis of CTR (n=7) and PE (n=5, EO n=3, LO n=2) confirmed higher (though not significant) phagocytosis of PE HBCs (Figure 4F). Furthermore, visualisation of phagocytosis allowed us to analyse morphology of the cells, calculating cell size - surface area (μM^2), which was lower in EO- and LO-PE groups, when compared to the surface area (μM^2) of CTRs (Supplementary Figure 6) confirming *in vitro* observations of smaller round cell morphologies of PE HBC (Supplementary Figures 1A–C).

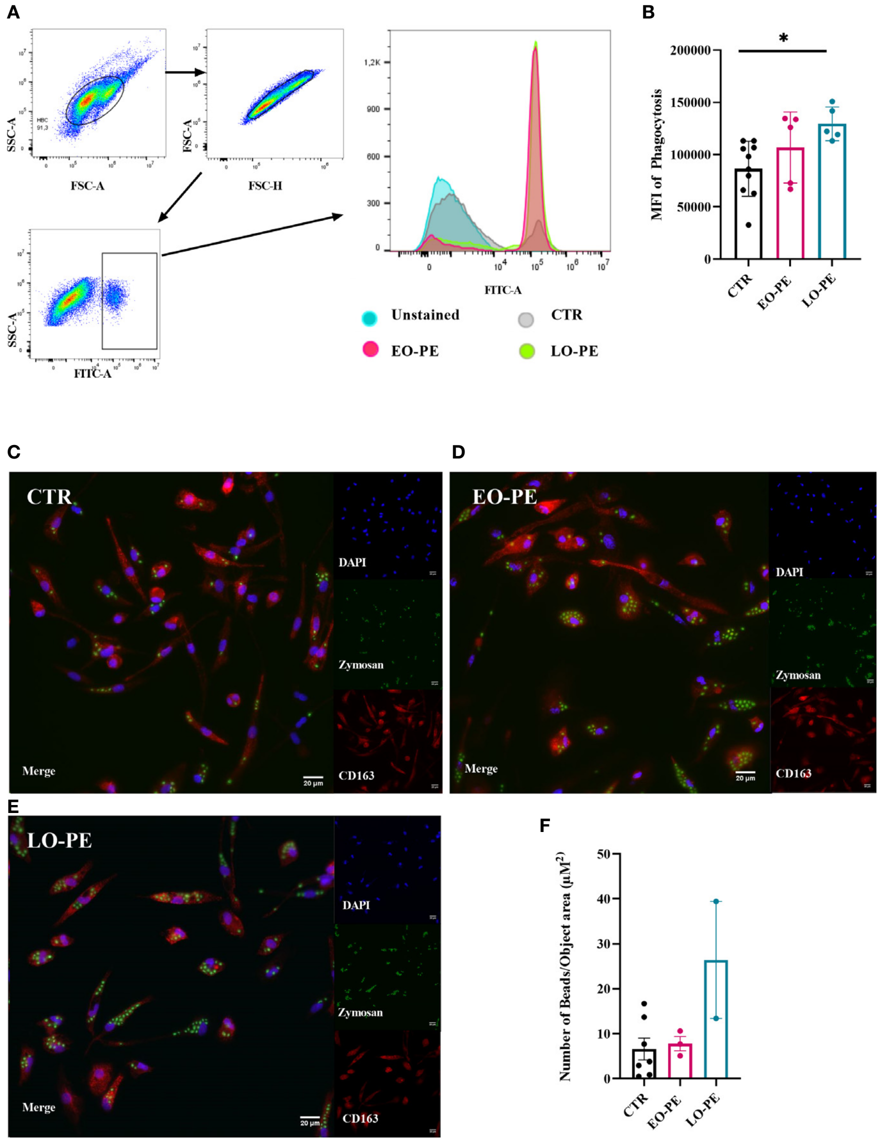

FIGURE 4
PE HBCs possess a higher phagocytic capacity. **(A)** Gating strategy cells for the measurement of phagocytosis. Cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination and gating against SSC-area and FITC -A fluorescent signal. Histograms of one representative experiment are shown, in total 10 CTR, 5 EO-PE and 5 LO-PE HBCs were used. **(B)** Quantification of median fluorescence intensity (MFI) of phagocytosis measured with FACS. **(C)** Visualization of phagocytosis with high content screening microscopy (HCS). HBCs were treated with zymosan beads (green) and co-stained with CD163 (red), DAPI was used to stain nuclei. Representative images of individual experiments are shown. To visualize phagocytosis CTR (**C**, n=7), EO-PE (**D**, n=3) and LO-PE (**E**, n=2) isolations were used. Scale bar represents 20µM. **(F)** Quantification of the phagocytosis measured with high content screening. Analysis was carried out with NisViewer Software, analyzing the number of beads within the CD163 positive HBC cell. All data in **(B, F)** are presented as mean ± S.E.M, ANCOVA with adjustment for gestational age with Sidak's *post-hoc* test was used for to test statistical significance. *$p \leq 0.05$.

3.6 PE attenuates MMP-9 activity of HBCs

In a normal placenta M2-polarized HBCs contribute to tissue remodeling and repair (73). To confirm strong upregulation of *MMP9* (Figure 3G), we additionally performed gelatin zymography to assess the activity of MMP-2 and MMP-9 (Figure 5A). As shown with *MMP2* mRNA expression (Supplementary Figure 4), detectable MMP-2 activity (Figure 5A) did not differ between studied groups (Figure 5B). In line with upregulation of *MMP9*, EO-PE HBCs displayed significantly ($p \leq 0.05$) higher MMP-9 activity as CTR. LO-PE HBCs MMP-9 activity was increased, but only by trend (Figure 5C).

Expression and production of MMPs are usually tightly regulated within the complex network of their four different tissue inhibitors of metalloproteinases 1-4 (TIMP). HBCs secretion of TIMP- (1, 2, 74, 75) was assessed using a multiplex ELISA-on-bead assay (Figure 5D). Production of TIMP-1, TIMP-2, TIMP-3 was unchanged in EO-PE group, we noticed a decreased production of TIMP-4 in the respective group. Furthermore, LO-PE HBCs secretion of TIMP-1 was significantly decreased, followed by trend in the reduced production of TIMP-2. Interestingly, release of TIMP-3 in LO-PE group was similar as in CTR. LO-PE HBCs production of TIMP-4 was elevated, but only by trend. To further explore the gelatinolytic activity of HBCs, the ratio between mRNA expression of *MMP2/TIMP1*,

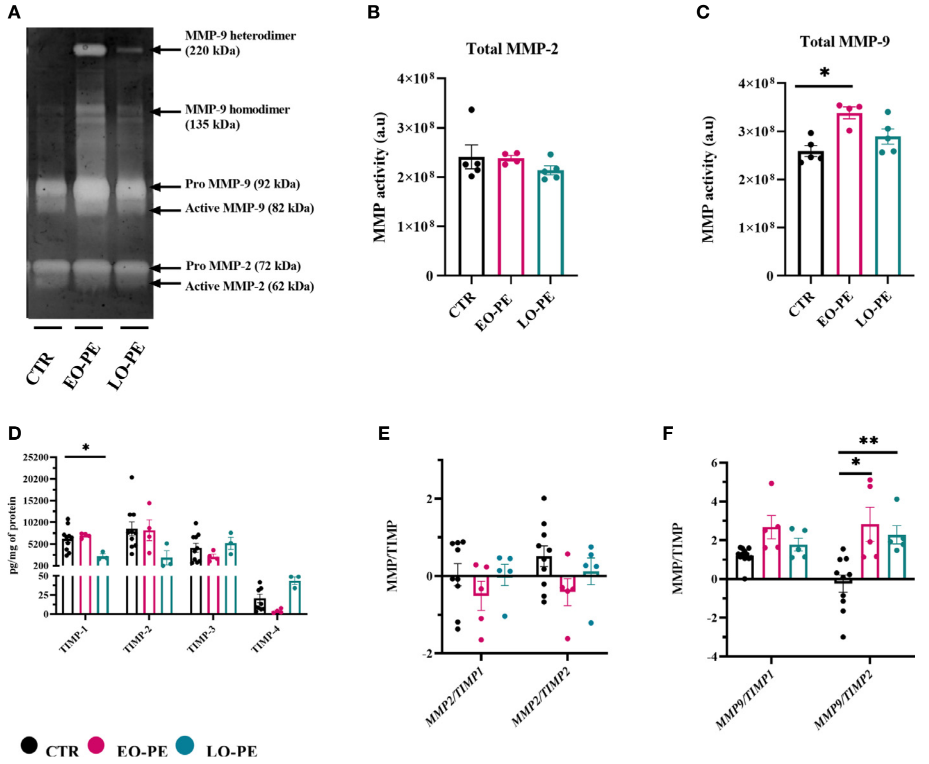

FIGURE 5
Gelatin zymography demonstrating gelatinolytic activity of HBCs. **(A)** A representative zymogram gel showing MMP-9 and MMP-2 activity of CTR (n=5), EO- (n=4) and LO-PE (n=5) HBCs. Activity was examined in the supernatants collected from CTR and PE HBCs on the 5th day post isolation. In total 15µg of protein measured in supernatants were loaded onto gelatin gels, respectively. To generate the comparable gelatinolytic bands, gelatin zymography was repeated twice. White bands of gelatinase activity observed at 92 and 82kDa are pro- and active form of MMP-9. Moreover, white bands at the size of 72 and 62kDa represents pro- and active form of MMP-2. Quantification of total (pro and active) form of MMP-9 **(B)** and MMP-2 **(C)** was performed as densitometric analysis of respective bands. **(D)** TIMP-1, TIMP-2, TIMP-3 and TIMP-4 levels measured in the cell culture supernatants using multiplex assay. In total CTR (n=10), EO- (n=4) and LO-PE (n=3) HBCs were used in technical duplicates. Secretion of respective TIMPs was normalized to the total protein content measured in the cell culture supernatants. **(E)** mRNA ratio between *MMP2* and *TIMP1* and *TIMP2*. **(F)** mRNA ratio between *MMP9* and *TIMP1* and *TIMP2*. mRNA ratio was calculated from the -ddCT values normalized to the corresponding housekeeping genes (*18S*, *RPL30* and *HRPT1*). The results are showed as a mean ± S.E.M of technical triplicates. Statistical significance was assessed using ANCOVA with adjustment for gestational age followed by Sidak's *post-hoc* test. *p ≤ 0.05. **p ≤ 0.01.

MMP2/TIMP2, *MMP9/TIMP1*, and *MMP9/TIMP2* was calculated. Normally, TIMPs regulate inhibition of MMPs by binding in 1:1 reversible complex with the MMPs (76, 77). The shift in MMP/TIMP balance in favor of MMPs reflects as increased extracellular matrix (ECM) proteolysis, or if in favor of TIMP as decreased proteolysis and protection of the ECM (78–80). In CTR, EO- and LO- PE HBCs the ratio between *MMP2* and *TIMP1* or *TIMP2* (Figure 5E) stayed unchanged. Similar *MMP9/TIMP1* ratio of investigated groups, was favoring the gelatinolytic activity, indicating that MMP-9 is the main gelatinase produced by HBCs. However, significant differences were demonstrated in *MMP-9/TIMP-2* of EO- and LO-PE HBCs, confirming higher gelatinolytic activity shown with gelatin zymography (Figures 5A, C, F).

4 Discussion

Hofbauer cells, play a pivotal but also a diverse role in placental physiology by maintaining tissue homeostasis and the tolerogenic environment (26, 36, 41, 81). HBCs plasticity is well characterized by an unique phenotype expressing both, M2 and M1 polarization markers (26, 30, 31). Preeclampsia is an inflammatory condition, accompanied by activation of both the innate and adaptive immune system. These alterations may directly influence the phenotype of HBCs and contribute to placental dysfunction (82). Due to the presumed distinct pathophysiological origins of EO- and LO-PE and the resulting different inflammatory burden within the PE placenta (5, 8, 9, 83), we aimed to determine the phenotypic and functional alterations between different HBCs. One of the more significant findings to emerge from this study is that both, EO- and LO-PE HBCs maintain a profound anti-inflammatory phenotype in the human placenta. In addition to HBCs general adaptive response to inflammatory stimuli, EO-PE HBCs cope differently with signals from their microenvironment.

The EO-PE placenta has been linked to placental malperfusion (84), leading to oxygen deficiency, increased inflammation and oxidative stress which all together deteriorate the mechanisms of placentation early in pregnancy. In contrast, LO-PE placenta has been linked to changes of systemic blood pressure in the mother leading to maternal endothelial dysfunction and oxidative stress, resulting in placental dysfunction (85, 86). Consequently, both subtypes of PE are characterized by excessive inflammation caused by oxidative stress and a hypoxic microenvironment, which affects the appearance and phenotypic composition of immune cells, particularly macrophages

(87). To investigate whether the number of HBCs differs between CTR and PE placenta, we first quantified the cells *in situ* using specific markers for tissue-resident macrophages. In agreement, with the findings of Tang et al., where they investigated CD163 positive HBCs in PE placentae (51), we found a decreased number of CD163 - positive macrophages in both EO- and LO-PE placentae. Similarly, Yang et al. observed a significantly reduced number of CD14-positive HBCs in PE (87). In addition, Broekhuizen et al., used combined staining for CD68 and CD163 and observed a significant decrease in double-positive HBCs in EO-PE, whereas the number of double-positive HBCs in LO-PE remained unchanged (18). We verified our immunohistochemical findings by analyzing the yield of primary isolated HBCs *in vitro*. Similar to *in situ*, we obtained a decreased number of primary HBCs isolated from EO- and LO-PE placental tissues. As PE placenta is characterized by an increased number of apoptotic trophoblasts (88, 89), it is likely that HBCs may undergo a similar apoptosis cascade, leading to a decreased number of vital HBCs in culture. According to our results, PE may exacerbate the participation of initial stages of apoptosis in placental tissue which in turn leads to a reduced anti-inflammatory and immunoregulatory capability of remaining HBCs.

Macrophage polarization is a complex process controlled by several factors and mechanisms (90). It is noteworthy that a subpopulation identified with the same markers may have different functions in different tissues and pathologies, adding to the complexity of defining phenotypes (27). At baseline, HBCs express M1 and M2 polarization markers (30, 31). Moreover, HBCs retain alternative M2 polarization in inflammatory complications such as in gestational diabetes mellitus (31), chorioamnionitis (91), or upon *in vitro* stimulation (30). Contrary to expectations, we observed differences in the expression pattern of polarization markers between EO- and LO-PE HBCs. In EO- HBC markers involved in M1 polarization such as TLR4, HLA-DR, CD40, CD80 and CD86 were upregulated, suggesting M1 phenotype. In contrast, in LO-PE HBCs the expression of before mentioned markers was either reduced or similar to CTRs. The anti-inflammatory phenotype of LO-PE HBCs is strongly underpinned by high expression of IRF4, which exerts important function in controlling local cytokine milieu thereby polarization (92). TLR signaling has been proposed as an important link between activation of innate immune system and PE, known to modulate the inflammatory responses (18, 93). Interestingly, Young et al. demonstrated the maintenance of the M2 phenotype of HBCs despite pro-inflammatory treatment and upregulation of TLR4 and increased secretion of pro-inflammatory cytokines (IL-6, IL-8) (37). We demonstrated an upregulation of TLR4 and its downstream mediators *NFKB1* and TNF-α in EO-PE, implying they give up M2 polarization and a shift towards M1. A change of phenotype toward M1 polarization is also suggested by increased expression of IRF5 and *NOS2*. IRF4 and IRF5, both regulators of the Myd88 pathway are crucial for the expression of M1/M2 genes (94). In particular, IRF5 regulates the expression of pro-inflammatory factors such as: TNF-α, IL-6, IL-12p70, CD86 (58). IRF4, on the other hand, is known to compete with IRF5 for interaction with Myd88 to activate the M2 program (56, 92). Its expression has been shown to be induced by anti-inflammatory IL-4 (92, 95). Interestingly, EO-PE HBCs, although favoring IRF5 activation, express IRF4 and consequently secrete both pro- and anti-inflammatory cytokines. A dysbalance of intracellular IRF4 and IRF5 regulating factors may control different phenotypes and the associated contributions to tissue inflammation.

It has been reported that in PE placentae CD163 and FR–β, expression is decreased (51). Although a reduction of CD163 on tissue levels could be confirmed, no differences of CD163 and FR-β expression on the primary isolated HBCs were detected. This inconsistency can be explained because CD163 is used as a tissue-resident marker for placental macrophages rather than only as an M2 marker. Among anti-inflammatory M2 markers, CD209 serves as one of the major M2 markers, moreover, CD209-positive HBCs have been shown to produce IL -10 an immunosuppressive cytokine one of the drivers of immunoregulatory M2 polarization (96). Our results suggest differential expression patterns of CD209 between EO- and LO-PE HBCs. Interestingly, the expression of CD209 was unchanged in CTRs and in the EO-PE group, whereas it was significantly reduced in the LO-PE group. In line, LO-PE HBCs tend to secrete lower levels of IL-10 supporting the notion that this polarization pattern favors regulatory properties of these cells. Yang et al. also reported lower numbers of CD209-positive HBCs in PE placental tissues, but they did not distinguish between onsets of PE (96)

Cytokines are important coordinators that likely promote phenotypic and functional changes of immune cells in inflammation (90, 97). We have shown that both, EO- and LO-PE HBCs produce different regulators of M2 polarization: IL-4, IL-10, IL-13 and TGF-β. Interestingly, whereas in EO-PE an increased production of TGF-β and IL-10 was observed, LO-PE HBCs secreted higher amounts of IL-4, IL-13, and TGF-β. IL-10 and IL-4, have been recognized as inducers of *ARG1*, which represents a hallmark of M2 polarization (97–100) IL-10 is a cytokine produced mainly by M2 macrophages (58) and its anti-inflammatory effect has been demonstrated by reduced production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12 (101, 102). Moreover, although pro-inflammatory M1 marker - IRF5 has been shown to inhibit transcription of IL-10 and TGF-β (58, 103), this regulation may be impaired in EO-PE, where basal secretion of IL-10 and TGF-β levels were increased. Enhanced expression of M1 polarization markers, upregulation of both M1 *NOS2* and M2 *ARG1*, accompanied by secretion of anti-inflammatory cytokines, indicate a development of a specific M1 and M2 phenotype of EO-PE HBCs. On the other hand, LO-PE HBCs downregulate *NOS2* and express *ARG1* at the same level as CTRs HBCs, suggesting their anti-inflammatory phenotype.

Phenotypic plasticity enables macrophages to perform a variety of functions required for maintenance of homeostasis and rapid termination of inflammation in their microenvironment (66). The resolution of acute inflammation is a well-orchestrated synergistic process and can be divided into three phases on the way back to cell homeostasis. First, inflammation is downregulated by the temporal switching of secreted lipid mediators, then the clearance of debris and apoptotic cells by phagocytically active macrophages, and finally, tissue repair and angiogenesis are stimulated by the pro-resolving phenotype of macrophages (20, 24, 54, 104). We demonstrated a higher phagocytic capacity of EO- and LO-PE compared to normal HBCs, with concomitantly significantly higher levels of TGF-β produced by both groups. Interestingly, TGF-β in monocytes and macrophages is known for its role in maintaining the resolution of inflammation by increasing phagocytosis and restoring tissue integrity (105–107). The increased phagocytosis and production of anti-inflammatory cytokines (90) may indicate one of the regulatory mechanisms that determine M2 phenotype of EO- and LO-PE HBCs by preventing a direct switch to M1 polarization.

Following clearance of cell-debris, M2 macrophages initiate events that are critical for tissue repair. These include the production of extracellular matrix (ECM), MMPs and the promotion of angiogenesis (108). Furthermore, MMPs make an important contribution to ECM repair (108), and macrophages require active MMP-9 for migration during an inflammatory response (109). TGF-β acts as a regulator of ECM production, reflecting its role in the tissue remodeling (110). We have shown compared to normal HBCs that both EO- and LO-PE HBCs secrete higher levels of MMP-9 and TGF-β. Interestingly, one of the many functions of MMP-9 is also to activate the inactive form of TGF-β (111), suggesting a possible mechanism driving polarization toward M2. MMP-9 is regulated by the expression of TIMPs, which has been shown to have pro-angiogenic features and is unique to M2 macrophages (112). Although the MMP9/TIMP2 ratio of EO- and LO-PE HBCs favors M2 polarization, we have shown that EO-PE HBCs were unable to stimulate proliferation of endothelial cells. Ability to enhance endothelial proliferation and consequently angiogenesis is one of the traits of M2 macrophages in the process of resolution of inflammation (104). The unsuccessful activation of proliferation of endothelial cells reveals another aspect of the pro-inflammatory M1 signature of EO-PE HBCs. It is more likely that they contribute to endothelial dysfunction instead of positive pro-angiogenic endothelial activation, but further investigation is needed.

Knowledge about the function of macrophages in PE is still insufficient. Our study focused on the *in vitro* polarization and functionality of EO- and LO-PE HBCs. Our results suggest that EO-PE HBCs develop a strong M1 signature, but despite the M1 features and PE inflammatory microenvironment, they still attempt to resolve inflammation by upregulating M2 anti-inflammatory factors and functions. Based on the fact that the expression of CD209 in EO-PE HBCs is at basal levels as in CTR HBCs, and expression of CD86 and secretion of TGF-β are increased, they might develop an immunoregulatory M2b and a tissue remodeling M2a phenotype with features of M1 polarization based on the increased expression of TLR4, HLA-DR, and IRF5. In contrast, LO-PE HBCs tend to develop a phagocytic M2 phenotype with increased production of IL-4, IL-13, and TGF-β. However, the higher expression of TLR1, TLR4, and CD80 and increased production of TNF-α indicate a specific pro-inflammatory pattern distinct from typical M2 polarization (Figure 6). Given the strength of our study, namely the use of a large number of clinically well-characterized samples from EO- and LO-PE placenta, there are some apparent limitations. First, macrophage polarization is a dynamic process, and therefore the choice of polarization markers included may vary among investigators. Because characterization of macrophage phenotype using polarization markers and cytokine release is rather descriptive, we chose to use functional assays to determine the relevant physiological functions of macrophages in addition to their phenotype. Second, primary HBCs might develop a different polarization pattern than *in vivo* because of the sensitivity of

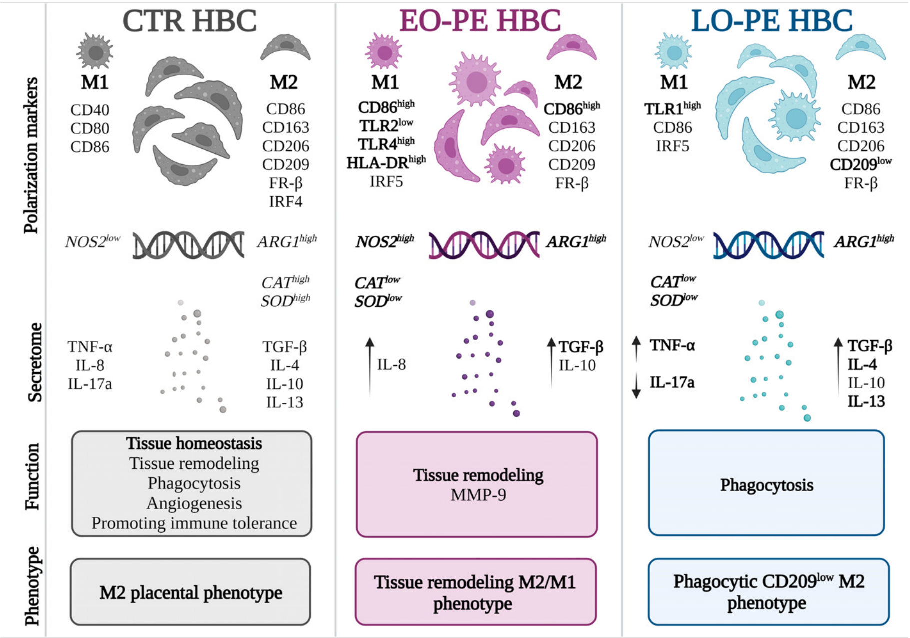

FIGURE 6
Different polarization patterns of CTR, EO - and LO-PE HBCs. HBCs isolated from CTR placenta develop a specific placental phenotype and express both M1 and M2 polarization markers. In CTR placenta, their M2 nature is reflected by increased expression of *ARG1*, *CAT*, and *SOD*, where HBCs promote tissue repair, angiogenesis, and homeostasis. Our results suggest that EO-PE HBCs develop an M2 phenotype that is strongly shifted toward M1 polarization. Their M2 phenotype is reflected in the upregulation of *ARG1*, secretion of TGF-β, and tissue remodeling function, whereas features of M1 polarization are seen in the increased expression of TLR4, HLA-DR, IRF5, and *NOS2*. In contrast, LO-PE HBCs tend to develop a phagocytic CD209^{low} M2 phenotype with increased production of IL-4, IL-13, and TGF-β. However, the higher expression of TLR1 and increased production of TNF-α indicate a specific pro-inflammatory pattern that differs from the typical M2 polarization. The figure was generated using BioRender. Differences that were significant different between the groups studied are printed in bold.

primary HBCs to the *in vitro* environment. However, we and others have shown that Hofbauer cells have a very stable phenotype *in vitro* that is difficult to alter and correlates with the phenotype in tissue *in vivo* (30, 37).

In conclusion, to the best of our knowledge, this is the first study to show a pivotal difference of the polarization pattern between EO - and LO-PE HBCs *in vitro*. We demonstrated that the inflammatory environment of PE causes the phenotypic changes observed between early and late PE HBCs. The changes in polarization patterns indicate different etiologies of PE, as EO-PE is associated with inflammation on the placental side, whereas LO-PE results from a maternal inflammatory response. Furthermore, since placental immune cells responds differently to the source of inflammation, PE could be identified as different entities with a common phenotype rather than a single disorder.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional ethics committee of the Medical University of Graz (29-319 ex 16/17). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MHM and CW conceived the study and designed the experiments. MHM performed the experiments and analysed the data. Reviewing and editing was done by CS and HF. MHM and CW wrote the original draft manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1095879/full#supplementary-material

References

1. Gestational hypertension and preeclampsia: ACOG practice bulletin, number 222. *Obstet Gynecol* (2020) 135:e237–60. doi: 10.1097/AOG.0000000000003891

2. Practice Bulletin No ACOG. 203: Chronic hypertension in pregnancy. *Obstet Gynecol* (2019) 133:e26–50. doi: 10.1097/AOG.0000000000003020

3. Brown MA, Magee LA, Kenny LC, Karumanchi SA, McCarthy FP, Saito S, et al. Hypertensive disorders of pregnancy. *Hypertension* (2018) 72:24–43. doi: 10.1161/HYPERTENSIONAHA.117.10803

4. Wójtowicz A, Zembala-Szczerba M, Babczyk D, Kołodziejczyk-Pietruszka M, Lewaczyńska O, Huras H. Early-and late-onset preeclampsia: A comprehensive cohort study of laboratory and clinical findings according to the new ISHHP criteria. *Int J Hypertens* (2019) 2019:4108271. doi: 10.1155/2019/4108271

5. von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. *Hypertens Pregnancy* (2003) 22:143–8. doi: 10.1081/PRG-120021060

6. Tranquilli AL, Brown MA, Zeeman GG, Dekker G, Sibai BM. The definition of severe and early-onset preeclampsia. statements from the international society for the study of hypertension in pregnancy (ISSHP). *Pregnancy Hypertension: Int J Women's Cardiovasc Health* (2013) 3:44–7. doi: 10.1016/J.PREGHY.2012.11.001

7. Huppertz B. Placental origins of preeclampsia: Challenging the current hypothesis. *Hypertension* (2008) 51:970–5. doi: 10.1161/HYPERTENSIONAHA.107.107607

8. Li XL, Guo PL, Xue Y, Gou WL, Tong M, Chen Q. An analysis of the differences between early and late preeclampsia with severe hypertension. *Pregnancy Hypertension: Int J Women's Cardiovasc Health* (2016) 6:47–52. doi: 10.1016/J.PREGHY.2015.12.003

9. Nelson DB, Ziadie MS, McIntire DD, Rogers BB, Leveno KJ. Placental pathology suggesting that preeclampsia is more than one disease. *Am J Obstet Gynecol* (2014) 210:66. doi: 10.1016/J.AJOG.2013.09.010

10. Stergiotou I, Crispi F, Valenzuela-Alcaraz B, Bijnens B, Gratacos E. Patterns of maternal vascular remodeling and responsiveness in early- versus late-onset preeclampsia. *Am J Obstet Gynecol* (2013) 209:558. doi: 10.1016/J.AJOG.2013.07.030

11. del Gaudio I, Sasset L, di Lorenzo A, Wadsack C. Sphingolipid signature of human feto-placental vasculature in preeclampsia. *Int J Mol Sci* (2020) 21(3):1019. doi: 10.3390/ijms21031019

12. Murthi P, Pinar AA, Dimitriadis E, Samuel CS. Inflammasomes–a molecular link for altered immunoregulation and inflammation mediated vascular dysfunction in preeclampsia. *Int J Mol Sci* (2020) 21:1406. doi: 10.3390/IJMS21041406

13. Borzychowski AM, Sargent IL, Redman CWG. Inflammation and pre-eclampsia. *Semin Fetal Neonatal Med* (2006) 11:309–16. doi: 10.1016/J.SINY.2006.04.001

14. Sharma A, Satyam A, Sharma JB. Leptin, IL-10 and inflammatory markers (TNF-α, IL-6 and IL-8) in pre-eclamptic, normotensive pregnant and healthy non-pregnant women. *Am J Reprod Immunol* (2007) 58:21–30. doi: 10.1111/J.1600-0897.2007.00486.X

15. Vishnyakova P, Poltavets A, Nikitina M, Muminova K, Potapova A, Vtorushina V, et al. Preeclampsia: inflammatory signature of decidual cells in early manifestation of disease. *Placenta* (2021) 104:277–83. doi: 10.1016/j.placenta.2021.01.011

16. Aneman I, Pienaar D, Suvakov S, Simic TP, Garovic VD, McClements L. Mechanisms of key innate immune cells in early- and late-onset preeclampsia. *Front Immunol* (2020) 11:1864/XML/NLM. doi: 10.3389/FIMMU.2020.01864/XML/NLM

17. Han X, Ghaemi MS, Ando K, Peterson LS, Ganio EA, Tsai AS, et al. Differential dynamics of the maternal immune system in healthy pregnancy and preeclampsia. *Front Immunol* (2019) 10:1305/BIBTEX. doi: 10.3389/FIMMU.2019.01305/BIBTEX

18. Broekhuizen M, Hitzerd E, van den Bosch TPP, Dumas J, Verdijk RM, van Rijn BB, et al. The placental innate immune system is altered in early-onset preeclampsia, but not in late-onset preeclampsia. *Front Immunol* (2021) 12:780043/BIBTEX. doi: 10.3389/FIMMU.2021.780043/BIBTEX

19. Ma Y, Ye Y, Zhang J, Ruan CC, Gao PJ. Immune imbalance is associated with the development of preeclampsia. *Med (United States)* (2019) 98(14):e15080. doi: 10.1097/MD.0000000000015080

20. Atri C, Guerfali FZ, Laouini D. Role of human macrophage polarization in inflammation during infectious diseases. *Int J Mol Sci* (2018) 19(6):1801. doi: 10.3390/IJMS19061801

21. Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, et al. Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol* (2018) 233:6425–40. doi: 10.1002/jcp.26429

22. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* (2014) 40:274. doi: 10.1016/J.IMMUNI.2014.01.006

23. Takiguchi H, Yang CX, Yang CWT, Sahin B, Whalen BA, Milne S, et al. Macrophages with reduced expressions of classical M1 and M2 surface markers in human bronchoalveolar lavage fluid exhibit pro-inflammatory gene signatures. *Sci Rep* (2021) 11:1–11. doi: 10.1038/s41598-021-87720-y

24. Porcheray F, Viaud S, Rimaniol A-C, Léone C, Samah B, Dereuddre-Bosquet N, et al. Macrophage activation switching: an asset for the resolution of inflammation. *Clin Exp Immunol* (2005) 142:481. doi: 10.1111/J.1365-2249.2005.02934.X

25. Oshi M, Tokumaru Y, Asaoka M, Yan L, Satyananda V, Matsuyama R, et al. M1 macrophage and M1/M2 ratio defined by transcriptomic signatures resemble only part of their conventional clinical characteristics in breast cancer. *Sci Rep* (2020) 10:1–12. doi: 10.1038/s41598-020-73624-w

26. Loegl J, Hiden U, Nussbaumer E, Schliefsteiner C, Cvitic S, Lang I, et al. Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis. *Reproduction* (2016) 152:447–55. doi: 10.1530/REP-16-0159

27. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* (2014) 6:13. doi: 10.12703/P6-13

28. Turco MY, Moffett A. Development of the human placenta. *Development* (2019) 146(22):dev163428. doi: 10.1242/DEV.163428

29. Reyes L, Wolfe B, Golos T. "Hofbauer cells: Placental macrophages of fetal origin". In: *Results Problems Cell Differentiation* (2017) 62:45–60. doi: 10.1007/978-3-319-54090-0_3

30. Schliefsteiner C, Ibesich S, Wadsack C. Placental hofbauer cell polarization resists inflammatory cues *In vitro*. *Int J Mol Sci* (2020) 21(3):736. doi: 10.3390/ijms21030736

31. Schliefsteiner C, Peinhaupt M, Kopp S, Lögl J, Lang-Olip I, Hiden U, et al. Human placental hofbauer cells maintain an anti-inflammatory M2 phenotype despite the presence of gestational diabetes mellitus. *Front Immunol* (2017) 8:888. doi: 10.3389/fimmu.2017.00888

32. Tang Z, Niven-Fairchild T, Tadesse S, Norwitz ER, Buhimschi CS, Buhimschi IA, et al. Glucocorticoids enhance CD163 expression in placental hofbauer cells. *Endocrinology* (2013) 154:471–82. doi: 10.1210/EN.2012-1575

33. Kim SY, Romero R, Tarca AL, Bhatti G, Kim CJ, Lee J, et al. Methylome of fetal and maternal monocytes and macrophages at the feto-maternal interface. *Am J Reprod Immunol* (2012) 68:8–27. doi: 10.1111/J.1600-0897.2012.01108.X

34. Goldstein J, Braverman M, Salafia C, Buckley P. The phenotype of human placental macrophages and its variation with gestational age(1988) (Accessed November 5, 2021).

35. Zulu MZ, Martinez FO, Gordon S, Gray CM. The elusive role of placental macrophages: The hofbauer cell. *J Innate Immun* (2019) 11:447–56. doi: 10.1159/000497416

36. Thomas JR, Appios A, Zhao X, Dutkiewicz R, Donde M, Lee CYC, et al. Phenotypic and functional characterization of first-trimester human placental macrophages, hofbauer cells. *J Exp Med* (2020) 218(1):e20200891. doi: 10.1084/JEM.20200891

37. Young OM, Tang Z, Niven-Fairchild T, Tadesse S, Krikun G, Norwitz ER, et al. Toll-like receptor-mediated responses by placental hofbauer cells (HBCs): A potential pro-inflammatory role for fetal M2 macrophages. *Am J Reprod Immunol* (2015) 73:22–35. doi: 10.1111/aji.12336

38. Svensson-Arvelund J, Mehta RB, Lindau R, Mirrasekhian E, Rodriguez-Martinez H, Berg G, et al. The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. *J Immunol* (2015) 194:1534–44. doi: 10.4049/JIMMUNOL.1401536

39. Cervar M, Blaschitz A, Dohr G, Desoye G. Paracrine regulation of distinct trophoblast functions *in vitro* by placental macrophages. *Cell Tissue Res* (1999) 295:297–305. doi: 10.1007/S004410051236

40. Khan S, Katabuchi H, Araki M, Nishimura R, Okamura H. Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation. *In Vitro Biol Reprod* (2000) 62:1075–83. doi: 10.1095/BIOLREPROD62.4.1075

41. Anteby EY, Natanson-Yaron S, Greenfield C, Goldman-Wohl D, Haimov-Kochman R, Holzer H, et al. Human placental hofbauer cells express sprouty proteins: a possible modulating mechanism of villous branching. *Placenta* (2005) 26:476–83. doi: 10.1016/J.PLACENTA.2004.08.008

42. Seval Y, Korgun ET, Demir R. Hofbauer cells in early human placenta: possible implications in vasculogenesis and angiogenesis. *Placenta* (2007) 28:841–5. doi: 10.1016/J.PLACENTA.2007.01.010

43. ben Amara A, Gorvel L, Baulan K, Derain-Court J, Buffat C, Vérollet C, et al. Placental macrophages are impaired in chorioamnionitis, an infectious pathology of the placenta. *J Immunol* (2013) 191:5501–14. doi: 10.4049/jimmunol.1300988

44. Vinnars M-TN, Rindsjö E, Ghazi S, Sundberg A, Papadogiannakis N. The number of CD68+ (Hofbauer) cells is decreased in placentas with chorioamnionitis and with advancing gestational age. *Pediatr Dev Pathol* (2010) 13:300–4. doi: 10.2350/09-03-0632-OA.1

45. Toti P, Arcuri F, Tang Z, Schatz F, Zambrano E, Mor G, et al. Focal increases of fetal macrophages in placentas from pregnancies with histological chorioamnionitis: Potential role of fibroblast monocyte chemotactic protein-1. *Am J Reprod Immunol* (2011) 65:470–9. doi: 10.1111/j.1600-0897.2010.00927.x

46. Kim JS, Romero R, Kim MR, Kim YM, Friel L, Espinoza J, et al. Involvement of hofbauer cells and maternal T cells in villitis of unknown aetiology. *Histopathology* (2008) 52:457–64. doi: 10.1111/j.1365-2559.2008.02964.x

47. Quicke KM, Bowen JR, Johnson EL, McDonald CE, Ma H, O'Neal JT, et al. Zika virus infects human placental macrophages. *Cell Host Microbe* (2016) 20:83–90. doi: 10.1016/J.CHOM.2016.05.015

48. Tang Z, Tadesse S, Norwitz E, Mor G, Abrahams VM, Guller S. Isolation of hofbauer cells from human term placentas with high yield and purity. *Am J Reprod Immunol* (2011) 66:336–48. doi: 10.1111/j.1600-0897.2011.01006.x

49. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. *Sci Rep* (2017) 7:1–7. doi: 10.1038/s41598-017-17204-5

50. Moldenhauer JS, Stanek J, Warshak C, Khoury J, Sibai B. The frequency and severity of placental findings in women with preeclampsia are gestational age dependent. *Am J Obstet Gynecol* (2003) 189:1173–7. doi: 10.1067/S0002-9378(03)00576-3

51. Tang Z, Buhimschi IA, Buhimschi CS, Tadesse S, Norwitz E, Niven-Fairchild T, et al. Decreased levels of folate receptor-β and reduced numbers of fetal macrophages (Hofbauer cells) in placentas from pregnancies with severe pre-eclampsia. *Am J Reprod Immunol* (2013) 70:104–15. doi: 10.1111/AJI.12112

52. McWhorter FY, Wang T, Nguyen P, Chung T, Liu WF. Modulation of macrophage phenotype by cell shape. *Proc Natl Acad Sci* (2013) 110:17253–8. doi: 10.1073/PNAS.1308887110

53. Porta C, Riboldi E, Ippolito A, Sica A. Molecular and epigenetic basis of macrophage polarized activation. *Semin Immunol* (2015) 27:237–48. doi: 10.1016/J.SMIM.2015.10.003

54. Wang Lx, Zhang S, Wu Hj, Rong X, Guo J. M2b macrophage polarization and its roles in diseases. *J Leukoc Biol* (2019) 106:345–58. doi: 10.1002/JLB.3RU1018-378RR

55. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. *Front Bioscience* (2008) 13:453–61. doi: 10.2741/2692/PDF

56. Negishi H, Ohba Y, Yanai H, Takaoka A, Honma K, Yui K, et al. Negative regulation of toll-like-receptor signaling by IRF-4. *Proc Natl Acad Sci* (2005) 102:15989–94. doi: 10.1073/PNAS.0508327102

57. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, Mizutani T, et al. Integral role of IRF-5 in the gene induction programme activated by toll-like receptors. *Nature* (2005) 434:243–9. doi: 10.1038/nature03308

58. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, et al. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat Immunol* (2011) 12:231–8. doi: 10.1038/ni.1990

59. Honma K, Udono H, Kohno T, Yamamoto K, Ogawa A, Takemori T, et al. Interferon regulatory factor 4 negatively regulates the production of proinflammatory cytokines by macrophages in response to LPS. *Proc Natl Acad Sci* (2005) 102:16001–6. doi: 10.1073/PNAS.0504226102

60. Raggi F, Pelassa S, Pierobon D, Penco F, Gattorno M, Novelli F, et al. Regulation of human macrophage M1–M2 polarization balance by hypoxia and the triggering receptor expressed on myeloid cells-1. *Front Immunol* (2017) 0:1097. doi: 10.3389/FIMMU.2017.01097

61. Makita N, Hizukuri Y, Yamashiro K, Murakawa M, Hayashi Y. IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration. *Int Immunol* (2015) 27:131–41. doi: 10.1093/INTIMM/DXU090

62. Fernando MR, Reyes JL, Iannuzzi J, Leung G, McKay DM. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PloS One* (2014) 9:e94188. doi: 10.1371/JOURNAL.PONE.0094188

63. Frank PG, Lisanti MP. ICAM-1: role in inflammation and in the regulation of vascular permeability. *Am J Physiol Heart Circ Physiol* (2008) 295:H926. doi: 10.1152/AJPHEART.00779.2008

64. Hu G, Su Y, Kang BH, Fan Z, Dong T, Brown DR, et al. High-throughput phenotypic screen and transcriptional analysis identify new compounds and targets for macrophage reprogramming. *Nat Commun* (2021) 12:1–14. doi: 10.1038/s41467-021-21066-x

65. Liu T, Zhang L, Joo D, Sun S-C. NF-κB signaling in inflammation. *Signal Transduction Targeted Ther* (2017) 2:1–9. doi: 10.1038/sigtrans.2017.23

66. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, et al. Macrophage activation and polarization: Nomenclature and experimental guidelines. *Immunity* (2014) 41:14–20. doi: 10.1016/J.IMMUNI.2014.06.008

67. Palanisamy V, Jakymiw A, van Tubergen EA, D'Silva NJ, Kirkwood KL. Control of cytokine mRNA expression by RNA-binding proteins and microRNAs. *J Dent Res* (2012) 91:651. doi: 10.1177/0022034512437372

68. Rath M, Müller I, Kropf P, Closs EI, Munder M. Metabolism *via* arginase or nitric oxide synthase: Two competing arginine pathways in macrophages. *Front Immunol* (2014) 0:532. doi: 10.3389/FIMMU.2014.00532

69. Zhang Y, Choksi S, Chen K, Pobezinskaya Y, Linnoila I, Liu Z-G. ROS play a critical role in the differentiation of alternatively activated macrophages and the occurrence of tumor-associated macrophages. *Cell Res* (2013) 23:898–914. doi: 10.1038/cr.2013.75

70. Rendra E, Riabov V, Mossel DM, Sevastyanova T, Harmsen MC, Kzhyshkowska J. Reactive oxygen species (ROS) in macrophage activation and function in diabetes. *Immunobiology* (2019) 224:242–53. doi: 10.1016/J.IMBIO.2018.11.010

71. Griess B, Mir S, Datta K, Teoh-Fitzgerald M. Scavenging reactive oxygen species selectively inhibits M2 macrophage polarization and their pro-tumorigenic function in part, *via* Stat3 suppression. *Free Radic Biol Med* (2020) 147:48–60. doi: 10.1016/J.FREERADBIOMED.2019.12.018

72. Klöditz K, Fadeel B. Three cell deaths and a funeral: macrophage clearance of cells undergoing distinct modes of cell death. *Cell Death Discov* (2019) 5:1–9. doi: 10.1038/s41420-019-0146-x

73. Reyes L, Golos TG. Hofbauer cells: Their role in healthy and complicated pregnancy. *Front Immunol* (2018) 9:2628. doi: 10.3389/fimmu.2018.02628

74. Raguema N, Moustadraf S, Bertagnolli M. Immune and apoptosis mechanisms regulating placental development and vascularization in preeclampsia. *Front Physiol* (2020) 11:98. doi: 10.3389/fphys.2020.00098

75. Phipps EA, Thadhani R, Benzing T, Karumanchi SA. Pre-eclampsia: pathogenesis, novel diagnostics and therapies. *Nat Rev Nephrol* (2019) 15:275–89. doi: 10.1038/s41581-019-0119-6

76. Brew K, Nagase H. The tissue inhibitors of metalloproteinases (TIMPs): An ancient family with structural and functional diversity. *Biochim Biophys Acta* (2010) 1803:55. doi: 10.1016/J.BBAMCR.2010.01.003

77. Cataldo DD, Gueders M, Munaut C, Rocks N, Bartsch P, Foidart J-M, et al. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. *Lab Invest* (2004) 84:418–24. doi: 10.1038/labinvest.3700063

78. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases. *Circ Res* (2003) 92:827–39. doi: 10.1161/01.RES.0000070112.80711.3D

79. Gardner K, Arnoczky SP, Caballero O, Lavagnino M. The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: An in vitro experimental study. *Disabil Rehabil* (2008). 30(20–22):1523–9. doi: 10.1080/09638280701785395

80. Arpino V, Brock M, Gill SE. The role of TIMPs in regulation of extracellular matrix proteolysis. *Matrix Biol* (2015) 44–46:247–54. doi: 10.1016/J.MATBIO.2015.03.005

81. Svensson J, Jenmalm MC, Matussek A, Geffers R, Berg G, Ernerudh J. Macrophages at the fetal–maternal interface express markers of alternative activation and are induced by m-CSF and IL-10. *J Immunol* (2011) 187:3671–82. doi: 10.4049/JIMMUNOL.1100130

82. Rana S, Lemoine E, Granger J, Karumanchi SA. Preeclampsia: Pathophysiology, challenges, and perspectives. *Circ Res* (2019) 124:1094–112. doi: 10.1161/CIRCRESAHA.118.313276/FORMAT/EPUB

83. Ren Z, Gao Y, Gao Y, Liang G, Chen Q, Jiang S, et al. Distinct placental molecular processes associated with early-onset and late-onset preeclampsia. *Theranostics* (2021) 11:5028–44. doi: 10.7150/THNO.56141

84. Kovo M, Schreiber L, Ben-Haroush A, Gold E, Golan A, Bar J. The placental component in early-onset and late-onset preeclampsia in relation to fetal growth restriction. *Prenat Diagn* (2012) 32:632–7. doi: 10.1002/PD.3872

85. Sohlberg S, Mulic-Lutvica A, Lindgren P, Ortiz-Nieto F, Wikström AK, Wikström J. Placental perfusion in normal pregnancy and early and late preeclampsia: A magnetic resonance imaging study. *Placenta* (2014) 35:202–6. doi: 10.1016/J.PLACENTA.2014.01.008

86. Marín R, Chiarello DI, Abad C, Rojas D, Toledo F, Sobrevia L. Oxidative stress and mitochondrial dysfunction in early-onset and late-onset preeclampsia. *Biochim Biophys Acta (BBA) - Mol Basis Dis* (2020) 1866:165961. doi: 10.1016/J.BBADIS.2020.165961

87. Saito S, Shiozaki A, Nakashima A, Sakai M, Sasaki Y. The role of the immune system in preeclampsia. *Mol Aspects Med* (2007) 28:192–209. doi: 10.1016/J.MAM.2007.02.006

88. DiFederico E, Genbacev O, Fisher SJ. Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall. *Am J Pathol* (1999) 155:293–301. doi: 10.1016/S0002-9440(10)65123-1

89. He G, Xu W, Chen Y, Liu X, Xi M. Abnormal apoptosis of trophoblastic cells is related to the up-regulation of CYP11A gene in placenta of preeclampsia patients. *PloS One* (2013) 8:e59609. doi: 10.1371/JOURNAL.PONE.0059609

90. Mor G, Abrahams VM. Potential role of macrophages as immunoregulators of pregnancy. *Reprod Biol Endocrinol* (2003) 1:1–8. doi: 10.1186/1477-7827-1-119/FIGURES/4

91. Joerink M, Rindsjö E, van Riel B, Alm J, Papadogiannakis N. Placental macrophage (Hofbauer cell) polarization is independent of maternal allergen-sensitization and presence of chorioamnionitis. *Placenta* (2011) 32:380–5. doi: 10.1016/J.PLACENTA.2011.02.003

92. Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol* (2010) 11:936–44. doi: 10.1038/ni.1920

93. Yeon MK, Romero R, Seo YO, Chong JK, Kilburn BA, Armant DR, et al. Toll-like receptor 4: A potential link between "danger signals," the innate immune system, and preeclampsia? *Am J Obstet Gynecol* (2005) 193:921.e1–8. doi: 10.1016/J.AJOG.2005.07.076

94. al Mamun A, Chauhan A, Qi S, Ngwa C, Xu Y, Sharmeen R, et al. Microglial IRF5-IRF4 regulatory axis regulates neuroinflammation after cerebral ischemia and impacts stroke outcomes. *Proc Natl Acad Sci USA* (2020) 117:1742–52. doi: 10.1073/PNAS.1914742117/-/DCSUPPLEMENTAL

95. el Chartouni C, Schwarzfischer L, Rehli M. Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming. *Immunobiology* (2010) 215:821–5. doi: 10.1016/J.IMBIO.2010.05.031

96. Yang SW, Cho EH, Choi SY, Lee YK, Park JH, Kim MK, et al. DC-SIGN expression in hofbauer cells may play an important role in immune tolerance in fetal chorionic villi during the development of preeclampsia. *J Reprod Immunol* (2017) 124:30–7. doi: 10.1016/J.JRI.2017.09.012

97. Deng B, Wehling-Henricks M, Villalta SA, Wang Y, Tidball JG. IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. *J Immunol* (2012) 189:3669–80. doi: 10.4049/JIMMUNOL.1103180

98. Sulahian TH, Högger P, Wahner AE, Wardwell K, Goulding NJ, Sorg C, et al. Human monocytes express cd163, which is upregulated by il-10 and identical TO p155. *Cytokine* (2000) 12:1312–21. doi: 10.1006/CYTO.2000.0720

99. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPβ. *Gene* (2005) 353:98–106. doi: 10.1016/J.GENE.2005.04.004

100. Wu WK, Georgiadis A, Copland DA, Liyanage S, Luhmann UFO, Robbie SJ, et al. IL-4 regulates specific arg-1+ macrophage sFlt-1–mediated inhibition of angiogenesis. *Am J Pathol* (2015) 185:2324–35. doi: 10.1016/J.AJPATH.2015.04.013

101. Lang R, Patel D, Morris JJ, Rutschman RL, Murray PJ. Shaping gene expression in activated and resting primary macrophages by IL-10. *J Immunol* (2002) 169:2253–63. doi: 10.4049/JIMMUNOL.169.5.2253

102. Schottelius AJG, Mayo MW, Balfour Sartor R, Baldwin AS. Interleukin-10 signaling blocks inhibitor of κB kinase activity and nuclear factor κB DNA binding *. *J Biol Chem* (1999) 274:31868–74. doi: 10.1074/JBC.274.45.31868

103. Dalmas E, Toubal A, Alzaid F, Blazek K, Eames HL, Lebozec K, et al. Irf5 deficiency in macrophages promotes beneficial adipose tissue expansion and insulin sensitivity during obesity. *Nat Med* (2015) 21:610–8. doi: 10.1038/nm.3829

104. Mosser DM, Hamidzadeh K, Goncalves R. Macrophages and the maintenance of homeostasis. *Cell Mol Immunol* (2020) 18:579–87. doi: 10.1038/s41423-020-00541-3

105. A-Gonzalez N, Bensinger SJ, Hong C, Beceiro S, Bradley MN, Zelcer N, et al. Apoptotic cells promote their own clearance and immune tolerance through activation of the nuclear receptor LXR. *Immunity* (2009) 31:245–58. doi: 10.1016/J.IMMUNI.2009.06.018

106. Kourtzelis I, Hajishengallis G, Chavakis T. Phagocytosis of apoptotic cells in resolution of inflammation. *Front Immunol* (2020) 11:553/BIBTEX. doi: 10.3389/FIMMU.2020.00553/BIBTEX

107. Gong D, Shi W, Yi S, Chen H, Groffen J, Heisterkamp N. TGFβ signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol* (2012) 13:1–10. doi: 10.1186/1471-2172-13-31

108. Oishi Y, Manabe I. Macrophages in inflammation, repair and regeneration. *Int Immunol* (2018) 30:511–28. doi: 10.1093/INTIMM/DXY054

109. Hanania R, Sun HS, Xu K, Pustylnik S, Jeganathan S, Harrison RE. Classically activated macrophages use stable microtubules for matrix metalloproteinase-9 (MMP-9) secretion. *J Biol Chem* (2012) 287:8468–83. doi: 10.1074/JBC.M111.290676/ATTACHMENT/13099993-EBF4-49E0-A71C-77C3089A9E06/MMC1.PDF

110. Xu X, Zheng L, Yuan Q, Zhen G, Crane JL, Zhou X, et al. Transforming growth factor-β in stem cells and tissue homeostasis. *Bone Res* (2018) 6:1–31. doi: 10.1038/s41413-017-0005-4

111. Kobayashi T, Kim HJ, Liu X, Sugiura H, Kohyama T, Fang Q, et al. Matrix metalloproteinase-9 activates TGF-β and stimulates fibroblast contraction of collagen gels. *Am J Physiol Lung Cell Mol Physiol* (2014) 306:L1006. doi: 10.1152/AJPLUNG.00015.2014

112. Zajac E, Schweighofer B, Kupriyanova TA, Juncker-Jensen A, Minder P, Quigley JP, et al. Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9. *Blood* (2013) 1122(25):4054–67. doi: 10.1182/BLOOD-2013-05-501494

Supplementary Material

Supplementary methods

Isolation of human arterial endothelial cells (fpEC)

Primary fpECs were isolated from chorionic arterial blood vessels (CTR n=5, EO-PE n=3) as described by Lang et al (51). Isolated fpECs were cultured at 12% oxygen and 37 °C on 1% gelatin-coated flasks (Sigma Aldrich) using Endothelial Cell Growth Medium MV (Promocell) supplemented with 5% FCS (Promocell), hydrocortisone (Promocell), ECGS (Promocell), and 1% gentamicin (Thermo Fischer Scientific).

Proliferation assay

BrDU

To measure the proliferation of EO-PE and CTR fpEC, BrDU colorimetric cell proliferation ELISA kit (Roche) was used according to the manufacturer´s recommendation. fpEC were seeded overnight at a cell density of $2x10^6$ cells/mL in Endothelial Cell Growth Medium MV (Promocell). The medium was then replaced with a pool of conditioned medium (CM) obtained from 10 different CTR and 5 EO-PE HBC isolations. CM was diluted 1:1 with full fpEC medium. Incubated macrophage medium without cells diluted with endothelial medium served as a control. After 24 hours of treatment with CM, 10 µL of BrDU reagent was added and incubated for 3 hours. After fixation, denaturation and incubation with the anti-BRdU- POD working solution, cells were intensively washed and in final step incubated with the substrate solution for 25 minutes. Afterwards, absorbance was measured at 492 nm using the SPECTROstar Nano plate reader (BMG Lab Technologies).

MTS

This assay (Cell titer 96 Aquaeous one solution cell, Promega) was applied according to the manufacturer's instructions. EO-PE and CTR fpEC were seeded in a gelatin-coated 96-well plate at a cell density of $1x10^6$ cells/mL in Endothelial Cell Growth Medium MV containing supplements (Promocell) and cultured overnight. The medium was then replaced with a pool of CM obtained from 10 different CTR and 5 EO-PE HBC isolations. The CM was diluted 1:1 with the complete fpEC medium. Incubated macrophage medium without HBCs, diluted with fpEC medium served as a control. After 24 hours of treatment, 20 µL of MTS reagent was added to the cells and incubated for 4 hours. Absorbance was measured at 492 nm using the SPECTROstar Nano plate reader (BMG Labtech).

Supplementary Tables

Table S1: List of antibodies and their concentrations used for the FACS analysis

Antibody	Distributor / Catalog number	Concentration
APC anti-human CD163 Antibody	BioLegend / 333609	15 µl / 100 µl
PE Mouse Anti-Human CD68	BD Pharmingen™ / 556078	10 µl / 100 µl
V450 Mouse Anti-Human CD11b	BD Pharmingen™ / 560481	5 µl / 100 µl
PE Mouse Anti-Human CD11c	BD Pharmingen™ / 560999	5 µl / 100 µl
FITC Mouse Anti-Human CD206	BD Pharmingen™ / 551135	5 µl / 100 µl
PerCP-Cy™5.5 Mouse Anti-Human CD209	BD Pharmingen™ / 558263	10 µl / 100 µl
V450 Mouse Anti-Human CD80	BD Pharmingen™ / 560442	10 µl / 100 µl
V450 Mouse Anti-Human CD86	BD Pharmingen™ / 560357	5 µl / 100 µl
FITC Anti-TIL/TLR1 antibody	Abcam / ab59702	5 µl / 100 µl
Human TLR4 PE-conjugated Antibody	R&D / FAB6248P	5 µl / 100 µl
Pacific Blue™ anti-human HLA-DR Antibody	BioLegend / 307633	5 µl / 100 µl
APC anti-human Folate Receptor β (FR-β) Antibody	BioLegend / 391705	2 µl / 100 µl
FITC Anti-TLR2 antibody	abcam / ab59711	3 µl / 100 µl
PE Mouse Anti- IRF4	BD Pharmingen™ / 566646	5 µl / 100 µl

IRF5 Monoclonal Antibody (ALYSCLN), eFluor 660	Invitrogen / 50-9698- 41	5 µl / 100 µl
FITC Mouse Anti-Human CD40	BD Pharmingen™ / 556624	5 µl / 100 µl
7-AAD	BD Pharmingen™ / 559925	5 µl / 100 µl
FITC Mouse IgG1 K Isotype Control	BD Pharmingen™/ 555748	5 µl / 100 µl
PerCP-Cy™5.5 Mouse IgG1 κ Isotype Control	BD Pharmingen™/ 550795	10 µl / 100 µl
Pacific Blue™ Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400151	5 µl / 100 µl
V450 Mouse IgG1/ κ Isotype Control	BD Pharmingen™ / 560373	5 µl / 100 µl
APC Mouse IgG1 K Isotype Control	BD Pharmingen™/ 555751	10 µl / 100 µl
PE Mouse IgG1 K Isotype Control	BD Pharmingen™/ 555749	5 µl / 100 µl

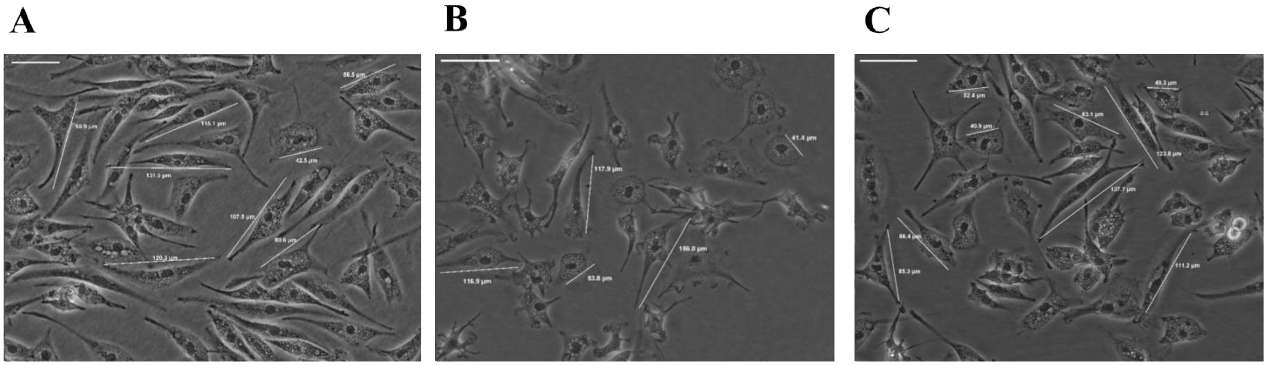
Table S2: List of primer assays used for the RT-qPCR analysis

Target	Assay	Supplier
RPL30	Hs_RPL30_1_SG QuantiTect Primer Assay (QT00056651)	Quiagen
HPRT1	Hs_HPRT1_1_SG QuantiTect Primer Assay (QT00059066)	Quiagen
MMP9	Hs_MMP9_1_SG QuantiTect Primer Assay (QT00040040)	Quiagen

MMP12	Hs_MMP12_1_SG QuantiTect Primer Assay (QT01004472)	Quiagen
TIMP1	Hs_TIMP1_1_SG QuantiTect Primer Assay (QT00084168)	Quiagen
TIMP2	Hs_TIMP2_1_SG QuantiTect Primer Assay (QT00017759)	Quiagen
CCL4	Hs_CCL4_1_SG QuantiTect Primer Assay (QT01008070)	Quiagen
IL6	Hs_IL6_1_SG QuantiTect Primer Assay (QT00083720)	Quiagen
CXCL8	Hs_CXCL8_1_SG QuantiTect Primer Assay (QT00000322)	Quiagen
ICAM	Hs_ICAM1_1_SG QuantiTect Primer Assay (QT00074900)	Quiagen
VCAM	Hs_VCAM1_1_SG QuantiTect Primer Assay (QT00018347)	Quiagen
TGFB1	Hs_TGFB1_1_SG QuantiTect Primer Assay (QT00000728)	Quiagen
IL10	Hs_IL10_1_SG QuantiTect Primer Assay (QT00041685)	Quiagen
18S	Forward (5'-3') CTACCACATCCAAGGAAGCA Reverse (5'-3') TTTTTCGTCACTACCTCCCCG	Sigma- Aldrich
NFKB1	KiCqStart™ Primer: H_NFKB1_1	Sigma- Aldrich
LEP	KiCqStart™ Primer: H_LEP_1	Sigma- Aldrich
HIF1A	KiCqStart™ Primer: H_HIF1A_1	Sigma- Aldrich
SOD	KiCqStart™ Primer: H_SOD1_1	Sigma- Aldrich

CAT	KiCqStart™ Primer: H_CAT_1	Sigma- Aldrich
ARG1	KiCqStart™ Primer: H_ARG1_1	Sigma- Aldrich
IL1A	KiCqStart™ Primer: H_IL1A_1	Sigma- Aldrich
IL1B	KiCqStart™ Primer: H_IL1B_1	Sigma- Aldrich
TNF	KiCqStart™ Primer: H_TNF_1	Sigma- Aldrich
FLT1	KiCqStart™ Primer: H_FLT1_1	Sigma- Aldrich
VEGFA	KiCqStart™ Primer: H_VEGFA_1	Sigma- Aldrich
KDR	KiCqStart™ Primer: H_KDR_1	Sigma- Aldrich
CDH2	KiCqStart™ Primer: H_CDH2_1	Sigma- Aldrich
CDH5	KiCqStart™ Primer: H_CDH5_1	Sigma- Aldrich
NOS2	KiCqStart™ Primer: H_NOS2_1	Sigma- Aldrich

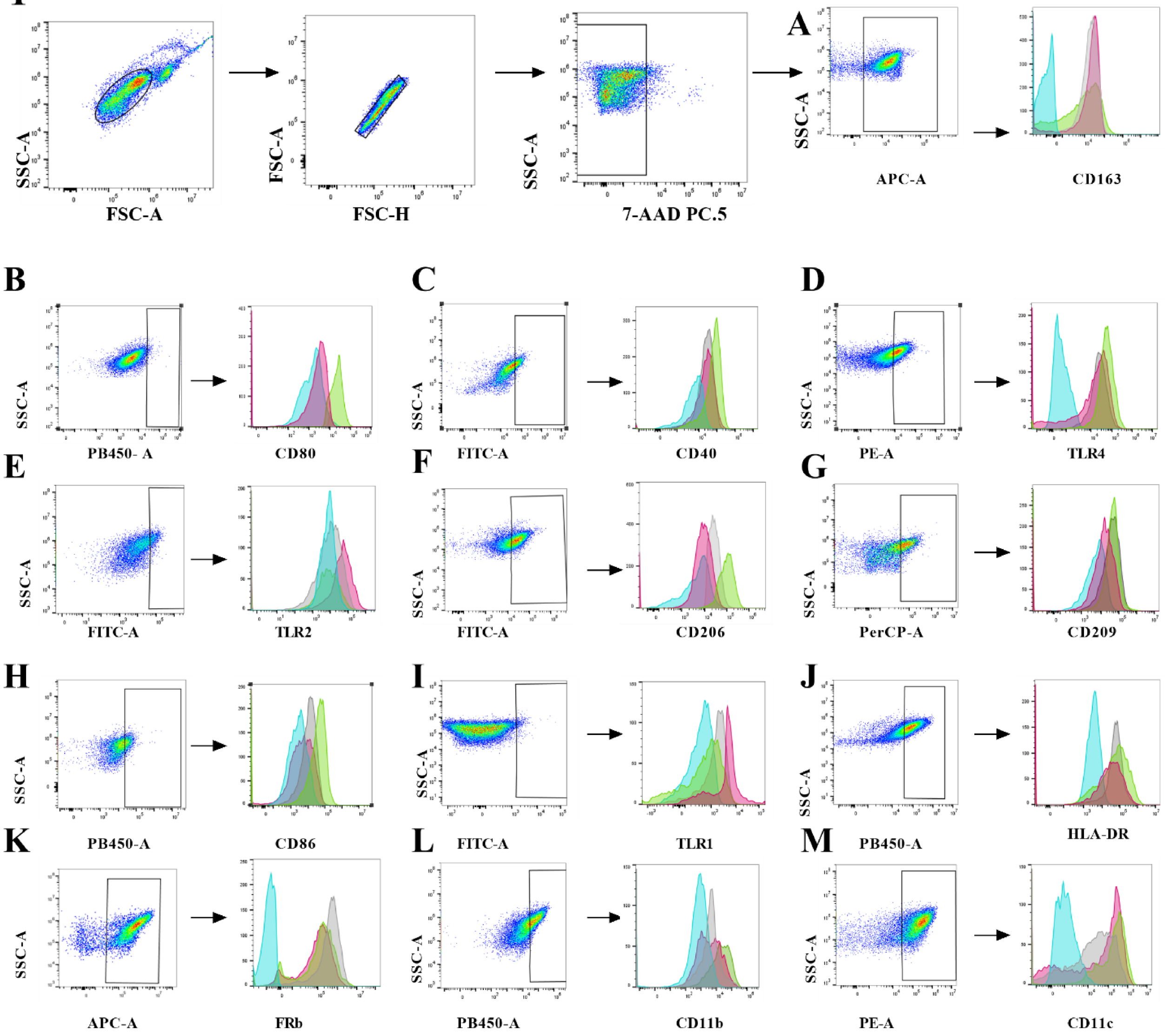
Supplementary Figures



CTR HBC **EO-PE HBC** **LO-PE HBC**

Figure S1: Cell morphology and characterization of CTR (A), early onset (EO-) (B) and late onset (LO-) PE (C) HBCs isolated from term placentae. Representative images of HBCs taken on the 5th day post isolation are shown. Scale bar represents 50µm, cell size was measured using CellSens measuring tool.

1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SSC-A
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SSC-A
PB450- A
CD80
C
SSC-A
FITC-A
CD40
D
SSC-A
PE-A
TLR4
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SSC-A
FITC-A
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SSC-A
PerCP-A
CD209
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SSC-A
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CD86
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SSC-A
FITC-A
TLR1
J
SSC-A
PB450-A
HLA-DR
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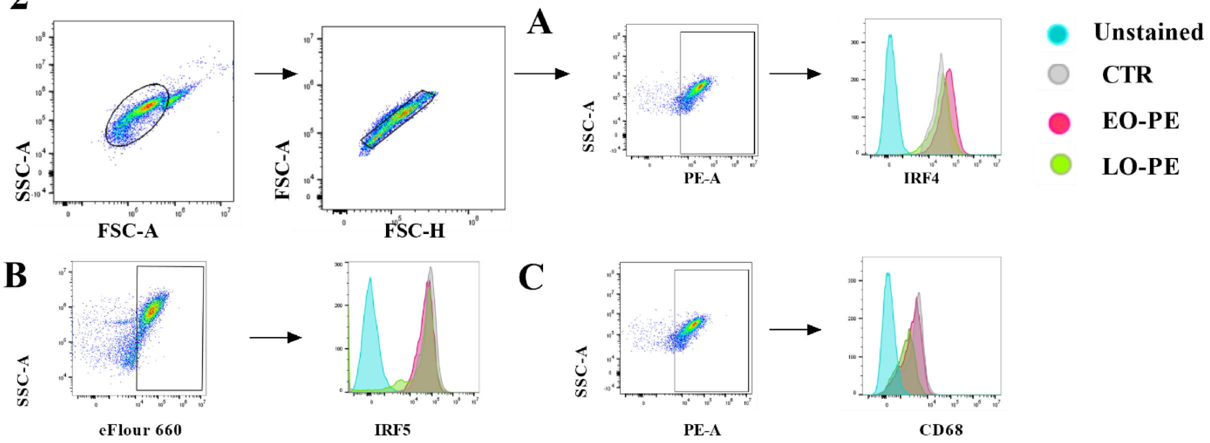
CD11c

2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SSC-A
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IRF4
Unstained
CTR
EO-PE
LO-PE
B
SSC-A
eFlour 660
IRF5
C
SSC-A
PE-A
CD68

Figure S2: Representative gating strategy to identify the expression of specific surface (1) and intracellular (2) polarization markers. (1A) CD163, (1B) CD80, (1C) CD40, (1D) TLR4, (1E) TLR2, (1F) CD206, (1G) CD209, (1H) CD86, (1I) TLR1, (1J) HLA-DR, (1K) folate receptor-β (FRb), (1L) CD11b, (1M) CD11c, respectively. (2) Staining against intracellular markers: (2A) IRF4, IRF5 (2B), and (2C) CD68, respectively. Blue histogram peaks represent unstained samples, grey represents CTR HBCs, red represents EO-PE HBCs and green represents LO-PE HBCs. Histograms of one representative experiment are shown, in total a number of isolations of CTR (n=14), EO (n=6) and LO (n=5) were used.

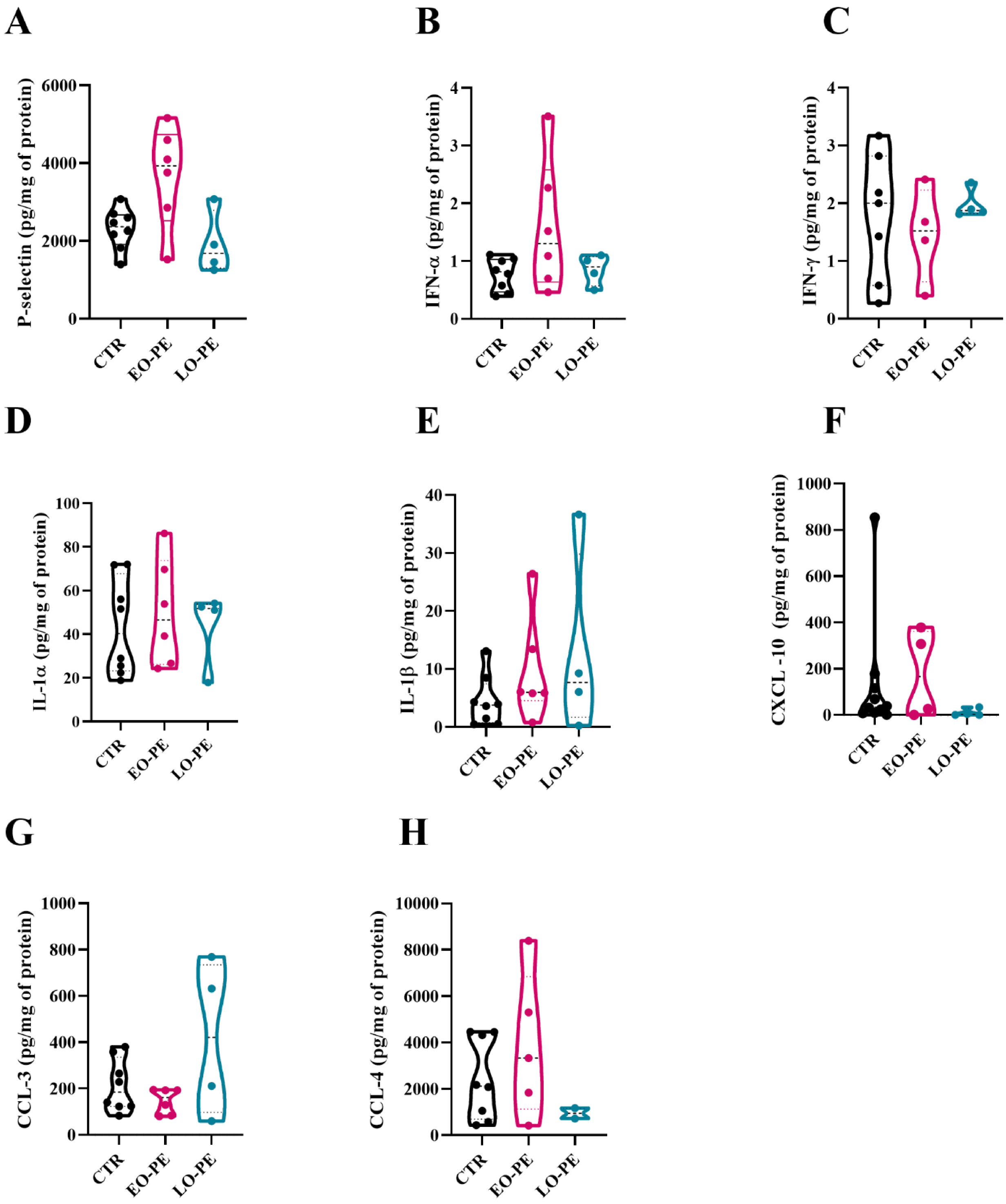

Figure S3: Secretion of pro- and anti-inflammatory cytokines CTR (n=8), EO- (n=6) and LO-PE (n=4) HBCs. Multiplex-ELISA-on-beads assay for followed pro-inflammatory and anti-inflammatory cytokines (A) P-selectin, (B) IFN-α, (C) IFN- γ, (D) IL-1β, (E) IL-17a, (F) CXCL-10, (G) CCL-3 and (H) CCL-4, respectively. Multiplex was performed in duplicates. Secretion of respective cytokines was normalized to the total protein content measured in the cell culture supernatants. Statistical significance was assessed using ANCOVA with adjustment for gestational age followed by Sidak´s post-hoc test. $^*p \leq 0.05$.

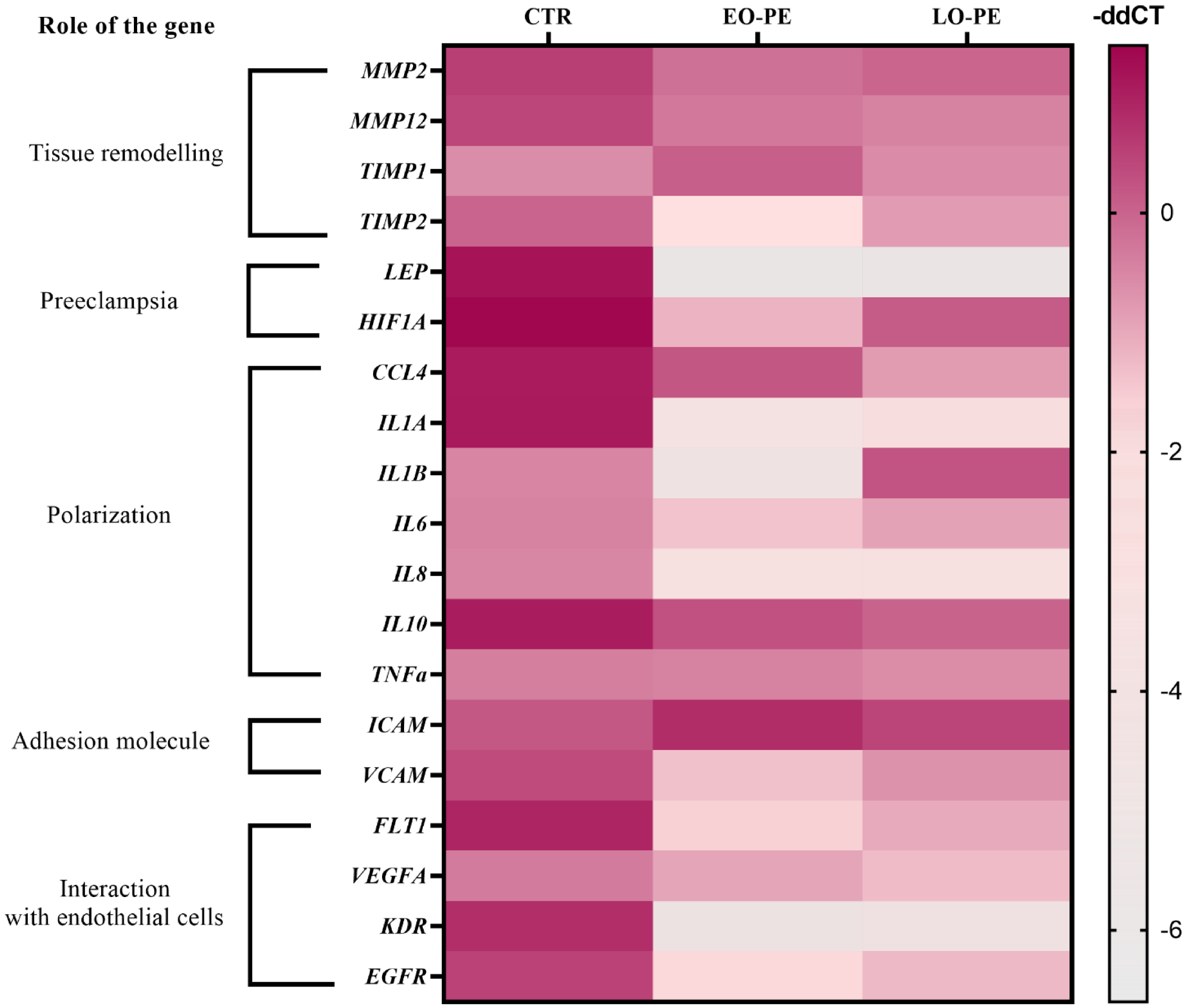

Figure S4: Preeclampsia alters inflammatory related gene expression in HBCs. Total RNA of cultivated HBCs was harvested on the fifth day post isolation and analysed by RT-qPCR. Genes associated with PE, inflammation and regulation of polarization are shown on the heat map. In total 12 CTR, 5 EO-PE and 5 LO-PE HBCs isolations in three technical replicates were used. Expression of target genes was normalized to the following housekeeping genes (*18S*, *RPL30* and *HPRT1*) using $2^{\Delta\Delta Ct}$ method. Statistical significance was tested using ANCOVA with adjustment for gestational age followed by Sidak's post-hoc test. $^*p \leq 0.05$.

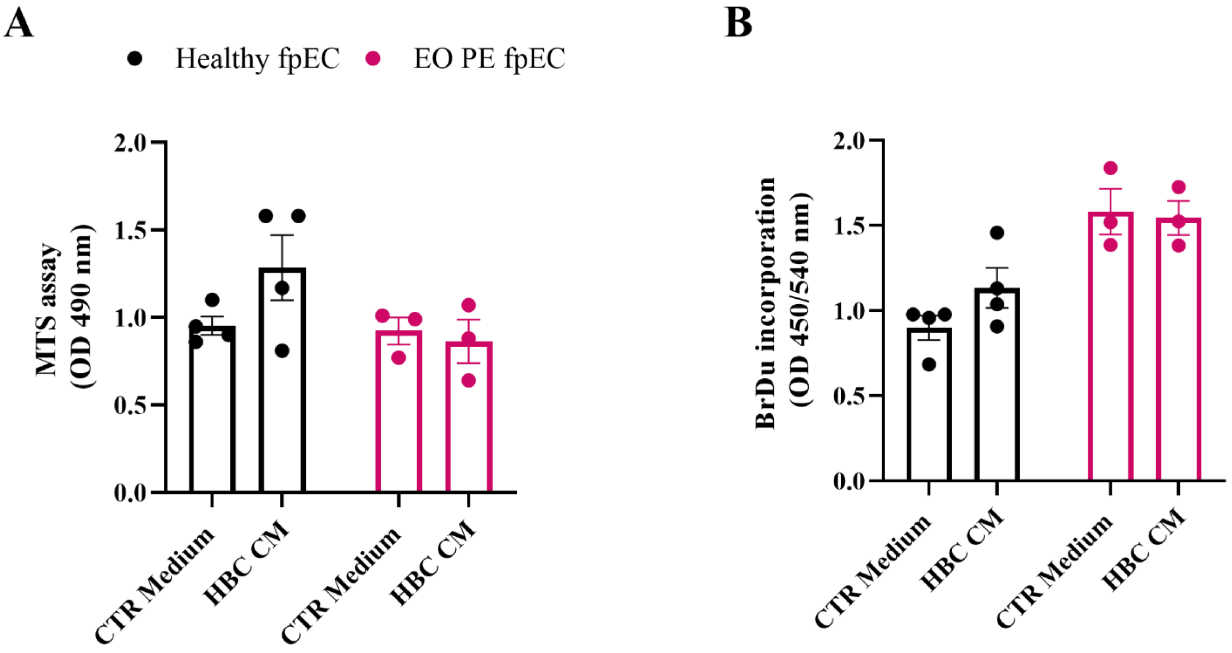

Figure S5: Effect of CTR and EO-PE conditioned medium (CM) of HBCs on the proliferation of CTR and PE fpECA. CM from 5 different HBC isolations was used for proliferation assays with CTR (n=4) and PE (n=3) fpECA. As a CTR medium cultivated Macrophage medium without cells was used. Healthy fpEC were treated with CM collected from healthy HBCs and PE fpEC with CM collected from EO-PE HBCs, respectively. CM of CTR HBCs tend to have an effect on proliferation of fpEC measured with MTS assay (A) and BrDU assay (B). Data are presented as the mean of the technical triplicates of the number of individual biological replicates of fpECA. To test statistical significance Two-way ANOVA with Sidak's post-hoc test was used. Data are shown as mean ± S.E.M. p-value ≤ 0.05 was considered statistically significant.

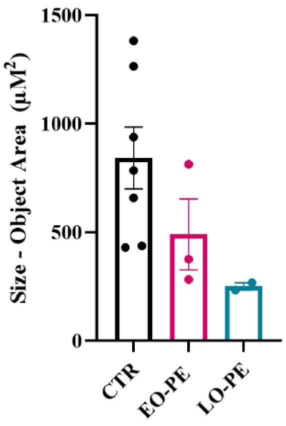

Figure S6: Measurement of morphology (size-object area, µM^2) using high content screening (HCS). Measurement of the size of the HBCs was performed using NisViewer software. All data in are presented as mean ± S.E.M, ANCOVA with adjustment for gestational age with Sidak's post-hoc test was used for to test statistical significance. *$p \leq 0.05$.

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TGFβ signalling: a nexus between inflammation, placental health and preeclampsia throughout pregnancy

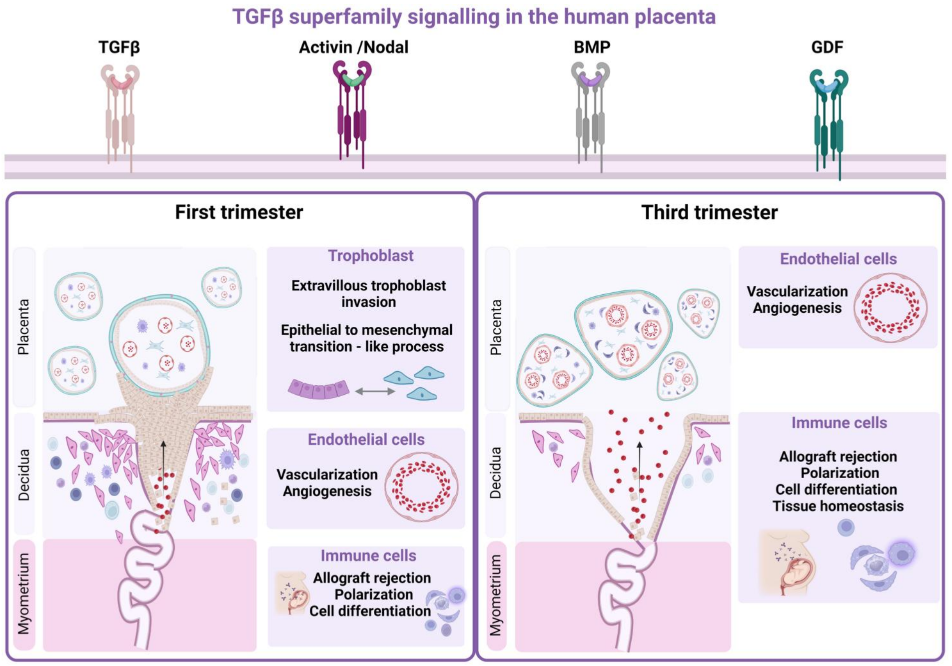
Monika Horvat Mercnik [1], Carolin Schliefsteiner [1], Gonzalo Sanchez-Duffhues [2], and Christian Wadsack [1,3,*]

[1]Department of Obstetrics and Gynaecology, Medical University of Graz, Graz, Austria
[2]Nanomaterials and Nanotechnology Research Center (CINN-CSIC), Tissue-Specific BMP Signalling ISPA-HUCA, Oviedo, Spain
[3]BioTechMed-Graz, Graz, Austria

*Correspondence address. Department of Obstetrics and Gynaecology, Medical University of Graz, Auenbruggerplatz 14, 8036 Graz, Austria. E-mail: christian.wadsack@medunigraz.at https://orcid.org/0000-0002-5589-8642

TABLE OF CONTENTS

GRAPHICAL ABSTRACT



TGFβ signalling governs essential processes in placental development, coordinating trophoblast invasion, vascularization, immune tolerance, and tissue remodelling across cell types, to ensure a healthy pregnancy outcome.

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ABSTRACT

BACKGROUND: The placenta is a unique and pivotal organ in reproduction, controlling crucial growth and cell differentiation processes that ensure a successful pregnancy. Placental development is a tightly regulated and dynamic process, in which the transforming growth factor beta (TGFβ) superfamily plays a central role. This family of pleiotropic growth factors is heavily involved in regulating various aspects of reproductive biology, particularly in trophoblast differentiation during the first trimester of pregnancy. TGFβ signalling precisely regulates trophoblast invasion and the cell transition from cytotrophoblasts to extravillous trophoblasts, which is an epithelial-to-mesenchymal transition-like process. Later in pregnancy, TGFβ signalling ensures proper vascularization and angiogenesis in placental endothelial cells. Beyond its role in trophoblasts and endothelial cells, TGFβ signalling contributes to the polarization and function of placental and decidual macrophages by promoting maternal tolerance of the semi-allogeneic foetus. Disturbances in early placental development have been associated with several pregnancy complications, including preeclampsia (PE) which is one of the severe complications. Emerging evidence suggests that TGFβ is involved in the pathogenesis of PE, thereby offering a potential target for intervention in the human placenta.

OBJECTIVE AND RATIONALE: This comprehensive review aims to explore and elucidate the roles of the major members of the TGFβ superfamily, including TGFβs, bone morphogenetic proteins (BMPs), activins, inhibins, nodals, and growth differentiation factors (GDFs), in the context of placental development and function. The review focusses on their interactions within the major cell types of the placenta, namely trophoblasts, endothelial cells, and immune cells, in both normal pregnancies and pregnancies complicated by PE throughout pregnancy.

SEARCH METHODS: A literature search was carried out using PubMed and Google Scholar, searching terms: 'TGF signalling preeclampsia', 'pregnancy TGF signalling', 'preeclampsia tgfβ', 'preeclampsia bmp', 'preeclampsia gdf', 'preeclampsia activin', 'endoglin preeclampsia', 'endoglin pregnancy', 'tgfβ signalling pregnancy', 'bmp signalling pregnancy', 'gdf signalling pregnancy', 'activin signalling pregnancy', 'Hofbauer cell tgfβ signalling', 'placental macrophages tgfβ', 'endothelial cells tgfβ', 'endothelium tgfβ signalling', 'trophoblast invasion tgfβ signalling', 'trophoblast invasion Smad', 'trophoblast invasion bmp', 'trophoblast invasion tgfβ', 'tgfβ preeclampsia', 'tgfβ placental development', 'TGFβ placental function', 'endothelial dysfunction preeclampsia tgfβ signalling', 'vascular remodelling placenta TGFβ', 'inflammation pregnancy tgfβ', 'immune response pregnancy tgfβ', 'immune tolerance pregnancy tgfβ', 'TGFβ pregnancy NK cells', 'bmp pregnancy NK cells', 'bmp pregnancy tregs', 'tgfβ pregnancy tregs', 'TGFβ placenta NK cells', 'TGFβ placenta tregs', 'NK cells preeclampsia', 'Tregs preeclampsia'. Only articles published in English until 2023 were used.

OUTCOMES: A comprehensive understanding of TGFβ signalling and its role in regulating interconnected cell functions of the main placental cell types provides valuable insights into the processes essential for successful placental development and growth of the foetus during pregnancy. By orchestrating trophoblast invasion, vascularization, immune tolerance, and tissue remodelling, TGFβ ligands contribute to the proper functioning of a healthy maternal–foetal interface. However, dysregulation of TGFβ signalling has been implicated in the pathogenesis of PE, where the shallow trophoblast invasion, defective vascular remodelling, decreased utero-placental perfusion, and endothelial cell and immune dysfunction observed in PE, are all affected by an altered TGFβ signalling.

WIDER IMPLICATIONS: The dysregulation of TGFβ signalling in PE has important implications for research and clinical practice. Further investigation is required to understand the underlying mechanisms, including the role of different ligands and their regulation under pathophysiological conditions, in order to discover new therapeutic targets. Distinguishing between clinically manifested subtypes of PE and studying TGFβ signalling in different placental cell types holistically is an important first step. To put this knowledge into practice, pre-clinical animal models combined with new technologies are needed. This may also lead to improved human research models and identify potential therapeutic targets, ultimately improving outcomes for affected pregnancies and reducing the burden of PE.

Keywords: preeclampsia / transforming growth factor / bone morphogenetic protein / TGFβ / signalling / trophoblast / endothelial cells / macrophages / human placenta / immune cells

Introduction

During pregnancy, the human placenta plays a unique role as the first organ to develop from cells originating from the blastocyst. The beginning of a healthy pregnancy relies on the intricate regulation between trophoblast cell invasion and foeto-maternal tolerance (Knöfler *et al.*, 2019). As the pregnancy advances, the successful maintenance of the pregnancy relies on the precise coordination, action, and interplay of various cell types in a highly orchestrated manner, to support the developing foetus and ensure a healthy pregnancy outcome (Huppertz, 2008a; Cindrova-Davies and Sferruzzi-Perri, 2022). TGFβ signalling plays a major part in embryonic and placental development and thereby effecting pregnancy outcome (Fig. 1) (Haider *et al.*, 2017, 2022; Li *et al.*, 2021; Yang *et al.*, 2021; Fang *et al.*, 2022). In the first trimester of pregnancy, TGFβ signalling is primarily involved in the regulation of trophoblast invasion, remodelling of the spiral arteries, and development of the placental vasculature (Dietrich *et al.*, 2022; Li *et al.*, 2023a). Disturbances in TGFβ levels in maternal plasma have been associated with miscarriages (Ogasawara *et al.*, 2000; Dirisipam *et al.*, 2023), but TGFβ levels in the placental bed do not seem to be altered under these circumstances (Ball *et al.*, 2007). Emerging evidence suggest that various components of the TGFβ pathways have been frequently reported as altered in the pathogenesis of preeclampsia (PE) (Venkatesha *et al.*, 2006; Haider *et al.*, 2017; Zhao *et al.*, 2020b; Deng *et al.*, 2023), and might be potential therapeutic targets for intervention. As the pregnancy progresses, TGFβs take up the role as immune suppressors, promoting maternal tolerance of the semi-allogenic allograft during gestation (Ingman and Robertson, 2009). Additionally, TGFβ family members are involved in regulating the function of placental endothelial cells (ECs) (Zhou *et al.*, 2019) and immune cells (Keskin *et al.*, 2007; Mercnik *et al.*, 2022; Vondra *et al.*, 2023). This comprehensive review highlights the TGFβ superfamily signalling and its key ligands, such as transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), activins, inhibins, nodals, and growth differentiation factors (GDFs), in the context of placental function in normal and PE-compromised pregnancies. In consequence, all major cell types of the early and late placenta namely, trophoblasts, ECs, and immune cells, are the subject of these summaries. By highlighting the indispensable role of TGFβ family members, this review emphasizes the inherent biological diversity of placental cells and related cell models, which are, in addition, influenced by non-placental cell types that produce vital TGFβ ligands. While placental cells are the primary source of TGFβ ligands (Jones *et al.*, 2006b; Chuva de Sousa Lopes *et al.*, 2020), it is also crucial to

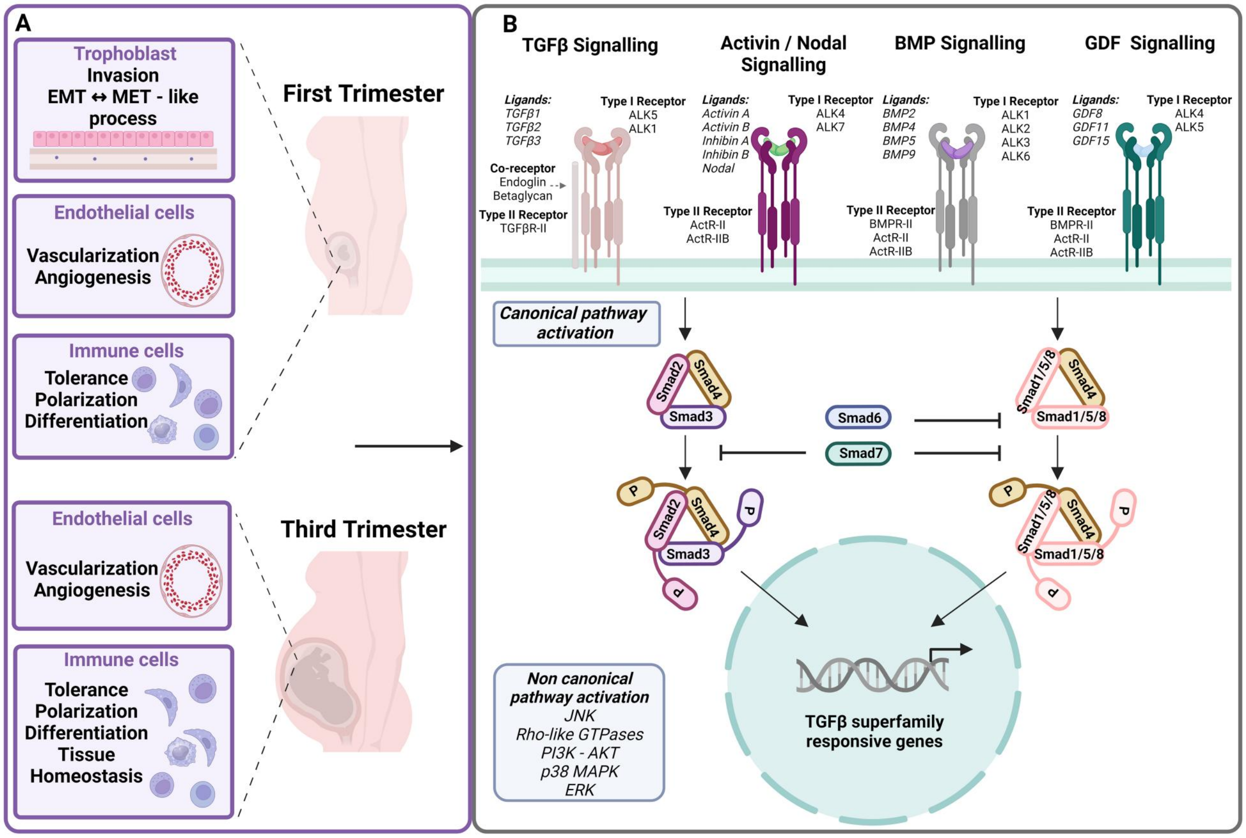

Figure 1. TGFβ signalling in the placenta. (**A**) TGFβ signalling in the first and third trimester placenta. TGFβ signalling is crucial for placental development and the functional regulation of placental cells. In trophoblasts, particularly during the first trimester, TGFβ signalling is involved in regulating cell invasion and the transition of CTs to EVTs through an EMT-like process. Additionally, some ligands of the TGFβ family stimulate the reverse process called MET. To ensure blood supply and placental function, TGFβ signalling contributes to vascularization by stimulating angiogenesis through placental ECs. During pregnancy, TGFβ signalling contributes to the establishment of the maternal tolerance to the semi-allogeneic foetus by limiting the immunogenic response. TGFβ also plays a central role in Tregs differentiation, regulation of NK cells functions, and the balance between M1 and M2 macrophages. (**B**) TGFβ family signalling activation pathways in the human placenta. TGFβ ligands interact with type I and type II receptors and induce downstream signalling through canonical and non-canonical pathways. Upon ligand-receptor binding, the receptor complex becomes phosphorylated and recruits the canonical intracellular mediators Smad2/3 or Smad1/5/8. Depending on the type I receptor activated, in the canonical signalling route, either Smad2/3 or Smad1/5/8 are trans-phosphorylated. TGFβ and Activins via ALK4/5/7 primarily activate Smad2/3, whereas Smad1/5/8 activation is induced by the BMP receptors ALK1/2/3/6. Of note, on certain occasions, TGFβ s, activins, and GDFs can activate both Smad1/5/8 and Smad2/3 signalling. Phosphorylated Smads form complexes with Smad4 and translocate to the nucleus to regulate gene expression. Inhibitory Smads, such as Smad6 and Smad7, act as negative feedback regulators to balance receptor activity. In addition to the canonical pathways, TGFβ ligand binding can activate non-canonical, Smad-independent signalling pathways. These pathways involve downstream effectors such as JNK, Rho-like GTPases, PI3K-AKT, p38 MAPK, and ERK. Non-canonical signalling regulates TGFβ responsive genes and mediates additional cellular responses.

note the involvement of other non-placental factors. For example, the release of TGFβ ligands from foetal or maternal tissues, that is the liver (Bidart et al., 2012) or the endometrium (Jones et al., 2006b), thereby, contributing to the complex network of TGFβ signalling in the human placenta (Table 1). In detail, this substantial contribution of TGFβ family ligands from maternal and foetal cell types beyond the placenta, influencing the maternal–placental interface, is essential for successful pregnancy initiation, and involves cellular interactions and networks, thereby intricately shaping placental function and development. Accounting for ligand bioavailability broadens the signalling window through the TGFβ receptor family, adding complexity to the understanding of the TGFβ cellular network and highlighting the critical importance of TGFβ signalling in the context of pregnancy.

Transforming growth factor beta signalling

Transforming growth factor beta (TGFβ) signalling is vital in tissue and cell homeostasis, regulating inflammatory and cellular processes such as proliferation, growth, differentiation, apoptosis, migration, or matrix formation (Moustakas et al., 2002; Tzavlaki and Moustakas, 2020). Since the discovery of the first ligand namely TGFβ1 (Assoian et al., 1983), more than thirty ligands have been described (Derynck and Budi, 2019). The characterization of TGFβ ligands has been based on structure and phylogeny (Heldin and Moustakas, 2016), sub-stratifying them into six major subfamilies: the TGFβ isoforms, BMPs, nodals, anti-Müllerian hormone (AMH), activins/inhibins, and GDFs (Derynck and Budi, 2019). This review focuses on the TGFβ subfamilies and their roles in healthy and preeclamptic placentas (Fig. 1). While AMH is vital for the ovarian reserve and for detecting polycystic ovary syndrome (Rudnicka et al., 2021), its role in placental development remains unclear. Although AMH and its receptor are present in placental tissue (Novembri et al., 2015), their direct significance and function in this context are yet to be understood, and therefore, this review will not delve into it.

All members of the TGFβ family act as homo- or heterodimers, in most cases formed through a disulfide bond (Heldin and Moustakas, 2016). Activation of the TGFβ superfamily begins

Table 1. Tissue sources of TGFβ family ligands.

TGFβ superfamily	Ligand	Compartment	Tissue/cell type	Reference
TGFβ	TGFβ1	Placenta	CT	Lash *et al.*, 2005; Jones *et al.*, 2006b; Forbes *et al.*, 2010
			EVT	Jones *et al.*, 2006b; Ma *et al.*, 2020b; Arutyunyan *et al.*, 2023; Vondra *et al.*, 2023
			ST	Jones *et al.*, 2006b
			dNK	Lash *et al.*, 2006; Fraser *et al.*, 2012
			dM	Ning *et al.*, 2016; Wang *et al.*, 2022
			HBC	Schliefsteiner *et al.*, 2017, 2020; Pavlov *et al.*, 2020; Azari *et al.*, 2021; Mercnik *et al.*, 2022
		Maternal tissue	Decidua, Endometrium, Uterus	Jones *et al.*, 2006b; Zhang *et al.*, 2019; Yang *et al.*, 2021
	TGFβ2	Placenta	CT	Jones *et al.*, 2006b; Lash *et al.*, 2005
			EVT	Lyall *et al.*, 2001
			dM	Vondra *et al.*, 2023
			HBC	Pavlov *et al.*, 2020
		Maternal tissue	Decidua, Endometrium, Uterus	Simpson *et al.*, 2002
	TGFβ3	Placenta	EVT	Caniggia *et al.*, 1999
			HBC	Pavlov *et al.*, 2020
		Maternal tissue	Decidua, Endometrium, Uterus	Jones *et al.*, 2006b; Simpson *et al.*, 2002
BMP	BMP2	Placenta	EVT	Yi *et al.*, 2021
			HBC	Deng *et al.*, 2023
		Maternal tissue	Decidua	Yi *et al.*, 2021
	BMP4	Foetal tissue	Extraembryonic ectoderm	Roberts *et al.*, 2022
	BMP9	Maternal tissue	Liver	Bidart *et al.*, 2012
NODAL	NODAL	Maternal tissue	Uterus	Park *et al.*, 2012
ACTIVIN/INHIBIN	ACTIVIN A	Placenta	CT	Debieve *et al.*, 2000; Bearfield *et al.*, 2005; Mylonas *et al.*, 2006; Barber *et al.*, 2023
		Maternal tissue	Decidua	Keelan *et al.*, 1998
		Foetal tissue	Foetal membranes	Petraglia *et al.*, 1993; Keelan *et al.*, 1998; Barber *et al.*, 2023
	ACTIVIN B	Placenta	CT	Jones *et al.*, 2006a,b; Adu-Gyamfi *et al.*, 2020
	INHIBIN A	Placenta	CT	Debieve *et al.*, 2000; Bearfield *et al.*, 2005
		Maternal tissue	Decidua	Riley *et al.*, 2000
		Foetal tissue	Foetal membranes	Petraglia *et al.*, 1993; Riley *et al.*, 2000
	INHIBIN B	Placenta	CT	Mylonas *et al.*, 2006
			ST	Mylonas *et al.*, 2006
		Maternal tissue	Decidua	Riley *et al.*, 2000
		Foetal tissue	Foetal membranes	Riley *et al.*, 2000

(continued)

Table 1. Continued

TGFβ superfamily	Ligand	Compartment	Tissue/cell type	Reference
GDF	GDF8	Placenta	CT	Peiris & Mitchell, 2012
			ST	Peiris & Mitchell, 2012
			EVT	Peiris *et al.*, 2014; Xie *et al.*, 2020
		Maternal tissue	Uterus, Endometrium	Peiris & Mitchell, 2012
	GDF11	Placenta	CT	Sugulle *et al.*, 2009
			ST	Sugulle *et al.*, 2009
	GDF15	Placenta	CT	Sugulle *et al.*, 2009
			ST	Marjono *et al.*, 2003; Jones *et al.*, 2006b; Sugulle *et al.*, 2009; Zeng *et al.*, 2023
			EVT	Segerer *et al.*, 2012; Zeng *et al.*, 2023
		Maternal tissues	Decidua	Marjono *et al.*, 2003; Segerer *et al.*, 2012

CT, cytotrophoblast; EVT, extravillous trophoblasts; ST, syncytiotrophoblast; dNK, decidual natural killer cells; dM, decidual macrophages; HBC, Hofbauer cells.

with ligand dimer binding to transmembrane serine–threonine kinase receptors, which are divided into type I and type II classes. There are seven type I receptors, known as activin receptor-like kinases (ALK1–ALK7) and five type II receptors: activin type II receptor A and B (ActRIIA, ActRIIB), BMP receptor II (BMPRII), transforming growth factor β receptor II (TGFβRII), and anti-Müllerian hormone receptor II (AMHRII) (Sierra-Filardi *et al.*, 2011; Heldin and Moustakas, 2016; Derynck and Budi, 2019; Tzavlaki and Moustakas, 2020). Co-receptors such as endoglin (Eng), BMP and activin membrane-bound inhibitor (BAMBI), betaglycan (TGFBRIII), cripto, CD109, repulsive guidance molecule b (RGMb), and neuropilin-1 further affect the signalling (Vogt *et al.*, 2011; Tzavlaki and Moustakas, 2020).

Canonical Smad pathway

TGFβ family members exhibit the conventional activation of canonical Smad (derived from the fusion of the Caenorhabditis elegans Sma genes and the Drosophila Mad—Mothers against decapentaplegic—genes) mediated pathways (Moustakas and Heldin, 2009; Derynck and Budi, 2019; Tzavlaki and Moustakas, 2020). The Smad family consist of eight Smad proteins, which are divided into three distinct subgroups: receptor-activated Smads (R-Smads: Smad1, Smad2, Smad3, Smad5, and Smad8), the common Smad (C-Smad4), and inhibitory Smads (I-Smads: Smad6 and Smad7) (Heldin *et al.*, 1997; Wrighton *et al.*, 2009).

To initiate the signalling cascade, a dimeric TGFβ ligand interacts with the specific type I and type II receptors to form a heterotetrameric complex. Upon a complex formation, type II receptors activate the type I receptor by trans-phosphorylation (Heldin and Moustakas, 2016). This phosphorylation occurs in the juxtamembrane domain of the type I receptor and activates its kinase function (Moustakas and Heldin, 2009; Heldin and Moustakas, 2016; Yan *et al.*, 2018). Followed by a successful receptor activation, the R-Smads are recruited and activated by the type I receptor to form cytosolic heteromeric trimers together with Smad4 (Tzavlaki and Moustakas, 2020). Smad4 functions as a cofactor that enhances ligand-induced transcription by stabilizing the interaction of R-Smads with DNA (Hill, 2016; Kamato *et al.*, 2020). Although Smad4 is not essential for nuclear transport, it is often translocated together with R-Smads. In the nucleus, Smads form transcriptional complexes with specific cofactors and regulatory proteins, thereby influencing the expression of target genes (Miyazono *et al.*, 2005). TGFβ ligands can activate both the Smad2/3 and Smad1/5/8 pathways in a context-dependent manner. In particular, Smad1, Smad5, and Smad8 are primarily R-Smads activated by BMP type I receptors, whereas Smad2 and Smad3 are known to be activated by activin, GDF, and TGFβ type I receptors (Rochette *et al.*, 2020). However, it is important to emphasize that activation of the Smad2/3 or the Smad1/5/8 pathway is context dependent and varies according to ligand binding, receptor activation, and conditions (Heldin and Moustakas, 2016; Rochette *et al.*, 2020; Tzavlaki and Moustakas, 2020).

Post-translational modifications of Smads fine-tune the responses to TGFβ ligands. Phosphorylation, ubiquitination, sumoylation, and other modifications occur to control Smad stability and function. In the nucleus, R-Smads are constantly dephosphorylated, leading to dissociation of Smad complexes and the export of inactive Smads to the cytoplasm (Derynck and Zhang, 2003; Vogt *et al.*, 2011). Moreover, Smad signalling is regulated by positive and negative regulators, including I-Smads, the co-repressors Ski and SnoN and the Smurf family of E3 ubiquitin ligases (Luo, 2017). The I-Smads are strongly induced by TGFβ receptor activation, and their induction provides an auto-inhibitory feedback mechanism for ligand-induced signalling (Yan *et al.*, 2016). Smad6 and Smad7 compete with R-Smads for receptor interaction. They can also induce receptor degradation or interfere with Smad DNA binding (Yan *et al.*, 2009, 2016). In addition, Smads are involved in crosstalk with other signalling pathways, such as the Wingless-related integration site (Wnt), Hippo, Notch, Hedgehog, and Nuclear Factor κB (NF-κB) pathways, often in a cell type-specific manner, which plays an important role in the regulation of various biological responses (Luo, 2017).

Non-canonical pathway

In addition to canonical Smad signalling, TGFβ-related ligands can also regulate other signalling pathways, that may influence both Smad-mediated and Smad-independent responses (Vogt *et al.*, 2011). Smad-independent signalling or non-canonical TGFβ signalling involves the activation of several downstream pathways, including: c-JUN N-terminal kinase (JNK), Rho-like GTPases, phosphatidylinositol 3-kinase (PI3K)—protein kinase B (Akt), p38 mitogen-activated kinase (MAPK), and extracellular signal-regulated kinase (ERK) MAPK (Luo, 2017; van Caam *et al.*, 2017). The MAPK and Smad pathways often directly or reciprocally regulate each other's activities and output. For example, ERK and GSK3b kinases can phosphorylate the R-Smads in the linker region, to either enhance or diminish Smad mediated transcriptional activation (Fuentealba *et al.*, 2007; Hough *et al.*, 2012). Moreover, activation of non-canonical signalling leads to the differential recruitment of cofactors to transcriptional complexes containing the Smads, thereby influencing the selectivity, potency, and longevity of Smads-induced gene expression (Abdollah *et al.*, 1997; Derynck and Budi, 2019). TGFβRI mediated phosphorylation of TAK1 (TGFβ activated kinase 1), which is an important mediator of p38 MAPK pathway, has been implicated in the regulation of apoptosis, cell migration, and cell differentiation (Wendt *et al.*, 2009; Derada Troletti *et al.*, 2019). TGFβ family ligands induce a rapid activation of Rho-like GTPases resulting in control of cytoskeletal dynamics and cell motility (Derynck and Zhang, 2003; Derynck and Budi, 2019). Moreover, TGFβ can indirectly activate PI3K-Akt signalling through miRNAs (Zhang, 2017). In turn, inhibition of PI3K-Akt signalling has been shown to prevent TGFβ-induced Smad3 activation and the association with Smad4 (Runyan *et al.*, 2004). PI3K-Akt signalling has been shown to antagonize TGFβ/Smad signalling to promote cell survival through an Akt kinase-dependent mechanism (Zhang, 2017).

TGFβ superfamily ligands

TGFβ subfamily

TGFβ exists in three known isoforms: TGFβ1, TGFβ2, and TGFβ3, all of which are secreted into the extracellular space in an inactive, latent form (Jenkins, 2008). The TGFβ isoforms contain highly conserved regions but differ in several amino acid sequences (Kubiczkova *et al.*, 2012; Poniatowski *et al.*, 2015). Despite sequence similarities, the isoforms bind differently to the receptors. TGFβ2 binds to the TGFβRII through different residues than TGFβ1 and TGFβ3, and presentation of TGFβ2 to the receptor requires the presence of a co-receptor, either betaglycan or Eng (Heldin and Moustakas, 2016), whereas TGFβ1 and TGFβ3 can bind directly to TGFβRII (Laverty *et al.*, 2009; Huang *et al.*, 2014). Although the isoforms signal via similar signalling pathways, the cellular effect and outcome may differ. TGFβ1 is the most abundant and ubiquitously expressed isoform, and is predominantly expressed in the cells of the immune system, where it acts as a potent immunoregulator (Yoshimura *et al.*, 2010; Kubiczkova *et al.*, 2012). Activation of latent TGFβ is an important checkpoint of TGFβ bioavailability (Jenkins, 2008). To prevent access to TGFβ receptors, ligands are synthesized as precursors (18) consisting of a pro-domain, called latency associated polypeptide (LAP) and the active TGFβ. The precursors can be cleaved into mature dimeric proteins, often linked by disulfide bonds during processing through the secretory pathway. Proteases play a role in the process of indirect or direct activation of extracellular TGFβ. Some matrix metalloproteinases (MMPs), such as MMP2 and MMP9 can directly cleave and activate latent TGFβ, whereas membrane-type matrix metalloproteinase (MT-MMP) interacts with integrin-mediated TGFβ activation pathways (Jenkins, 2008). Active TGFβ is involved in the synthesis of many of its own activators, such as thrombospodin-1 (TSP-1), furins, and metalloproteinases (De Caestecker *et al.*, 1998), creating autocrine feedback loops in TGFβ bioavailability and signalling.

Upon activation of the ligands, they bind to their respective receptors. TGFBRII is primarily involved in the basic signal transduction of the TGFβ subfamily as a type II receptor (Moustakas and Heldin, 2009; Heldin and Moustakas, 2016). In most cell types, TGFβ signals via ALK5 or TGFβRI as a type I receptor, whereas in ECs signal transduction can occur via both ALK5 and ALK1 (Goumans *et al.*, 2002, 2003). Signalling via ALK5 activates Smad2/3 signalling (Tzavlaki and Moustakas, 2020), which is considered the most canonical TGFβ signalling, while stimulation of ALK1 leads to activation of Smad1/5/8 (Goumans *et al.*, 2003). The coreceptor Eng is required for TGFβ signalling via ALK1. Other coreceptors, like betaglycan or TGFBRIII, may also regulate signalling by either facilitating or inhibiting ligand–receptor interaction, adding another layer of regulation and specificity to the signalling cascade (Heldin *et al.*, 1997; Moustakas and Heldin, 2009; Heldin and Moustakas, 2016; Tzavlaki and Moustakas, 2020).

BMP subfamily

Since their discovery as ectopic bone inducers, BMPs have been shown to affect a wide variety of cell types and processes beyond bone physiology (Wang *et al.*, 2014). They are important morphogens in embryogenesis and development and have also been shown to regulate adult tissue homeostasis (Wang *et al.*, 2014). Many processes in early development depend on BMP signalling gradients for cell growth, apoptosis, and differentiation (Zou and Niswander, 1996; Kobayashi *et al.*, 2005; Jones *et al.*, 2006b; Stewart *et al.*, 2010; Wang *et al.*, 2014). Based on the sequence similarity and their function, BMPs are divided into several subgroups: (i) BMP2/4, (ii) BMP5/6/7/8a/8b, (iii) BMP9/10, and (iv) BMP12/13/14 (Bragdon *et al.*, 2011). Normally, BMPs are synthesized as pro-peptides, and after secretion and cleavage, BMPs bind to either the extracellular matrix, soluble antagonists, co-receptors, or transmembrane serine/threonine kinase receptors (Sieber *et al.*, 2009). Similar to TGFβ, BMPs bind to heteromeric receptor complexes composed of type I and type II (BMPRII, ActRII, ActRIIB) transmembrane serine/threonine kinase receptors and activate the corresponding signalling pathway (Von Bubnoff and Cho, 2001; Miyazono *et al.*, 2005; Xiao *et al.*, 2007; Sieber *et al.*, 2009). In general, BMPs are thought to bind to the type I receptors ALK1/2/3/6 and activate the Smad1/5/8 pathway (Tzavlaki and Moustakas, 2020).

Nodal subfamily

Nodal is a multifunctional cytokine that plays a central role in the embryonic stages of mammals and is important in regulating placental development. It does this by suppressing trophoblast proliferation and inhibiting both extravillous trophoblast (EVT) formation and invasion of the decidua (Sarkar *et al.*, 2015). Nodal binds to the type I receptors ALK4/ALK7 and the type II receptors ActRIIA and ActRIIB to induce Smad2/3 activation (Reissmann *et al.*, 2001; Nadeem *et al.*, 2011). The signalling process is modulated by cripto (TDGF1), which acts as a co-receptor for nodal. The regulation of nodal activity by cripto is complex, as cripto can act both as a membrane-bound and a soluble co-receptor, with opposing effects on nodal-induced signal transduction (Reissmann *et al.*, 2001; Yeo and Whitman, 2001; Pauklin and Vallier, 2015).

Activin/inhibin subfamily

Activins and inhibins, which belong to the TGFβ subfamily, are versatile proteins with a wide range of physiological functions, including gonadal function, hormonal homeostasis, development, reproduction, and tissue homeostasis (Jones *et al.*, 2006b; Tsuchida *et al.*, 2009; Namwanje and Brown, 2016). Both activins and inhibins are composed of β-subunits, with activins forming dimers of two β-subunits and inhibins forming heterodimers of α- and β-subunits (Pryor-Koishi *et al.*, 2007). Heteromeric complexes of type II receptors (ActRIIA and ActRIIB) and type I receptors (mainly ALK4, but also ALK2 and ALK7) are involved in activin signal transduction (Schneider-Kolsky *et al.*, 2002). Upon binding of activin to the respective type II receptor, the type I receptors are activated and initiate the phosphorylation of intracellular Smad2/3. These newly formed complexes, together with Smad4, are translocated to the nucleus and modulate the expression of specific target genes (Moustakas and Heldin, 2009). Inhibins counteract activin signalling by binding one of the type II receptors to the type III receptor, betaglycan. This interaction binds the II receptors in an inactive complex, inhibiting further signal transduction (Namwanje and Brown, 2016).

GDF subfamily

Within the GDF subfamily of TGFβ signalling, a limited number of factors, in particular GDF8 and GDF11 play critical roles in early placental development and function. GDF8 and GDF11, due to their structural similarities (Wu *et al.*, 2022), primarily use the ActRIIA and ActRIIB type II receptors, which are coupled to the ALK4 and ALK5 type I receptors and activate the Smad2/3 signalling pathways. In addition, their signalling is modulated by extracellular antagonists, in particular follistatin-related protein-3 (FSTL3) (Walker *et al.*, 2017). On the other hand, GDF15, also known as macrophage inhibitory cytokine 1 (MIC-1), is a distant member of the family and its mechanism of action seems not to involve TGFβ receptors activation (Mullican *et al.*, 2017; Olsen *et al.*, 2017). The main effects of GDF15 have been related to metabolic regulation through the regulation of PI3K/Akt/ERK kinases (Rochette *et al.*, 2020).

TGFβ signalling in PE

PE is an inflammatory syndrome, and its incidence is on the rise globally. According to the World Health Organization (WHO), the incidence of PE ranges between 2% and 10% of pregnancies worldwide (Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222, 2020). It is a major cause of preterm birth and intrauterine growth restriction (Phipps *et al.*, 2019; Raguema *et al.*, 2020). PE is a complex and heterogeneous condition and the underlying pathophysiology is not yet fully understood (Huppertz, 2008b; Than *et al.*, 2018). The syndrome is classified according to the time of diagnosis, before or after 34-week gestation, and subdivided into early-onset (EO) and late-onset (LO) PE (von Dadelszen *et al.*, 2009; Tranquilli *et al.*, 2013; Wójtowicz *et al.*, 2019). While EOPE and LOPE share a number of common, mainly clinical, characteristics, they have different maternal and foetal outcomes, suggesting different underlying etiologies (Tranquilli *et al.*, 2013; Rana *et al.*, 2019; Parada-Niño *et al.*, 2022a). EOPE is often considered as a more severe form, associated with inadequate early placentation, placental dysfunction, reduced placental volume, intrauterine growth restriction and immune maladaptation, and carries a higher risk of future disease for the mother and offspring (Valensise *et al.*, 2008; Li *et al.*, 2014b, 2022). LOPE, on the other hand, is primarily considered a maternal disorder with milder complications, associated with maternal endothelial dysfunction and is generally associated with a more favourable foetal outcome (Valensise *et al.*, 2008). Despite these differences, the placental-derived molecular mechanisms of EO and LOPE remain unknown, and consequently there is a lack of sufficient molecular criteria to distinguish between the PE subtypes (Valensise *et al.*, 2008; Wu *et al.*, 2019; Stepan *et al.*, 2020). One of the key features associated with PE is impaired trophoblast invasion and endothelial dysfunction, both of which contribute to abnormal placental development (Huppertz, 2008b; Than *et al.*, 2018). Studies have shown that villous trophoblasts of PE placentas develop an immature phenotype, both ultrastructurally and biochemically, compared to normal placentas. In particular, EVT cells in the decidua of PE women have a less invasive and more proliferative phenotype than those from uncomplicated pregnancies (Redline and Patterson, 1995).

Placentation, the process of formation and development of the placenta, is crucial for a successful pregnancy. Early in a healthy pregnancy, EVTs invade deep into the endometrial lining of the uterus and remodel the uterine spiral arteries into highly conductive vessels (Pijnenborg *et al.*, 1991; Vanwijk *et al.*, 2000; Brosens *et al.*, 2011). This remodelling ensures an optimal and high flow of oxygenated blood to the uteroplacental bed, which is critical for placental and foetal development (Pijnenborg *et al.*, 1991; Meekins *et al.*, 1994; Brosens *et al.*, 2011; Lyall *et al.*, 2013). In normal placental development, the spiral arteries undergo significant changes during remodelling, including an increase in terminal luminal diameter and loss of elastic and muscular components. These changes extend into the inner third of the myometrium and result in a loss of vascular smooth muscle condensation near the myometrial decidual junction (Vanwijk *et al.*, 2000; Brosens *et al.*, 2011). However, this process is incomplete in PE. Abnormal placentation in PE is characterized by impaired trophoblast invasion, particularly EVT dysfunction, resulting in shallow invasion and a failure to remodel the spiral arteries (Pijnenborg *et al.*, 1991; Lyall *et al.*, 2013; Lin *et al.*, 2020). Terminal dilatation of the spiral arteries is less extensive and smooth muscle removal is inadequate and does not extend beyond the decidua (Brosens *et al.*, 2011). This failed remodelling of the spiral arteries contributes to placental hypoxia, oxidative stress, and endoplasmic reticulum stress, disrupting normal blood flow patterns and reducing nutrient and oxygen delivery to the placenta (Burton *et al.*, 2009b). The resulting ischaemia and hypoxia of the placenta are important factors in the development of PE. The impaired blood flow and inadequate nutrient supply trigger a cascade of events leading to the induction of the possible pathophysiological features that may later develop into the clinical symptoms of PE (Burton *et al.*, 2009a; Brosens *et al.*, 2011). Inadequate placentation, shallow trophoblast invasion, and impaired immune (Mercnik *et al.*, 2022) and endothelial function (Zhou *et al.*, 2019) in PE emphasize its complexity and the need to understand the underlying mechanisms driving its development.

TGFβ signalling has been implicated in the pathophysiology of PE, but its exact role is still not understood. Several TGFβ ligands and cofactors have been suggested as potential biomarkers in the development of PE. Endoglin (Eng) has received much attention, as elevated levels of soluble Eng (sEng) are found in both the serum and placenta of PE patients. These elevated levels correlate with the severity of the syndrome, and can be detected before the onset of clinical manifestations (Venkatesha *et al.*, 2006; Perucci *et al.*, 2014; Leavey *et al.*, 2016). Different mechanisms have been proposed to the increase in soluble Eng expression in PE. For example, the PE placenta is associated with a deficiency of antioxidants such as heme-oxygenase, superoxide dismutase, and catalase. This deficiency leads to in increased oxidative stress, which in turn increases the production of Eng and secretion of its soluble form in the PE placenta (Gregory *et al.*, 2014). Additionally, oxysterols, agonists of liver X receptors have been shown to induce Eng expression in the placenta affected by PE (Henry-Berger *et al.*, 2008; Margioula-Siarkou *et al.*, 2021). While Eng is best known for its interaction with TGFβ1 and TGFβ3 isoforms, it has the ability to interact with other ligands of the TGFβ superfamily, such as BMPs (Barbara *et al.*, 1999).

The literature on the cellular origin, expression, and function of TGFβ family ligands in PE remains controversial and incomplete (Table 2). Contradictory published data on TGFβ isoform levels in combination with a not always well-defined classification of the PE samples used do not allow a selective role of TGFβ in this pathophysiology. Interestingly in placental tissue, Xu *et al.* (2016) observed a significant upregulation of the Smad2/3 pathway induced by TGFβ in the placenta of EOPE, suggesting a potential role of TGFβ ligands in the pathology of this condition. TGFβ1 is elevated in the plasma and serum of PE patients (Djurovic *et al.*, 1997; Benian *et al.*, 2002; Hennessy *et al.*, 2002; Shaarawy *et al.*, 2016), and has been associated with an increase in diastolic blood pressure (Benian *et al.*, 2002). Consistent with this, higher levels of active TGFβ1 have been observed in both, EO- and LO-PE subtypes of PE, when compared to normotensive controls (Djurovic *et al.*, 1997). However, Hennessy *et al.* (2002) found lower serum TGFβ1 levels in women with PE, possibly due to the differences in sampling methods. Similar to TGFβ1, TGFβ2 is elevated in the serum of patients with PE (Shaarawy *et al.*, 2016). The expression of GDF8 and its antagonist FSTL3 was significantly increased in the maternal serum of PE subjects compared to controls (Pryor-Koishi *et al.*, 2007). The upregulation of FSTL3 and GDF8 was also confirmed in the PE placenta, suggesting that the tissue itself may be a source of circulating GDF8 and FSTL3 in PE (Guo *et al.*, 2012). Of note, inhibin A and activin A, but not inhibin B, are elevated in women with PE (Yair *et al.*, 2001; Mylonas *et al.*, 2006). As activin A levels are also elevated in PE placentas, it is reasonable to speculate that secreted placental activin A contributes, at least in part, to these differences observed in the maternal circulation (Manuelpillai *et al.*, 2001). Using an optimized method for co-expression network analysis, Tejera *et al.*, identified a number of TGFβ superfamily genes that are upregulated in PE placenta. In addition to *ENG*, an increased expression of *INHBA* (encoding inhibin A) and *ACVR1* (encoding the BMP receptor ALK2) was quantified (Tejera *et al.*, 2013). *ACVR1*, as well as *ACVR2A* (encoding ActRIIA), was already found to be upregulated in PE placenta and maternal circulation (Inkeri Lokki *et al.*, 2017; Li *et al.*, 2022). In particular, *ACVR1* mRNA levels in decidua correlate with the severity of PE (Yong *et al.*, 2014). Furthermore, in a study conducted in a Brazilian cohort, *ACVR2A* was associated with the development of EOPE (Ferreira *et al.*, 2015). Further analysis using single-nucleotide polymorphisms identified a common maternal PE susceptibility locus on chromosome 2q22-23, that affects ACVR2A expression and contributes to the development of PE (Roten *et al.*, 2008). Despite recent developments, it is still crucial to further elucidate the role of TGFβ signalling and dissect the interactions within the different branches of the TGFβ pathway to gain a deeper understanding of their contribution to the pathogenesis of PE, and to unravel their potential as targets or biomarkers for this critical condition. Given the proposed different subtypes of PE, it is highly recommended to study the TGFβ signalling pathways in early- and late-onset PE cohorts separately in future (Valensise *et al.*, 2008; Wu *et al.*, 2019). This focused approach may deepen our understanding of the disease, elucidate specific mechanisms, and reveal potential therapeutic targets specific to each subgroup.

The TGFβ superfamily and placental cells: from early weeks until pregnancy term

Trophoblast cells

Considering the complex role of the placenta in foetal–maternal communication, it may not be surprising that its diverse functions at different stages of normal and abnormal pregnancies involve the coordinated actions of several cell types. Within the first weeks of pregnancy, the human placenta generates epithelial trophoblasts with diverse biological roles including in the attachment of the conceptus to the uterine wall, the establishment of early histotrophic response, and the adaption of the maternal uterine vasculature. Different types of trophoblasts, including stem cells, progenitors, and differentiated subtypes with multiple functions develop (Knöfler *et al.*, 2019). Cytotrophoblasts (CTs), progenitor cells located in the first trimester placental villi, fuse to form the multinucleated syncytium, which plays a key role in nutrient transport and hormone production (Gauster *et al.*, 2022). Thereafter CTs undergo a differentiation process leading to the formation of EVTs through a process similar to that which is well documented in cancer biology, known as the epithelial-to-mesenchymal transition (EMT) (Gonzalez and Medici, 2014; Davies *et al.*, 2016). During the EMT-like differentiation, EVTs partially lose their well-organized epithelial phenotype and transition to a migratory and invasive mesenchymal phenotype. Key features of this differentiation include changes in cell polarity and adhesion (Kokkinos *et al.*, 2010). Essentially, trophoblasts acquire mesenchymal characteristics by exhibiting migratory and invasive capabilities, upregulation of EMT transcription factors such as Snail, increased Wnt signalling, and downregulation of epithelial polarity genes such as Occludin and ZO1 (Knöfler and Pollheimer, 2013). It is important to emphasize that trophoblasts simultaneously maintain certain epithelial features, characterized by the expression of cytokeratin 7 and the absence of mesenchymal vimentin induction (Davies *et al.*, 2016; Haider *et al.*, 2017). These changes are critical for successful embryo implantation in the early weeks of pregnancy (Davies *et al.*, 2016; Haider *et al.*, 2017). These processes involve the migration of EVT cells into the maternal decidua and the remodelling of the maternal spiral arteries to ensure an adequate blood supply to the developing placenta (Haider *et al.*, 2022). Dysregulation of trophoblast proliferation and EVT invasion may contribute to conditions like PE, placenta accreta, and recurrent miscarriage (Incebiyik *et al.*, 2014). Among the signalling molecules involved in regulating trophoblast function, members of the TGFβ superfamily play significant and distinct roles during the first trimester of pregnancy (Fig. 2). Research on the TGFβ superfamily and trophoblast function at pregnancy term is relatively limited, and further investigation is necessary to gain a comprehensive understanding of the pregnancy progression and its potential implications for maternal and foetal health.

TGFβ signalling in trophoblasts

The balance between factors that promote or inhibit cell invasion is essential for a normal placentation. The TGFβ ligands TGFβ1, TGB2, and TGFβ3, exert several biological effects in trophoblasts and in the regulation of placental invasion (Jones *et al.*, 2006b). As mentioned above, TGFβs bind to the type I receptor ALK5 and activate the Smad2/3 signalling cascade (Budi *et al.*, 2017). The expression of some TGFβ signalling components, Smad2/3/4, TGFβRI, TGFβRII, TGFβ1, and TGFβ2, is highest in trophoblasts during the first trimester and decreases during pregnancy (Xuan *et al.*, 2007). All three forms of TGFβ contribute, at least in part, through different mechanisms to the inhibition of trophoblast invasion and migration (Irving and Lala, 1995; Caniggia *et al.*, 1999; Karmakar and Das, 2002; Tse *et al.*, 2002; Jones *et al.*, 2006b). Consistently, TGFβ3 expression decreases around the ninth week of gestation, correlating with a switch from trophoblast invasion and migration to a proliferative state, characterized by increased

Table 2. Systemic and tissue levels of TGFβ superfamily ligands in PE.

TGFβ superfamily ligand	Subtype of PE	Type of control	Sample	Changes of ligand levels in PE	Reference
TGFβ1	Mild/Severe EO/LOPE	Normotensive pregnancy (Unmatched for GA)	Plasma	Elevated in all PE subtypes	Perucci *et al.*, 2014
	PE	Normotensive pregnancy (Matched for GA)	Plasma	Elevated	Benian *et al.*, 2002; Peraçoli *et al.*, 2008
	Mild/Severe/ PE+ FGR	Normotensive pregnancy (Unmatched for GA)	Plasma	Active TGFβ elevated in all PE subtypes	Djurovic *et al.*, 1997
	PE	Normotensive pregnancy (Matched for GA)	Serum	No difference in total or active TGFβ1	Huber *et al.*, 2002
	PE	Normotensive pregnancy (Unmatched for GA)	Serum	No difference in total or active TGFβ1	Hennessy *et al.*, 2002
	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Serum	Decreased levels in mild PE, elevated in severe PE	Xu *et al.*, 2017
	PE	Normotensive pregnancy (Matched for GA)	Placenta	Elevated	Benian *et al.*, 2002
	PE	Normotensive pregnancy (Unmatched for GA)	Placenta	Elevated	Zhang *et al.*, 2019
	PE	Preterm normotensive pregnancy	Placenta	Elevated	Yi *et al.*, 2021
	PE	Normotensive pregnancy (Unmatched for GA)	Placenta	No difference	Hennessy *et al.*, 2002
TGFβ2	Mild/Severe PE/Eclampsia	Normotensive pregnancy (Matched for GA)	Serum	Elevated in Severe PE and Eclampsia	Shaarawy *et al.*, 2016
TGFβ3	PE	Normotensive pregnancy (Matched for GA)	Placenta	Elevated	Caniggia *et al.*, 1999
	EOPE	Preterm normotensive pregnancy	Placenta	Reduced	Yi *et al.*, 2021
BMP2	EOPE	Preterm normotensive pregnancy	Placenta	Reduced	Yi *et al.*, 2021
BMP4	PE	Normotensive pregnancy (Unmatched for GA)	Serum	No difference	Gallardo-Vara *et al.*, 2020
	EOPE	Preterm normotensive pregnancy	Placenta	Reduced	Yi *et al.*, 2021
BMP5	EOPE	Preterm normotensive pregnancy	Placenta	Reduced	Yi *et al.*, 2021
Nodal	PE	Preterm normotensive pregnancy (Matched for GA)	Placenta	Elevated	Nadeem *et al.*, 2011
Activin A	Mild/Severe PE/ Gestational hypertension	Normotensive pregnancy (Matched for GA)	Serum	Elevated in severe PE > mild PE and gestational hypertension	Xu *et al.*, 2017
	PE	Normotensive pregnancy (Matched for GA)	Serum	Elevated	Yair *et al.*, 2001
	PE	Normotensive pregnancy (Unmatched for GA)	Serum	Elevated	Manuelpillai *et al.*, 2001
	PE	Normotensive pregnancy (Unmatched for GA)	Placenta	Elevated	Manuelpillai *et al.*, 2001
Inhibin A	Mild/Severe PE/ Gestational hypertension	Normotensive pregnancy (Matched for GA)	Serum	Elevated in severe PE > mild PE and gestational hypertension	Xu *et al.*, 2017
	PE	Normotensive pregnancy (Matched for GA)	Serum	Elevated	Yair *et al.*, 2001
	EOPE	Preterm normotensive pregnancy	Placenta	Elevated	Yi *et al.*, 2021
Inhibin B	PE	Normotensive pregnancy (Matched for GA)	Serum	No difference	Yair *et al.*, 2001
	EOPE	Preterm normotensive pregnancy	Placenta	Elevated	Yi *et al.*, 2021

(continued)

Table 2. Continued

TGFβ superfamily ligand	Subtype of PE	Type of control	Sample	Changes of ligand levels in PE	Reference
Myostatin (GDF8)	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Serum	Elevated	Guo *et al.*, 2012
	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Placenta	Elevated	Guo *et al.*, 2012
Endoglin	Mild/Severe PE	Normotensive pregnancy (Unmatched for GA)	Plasma	Increasing with the severity of PE	Perucci *et al.*, 2014
	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Serum	Increasing with the severity of PE	Venkatesha *et al.*, 2006; Xu *et al.*, 2017; Zhang *et al.*, 2020
	Mild/Severe PE	Normotensive pregnancy (Unmatched for GA)	Serum	Increasing with the severity of PE	Leaños-Miranda *et al.*, 2019; Gallardo-Vara *et al.*, 2020
	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Placenta	Increasing with the severity of PE	Venkatesha *et al.*, 2006
Follistatin related gene protein	PE	Normotensive pregnancy (Matched for GA)	Serum	Elevated	Pryor-Koishi *et al.*, 2007
	PE	Normotensive pregnancy (Matched for GA)	Placenta	Elevated	Pryor-Koishi *et al.*, 2007
FSTL3	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Serum	Elevated	Guo *et al.*, 2012
	Mild/Severe PE	Normotensive pregnancy (Matched for GA)	Placenta	Elevated	Guo *et al.*, 2012

EOPE, early onset PE; LOPE, late onset PE; FGR, fetal growth restriction; GA, gestational age.

fibronectin synthesis (Caniggia *et al.*, 1999). For successful invasion, trophoblasts secrete proteases such as matrix metalloproteases (MMPs), urokinase-type plasminogen activators (uPA), and cysteine proteases such as cathepsins (Lash *et al.*, 2005; Li *et al.*, 2021). The activity of trophoblast proteases is tightly regulated by natural inhibitors (Librach *et al.*, 1994; Karmakar and Das, 2002), such as plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitors of metalloproteinases (TIMPs). TGFβ1, TGFβ2, and TGFβ3 induce the activation of PAI-1 and TIMPs (Lash *et al.*, 2005), thereby increasing cell adhesion to the extracellular matrix (ECM) (Irving and Lala, 1995). Local inflammatory mediators, such as IL1β and TGFβ1, are balanced to further regulate the activity of these enzymes. In this sense, IL-1β promotes the expression of uPA and MMP9, thereby promoting invasion, whereas TGFβ1 upregulates TIMP-1, TIMP-2, and PAI-1, which in contrast inhibit the action of uPA and MMP9 (Laiho *et al.*, 1987; Graham, 1997). Additionally, IL1β induces the expression of MT-MMP1 or MMP14, a collagenase involved in ECM degradation, but exposure to TGFβ1 abolishes the expression of MT-MMP in JEG-3 cells (Librach *et al.*, 1994; Karmakar and Das, 2002).

Another important milestone towards a successful pregnancy is the proper invasion of the EVT. Using RNAseq analysis, Haider *et al.* (2022) identified that TGFβ/Smad2/3 signalling governs the differentiation of placental EVTs, as demonstrated in primary EVTs and trophoblast organoids. The same group characterized different Smad2/3 phosphorylated residues in trophoblast subtypes. Thus, while C-terminal Smad2/3 phosphorylation was proposed to regulate EVT function and differentiation, Smads2 linker phosphorylation was mainly associated with proliferative primary CTs (Haider *et al.*, 2017). In immortalized HTR8/SVneo cells, cell invasion required the expression of the cell membrane junction protein VE-cadherin. Notably, incubation with TGF-β1 induced the activation of the Smad2/3 signalling and the expression of the Snail and Slug transcription factors, and genetic inhibition of Snail was sufficient to prevent invasion of primary trophoblasts and the HTR8/SVneo cell line (Cheng *et al.*, 2013). Conversely, a recent study showed how TGFβ1 can activate the non-canonical ALK5-ERK1/2 pathway and inhibit invasion in primary EVTs and HTR8/SVneo cells, by inducing downstream kisspeptins (Fang *et al.*, 2022). Interestingly, elevated levels of kisspeptin-1 have been observed in PE placenta, suggesting their potential contribution to disrupted PE pathology (Kapustin *et al.*, 2020). In addition, miR142-3p, which is highly upregulated in PE placenta, has been shown to induce trophoblast invasion by activating the TGFβ1/Smad3 signalling pathway in the HTR8/SVneo cell line (Liu *et al.*, 2019). As mentioned above, in some cell types the TGFβ co-receptor Eng is able to balance Smad signalling between ALK1-Smad1/5/8 and ALK5-Smad2/3. In response to TGFβ1 and/or TGFβ3, Eng was shown to be essential to prevent EVT invasion, and Eng inhibition triggered EVT outgrowth, migration, and fibronectin production in villous explants (Caniggia *et al.*, 1997b). Indeed, TGFβ3 triggers the premature invasive phenotype of the trophoblast, and its downregulation rescues the abnormal invasive phenotype observed in PE placentas (Caniggia *et al.*, 1999; Cowden Dahl *et al.*, 2005). Furthermore, a critical link between TGFβ3 and hypoxia is emerging in normal placental development, where elevated oxygen levels at 10–12 weeks of gestation downregulate HIF-1α and TGFβ3 thereby regulating trophoblast invasiveness (Tal, 2012). However, as demonstrated in JAR, JEG-3, and villous explants in PE, unsuccessful suppression of HIF-1α increases placental TGFβ3 expression, leading to the aberrant trophoblast invasion and persistent placental hypoxia, causing a detrimental loop where chronic/continuous hypoxia further stimulates HIF-1α and TGFβ3 expression (Caniggia *et al.*, 1999; Schäffer *et al.*, 2003; Nishi *et al.*, 2004; Xu *et al.*, 2019).

ECM remodelling is a critical aspect of a successful pregnancy, intricately regulating trophoblast invasion and placental development (Huppertz, 2008b). Furthermore, the composition of ECM determines its effect on trophoblast invasion (Huppertz, 2008a). A study by Xu *et al.* (2019) investigated lysyl oxidase (LOX) and

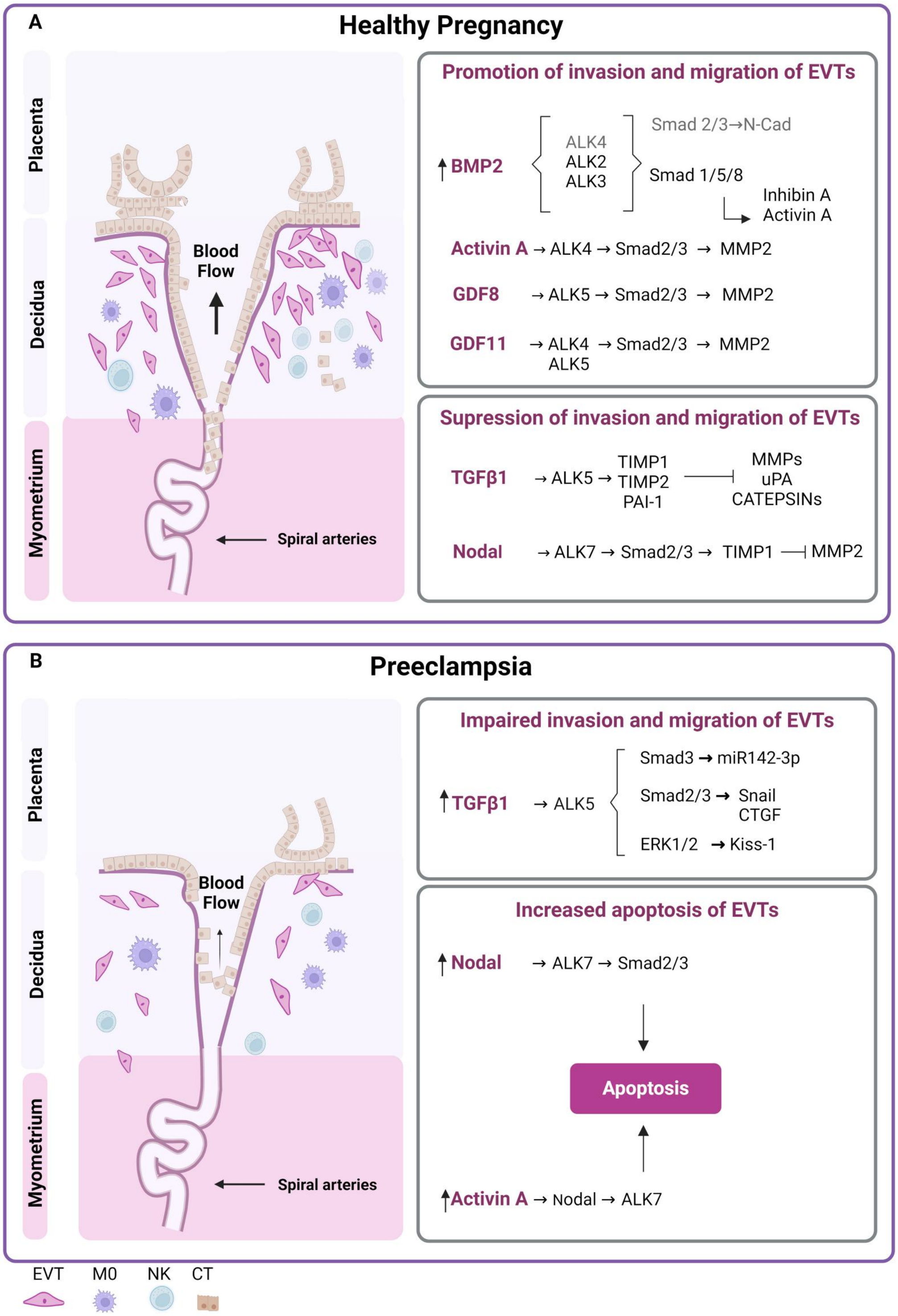

Figure 2. Summary of the TGFβ family induced signalling in EVT. During placentation, the coordinated migration and invasion of EVTs is critical for the maintenance of a successful pregnancy. During the first trimester, CTs undergo an EMT-like process and differentiate into EVTs. These EVTs migrate and invade the decidua and maternal spiral arteries in the myometrium. Highly regulated interactions between EVTs and surrounding cells, including NK cells, macrophages (M0), and stromal cells, play an important role in the immunological acceptance and depth of trophoblast invasion. In a normal pregnancy (**A**), TGFβ signalling regulates the process of trophoblast invasion. Different ligands, such as BMPs, activins, and GDFs, are involved in promoting EVT invasion and migration. For example, BMP2 can directly or indirectly activate different receptors (ALK2, ALK3, or ALK4), subsequently inducing Smad1/5/8 or Smad2/3 activation, depending on the context of signalling and receptor binding. Activin A binds to ALK4 and activates Smad2/3, leading to the activation of metalloproteinase 2 (MMP2). GDF8 and GDF11 also activate Smad2/3 and downstream MMP2. To maintain a balance in trophoblast invasion, the secretion of inhibitors or molecules that attenuate their activity regulates the activity of trophoblast proteases. TGFβ and Nodal subfamilies are involved in the induction of TIMPs, with TGFBs signalling through ALK5 and Nodals signalling through ALK7. In PE (**B**), trophoblasts fail to invade the spiral arteries adequately, resulting in reduced blood flow and impaired utero-placental perfusion. The impaired invasion appears to be the first change on cellular level that contributes to the pathogenesis of PE. TGFβ1 inhibits cell invasion through ALK5 and Smad2/3 induction, and contributes to the upregulation of miR142-3p and Snail expression in PE. Non-canonical ERK1/2 signalling may also be involved in inhibiting invasion by inducing Kiss-1 expression. Interestingly, elevated levels of Activin A in PE trigger apoptosis via the Nodal/ALK7 pathway rather than by inducing MMP expression. Elevated levels of Nodal, on the other hand, do not inhibit EVT migration but rather induce apoptosis via the ALK7 pathway. M0, macrophages.

LOX-like protein 2 (LOX2L) signalling components involved in ECM remodelling and HTR8/SVneo trophoblast invasion in PE placentas. The TGFβ1/Smad3 pathway has been shown to inhibit LOX and LOXL2-dependent induction of collagen production, thereby suppressing the migration and invasion of trophoblasts. Downregulation of LOX and LOXL2 has also been observed in PE placenta (Xu *et al.*, 2019). Additionally, connective tissue growth factor (CTGF), also known as CNN2, is an important downstream target of TGFβ-Smad2/3 signalling and plays a role in extracellular matrix deposition. Elevated levels of CTGF have been detected in the placenta and serum of patients with severe PE and foetal growth restriction, suggesting its potential involvement in regulating of trophoblast invasion in PE. Importantly, genetic knockdown of CTGF partially prevented TGFβ1-induced inhibition of invasion of primary trophoblasts and the HTR8/SVneo cell line (Oh *et al.*, 2009; Chen *et al.*, 2012; Cheng *et al.*, 2017).

In summary, studies have implicated various components of the TGFβ signalling pathway, including Smad2/3, TGFβ receptors, and downstream effectors such as VE-cadherin and kisspeptins, in trophoblast differentiation and invasion. Dysregulation of TGFβ signalling, as seen in PE, may contribute to impaired trophoblast invasion and placental dysfunction.

BMP signalling in trophoblasts

The BMP subfamily is one of the most important signalling pathways in developmental processes, as it is involved in trophoblast differentiation and invasion, cell growth, and apoptosis (Jones *et al.*, 2006b). BMP2 and BMP4 play important roles in placental development and particularly in trophoblast invasion. BMP2 is localized to all trophoblast subtypes and to the decidua during the first trimester of pregnancy, with its levels increasing during this period (Yi *et al.*, 2021). *In vitro* models such as the use of primary trophoblasts and the cell line HTR8/SVneo have demonstrated the action of BMP2 in regulating human trophoblast invasion and spiral artery remodelling (Zhao *et al.*, 2018b, 2020a,b). During normal pregnancy, there are increased serum levels of BMP2, some of which is secreted by trophoblasts (You *et al.*, 2021). Of note, a recent study identified Hofbauer cells (HBCs) as an additional source of BMP2. Crosstalk between HBCs and trophoblast leads to upregulation of BMP6 via ALK3-Smad2/3-Smad4 signalling in primary trophoblasts, promoting their invasion and vascular mimicry (Deng *et al.*, 2023). In trophoblasts, BMP2 has been shown to regulate EMT-like process, cell adhesion, and invasion (Luo, 2017). To promote trophoblast differentiation, BMP2 regulates the expression of adhesion molecules and genes involved in ECM remodelling in primary trophoblast and HTR8/SVneo (Yi *et al.*, 2021). BMP2 upregulates the expression of ECM-associated genes, including *COL6A1, COL7A1, ITGA2, ITGA6, ADAM12*, and *MMP11* (Yi *et al.*, 2021). The expression of the EMT transcription factor Snail (Luo, 2017) is induced by BMP2 through the induction of lncRNA NR026833.1, which binds to miR502-5p and upregulates Snail expression in primary trophoblasts (You *et al.*, 2021). Snail, in turn, induces *MMP2*, a key molecule involved in trophoblast invasion at 6–8 weeks of gestation (You *et al.*, 2021). In primary trophoblasts and in the HTR8/SVneo cell line, BMP2 also promotes invasion and endothelial-like tube formation through an *ID1*–IGFBP3–Slug axis (Zhao *et al.*, 2020b).

The mechanisms by which BMP2 regulates trophoblast invasion are complex. BMP2 binding activates non-canonical Smad2/3 signalling leading to the induction of N-cadherin in primary EVTs and HTR8/SVneo cells (Zhao *et al.*, 2018b). Pharmacological inhibition of ALK2/3, but not ALK4/5/7, effectively diminished the induction of Smad2/3 phosphorylation in response to BMP2 (Zhao *et al.*, 2018a,b). Interestingly, ALK3 is required for BMP2 to induce inhibin A and activin A production in primary EVTs, which may act in an autocrine manner (Zhao *et al.*, 2018a). Other factors, such as the adhesion molecule AMIGO2 and the membrane-bound BMP and activin inhibitor (BAMBI), are involved in BMP2-induced trophoblast invasion and WNT/β-catenin signalling crosstalk in primary trophoblast and HTR8/SVneo (Zhao *et al.*, 2020a; Yi *et al.*, 2021). In EOPE placenta, particularly in trophoblasts, BMP2 levels are decreased, as are the ligands BMP4 and BMP5. In contrast, the expression of the BMP type II receptor BMPR2 is increased (Yi *et al.*, 2021), perhaps as a compensatory mechanism. Consistent with the downregulation of *BMP2* mRNA levels in EOPE trophoblasts (Yi *et al.*, 2021), the expression of lncRNA NR026833.1 is downregulated in EOPE (You *et al.*, 2021). The downregulation of *BMP2* mRNA and lncRNA NR026833.1 expression in EOPE suggests potential mechanisms contributing to trophoblast dysfunction in PE.

BMP4 is used to stimulate the differentiation of various human pluripotent stem cells (hPSCs) origins, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), into different trophoblast cell models (Kojima *et al.*, 2017; Jang *et al.*, 2022; Soncin *et al.*, 2022). To introduce BMP4 to hPSCs induces the formation of self-renewing trophoblast stem cells (TSCs), which possess the capability to undergo further differentiation into EVTs and ST (Jang *et al.*, 2022; Soncin *et al.*, 2022). Notably, Kojima *et al.* (2017) have demonstrated that the concentration-dependent stimulation of BMP4 in human-induced pluripotent stem cells (hiPSCs) leads to the direct differentiation of cells into EVTs and STs without the self-renewing TSC stage. Due to the challenges of obtaining human first trimester tissue samples and the potential biological differences between species, differentiated hPSCs have been used as an alternative model to study trophoblast biology. Successful differentiation of hPSCs into trophoblast-like stem cells requires the inhibition of activin/nodal signalling by BMP4 stimulation (Xu *et al.*, 2002). Furthermore, inhibition of activin/nodal signalling via BMP4 activates FGR signalling, leading to hPSC differentiation into the β-human chorionic gonadotropin (βhCG) hormone-secreting multinucleated ST (Sudheer *et al.*, 2012). In particular, BMP4 activates amniotic and/or mesodermal markers under certain conditions. Therefore, its utilization as a differentiation agent remains controversial, and different models and protocols have been proposed (Roberts *et al.*, 2018; Knöfler *et al.*, 2019; Io *et al.*, 2021).

In summary, the BMP subfamily, particularly BMP2 and BMP4, plays a critical role in placental development, trophoblast invasion and differentiation. The understanding of BMP signalling in PE trophoblasts remains poor, and further studies are needed to clarify the role of BMPs in trophoblast function.

Nodal signalling in trophoblasts

Nodal is a multifunctional cytokine, of key importance during mammalian embryonic development, and involved in the regulation of placental development by inhibiting trophoblast proliferation, EVT formation, and EVT invasion of the decidua (Sarkar *et al.*, 2015). During early pregnancy, nodal and ALK7 are expressed in villous and EVT cells, and their levels are strongly upregulated in PE placentas (Nadeem *et al.*, 2011). In a healthy pregnancy, high levels of nodal/ALK7 inhibit trophoblast proliferation and invasion, and may contribute to syncytialization. Subsequently, reduced levels of nodal/ALK7 allow trophoblast proliferation and invasion. Mechanistically, in HTR8/SVneo and villous explants, Nodal/ALK7 activates the Smad2/3 signalling cascade and upregulates TIMP-1, which subsequently inhibits MMP2 and MMP9 (Nadeem *et al.*, 2011).

Nodal levels are regulated by molecules involved in trophoblast proliferation, such as Lefty (Li et al., 2018), miR-378a-5p (Parada-Niño et al., 2022), miR-376 (Fu et al., 2013), and miR-454 (Adu-Gyamfi et al., 2020). miR-376c inhibits both ALK5 and ALK7 and consequently impairs TGFβ/nodal signalling, leading to increased cell proliferation and invasion. Interestingly, miR376c levels are reduced in placentas and plasma of women with PE (Fu et al., 2013). In PE, where nodal/ALK7 is overexpressed, the activation of Smad2/3 signalling leads to impaired trophoblast invasion and increased apoptosis as shown in HTR8/SVneo and villous explants (Nadeem et al., 2011). The inhibition of JEG-3, JAR, and Bewo proliferation by Nodal is partly mediated by the p27-cyclin E/Cdk2 pathway, resulting in G1-cell cycle arrest (Munir et al., 2004). TGFβ1 can inhibit activin/nodal-induced EVT formation shown in TSC model (Sarkar et al., 2015), while activin A enhances nodal signalling (Yu et al., 2012). Maternal Nodal can induce foeto-placental nodal signalling and modulate the PE susceptibility gene *STOX1* through activin A secretion at the maternal interface (Thulluru et al., 2013). As both activin A and nodal have been shown to be elevated in PE, it has been suggested that their interaction promotes trophoblast apoptosis through activation of the nodal/ALK7 signalling in primary trophoblasts and HTR8/SVneo cell line (Yu et al., 2012).

To sum up, nodal plays a critical role in placental development by regulating trophoblast proliferation, invasion, and apoptosis.

Activin and inhibin signalling pathways in trophoblasts

Activins and inhibins are members of the TGFβ subfamily and have a similar structure (Pryor-Koishi et al., 2007). Activin A is the predominant form of activins in pregnancy and is expressed in the syncytiotrophoblast (ST) and underlying CT layers throughout pregnancy. Activin A and inhibin A are secreted by the placenta, decidua, and foetal membranes and are found in the maternal circulation, amniotic fluid, and umbilical cord blood (Florio et al., 2004). Its type I receptors, ALK4 and ALK7, are expressed in the ST during the first and second trimesters and predominantly in the ECs of the villi during the third trimester (Schneider-Kolsky et al., 2002).

Activin A is highly involved in the placentation, acting in an autocrine or paracrine manner, being expressed in the newly formed ST and/or playing a role in the aggregation and syncytialization of CT cells. In the first trimester, activin A stimulates primary trophoblast differentiation towards an invasive phenotype (Bearfield et al., 2005). Villous explant studies have demonstrated that activin A promotes CT outgrowth and fusion, EVT formation, and EVT invasion into the decidua (Caniggia et al., 1997a). To facilitate the foeto-maternal interface, CT differentiate towards the invasive and migratory EVT phenotype in a complex and regulated EMT-like process (Kokkinos et al., 2010). Molecules of the TGFβ superfamily, including activin A, are recognized as potent inducers of the EMT-like process (Li et al., 2014c; Tzavlaki and Moustakas, 2020). Indeed, BMP2 can induce the production, of activin A, leading to the upregulation of N-cadherin, an important marker of EMT (Zhao et al., 2018a,b). Activin A mediated invasion of primary EVTs and HTR8/SVneo is linked to the ALK4-Smad2/3 signalling pathway, leading to the activation of either Snail or Slug and their downstream molecules MMP2, MMP9, and MMP26 (Li et al., 2015a; Adu-Gyamfi et al., 2020; Zhu et al., 2021). Interestingly, excessive levels of activin A can induce apoptosis via the nodal-ALK7 pathway rather than promoting MMP expression (Adu-Gyamfi et al., 2020).

Notably, as shown in primary EVTs and HTR8/SVneo during trophoblast invasion, both TGFβ1 and activin A are capable of signalling through ALK5 and ALK4 receptors, respectively, thereby inducing the Snail transcription factor. Of note, TGFβ has been shown to activate Smad-independent signalling pathways, including MAPK (ERK, p38, and JNK), PI3K, and Rho-like GTPases, whereas activin A preferentially signals via Smad dependent pathways (Matsuzaki, 2011; Li et al., 2015a; Zhu et al., 2021).

Inhibin A, produced by the CT, acts as a feedback mechanism in EVT invasion by antagonizing the production of activin A-activated metalloproteinases (MMP2, MMP7, and MMP9) (Jones et al., 2006a; Adu-Gyamfi et al., 2020). Follistatin, produced by the developing placenta, is an activin A soluble antagonist that regulates EVT outgrowth in villous explants (Caniggia et al., 1997a; Petraglia et al., 1994). Another regulator of activin A is follistatin-related protein (FLRG), which is primarily expressed by the decidua and placental ECs (Ciarmela et al., 2003). FLRG levels are increased in the ST of PE placentas and are associated with low birth weight (Pryor-Koishi et al., 2007). Although follistatin and FLRG give similar signals, their expression differs during pregnancy. While follistatin expression decreases throughout the pregnancy, FLRG expression continues to increase until delivery (Wang et al., 2003; Pryor-Koishi et al., 2007). In PE, placental levels of both activin A and inhibin A are elevated (Yi et al., 2021). Interestingly, hypoxia can enhance the activity of both Inhibin A and Activin A, but their expression is not influenced under low oxygen levels in placental cells (Manuelpillai et al., 2003), suggesting that triggers other than hypoxic conditions may modulate the expression of activin and inhibin ligands during PE.

Both TGFβ1 and activin A are involved in the regulation of βhCG production by primary trophoblasts, although their effects may vary depending on the signalling pathways that they induce and the stage of pregnancy. TGFβ1 has an inhibitory effect, whereas activin A stimulates the expression and secretion of βhCG (Song et al., 1996). The effects of activin A are tightly controlled by the antagonistic effects of inhibin A (Petraglia et al., 1989).

GDF signalling cascade

Among the GDF subfamily of the TGFβ signalling, only a few factors have been identified to play a role in the development and function of the early placenta, including GDF8, GDF11, and GDF15. GDF8, also known as myostatin, and its receptor ACVR2B are localized in EVTs in the first and third trimester placenta and contribute to the regulation of normal placentation, by inducing proliferation and migration of primary EVTs and HTR8/SVneo (Peiris et al., 2014). Interestingly, GDF8 can induce the expression of FSTL3, an antagonist of GDF8 signalling, to further enhance invasion in primary EVTs and the HTR8/SVneo cell line via the ALK5-Smad2/3 pathway (Xie et al., 2020). Additionally, the activated ALK5-Smad2/3 pathway induces the expression of *MMP2* in HTR8/SVneo cell line (Fang et al., 2021), thereby stimulating EVTs invasion, providing insight into early placental development, as MMP2 is primarily active between 6–8 weeks of gestation.

GDF11, which is structurally similar to GDF8, induces cell invasion by using the ALK4 and ALK5 type I receptors and subsequently activates Smad2/3 and the expression of *MMP2* in primary EVTs and in HTR8/SVneo cells (Wu et al., 2022). In addition, GDF11 regulates the transcriptional regulator ID2, which is required for MMP2 expression and invasion of EVTs (Wu et al., 2022).

Another member of the GDF subfamily, GDF15, also known as MIC-1, is abundantly expressed in the placenta, and has been localized to the CT and ST in particular (Sugulle et al., 2009). Using the HTR-8/Neo cell line and villous explants, fragile X-related protein 1 (FXR1) was been shown to regulate GDF15 expression

(Hong *et al.*, 2022). The maturation and processing of GDF15 are mediated by the activity of MMP26, which is co-localized on villous and EVT cells. GDF15 has been shown to promote EVT apoptosis and inhibit proliferation in the HTR8/SVneo cell line (Li *et al.*, 2014a). However, the mechanisms by which GDF15 modulates TGFβ signalling responses in the placenta remain unclear.

In summary, GDFs are mainly involved in the regulation of EVT invasion. Indeed, the exact mechanisms and the significance in healthy and pathological pregnancies are still unknown and require further investigation.

Trophoblast models to study TGFβ signalling

To comprehensively study TGFβ superfamily signalling in trophoblasts, several placental *in vitro* and *ex vivo* models are used, each with unique strengths and limitations (Table 3). These models, including primary trophoblasts, cell lines, placental explants, TSCs, organoids, placenta-on-chip, and animal models, provide valuable insights into trophoblast biology (reviewed in Horii *et al.*, 2020; Sheridan *et al.*, 2020; Haider and Beristain, 2023; Li *et al.*, 2023b; Liu *et al.*, 2023). The choice of the appropriate cell model is crucial, depending on the experimental design and specific research questions related to trophoblast development, differentiation, and function (Table 4). Despite the challenges in achieving universally reproducible models, understanding these models is essential for interpreting TGFβ-induced placental disorders. Each model has strengths and limitations that must be carefully considered when interpreting the TGFβ signalling dynamics. Given the ethical constraints of studying first trimester placental tissue, continued progress in placental research is essential. In addition, the development of new 2D and 3D models is needed to better mimic the multicellular environment as *in vivo* and improve our understanding of TGFβ signalling in trophoblasts.

Primary trophoblasts isolated from the first or third trimester placenta are life-like cells for studying TGFβ signalling in placental development, as they retain their original genetic properties (Li *et al.*, 2023b). However, their limited proliferation and lack of self-renewal make genetic manipulation difficult (Tannetta *et al.*, 2008). To overcome this, researchers often combine them with cell lines such as JEG-3, Bewo, JAR, and HTR8/SVneo. Although originated from choriocarcinoma (JEG-3, Bewo, JAR) or transfected with simian virus 40 large T antigen (HTR8/SVneo), these cell lines are often used to study trophoblast differentiation and invasion, because of their ease of maintenance, stability, and reproducibility by genetic manipulation (Li *et al.*, 2023b; Liu *et al.*, 2023). However, it is important to note that immortalized cell lines may have genetic alterations and phenotypic differences compared to primary trophoblasts (Abou-Kheir *et al.*, 2017; Pastuschek *et al.*, 2021). Their *in vitro* properties may not fully reflect *in vivo* signalling, although they are genetically manipulated and reproducible (Msheik *et al.*, 2019; Pastuschek *et al.*, 2021). On the other hand, placental explants, retain the *in vivo* structure for studying cell-to-cell interactions in healthy and pathological pregnancies, such as PE (Li *et al.*, 2023b). When exposed to flow, placental explants retain tissue integrity, further expanding their potential uses (Kupper *et al.*, 2021). However, explants have limitations in terms of difficulties in gene manipulation, lack of self-renewal, and shorter incubation times in static culture (Li *et al.*, 2023b). In addition, explant cultures maintain cells in their tissue context. This offers the advantage to mimic more closely the *in vivo* situation, but may require additional work to attribute observed differences and changes to a specific cell type within the tissue.

TSCs are emerging as a valuable 2D *in vitro* model for studying trophoblast biology. TSCs can be derived from two primary sources: first trimester chorionic villi (CT) and blastocysts (Okae *et al.*, 2018). TSCs are cultured in a specific medium containing activin/nodal/TGFβ pathway inhibitors (e.g. A83-01), Rho-associated protein inhibitors (e.g. Y27632), histone deacetylase inhibitors (e.g. valproic acid), Wnt pathway inducers (e.g. CHIR99021), and epidermal growth factor (EGF) (Okae *et al.*, 2018). This facilitates the long-term expansion of CT-TSCs, which show transcriptional similarities to primary trophoblasts. Forskolin induces TSC fusion into syncytia, and upon removal of Wnt activator, TSCs differentiate into invasive matrix-degrading HLA-G+ EVTs (Okae *et al.*, 2018). Alternatively, TSCs can be derived from hPSCs, including ESCs, iPSCs, or expanded potential stem cells (ePSCs) (Li *et al.*, 2023b). As outlined in the earlier section on BMP signalling in trophoblasts, differentiation induced by BMP4 and activin inhibitors yields TSCs with characteristics similar to primary trophoblasts, although HLA expression patterns may differ from primary trophoblast (Xu *et al.*, 2002; Amita *et al.*, 2013; Koel *et al.*, 2017; Roberts *et al.*, 2018; Horii *et al.*, 2019; Sheridan *et al.*, 2021). Of note, TSCs exhibit normal karyotypes, unlimited proliferation, and versatile differentiation potential, making them adaptable for straightforward genetic manipulation, despite their potential genetic heterogeneity (Li *et al.*, 2023b; Liu *et al.*, 2023).

Development of trophoblast organoids provide an efficient *in vitro* model to study human placental development (Haider *et al.*, 2018; Turco *et al.*, 2018). Trophoblast organoids derived from primary CT cells in early pregnancy show differentiation into EVTs and STs as well as self-renewal capacity (Haider *et al.*, 2018). These organoids secrete placenta-specific peptides such as hCG and GDF15 and closely resemble first trimester trophoblasts (Turco *et al.*, 2018; Sheridan *et al.*, 2021). Cultured on matrigel with TGFβ and BMP signalling inhibitors (A83-01 and Noggin, respectively), EGF, and enhanced Wnt signalling, the organoids show prolonged expansion potential (Haider *et al.*, 2018). Similar to TSCs, removal of the Wnt inducer leads to re-differentiation of ST organoids into HLA-G+ EVTs (Haider *et al.*, 2018). Inhibition of TGFβ supports early invasive EVT development, whereas promotion of TGFβ signalling is critical for mature decidual EVT expression, highlighting the central role of decidual cell-derived TGFβ in controlling the EVT environment (Haider *et al.*, 2022). Trophoblast organoids present notable advantages, including continuous proliferative capacity and the capability to be cryopreserved and thawed (Sheridan *et al.*, 2020). Despite these benefits, a notable limitation involves the inverted architecture of CT and ST within organoids compared to placental villi (Haider *et al.*, 2018). However, this issue can be addressed by cultivating organoids in suspension culture with gentle agitation (Yang *et al.*, 2023). Furthermore, organoids mirror the expression profile of first-trimester placental tissue, potentially limiting their suitability as a model for third-trimester placenta. However, the complex culturing system they require provides an opportunity to study placental pathologies (Chuva de Sousa Lopes *et al.*, 2020; Karvas *et al.*, 2022).

In addition to 3D organoid models, placenta-on-chip models offer precise control of the microenvironment for real-time trophoblast differentiation and placental barrier studies (Lee *et al.*, 2016). While maintaining a placenta-on-a-chip system can increase the costs, the fluid shear stress environment within the system closely mimics *in vivo* conditions and provides an alternative to static cultures. This system allows the study of co-cultures with different cell types to investigate cell-to-cell communication and the placental barrier (Park *et al.*, 2022; Li *et al.*, 2023b; Liu *et al.*, 2023). Unlike the aforementioned organoids, in these on-chip models, first trimester and term placental

Table 3. Summary of published models to study TGFβ signalling in trophoblasts.

TGFβ superfamily	Ligand	Model system		Studied trophoblast function	Reference
TGFβ	TGFβ1	Primary trophoblast		Differentiation of placental EVTs into interstitial EVTs	Haider *et al.*, 2022
				TGFβ targets expression	Haider *et al.*, 2017
				Invasion	Oh *et al.*, 2009; Chen *et al.*, 2012; Cheng *et al.*, 2013, 2017; Fang *et al.*, 2022
		Placental explants		Invasion	Caniggia *et al.*, 1997b
		Cell lines	JEG-3	Invasion	Karmakar & Das, 2002
			HTR8/SVneo	Invasion	Oh *et al.*, 2009; Chen *et al.*, 2012; Cheng *et al.*, 2013, 2017; Liu *et al.*, 2019; Xu *et al.*, 2019; Fang *et al.*, 2022
		Organoids		Differentiation of placental EVTs into interstitial EVTs	Haider *et al.*, 2022
	TGFβ3	Cell lines	JAR	Invasion	Nishi *et al.*, 2004
			JEG-3	Invasion	Nishi *et al.*, 2004
		Placental explants		Invasion	Caniggia *et al.*, 1997b, 1999
BMP	BMP2	Primary trophoblasts		Invasion	Zhao *et al.*, 2018a,b, 2020a,b; You *et al.*, 2021; Deng *et al.*, 2023
				Differentiation	Yi *et al.*, 2021; You *et al.*, 2021
		Cell lines	HTR8/SVneo	Invasion	Zhao *et al.*, 2018b, 2020a,b
				Endothelial-like tube formation	Zhao *et al.*, 2020b
	BMP4	Trophoblast stem cells (TSC)		Derivation of TSCs from pluripotent stem cells	Sudheer *et al.*, 2012; Kojima *et al.*, 2017; Jang *et al.*, 2022; Soncin *et al.*, 2022
Nodal	Nodal	Primary trophoblasts		Apoptosis	Yu *et al.*, 2012
		Placental explants		Invasion	Nadeem *et al.*, 2011; Fu *et al.*, 2013
				Proliferation	Fu *et al.*, 2013
		Cell lines	JAR	Proliferation	Munir *et al.*, 2004

(continued)

Table 3. Continued

TGFβ superfamily	Ligand	Model system		Studied tropho-blast function	Reference
			JEG-3	Proliferation	Munir *et al.*, 2004
			Bewo	Proliferation	Munir *et al.*, 2004
			HTR8/SVneo	Invasion	Nadeem *et al.*, 2011; Fu *et al.*, 2013
				Proliferation	Fu *et al.*, 2013; Li *et al.*, 2018
				Apoptosis	Yu *et al.*, 2012
		Trophoblast stem cells		EVT formation	Sarkar *et al.*, 2015
Activin/Inhibin	Activin A	Primary trophoblasts		Differentiation	Caniggia *et al.*, 1997a; Bearfield *et al.*, 2005
				Invasion	Li *et al.*, 2015a; Adu-Gyamfi *et al.*, 2020; Zhu *et al.*, 2021
				Apoptosis	Yu *et al.*, 2012
				Hormone production	Song *et al.*, 1996
		Placental explants		Differentiation	Caniggia *et al.*, 1997a
				EVT formation	Caniggia *et al.*, 1997a
				Invasion	Caniggia *et al.*, 1997a
		Cell lines	HTR8/SVneo	Invasion	Li *et al.*, 2015a; Adu-Gyamfi *et al.*, 2020; Zhu *et al.*, 2021
				Apoptosis	Yu *et al.*, 2012
	Inhibin A	Placental explants		EVT growth	Caniggia *et al.*, 1997a; Petraglia *et al.*, 1994
GDF	GDF8	Primary trophoblast		Invasion	Xie *et al.*, 2020
		Cell lines	HTR8/SVneo	Invasion	Xie *et al.*, 2020; Fang *et al.*, 2021; Chen *et al.*, 2023
	GDF11	Primary trophoblast		Invasion	Wu *et al.*, 2022
		Cell lines	HTR8/SVneo	Invasion	Wu *et al.*, 2022
	GDF15	Placental Explants		Ligand expression	Hong *et al.*, 2022
		Cell lines	HTR8/SVneo	Ligand expression	Hong *et al.*, 2022
				Apoptosis	Li *et al.*, 2014a
				Proliferation	Li *et al.*, 2014a

EVT, extravillous trophoblasts; TSC, trophoblast stem cells.

Table 4. Comparison of known models to study TGFβ biology in the placenta trophoblast biology.

Model	Origin	Strengths	Limitations	References
Primary trophoblasts	First and third trimester placenta	• Preserve original genetic characteristics • Closely resemble the *in vivo* status • Accessible samples obtained from term healthy and pathophysiological pregnancies • Well established isolation, characterization, and cryopreservation protocols.	• Limited proliferation ability • Limited self-renewal • Mixed cell populations (CTs, STs …) • Reduced invasiveness and motility of EVTs at term • Difficult to acquire second trimester placental samples • Lack of physiological microenvironment and other cell types	Tannetta *et al.*, 2008; Li *et al.*, 2023b; Liu *et al.*, 2023
Placental explants	First and third trimester placenta	• Retain the *in vivo* tissue structure and integrity • Study cell-to-cell interactions and responses • Investigate cellular metabolism under healthy and pathophysiological conditions • Flow-cultured explants preserve the tissue intactness	• Shorter cultivation time in the static settings • Limited self-renewal • Genetic manipulation	Kupper *et al.*, 2021; Li *et al.*, 2023b; Liu *et al.*, 2023
Cell lines	Derived from: a) Choriocarcinoma: • JAR • JEG-3 • Bewo b) Primary first trimester extravillous trophoblasts transfected with simian virus 40 large T-antigen (SV40T): • HTR8/SVneo	• Display (some) properties of EVTs • Easy to maintain • Genetic manipulation • Stable and reproducible • Time-dependent experimental settings allow dynamic studies of placental development • Used to study invasion, proliferation and regulation	• Immortalized cells • Genetic alterations and phenotypical differences as primary trophoblasts • Mimic specific phenotypes of EVTs or STs • Limited functional differentiation • Cell lines observed signalling do not completely manifest the pathway signalling in primary trophoblasts	Abou-Kheir *et al.*, 2017; Msheik *et al.*, 2019; Pastuschek *et al.*, 2021; Li *et al.*, 2023b; Liu *et al.*, 2023
Trophoblast stem cells (TSC)	Derived from: a) Human placental trophoblast tissue b) Human pluripotent stem cells (PSCs)/human embryonic stem cells (ESCs)/human-induced pluripotent stem cells (iPSCs) and human ePSCs stimulated with inducing factors as BMP4	• Normal karyotype • Unlimited proliferation • Multi-directional differentiation • Similar gene expression as primary trophoblasts • Studies on pathophysiological cells as in PE • Easy genetic manipulation • Cell fusion/differentiation is inducible	• Genetic heterogeneity • Widespread imprint erasure • High demanding complex culturing process • Higher costs	Xu *et al.*, 2002; Koel *et al.*, 2017; Okae *et al.*, 2018; Dong *et al.*, 2020; Sheridan *et al.*, 2021; Liu *et al.*, 2023
Trophoblast organoids	First trimester primary trophoblasts/JEG-3/third trimester trophoblasts/TSC	• Structural and transcriptional similarity to placental villi, allowing biological dynamics to mimic developmental processes • Capable of proliferation and self-renewal, fusion and secretion of placental hormones	• Organoids represent expression profile of first trimester placental tissue, therefore might not be suitable as a model for third trimester placenta • Combination of primary cells and immortalized cell lines • Complex culturing system	Haider *et al.*, 2018; Turco *et al.*, 2018; Chuva de Sousa Lopes *et al.*, 2020; Sheridan *et al.*, 2020; Dietrich *et al.*, 2021; Karvas *et al.*, 2022

(continued)

Table 4. Continued

Model	Origin	Strengths	Limitations	References
		• Long-term culturing and cryopreservation	• Costly	
Placenta-on-chip	Primary trophoblast/ JEG-3, Co-culture with endothelial cells, fibroblasts	• Microfluidics system • Control of culture microenvironment • Real-time trophoblast differentiation • Study of placental barrier, metabolism, transport, and cell-to-cell interactions	• Combination of primary cells and immortalized cell lines • Costly	Lee *et al.*, 2016; Park *et al.*, 2022; Li *et al.*, 2023b; Liu *et al.*, 2023
Animal models	Rodents, non-human primates	• Study TGFβ signalling in the context of tissue micro-environment • Investigate *in vivo* responses to TGFβ signalling provides insights into systemic effects	• Anatomical and physiological differences between species • The structural and functional differences in placental anatomy across pregnancy • Genetic manipulation • Mimic pathophysiological conditions as PE • Costly • Ethics	Makris *et al.*, 2016; Marshall *et al.*, 2018; Carter, 2020; Li *et al.*, 2023b

EVT, extravillous trophoblast.

cells can be used alike, making them a versatile tool for different stages of pregnancy. Animal models, including rodents and non-human primates, allow the study of *in vivo* responses and provide critical insight into the systemic effects of signalling pathways within the tissue environment (Li *et al.*, 2023b). Despite their widespread use, it is important to recognize potential limitations. These can arise from species differences in anatomy and physiology, resulting in structural and functional differences in placental architecture (Carter, 2020). In addition, the challenges of genetic modification, higher costs, and ethical concerns should be considered when using animal models (Makris *et al.*, 2016; Marshall *et al.*, 2018).

Endothelial cells

The human placenta is a unique source of ECs that are specifically adapted to support vascular needs of the developing foetus. Indeed, several distinct subtypes of first trimester placental EC have been identified, highlighting their functional and metabolic heterogeneity (Zhou *et al.*, 2019). Upregulation of specific TGFβ signalling components (i.e. Smad1, Smad4, BMPR2) has demonstrated the importance of TGFβ in determining EC subtypes and an angiogenic functions (Li *et al.*, 2023a). In general, the TGFβ family maintains the endothelium in a quiescent state and regulates vascular development and barrier function (Possomato-Vieira and Khalil, 2016). Dysregulated TGFβ signalling, together with other factors, leads to the endothelial dysfunction, increased vascular tone, and altered vascular permeability observed in PE (Venkatesha *et al.*, 2006). Among the TGFβ family members, TGFβ1 is one of the most studied factors in endothelial biology, being involved in angiogenesis, cell migration, and blood vessel formation and repair, by inducing tube formation and sprouting (Lebrin *et al.*, 2004; Li *et al.*, 2023a). Indeed, neutralization of TGFβ1 leads to impaired endothelium-mediated vasodilation and increased expression of surface adhesion molecules, resulting in increased leukocyte adhesion (Walshe *et al.*, 2009). Under healthy conditions, TGFβ induces the activation of Smad2/3 signalling via ALK5 and TGFBRII (Goumans *et al.*, 2003). In addition, the membrane-anchored co-receptor Eng is able to fine tune this complex, by enhancing the affinity for ALK1 and promoting a mixed receptor complex containing ALK1/ALK5 (Goumans *et al.*, 2003; Lebrin *et al.*, 2004). This complex leads to the activation of downstream Smad1/5/8 signalling, thereby promoting angiogenesis (Goumans *et al.*, 2002; Lebrin *et al.*, 2004, 2005). By stimulating different Smad pathways, ALK1 and ALK5 have opposite effects on the behaviour of ECs (Goumans *et al.*, 2002). While ALK5 induces PAI-1, a negative regulator of proliferation and migration, and consequently mediates the production of fibronectin and collagen (Lebrin *et al.*, 2005), ALK1 induces the expression of ID-1, which promotes EC migration and proliferation (Goumans *et al.*, 2002; Lebrin *et al.*, 2005). The interplay between ALK5 and ALK1 allows fine regulation of endothelial function in physiological and pathological processes.

The dysregulation of certain members of the TGFβ family in placental EC biology has attracted considerable attention, particularly with the regard to Eng, a highly upregulated TGFβ accessory receptor that may be associated with endothelial dysfunction (Venkatesha *et al.*, 2006; Leaños-Miranda *et al.*, 2019). Although Eng is essential for vascular homeostasis, its elevated levels in certain inflammatory pathological conditions, such as in PE, contribute to impaired angiogenesis and aberrant vascular development (Venkatesha *et al.*, 2006). During inflammation, MMP12 and/or MMP14 cleave the extracellular domain of Eng, which triggers the excessive release of soluble Eng (sEng) in ECs (Tzavlaki and Moustakas, 2020). By interfering with the binding of TGFβ1 to its receptors TGFβRI and TGFβRII, sEng interrupts with downstream signalling events including inhibiting eNOS activation and vasodilation (Venkatesha *et al.*, 2006). Further, pro-angiogenic factors, such as angiopoietin 1 (Ang-1), vascular endothelial growth factor A (VEGF-A), and fibroblast growth factor receptor 2 (FGR-2) prevent the release of sEng through Akt signalling, a key upstream pathway of VEGF-A (Cudmore *et al.*, 2012). However, in PE, there is an increase in sEng levels, accompanied by increased sFlt-1 (a soluble form of the VEGFR1, encoded by the *FLT1* gene) and decreased Akt phosphorylation, further exacerbating the condition by antagonizing VEGF-A activity and compromising EC (Venkatesha *et al.*, 2006; Cudmore *et al.*, 2012).

The balance between the endothelial BMP and TGFβ signalling pathways is essential in many cardiovascular diseases (Goumans and ten Dijke, 2018). In addition to regulating TGFβ activity, sEng can promote the interaction of BMP9 and BMP10 with ALK1 in EC (Lawera *et al.*, 2019). Interestingly, BMP9 upregulates *ENG* mRNA expression and protein expression in EC, where Eng modulates TGFβ1 function, highlighting the role of Eng as a mediator between BMP and TGFβ signalling in EC (Venkatesha *et al.*, 2006). Although sEng was initially thought to be a ligand trap for BMP9 signalling, preventing BMP9 signalling (Castonguay *et al.*, 2011), Lawera *et al.* (2019) have shown that sEng does not inhibit BMP9 signalling but rather binds BMP9 in a complex and increases the affinity for ALK1, thereby enhancing Smad1/5/8 signalling in ECs. Of note, the sEng/BMP9 complex is highly expressed in PE subjects compared with to normotensive controls. Notably, a negative feedback mechanism has been described whereby sEng stimulates BMP4 expression, which is inhibited by the natural BMP antagonist Noggin. Given that BMP4 contributes to endothelial dysfunction, sEng-BMP4 may be an interesting target for hypertension therapeutics (Gallardo-Vara *et al.*, 2020).

Inflammatory conditions, such as those observed in PE, can trigger signalling pathways and molecular changes that induce endothelial-to-mesenchymal transition (EndoMT) (Derada Troletti *et al.*, 2019). EndoMT is characterized by profound morphological, functional, and molecular changes in the endothelial phenotype (Sánchez-Duffhues *et al.*, 2019). The morphological changes are driven by changes in cell polarity and cytoskeletal rearrangement as ECs differentiate into mesenchymal cells (Ma *et al.*, 2021). During the process of EndoMT, the expression of endothelial markers such as CD31, TIE-1, TIE-2, and VE-cadherin is downregulated, whereas the expression of mesenchymal Vimentin, N-Cadherin, and fibronectin is upregulated (Cho *et al.*, 2018). The phenotypic shift is accompanied by functional changes, such as increased cell migration, and decreased barrier function. It should be noted that EndoMT is a gradual, reversible, and dynamic process, often triggered by paracrine molecules (Ma *et al.*, 2020a). TGFβ signalling is one of the most potent inducers of EndoMT (Ma *et al.*, 2021). Specifically, in human umbilical cord vein ECs (HUVEC), TGFβ1 initiates EndoMT through the phosphorylation of Smad3, leading to the development of the mesenchymal phenotype (Evrard *et al.*, 2016). For example, co-culture of trophoblasts derived from PE placentas and HUVEC of healthy placentas had an effect on the disrupted barrier integrity of the ECs. In addition to observed changes in cell polarity and morphology, the disruption was manifested by the loss of VE-cadherin and occludin, key adhesion molecules (Li *et al.*, 2015b; Wang *et al.*, 2004). Understanding the molecular pathways underlying EndoMT in the context of PE will provide new insights into this pathology that may be exploited for therapeutic gain.

The role of foetal sex linked to effects on the placental endothelium and the development of PE is a subject of increasing research interest. Interestingly, a study conducted by Zhou *et al.* has demonstrated that female HUVECs are more susceptible to the effects of PE, than male HUVECs. This sexual dimorphism of PE affects the endothelium, with PE dysregulating EC migration, impairing vascularization, and disrupting angiogenesis in female foetal ECs. Conversely, in male foetal ECs, PE upregulates genes associated with cell growth failure, blood pressure, and chronic heart failure (Zhou *et al.*, 2019). Remarkably, a manuscript by Zhou showed that TGFβ1 stimulation resulted in increased cell proliferation only in female PE ECs, whereas normotensive controls showed a decreased response. On the other hand, stimulation with TGFβ1 led to a reduction in cell permeability in normotensive female foetal ECs, and not in PE female ECs (Zhou *et al.*, 2019). The identification of sex-specific PE dysregulated gene networks in the foetus has significant implications for early foetal programming and may be associated with the development of cardiovascular disease in children born from PE pregnancies. As endothelial dysfunction is a hallmark of several cardiovascular diseases, an early identification of molecular networks may have a therapeutic potential to prevent long-term cardiovascular risk in individuals exposed to PE during pregnancy.

Immune cells at the foeto-maternal interface and within the foeto-placental unit

Successful pregnancy requires tolerance between the immune system and the allogeneic foetus (Svensson-Arvelund *et al.*, 2015). Key immune populations at the foeto-maternal interface include regulatory T cells (Tregs), natural killer (NK) cells, and decidual macrophages (dM), while placental macrophages (HBCs) are located in the foetoplacental unit. Despite their location, immune cells coordinate their actions by secreting cytokines to establish immunological tolerance to the foetus, and contribute to vascular remodelling, yet maintain tissue homeostasis (Yagel, 2009; Alijotas-Reig *et al.*, 2014; Reyes *et al.*, 2017; Vondra *et al.*, 2023). TGFβ serves as an important immune regulator during pregnancy (Svensson-Arvelund *et al.*, 2015; Yang *et al.*, 2021). In the first trimester, TGFβ signalling is central to promoting differentiation of Tregs, regulating NK cell function and maintaining the balance between M1 and M2 macrophages at the foeto-maternal interface (Yang *et al.*, 2021). Furthermore, during the third trimester of pregnancy, TGFβ is essential for maintaining tissue homeostasis by regulating an anti-inflammatory phenotype of HBCs (Schliefsteiner *et al.*, 2017, 2020; Mercnik *et al.*, 2022). Perturbances in these regulatory mechanisms are associated with pregnancy complications such as PE or miscarriage (Reyes and Golos, 2018; Yang *et al.*, 2021). Understanding these immune cell interactions and functions is critical to understanding the immune dynamics during pregnancy and their impact on a successful and healthy outcome.

Regulatory T-cells

Tregs are pivotal to a successful pregnancy outcome, as they orchestrate maternal tolerance by 'education' of other immune cells (Alijotas-Reig *et al.*, 2014). They exist as thymus-derived and peripherally induced Tregs. In pregnancy, specific recruitment of peripheral maternal Tregs to the decidua seems to take place (Tilburgs *et al.*, 2009); after an initial influx of Tregs to the decidua in the first trimester, where Tregs make up about 10% of the leukocyte population, the Treg density plateaus and even decreases again towards term (Williams *et al.*, 2009).

Specifically for the induction of peripheral Tregs, TGFβ is needed (Wilczynski *et al.*, 2008; Yang *et al.*, 2021). FOXP3 expression is hallmark of Tregs, although FOXP3− subsets exist (Krop *et al.*, 2020). TGFβ1induces the production of CD4+/FOXP3+ Tregs, and these Tregs can in turn secrete TGFβ1and participate in immune regulation by acting on other cells and themselves, in para- and autocrine manners (Huang *et al.*, 2020). Of note, several foeto-placental cell types appear to be able to induce Tregs via TGFβ. Ma *et al.* (2020b) found that endovascular EVT released TGFβ which was able to induce maternal Treg formation. Oettel *et al.* (2016) demonstrated that even umbilical ECs release TGFβ which turned maternal T-cells into Tregs, and observed interesting sex-specific differences, with female HUVECs showing a stronger capacity to do so.

Furthermore, TGFβ balances the ratio of Tregs to other T-cell populations; specifically, a tight ratio between Th17 cells and Tregs at the foeto-maternal interface must be maintained for successful pregnancy, as observed from shifts in the Th17/Tregs ratio in patients with recurrent spontaneous miscarriages, and TGFβ is an important factor in its regulation (Wu *et al.*, 2014). A similar shift in TH17/Treg subsets has been observed in PE too, but was not linked to TGFβ in this study (Eghbal-Fard *et al.*, 2019).

In women suffering from PE, changes in Treg frequency and distribution between decidual tissue and periphery have been found (Sasaki *et al.*, 2007). Similar to NK cells, most research on Tregs focusses on Tregs accumulating in the decidua. Quinn *et al.* (2011) specifically investigated decidua from early-onset as opposed to late-onset PE and controls, and found that Tregs were lowest in EOPE. Little data are available as to the presence Tregs within villous tissue. In a recent histopathological study of different placental pathologies, it was found that FOXP3− reactivity within villous tissue was reduced in PE and IUGR compared to controls, while being increased in gestational diabetes (Emirdar *et al.*, 2021).

In summary, Tregs are pivotal for establishing maternal tolerance during pregnancy, orchestrating the immune balance through recruitment and modulation via TGFβ signalling. Imbalances in Treg populations observed in PE, possibly linked with TGFβ signalling, emphasize the crucial role of Treg regulation in maintaining a successful pregnancy.

NK cells

NK cells are an integral part of the endometrial innate immune milieu and play an important role in maintaining pregnancy (Yagel, 2009). During pregnancy, the majority of leukocytes are decidual natural killer (dNK) cells. These cells are recruited from the periphery in a CXCR4/CXCL12 dependent manner by EVT cells (Hanna *et al.*, 2003) and then are converted to CD56$^{\mathrm{bright}}$/CD16− cells by TGFβ, which is secreted, for example, by decidual stromal cells (Keskin *et al.*, 2007). dNK cells exhibit low cytotoxicity compared to other NK cell subsets and rather contribute to vascular remodelling at the foeto-maternal interface by autocrine release of TGFβ, along with VEGF, angiopoietin, and MMP2 and MMP9 (Lash *et al.*, 2006; Fraser *et al.*, 2012). PE is associated with abnormal TGFβ levels in the decidua (Yang *et al.*, 2021); these elevated levels serve as influential factors modulating dNK cell phenotype and function. Abnormal TGFβ levels, particularly TGFβ1 of Tregs, can disrupt dNK cell function and potentially influence the pathophysiology of PE (Zhang *et al.*, 2019). Further comprehensive insights on dNK cells, particularly their involvement in PE, can be found in recent reviews (Liu *et al.*, 2021; Wei and Yang, 2023). Here we have focused on understanding the role of TGFβ signalling in the context of dNK cells.

However, the plethora of research on NK cells in pregnancy has not focussed on NK cells in the decidua or placenta, but on peripheral NK cells in maternal (and less frequently, foetal cord) blood. For the maternal circulation, there are conflicting data on whether the number of peripheral NK cells is increased, decreased, or maintained at PE, and whether these NK cells are more, less, or as cytotoxic as their counterparts from healthy pregnancies (reviewed in Wei and Yang, 2023). Concerning the foetal circulation, two independent studies supported that in cord blood from PE neonates, activated NK cells appear to be expanded compared to regulatory NK cells, thus initiating an inflammatory response (Sohlberg *et al.*, 2014; Loewendorf *et al.*, 2015). However, neither of these studies looked specifically at TGFβ or BMP signalling and are only mentioned here for completeness.

Macrophages

In pregnancy, placental macrophages are a heterogeneous population of immune cells, involved in the maternal–foetal immune regulation, placental cell invasion, angiogenesis, and tissue remodelling (Houser *et al.*, 2011; Reyes and Golos, 2018). Two main types of macrophages are present at all stages of pregnancy. The first type is the decidual macrophages (dM), located in either in the decidua basalis (dBAM) or in the decidua parientalis (dPAM), and the second type are the foeto-placental macrophages, called HBCs (Houser *et al.*, 2011). Regardless of their specific location, their main function is to act as gatekeepers of placental homeostasis (Aneman *et al.*, 2020). Macrophages are keen to polarize into different polarization states in response to microenvironmental stimuli, namely pro-inflammatory M1 and anti-inflammatory M2 (Murray and Wynn, 2011; Murray *et al.*, 2014). The polarization state determines functionality of HBCs, and both dM and HBCs shifts during pregnancy as a function of placental requirements. As the pregnancy progresses, both dM and HBCs develop specific M1/M2 phenotypes, with macrophages in the first trimester tending to exhibit a more pro-inflammatory polarization (Mezouar *et al.*, 2021), but there is a shift towards M2 as pregnancy progresses towards term (Yao *et al.*, 2019). Inappropriate macrophage activation and polarization has been associated with pregnancy disorders such as PE, recurrent pregnancy loss, or chorioamnionitis (Reyes and Golos, 2018). During the first trimester, activated dM secrete a variety of molecules, including tumour necrosis factor (TNF)α and TGFβ1, which may affect regulatory functions of surrounding trophoblasts. In PE, an accumulation of activated dM is observed in proximity of the spiral arteries (Ning *et al.*, 2016). This suggests a potential role for dM in inhibiting trophoblast invasion. M2 polarized dM, secrete high levels of TGFβ1. As discussed above, TGFβ1 is a potent inhibitor of trophoblast invasion and affects the production of MMPs, which are enzymes involved in tissue remodelling (Ning *et al.*, 2016). Conversely, the secretome of EVTs has been shown to regulate the function of dM. In a study by Vondra *et al.* (2023), the secretome of EVTs containing TGFβ induced the proliferation of dBAM, but not dPAM, through the upregulation of macrophage colony stimulating factor (M-CSF). Additionally, factors secreted by EVT such as TGFβ, M-CSF, and IL10 have been implicated in the regulation of M2 polarization of dM (Aldo *et al.*, 2014; Svensson-Arvelund *et al.*, 2015). Trophoblast-derived IL6 has also been shown to induce a specific M2 polarization of dM by activating the STAT3 signalling, resulting in high expression of CD206, CCL18, IL10, and TGFβ (Ding *et al.*, 2021). Furthermore, abnormal levels of decorin, a molecule produced by first trimester dM, have been associated with the development of pregnancy related disorders such as PE, recurrent pregnancy loss, and foetal growth restriction (Nandi *et al.*, 2016). Decorin regulates the toll like receptor 2 (TLR2)/TLR4-MyD88-NF-κB signalling pathway (Wang *et al.*, 2022), which alters mitochondrial metabolism, and promotes a pro-inflammatory M1 phenotype switch of dM, and reduced TGFβ secretion (Wang *et al.*, 2022).

In summary, studies performed during the first trimester highlight the dynamic interaction between EVTs and dM, where both cell types produce high levels of TGFβ and mutually influence each other's function. Investigating the interplay between EVTs and dM and their modulation of the TGFβ signalling may provide insights into the early pathogenesis of PE, where TGFβ signalling is impaired.

During the third trimester of pregnancy, TGFβ acts as vital immunoregulator, preserving the homeostatic phenotype of macrophages (Svensson-Arvelund *et al.*, 2015). TGFβs, as major drivers of M2 polarization, are regulators of the function of dM and HBCs (Murray *et al.*, 2014). Both, dM and HBCs constitutively express TGFβ1 (Schliefsteiner *et al.*, 2017, 2020; Pavlov *et al.*, 2020; Mercnik *et al.*, 2022). While dMs only produce the pro-form of TGFβ1, HBCs also secrete the active form of TGFβ1 (Schliefsteiner *et al.*, 2017; Pavlov *et al.*, 2020; Mercnik *et al.*, 2022). In addition, dPAM and dBAM also secrete TGFβ2 (Vondra *et al.*, 2023). HBCs secrete TGFβ2 and TGFβ3, although TGFβ1 is the major ligand secreted by HBCs (Pavlov *et al.*, 2020). The contribution of TGFβ to the tolerogenic and homeostatic functions of HBCs has been demonstrated particularly in the context of infections and inflammatory pathologies that occur during the pregnancy (Azari *et al.*, 2021). For example, during infection with *Lysteria monocytogeneses*, HBCs undergo pro-inflammatory reprograming. At the same time however, HBCs increase the secretion of anti-inflammatory TGFβ1, which serves to prevent maternal anti-foetal adaptive immunity and further promotes the anti-inflammatory environment (Azari *et al.*, 2021). Additionally, the immunoregulatory actions of TGFβ1 by HBCs have been found to limit HIV-1 replication in HBCs when exposed to exogenous TGFβ (Johnson and Chakraborty, 2012). Furthermore, the increased secretion of TGFβ1 in EO—and LOPE HBCs is associated with the maintenance of anti-inflammatory phenotype of both inflammatory conditions, as TGFβ is widely recognized for its role in resolving inflammation (Mercnik *et al.*, 2022).

These findings highlight the important role of TGFβ signalling in modulating the immune responses and maintaining the immune homeostasis in HBCs during the pregnancy and in providing protection against excessive inflammation such as in PE.

Conclusion and future perspectives

We have summarized how TGFβ signalling plays an essential spatio-temporal role in regulating the function of all placental cells, including trophoblasts, foeto-placental EC, and immune cells. TGFβ ligands contribute to processes such as trophoblast invasion, vascularization, immune tolerance, and tissue remodelling, to ensure successful placental development during pregnancy (Dietrich *et al.*, 2022; Li *et al.*, 2023a). Dysregulation of TGFβ signalling has been linked to the pathogenesis of PE, a complex disorder characterized by shallow trophoblast invasion, defective vascular remodelling, decreased uteroplacental perfusion (Haider *et al.*, 2017), and impaired EC (Zhou *et al.*, 2019) and macrophages (Mercnik *et al.*, 2022). Despite significant progress, there are still many unknowns and challenges in understanding TGFβ signalling in the context of PE (Fig. 3). In trophoblasts, the specific mechanisms by which TGFβ ligands regulate invasion and EMT-

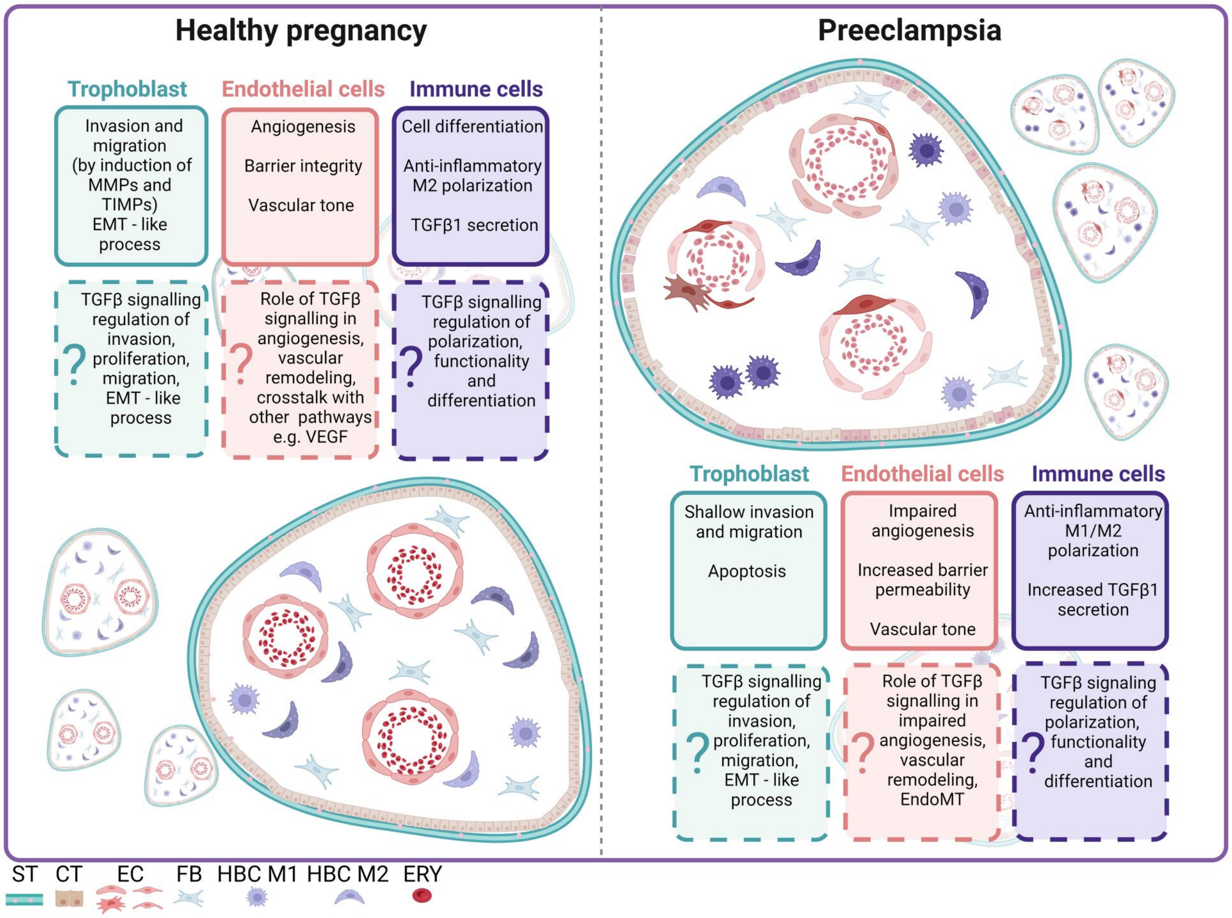

Figure 3. The unequal importance of TGFβ signalling in PE-compromised placental cells. The figure provides a cross-sectional representation of the placental villous structure, illustrating a comparison between a healthy (CTR) placenta and a preeclamptic (PE) placenta. The main placental cell types, including trophoblasts, ECs, and immune cells, are depicted within the structure, highlighting their specific known roles in the respective placenta. Question marks (?) indicate the unknown and challenging aspects of TGFβ signalling in these specific cell types. These questions relate to the precise mechanisms and effects of TGFβ signalling in trophoblast function, the impaired vascular remodelling and angiogenesis of ECs, and the phenotypic changes observed in immune cells during PE. FB, fibroblasts; HBC M1, M1 polarized Hofbauer cells; HBC M2, M2 polarized Hofbauer cells; ERY, erythrocytes.

like process remain poorly understood. In EC, the precise role of TGFβ signalling in impaired vascular remodelling, angiogenesis, and possible endothelial-to-mesenchymal transition (EndoMT) requires further investigation. TGFβ serves as an important immune regulator during pregnancy and plays a central role in Treg differentiation and regulation of NK cell function, which is poorly understood. In macrophages, the influence of TGFβ signalling on polarization, cytokine production, and tissue remodelling during PE remains to be elucidated. Further research is needed to unravel the dysregulation of TGFβ ligands, the regulation of receptor activation, the crosstalk with other signalling pathways, and the epigenetic regulation in PE. Of note, distinguishing between the different clinically manifest subtypes of PE is critical to advancing our understanding of TGFβ signalling in this syndrome. Understanding the interactions and dysregulation of TGFβ signalling in these cell types is essential to uncover the underlying mechanisms that contribute to the development of PE. This knowledge may facilitate the development of improved *in vitro* and *in vivo* models to study PE and identify potential therapeutic targets within the TGFβ signalling pathway.

Data availability

There are no new data associated with this article.

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All figures have been generated using BioRender (Laboratory licence is given).

Authors' roles

M.H.M. and C.W. designed the concept of the manuscript. M.H.M. and C.S. prepared the original draft. M.H.M. prepared the figures and the tables. The draft was critically revised and edited by M.H.M., C.S., G.S.-D., and C.W. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Abdollah S, Macías-Silva M, Tsukazaki T, Hayashi H, Attisano L, Wrana JL. TβRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem* 1997;**272**:27678–27685.

Abou-Kheir W, Barrak J, Hadadeh O, Daoud G. HTR-8/SVneo cell line contains a mixed population of cells. *Placenta* 2017;**50**:1–7.

Adu-Gyamfi EA, Ding YB, Wang YX. Regulation of placentation by the transforming growth factor beta superfamily. *Biol Reprod* 2020;**102**:18–26.

Aldo PB, Racicot K, Craviero V, Guller S, Romero R, Mor G. Trophoblast induces monocyte differentiation into CD14+/CD16+ macrophages. *Am J Reprod Immunol* 2014;**72**:270–284.

Alijotas-Reig J, Llurba E, Gris JM. Potentiating maternal immune tolerance in pregnancy: a new challenging role for regulatory T cells. *Placenta* 2014;**35**:241–248.

Amita M, Adachi K, Alexenko AP, Sinha S, Schust DJ, Schulz LC, Roberts RM, Ezashi T. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci USA* 2013;**110**:E1212–E1221.

Aneman I, Pienaar D, Suvakov S, Simic TP, Garovic VD, McClements L. Mechanisms of key innate immune cells in early- and late-onset preeclampsia. *Front Immunol* 2020;**11**:1864.

Arutyunyan A, Roberts K, Troulé K, Wong FCK, Sheridan MA, Kats I, Garcia-Alonso L, Velten B, Hoo R, Ruiz-Morales ER *et al.* Spatial multiomics map of trophoblast development in early pregnancy. *Nature* 2023;**616**:143–151.

Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem* 1983;**258**:7155–7160.

Azari S, Johnson LJ, Webb A, Kozlowski SM, Zhang X, Rood K, Amer A, Seveau S. Hofbauer cells spread listeria monocytogenes among placental cells and undergo pro-inflammatory reprogramming while retaining production of tolerogenic factors. *mBio* 2021;**12**:e0184921.

Ball E, Robson SC, Ayis S, Lyall F, Bulmer JN. Expression of TGF beta in the placental bed is not altered in sporadic miscarriage. *Placenta* 2007;**28**:965–971.

Barbara NP, Wrana JL, Letarte M. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-β superfamily. *J Biol Chem* 1999;**274**:584–594.

Barber CV, Yo JH, Rahman RA, Wallace EM, Palmer KR, Marshall SA. Activin A and pathologies of pregnancy: a review. *Placenta* 2023;**136**:35–41.

Bearfield C, Jauniaux E, Groome N, Sargent IL, Muttukrishna S. The secretion and effect of inhibin A, activin A and follistatin on first-trimester trophoblasts *in vitro*. *Eur J Endocrinol* 2005;**152**:909–916.

Benian A, Madazli R, Aksu F, Uzun H, Aydin S. Plasma and placental levels of interleukin-10, transforming growth factor-β1, and epithelial-cadherin in preeclampsia. *Obstet Gynecol* 2002;**100**:327–331.

Bidart M, Ricard N, Levet S, Samson M, Mallet C, David L, Subileau M, Tillet E, Feige JJ, Bailly S. BMP9 is produced by hepatocytes and circulates mainly in an active mature form complexed to its prodomain. *Cell Mol Life Sci* 2012;**69**:313–324.

Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, Nohe A. Bone morphogenetic proteins: a critical review. *Cell Signal* 2011;**23**:609–620.

Brosens I, Pijnenborg R, Vercruysse L, Romero R. The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. *Am J Obstet Gynecol* 2011;**204**:193–201.

Budi EH, Duan D, Derynck R. Transforming Growth Factor-β Receptors and Smads: Regulatory Complexity and Functional Versatility. *Trends Cell Biol* 2017;**27**:658–672.

Burton GJ, Woods AW, Jauniaux E, Kingdom JCP. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta* 2009a;**30**:473–482.

Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS. Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta* 2009b;**30**(Suppl A):S43–S48.

Caniggia I, Grisaru-Gravnosky S, Kuliszewsky M, Post M, Lye SJ. Inhibition of TGF-beta 3 restores the invasive capability of extravillous trophoblasts in preeclamptic pregnancies. *J Clin Invest* 1999;**103**:1641–1650.

Caniggia I, Lye SJ, Cross JC. Activin is a local regulator of human cytotrophoblast cell differentiation. *Endocrinology* 1997a; **138**:3976–3986.

Caniggia I, Taylor CV, Ritchie JWK, Lye SJ, Letarte M. Endoglin regulates trophoblast differentiation along the invasive pathway in human placental villous explants. *Endocrinology* 1997b; **138**:4977–4988.

Carter AM. Animal models of human pregnancy and placentation: alternatives to the mouse. *Reproduction* 2020;**160**:R129–R143.

Castonguay R, Werner ED, Matthews RG, Presman E, Mulivor AW, Solban N, Sako D, Pearsall RS, Underwood KW, Seehra J *et al.* Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. *J Biol Chem* 2011;**286**:30034–30046.

Chen J, Song T, Yang S, Meng Q, Han X, Wu Z, Cheng JC, Fang L. Snail mediates GDF-8-stimulated human extravillous trophoblast cell invasion by upregulating MMP2 expression. *Cell Commun Signal* 2023;**21**:1–13.

Chen X, Liu Y, Xu X, Chen H. Correlation of Cyr61 and CTGF in placentas from the late pre-eclamptic pregnancy. *J Perinat Med* 2012; **40**:199–200.

Cheng JC, Chang HM, Leung PC. TGF-β1 inhibits human trophoblast cell invasion by upregulating connective tissue growth factor expression. *Endocrinology* 2017;**158**:3620–3628.

Cheng JC, Chang HM, Leung PCK. Transforming growth factor-β1 inhibits trophoblast cell invasion by inducing snail-mediated down-regulation of vascular endothelial-cadherin protein. *J Biol Chem* 2013;**288**:33181–33192.

Cho JG, Lee A, Chang W, Lee MS, Kim J. Endothelial to mesenchymal transition represents a key link in the interaction between inflammation and endothelial dysfunction. *Front Immunol* 2018; **9**:294.

Chuva de Sousa Lopes SM, Alexdottir MS, Valdimarsdottir G. The TGFβ family in human placental development at the fetal-maternal interface. *Biomolecules* 2020;**10**:453.

Ciarmela P, Florio P, Toti P, Franchini A, Maguer-Satta V, Ginanneschi C, Ottavian E, Petraglia F. Human placenta and fetal membranes express follistatin-related gene mRNA and protein. *J Endocrinol Invest* 2003;**26**:641–645.

Cindrova-Davies T, Sferruzzi-Perri AN. Human placental development and function. *Semin Cell Dev Biol* 2022;**131**:66–77.

Cowden Dahl KD, Fryer BH, Mack FA, Compernolle V, Maltepe E, Adelman DM, Carmeliet P, Simon MC. Hypoxia-inducible factors 1α and 2α regulate trophoblast differentiation. *Mol Cell Biol* 2005; **25**:10479–10491.

Cudmore MJ, Ahmad S, Sissaoui S, Ramma W, Ma B, Fujisawa T, Al-Ani B, Wang K, Cai M, Crispi F *et al.* Loss of Akt activity increases circulating soluble endoglin release in preeclampsia: identification of inter-dependency between Akt-1 and heme oxygenase-1. *Eur Heart J* 2012;**33**:1150–1158.

De Caestecker MP, Parks WT, Frank CJ, Castagnino P, Bottaro DP, Roberts AB, Lechleider RJ. Smad2 transduces common signals from receptor serinethreonine and tyrosine kinases. *Genes Dev* 1998;**12**:1587–1592.

Debieve F, Pampfer S, Thomas K. Inhibin and activin production and subunit expression in human placental cells cultured *in vitro*. *Mol Hum Reprod* 2000;**6**:743–749.

Deng J, Zhao HJ, Zhong Y, Hu C, Meng J, Wang C, Lan X, Wang X, Chen ZJ, Yan J *et al.* H3K27me3-modulated Hofbauer cell BMP2 signalling enhancement compensates for shallow trophoblast invasion in preeclampsia. *EBioMedicine* 2023;**93**:104664.

Derada Troletti C, Fontijn RD, Gowing E, Charabati M, van Het Hof B, Didouh I, van der Pol SMA, Geerts D, Prat A, van Horssen J *et al.* Inflammation-induced endothelial to mesenchymal transition promotes brain endothelial cell dysfunction and occurs during multiple sclerosis pathophysiology. *Cell Death Dis* 2019;**10**:45–13.

Derynck R, Budi EH. Specificity, versatility, and control of TGF-B family signaling. *Sci Signal* 2019;**12**:eaav5183.

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-B family signalling. *Nature* 2003;**425**:577–584.

Dietrich B, Haider S, Meinhardt G, Pollheimer J, Knöfler M. WNT and NOTCH signaling in human trophoblast development and differentiation. *Cell Mol Life Sci* 2022;**79**:1–16.

Dietrich B, Kunihs V, Haider S, Pollheimer J, Knöfler M. 3-Dimensional JEG-3 choriocarcinoma cell organoids as a model for trophoblast expansion and differentiation. *Placenta* 2021; **104**:243–246.

Ding J, Yang C, Cheng Y, Wang J, Zhang S, Yan S, He F, Yin T, Yang J. Trophoblast-derived IL-6 serves as an important factor for normal pregnancy by activating Stat3-mediated M2 macrophages polarization. *Int Immunopharmacol* 2021;**90**:106788.

Dirisipam K, Madduru D, Jahan P, Gujrati D. Can circulating levels of transforming growth factor-β1 in early pregnancy serve as a predictive marker of unfavourable outcome? *Placenta* 2023; **137**:65–69.

Djurovic S, Schjetlein R, Wisløff F, Haugen G, Husby H, Berg K. Plasma concentrations of Lp(a) lipoprotein and TGF-β1 are altered in preeclampsia. *Clin Genet* 1997;**52**:371–376.

Dong C, Beltcheva M, Gontarz P, Zhang B, Popli P, Fischer LA, Khan SA, Park KM, Yoon EJ, Xing X *et al.* Derivation of trophoblast stem cells from naïve human pluripotent stem cells. *Elife* 2020; **9**:e52504.

Davies JE, Pollheimer J, Yong HEJ, Kokkinos MI, Kalionis B, Knöfler M, Murthi P. Epithelial-mesenchymal transition during extravillous trophoblast differentiation. *Cell Adh Migr* 2016;**10**:310–321.

Eghbal-Fard S, Yousefi M, Heydarlou H, Ahmadi M, Taghavi S, Movasaghpour A, Jadidi-Niaragh F, Yousefi B, Dolati S, Hojjat-Farsangi M *et al.* The imbalance of Th17/Treg axis involved in the pathogenesis of preeclampsia. *J Cell Physiol* 2019;**234**:5106–5116.

Emirdar V, Ekizceli G, Dilber Y, Inan S, Sanci M. Immunolocalization of FOXP3, JAK1 and STAT5 in preeclamptic, intrauterine growth restricted and gestational diabetic human placentas. *Aegean J Obstet Gynecol* 2021;**3**:73–77.

Evrard SM, Lecce L, Michelis KC, Nomura-Kitabayashi A, Pandey G, Purushothaman K-R, d'Escamard V, Li JR, Hadri L, Fujitani K *et al.* Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. *Nat Commun* 2016;**7**:11853–11816.

Fang L, Wang Z, Wu Z, Yan Y, Gao Y, Li Y, Cheng JC, Sun YP. GDF-8 stimulates trophoblast cell invasion by inducing ALK5-SMAD2/3-mediated MMP2 expression. *Reproduction* 2021;**162**:331–338.

Fang L, Yan Y, Gao Y, Wu Z, Wang Z, Yang S, Cheng JC, Sun YP. TGF-β1 inhibits human trophoblast cell invasion by upregulating kisspeptin expression through ERK1/2 but not SMAD signaling pathway. *Reprod Biol Endocrinol* 2022;**20**:1–12.

Ferreira LC, Gomes CEM, Araújo ACP, Bezerra PF, Duggal P, Jeronimo SMB. Association between ACVR2A and early-onset preeclampsia: replication study in a Northeastern Brazilian population. *Placenta* 2015;**36**:186–190.

Florio P, Luisi S, Ciarmela P, Severi FM, Bocchi C, Petraglia F. Inhibins and activins in pregnancy. *Mol Cell Endocrinol* 2004;**225**:93–100.

Forbes K, Souquet B, Garside R, Aplin JD, Westwood M. Transforming growth factor-β (TGFβ) receptors I/II differentially regulate TGFβ1 and IGF-binding protein-3 mitogenic effects in the human placenta. *Endocrinology* 2010;**151**:1723–1731.

Fraser R, Whitley GS, Johnstone AP, Host AJ, Sebire NJ, Thilaganathan B, Cartwright JE. Impaired decidual natural killer cell regulation of vascular remodelling in early human pregnancies with high uterine artery resistance. *J Pathol* 2012; **228**:322–332.

Fu G, Ye G, Nadeem L, Ji L, Manchanda T, Wang Y, Zhao Y, Qiao J, Wang YL, Lye S *et al.* MicroRNA-376c impairs transforming growth factor-β and nodal signaling to promote trophoblast cell proliferation and invasion. *Hypertension* 2013;**61**:864–872.

Fuentealba LC, Eivers E, Ikeda A, Hurtado C, Kuroda H, Pera EM, De Robertis EM. Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. *Cell* 2007;**131**:980–993.

Gallardo-Vara E, Gamella-Pozuelo L, Perez-Roque L, Bartha JL, Garcia-Palmero I, Casal JI, López-Novoa JM, Pericacho M, Bernabeu C. Potential role of circulating endoglin in hypertension via the upregulated expression of BMP4. *Cells* 2020;**9**:988.

Gauster M, Moser G, Wernitznig S, Kupper N, Huppertz B. Early human trophoblast development: from morphology to function. *Cell Mol Life Sci* 2022;**79**:1–18.

Gestational Hypertension and Preeclampsia: ACOG Practice Bulletin, Number 222. *Obstet Gynecol* 2020;**135**:e237–e260.

Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. *Sci Signal* 2014;**7**:re8.

Goumans MJ, ten Dijke P. TGF-β signaling in control of cardiovascular function. *Cold Spring Harb Perspect Biol* 2018;**10**:a022210.

Goumans MJ, Valdimarsdottir G, Itoh S, Lebrin F, Larsson J, Mummery C, Karlsson S, ten Dijke P. Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFβ/ALK5 signaling. *Mol Cell* 2003;**12**:817–828.

Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, Ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-β type I receptors. *EMBO J* 2002;**21**:1743–1753.

Graham CH. Effect of transforming growth factor-β on the plasminogen activator system in cultured first trimester human cytotrophoblasts. *Placenta* 1997;**18**:137–143.

Gregory AL, Xu G, Sotov V, Letarte M. Review: the enigmatic role of endoglin in the placenta. *Placenta* 2014;**35**(Suppl):S93–S99.

Guo J, Tian T, Lu D, Xia G, Wang H, Dong M. Alterations of maternal serum and placental follistatin-like 3 and myostatin in pre-eclampsia. *J Obstet Gynaecol Res* 2012;**38**:988–996.

Haider S, Beristain AG. Human organoid systems in modeling reproductive tissue development, function, and disease. *Hum Reprod* 2023;**38**:1449–1463.

Haider S, Kunihs V, Fiala C, Pollheimer J, Knöfler M. Expression pattern and phosphorylation status of Smad2/3 in different subtypes of human first trimester trophoblast. *Placenta* 2017; **57**:17–25.

Haider S, Lackner AI, Dietrich B, Kunihs V, Haslinger P, Meinhardt G, Maxian T, Saleh L, Fiala C, Pollheimer J. Transforming growth factor-β signaling governs the differentiation program of extravillous trophoblasts in the developing human placenta. *Proc Natl Acad Sci USA* 2022;**119**:e2120667119.

Haider S, Meinhardt G, Saleh L, Kunihs V, Gamperl M, Kaindl U, Ellinger A, Burkard TR, Fiala C, Pollheimer J *et al.* Self-renewing trophoblast organoids recapitulate the developmental program of the early human placenta. *Stem Cell Reports* 2018;**11**:537–551.

Hanna J, Wald O, Goldman-Wohl D, Prus D, Markel G, Gazit R, Katz G, Haimov-Kochman R, Fujii N, Yagel S *et al.* CXCL12 expression by invasive trophoblasts induces the specific migration of CD16− human natural killer cells. *Blood* 2003;**102**:1569–1577.

Heldin C-H, Miyazono K, ten Dijke P. TGF-signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;**390**:465–471.

Heldin CH, Moustakas A. Signaling receptors for TGF-β family members. *Cold Spring Harb Perspect Biol* 2016;**8**:a022053.

Hennessy A, Orange S, Willis N, Painter DM, Child A, Horvath JS. Transforming growth factor-β1 does not relate to hypertension in pre-eclampsia. *Clin Exp Pharmacol Physiol* 2002;**29**:968–971.

Henry-Berger J, Mouzat K, Baron S, Bernabeu C, Marceau G, Saru JP, Sapin V, Lobaccaro JMA, Caira F. Endoglin (CD105) expression is regulated by the liver X receptor alpha (NR1H3) in human trophoblast cell line JAR. *Biol Reprod* 2008;**78**:968–975.

Hill CS. Transcriptional control by the SMADs. *Cold Spring Harb Perspect Biol* 2016;**8**:a022079.

Hong W, Chen J-H, Ma H-J, Li, X-C, Li-Li. Fragile X-related protein 1 (FXR1) promotes trophoblast migration at early pregnancy via downregulation of GDF-15 expression. *Reprod Sci* 2022;**29**:110–121.

Horii M, Bui T, Touma O, Cho HY, Parast MM. An improved two-step protocol for trophoblast differentiation of human pluripotent stem cells. *Curr Protoc Stem Cell Biol* 2019;**50**:e96.

Horii M, Touma O, Bui T, Parast MM. Modeling human trophoblast, the placental epithelium at the maternal fetal interface. *Reproduction* 2020;**160**:R1–R11.

Hough C, Radu M, Doré JJE. TGF-beta induced Erk phosphorylation of Smad linker region regulates Smad signaling. *PLoS One* 2012; **7**:e42513.

Houser BL, Tilburgs T, Hill J, Nicotra ML, Strominger JL. Two unique human decidual macrophage populations. *J Immunol* 2011; **186**:2633–2642.

Huang N, Chi H, Qiao J. Role of regulatory T cells in regulating fetal-maternal immune tolerance in healthy pregnancies and reproductive diseases. *Front Immunol* 2020;**11**:1023.

Huang T, Schor SL, Hinck AP. Biological activity differences between TGF-β1 and TGF-β3 correlate with differences in the rigidity and arrangement of their component monomers terms of use. *Biochemistry* 2014;**53**:5749.

Huber A, Hefler L, Tempfer C, Zeisler H, Lebrecht A, Husslein P. Transforming growth factor-beta 1 serum levels in pregnancy and pre-eclampsia. *Acta Obstet Gynecol Scand* 2002;**81**:168–171.

Huppertz B. The anatomy of the normal placenta. *J Clin Pathol* 2008a; **61**:1296–1302.

Huppertz B. Placental origins of preeclampsia: challenging the current hypothesis. *Hypertension* 2008b;**51**:970–975.

Incebiyik A, Kocarslan S, Camuzcuoglu A, Hilali NG, Incebiyik H, Camuzcuoglu H. Trophoblastic E-cadherin and TGF-beta expression in placenta percreta and normal pregnancies. *J Matern Fetal Neonatal Med* 2014;**29**:126–129.

Ingman WV, Robertson SA. The essential roles of TGFB1 in reproduction. *Cytokine Growth Factor Rev* 2009;**20**:233–239.

Io S, Kabata MIO, Iemura Y, Semi K, Morone N, Minagawa A, Wang BO, Okamoto I, Nakamura T, Kojima Y *et al.* Capturing human trophoblast development with naive pluripotent stem cells in vitro. *Cell Stem Cell* 2021;**28**:1023–1039.e13.

Irving JA, Lala PK. Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-β, IGF-II, and IGFBP-1. *Exp Cell Res* 1995;**217**:419–427.

Jang YJ, Kim M, Lee BK, Kim J. Induction of human trophoblast stem-like cells from primed pluripotent stem cells. *Proc Natl Acad Sci USA* 2022;**119**:e2115709119.

Jenkins G. The role of proteases in transforming growth factor-β activation. *Int J Biochem Cell Biol* 2008;**40**:1068–1078.

Johnson EL, Chakraborty R. Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of immunoregulatory cytokines. *Retrovirology* 2012;**9**:101–111.

Jones RL, Findlay JK, Farnworth PG, Robertson DM, Wallace E, Salamonsen LA. Activin A and inhibin A differentially regulate human uterine matrix metalloproteinases: potential interactions during decidualization and trophoblast invasion. *Endocrinology* 2006a;**147**:724–732.

Jones RL, Stoikos C, Findlay JK, Salamonsen LA. TGF-β superfamily expression and actions in the endometrium and placenta. *Reproduction* 2006b;**132**:217–232.

Kamato D, Bich Do H, Osman N, Ross BP, Mohamed R, Xu S, Little PJ. Smad linker region phosphorylation is a signalling pathway in its own right and not only a modulator of canonical TGF-β signalling. *Cell Mol Life Sci* 2020;**77**:243–251.

Kapustin RV, Drobintseva AO, Alekseenkova EN, Onopriychuk AR, Arzhanova ON, Polyakova VO, Kvetnoy IM. Placental protein expression of kisspeptin-1 (KISS1) and the kisspeptin-1 receptor (KISS1R) in pregnancy complicated by diabetes mellitus or preeclampsia. *Arch Gynecol Obstet* 2020;**301**:437–445.

Karmakar S, Das C. Regulation of trophoblast invasion by IL-1β and TGF-β1. *Am J Reprod Immunol* 2002;**48**:210–219.

Karvas RM, Khan SA, Verma S, Yin Y, Kulkarni D, Dong C, Park K, Mi Chew B, Sane E, Fischer LA *et al.* Stem-cell-derived trophoblast organoids model human placental development and susceptibility to emerging pathogens. *Cell Stem Cell* 2022;**29**:810–825.e8.

Keelan JA, Groome NP, Mitchell MD. Regulation of activin-A production by human amnion, decidua and placenta *in vitro* by pro-inflammatory cytokines. *Placenta* 1998;**19**:429–434.

Keskin DB, Allan DSJ, Rybalov B, Andzelm MM, Stern JNH, Kopcow HD, Koopman LA, Strominger JL. TGFβ promotes conversion of CD16+ peripheral blood NK cells into CD16-NK cells with similarities to decidual NK cells. *Proc Natl Acad Sci USA* 2007;**104**:3378–3383.

Knöfler M, Haider S, Saleh L, Pollheimer J, Gamage TKJB, James J. Human placenta and trophoblast development: key molecular mechanisms and model systems. *Cell Mol Life Sci* 2019;**76**:3479–3496.

Knöfler M, Pollheimer J. Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. *Front Genet* 2013;**4**:190.

Kobayashi T, Lyons KM, McMahon AP, Kronenberg HM. BMP signaling stimulates cellular differentiation at multiple steps during cartilage development. *Proc Natl Acad Sci USA* 2005;**102**:18023–18027.

Koel M, Võsa U, Krjutškov K, Einarsdottir E, Kere J, Tapanainen J, Katayama S, Ingerpuu S, Jaks V, Stenman UH *et al.* Optimizing bone morphogenic protein 4-mediated human embryonic stem cell differentiation into trophoblast-like cells using fibroblast growth factor 2 and transforming growth factor-β/activin/nodal signalling inhibition. *Reprod Biomed Online* 2017;**35**:253–263.

Kojima J, Fukuda A, Taira H, Kawasaki T, Ito H, Kuji N, Isaka K, Umezawa A, Akutsu H. Efficient production of trophoblast lineage cells from human induced pluripotent stem cells. *Lab Invest* 2017;**97**:1188–1200.

Kokkinos MI, Murthi P, Wafai R, Thompson EW, Newgreen DF. Cadherins in the human placenta—epithelial–mesenchymal transition (EMT) and placental development. *Placenta* 2010;**31**:747–755.

Krop J, Heidt S, Claas FHJ, Eikmans M. Regulatory T cells in pregnancy: it is not all about FoxP3. *Front Immunol* 2020;**11**:1182.

Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-β – an excellent servant but a bad master. *J Transl Med* 2012;**10**:1–24.

Kupper N, Pritz E, Siwetz M, Guettler J, Huppertz B. Placental villous explant culture 2.0: flow culture allows studies closer to the in vivo situation. *Int J Mol Sci* 2021;**22**:7464.

Laiho M, Saksela O, Keski-Oja J. Transforming growth factor-beta induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. *J Biol Chem* 1987;**262**:17467–17474.

Lash GE, Otun HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Inhibition of trophoblast cell invasion by TGFB1, 2, and 3 is associated with a decrease in active proteases. *Biol Reprod* 2005;**73**:374–381.

Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, Robson SC, Bulmer JN. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol* 2006;**80**:572–580.

Laverty HG, Wakefield LM, Occleston NL, O'Kane S, Ferguson MWJ. TGF-β3 and cancer: a review. *Cytokine Growth Factor Rev* 2009;**20**:305–317.

Lawera A, Tong Z, Thorikay M, Redgrave RE, Cai J, van Dinther M, Morrell NW, Afink GB, Charnock-Jones DS, Arthur HM *et al.* Role of soluble endoglin in BMP9 signaling. *Proc Natl Acad Sci USA* 2019;**116**:17800–17808.

Leaños-Miranda A, Navarro-Romero CS, Sillas-Pardo LJ, Ramírez-Valenzuela KL, Isordia-Salas I, Jiménez-Trejo LM. Soluble endoglin as a marker for preeclampsia, its severity, and the occurrence of adverse outcomes. *Hypertension* 2019;**74**:991–997.

Leavey K, Benton SJ, Grynspan D, Kingdom JC, Bainbridge SA, Cox BJ. Unsupervised placental gene expression profiling identifies clinically relevant subclasses of human preeclampsia. *Hypertension* 2016;**68**:137–147.

Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-β receptor function in the endothelium. *Cardiovasc Res* 2005;**65**:599–608.

Lebrin F, Goumans MJ, Jonker L, Carvalho RLC, Valdimarsdottir G, Thorikay M, Mummery C, Arthur HM, Ten Dijke P. Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. *EMBO J* 2004;**23**:4018–4028.

Lee JS, Romero R, Han YM, Kim HC, Kim CJ, Hong JS, Huh D. Placenta-on-a-chip: a novel platform to study the biology of the human placenta. *J Matern Fetal Neonatal Med* 2016;**29**:1046–1054.

Li H, Cao R, Bai L, Qiao XM, Zhao YQ. Lefty promotes the proliferation and invasion of trophoblast cells by inhibiting nodal expression. *Cell Biol Int* 2018;**42**:1259–1264.

Li H, Peng H, Hong W, Wei Y, Tian H, Huang X, Jia L, Zheng J, Duan T, He Q *et al.* Human placental endothelial cell and trophoblast heterogeneity and differentiation revealed by single-cell RNA sequencing. *Cells* 2023a;**12**:87.

Li S, Wang Y, Cao B, Wu Y, Ji L, Li YX, Liu M, Zhao Y, Qiao J, Wang H *et al.* Maturation of growth differentiation factor 15 in human placental trophoblast cells depends on the interaction with matrix metalloproteinase-26. *J Clin Endocrinol Metab* 2014a;**99**:E2277–E2287.

Li X, Li ZH, Wang YX, Liu TH. A comprehensive review of human trophoblast fusion models: recent developments and challenges. *Cell Death Discov* 2023b;**9**:1–14.

Li X, Liu L, Whitehead C, Li J, Thierry B, Le TD, Winter M. Identifying preeclampsia-associated genes using a control theory method. *Brief Funct Genomics* 2022;**21**:296–309.

Li XL, Chen TT, Dong X, Gou WL, Lau S, Stone P, Chen Q. Early onset preeclampsia in subsequent pregnancies correlates with early onset preeclampsia in first pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2014b;**177**:94–99.

Li Y, Klausen C, Cheng JC, Zhu H, Leung PCK. Activin A, B, and AB increase human trophoblast cell invasion by up-regulating N-cadherin. *J Clin Endocrinol Metab* 2014c;**99**:E2216–E2225.

Li Y, Klausen C, Zhu H, Leung PCK. Activin A increases human trophoblast invasion by inducing SNAIL-mediated MMP2 up-regulation through ALK4. *J Clin Endocrinol Metab* 2015a; **100**:E1415–E1427.

Li Y, Yan J, Chang HM, Chen ZJ, Leung PCK. Roles of TGF-β superfamily proteins in extravillous trophoblast invasion. *Trends Endocrinol Metab* 2021;**32**:170–189.

Li Y, Zhao YJ, Zou QY, Zhang K, Wu YM, Zhou C, Wang K, Zheng J. Preeclampsia does not alter vascular growth and expression of CD31 and vascular endothelial cadherin in human placentas. *J Histochem Cytochem* 2015b;**63**:22–31.

Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y, Quigley JP, French DL, Fisher SJ. Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. *J Biol Chem* 1994;**269**:17125–17131.

Lin C, He H, Cui N, Ren Z, Zhu M, Khalil RA. Decreased uterine vascularization and uterine arterial expansive remodeling with reduced matrix metalloproteinase-2 and -9 in hypertensive pregnancy. *Am J Physiol Heart Circ Physiol* 2020;**318**:H165–H180.

Liu E, Liu Z, Zhou Y, Chen M, Wang L, Li J. MicroRNA-142-3p inhibits trophoblast cell migration and invasion by disrupting the TGF-β1/Smad3 signaling pathway. *Mol Med Rep* 2019;**49**:3775–3782.

Liu X, Wang G, Huang H, Lv X, Si Y, Bai L, Wang G, Li Q, Yang W. Exploring maternal-fetal interface with in vitro placental and trophoblastic models. *Front Cell Dev Biol* 2023;**11**:1279227.

Liu Y, Gao S, Zhao Y, Wang H, Pan Q, Shao Q. Decidual natural killer cells: a good nanny at the maternal-fetal interface during early pregnancy. *Front Immunol* 2021;**12**:663660.

Loewendorf AI, Nguyen TA, Yesayan MN, Kahn DA. Preeclampsia is characterized by fetal NK cell activation and a reduction in regulatory T cells. *Am J Reprod Immunol* 2015;**74**:258–267.

Lokki AI, Daly E, Triebwasser M, Kurki MI, Roberson EDO, Häppölä P, Auro K, Perola M, Heinonen S, Kajantie E *et al.* Protective low-frequency variants for preeclampsia in the Fms related tyrosine kinase 1 gene in the Finnish population. *Hypertension* 2017; **70**:365–371.

Luo K. Signaling cross talk between TGF-β/Smad and other signaling pathways. *Cold Spring Harb Perspect Biol* 2017;**9**:a022137.

Lyall F, Robson SC, Bulmer JN. Spiral artery remodeling and trophoblast invasion in preeclampsia and fetal growth restriction relationship to clinical outcome. *Hypertension* 2013;**62**:1046–1054.

Lyall F, Simpson H, Bulmer JN, Barber A, Robson SC. Transforming growth factor-β expression in human placenta and placental bed in third trimester normal pregnancy, preeclampsia, and fetal growth restriction. *Am J Pathol* 2001;**159**:1827–1838.

Ma J, Sanchez-Duffhues G, Goumans MJ, ten Dijke P. TGF-β-induced endothelial to mesenchymal transition in disease and tissue engineering. *Front Cell Dev Biol* 2020a;**8**:260.

Ma J, van der Zon G, Gonçalves MAFV, van Dinther M, Thorikay M, Sanchez-Duffhues G, ten Dijke P. TGF-β-induced endothelial to mesenchymal transition is determined by a balance between SNAIL and ID factors. *Front Cell Dev Biol* 2021;**9**:616610.

Ma Y, Yang Q, Fan M, Zhang L, Gu Y, Jia W, Li Z, Wang F, Li Y, Xia Wang J *et al.* Placental endovascular extravillous trophoblasts (enEVTs) educate maternal T-cell differentiation along the maternal-placental circulation. *Cell Prolif* 2020b;**53**:e12802.

Makris A, Yeung KR, Lim SM, Sunderland N, Heffernan S, Thompson JF, Iliopoulos J, Killingsworth MC, Yong J, Xu B *et al.* Placental growth factor reduces blood pressure in a uteroplacental ischemia model of preeclampsia in nonhuman primates. *Hypertension* 2016;**67**:1263–1272.

Manuelpillai U, Schneider-Kolsky M, Dole A, Wallace EM. Activin A and activin receptors in gestational tissue from preeclamptic pregnancies. *J Endocrinol* 2001;**171**:57–64.

Manuelpillai U, Schneider-Kolsky M, Thirunavukarasu P, Dole A, Waldron K, Wallace EM. Effect of hypoxia on placental activin a, inhibin a and follistatin synthesis. *Placenta* 2003;**24**:77–83.

Margioula-Siarkou G, Margioula-Siarkou C, Petousis S, Margaritis K, Vavoulidis E, Gullo G, Alexandratou M, Dinas K, Sotiriadis A, Mavromatidis G. The role of endoglin and its soluble form in pathogenesis of preeclampsia. *Mol Cell Biochem* 2021;**477**:479–491.

Marjono AB, Brown DA, Horton KE, Wallace EM, Breit SN, Manuelpillai U. Macrophage inhibitory cytokine-1 in gestational tissues and maternal serum in normal and pre-eclamptic pregnancy. *Placenta* 2003;**24**:100–106.

Marshall SA, Hannan NJ, Jelinic M, Nguyen TPH, Girling JE, Parry LJ. Animal models of preeclampsia: translational failings and why. *Am J Physiol Regul Integr Comp Physiol* 2018;**314**:R499–R508.

Matsuzaki K. Smad phosphoisoform signaling specificity: the right place at the right time. *Carcinogenesis* 2011;**32**:1578–1588.

Meekins JW, Pijnenborg R, Hanssens M, MCFadyen IR, van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. *Br J Obstet Gynaecol* 1994;**101**:669–674.

Mercnik MH, Schliefsteiner C, Fluhr H, Wadsack C. Placental macrophages present distinct polarization pattern and effector functions depending on clinical onset of preeclampsia. *Front Immunol* 2022;**13**:1095879.

Mezouar S, Katsogiannou M, Ben Amara A, Bretelle F, Mege JL. Placental macrophages: origin, heterogeneity, function and role in pregnancy-associated infections. *Placenta* 2021;**103**:94–103.

Miyazono K, Maeda S, Imamura T. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* 2005;**16**:251–263.

Moustakas A, Heldin CH. The regulation of TGFβ signal transduction. *Development* 2009;**136**:3699–3714.

Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-β signaling in regulation of cell growth and differentiation. *Immunol Lett* 2002;**82**:85–91.

Msheik H, El Hayek S, Bari MF, Azar J, Abou-Kheir W, Kobeissy F, Vatish M, Daoud G. Transcriptomic profiling of trophoblast fusion using BeWo and JEG-3 cell lines. *Mol Hum Reprod* 2019; **25**:811–824.

Mullican SE, Lin-Schmidt X, Chin CN, Chavez JA, Furman JL, Armstrong AA, Beck SC, South VJ, Dinh TQ, Cash-Mason TD *et al.* GFRAL is the receptor for GDF15 and the ligand promotes weight loss in mice and nonhuman primates. *Nat Med* 2017;**23**:1150–1157.

Munir S, Xu G, Wu Y, Yang B, Lala PK, Peng C. Nodal and ALK7 inhibit proliferation and induce apoptosis in human trophoblast cells. *J Biol Chem* 2004;**279**:31277–31286.

Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T *et al.* Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014;**41**:14–20.

Murray PJ, Wynn TA. Obstacles and opportunities for understanding macrophage polarization. *J Leukoc Biol* 2011;**89**:557–563.

Mylonas I, Schiessl B, Jeschke U, Vogl J, Makrigiannakis A, Kuhn C, Kunze S, Schulze S, Kainer F, Friese K. Expression of inhibin/activin subunits alpha (-α), beta A (-β A) and beta B (-β B) in placental tissue of normal and intrauterine growth restricted (IUGR) pregnancies. *J Mol Histol* 2006;**37**:43–52.

Mylonas I, Schiessl B, Jeschke U, Vogl J, Makrigiannakis A, Kuhn C, Schulze S, Kainer F, Friese K. Expression of inhibin/activin subunits alpha (-α), betaA (-βA), and betaB (-βB) in placental tissue of normal, preeclamptic, and HELLP pregnancies. *Endocr Pathol* 2006;**17**:19–33.

Nadeem L, Munir S, Fu G, Dunk C, Baczyk D, Caniggia I, Lye S, Peng C. Nodal signals through activin receptor-like kinase 7 to inhibit trophoblast migration and invasion: implication in the pathogenesis of preeclampsia. *Am J Pathol* 2011;**178**:1177–1189.

Namwanje M, Brown CW. Activins and inhibins: roles in development, physiology, and disease. *Cold Spring Harb Perspect Biol* 2016;**8**:a021881.

Nandi P, Siddiqui MF, Lala PK. Restraint of trophoblast invasion of the uterus by decorin: role in pre-eclampsia. *Am J Reprod Immunol* 2016;**75**:351–360.

Ning F, Liu H, Lash GE. The role of decidual macrophages during normal and pathological pregnancy. *Am J Reprod Immunol* 2016;**75**:298–309.

Nishi H, Nakada T, Hokamura M, Osakabe Y, Itokazu O, Huang LE, Isaka K. Hypoxia-inducible factor-1 transactivates transforming growth factor-β3 in trophoblast. *Endocrinology* 2004;**145**:4113–4118.

Novembri R, Funghi L, Voltolini C, Belmonte G, Vannuccini S, Torricelli M, Petraglia F. Placenta expresses anti-Müllerian hormone and its receptor: sex-related difference in fetal membranes. *Placenta* 2015;**36**:731–737.

Oettel A, Lorenz M, Stangl V, Costa SD, Zenclussen AC, Schumacher A. Human umbilical vein endothelial cells foster conversion of CD4+ CD25− FOXP3− T cells into CD4+ FOXP3+ regulatory T cells via transforming growth factor-β. *Sci Rep* 2016;**6**:23278.

Ogasawara MS, Aoki K, Aoyama T, Katano K, Iinuma Y, Ozaki Y, Suzumori K. Elevation of transforming growth factor-beta1 is associated with recurrent miscarriage. *J Clin Immunol* 2000;**20**:453–457.

Oh SY, Song SE, Seo ES, Kim KH, Choi SJ, Suh YL, Sadovsky Y, Roh CR. The expression of connective tissue growth factor in pregnancies complicated by severe preeclampsia or fetal growth restriction. *Placenta* 2009;**30**:981–987.

Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, Kabayama Y, Suyama M, Sasaki H, Arima T. Derivation of human trophoblast stem cells. *Cell Stem Cell* 2018;**22**:50–63.e6.

Olsen OE, Skjærvik A, Størdal BF, Sundan A, Holien T. TGF-β contamination of purified recombinant GDF15. *PLoS One* 2017;**12**:e0187349.

Parada-Niño L, Castillo-León LF, Morel A. Preeclampsia, natural history, genes, and miRNAs associated with the syndrome. *J Pregnancy* 2022;**2022**:3851225.

Park CB, DeMayo FJ, Lydon JP, Dufort D. NODAL in the uterus is necessary for proper placental development and maintenance of pregnancy. *Biol Reprod* 2012;**86**:194.

Park JY, Mani S, Clair G, Olson HM, Paurus VL, Ansong CK, Blundell C, Young R, Kanter J, Gordon S *et al.* A microphysiological model of human trophoblast invasion during implantation. *Nat Commun* 2022;**13**:1–18.

Pastuschek J, Nonn O, Gutiérrez-Samudio RN, Murrieta-Coxca JM, Müller J, Sanft J, Huppertz B, Markert UR, Groten T, Morales-Prieto DM. Molecular characteristics of established trophoblast-derived cell lines. *Placenta* 2021;**108**:122–133.

Pauklin S, Vallier L. Activin/Nodal signalling in stem cells. *Development* 2015;**142**:607–619.

Pavlov OV, Selutin AV, Pavlova OM, Selkov SA. Two patterns of cytokine production by placental macrophages. *Placenta* 2020;**91**:1–10.

Peiris HN, Mitchell MD. The expression and potential functions of placental myostatin. *Placenta* 2012;**33**:902–907.

Peiris HN, Salomon C, Payton D, Ashman K, Vaswani K, Chan A, Rice GE, Mitchell MD. Myostatin is localized in extravillous trophoblast and up-regulates migration. *J Clin Endocrinol Metab* 2014;**99**:E2288–E2297.

Peraçoli MTS, Menegon FTF, Borges VTM, De Araújo Costa RA, Thomazini-Santos IA, Peraçoli JC. Platelet aggregation and TGF-beta(1) plasma levels in pregnant women with preeclampsia. *J Reprod Immunol* 2008;**79**:79–84.

Perucci LO, Gomes KB, Freitas LG, Godoi LC, Alpoim PN, Pinheiro MB, Miranda AS, Teixeira AL, Dusse LM, Sousa LP. Soluble endoglin, transforming growth factor-beta 1 and soluble tumor necrosis factor alpha receptors in different clinical manifestations of pre-eclampsia. *PLoS One* 2014;**9**:e97632.

Petraglia F, Anceschi MM, Calza L, Garuti GC, Fusaro P, Giardino L, Genazzani AR, Vale W. Inhibin and activin in human fetal membranes: evidence for a local effect on prostaglandin release. *J Clin Endocrinol Metab* 1993;**77**:542–548.

Petraglia F, Gallinelli A, Grande A, Florio P, Ferrari S, Genazzani AR, Ling N, DePaolo LV. Local production and action of follistatin in human placenta. *J Clin Endocrinol Metab* 1994;**78**:205–210.

Petraglia F, Vaughan J, Vale W. Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placental cells. *Proc Natl Acad Sci USA* 1989;**86**:5114–5117.

Phipps EA, Thadhani R, Benzing T, Karumanchi SA. Pre-eclampsia: pathogenesis, novel diagnostics and therapies. *Nat Rev Nephrol* 2019;**15**:275–289.

Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L, van Assche A. Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Br J Obstet Gynaecol* 1991;**98**:648–655.

Poniatowski AA, Wojdasiewicz P, Gasik R, Szukiewicz D. Transforming growth factor beta family: insight into the role of growth factors in regulation of fracture healing biology and potential clinical applications. *Mediators Inflamm* 2015;**2015**:137823.

Possomato-Vieira JS, Khalil RA. Mechanisms of endothelial dysfunction in hypertensive pregnancy and preeclampsia. *Adv Pharmacol* 2016;**77**:361–431.

Pryor-Koishi K, Nishizawa H, Kato T, Kogo H, Murakami T, Tsuchida K, Kurahashi H, Udagawa Y. Overproduction of the follistatin-related gene protein in the placenta and maternal serum of women with pre-eclampsia. *BJOG* 2007;**114**:1128–1137.

Quinn KH, Lacoursiere DY, Cui L, Bui J, Parast MM. The unique pathophysiology of early-onset severe preeclampsia: role of decidual T regulatory cells. *J Reprod Immunol* 2011;**91**:76–82.

Raguema N, Moustadraf S, Bertagnolli M. Immune and apoptosis mechanisms regulating placental development and vascularization in preeclampsia. *Front Physiol* 2020;**11**:98.

Rana S, Lemoine E, Granger J, Karumanchi SA. Preeclampsia: pathophysiology, challenges, and perspectives. *Circ Res* 2019;**124**:1094–1112.

Redline RW, Patterson P. Pre-eclampsia is associated with an excess of proliferative immature intermediate trophoblast. *Hum Pathol* 1995;**26**:594–600.

Reissmann E, Jörnvall H, Blokzijl A, Andersson O, Chang C, Minchiotti G, Persico MG, Ibáñez CF, Brivanlou AH. The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by nodal proteins during vertebrate development. *Genes Dev* 2001;**15**:2010–2022.

Reyes L, Golos TG. Hofbauer cells: their role in healthy and complicated pregnancy. *Front Immunol* 2018;**9**:2628.

Reyes L, Wolfe B, Golos T. Hofbauer cells: placental macrophages of fetal origin. *Results Probl Cell Differ* 2017;**62**:45–60.

Riley SC, Leask R, Balfour C, Brennand JE, Groome NP. Production of inhibin forms by the fetal membranes, decidua, placenta and fetus at parturition. *Hum Reprod* 2000;**15**:578–583.

Roberts RM, Ezashi T, Sheridan MA, Yang Y. Specification of trophoblast from embryonic stem cells exposed to BMP4. *Biol Reprod* 2018;**99**:212–224.

Roberts RM, Ezashi T, Temple J, Owen JR, Soncin F, Parast MM. The role of BMP4 signaling in trophoblast emergence from pluripotency. *Cell Mol Life Sci* 2022;**79**:1–22.

Rochette L, Zeller M, Cottin Y, Vergely C. Insights into mechanisms of GDF15 and receptor GFRAL: therapeutic targets. *Trends Endocrinol Metab* 2020;**31**:939–951.

Roten LT, Johnson MP, Forsmo S, Fitzpatrick E, Dyer TD, Brennecke SP, Blangero J, Moses EK, Austgulen R. Association between the candidate susceptibility gene ACVR2A on chromosome 2q22 and pre-eclampsia in a large Norwegian population-based study (the HUNT study). *Eur J Hum Genet* 2008;**17**:250–257.

Rudnicka E, Kunicki M, Calik-Ksepka A, Suchta K, Duszewska A, Smolarczyk K, Smolarczyk R. Anti-Müllerian hormone in pathogenesis, diagnostic and treatment of PCOS. *Int J Mol Sci* 2021; **22**:12507.

Runyan CE, Schnaper HW, Poncelet AC. The phosphatidylinositol 3-kinase/Akt pathway enhances Smad3-stimulated mesangial cell collagen i expression in response to transforming growth factor-β1. *J Biol Chem* 2004;**279**:2632–2639.

Sánchez-Duffhues G, García de Vinuesa A, van de Pol V, Geerts ME, de Vries MR, Janson SGT, van Dam H, Lindeman JH, Goumans MJ, ten Dijke P. Inflammation induces endothelial-to-mesenchymal transition and promotes vascular calcification through downregulation of BMPR2. *J Pathol* 2019;**247**:333–346.

Sarkar P, Randall SM, Collier TS, Nero A, Russell TA, Muddiman DC, Rao BM. Activin/nodal signaling switches the terminal fate of human embryonic stem cell-derived trophoblasts. *J Biol Chem* 2015; **290**:8834–8848.

Sasaki Y, Darmochwal-Kolarz D, Suzuki D, Sakai M, Ito M, Shima T, Shiozaki A, Rolinski J, Saito S. Proportion of peripheral blood and decidual CD4+ CD25(bright) regulatory T cells in pre-eclampsia. *Clin Exp Immunol* 2007;**149**:139–145.

Schäffer L, Scheid A, Spielmann P, Breymann C, Zimmermann R, Meuli M, Gassmann M, Marti HH, Wenger RH. Oxygen-regulated expression of TGF-β3, a growth factor involved in trophoblast differentiation. *Placenta* 2003;**24**:941–950.

Schliefsteiner C, Ibesich S, Wadsack C. Placental Hofbauer cell polarization resists inflammatory cues in vitro. *Int J Mol Sci* 2020; **21**:736.

Schliefsteiner C, Peinhaupt M, Kopp S, Lögl J, Lang-Olip I, Hiden U, Heinemann A, Desoye G, Wadsack C. Human placental Hofbauer cells maintain an anti-inflammatory M2 phenotype despite the presence of gestational diabetes mellitus. *Front Immunol* 2017; **8**:888.

Schneider-Kolsky ME, Manuelpillai U, Waldron K, Dole A, Wallace EM. The distribution of activin and activin receptors in gestational tissues across human pregnancy and during labour. *Placenta* 2002;**23**:294–302.

Segerer SE, Rieger L, Kapp M, Dombrowski Y, Müller N, Dietl J, Kämmerer U. MIC-1 (a multifunctional modulator of dendritic cell phenotype and function) is produced by decidual stromal cells and trophoblasts. *Hum Reprod* 2012;**27**:200–209.

Shaarawy M, Meleigy ME, Rasheed K. Maternal serum transforming growth factor beta-2 in preeclampsia and eclampsia, a potential biomarker for the assessment of disease severity and fetal outcome. *J Soc Gynecol Investig* 2016;**8**:27–31.

Sheridan MA, Fernando RC, Gardner L, Hollinshead MS, Burton GJ, Moffett A, Turco MY. Establishment and differentiation of long-term trophoblast organoid cultures from the human placenta. *Nat Protoc* 2020;**15**:3441–3463.

Sheridan MA, Zhao X, Fernando RC, Gardner L, Perez-Garcia V, Li Q, Marsh SGE, Hamilton R, Moffett A, Turco MY. Characterization of primary models of human trophoblast. *Development* 2021; **148**:dev199749.

Sieber C, Kopf J, Hiepen C, Knaus P. Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev* 2009;**20**:343–355.

Sierra-Filardi E, Puig-Kröger A, Blanco FJ, Nieto C, Bragado R, Palomero MI, Bernabéu C, Vega MA, Corbí AL. Activin A skews macrophage polarization by promoting a proinflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers. *Blood* 2011;**117**:5092–5101.

Simpson H, Robson SC, Bulmer JN, Barber A, Lyall F. Transforming growth factor β expression in human placenta and placental bed during early pregnancy. *Placenta* 2002;**23**:44–58.

Sohlberg E, Saghafian-Hedengren S, Bachmayer N, Hamad RR, Bremme K, Holmlund U. Pre-eclampsia affects cord blood NK cell expression of activation receptors and serum cytokine levels but not CB monocyte characteristics. *Am J Reprod Immunol* 2014; **71**:178–188.

Soncin F, Morey R, Bui T, Requena DF, Cheung VC, Kallol S, Kittle R, Jackson MG, Farah O, Chousal J *et al.* Derivation of functional trophoblast stem cells from primed human pluripotent stem cells. *Stem Cell Reports* 2022;**17**:1303–1317.

Song Y, Keelan J, France JT. Activin-A stimulates, while transforming growth factor β1 inhibits,chorionic gonadotrophin production and aromatase activity in cultured human placental trophoblasts. *Placenta* 1996;**17**:603–610.

Stepan H, Hund M, Andraczek T. Combining biomarkers to predict pregnancy complications and redefine preeclampsia. *Hypertension* 2020;**75**:918–926.

Stewart A, Guan H, Yang K. BMP-3 promotes mesenchymal stem cell proliferation through the TGF-beta/activin signaling pathway. *J Cell Physiol* 2010;**223**:658–666.

Sudheer S, Bhushan R, Fauler B, Lehrach H, Adjaye J. FGF inhibition directs BMP4-mediated differentiation of human embryonic stem cells to syncytiotrophoblast. *Stem Cells Dev* 2012; **21**:2987–3000.

Sugulle M, Dechend R, Herse F, Weedon-Fekjaer MS, Johnsen GM, Brosnihan KB, Anton L, Luft FC, Wollert KC, Kempf T *et al.* Circulating and placental growth-differentiation factor 15 in preeclampsia and in pregnancy complicated by diabetes mellitus. *Hypertension* 2009;**54**:106–112.

Svensson-Arvelund J, Mehta RB, Lindau R, Mirrasekhian E, Rodriguez-Martinez H, Berg G, Lash GE, Jenmalm MC, Ernerudh J. The human fetal placenta promotes tolerance against the semiallogeneic fetus by inducing regulatory T cells and homeostatic M2 macrophages. *J Immunol* 2015;**194**:1534–1544.

Tal R. The role of hypoxia and hypoxia-inducible factor-1alpha in preeclampsia pathogenesis. *Biol Reprod* 2012;**87**:134–135.

Tannetta DS, Sargent IL, Linton EA, Redman CWG. Vitamins C and E inhibit apoptosis of cultured human term placenta trophoblast. *Placenta* 2008;**29**:680–690.

Tejera E, Bernardes J, Rebelo I. Co-expression network analysis and genetic algorithms for gene prioritization in preeclampsia. *BMC Med Genomics* 2013;**6**:51–10.

Than NG, Romero R, Tarca AL, Kekesi KA, Xu Y, Xu Z, Juhasz K, Bhatti G, Leavitt RJ, Gelencser Z *et al.* Integrated systems biology approach identifies novel maternal and placental pathways of preeclampsia. *Front Immunol* 2018;**9**:1661.

Thulluru HK, Park C, Dufort D, Kleiverda G, Oudejans C, van Dijk M. Maternal nodal inversely affects NODAL and STOX1 expression in the fetal placenta. *Front Genet* 2013;**4**:170.

Tilburgs T, Scherjon SA, van der Mast BJ, Haasnoot GW, Versteeg-V D, Voort-Maarschalk M, Roelen DL, van Rood JJ, Claas FHJ. Fetal–maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. *J Reprod Immunol* 2009;**82**:148–157.

Tranquilli AL, Brown MA, Zeeman GG, Dekker G, Sibai BM. The definition of severe and early-onset preeclampsia. Statements from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Pregnancy Hypertens* 2013;**3**:44–47.

Tse WK, Whitley GSJ, Cartwright JE. Transforming growth factor-β1 regulates hepatocyte growth factor-induced trophoblast motility and invasion. *Placenta* 2002;**23**:699–705.

Tsuchida K, Nakatani M, Hitachi K, Uezumi A, Sunada Y, Ageta H, Inokuchi K. Activin signaling as an emerging target for therapeutic interventions. *Cell Commun Signal* 2009;**7**:1–11.

Turco MY, Gardner L, Kay RG, Hamilton RS, Prater M, Hollinshead MS, McWhinnie A, Esposito L, Fernando R, Skelton H *et al.* Trophoblast organoids as a model for maternal–fetal interactions during human placentation. *Nature* 2018;**564**:263–267.

Tzavlaki K, Moustakas A. TGF-β signaling. *Biomolecules* 2020;**10**:487.

Valensise H, Vasapollo B, Gagliardi G, Novelli GP. Early and late preeclampsia. *Hypertension* 2008;**52**:873–880.

van Caam A, Madej W, Garcia de Vinuesa A, Goumans MJ, ten Dijke P, Blaney Davidson E, van der Kraan P. TGFβ1-induced SMAD2/3 and SMAD1/5 phosphorylation are both ALK5-kinase-dependent in primary chondrocytes and mediated by TAK1 kinase activity. *Arthritis Res Ther* 2017;**19**:112.

Vanwijk MJ, Kublickiene K, Boer K, VanBavel E. Vascular function in preeclampsia. *Cardiovasc Res* 2000;**47**:38–48.

Venkatesha S, Toporsian M, Lam C, Hanai JI, Mammoto T, Kim YM, Bdolah Y, Lim KH, Yuan HT, Libermann TA *et al.* Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* 2006; **12**:642–649.

Vogt J, Traynor R, Sapkota GP. The specificities of small molecule inhibitors of the TGFß and BMP pathways. *Cell Signal* 2011; **23**:1831–1842.

von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. *Hypertens Pregnancy* 2009;**22**:143–148. https://doi.org/101081/PRG-120021060

Vondra S, Höbler AL, Lackner AI, Raffetseder J, Mihalic ZN, Vogel A, Saleh L, Kunihs V, Haslinger P, Wahrmann M *et al.* The human placenta shapes the phenotype of decidual macrophages. *Cell Rep* 2023;**42**:111977.

Walker RG, Czepnik M, Goebel EJ, McCoy JC, Vujic A, Cho M, Oh J, Aykul S, Walton KL, Schang G *et al.* Structural basis for potency differences between GDF8 and GDF11. *BMC Biol* 2017;**15**:19–22.

Walshe TE, Dole VS, Maharaj ASR, Patten IS, Wagner DD, D'Amore PA. Inhibition of VEGF or TGF-β signaling activates endothelium and increases leukocyte rolling. *Arterioscler Thromb Vasc Biol* 2009; **29**:1185–1192.

Wang HQ, Takebayashi K, Tsuchida K, Nishimura M, Noda Y. Follistatin-related gene (FLRG) expression in human endometrium: sex steroid hormones regulate the expression of FLRG in cultured human endometrial stromal cells. *J Clin Endocrinol Metab* 2003;**88**:4432–4439.

Wang L, Wang H, Luo J, Xie T, Mor G, Liao A. Decorin promotes decidual M1-like macrophage polarization via mitochondrial dysfunction resulting in recurrent pregnancy loss. *Theranostics* 2022; **12**:7216–7236.

Wang RN, Green J, Wang Z, Deng Y, Qiao M, Peabody M, Zhang Q, Ye J, Yan Z, Denduluri S *et al.* Bone morphogenetic protein (BMP) signaling in development and human diseases. *Genes Dis* 2014; **1**:87–105.

Wang Y, Lewis DF, Gu Y, Zhang Y, Alexander JS, Granger DN. Placental trophoblast-derived factors diminish endothelial barrier function. *J Clin Endocrinol Metab* 2004;**89**:2421–2428.

Wei X, Yang X. The central role of natural killer cells in preeclampsia. *Front Immunol* 2023;**14**:1009867.

Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-β. *Future Oncol* 2009; **5**:1145–1168.

Wilczynski JR, Radwan M, Kalinka J. The characterization and role of regulatory T cells in immune reactions. *Front Biosci* 2008; **13**:2266–2274.

Williams PJ, Searle RF, Robson SC, Innes BA, Bulmer JN. Decidual leucocyte populations in early to late gestation normal human pregnancy. *J Reprod Immunol* 2009;**82**:24–31.

Wójtowicz A, Zembala-Szczerba M, Babczyk D, Kołodziejczyk-Pietruszka M, Lewaczyńska O, Huras H. Early- and late-onset preeclampsia: a comprehensive cohort study of laboratory and clinical findings according to the new ISHHP criteria. *Int J Hypertens* 2019;**2019**:4108271.

Wrighton KH, Lin X, Feng XH. Phospho-control of TGF-β superfamily signaling. *Cell Res* 2009;**19**:8–20.

Wu K, Liu F, Wu W, Chen Y, Zhang W. Bioinformatics approach reveals the critical role of TGF-β signaling pathway in preeclampsia development. *Eur J Obstet Gynecol Reprod Biol* 2019; **240**:130–138.

Wu L, Luo LH, Zhang YX, Li Q, Xu B, Zhou GX, Luan HB, Liu YS. Alteration of Th17 and Treg cells in patients with unexplained recurrent spontaneous abortion before and after lymphocyte immunization therapy. *Reprod Biol Endocrinol* 2014;**12**:74.

Wu Z, Fang L, Yang S, Gao Y, Wang Z, Meng Q, Dang X, Sun YP, Cheng JC. GDF-11 promotes human trophoblast cell invasion by increasing ID2-mediated MMP2 expression. *Cell Commun Signal* 2022;**20**:13.

Xiao YT, Xiang LX, Shao JZ. Bone morphogenetic protein. *Biochem Biophys Res Commun* 2007;**362**:550–553.

Xie J, Zhu H, Chang HM, Klausen C, Dong M, Leung PCK. GDF8 promotes the cell invasiveness in human trophoblasts by upregulating the expression of follistatin-like 3 through the ALK5-SMAD2/3 signaling pathway. *Front Cell Dev Biol* 2020;**8**:573781.

Xu Y-T, Shen M-H, Jin A-Y, Zhu R. Maternal circulating levels of transforming growth factor-β superfamily and its soluble receptors in hypertensive disorders of pregnancy. *Int J Gynaecol Obstet* 2017;**137**:246–252.

Xu J, Sivasubramaniyam T, Yinon Y, Tagliaferro A, Ray J, Nevo O, Post M, Caniggia I. Aberrant TGFβ signaling contributes to altered trophoblast differentiation in preeclampsia. *Endocrinology* 2016; **157**:883–899.

Xu RH, Chen X, Li DS, Li R, Addicks GC, Glennon C, Zwaka TP, Thomson JA. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 2002;**20**:1261–1264.

Xu XH, Jia Y, Zhou X, Xie D, Huang X, Jia L, Zhou Q, Zheng Q, Zhou X, Wang K. Downregulation of lysyl oxidase and lysyl oxidase-like protein 2 suppressed the migration and invasion of trophoblasts by activating the TGF-β/collagen pathway in preeclampsia. *Exp Mol Med* 2019;**51**:1–12.

Xuan YH, Choi YL, Shin YK, Ahn GH, Kim KH, Kim WJ, Lee HC, Kim SH. Expression of TGF-beta signaling proteins in normal placenta and gestational trophoblastic disease. *Histol Histopathol* 2007; **22**:227–234.

Yagel S. The developmental role of natural killer cells at the fetal-maternal interface. *Am J Obstet Gynecol* 2009;**201**:344–350.

Yair D, Eshed-Englender T, Kupferminc MJ, Geva E, Frenkel J, Sherman D. Serum levels of inhibin B, unlike inhibin A and activin A, are not altered in women with preeclampsia. *Am J Reprod Immunol* 2001;**45**:180–187.

Yan X, Liao H, Cheng M, Shi X, Lin X, Feng XH, Chen YG. Smad7 protein interacts with receptor-regulated Smads (R-Smads) to inhibit transforming growth factor-β (TGF-β)/Smad signaling. *J Biol Chem* 2016;**291**:382–392.

Yan X, Liu Z, Chen Y. Regulation of TGF-β signaling by Smad7. *Acta Biochim Biophys Sin (Shanghai)* 2009;**41**:263–272.

Yan X, Xiong X, Chen Y-G. Feedback regulation of TGF-β signaling. *Acta Biochim Biophys Sin (Shanghai)* 2018;**50**:37–50.

Yang D, Dai F, Yuan M, Zheng Y, Liu S, Deng Z, Tan W, Chen L, Zhang Q, Zhao X *et al.* Role of transforming growth factor-β1 in regulating fetal-maternal immune tolerance in normal and pathological pregnancy. *Front Immunol* 2021;**12**:689181.

Yang L, Liang P, Yang H, Coyne CB. Trophoblast organoids with physiological polarity model placental structure and function. bioRxiv. 2023.01.12.523752, 2023, preprint: not peer reviewed.

Yao Y, Xu XH, Jin L. Macrophage polarization in physiological and pathological pregnancy. *Front Immunol* 2019;**10**:792.

Yeo CY, Whitman M. Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* 2001; **7**:949–957.

Yi Y, Zhu H, Klausen C, Chang HM, Inkster AM, Terry J, Leung PCK. Dysregulated BMP2 in the placenta may contribute to early-onset preeclampsia by regulating human trophoblast expression of extracellular matrix and adhesion molecules. *Front Cell Dev Biol* 2021;**9**:768669.

Yong HEJ, Murthi P, Borg A, Kalionis B, Moses EK, Brennecke SP, Keogh RJ. Increased decidual mRNA expression levels of candidate maternal pre-eclampsia susceptibility genes are associated with clinical severity. *Placenta* 2014;**35**:117–124.

Yoshimura A, Wakabayashi Y, Mori T. Cellular and molecular basis for the regulation of inflammation by TGF-β. *J Biochem* 2010;**147**:781–792.

You J, Wang W, Chang HM, Yi Y, Zhao H, Zhu H, Sun Y, Tang M, Wang C, Sang Y *et al.* The BMP2 signaling axis promotes invasive differentiation of human trophoblasts. *Front Cell Dev Biol* 2021; **9**:607332.

Yu L, Li D, Liao QP, Yang HX, Cao B, Fu G, Ye G, Bai Y, Wang H, Cui N *et al.* High levels of activin A detected in preeclamptic placenta induce trophoblast cell apoptosis by promoting nodal signaling. *J Clin Endocrinol Metab* 2012;**97**:E1370–E1379.

Zeng YT, Liu WF, Zheng PS, Li S. GDF15 deficiency hinders human trophoblast invasion to mediate pregnancy loss through downregulating Smad1/5 phosphorylation. *iScience* 2023;**26**:107902.

Zhang L, Li X, Zhou C, You Z, Zhang J, Cao G. The diagnosis values of serum STAT4 and sEng in preeclampsia. *J Clin Lab Anal* 2020; **34**:e23073.

Zhang J, Dunk CE, Shynlova O, Caniggia I, Lye SJ. TGFb1 suppresses the activation of distinct dNK subpopulations in preeclampsia. *EBioMedicine* 2019;**39**:531–539.

Zhang YE. Non-Smad signaling pathways of the TGF-β family. *Cold Spring Harb Perspect Biol* 2017;**9**:a022129.

Zhao HJ, Chang HM, Klausen C, Zhu H, Li Y, Leung PCK. Bone morphogenetic protein 2 induces the activation of WNT/β-catenin signaling and human trophoblast invasion through up-regulating BAMBI. *Cell Signal* 2020a;**67**:109489.

Zhao HJ, Chang HM, Zhu H, Klausen C, Li Y, Leung PCK. Bone morphogenetic protein 2 promotes human trophoblast cell invasion by inducing activin A production. *Endocrinology* 2018a; **159**:2815–2825.

Zhao HJ, Klausen C, Li Y, Zhu H, Wang YL, Leung PCK. Bone morphogenetic protein 2 promotes human trophoblast cell invasion by upregulating N-cadherin via non-canonical SMAD2/3 signaling. *Cell Death Dis* 2018b;**9**:1–12.

Zhao HJ, Klausen C, Zhu H, Chang HM, Li Y, Leung PCK. Bone morphogenetic protein 2 promotes human trophoblast cell invasion and endothelial-like tube formation through ID1-mediated upregulation of IGF binding protein-3. *FASEB J* 2020b;**34**:3151–3164.

Zhou C, Yan Q, Zou QY, Zhong XQ, Tyler CT, Magness RR, Bird IM, Zheng J. Sexual dimorphisms of preeclampsia-dysregulated transcriptomic profiles and cell function in fetal endothelial cells. *Hypertension* 2019;**74**:154–163.

Zhu S, Li Z, Cui L, Ban Y, Leung PCK, Li Y, Ma J. Activin A increases human trophoblast invasion by upregulating integrin β1 through ALK4. *FASEB J* 2021;**35**:e21220.

Zou H, Niswander L. Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* 1996;**272**:738–741.

Human Reproduction Update, 2024, 30, 442–471
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Unlocking the secrets of Hofbauer cells in placental (patho-) physiology: Isolation and quality assessment in human term placenta

Monika Horvat Mercnik [a], Christian Wadsack [a,b,*], Carolin Schliefsteiner [a]

[a] Medical University of Graz, Austria. Department of Obstetrics and Gynecology, Research Unit, Austria
[b] BioTechMed-Graz, Graz, Austria

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ABSTRACT

Introduction: Hofbauer cells (HBCs) are macrophages of fetal origin that reside in the villous tissue. They are the only immune cells within healthy villi. While HBCs perform innate immune functions such as phagocytosis and antigen presentation, they are increasingly recognized for their diverse roles in placental physiology e.g. vascular functionality, tissue homeostasis, tolerance. Consequently, HBCs are of utmost interest in a variety of non-physiological placental conditions.
Isolation: Villous tissue is collected freshly after delivery and finely minced. The resulting tissue is digested in a two-step process, using Trypsin/DNase to separate cytotrophoblasts and collagenase/DNase to penetrate deeper into the villous stroma, containing HBCs, and obtain a single cell suspension. After a density gradient centrifugation, the corresponding cell layer is collected and subjected to negative immune selection of HBCs, yielding unaffected cells that have not been activated during the isolation process.
Quality control: In addition to a classical immunocytochemistry (ICC) approach including macrophage markers, and markers for potentially contaminating cell types (e.g. fibroblasts, muscle, mesenchymal cells), we have developed a multi-color flow cytometry (FC) panel. This panel assesses Hofbauer cell purity and polarization states more accurately and comprehensively than qualitative ICC, using percentage analysis of parent cells to estimate the expression levels of specific markers.
Discussion: The presented protocol allows us to isolate HBCs in significant numbers and high purity, even from placentae compromised by preeclampsia (PE) with limited placental volume. We have successfully developed and implemented this protocol to study healthy, diabetic and PE macrophages, aiding a better understanding of the underlying placental pathophysiology at the cellular level.

1. Introduction

Hofbauer cells (HBCs) are macrophages of the feto-placental unit, residing in placental stroma. They were first described in 1095 by Austrian-American gynecologist Hofbauer, hence their name. It took until the 1980s, however, until these cells were studied intensively. Thanks to sex-chromosome specific genotyping, it is now widely accepted that HBCs are of fetal origin [1]. They perform pleiotropic functions such as microbicidal and phagocytic activities (reviewed in Ref. [2]), scavenging of b-hCG [3] and iron [4], paracrine signaling to the trophoblast [5,6], as well as homeostatic properties in tissue remodeling and angiogenesis [7,8], and likely facilitate tolerance towards the fetus [9,10]. However, they also have a role in the vertical transmission of infection from mother to child [11,12]. Changes of HBC numbers in different pregnancy pathologies have been investigated (reviewed in: [13]). Differences in macrophage polarization along the M1/M2 spectrum in different placental pathologies have also been studied [8,14–19].

However, many of the studies to date have worked on histological sections of placental tissue [20,21] rather than with isolated macrophages, therefore making these studies more descriptive in nature. Here, we present a protocol for macrophage isolation from term placentas, which we apply to both healthy and pathological placentas, yielding high numbers of isolated cells. These primary cells can be used for *in vitro* cell culture and downstream analysis to investigate RNA or protein levels, as well as secreted factors and functional analyses (phagocytosis, etc.). While the protocol is modified from a previously published one by Tang et al. [22], we additionally present a recently established FC

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* Corresponding author. Medical University of Graz, Austria. Department of Obstetrics and Gynecology, Research Unit, Austria.
E-mail address: christian.wadsack@medunigraz.at (C. Wadsack).

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strategy for quality control and rapid assessment of macrophage polarization. The isolation itself is tedious and challenging, and we share our experience and recommendations here, which we hope will be of interest to other research groups.

2. Methods and results

All women who donated their placenta gave written informed consent and the study protocol was approved of by the local ethics board of the Medical University of Graz (29–319 ex 16/17). Placentae from singleton pregnancies, and both spontaneous vaginal births and cesarean sections were used. To obtain HBCs from gestational diabetes mellitus (GDM) pregnancies, only placentae from women with the mildest form of GDM (Classification White A), receiving dietary counseling but no medication were used (also see Ref. [19]). To obtain HBCs from pre-eclamptic (PE) pregnancies, women with both early and late-onset PE were included, but treated them as separate groups (also see Ref. [17]). We excluded PE placentae if newborns presented with growth restriction (birth weight <3rd percentile).

2.1. Protocol of HBC isolation

2.1.1. Tissue Preparation for HBC isolation from term placenta

- The placenta should be obtained within 30min after delivery, ideally. If necessary, it can be stored short term (<2h) on 4 °C before you start.
- Place the placenta on a tray, fetal side up. Using sterile tweezer, sterile scissors and gentle pulling with your fingers, remove the placental amnion and cut off the umbilical cord.

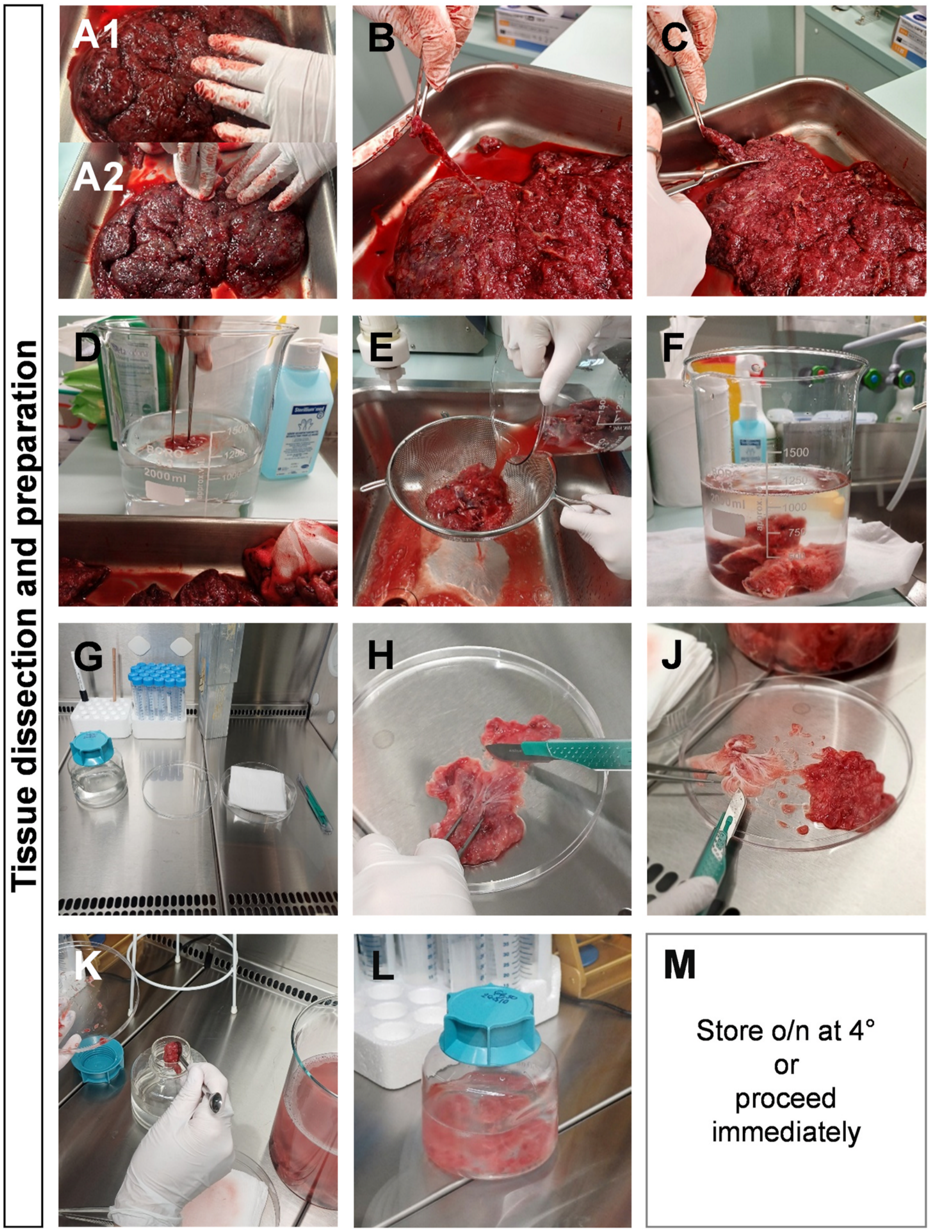

Fig. 1. Tissue Preparation for HBC isolation from term placenta. Tissue is turned maternal side up and wiped down with disinfectant (A1), which turns the surface grey (A2). Decidual remnants are removed with tweezers and scissors (B) and the tissue is cut into 4 × 4cm junks (C). The junks are washed extensively in saline, the tissue is strained and the washing solution is replaced until no excess blood remains (D–F). Prepare a flask of PBS, a dish, gauze and a scalpel in the sterile bench (G). The junks are dip-dryed on gauze, and the villous tissue is scraped off the vessels (H and J). The tissue is placed in PBS, weighed and either stored overnight at 4 °C or used immediately (M).

- Turn the placenta so that the maternal side is up. Remove any blood clots with sterile gauze swabs (Lohmann&Rausch, Gazin, 16 plies, 17 threads strength, Cat.No. 18515) and povidone-iodine (11 % bioavailable iodine). The upper layer will appear slightly greyish (Fig. 1 A1 and A2). Use the tweezers and scissors to remove this upper layer of maternal decidua basalis (Fig. 1 B).
- Once all decidual remnants have been removed, wipe again with gauze swabs soaked in physiological saline solution (0.9 % NaCl). Cut the placental tissue into smaller pieces (approx. 4 × 4cm; Fig. 1C)
- Place the pieces in a beaker, wash in physiologic saline solution to remove excess blood (Fig. 1 D). Repeat this several times, using a strainer to conveniently change the saline solution (Fig. 1 E). Once the saline solution is mostly clear (Fig. 1 F), move to the sterile workbench (Fig. 1G). From this step on, handle tissue only within the sterile bench and use sterile supplies and reagents.
- Place approximately 100–150 ml of sterile PBS solution in a sterile cup and weigh.
- Dip-dry a piece of tissue on gauze, using tweezers to hold and a scalpel to finely mince the chunks by scraping the villous tissue off of the vascular trees (Fig. 1H and J).
- Transfer the minced tissue to the sterile cup containing the PBS (Fig. 1 K). Repeat this procedure for all tissue chunks, then reweigh the tissue in PBS (Fig. 1 L) and calculate back the tissue weight. Typically, around 100–150g should be obtained.
- At this point the tissue can be stored overnight at 4 °C. Alternatively, one can proceed directly with the isolation protocol (Fig. 1 M) → also see Supplementary Materials.

2.1.2. Trypsin digestion of placental tissue

- Prepare the Trypsin/DNase digestion solution (see Supplementary Materials: 1. Buffer recipes and required initial preparations).
- Cover a beaker with sterile surgical dressing cloth (Lohmann&Rausch, Toptex Lite RK, 40 × 40cm, 6 plies, Cat.No. 165003) and secure with a rubber band. Gently form a hollow to accommodate the tissue (Fig. 2 A) by pressing the cloth into the beaker using a sterile spoon or spatula. Pour the tissue and PBS onto it, tissue will be retained by the cloth (Fig. 2 B). Try to remove as much PBS as possible so that the digestion solution remains largely undiluted in the following step.
- Transfer tissue to a bioreactor bottle (Fig. 2C; Techne F7690, 350 ml, made of glass) by using a spoon and add 150 ml of Trypsin/DNaseI digest solution (Fig. 2 D).

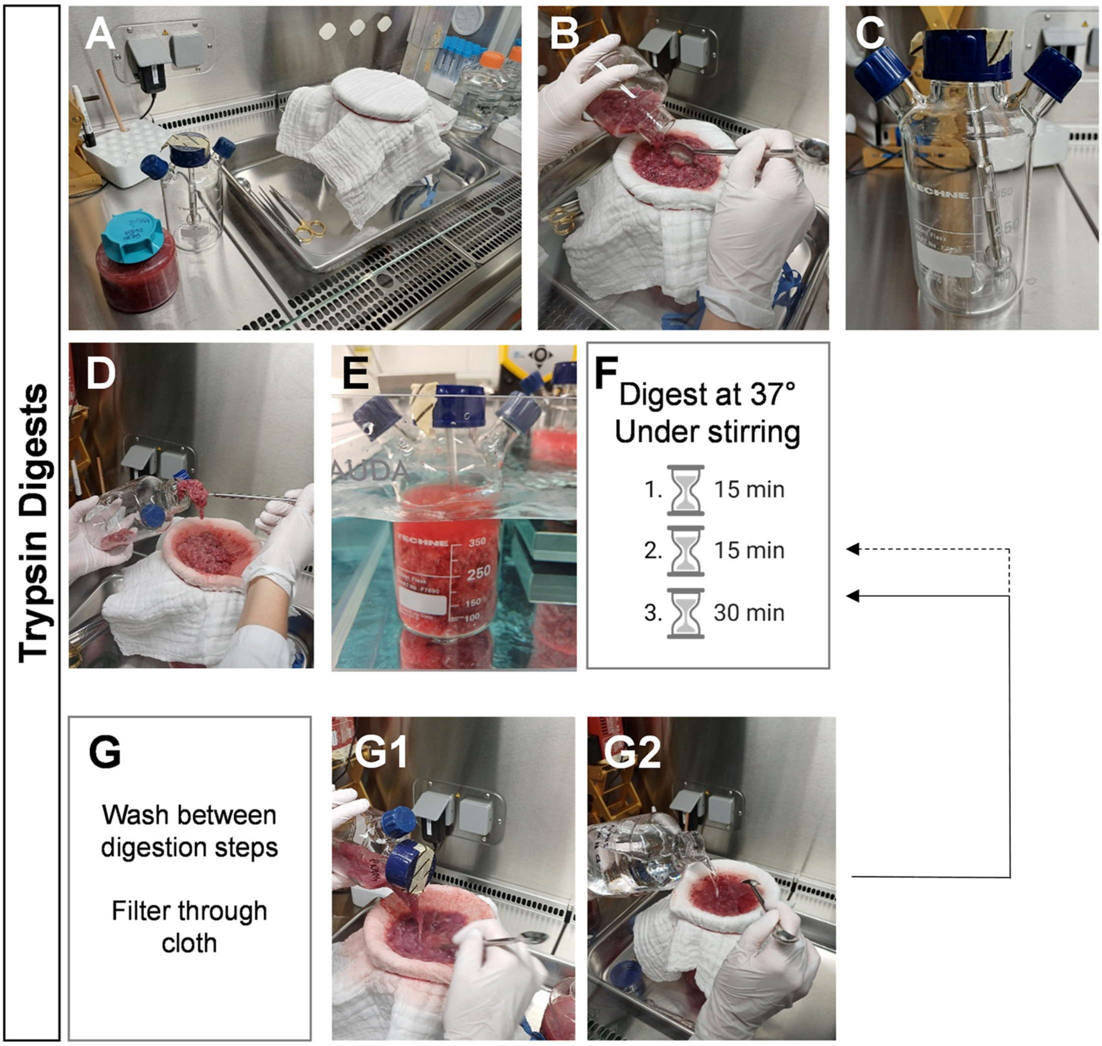

Fig. 2. Trypsin digestion of placental tissue; to remove excess PBS, a beaker covered with a cotton cloth is prepared to strain the tissue from the liquid (A and B). The tissue is transferred to a bioreactor bottle which allows magnetic stirring in a water bath and Trypsin solution is added (C–E). Three subsequent digestions of 15 min each are carried out, twice for 15 min and once for 30 min (F). Between digestion steps, the tissue is strained through a cloth and washed on the cloth with PBS by adding it and stirring (G1-G2).

- Place in a 37 °C water bath for 15 min (Fig. 2 E), stirring at 50 rpm. NOTE: the meniscus of the liquid inside the bioreactor should be below the water bath, to ensure optimal temperature. The tissue should be stirred during digestion; either use a bioreactor with a stirring magnet and a matching built-in stirrer in your water bath, or use an autoclaved magnet and submerge a waterproof stirring plate in the water bath. Another alternative is to use a water bath with an orbital shaking plate.
- After 15 min, filter the tissue again through a layer of tissue cloth (Fig. 2 G1-G2). Wash again with PBS by adding some to the hollow

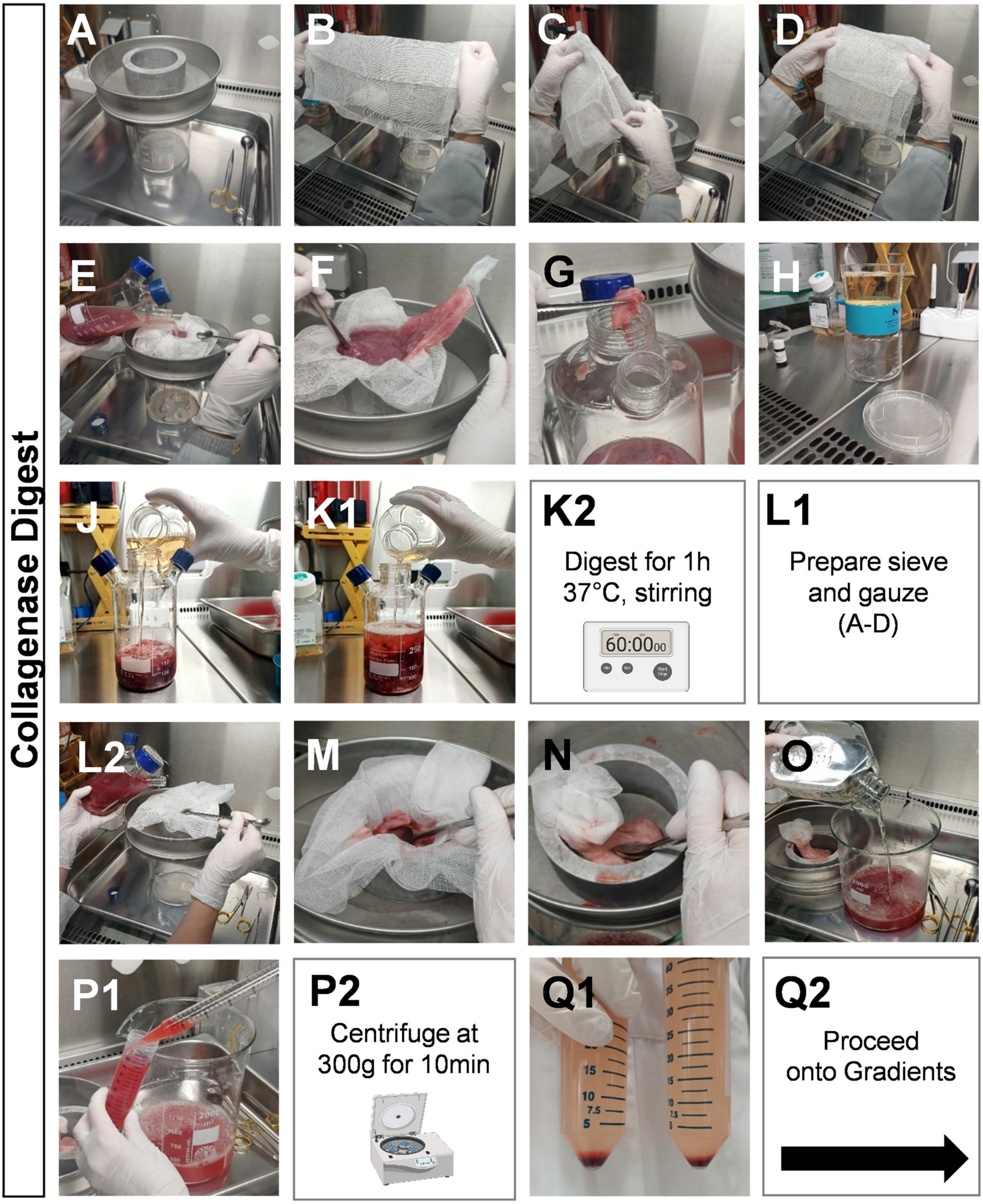

Fig. 3. Collagenase digestion of placental tissue; after the last Trypsin digestion, prepare a fresh beaker with a 70 µm mesh sieve. Depending on the size of the beaker and the sieve, it may be necessary to add a metal ring to limit the surface area (A). Cover the ring or the edge of the sieve with 4 layers of gauze as folded from a single sheet in (B–D). Filter the trypsinised tissue (E), which is now viscous (F). Put as much tissue as possible back into the bioreactor bottle and also cut the gauze with adhering tissue into small pieces and add them (G) to the collagenase digestion solution in the bioreactor (H-K). Digest for 1h, prepare the same setup as before, strain the cell solution through gauze and sieve (L–N). Add RPMI with 10 % FCS to stop enzymatic activity, transfer to centrifuge tubes and spin down cells (O–Q). Remove the supernatant, resuspend the pellets and continue with the Percoll gradients.

formed in the cloth and gently stir with a spoon to drain off the liquid (Fig. 2 G1-G2).

- Return the tissue to the bioreactor bottle and add a further 150 ml of Trypsin/DNaseI digest solution, digest for 30 min, stirring at 50 rpm.
- Filter through cloth and wash with PBS as before, return to the bioreactor bottle and add the remaining 200 ml of Trypsin/DNaseI digest solution. Digest for further 30 min at 37 °C, stirring at 50 rpm.

NOTE: Alternatively, wash the tissue by sedimentation (→ Supplementary Material)

2.1.3. *Collagenase digestion of placental tissue*

- Prepare a fresh sterile beaker, place a 70 μm nylon mesh sieve (Retsch analytical sieves, Cat. No. 9226661) on top and cover with four layers of gauze swabs (Fig. 3A–D).
- Filter the Trypsin-digested tissue (Fig. 3 E) and SAVE IT. If desired, collect the flow-through of this last trypsin digestion step (and if desired also the second digest), as it can be used for cytotrophoblast isolation [22].
- Proceed with the undigested tissue: wash briefly with PBS, then transfer as much of it as possible back into the bioreactor bottle; as it has become typically viscous (Fig. 3 F), it is not easy to scrape it off the gauze. Using sharp scissors, cut the gauze swabs containing the remaining viscous tissue slurry into small pieces (2 × 2 cm) and add these to the bioreactor bottle (Fig. 3 G).
- Add 100 ml of the prepared Collagenase/DNaseI digestion solution (see Supplementary Information 1. Buffer Recipes and Required Preparations; Fig. 3H–K), digest for 1 h at 37 °C with gentle stirring/agitation (50 rpm).
- Again, prepare a fresh sterile beaker, place the 70 μm mesh sieve on top and add four86 layers of gauze swabs (repeat as in Fig. 3A–D).

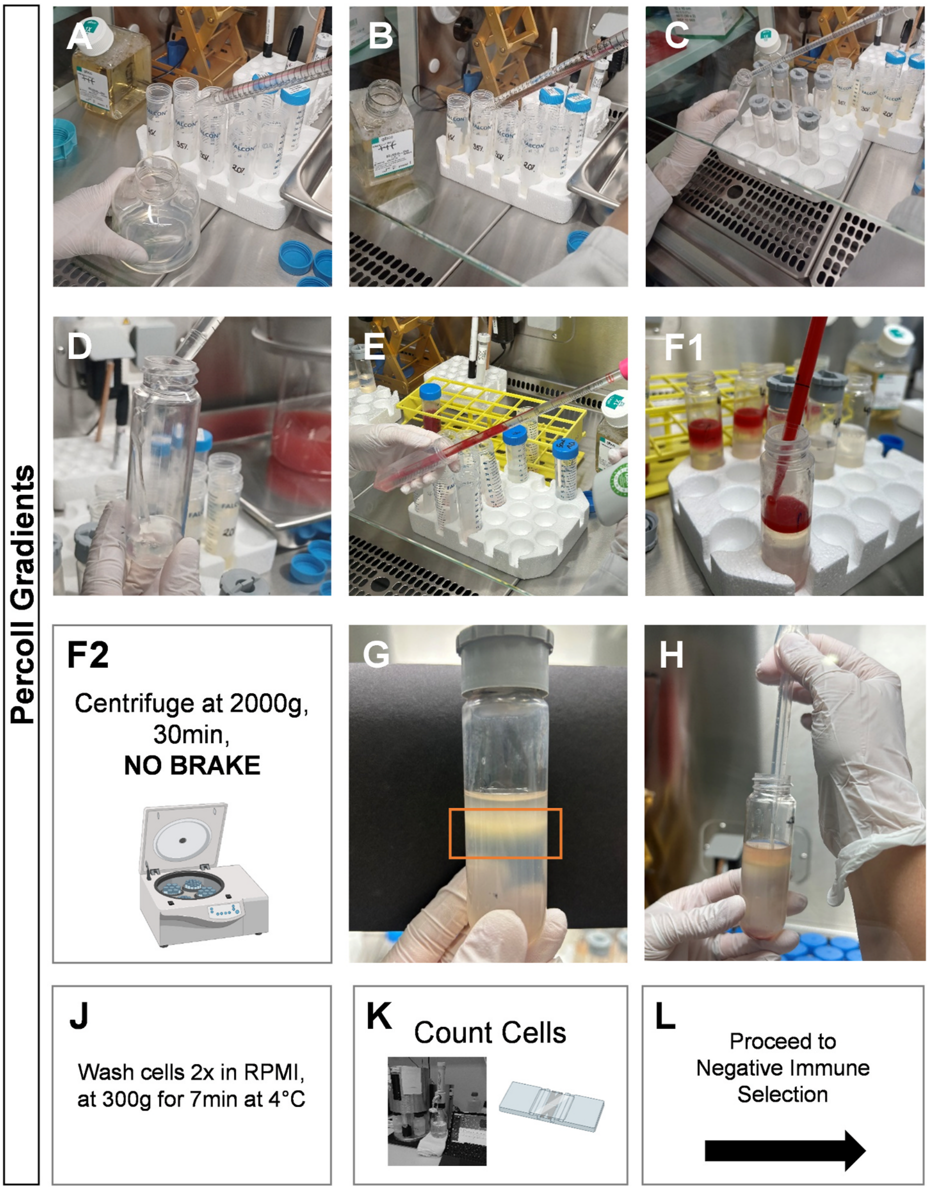

Fig. 4. Percoll gradient density centrifugation; prepare Percoll gradients by diluting Percoll in RPMI with 10 % FCS from 90 % to 40, 35, 30 and 20 %, respectively (A and B). Layer the solutions on top of sterile centrifuge tubes, starting with the highest percentage and ending with the lowest; pipette gently, allowing each solution run slowly down the tube wall onto the previous layer (C and D). Add the cell suspension obtained from the digestion steps to the 20 % Percoll layer (E and F) and centrifuge the gradients with the brake turned off. Macrophages are found where the 30 and 35 % Percoll layers were before centrifugation (G). Gently aspirate this fraction with a Pasteur pipette (H). Wash the cells twice (J), count (K) and continue with the immune selection (L).

- Filter the now liquid cell suspension (Fig. 3 L1-L2), squeeze the gauze swabs with a spoon or spatula (basically like a teabag) to get most of the liquid from the top into the flow through (Fig. 3 M and N).
- Continue with the flow-through: add RPMI 1640 with 10 % FBS to inactivate the enzymes to a total volume of approx. 240 ml (Fig. 3 O).

Distribute the cell suspension into 50 ml Falcon tubes and centrifuge at 300 g, 10 min (Fig. 3 P1-P2). *NOTE: The liquid on top of the pellet will not become completely clear, even with longer centrifugation (Fig. 3 Q1-Q2).*

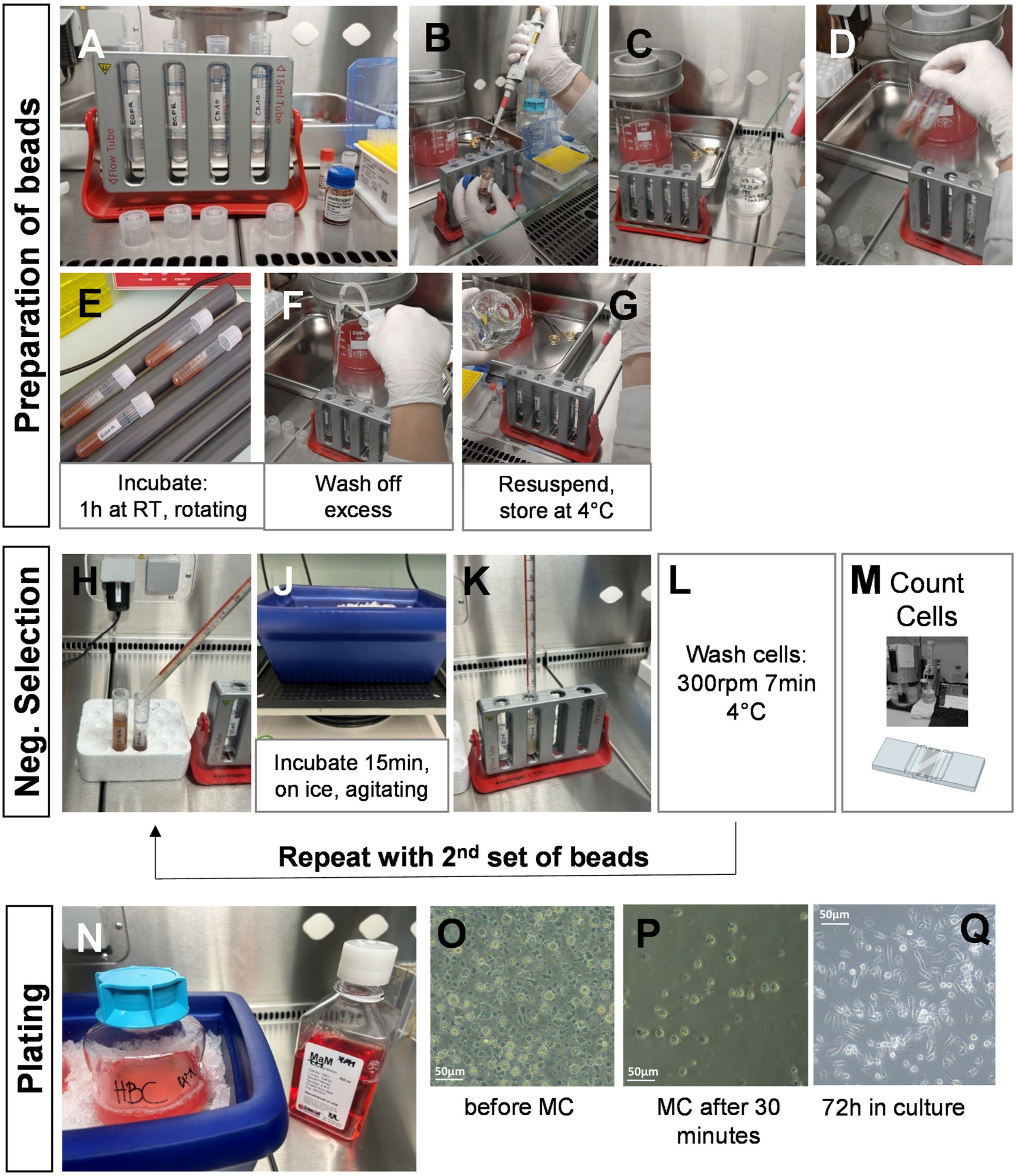

Fig. 5. Immune selection and plating; antibodies against EGFR and CD10 are conjugated to magnetic IgG Dynabeads, excess antibody is removed and beads are stored at 4 °C until use (A–G). 100 × 10ˆ6 cells are added to 100ul of anti-EGFR beads and incubated for 15min on ice under gentle agitation (H and J). Tubes are placed in the magnetic rack, Dynabeads with cells carrying EGFR are pulled to the tube wall and the cell suspension is collected (K), cells are washed in RPMI with 10%FCS (L). Cells are added to the second set of beads and the process is repeated. Finally, the cells are counted (M). Plate 1 × 10ˆ6/ml of MaM medium into appropriate culture dishes (N), which will appear full and heterogeneous in cell population (O). After 30min perform a media change, remove the contaminating cells and adhere the HBCs, which appear round and rich in vacuoles, firmly to the plastic (P). After 48–72h in culture, HBCs begin to elongate and lose their round appearance (Q).

2.1.4. Percoll gradient density centrifugation

- Aspirate the supernatant, resuspend the pellets in cold RPMI medium to wash them, centrifuge again at 300 g for 10 min. Resuspend the pellets in 24 ml of cold RPMI and load evenly onto previously prepared Percoll gradients (see Supplementary Materials 1.Buffer recipes and required preparations; Fig. 4A–F).
- Centrifuge at 1000×g for 30min, 4 °C, turn the BRAKE OFF (Fig. 4 F2).
- After centrifugation, the erythrocytes will have settled to the bottom of the tubes. The leukocytes should appear as a white condensed band in the area of the 30–35 % Percoll layer (Fig. 4 G, and Supplementary Fig. 2). Using a plastic Pasteur pipette, gently push through the top layer (20 %) and collect the band corresponding to the cell fraction of interest (ca. 7ml/tube; Fig. 4 G and H, Supplementary Fig. 2). Do not disturb the upper layer.
- Transfer the collected cell fraction to tubes containing cold RPMI medium (see Supplementary Materials 1. Buffer recipes and required preparations), wash cells once with RPMI medium by centrifugation at 300×g for 10min, 4 °C. Keep the cells on ice from this step on, as they will quickly adhere to plastic ware at warmer temperatures (Fig. 4 J).
- Use a hemocytometer of your choice (automated or manual) to assess the number of viable cells recovered from the gradients (Fig. 4K–L).

2.1.5. Immune selection and plating

- For negative immune selection, add 100 μl of Dynabeads (Invitrogen, Cat #11033) coated with antibody against EGFR (see Supplementary Material 1. Buffer recipes and required preparations; Fig. 5A–G) per 100 × 10ˆ6 cells and incubate for 15 min on ice with shaking (Fig. 5H and J).
- Collect the cell suspension by transferring the solution containing the anti-EGFR Dynabeads to a magnetic separator. Discard the anti-EGFR Dynabeads. Transfer the cell suspension to anti-CD10 conjugated Dynabeads (see Supplementary Materials 1. Buffer recipes and required preparations) and incubate for 15 min on ice with shaking as before (Fig. 5H and L).
- Collect the cell suspension by transferring the solution containing anti-CD10 Dynabeads to a magnetic separator. Discard the anti-CD10 Dynabeads (Fig. 5H, J) and proceed to count the viable cells again using a hemocytometer of your choice (Fig. 5 M).
- Resuspend in Macrophage Medium (MaM, ScienCell, Cat #SC1921) to give 1 × 10ˆ6 cells/ml (Fig. 5 N) and plate in appropriate culture dishes according to your experimental requirements.
- Incubate for 30min at 37 °C, 21 % O_2; thereafter change to fresh MaM medium. HBCs typically adhere to plastic culture dishes within 30min. Therefore, this is a final purification step to eliminate possible contaminating cells (compare Fig. 5 O – Q) which have not attached yet.
- Under the microscope, HBCs should now appear as rounded cells, densely packed with granular vacuoles (Fig. 5 P). Within 48–72h, cells will begin to elongate and take on a more edged, spindle-like shape (Fig. 5 Q).

NOTE: Supplementary Table 1 provides a list of possible pitfalls in the isolation process as well as suggestions how to troubleshoot in each case.

Our HBC isolation protocol is modified from a previously published method [22]. We use straining of the tissue for washing the tissue between digestion steps, thereby removing more excess PBS which would dilute the digestion solutions and at the same time the washing steps can be performed faster. Also we reduced some washing steps after the gradient centrifugation, and perform the last selection step, a final medium change, after 30min vs. 60min. In total about 1.5–2h hands-on time can be saved by these slight modifications; nevertheless, the yield and viability improve/remain comparable, respectively (see Supplementary Fig. 3). We have successfully applied it to healthy placentas and those affected by gestational diabetes mellitus (GDM) [19] and pre-eclampsia (PE) [17]. Isolation of HBCs is a rigorous process involving multiple enzymatic digestions and subsequent immunopurification steps. The initial enzymatic digestion prior to immunopurification yields a mixed cell fraction containing HBCs and various placental cell subtypes such as trophoblasts and fibroblasts (Fig. 6A). After immunopurification, there is a significant decrease in isolated HBCs under all conditions (CTR, GDM, and PE), yet viability and function remain intact even after culturing. Notably, viability after immunopurification increases significantly in CTR (+3.8 %) and GDM (+7.7 %) isolations, but decreases in PE by 7.3 % (Fig. 6B), likely due to the pathology.

2.1.6. Cryopreservation of Hofbauer cells

Despite achieving high levels of viability and purity in isolated primary HBCs, the challenge of working with freshly isolated HBCs within a tight seven to ten day timeframe remains. Despite extensive experimentation with various freezing media and culture dish coatings to improve cryopreservation success, the recovery of viable HBCs after thawing remains elusive. Our efforts to explore ways to cryopreserve HBCs have included the use of various commonly suggested cryoprotective agents (CPAs) such as 1) MaM culture medium with an increased amount of FBS to 20 % and 10 % DMSO, 2) 90 % FBS and 10 % DMSO [23], or sugars as osmoprotectants in combination with 10%DMSO such as 50 mM trehalose [24], 60 mM sucrose [25] and 1 % methylcellulose [26]. A commercially available freezing medium specifically designed for the cryopreservation of macrophages and microglia (Cell Applications Cat. No. 043–50) was also tested. For cryopreservation, cells were harvested at a minimum density of 3 × 10ˆ6/ml, resuspended in the respective freezing media and cooled down in Biocision CoolCell LX devices placed at −80 °C. After 24–48h, the cells were stored in liquid nitrogen. To thaw the cells, cryotubes were rapidly thawed using the Biocision ThawStar device. The cell suspension was diluted in four volumes of warm MaM, centrifuged at 300g for 7min, the supernatant containing CPA was removed and the pellet was resuspended in an equivalent volume of warm MaM. Cells were cultured in 6-well plates at a minimum density of 3 × 10ˆ6 cells/well. A media change was performed after 24h. The results of these efforts are detailed in Supplementary Fig. 1, and demonstrates that, regardless of the freezing medium used, frozen HBCs undergo cellular changes, including size reduction and reduced adherence to culture surfaces, hindering their ability to differentiate into their characteristic morphology. To overcome the reduced adherence to culture dishes, different coatings with extracellular matrix proteins were tested: 1) 1 % gelatine coating, 2) fibronectin coating at 3 μg/cm^2, and 3) collagen I at 3 μg/cm^2. However, none of these coatings improved the adherence of HBCs to culture ware after thawing (Supplementary Fig. 2). The lack of successful cryopreservation of HBCs is a limitation that significantly impedes the versatility of these cells in experimental design and execution.

2.2. Quality control (QC) of isolated HBCs

After successful isolation of high-yield, high-viability HBCs, assessing their quality is crucial for studying their function. We use various methods such as flow cytometry (FC) and immunocytochemistry (ICC) to detect contaminants and confirm HBC identity. Our quality control panel includes CD163 as a specific HBC marker with an average positivity of 91 %. In addition, markers for fibroblasts (VIM, CD90) and muscle cells (SMA) show low positivity (<10 %), indicating the purity of our HBC isolation protocol (Fig. 7A–B). In parallel, ICC staining confirms CD163 positivity, while VIM, CD90 and SMA were stained negative (Fig. 7C). In addition, macrophage activity can be assessed using a phagocytosis assay [8,17] which reflects the successful isolation of functional phagocytes. This assay, using zymosan-labelled beads, revealed visible engulfed beads within HBC vacuoles after 3 h, as

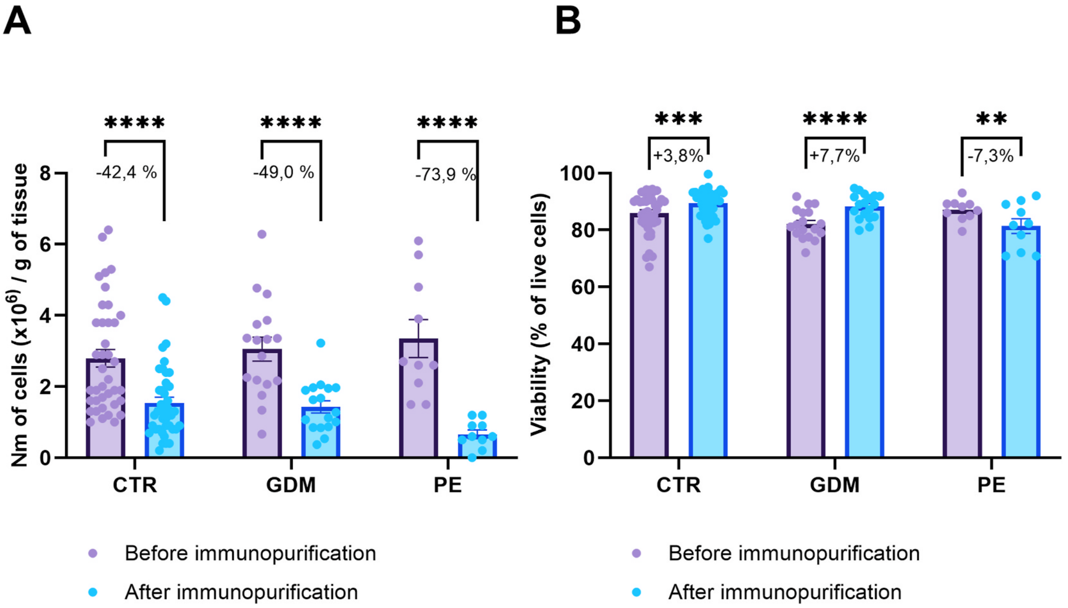

Fig. 6. (A) Average yield of isolated cells, adjusted to the wet weight (in grams) of minced tissue for each condition before and after immunopurification. Differences in percentages (%) represents the performance and enrichment in the yield of HBCs. (B) Average viability of isolated cells from CTR, GDM or PE tissue before and after immunopurification. % on graph represents the selection output and enrichment in the viability of isolated HBCs. To test yield and viability of isolated cells CTR n = 40, GDM n = 17, PE n = 10 were used. Data on all graphs are presented as mean ± S.E.M. To test statistical significance in (A) and (B) 2-way ANOVA followed by Sidak's *post-hoc* test was used $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.000$.

observed by brightfield and fluorescence microscopy (Fig. 7D).

2.2.1. Flow cytometry (FC)

In the intracellular FC approach, we use two different types of gating strategies. First, we check the quality of HBCs and possible contamination for fibroblasts and muscle cells, while the second gating setup allows us to efficiently detect macrophage activation and polarization. Intracellular FC staining offers advantages such as optimized cell utilization, comprehensive marker detection, and flexible measurement timing.

2.2.2. Intracellular FC staining

- It is recommended to perform FC measurements on day 4 or 5 after isolation, as HBCs will have recovered well from the isolation process and will be most metabolically active [17–19].
- Treat all HBCs isolated from either CTR or pathological placenta in the same way for FC measurement.
- After isolating the HBCs, seed the cells at a density of 5 × 10ˆ6 cells into a T25 cm^2 flask without coating. Cultivate the cells in complete macrophage medium (MaM, ScienCell, Cat #SC1921).
- Harvest the cells using TrypLE solution (Thermo Fischer Scientific, Cat # 12604013) and gentle scraping.
- Determine the viability and number of cells after scraping using a CASY cell counter model TT (Innovatis, Bielefeld) or a comparable method.
- Use a minimum of 2.5 × 10ˆ5 viable cells per FC tube.
- Wash the cells with FC Staining and Wash Buffer (see Supplementary Materials 1. Buffer Recipes and Required Preparations) and centrifuge at 300 g for 5 min. After the washing and centrifugation steps, carefully remove the supernatant by hand or using a suction pump.
- Resuspend the cells in 1 mL/tube of 3 % FCS-HBSS solution for 10 min at room temperature to block Fc receptors and reduce non-specific binding.
- Repeat the centrifugation step and remove the supernatant.
- Fix the cells for 20 min in the fridge at 4^{O}C using 1 mL/tube of BD Cytofix (BD Biosciences, Cat #554714).
- After fixation, repeat the centrifugation step, remove the supernatant, and wash the cells in 1 mL CytoPerm solution (BD Biosciences, Cat #554714). Repeat the centrifugation step and remove the supernatant.
- Add the appropriate volume of antibody and stain for 30 min in the dark at 4^{O}C.
- Wash the cells twice in 2 mL FC Staining and Wash Buffer and add 200 μL of the buffer to each tube after the final centrifugation step.
- Measure on the flow cytometer.

Cell measurement was performed using a CytoFLEX flow cytometer (Beckman Coulter) and analysed using FlowJoTM v10.10 software for gate setting and data analysis. Graph Pad Prism 10.2.1 software (GraphPad Software Inc.) was used for statistical calculations and graph generation. The Shapiro-Wilk test was used to test for normal distribution. Skewed data were transformed to natural logarithm (ln) before statistical analysis and re-transformed for graphical presentation. One-way ANOVA with Sidak's post hoc test was used to assess statistical differences between more than two groups. When normality was not achieved, the Kruskal-Wallis test with Dunn's multiple comparison test was used instead. Two-way ANOVA with Sidak's post hoc test was used to compare the effect of immunopurification on HBCs from CTR, GDM and PE placenta. All values are expressed as mean ± S.E.M. p-values ≤0.05 were considered statistically significant.

2.2.3. Staining and gating for QC of isolated HBCs

- Prepare the samples as described above.
- Next to the tubes stained for respective fluorochrome conjugated antibodies (CD163, Vimentin (Vim), CD90 and Smooth muscle actin (SMA)), prepare unstained tubes and tubes containing the isotype controls. Antibodies used for FC analysis and their corresponding dilutions are listed in the Supplementary Table 2.
- Count a minimum of total 1 × 10ˆ4 events per sample with flow cytometer.
- To investigate the expression of intracellular markers, cells were separated by size using forward and size scatter (FSC and SSC,

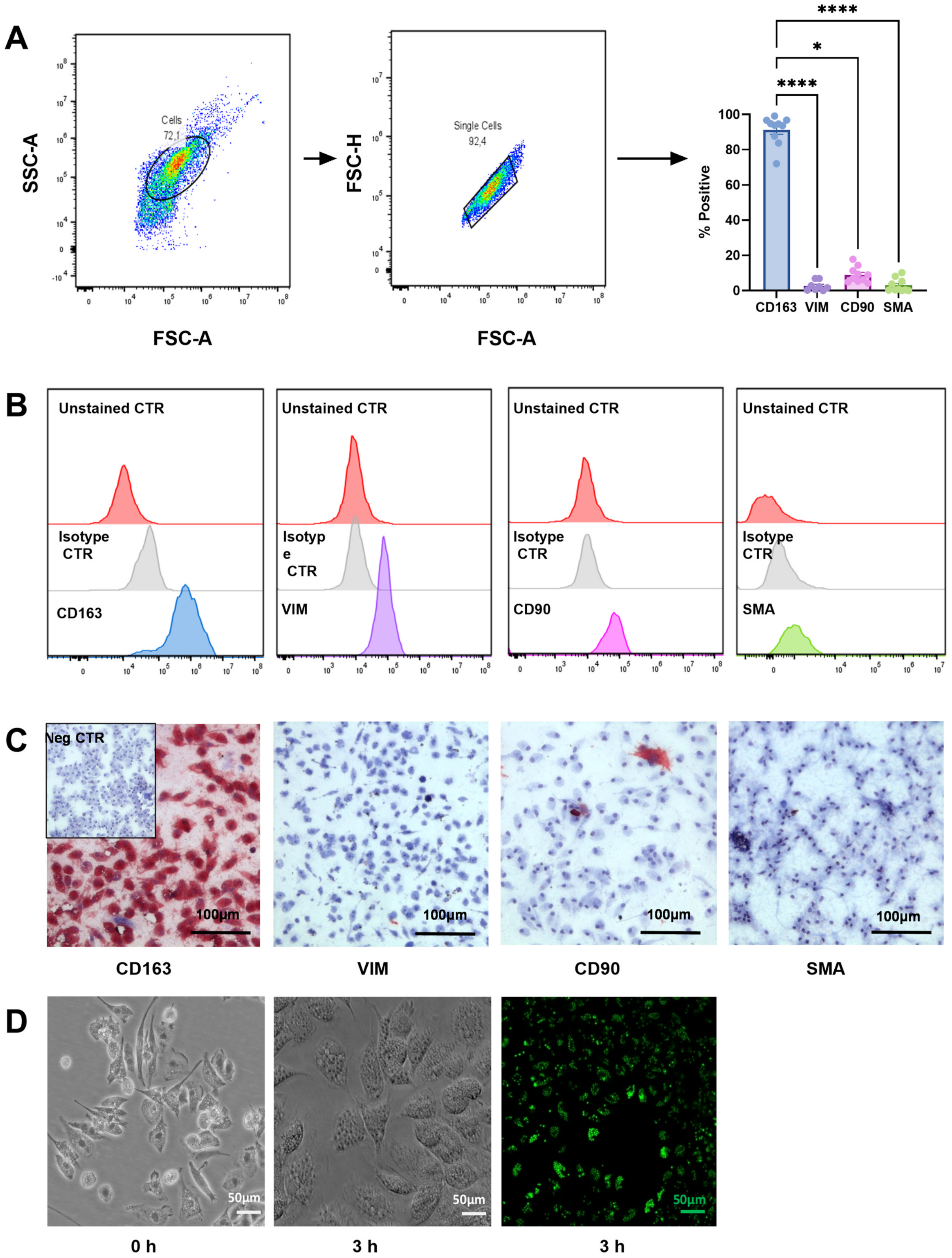

Fig. 7. (A) Representative gating sequence for quality control of isolated primary HBCs. Cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination and gating against the SSC area and the corresponding marker. Data on all graphs are presented as mean ± S.E.M. One-way ANOVA followed by Dunnett's post-hoc test was used to test statistical significance. $^*p \leq 0.05$ and $^{****}p \leq 0.0001$. (CTR isolations, n = 4) (B) Representative histograms of FACS; in red unstained CTR, in grey isotype CTR, followed by histograms for each marker examined, namely CD163, VIM, CD90 and SMA. (C) Immunocytochemistry (ICC) of a representative CTR HBC isolation. The insert in the upper right corner of the CD163-stained image shows the negative control staining (blue). Images were acquired using CellSens standard software and an Olympus BX53 light microscope with an Olympus UC90 camera. The scale bar represents 100 µm. (D) Visualization of phagocytosis using high content screening (HCS) microscopy. HBCs were treated with zymosan beads (green) for 3 h. Images were acquired using a Nikon microscope with the Zyla sCMON camera. Representative images of single experiments are shown. The scale bar represents 50 µm.

- respectively), followed by doublet discrimination and gating against the SSC area and the respective marker. Cells for each marker were then gated against the SSC area (Fig. 7A).
- Isotype controls corresponding to each fluorochrome used in the experiment were used to detect non-specific positive signals (Fig. 7B).

2.2.4. Immunocytochemical staining (ICC)

- Seed HBCs at a density of 1 × 10ˆ5 cells/well onto 4 well glass chamber slides.
- Wash cells twice in cold 1x HBSS, dry slides and fix in ice-cold acetone for 3 min.
- Rehydrate cells by washing in TBE +0.1 % Tween.
- Apply primary antibodies (CD163, Vim, CD90, SMA, negative control) diluted in Dako Antibody Diluent (Agilent, Dako, Cat #S0809) and incubate for 30 min at room temperature. Antibodies used for ICC and their corresponding dilutions are listed in Supplementary Table 2
- After an intensive wash step, apply Primary Antibody Enhancer (Thermo Fischer Scientific, Cat #TL125HL) for 15 min.
- Repeat wash step and apply Large HRP Polymer solution (Thermo Fisher Scientific, Cat #TL125HL) for 20 min.
- After washing, incubate the slides in AEC Chromogen Solution (Abcam, Cat #64252) for 10 min.
- Wash slides in distilled water and counterstain with haematoxylin (Gatt-Koller Cat #401296170) for 5 min.
- Mount the slides using mounting medium.

2.2.5. Functional assay – phagocytosis

- Use the Phagocytosis Assay Kit (Abcam, Cat #ab234053) according to the manufacturer's instructions.
- Seed HBCs at a density of 0.5 × 106 cells/ml into 24-well black/clear bottom plates.
- Add 5 μL of zymosan slurry to the cells and incubate for 3 h at 21 % oxygen and 37 °C, followed by a wash step.
- Fix the cells in a plate containing 4 % neutral buffered paraformaldehyde solution, followed by an intensive wash step with TBE buffer containing 1x TBE and 0.1 % Tween. Add 300 μL of PBS to each well and store the plates at 4 °C.

2.2.6. FC staining and gate setting to determine HBC polarization

- Prepare samples as described in the intracellular FC staining section.
- For a multicolor FC staining compensate the fluorochrome conjugated antibodies by individual staining (Thermo Fischer Scientific, Cat #01-1111-42). Include fluorescence-minus-one (FMO) controls to set appropriate gates.
- Count a minimum of total 4 × 10ˆ4 events per sample by flow cytometer.
- To determine polarization of isolated HBCs, the following polarization markers are recommended: IRF4, IRF5, CD40, CD80, CD86, CD163, CD206 and CD209. Antibodies used for FC analysis and their corresponding dilutions are listed in Supplementary Table 2.
- To investigate the expression of intracellular markers, cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination and gating against the SSC area and CD163, marker for tissue-resident HBC (Fig. 8A).
- Next, IRF4 and IRF5 antibodies were used to discriminate between CD163 + IRF4+ positive M2 HBCs (population a on Fig. 8B) and CD163 + IRF4+ IRF5+ M1 HBCs (population b on Fig. 8B).
- To define the sub-polarization of each respective polarization, we recommend using a panel of CD40, CD86, CD206 and CD209 to determine M2 polarization and a combination of CD40, CD80 and CD86 for M1 polarization. The gating strategy is shown in Fig. 8C and D.

To assess HBC polarization patterns, we developed a FC panel using common polarization markers. We aimed to determine the ratio of M1 and M2 polarized HBCs by gating CD163+ cells for IRF4 and IRF5. We found that 86.5 % of isolated HBCs are IRF4+ (M2 polarized, anti-inflammatory), with only 0.36 % identified as pure M1 cells and 13.12 % showing a shift towards M1 polarization (Fig. 8B). For M2 polarization, CD163+IRF4+ gated HBCs showed high co-expression of CD40 and CD86. The use of CD206 and CD209 helped us to differentiate between M2a and M2c subtypes, with 41 % of HBCs showing M2c CD206+ polarization and 35 % showing M2a CD206+CD209+ phenotype (Fig. 8C). To identify M1 polarized HBCs (CD163+IRF4+IRF5+ positive HBCs), we use CD40, CD80 and CD86 markers. Our data reveal that a small fraction of cells express only CD80, indicating that the majority of HBCs are not M1 polarized under control (CTR) conditions (Fig. 8D). It is important to note that the suggested panel of markers provides a current snapshot of HBCs polarization and activation along the IRF5-IRF4 regulatory axis. Polarization occurs along a balanced spectrum and there is no distinct separation between M1 and M2 HBCs seen in normal placentas, but rather various intermediate polarized cells. Despite the isolation process taking up to 48 h, this simple quality control assessment confirms that the isolated primary cells remain biologically active and retain their M2 phenotype.

3. Discussion

Hofbauer cells (HBCs) are fetal macrophages that residue within the connective tissue of the placental villous tree (Benirschke 2012, Castellucci 1980, 1984). Despite the growing interest in the field, the lack of one accepted isolation protocol has hindered a comprehensive understanding of HBCs physiology. Various protocols have been used to isolate HBCs from first and term placentas, including enzymatic digestion, density gradients, immunopurification and fluorescence activated cell sorting (FACS). However, challenges remain in achieving best possible yield and purity, highlighting the need for an optimized protocol and rigorous quality control measures to advance our understanding of HBC functions. This discussion will detail our ongoing efforts to refine the HBCs isolation protocol, guided by lessons learned from the work of Tang et al. [22]. Subsequently, the protocol presented here highlights the additive need and importance of established FC quality control panel to increase the efficiency of HBC isolation. Together, these additional information contribute to a more nuanced understanding of HBC-cell biology in normal and pathophysiological conditions.

The here presented HBC isolation protocol is very similar to a previously published method by Tang et al. [22], but with notable improvements. We have achieved higher yields, without a reduction in viability or purity, mainly by altering/reducing the washing steps and also shortening some incubation times. In an effort to optimize the isolation process, researchers have explored different digestion techniques, including the use of different types of enzymes such as trypsin or collagenase [27–29], as well as shortening digestion times [30]. The use of advanced technology has facilitated FACS sorting, allowing isolation of characteristic cell populations. In particular, sorting techniques based on specific human leukocyte antigen typing of maternal cells in first and third trimester placentas provide a more refined approach compared to sex chromatin determination [30,31]. Notably, FACS sorting applied to first trimester placental cells allowed the differentiation of three distinct macrophage populations; HBCs, maternal villous macrophages (PAMM1) and maternal decidual macrophages (PAMM2) [31]. We are aware of protocols that use either positive immune selection via antibody-conjugated magnetic beads [32], as well as those that omit immune selection altogether [33]. We do not consider the use of positive selection to be feasible, as it could induce immunological activation of the obtained macrophages. The authors who avoided immunoselection

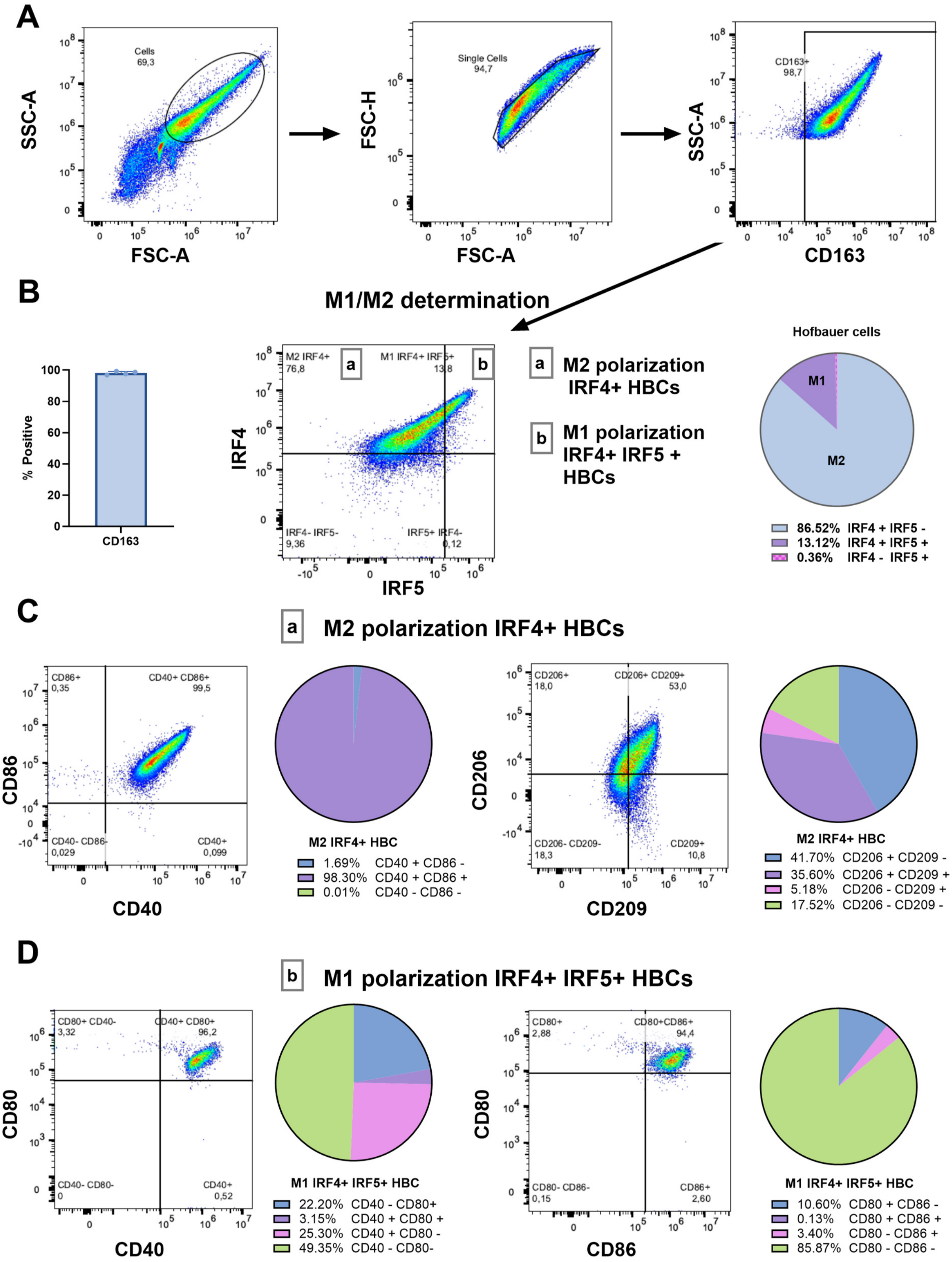

Fig. 8. (A) Representative gating strategy to determine polarization of isolated primary HBCs. Cells were separated by size using forward and size scatter (FSC and SSC, respectively), followed by doublet discrimination and gating against the SSC area and the CD163 marker. (B) The scatter plot on the left shows the % of the positive population for CD163. The CD163+ population was further gated for IRF4+ and IRF4+IRF5+ positive HBCs to identify M1 or M2 polarized HBCs. Pie charts show the ratio of IRF4+, IRF4+IRF5+ and IRF5+ HBCs. (C) Differential gating for further clarification of M2 IRF4+ polarization using CD40, CD86, CD206 and CD209 polarizations markers. Pie charts represent the ratio of either CD40 and CD86 positive or CD206 and CD209 positive HBCs. (D) Differential gating to further elucidate M1 IRF4+ IRF5 polarizations using CD40, CD80 and CD86 polarization markers. Pie charts represent the ratio of either CD40 and CD80 positive or CD80 and CD86 positive HBCs. For pie charts in (B, C and D) n = 4 of CTR HBCs were used.

altogether, stated themselves that their intention was to perform rapid flow cytometric analyses rather than to cultivate the obtained HBCs [33]. However, this limits the experimenter to descriptive studies of isolated HBCs and does not allow the study of HBCs over time, in response to different stimuli, or to combine HBCs in co-cultures or use them in *in vitro* assays. As we are interested in studying different functional readouts of HBC cultures, we consider the bead selection necessary to eliminate contaminating cell types to be rigorous and we believe that negative immune selection is preferable.

Hofbauer cells (HBCs) exhibit remarkable plasticity, and rapidly respond to microenvironmental stimuli by switching between polarizing patterns. The range of pro-inflammatory M1 and anti-inflammatory M2 polarizing spectrum highlights HBCs functional versatility [13,34,35]. Despite different isolation protocols, the implementation of reliable quality control measures and the assessment of polarization patterns are crucial. Here, we present a streamlined flow cytometry (FC) panel tailored for rapid differentiation of M1 and M2 polarized states of HBCs. While some rely solely on CD68 for HBC identification and cytokeratin 7 (CK7) for trophoblast contamination, the limitations of CD68 prompt the exploration of more refined approaches [22,27,32,33]. Our FC protocol not only serves as a quality control but also as a quantitative assessment of HBC polarization towards the M1 or M2 phenotype. Based on the high expression of CD163, a tissue resident macrophage marker, we incorporated this marker into our protocol. In addition, we introduced IRF4 and IRF5, regulators of the Myd88 pathway, which controls the expression of M1/M2 genes in immune cells and inflammatory diseases [36,37]. IRF4 is associated with anti-inflammatory M2 programming, while IRF5 controls pro-inflammatory M1 factors [37,38]. Despite the use of different markers to determine the M1/M2 phenotype, it is important to recognize that due to the complexity of polarization, a combination of approaches including the study of the secretome, gene expression and functionality is required for accurate determination of HBC phenotype [34,36]. Our FC protocol serves as an initial guide to aid experimental design. As characterization of macrophage phenotype using polarization markers and cytokine release remains descriptive, we recommend incorporating functional assays alongside phenotypic analysis to provide a comprehensive understanding of HBC physiology.

While our modified and optimized isolation protocol for HBCs together with the FC based quality control provides a convenient and reliable overall method, we need to address some limitations. First, HBCs show strong adherence to culture dishes, which requires careful experimental design. If yields are lower than expected, experiments may need to be postponed or adapted. HBCs are typically plated directly into the dish intended for the experiment. Attempts to use CellUp dishes, which are surface treated and should release cells by temperature change rather than enzymatically [39], have been unsuccessfully tested. Instead, treatment with TrypLE Select or Accutase for 2–5 min, followed by gentle scraping is the preferred method to detach HBCs, despite some cell death and debris. This approach is preferable to higher concentrations of trypsin-EDTA or longer exposure times as it minimizes shedding of surface molecules [22]. It has also been suggested to use Teflon-coated bags to plate monocytes/macrophages so that they do not adhere during differentiation [40]. We have not yet tried this. Secondly, HBCs cannot be thawed viably after freezing, which limits their use to within ten days of isolation. We recommend completing experiments within seven days to avoid significant changes in morphology and potential changes in metabolism or polarizations. Despite efforts to investigate different freezing media and culture dish coatings [24,25, 41–43], viable cells were not obtained in significant numbers after thawing. Cell counts showed that while viability remained at around 90 % after thawing, HBCs size decreased, indicating possible cell shrinkage upon freezing. In addition, the cells show reduced adherence to culture dishes after thawing, which further complicates their use. This limitation requires immediate use of the isolated cells, which is an inconvenience in experimental design. Eventually, different cooling rates could be tested in the future, as shrinkage suggests that cells were frozen too slowly [44]. Furthermore, protocols encapsulating cells into hydrogels before freezing are emerging as solution to cryopreservation challenges, also specifically of macrophages [43].

Despite the limitations noted, our refined protocol allows isolation of HBCs not only from healthy placentas but also from those affected by gestational diabetes mellitus (GDM) [19] and preeclampsia (PE) [17]. An obvious progression of this study is to analyze HBCs homeostasis in pregnancy associated disorders that induce placental inflammation. In addition, further studies should analyze HBCs along with feto-placental endothelial cells and their expression of angiogenic markers and chemokines in the context of their proximity. Nevertheless, this protocol to isolate HBCs at high purity increases the feasibility of performing functional and metabolic assays as outlined, thereby contributing to a more comprehensive understanding of HBC biology in both healthy and pathophysiological placentas.

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CRediT authorship contribution statement

Monika Horvat Mercnik: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Christian Wadsack:** Writing – review & editing, Supervision, Funding acquisition. **Carolin Schliefsteiner:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2024.07.004.

References

[1] L. Reyes, B. Wolfe, T. Golos, Hofbauer cells: placental macrophages of fetal origin, in: Results Probl Cell Differ, Springer Verlag, 2017, pp. 45–60, https://doi.org/10.1007/978-3-319-54090-0_3.

[2] G. Fakonti, P. Pantazi, V. Bokun, B. Holder, Placental macrophage (hofbauer cell) responses to infection during pregnancy: a systematic scoping review, Front. Immunol. 12 (2022), https://doi.org/10.3389/fimmu.2021.756035.

[3] H. Katabuchi, T. Ohba, Human chorionic villous macrophages as a fetal biological shield from maternal chorionic gonadotropin, Dev. Growth Differ. 50 (2008) 299–306, https://doi.org/10.1111/j.1440-169X.2008.01030.x.

[4] C. Cao, M.D. Fleming, The placenta: the forgotten essential organ of iron transport, Nutr. Rev. 74 (2016) 421–431, https://doi.org/10.1093/nutrit/nuw009.

[5] M. Cervar, A. Blaschitz, G. Dohr, G. Desoye, Paracrine regulation of distinct trophoblast functions in vitro by placental macrophages, Cell Tissue Res. 295 (2 295) (1999) 297–305, https://doi.org/10.1007/S004410051236, 1999.

[6] S. Khan, H. Katabuchi, M. Araki, R. Nishimura, H. Okamura, Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. http://www.biolreprod.org, 2000.

[7] Y. Seval, E.T. Korgun, R. Demir, Hofbauer cells in early human placenta: possible implications in vasculogenesis and angiogenesis, Placenta 28 (2007) 841–845, https://doi.org/10.1016/J.PLACENTA.2007.01.010.

[8] J. Loegl, U. Hiden, E. Nussbaumer, C. Schliefsteiner, S. Cvitic, I. Lang, C. Wadsack, B. Huppertz, G. Desoye, Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis, Reproduction 152 (2016) 447–455, https://doi.org/10.1530/REP-16-0159.

[9] S.W. Yang, E.H. Cho, S.Y. Choi, Y.K. Lee, J.H. Park, M.K. Kim, J.Y. Park, H.J. Choi, J.I. Lee, H.M. Ko, S.H. Park, H.S. Hwang, Y.S. Kang, DC-SIGN expression in Hofbauer cells may play an important role in immune tolerance in fetal chorionic

villi during the development of preeclampsia, J. Reprod. Immunol. 124 (2017) 30–37, https://doi.org/10.1016/j.jri.2017.09.012.

[10] S. Matsubara, T. Takayama, T. Yamada, R. Usui, A. Izumi, T. Watanabe, A. Ohkuchi, H. Shibahara, I. Sato, M. Suzuki, Hofbauer cell activation and its increased glucose-6-phosphate dehydrogenase activity in second trimester-spontaneous abortion: an ultrastructural dual staining enzyme-cytochemical study, Am. J. Reprod. Immunol. 49 (2003) 202–209, https://doi.org/10.1034/j.1600-0897.2003.01180.x.

[11] K.M. Quicke, J.R. Bowen, E.L. Johnson, C.E. McDonald, H. Ma, J.T. O'Neal, A. Rajakumar, J. Wrammert, B.H. Rimawi, B. Pulendran, R.F. Schinazi, R. Chakraborty, M.S. Suthar, Zika virus infects human placental macrophages, Cell Host Microbe 20 (2016) 83–90, https://doi.org/10.1016/j.chom.2016.05.015.

[12] E.L. Johnson, R. Chakraborty, Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of immunoregulatory cytokines, Retrovirology 9 (2012) 101, https://doi.org/10.1186/1742-4690-9-101.

[13] L. Reyes, T.G. Golos, Hofbauer cells: their role in healthy and complicated pregnancy, Front. Immunol. 9 (2018) 1–8, https://doi.org/10.3389/fimmu.2018.02628.

[14] M.B. Brown, M. von Chamier, A.B. Allam, L. Reyes, M1/M2 macrophage polarity in normal and complicated pregnancy, Front. Immunol. 5 (2014) 1–10, https://doi.org/10.3389/fimmu.2014.00606.

[15] Y.-H. Zhang, M. He, Y. Wang, A.-H. Liao, Modulators of the balance between M1 and M2 macrophages during pregnancy, Front. Immunol. 8 (2017) 1–12, https://doi.org/10.3389/fimmu.2017.00120.

[16] Y. Yao, X.H. Xu, L. Jin, Macrophage polarization in physiological and pathological pregnancy, Front. Immunol. 10 (2019), https://doi.org/10.3389/fimmu.2019.00792.

[17] M.H. Mercnik, C. Schliefsteiner, H. Fluhr, C. Wadsack, Placental macrophages present distinct polarization pattern and effector functions depending on clinical onset of preeclampsia, Front. Immunol. 13 (2023) 8168, https://doi.org/10.3389/FIMMU.2022.1095879/BIBTEX.

[18] C. Schliefsteiner, S. Ibesich, C. Wadsack, Placental hofbauer cell polarization resists inflammatory cues in vitro, Int. J. Mol. Sci. 21 (2020), https://doi.org/10.3390/ijms21030736.

[19] C. Schliefsteiner, M. Peinhaupt, S. Kopp, J. Lögl, I. Lang-Olip, U. Hiden, A. Heinemann, G. Desoye, C. Wadsack, Human placental Hofbauer cells maintain an anti-inflammatory M2 phenotype despite the presence of gestational diabetes mellitus, Front. Immunol. 8 (2017), https://doi.org/10.3389/fimmu.2017.00888.

[20] M. Joerink, E. Rindsjö, B. Van Riel, J. Alm, N. Papadogiannakis, Placental macrophage (Hofbauer cell) polarization is independent of maternal allergen-sensitization and presence of chorioamnionitis, Placenta 32 (2011) 380–385, https://doi.org/10.1016/J.PLACENTA.2011.02.003.

[21] C. Grigoriadis, A. Tympa, M. Creatsa, P. Bakas, A. Liapis, A. Kondi-Pafiti, G. Creatsas, Hofbauer cells morphology and density in placentas from normal and pathological gestations, Rev. Bras. Ginecol. Obstet. 35 (2013) 407–412, https://doi.org/10.1590/S0100-72032013000900005.

[22] Z. Tang, S. Tadesse, E. Norwitz, G. Mor, V.M. Abrahams, S. Guller, Isolation of hofbauer cells from human term placentas with high yield and purity, Am. J. Reprod. Immunol. 66 (2011) 336–348, https://doi.org/10.1111/j.1600-0897.2011.01006.x.

[23] P. Haider, T. Hoberstorfer, M. Salzmann, M.B. Fischer, W.S. Speidl, J. Wojta, P. J. Hohensinner, Quantitative and functional assessment of the influence of routinely used cryopreservation media on mononuclear leukocytes for medical research, Int. J. Mol. Sci. 23 (2022) 1881, https://doi.org/10.3390/ijms23031881.

[24] Y. Hu, X. Liu, F. Liu, J. Xie, Q. Zhu, S. Tan, Trehalose in biomedical cryopreservation–properties, mechanisms, delivery methods, Applications, benefits, and problems, ACS Biomater. Sci. Eng. 9 (2023) 1190–1204, https://doi.org/10.1021/acsbiomaterials.2c01225.

[25] I. V Arutyunyan, E.Yu Kananykhina, A. V Elchaninov, T.Kh Fatkhudinov, Influence of sucrose on the efficiency of cryopreservation of human umbilical cord-derived multipotent stromal cells with the use of various penetrating cryoprotectants, Bull. Exp. Biol. Med. 171 (2021) 150–155, https://doi.org/10.1007/s10517-021-05187-3.

[26] J.-C. Volbers, L. Lauterböck, N. Hofmann, B. Glasmacher, Cryopreservation of cells using defined serum-free cryoprotective agents, Current Directions in Biomedical Engineering 2 (2016) 315–318, https://doi.org/10.1515/cdbme-2016-0070.

[27] O.V. Pavlov, A.V. Selutin, O.M. Pavlova, S.A. Selkov, Two patterns of cytokine production by placental macrophages, Placenta 91 (2020) 1–10, https://doi.org/10.1016/J.PLACENTA.2020.01.005.

[28] E.L. Johnson, R. Chakraborty, Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of immunoregulatory cytokines, Retrovirology 9 (2012) 1–11, https://doi.org/10.1186/1742-4690-9-101/FIGURES/6.

[29] B. Wetzka, D.E. Clark, D.S. Charaock-Jones, H.P. Zahradnik, S.K. Smith, Isolation of macrophages (Hofbauer cells) from human term placenta and their prostaglandin E2 and thromboxane production, Hum. Reprod. 12 (1997) 847–852, https://doi.org/10.1093/HUMREP/12.4.847.

[30] A. Appios, J.R. Thomas, N. McGovern, Isolation of first-trimester and full-term human placental Hofbauer cells, Bio Protoc 11 (2021), https://doi.org/10.21769/BIOPROTOC.4044.

[31] J.R. Thomas, A. Appios, X. Zhao, R. Dutkiewicz, M. Donde, C.Y.C. Lee, P. Naidu, C. Lee, J. Cerveira, B. Liu, F. Ginhoux, G. Burton, R.S. Hamilton, A. Moffett, A. Sharkey, N. McGovern, Phenotypic and functional characterization of first-trimester human placental macrophages, Hofbauer cells, J. Exp. Med. 218 (2020), https://doi.org/10.1084/JEM.20200891.

[32] S. Mezouar, A. Ben Amara, C. Chartier, L. Gorvel, J.L. Mege, A fast and reliable method to isolate human placental macrophages, Curr. Protoc. Im. 125 (2019) e77, https://doi.org/10.1002/CPIM.77.

[33] M. Lasch, K. Sudan, C. Paul, C. Schulz, T. Kolben, J. van Dorp, S. Eren, S. Beyer, L. Siniscalchi, S. Mahner, U. Jeschke, S. Meister, Isolation of decidual macrophages and hofbauer cells from term placenta—comparison of the expression of CD163 and CD80, Int. J. Mol. Sci. 23 (2022), https://doi.org/10.3390/ijms23116113.

[34] F.O. Martinez, A. Sica, A. Mantovani, M. Locati, Macrophage activation and polarization, Front. Biosci. 13 (2008) 453–461, https://doi.org/10.2741/2692/PDF.

[35] M.B. Brown, M. von Chamier, A.B. Allam, L. Reyes, M1/M2 macrophage polarity in normal and complicated pregnancy, Front. Immunol. 5 (2014) 1–10, https://doi.org/10.3389/fimmu.2014.00606.

[36] P.J. Murray, J.E. Allen, S.K. Biswas, E.A. Fisher, D.W. Gilroy, S. Goerdt, S. Gordon, J.A. Hamilton, L.B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F.O. Martinez, J.L. Mege, D.M. Mosser, G. Natoli, J.P. Saeij, J.L. Schultze, K.A. Shirey, A. Sica, J. Suttles, I. Udalova, J.A. vanGinderachter, S.N. Vogel, T.A. Wynn, Macrophage activation and polarization: nomenclature and experimental guidelines, Immunity 41 (2014) 14–20, https://doi.org/10.1016/J.IMMUNI.2014.06.008.

[37] T. Krausgruber, K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, I.A. Udalova, IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses, Nat. Immunol. 12 (3 12) (2011) 231–238, https://doi.org/10.1038/ni.1990, 2011.

[38] C. El Chartouni, L. Schwarzfischer, M. Rehli, Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming, Immunobiology 215 (2010) 821–825, https://doi.org/10.1016/J.IMBIO.2010.05.031.

[39] K. Rennert, M. Nitschke, M. Wallert, N. Keune, M. Raasch, S. Lorkowski, A. S. Mosig, Thermo-responsive cell culture carrier: effects on macrophage functionality and detachment efficiency, J. Tissue Eng. 8 (2017), https://doi.org/10.1177/2041731417726428.

[40] K. Menck, D. Behme, M. Pantke, N. Reiling, C. Binder, T. Pukrop, F. Klemm, Isolation of human monocytes by double gradient centrifugation and their differentiation to macrophages in Teflon-coated cell culture bags, JoVE (2014), https://doi.org/10.3791/51554.

[41] T. Ohno, K. Kurita, S. Abe, N. Eimori, Y. Ikawa, A simple freezing medium for serum-free cultured cells, Cytotechnology 1 (1988) 257–260, https://doi.org/10.1007/BF00145029.

[42] Cryopreserved Human Macrophages Instruction manual, n.d. www.promocell.com/coa.

[43] N.A. Ortiz Silva, S. Denis, J. Vergnaud, H. Hillaireau, Controlled hydrogel-based encapsulation of macrophages determines cell survival and functionality upon cryopreservation, Int. J. Pharm. 650 (2024) 123491, https://doi.org/10.1016/j.ijpharm.2023.123491.

[44] D.E. Pegg, in: Principles of Cryopreservation, 2007, pp. 39–57, https://doi.org/10.1007/978-1-59745-362-2_3.

Unlocking the secrets of Hofbauer cells in placental (patho-) physiology: Isolation and Quality Assessment in Human Term Placenta

Authors: Monika Horvat Mercnik[1], Christian Wadsack[1;2*], Carolin Schliefsteiner[1]

[1] Medical University of Graz, Austria. Univ. Clinic for Obstetrics and Gynecology, Research Unit

[2] BioTechMed-Graz, Graz, Austria

* Address correspondence to: christian.wadsack@medunigraz.at

Supplementary Material

1. Buffer recipes and required initial preparations

Trypsin DNase I Digestion Solution

Reagent	Volume (ml)
HBSS (10x)	50
HEPES (1M)	12.5
CaCl2 (1M)	1
Mg2SO4 (0.4M)	1
NaOH (2N)	2.5
DNaseI (20mg/ml; Roche, Cat #10104159001)	2
Trypsin (2.5%, 10x; Sigma Aldrich, Cat #T4549)	50
Distilled H2O	Ad 500
Total:	**500**

Mix reagents together (NOTE: addition of 2N NaOH might cause precipitate formation, precipitate will dissolve again once water is added) and filter through a sterile filtration unit.

Complete RPMI

Reagent	Volume (ml)
RPMI 1640 without phenol red	500ml
Fetal Bovine Serum	50ml
HEPES, 1M	12.5ml
Penicillin/Streptomycin Mix (10.000IU/ml)	5ml

Simply add FBS, HEPES and Pen/Strep to the RPMI. Prepare at least 2 bottles per isolation. We recommend always keeping one bottle on 4°C and exchange bottles every other working step, to always have cold medium in use. HBCs stick to plastic easily and maintaining them in cold medium and on ice after taking them off the Percoll gradient improves the yields.

Using RPMI 1640 that does not contain phenol red is crucial, as the pH indicator might affect the gradient centrifugation and make recovery of the desired cell layer harder.

Collagenase A/DNaseI Digestions Solution

Reagent	Amount
Collagenase A (Roche, Cat #10103586001)	100mg
DNaseI (20mg/ml; Roche, Cat #10104159001)	2ml
RPMI 1640	2x 50ml
Total:	**100ml**

Dissolve 100mg of Collagenase A in 2-5ml of complete RPMI medium. It is hard to solve, you will need to flick the tube intensely to get all the powder into solution. Fill the volume up to 50ml. Add 2ml of

DNaseI (20mg/ml) to another 50ml of RPMI medium. Filter both solutions through 50ml Stericup units. Pool together when adding it to the tissue in the bioreactor bottle will provide the complete digestion buffer.

Washing by sedimentation

First, allow the tissue to settle in the bioreactor bottle and remove most of the supernatant using a sterile pipette. Pour the tissue into a fresh beaker, add PBS, stir, and allow the tissue to sediment down again (Supplementary Figure 2). Remove the PBS on top of the sedimented tissue by careful pouring or pipetting and repeat. This option takes a more time and might increase the volume of PBS remaining with the tissue, therefore diluting the digestion solution further. We recommend straining the tissue through a cloth, as it shortens hands-on time and yields more cells.

Percoll gradient solutions

To yield a 90% Percoll solution from the stock, mix 63ml of Percoll with 7ml of 10xHBSS solution. Starting from this 90% solution, prepare the following diluted solutions and manually pipette the gradient layers starting with the highest concentration and going to the lowest concentration. Prepare 6-8 centrifugal tubes (depending on how much tissue you initially started with) and distribute Percoll solutions evenly (e.g. 5ml per layer for eight tubes, 7ml per layer for six tubes). If desired, draw a line on the outside of the tube after each layer is added, to have a better estimate where the desired cells will accumulate.

Fin. Conc. v/v	90% Percoll Solution	RPMI
40% Percoll	20ml	25ml
35% Percoll	17.5ml	27.5ml
30% Percoll	15ml	30ml
20% Percoll	10ml	35ml

Alternatively, if you have access to an ultracentrifuge and sterile ultracentrifuge tubes, gradients can be prepared in one step. Mix 80ml Percoll with 100ml RPMI and 20ml 10x HBSS solution. Distribute the volume evenly onto 8 centrifuge tubes (25ml/tube) and centrifuge at 15000xg for 50min at 4°C, brake OFF. Then put cell suspension gently on top and proceed as described in the general protocol.

Preparation of magnetic beads for Immunopurification

For negative immune selection, magnetic beads are coated with antibodies targeting EGFR and CD10. EGFR is readily present on potentially contaminating cytotrophoblast cells. CD10 (now more commonly referred to as neprilysin) is also expressed on CT but also on fibroblasts, as well as smooth muscle cells. Therefore, negative selection against these cellular markers should yield mostly pure and untouched, i.e. immunologically non-activated, HBCs.

The ratio of cells to beads should be 100ul of beads suspension per 100mio cells. Given the typically obtained yields at the first viable cell count after cells are recovered from the percoll gradient, we recommend to prepare 200ul of anti-EGFR labeled and anti-CD10 labeled beads each. Proceed as follows:

- To make sure to get a defined amount of beads and not mostly storage buffer, gently flick the bead stock solution to mix well.
- Add 200ul of beads (Dynabeads goat anti-mouse IgG, Invitrogen) to a 14ml Falcon tube fitting into the magnetic tube rack. Add 2-4ml of Bead Buffer to remove sodium-azide contained

within the storage solution. Place into magnetic rack, wait for beads to attach to the tube walls and aspirate.

- Wash once more as before.
- Add 200ul of Bead Buffer to each tube and flick to mix with washed beads.
- For anti-EGFR conjugated beads add 40ul (1:5 dilution) of anti-EGFR antibody (Santa Cruz, Cat # sc-120). For anti-CD10 conjugated beads, add 8ul (1:25 dilution) of anti-CD10 antibody (Sigma/Merck #SAB4700440, Clone MEM78).
- Leave to conjugate for 1h at RT under gentle rotation.
- Wash as described before using bead buffer to remove excess antibodies.
- Then add 200ul of Bead Buffer and store at 4°C until further use.

Bead Buffer/FACS staining and washing buffer

Reagent	Amount
PBS (1x)	500ml
EDTA (2mM)	0.34g
BSA (1% w/v)	0.5g
Total:	**500ml**

FcR-receptor Block Solution

Reagent	Volume (mL)
1x HBSS	500 ml
Fetal bovine serum	15 ml

Supplementary Table 1: Troubleshooting – possible troubles and explanations during isolation process

Troubleshooting	Explanation and problem solving
Long isolation process, time constraints	• Store minced placental tissue at 4oC overnight and continue isolation the following day.
Inadequate tissue digestion (tissue too sticky or dissolves completely)	• Use the appropriate concentration of collagenase I (100 mg / 100 g tissue). • Adjust the volume/concentration of digestion enzymes to the wet weight of the minced tissue (max. 150g tissue at given concentrations). • Use the bioreactor with stirring function for the digestion steps. • Ensure that the entire sample is submerged in the water bath and that the water temperature is 37°C. • If the tissue is too sticky/junky and cannot be filtered through the cloth, wash the tissue by sedimentation.
Insufficient Percoll – cell separation	• Centrifuge without brake. • Volume of Percoll layers is too low (Use at least 5 mL of the appropriate Percoll solution per layer.

	• Number of Percoll tubes is too small (use 6-8 Percoll tubes per 100-150 g tissue/isolation).
Insufficient removal of Percoll from the cell suspension	• Increase the volume of RPMI medium per tube, repeat centrifugation step for 10 min.
Erythrocyte contamination	• Increase the number of wash steps during the digestion process. • If contamination occurs after HBC seeding, wash away the erythrocytes by changing the medium.
Trophoblast/fibroblast/muscle cell contamination	• Change the cloth when filtering the tissue digest to avoid contamination. • Adjust the concentration of CD10 and EGFR antibodies for immune purification. • After collecting the HBC fraction, check the beads on the side of the tube walls. If they are covered with non-HBCs, they will appear swollen with cellular material, resembling a 'slurry'.
Low yield of HBCs	• Obtain and mince placental tissue within 30-60 minutes of delivery. Once minced, the tissue can be stored in PBS overnight in the refrigerator. • Check that the placental tissue is finely minced. • Adjust the volume/concentration of the digestive enzymes to the wet weight of the minced tissue (max. 150g at given enzyme concentrations). • The more experience with the protocol and handling, the higher the yields. Be patient and practice the isolation process.
Low viability of HBCs	• Check that the placental tissue is finely minced. • Adjust the volume/concentration of the digestive enzymes to the wet weight of the minced tissue (see above). • HBCs are sticky cells. To avoid sticking to the plastic, place the cells on ice during immune purification/when counting etc.
Detachment and dead HBCs after seeding	• It is essential to change the medium 30-60 minutes after seeding to prevent the HBCs from becoming detached. • The number of HBCs seeded was too low.

HBC differentiation problems	- HBCs lack cell-cell contact and therefore do not differentiate into elongated morphology. - The number of HBCs seeded was too low, increase the concentration of seeded cells.
Problems releasing HBCs for subsequent assays	- Increase the time of Accutase treatment (5 min). - Use trypsin or trypsin EDTA - surface molecules may be detached. - Gently scrape cells on ice after Accutase incubation.
Undetectable HBCs on FACS	- Use aspiration pump to control supernatant washout. - Increase the number of cells for FACS experiments
Non-specific binding on FACS	- Increase wash steps during staining - Include appropriate isotype/FMO controls.

Supplementary Table 2: Used antibodies for Isolation, FACS and ICC analysis.

Antibody	**Distributor / Catalog number**	**Volume (µl) / dillution**
IRF5 Monoclonal Antibody (ALYSCLN), eFluor 660, eBioscience™	Invitrogen / 50-9698-42	2,5 / 100 µl
PE Mouse Anti- IRF4 Clone Q9-343	BD Pharmingen™ / 566646	2,5 / 100 µl
FITC anti-human CD68 Antibody	BioLegend / 333806	2 / 100 µl
Brilliant Violet 421™ anti-human CD209 (DC-SIGN) Antibody	BioLegend / 330118	2 / 100 µl
Brilliant Violet 510™ anti-human CD206 (MMR) Antibody	BioLegend / 321138	2 / 100 µl
Brilliant Violet 650™ anti-human CD80 Antibody	BioLegend / 305227	2 / 100 µl
PE/Dazzle™ 594 anti-human CD86 Antibody	BioLegend / 374218	2 / 100 µl
APC/Cyanine7 anti-human CD40 Antibody	BioLegend / 334324	2 / 100 µl
FITC anti-human CD163 Antibody	BioLegend / 333618	2 / 100 µl
APC anti-human CD163 Antibody	BioLegend / 333609	10 / 100 µl
PE anti-Vimentin Antibody	Abcam / ab49918	5 / 100 µl
Alpha-Smooth Muscle Actin Monoclonal Antibody (1A4), eFluor™ 660, eBioscience	Invitrogen / 50-9760-82	5 / 100 µl
PE anti-human CD90 (Thy1) Antibody	Biolegend / 328109	5 / 100 µl
PE Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400112	2,5 / 100 µl
APC Mouse IgG1 κ Isotype Ctrl Antibody	BD Pharmingen™ / 555751	2,5 / 100 µl
FITC Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400138	2 / 100 µl
Brilliant Violet 510™ Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400171	2 / 100 µl

Brilliant Violet 421 Mouse IgG1, κ isotype Ctr Antibody	BioLegend / 400158	2 / 100 µl
APC/Cyanine7 Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400128	2 / 100 µl
Mouse IgG1 Isotype Control [Alexa Fluor® 594] Antibody	Novus Biologicals / IC002T	2 / 100 µl
Brilliant Violet 650™ Mouse IgG1, κ Isotype Ctrl Antibody	BioLegend / 400163	2 / 100 µl
FITC Mouse IgG2b, κ Isotype Ctrl Antibody	BioLegend / 402208	2 / 100 µl
CD163 Polyclonal Antibody	Thermo / PA5-83817	1:200
Anti-Vimentin Antibody	Dako / MO725	1:100
Smooth Muscle Actin Antibody, Clone 1A4	Dako / M085129-2	1:100
Anti-Fibroblasts (CD90, Thy-1) (Hu) from Mouse (AS02)	Dianova / DIA100	1:100
Negative Control Mouse IgG1. Monoclonal, clone DAK-GO1	Dako / X093101-2	1:100
anti-EGFR Antibody	Santa Cruz / sc-120	40 µl
anti-CD10 Antibody, Clone MEM78	Sigma / SAB4700440	8 µl

Supplementary Figure 1: Washing of tissue between digestion steps by sedimentation.

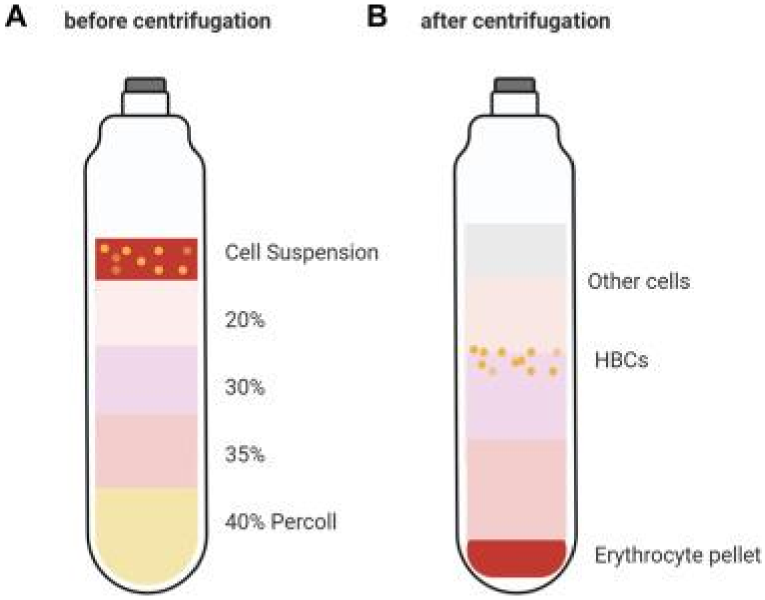
Supplementary Figure 2: Schematic drawing of Percoll gradients before (A) and after (B) centrifugation for better illustrations of HBC containing fraction.

Supplementary Figure 3: Comparison of yield (A) and viability (B) in a subset of HBCs isolated by the protocol according to Ref. 22 or with our modifications.

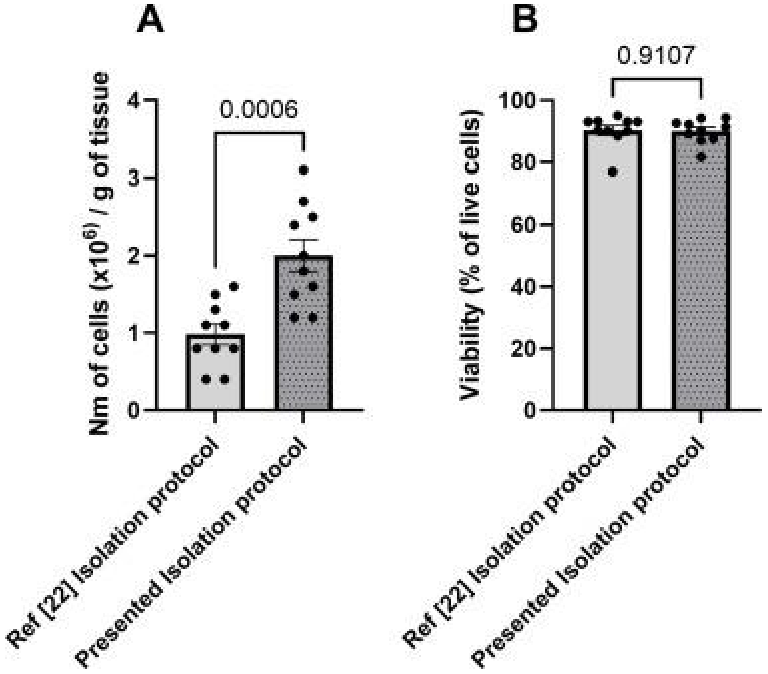
Supplementary Figure 4: Freezing Media tested for Cryopreservation of HBCs. Cells were frozen in MaM with cryoprotective agents namely: (A) 20% FBS + 10% DMSO, (B) 90% FBS + 10% DMSO, (C) 50mM Trehalose + 10% DMSO, (D) 60mM Sucrose + 10% DMSO, (E) 1% Methylcellulose + 10% DMSO. In addition, a commercially available freezing medium for macrophages and microglia by Cell Applications (F) was tested. Upon thawing, cells were plated on 6-well dishes with „No ECM“, i.e. uncoated culture ware (Nunclone Delta Surface) or Fibronectin (3µg/cm^2). Cells were checked under the microscope after 2h, 6h, 24h at which time also a media change was performed, and 48h. Representative images of 2h and 48h timepoints are shown. Images were acquired using CellSens standard software and an Olympus BX53 light microscope with an Olympus UC90 camera. Scale bar = 100µm.

Supplementary Figure 5: ECM coatings for attachment of HBCs after cryopreservation. HBCs frozen in 70% MaM / 20% FBS / 10% DMSO were rapidly thawed and plated at a density of 2x10^6/ml on uncoated culture ware (No ECM), or culture ware coated with Fibronectin (3µg/cm2), rat tail Collagen I (3µg/cm2) or 1% porcine skin gelatine. Cells were checked under the microscope for attachment after 2h, 6h, 24h at which time also a media change was performed, and 48h. Representative images of 2h and 48h timepoints are shown. Images were acquired using CellSens standard software and an Olympus BX53 light microscope with an Olympus UC90 camera. Scale bar = 100µm.

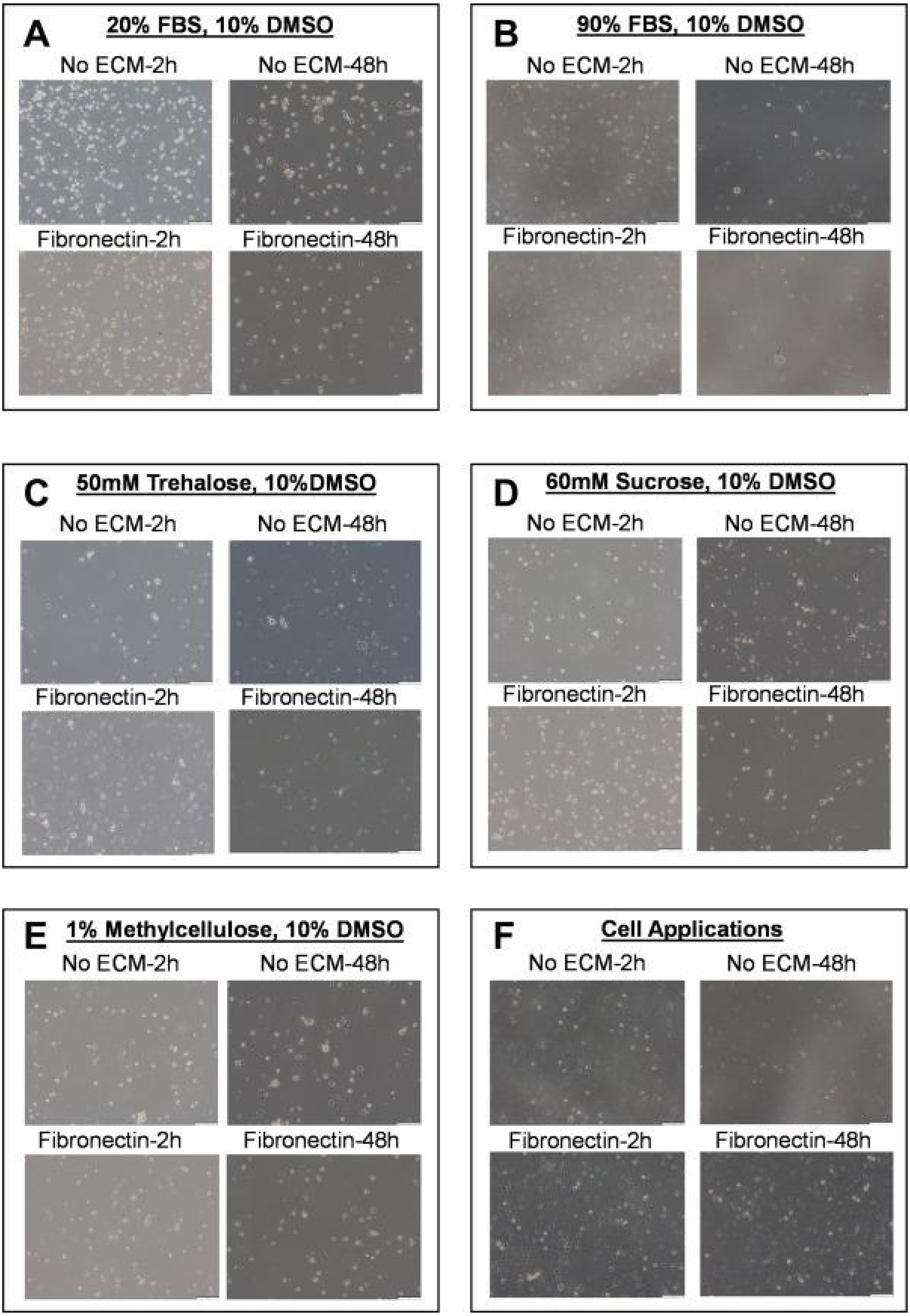
figs1. **Supplementary Figure 1:** Washing of tissue between digestion steps by sedimentation.



figs2. **Supplementary Figure 2:** Schematic drawing of Percoll gradients before (A) and after (B) centrifugation for better illustrations of HBC containing fraction.



figs3. **Supplementary Figure 3:** Comparison of yield (A) and viability (B) in a subset of HBCs isolated by the protocol according to Ref. 22 or with our modifications.



figs4. **Supplementary Figure 4:** Freezing Media tested for Cryopreservation of HBCs. Cells were frozen in MaM with cryoprotective agents namely: (A) 20% FBS + 10% DMSO, (B) 90% FBS + 10% DMSO, (C) 50mM Trehalose + 10% DMSO, (D) 60mM Sucrose + 10% DMSO, (E) 1% Methylcellulose + 10% DMSO. In addition, a commercially available freezing

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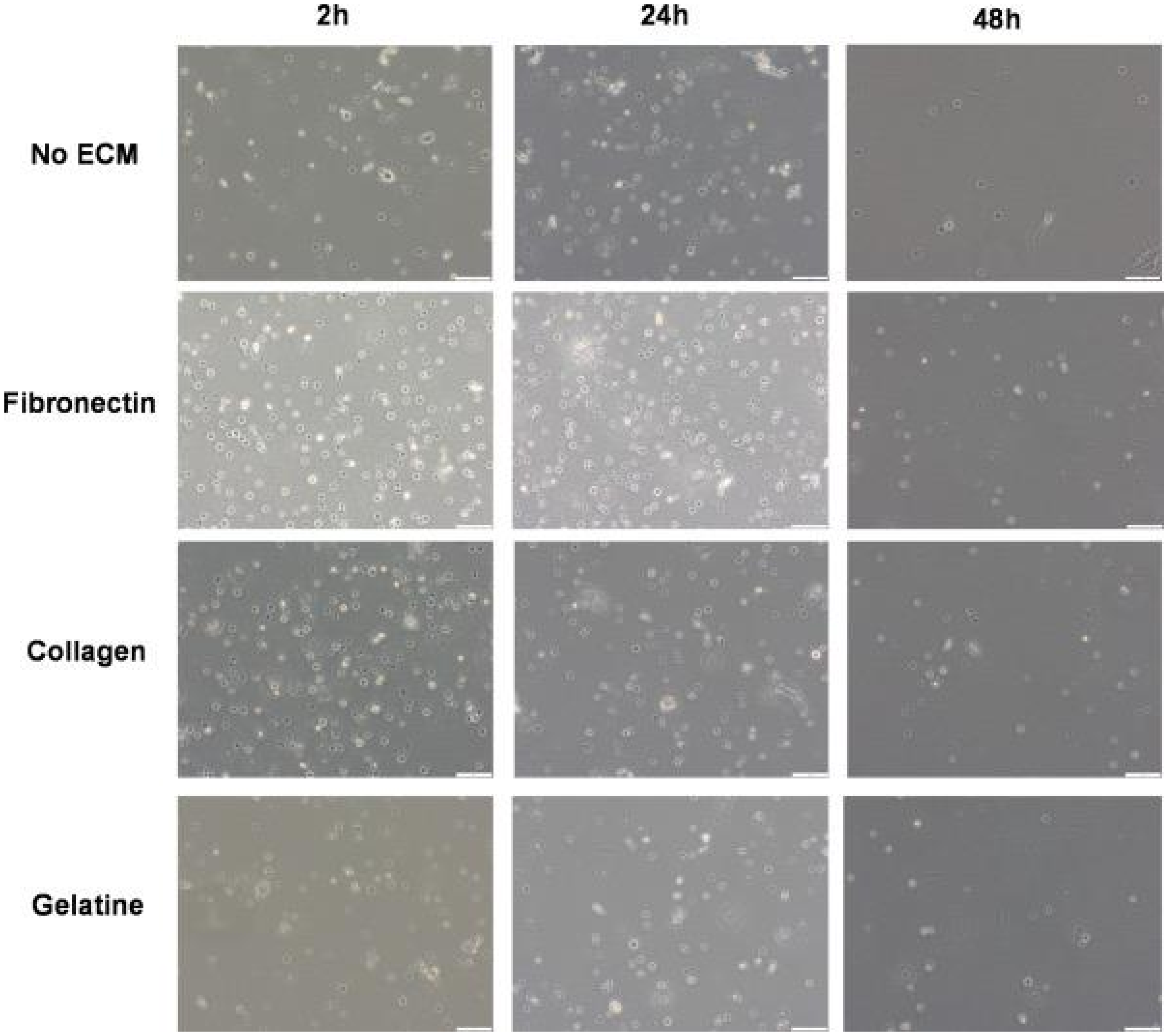

figs5. **Supplementary Figure 5:** ECM coatings for attachment of HBCs after cryopreservation. HBCs frozen in 70% MaM / 20% FBS / 10% DMSO were rapidly thawed and plated at a density of 2x10^6/ml on uncoated culture ware (No ECM), or culture ware coated with Fibronectin (3µg/cm2), rat tail Collagen I (3µg/cm2) or 1% porcine skin gelatine. Cells were checked under the microscope for attachment after 2h, 6h, 24h at which time also a media change was performed, and 48h. Representative images of 2h and 48h timepoints are shown. Images were acquired using CellSens standard software and an Olympus BX53 light microscope with an Olympus UC90 camera. Scale bar = 100µm.