Role of platelets in placental dysfunction

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Abstract

Placental dysfunction is a major cause of pregnancy complications such as preeclampsia (PE) and intra-uterine growth restriction (IUGR) which is associated with feto-maternal morbidity and mortality. The mechanisms underlying these diseases remain obscure and efficient therapies are lacking. Hyperactivation of the hemostatic system, platelet-activation and increased microparticles (MP) formation are commonly associated with pregnancy complications. However, thrombotic occlusion of the placental vascular bed is rarely observed. Accordingly, dispensability of embryonic platelets during intrauterine development has been shown using platelet deficient p45 NF-E2 deficient mice. These mice display IUGR independent of embryonic platelets through a partially unknown pathway. On the other hand, the mechanistic relevance of MP and maternal platelet activation for placental dysfunction remains unknown. Here we show that MP within the mother induce a thrombo-inflammatory response specifically in the placenta. Following MP-injection, activated platelets accumulate particularly within the placental vascular bed. MP cause ATP release from platelets and inflammasome activation within trophoblast cells through purinergic signaling. Inflammasome activation in trophoblast cells triggers a PE-like phenotype, characterized by pregnancy failure, elevated blood pressure, increased plasma sFlt-1, and renal dysfunction. Intriguingly, genetic inhibition of inflammasome activation specifically in the placenta, pharmacological inhibition of inflammasome or purinergic signaling, or genetic inhibition of maternal platelet-activation abolishes the PE-like phenotype. Inflammasome activation in trophoblast cells of women with preeclampsia corroborates the translational relevance of these findings. These results establish that MP within the maternal circulation cause placental sterile inflammation and PE through activation of maternal platelets and purinergic inflammasome activation in trophoblast cells, uncovering a novel thrombo-inflammatory mechanism at the maternal-embryonic interface. The other aspect of the study shows that within the embryonic placental compartment, p45 NF-E2 regulates syncytiotrophoblast formation and IUGR in humans. This process is regulated by CBP dependent Gcm-1 acetylation and SENP1 mediated Gcm-1 desumoylation in human trophoblast cells, corroborating a function of embryonic p45 NF-E2 independent of platelets. Collectively, these data provide novel insights into the developmental function of platelets.

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Zusammenfassung

Plazentare Dysfunktion ist eine der Hauptursachen für Schwangerschaftskomplikationen wie Präeklampsie (PE) und intrauterine Wachstumsrestriktion (IUGR) und ist mit mütterlicher und fetaler Morbidität und Mortalität verbunden. Die Mechanismen, die diesen Krankheiten zugrunde liegen, sind noch weitestgehend unverstanden und effiziente Therapien fehlen. Hyperaktivierung des hämostatischen Systems, Thrombozyten Aktivierung und vermehrte Mikropartikel (MP) - Generierung werden mit Komplikationen während der Schwangerschaft in Verbindung gebracht. Eine pathogenetische Relevanz thrombotischer Verschlüsse der plazentaren Gefäße ließ sich jedoch nicht nachweisen. Untersuchungen Thrombozyten-defizienter p45 NF-E2-Mäuse zeigten dementsprechend, dass embryonalen Thrombozyten während der intrauterinen Entwicklung entbehrlich sind. Diese Mäuse zeigen eine IUGR, welche unabhängig von embryonalen Thrombozyten ist, deren Pathogenese aber nur zum Teil aufgeklärt ist. Auf der anderen Seite bleibt die mechanistische Relevanz mütterlicher und deren Aktivierung durch MP für plazentare Dysfunktion unbekannt. Hier zeigen wir, dass MP innerhalb der Mutter eine thrombo-inflammatorische Antwort speziell in der Plazenta induziert. Im Anschluss akkumulieren durch MP-Injektion aktivierte Thrombozyten vor allem im plazentaren Gefäßbett. MP verursachen eine ATP-Freisetzung aus Thrombozyten und eine Inflammasom Aktivierung innerhalb von Trophoblastenzellen durch Aktivierung purinerger Rezeptoren. Die Inflammasom-Aktivierung in Trophoblastenzellen führt zu einem PE-ähnlichen Phänotyp, der durch eine erhöhte Abortrate, erhöhtem Blutdruck, erhöhte Plasma-sFlt-1 und eine Nierenfunktionsstörungen gekennzeichnet ist. Eine genetische Inaktivierung der Inflammasom Aktivierung speziell in der Plazenta, die pharmakologische Hemmung des Inflammasoms oder der Aktivierung purinerger Rezeptoren, oder eine Hemmung der mütterlichen Thrombozyten Aktivierung verhindern den PE-ähnlichen Phänotyp. Der Nachweis einer Inflammasom-Aktivierung in Trophoblastenzellen von Frauen mit Präeklampsie bestätigt die translationale Relevanz dieser Befunde. Diese Ergebnisse zeigen, dass MP im mütterlichen Kreislauf durch eine Aktivierung von Thrombozyten und purinerger Rezeptoren das Inflammasom in Trophoblastenzellen aktiveren und damit eine sterile Entzündung der Plazenta und Präeklampsie verursachen, Diese Daten weisen damit einen neuartigen thrombo-inflammatorischen Mechanismus an der mütterlich-embryonale Grenzfläche

nach. Untersuchungen zum anderen Gegenstand der Studie zeigen, dass innerhalb des embryonalen Plazentaanteiles p45 NF-E2 die Synzytiotrophoblasten Bildung und IUGR beim Menschen reguliert. Dieser Prozess wird durch CBP abhängige Gcm-1 Acetylierung und SENP1 vermittelte Gcm-1 Desumoylierung in humanen Trophoblastzellen unabhängig von Thrombozyten reguliert. Zusammengefasst geben diese Studien neue Einblicke in die entwicklungsbiologischen Funktionen von Thrombozyten.

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Abbreviations

ASC	Apoptosis-associated Speck-like protein with a caspase-recruitment domain
Casp-1	Caspase-1
CBP	CREB binding protein
DAMP	Danger associated molecular patterns
EVT	Extravillous trophoblast
EPCR	Endothelial Cell Protein C Receptor
Esx-1	Extraembryonic, spermatogenesis, homeobox 1
FVL	Factor-V Leiden
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glomerular basement membrane
Gcm-1	Glial cells missing homolog 1
GP1bα	Glycoprotein Ib (platelet) α-subunit
hCG-β	Human chorionic gonadotropin β
HELLP	hemolysis, elevated liver enzymes, low platelet count
IL-1β	Interleukin-1β
IL-1R	Interleukin-1 receptor
IUGR	Intra-uterine growth restriction
MP	Microparticles
NF-E2	Nuclear factor erythroid derived 2
NLRP3	NACHT, LRR and PYD domains-containing protein 3
PAR	Protease activated receptor
PL-II	Placental lactogen-II
PLA	Proximity ligation assay
PPP	Platelet poor plasma
PRP	Platelet rich plasma
p.c.	post-coitus
PE	Preeclampsia
ROTEM	Rotational thromboelastometry
SENP	Sentrin/SUMO specific protease
sFlt-1	Soluble fms-like tyrosine kinase-1

SUMO	Small Ubiquitin-like Modifier
Syn-1	Syncytin-1
TAFI	Thrombin-activatable fibrinolysis inhibitor
TEM	Transmission electron microscopy
TF	Tissue factor
TM	Thrombomodulin
Tpbpa	Trophoblast specific protein alpha

1.0 Introduction

1.1 Placental Dysfunction

The placenta is an organ specific for the mammalian pregnancy, which connects the developing fetus to the maternal uterine wall and serves as a transport organ allowing nutrient uptake, waste elimination and gaseous exchange by the fetus. In addition it secretes hormones which are required for successful maintenance of pregnancy. Placentation stems from the embryonic trophoblast which is composed of an outer multi-nucleate syncytiotrophoblast underlined by the proliferating cytotrophoblast [1]. These coordinately develop and form villous trees. The syncytiotrophoblast is broken through by the cytotrophoblast which differentiate into extravillous trophoblasts (EVTs), which invade the uterine stroma [2]. The EVTs migrate into the maternal decidual tissues and blood vessels and are directed mainly towards the uterine spiral arteries [3]. Proliferating EVTs which migrate along the vascular lumen of spiral arterioles form the endovascular trophoblast. The interstitial EVTs migrate around the spiral arteries and replace the muscular linings. These processes prevent the constriction of maternal blood vessels and enable adequate perfusion of the intervillous space in the placenta [4]. Inappropriate placental development or vascularization leads to placental dysfunction and is a cause of two frequent pregnancy complications - preeclampsia (PE) in the mother and intra-uterine growth restriction (IUGR) of the embryo [5, 6].

1.2 Preeclampsia

Preeclampsia is a hypertensive disorder of pregnancy affecting 3-5% of pregnancies worldwide and is a leading cause of maternal and fetal morbidity and mortality. PE is of placental origin and the delivery of placenta remains the only effective remedy resulting in pre-term delivery [7, 8]. The causes and mechanisms involved in the origin and progression of PE remain enigmatic and accordingly efficient and specific therapies are lacking [9]. PE is characterized by defective invasion of EVTs into maternal spiral arteries and impaired spiral artery remodeling. This causes impaired placental vascularization and is often associated with IUGR of the fetus [10]. Hypertension and Proteinuria are two characteristics symptoms implicating the occurrence of preeclampsia [11, 12]. Hypertension is defined as a systolic blood pressure $\geq$ 140 and/or a diastolic blood pressure $\geq$ 90 mmHg measured at two occasions with at least 4 h in between. Proteinuria is defined excretion of as $\geq$ 300 mg protein in urine per day and is usually associated with other renal complications which include glomerular endotheliosis and podocyturia (loss of

podocytes in the urine) [13]. Development of both hypertension and renal pathology implicate the associate of endothelial dysfunction in PE. Clinical studies have shown that factors associated with an injured or activated endothelium are prevalent in women affected with PE. These include sFlt-1, sEnogelin-1, endothelin-1, fibronectin, von Willebrand factor, thrombomodulin, markers of oxidative stress and inflammatory cytokines [14-19]. This makes PE an inflammatory placental disorder affecting the maternal endothelium. The manifestation of the disease is accompanied with several changes in the hemostatic system and thrombotic events resulting in pregnancy failure. A severe form of PE results in HELLP-syndrome (hemolysis, elevated liver enzymes, low platelet count). Therefore PE is a multi-factorial disease of the pregnancy associated with the placenta and affects several organ systems in the mother which ultimately lead to maternal and fetal morbidity.

1.3 Intra-uterine growth restriction

IUGR is a pregnancy complication resulting in the failure of the fetus or new born to achieve its growth potential. This increases the risk of perinatal morbidity and mortality and predisposes to diseases in later life such as diabetes mellitus or cardiovascular complications. Altered hemostatic system during pregnancy, maternal and paternal genetic factors, immunological factors and hypertensive disorders of PE can cause IUGR [20]. Placental insufficiency is a major cause of insufficient embryonic growth. An insufficient placenta failing to provide sufficient nutrients and gaseous exchange manifests into fetal metabolic, endocrine and vascular responses leading to developmental failure and still birth. There are meager mechanistic studies exploring the causes of IUGR and need to develop efficient therapies prevails [21].

1.4 Gestational hemostasis

Changes in the hemostatic system during pregnancy are well established [22, 23]. During pregnancy the mother acquires a slight pro-thrombotic state, which increases the risk of thrombotic events. This potential disadvantage is thought to be outweighed by a decreased risk of hemorrhage and excessive blood loss during delivery. However the physiological relevance of the acquired pregnancy associated hypercoagulable state remains unknown. Hypercoagulability is associated with an increase of pro-coagulant coagulation factors (e.g. factors I, V, VII, VIII, IX, X), increased platelet activation paralleled by a decreased number of circulating platelets, and increased microparticles (MP) [24-26]. The coagulation system at the feto-maternal interface which is balanced by both maternal and embryonic factors works in close coordination with the

platelets and regulates appropriate transfer of material to the embryo and its development. Hence it is highly important to dissect these components in order to understand their individual relevance and their impact on platelet function. Additionally it provides important clues for the development of therapeutic strategies which are safe and efficient in terms of maternal health and fetal development.

1.5 Platelets in Pregnancy

Platelets are a key element of the blood and play an essential role in primary thrombogenesis and hemostasis through mechanisms which include adhesion, aggregation, secretion and expression of procoagulant activity. They play an important physiological and pathological role during pregnancy. Thrombocytopenia (low platelet counts) and/or platelet-dysfunction have been associated with pregnancy complications like preeclampsia and HELLP-syndrome [27]. It is usually a common phenomenon for the platelet count to decrease as the pregnancy progresses. This may be physiologically normal but in certain disorders this may be more frequent and cause a complication during pregnancy affecting the development of the fetus. In these situations, the platelet volume and life span is reduced and platelets tend to be activated and aggregate more readily compared to a normal pregnancy. These changes in platelet function and reactivity during pregnancy and its associated complications are poorly understood. It can however not be ignored that a balanced homeostasis of platelet function is essential for the normal development and success of pregnancy.

Gestational thrombocytopenia or low platelet counts (<150 000/µl) during pregnancy is a common phenomenon (~8%-10% of pregnancies) occurring usually in the third trimester [28-30]. This is usually not associated with adverse maternal or fetal outcomes and may reflect an increased platelet turnover or a relative dilution due to an increased plasma volume. However, in the context of other pathologies, such as immune thrombocytopenic purpura (ITP), HELLP-syndrome (hemolysis, elevated liver enzymes, low platelet count), or heparin-induced thrombocytopenia, a fall of the platelet count indicates a potential harmful pregnancy associated disease process and the loss of platelet function itself may indeed become harmful.

1.5.1 Platelets in placental dysfunction

Hypertensive disorders of pregnancy such as preeclampsia and HELLP syndrome are a major cause of maternal, fetal and neonatal morbidity and mortality affecting approximately 5%-7% of pregnancies [31]. These pregnancy-associated diseases are associated with endothelial

dysfunction and a low platelet count. One of the key diagnostic criteria of HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count) is a drop of platelet numbers in the peripheral blood (below 100,000/µl)[32]. An early drop of platelet numbers before the onset of renal changes has been demonstrated, compatible with a causative role of platelet activation [33]. Pregnancy in general, and hypertensive disorders of pregnancy in particular, are associated with increased activity of the hemostatic system, but to which extent the activated coagulation system contributes to the drop in platelet numbers is not known. However, hypertensive disorders are likewise associated with an imbalance of prostacyclin (a vasodilator and platelet inhibitor) and thromboxane (a platelet activator and vasoconstrictor) and hence changes in platelet numbers and activation may be simply the consequence of an underlying disorder. Additionally, platelet properties change in the context of hypertensive disorders of pregnancy, as they display an increase in membrane fluidity, cholesterol concentration (independent of plasma levels), and an increase ratio between unsaturated and saturated fatty acids [34, 35].

Markers of platelet activation such as β-thromboglobulin, thromboxane β2, platelet factor-4, or soluble P-selectin are associated with hypertensive disorders of pregnancy, reflecting increased platelet activation in these disorders [36-39]. Interestingly, platelet reactivity and the propensity to secrete ATP also increase in HDPs [40, 41]. Further byproducts of platelet activation are microparticles. While the association of MP and platelet activation with PE is known, their potential interaction and the mechanistic relevance of MP and platelet activation for PE remains unresolved.

1.5.2 Microparticles in Pregnancy complications

Microparticles (MP) are small (100 – 1000 nm) membrane-bound extracellular vesicles that are released from cells during activation or cell death (Fig. 1.1) [42]. They can transmit intercellular signals both locally and systemically via the circulation. An important step in MP formation is the loss of plasma membrane asymmetry leading to the exposure of phosphatidylserine (PS) [42]. This and the frequently observed presence of TF on MP provide MP with procoagulant properties. Intriguingly, MP isolated from pregnant women with PE differ phenotypically and functionally from those isolated from healthy pregnant controls [43, 44].

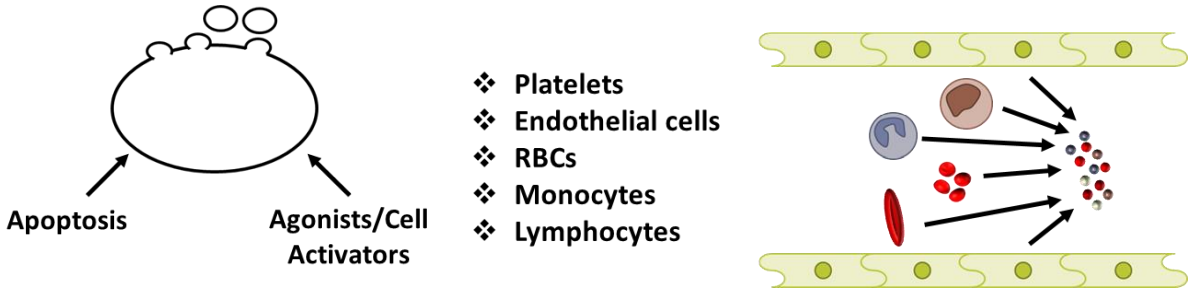


Fig. 1.1: Schematic representation showing MP can be generated from a cell which is undergoing apoptosis or has been activated using an agonist. In blood circulation, MP can be generated from different cell types which include but are not limited to platelets, endothelial cells, erythrocytes, monocytes or lymphocytes.

Several studies have suggested an association of pro-coagulant microparticles with pregnancy complications and fetal loss [25, 45]. *in vitro* microparticles isolated from women with pregnancy complications cause endothelial dysfunction in isolated myometrial arteries from healthy pregnant women [46]. Of note, not only platelet derived microparticles, but also microparticles of other cellular origin are associated with impaired pregnancy outcome [47]. Indeed, syncytiotrophoblast derived MP interact with thrombin activated platelets, and this interaction increases when using syncytiotrophoblast derived MP from pregnant women with preeclampsia compared to those from women without preeclampsia [48]. The occurrence of microparticles may hence reflect general cell-activation and maybe part of a self-propagating disease process. Accordingly, endothelial cell activation is well established in hypertensive pregnancy disorders, which likely contributes to MP formation and itself may cause platelet activation [49-51].

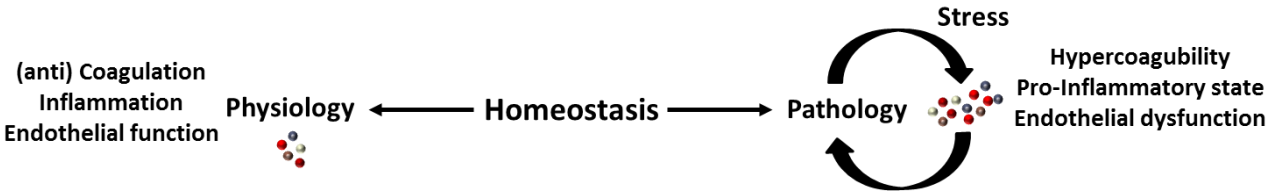


Fig. 1.2: Schematic representation showing that MP are involved in physiological and pathological processes.

Collectively, these studies imply a function of platelet activation and potentially microparticles for pregnancy associated vascular dysfunction and fetal loss. It is conceivable that platelets either directly cause or propagate the disease process. However causality and potential mechanisms remain to be established. On the other hand, MP may not necessarily have deleterious functions (Fig. 1.2). They have been shown to be involved in physiological processes which include, but may not be restricted to, intercellular communication, cellular waste management, improving

endothelial function and anti-inflammatory functions [52-56]. This shows that a balance between the physiological and pathological functions of MP is required to maintain cellular homeostasis.

1.5.3 Relevance of embryonic platelets in development

Early megakaryocyte like cells can be found as early as day 7.5 post-coitum (p.c.) [57]. Thus megakaryocyte and potentially platelets are present in the embryo during the establishment of a cardiovascular system and formation of the hemochorial placenta. This raises the question as to whether platelets may be required for the development of the cardiovascular system or the placenta. Qualitative defects with less responsive platelets owing to absence of the Gαq subunit or the thrombin receptors, protease activated receptor-4 (PAR4) or PAR3, have no effect on placentation or pregnancy outcome [58, 59]. Unlike in human platelets, in which PAR1 is the pivotal thrombin receptor required for thrombin dependent platelet activation, PAR4 activation by thrombin primarily fulfills this function in rodents, while PAR1 is dispensable. PAR3 is an accessory thrombin receptor on rodent platelets, enhancing PAR4 activation by thrombin, but it is not strictly required [60]. As both PAR3 and PAR4 deficiency does not impede placental or embryonic development, similar to that observed in Gαq deficient mice, qualitative platelet activation defects are entirely compatible with normal embryonic development and placentation in mice.

However, a severe quantitative platelet deficiency owing to the inactivation of the p45 subunit of the NF-E2 (Nuclear factor erythroid derived 2) transcription factor, which regulates megakaryopoiesis and is required for pro-platelet formation, does result in a growth retardation of the embryo and placental malperfusion [61-63].

The transcription factor nuclear factor erythroid derived-2 (NF-E2) belongs to a basic leucine-zipper family of transcription factors and is composed of a heterodimer formed of a tissue-restricted 45 kDa (p45) and widely expressed 18 kDa (p18) subunits [64]. The *in vivo* role of NF-E2, which was thought to be restricted to hematopoietic cells and relevant for erythropoiesis, was studied in mice lacking p45 subunit. Somewhat unexpectedly, these mice had almost normal erythropoiesis, but failed to complete the process of megakaryocyte maturation, resulting in thrombocytopenia, and an intrauterine growth restriction [63].

The absence of embryonic or placental defects in Gαq or PAR4 deficiency already indicates a function of platelets for placentation independent of their activation potential, or a specific function of p45 NF-E2 independent of platelets for placentation. Specific restoration of p45 NF-

E2 expression in trophoblast cells by tetraploid aggregation or lentiviral infection of trophectoderm paired with platelet restoration experiments excluded a function of embryonic platelets and established a function of the transcription factor p45 NF-E2 in trophoblast cells for placentation [62]. Trophoblast and placenta specific deficiency of p45 NF-E2 in mice is responsible for excess syncytiotrophoblast formation and this process is regulated through acetylation of Gcm-1 [62]. Further, p45 NF-E2 modulates Gcm-1 expression by increasing acetylation of JunD and thus its binding to the Gcm-1 promoter [65]. These studies clearly establish that p45 NF-E2 has a cell-autonomous function in trophoblast cells, modulating their differentiation, while embryonic platelets are dispensable for placentation and embryonic development. It however remains to be identified whether these effects seen in mice are of translational relevance to humans. This would help in identification of candidate gene which might serve as a biomarker for prediction of such pregnancy associated complications aiding in therapeutic development.

In addition, the mechanism by which p45 NF-E2 regulates Gcm-1 is not understood. Gcm-1 is known to regulate trophoblast fusion by transcriptionally activating Syncytin-1 and Syncytin-2 [66, 67]. The transcriptional activity of Gcm-1 is in-turn regulated by CBP which acetylates Gcm-1 at Lys367, Lys406, and Lys409 thereby preventing its ubiquitination and enhancing its stability [68]. Gcm-1 activity and placental cell fusion has been shown to be dependent on Gcm-1 desumoylation. SUMO proteins form an isopeptide bond with the target protein and result in sumoylation which can modify protein function. SENP1 is a sentrin/SUMO-specific cysteine protease which interacts with Gcm-1 and results in desumoylation of Gcm-1 promoting placental cell fusion. Whether CBP and / or SENP1 are involved in the p45 NF-E2 dependent regulation of Gcm-1 and whether p45 NF-E2 regulates Gcm-1 in human trophoblast cells and the potential relevance of this for pregnancy complications, in particular IUGR, in humans remains unidentified.

1.5.4 Relevance of maternal platelets in development

Unlike the dispensability of embryonic platelets, maternal platelets are required during placentation. Placental tissues from p45 NF-E2 null mothers display large blood pools indicating hemorrhage into the placenta, which, however, does not impair maternal or embryonic survival or embryonic development. This phenotype is specific to maternal platelet deficiency and independent of the embryonic platelet deficiency (Fig. 1.3). Strikingly, maternal fibrinogen

deficiency causes excessive bleeding into the placenta and the uterine cavity, resulting in abortion and hemorrhagic death of the mother in mid-gestation [69]. These contrasting phenotypes of p45NF-E2 and fibrinogen deficient mice indicate that fibrinogen mediates a crucial function of placentation beyond blood-clotting. It is conceivable, but remains to be shown, that fibrinogen, fibrin, or fibrinogen derived peptides have a function during placentation independent of blood clotting, such as tissue-remodeling or cell-adhesion. Intriguingly, low-TF (tissue factor) activity in pregnant female mice results in placental hemorrhage, but also subtle structural alterations within the placenta [70]. Whether the impaired TF-activity and loss of fibrinogen share a common mechanism in placentation and whether maternal platelets contribute to these functions remains unknown.

1.5.4.1 Maternal platelets in TM-EPCR pathway during development

While the question as to whether fibrinogen has a function in placentation independent of blood-clotting (e.g. the fibrin-platelet aggregate formation) remains unknown, a function of the cell-surface expressed coagulation regulators thrombomodulin (TM) and endothelial protein C receptor (EPCR) independent of blood-clotting is established [71, 72]. Loss of TM is embryonic lethal at day 8.5 p.c., causing rapid resorption until day 9.5 p.c. Likewise, loss of EPCR causes embryonic death around day 8.5 p.c., but the phenotype is less protruding and resorption of death embryos is protracted [71, 72]. The rapid resorption of TM-deficient embryos reflects the dual role of TM both in activating protein C and inhibiting fibrinolysis through activation of thrombin activatable fibrinolysis inhibitor (TAFI, aka as carboxypeptidase N) [73, 74]. Increased fibrinolysis and generation of fibrin-split products in TM-null placentae with reduced TAFI activation induces trophoblast cell death and hence rapid resorption [74]. The TM-null and EPCR-null embryonic lethal phenotype can be both rescued by trophoblast specific expression of TM or EPCR, respectively, establishing that the expression of both anticoagulant regulators is required on embryonic trophoblast cells at the embryonic maternal interface [75, 76] (Fig. 3, 4). The developmental function of TM is related to its capacity to initiate coagulation through a TF mediated pathway. Expression of tissue factor on the placental trophoblast cells provides a constitutive procoagulant stimulus in the placental vascular bed which would be suppressed by the activation of TM-EPCR pathway. A complete absence of TF in TM null embryos causes intrauterine lethality owing to vascular defect of the yolk sac but the embryos survive at least to E10.5. This TF dependent resorption of TM null embryos is accompanied by TF initiated

generation of thrombin and conversion of fibrinogen to fibrin and its degradation products. High levels of thrombin generation in these mice could potentially activate PAR receptors on trophoblast cells of the placenta. Deficiency of PAR1 or PAR2 from the trophoblast and embryo is, however, not protective in TM null embryos, establishing that the placental defect in TM-null embryos is independent of excessive PAR1 or PAR2 activation [77]. Intriguingly, maternal – but not embryonic – PAR4 deficiency is able to partial rescue TM null embryos [77]. Additionally, maternal platelet deficiency in p45 NF-E2-null mice or platelet depletion by anti-platelet antibodies likewise partially rescues TM-null embryos [77]. These studies establish a role of maternal platelets and platelet activation in regulating the lethality of TM-null embryos (Fig. 1.3, 1.4). The only partial rescue of TM-null embryos in the presence of maternal platelet deficiency indicates the involvement of other unidentified mechanisms or simply partial efficacy in platelet depletion. As excessive blood clotting has not been detected in TM-null embryos the death promoting mechanism of TM-null embryos must be independent of occlusive blood clot, but related to a blood-clot-independent interaction between TM and TF. Of note, as platelet deficiency improves survival of TM-null embryos, the mechanism must depend on platelets.

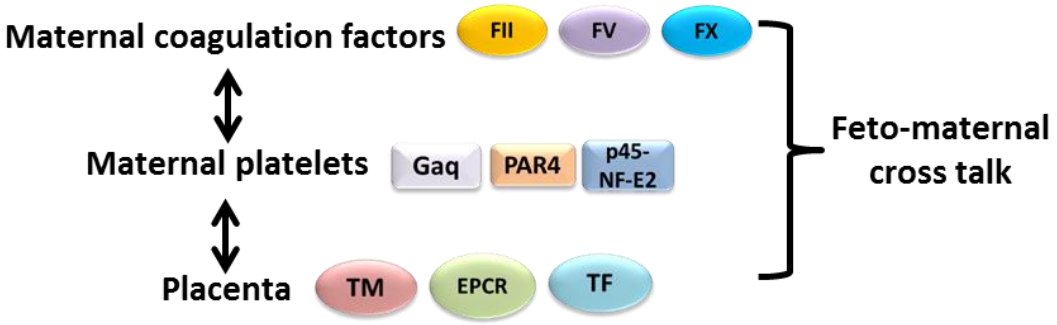


Fig. 1.3: Maternal coagulation factors and coagulation protease signaling regulated by the TM-EPCR system on trophoblast cells maintain the feto-maternal cross talk at the placental vascular bed and are required for normal placentation, placental function and embryonic platelets. Maternal platelets play an important mediator in modulating this hemostatic balance and their activation can lead to placental dysfunction and pregnancy failure. Factor II (FII); Factor V (FV); Factor X (FX); Gαq (Gαq receptor); protease activated receptor 4 (PAR4); p45 subunit Nuclear Factor erythroid derived 2 (p45-NF-E2); Thrombomodulin (TM); Endothelial Protein C Receptor (EPCR); Tissue Factor (TF).

The translational relevance of these insights is emphasized by the interaction of a frequent thrombophilic risk factor, the factor V Leiden (FVL) mutation, resulting in resistance to activated protein C, with hypomorphic embryonic TM expressed within the placenta and maternal platelets. The FVL mutation is associated with fetal loss and adverse pregnancy outcomes and the

combined presence of maternal FVL and partial loss of TM-dependent protein C activation on trophoblast (by expression of the G404P TM-mutant, TM^{Pro}, with markedly reduced ability to activate protein C) is embryonic lethal [78]. For example, intercrossing mice with homozygous FVL-mutation along with one TM^{Pro} allele ($TM^{Pro/+}$) shows a complete lethality of $Fv^{qq}TM^{ProPro}$ and fewer $Fv^{qq}TM^{Pro+}$ embryos. TM^{ProPro} embryos in $Fv^{qq}TM^{Pro+}$ mothers possess developmental defects at day 9.5 p.c. and are lost by day 10.5 p.c. The placentae of these embryos are smaller and lack a well-formed labyrinth layer, reflecting a failure of vascular remodeling and potential invasion of embryonic blood vessels into the developing placenta. This placental phenotype is, however, not associated with signs of thrombosis or impaired placental perfusion secondary to thrombosis [78]. Importantly, maternal platelet depletion starting a day 7.5 p.c., but not at day 9.5 p.c., prevents embryonic loss [78]. Similar to the results obtained in TM-null embryos, PAR4 deficiency, resulting in platelets unresponsive to thrombin, rescues the developmental block of TM^{ProPro} embryos in $FV^{qqTMPro+}$ mothers. This suggests that increased platelet activation during early placentation (~day 7.5 p.c.) impairs placental development and / or function and proper embryonic development in a pro-thrombotic maternal environment. Intriguingly, the authors consistently were unable to detect blood clots within the placenta. Nevertheless, low molecular weight heparin (LMWH) treatment, which is expected to dampen coagulation and hence platelet activation, is protective in this model of platelet dependent abortion. Strikingly, while the authors concluded that thrombin mediated maternal platelet activation is central for placental failure, other anticoagulants, including the direct thrombin inhibitor lepirudin, failed to be protective [79]. Hence, the precise mechanism causing platelet activation and subsequent placental failure remains unknown.

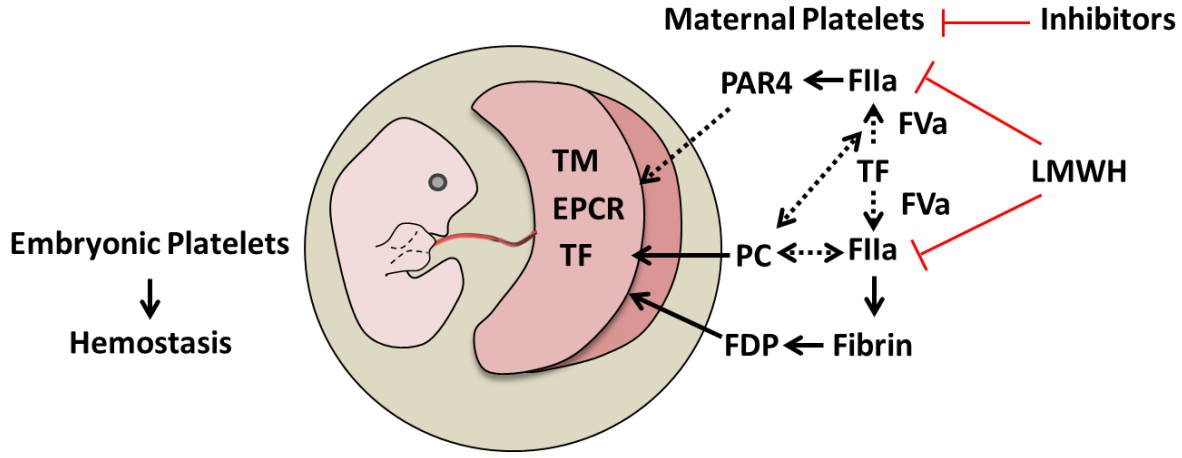


Fig. 1.4: Scheme summarizing the effects of platelets in embryonic development. Embryonic platelets are not required during intrauterine development. However, postnatally they are required for normal hemostasis. Conversely,

maternal platelets are required for proper placental function. Through poorly defined mechanisms they contribute to normal placentation. Excess platelet activation via PAR4, e.g. secondary to a disturbed interaction of embryonic (thrombomodulin, TM, endothelial protein C receptor, EPCR, tissue factor, TF) and maternal (thrombin, FIIa; fibrinogen and fibrin, activated factor V, FVa) coagulation regulators at the placental fetal-maternal interface cause placental dysfunction and embryonic demise. These effects can be improved by treatment with low-molecular weight heparin (LMWH) or inhibition of platelet-activation.

1.6 Inflammatory function of Platelets

A pro-inflammatory function of platelets, which is at least in part independent of blood-clotting, has been increasingly recognized in recent years [80]. Platelets have shown to have a vital role as innate immune cells causing inflammation. An injury of the vascular endothelium promotes the adhesion of platelets on the endothelial surface and attracts leukocytes. The adhesion of leukocytes on the endothelium is further mediated by endothelial-adherent platelets forming a bridge which is a site for thrombo-inflammatory injury. Although, platelets do not have a nucleus, but they possess RNA molecules which can be processed through post-transcriptional processes such as spliceosome machinery which is present in the platelets. This gives them a unique mechanism to mediate inflammatory effects. These pro-inflammatory processes may be related to the release of platelet derived bioactive mediators, such as ADP or IL-1β, or the generation of microparticles [81-83]. Such a thrombo-inflammatory function of platelets in pregnancy complications has not been explored.

1.7 Sterile Inflammation

An inflammation triggered by sterile stimuli including mechanical trauma, ischemia, toxins, minerals, crystals, chemicals, and antigens but not by an infectious agent such as bacteria or virus etc. is termed as sterile inflammation. These sterile stimuli typically trigger signaling pathways which ultimately activate the interleukin-1 (IL-1) pathway. Three major forms of IL-1 – IL-1α, IL-1β and IL-1ra are known. IL-1α and IL-1β both bind to the IL-1 receptor (IL-1R) whereas IL-1ra is a competitive antagonist for this receptor. IL-1β is the most studied pro-inflammatory cytokine involved in sterile inflammation. It is first produced in the cytosol as an inactive precursor form (pro-IL-1β) which is then cleaved by active caspase-1 to convert into mature IL-1β (Fig. 1.5). This mature form the cytokine is secreted by the cells and further activates IL-1R signaling. Activation of caspase-1 further requires formation of a complex known as the inflammasome. This complex majorly consists of Nod-like receptor (NLR) containing proteins

such as NLRP1, NLRP3, NLRC4 and AIM-2 NLR protein. NLRP3 complex is well-studied and implicated in responses to diverse sterile stimuli [84].

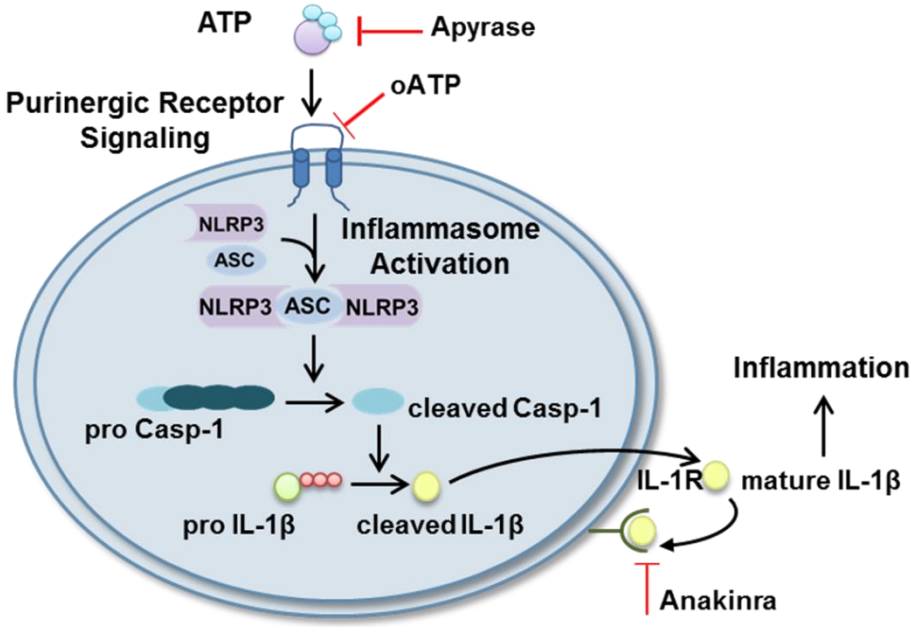


Fig. 1.5: Schematic representation of purinergic inflammasome signaling. Extracellular ATP activates purinergic receptors which mediate the assembly of NLRP3-ASC inflammasome complex. This results in Caspase-1 cleavage and activation which further cleaved IL-1β. Cleaved IL-1β is then secreted by the cell which mediates the inflammatory effects through IL-1R signaling. Apyrase, oATP and Anakinra can block the pathway at different levels and mediate protection.

The NLRP3 inflammasome is activated in response to stimuli generated during tissue damage, metabolic dysregulation, infection etc. These stimuli are known as danger associated molecular patterns (DAMPs) and include, among others, ATP, ADP, cholesterol, biglycans. These activate cell receptors which trigger the assembly of inflammasome complex. One such receptor signaling known for pathological sterile inflammation is the purinergic receptor signaling. Purinergic receptors are present on the surface of various cell types and activated by nucleotide based DAMPs such as ATP/ADP [85]. Activation of purinergic receptor triggers the NLRP inflammasome complex formation which consists of NLRP3 and ASC molecules. This complex cleaves the inactive (pro) form of caspase-1 into active (cleaved) caspase-1 which further cleaves pro IL-1β (Fig. 1.5). Placental dysfunction such as PE is associated with inflammatory condition in the placenta and systemic inflammation in the mother but if sterile inflammation and inflammasome activation contributes to these is not known.

1.8 Aim of the study

In order to define the role of platelets in placental dysfunction, we first aimed at studying the role of maternal platelets. A qualitative platelet deficiency ("loss of function") has been shown earlier to be entirely compatible with normal embryonic development in mice. However, the role of enhanced activation of maternal platelets ("gain of function") in pregnancy complications remains obscure. A proinflammatory function of platelets is increasingly recognized but is not understood in PE. Procoagulant microparticles are elevated in PE, but their potential interaction with platelets and its mechanistic relevance has not been studied. Furthermore, how MP and platelet activation, which can be systemically detected, may promote a placental specific thrombo-inflammatory disease process, is not understood. We therefore aimed to study the pathogenetic role of MP and platelet-activation and the associated inflammatory micro-milieu involved in pregnancy complications.

Another aspect of the study was aimed at investigating the role of transcription factor p45 NF-E2 in placental dysfunction. The loss of transcription factor p45 NF-E2 has been shown to have IUGR and placental insufficiency in mice independent of quantitative platelet deficiency of these embryos. While these studies provided strong evidence for a role of p45 NF-E2 independent of embryonic platelets, the mechanisms involved in causing these effects partially remain unknown. Furthermore, the relevance of these mechanisms in human pregnancy complications remains completely unresolved. We therefore investigated the role of p45 NF-E2 in human trophoblast cells and placenta.

2.0 Methods

2.1 Mice

Wild type C57BL/6 mice and constitutive NLRP3 and Casp-1 knockout mice were obtained from The Jackson Laboratory. p45-NFE-2 and Gαq knockout mice have been previously published [58]. The presence of targeted genes and transgenes was routinely determined by PCR analyses of tail DNA. To ensure randomization no pre-selection was conducted. Mice were bred within the colony and randomly allocated to experimental groups upon availability. Great care was taken to allocate littermates to different experimental groups and mating of littermates for experimental breeding was strictly avoided.

2.2 Human Tissues

Human placenta samples from pregnancies complicated with preeclampsia, normotensive IUGR and normotensive control pregnancies were provided by Universitätsklinikum Erlangen Frauenklinik (Erlangen, Germany), Universitätsfrauenklinik Magdeburg and Klinkum Olvenstedt Magdeburg (Magdeburg, Germany) in accordance with the guidelines and with the approval of the local ethics committee and after obtaining informed consent of patients. Preeclampsia was characterized by the development of hypertension, proteinuria, and an increased sFlt-1/PIGF ratio (>38). IUGR was defined as a growth below the 5% percentile.

2.3 Cell culture

Mouse trophoblast stem (TS) cells were obtained from J. Rossant (Hospital for Sick Children, Toronto, ON, Canada) and were maintained as stem cells or induced to differentiate [74, 86]. Briefly, TS cells were maintained in TS cells growth medium with FGF-4, at 37°C, in a humidified incubator with 5% CO_2. This was replaced by TS cells differentiation medium and the cells were allowed to differentiate for 5 days before analysis.

Human trophoblast like cells BeWo were obtained from ATCC and were cultured at 37°C, in a humidified incubator with 5% CO_2 in Ham's F-12K nutrient mixture with 10% fetal bovine serum and 1% Pencillin-Streptomycin.

Human trophoblast like cells JEG-3 were obtained from ATCC and were cultured at 37°C, in a humidified incubator with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% Pencillin-Streptomycin.

Mouse derived SVEC cells (mouse endothelial cells) were obtained from ATCC and were cultured at 37^{o}C, in a humidified incubator with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 1% Pencillin-Streptomycin.

Human derived HUVEC cells (human endothelial cells) were obtained from PromoCell and were grown on 0.2% gelatin-coated plates and maintained at 37 °C in a humidified 5% CO_2 incubator using endothelial growth medium in the presence of growth factors and supplements.

2.4 Generation and purification of procoagulant microparticles

Mouse derived SVEC cells or human derived HUVEC cells were serum starved for 72 hours to generate MPs. Cell culture supernatant was collected, centrifuged at 200xg for 10 minutes, followed by high speed centrifugation at 20000xg for 45 minutes to pellet endothelial cell derived MPs. For isolation of human or mouse platelet derived MPs citrated whole blood was collected and platelet rich plasma separated after centrifugation at 160xg for 20 minutes was activated using thrombin (1nM) for half an hour followed by addition of excess hirudin to inactivate thrombin. Platelets were separated by centrifugation at 2500xg for 20 minutes and the supernatant was further centrifuged at 20000xg for 45 minutes to pellet platelet derived MPs. After centrifugation the MP pellet (for both MP types) was washed twice with PBS followed by centrifugation each time. The MP pellet was finally re-suspended in PBS, aliquoted and stored at -80°C until further use. Supernatant from the last wash was used as control for all experiments. Procoagulant activity (thrombin generation potential in nM) of MPs was assessed using Zymuphen MP-Activity ELISA. MP used for the determination of procoagulant activity or for injection were only thawed once. MP-concentration was adjusted to 600 nM/kg bodyweight pro-coagulant activity before injection. Human-cell derived MP were only used for studies with human trophoblast cells, while mouse-cell derived MP were used for studies with mouse trophoblast cells and in mice..

2.5 in vitro interventions

The role of MP and platelets was studied on trophoblast cells. To generate platelet rich plasma (PRP) citrated blood was centrifuged at 160xg for 20 minutes and separated into two equal parts. One part was used directly as PRP and the other part was further centrifuged at 2500xg for 10 minutes to separate remaining platelets and collect platelet poor plasma (PPP). Equal amounts of either preparation (PRP or PPP) were added to cells along with MP (5nM thrombin equivalent). Mouse trophoblast stem (TS) cells were treated with MP alone or MP with PRP or PPP and in

some experiments with apyrase, or oATP along with MP and PRP for 24 hrs in differentiation media and were then allowed to further differentiate for 4 days. Human trophoblast like cells (BeWo, JEG-3) were treated with MP alone or MP with PRP or PPP. In some experiments apyrase or oATP were used along with MP and PRP for 24 hrs. For studying the effect of aspirin, PRP was obtained from healthy volunteers who had taken aspirin (500mg/day) for three consecutive days and blood was obtained 1 hour after the last dose. RNA or protein was then isolated for qPCR and western blotting, respectively.

To study the role of transcription factor p45NF-E2 in syncytiotrophoblast formation, fusogenic human trophoblast like cells BeWo were treated with 250μM 8-Br-cAMP to induce syncytiotrophoblast formation. In some experiments, BeWo cells were treated with 8-Br-cAMP along with 5μM C646 (CBP acetyl-transferase inhibitor). Knockdown and overexpression of p45NF-E2 was done using electroporation of plasmids as described below.

2.6 p45 NF-E2 knockdown and overexpression

Human p45NF-E2 (pLKO1-NF-E2) knockdown constructs containing shRNA for p45NF-E2 was purchased from thermoscientific. The p45NF-E2-expressing plasmid pCMV-NF-E2-cMyc was generated by sub-cloning pOTB7-NF-E2 under the regulation of CMV promoter. In detail, a 260bp PCR fragment was amplified from pOTB7-NF-E2 using primers carrying EcoRI overhang and designed at the start codon (forward, mutated to GGG) and spanning SacI site (reverse) within the gene sequence. This was further cloned into a TOPO vector and then cloned back into pOTB7-NFE-2 using EcoRI/SacI digest. The gene sequence containing the mutated start site was then cloned using EcoRI/XhoI digest into pCMV-cMyc vector generating the p45NF-E2 overexpressing construct. Plasmids were electroporated into BeWo cells using BTX electroporator using a single high voltage pulse of 250V and 960 μF.

2.7 Timed matings and in vivo interventions

Plugged female mice were separated from males and injected at day 10.5p.c. and 11.5p.c. with 600 nM/Kg bodyweight (BW) (pro-coagulant activity) endothelial or platelet-derived MPs intravenously and the pregnancy outcome was analyzed at day 12.5p.c. (Fig. 2.1) Control mice were injected with an equal volume of supernatant from the last PBS wash of MPs during the isolation procedure.

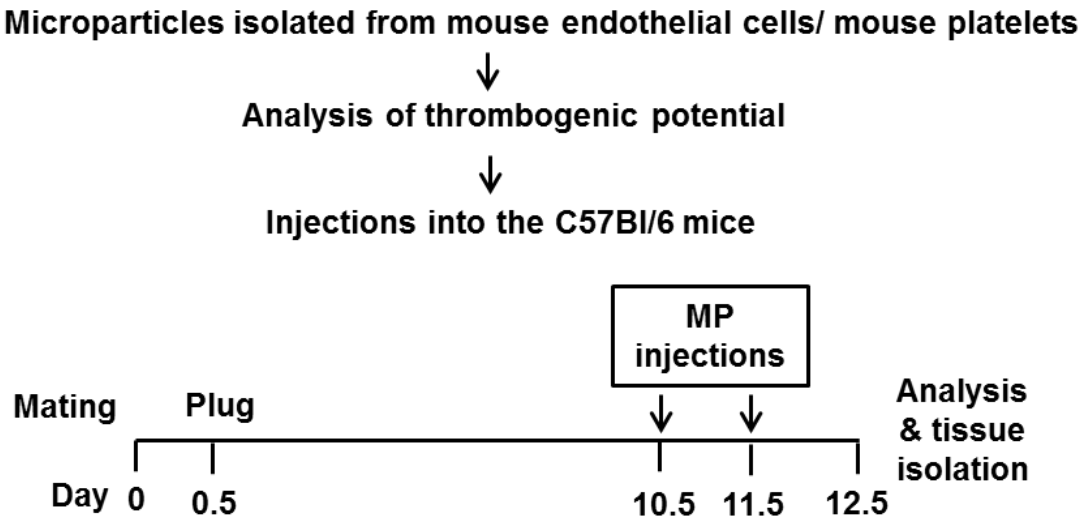


Fig. 2.1: Experimental approach: Experimental scheme reflecting MP isolation and treatment procedure.

For platelet depletion anti-GP1bα antibody (4mg/kg; polyclonal nonimmune IgG served as control) were injected intravenously on day 8.5p.c. Aspirin (100 mg/Kg), Apyrase (200U/kg BW), oATP (9mM/kg BW), or Anakinra (20mg/kg BW) were injected intraperitoneally 30 minutes prior to each MP injection. For analyses at day 12.5p.c., mice were anaesthetized followed by blood and organ collection. Embryos viability was ascertained by visible heart beat or pulsatile blow flow from the umbilical cord. The developmental stage was identified using Theiler staging criteria for mouse embryo development [87-89]. The placentae were separated from the embryos and half of the placental tissue was fixed in 4% buffered formalin while the other half was further divided and stored either in RNAlater (Ambion) or snap frozen in liquid nitrogen. After isolation of the uterus animals were perfused with ice-cold PBS and then with 4% buffered paraformaldehyde.

2.8 Determination of proteinuria

Spot urine samples were collected from mice at three different time points at day 12.5 p.c. We determined proteinuria by determining the total protein content in the urine using BCA assay (Pierce). Creatinine content was measured and used to normalize the protein concentration in the urine and to calculate Protein:Creatinine ratio (P/C) for analysis [90].

2.9 Determination of sFlt-1

sFlt-1 was determined in plasma samples using mouse VEGF R1/Flt-1 Quantikine ELISA Kit (R&D systems, Germany) as described by the manufacturer. Briefly samples were diluted (1:2),

added to the coated microplates, and incubated for 2 hrs at room temperature on an orbital shaker. This was followed by incubation with an antibody against mouse VEGF R1 conjugated to horseradish peroxidase for 2 hrs at room temperature. Following washing, hydrogen peroxide and tetramethylbenzidine were added for color development. Reaction was stopped by adding diluted hydrochloric acid and absorbance was measured at 450nm after 10 minutes.

2.10 Blood pressure Measurement

Blood pressure was measured using a non-invasive mouse tail-cuff blood pressure analysis system (CODA, Kent Scientific Corporation, USA) [91, 92]. Mice were first trained for two weeks to acclimate them to the tail-cuff procedure. After conditioning the mice to the system they were placed in cages along with males for mating. Pregnant mice were injected with MP at day 10.5 and 11.5p.c. Non-pregnant mice were trained and injected in parallel. The animals were placed on a warm plate and blood pressure was then measured using the tail-cuff system using 5 acclimatization and 10 regular cycles with a rest period of 2 seconds in between. Readings for systolic, diastolic, and mean blood pressure were recorded.

2.11 Histology

Tissues were fixed in 4% buffered paraformaldehyde for 2 days, embedded in paraffin and processed for sectioning. Placental morphology was analyzed on hematoxylin and eosin (H & E) stained sections.

Murine placentae sections were studied for maternal and fetal vascularization. In each section at least ten randomly selected microscopic fields within the labyrinthine region from three non-consecutive placental sections (magnification 40x) were acquired. The vascular spaces were outlined using NIH ImageJ free hand tool and added using ROI manager. For maternal vascular area blood vessels with enucleated erythrocytes were outlined, while for fetal vascular area blood vessels with nucleated erythrocytes were outlined. The coverage percentage was calculated as the ratio between the number of pixels covered by the area defined by the selection and the overall number of pixels in the image. The average area for each placenta was used to calculate the significance. Analyses were performed by a blinded investigator.

Human placentae sections were studied for syncytial knot formation. In each section at least ten randomly selected microscopic fields from three non-consecutive placental sections (magnification 40x) were acquired. Syncytial knots were identified as aggregates of syncytial

nuclei at the surface of terminal villi. Number of syncytial knots in each image was counted and added for different images and different section from each sample.

Murine glomerular histology and diameter were assessed by periodic acid–Schiff (PAS) staining [93]. A blinded investigator measured the diameter of the glomerular major axis and minor axis using NIH Image J software from at least 5 serial sections (100 glomeruli in total) for each sample in a blinded fashion and the average was taken for calculations. The average diameter of all glomeruli from each mouse was used for calculating significance.

2.12 Immunohistochemistry

Immunohistochemistry was performed on mouse placenta sections using antibodies for NLPR3, cleaved caspase-1 or fibrin(ogen). Immunofluorescence was performed on mouse placenta sections for Gcm-1, CD41, GP1bα or CD62P and on human placenta sections for p45NF-E2. Briefly, sections were de-paraffinized and hydrated followed by post-fixation in ice cold acetone for 1 min and washing in PBS for 10 min. Antigen retrieval was performed using antigen unmasking solution (Vector) and unspecific peroxidase activity was blocked by incubating the section in 3% H_2O_2. This was followed by blocking (blocking solution: 1.5% serum, 3% BSA prepared in PBS containing 0.05% Tween-20) for 1 h. Tween-20 was not used for CD62P staining. Sections were then incubated overnight at 4°C with primary antibodies against NLRP3, cleaved caspase-1 or fibrin(ogen). Sections incubated without primary antibodies were used as negative controls. Following washing with PBS for 10 minutes, corresponding HRP conjugated secondary antibodies were added for 120 min and then the sections were rinsed twice in PBS. Staining was developed using DAB reagent (Vector) for equal time in all groups (including negative controls). Nuclear counterstaining was conducted using hematoxylin. Slides were then dehydrated, mounted and analyzed using a light microscope. For immunofluorescence, following antigen unmasking and blocking, the sections were incubated overnight at 4°C with primary antibodies against Gcm-1, CD41, GP1bα, CD62P or p45NF-E2. Following washing in PBS for 10 minutes, sections were incubated for 120 min with corresponding fluorophore-labelled secondary antibody. Sections incubated without primary antibodies were used as negative controls. Mouse kidney sections were stained with FITC-Lectin (2µg/ml) for 30 min after overnight incubation with CD62P primary antibody. Sections were then rinsed twice for 10 min each in PBS and mounted in Vectashield containing DAPI and visualized using a fluorescence microscope. Image exposure and acquisition settings were set using negative controls (without primary antibodies) and similar settings were used for all sections.

2.13 Immunocytochemistry

Immunostaining for E-Cadherin was performed on BeWo cells to assess *in vitro* syncytia formation. Cells were fixed with ice-cold methanol for 10 minutes followed by PBS wash for 2x5 mins. This was followed by blocking for 1 hour in blocking solution (3% BSA prepared in PBS containing 0.05% Tween-20). Anti-E-Cadherin antibody prepared in blocking solution was then added and incubated overnight at 4°C. Following washing in PBS for 10 minutes, cells were incubated for 120 min with TRITC-labelled secondary antibody. Cells were then rinsed twice for 10 min each in PBS and mounted in Vectashield containing DAPI and visualized using a fluorescence microscope. Multinucleate cells (containing more than 3 nuclei) were regarded as once syncytia. Syncytia formation was calculated as a percentage ratio between the number of nuclei within the syncytia and number of nuclei outside the syncytia.

2.14 Reverse transcription-quantitative polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Ambion) and quality of RNA was assessed using gel-electrophoresis. Reverse-transcription was done by RevertAid First Strand cDNA Synthesis Kit (Thermoscientific, Germany). Quantitative polymerase chain reaction was performed in a Bio-Rad real time system (CFX-Connect) using SYBR Green (Thermoscientific, Germany). The mRNA levels of the genes tested were normalized to GAPDH as an internal control. The primer sequences were:

Mouse Gcm1: Forward 5'-GCT CTT GTG GCC CGA GTT C-3' and Reverse 5'-GTT TTC ACG TTC TGA GGC AGT T-3'; Mouse Esx1: Forward 5'-CAT CTG CTT CAC CCC GAT CC-3' and Reverse 5'-TCT GAA ACC AAA CCT GCA CTC T-3'; Mouse Tpbpa: Forward 5'-GAA ATG AGT GCC TCC GGT CA-3' and Reverse 5'-TGT CCA TGT TAC TGT GGC TGA TT-3'; Mouse PL-II : Forward 5'-CCA ACG TGT GAT TGT GGT GT-3' and Reverse 5'-TCT TCC GAT GTT GTC TGG TGG-3'; Mouse GAPDH: Forward 5'-AGT GTT TCC TCG TCC CGT AG-3' and Reverse 5'-GCC GTT GAA TTT GCC GTG AG-3'.

2.15 Transmission electron microscopy

Transmission electron microscopy (TEM) was performed at the Institute for Clinical Chemistry and Pathobiochemistry, Otto-von-Guericke-University, Magdeburg as described earlier [62, 65]. Renal cortex tissues were fixed with 2.5% glutaraldehyde, 2.5% polyvidone 25, 0.1 M sodium cacodylate pH 7.4. After washing with 0.1 M sodium cacodylate buffer (pH 7.4), samples were post-fixed in the same buffer containing 2% osmium tetroxide and 1.5% potassium ferrocyanide

for 1 hr, washed in water, contrasted *en bloc* with uranyl acetate, dehydrated using an ascending series of ethanol and embedded in glycidyl ether 100-based resin. Ultrathin sections were cut with a Reichert Ultracut S ultramicrotome (Leica Microsystems, Wetzlar, Germany), contrasted with uranyl acetate and lead citrate, and were viewed with an EM 10 CR electron microscope (Carl Zeiss NTS, Oberkochen, Germany). The glomerular basement membrane (GBM) thickness was analyzed by NIH-ImageJ software.

2.16 In-situ proximity ligation assay

Duolink *in-situ* PLA Kit was used for *in situ* proximity-ligation assay on human placenta sections according to the manufacturer's instructions (Sigma Aldrich, Germany) [94]. Briefly, following deparaffinization, rehydration, antigen retrieval, and blocking double immunolabeling with primary antibodies against NLRP3 and ASC was performed as described above, followed by incubation with PLA probes against the primary antibodies, ligation, and amplification using polymerase to detect the NLRP3 and ASC protein complexes. For quantification of PLA data, images from each section were acquired with identical exposure settings for each sample. Appropriate settings were established using negative controls (without primary antibodies). Twenty images from a single focal plane were captured per section. To count the PLA-positive signals per image, NIH-Image J Point Picker plugin was used and were added using ROI Manager. The number of PLA positive signals were normalized to the total nuclear area calculated using threshold analysis for DAPI channel. The percentage of PLA positive signals per sample was then calculated with respect to control sections.

2.17 Immunoblotting

Cell lysates were prepared using RIPA buffer containing 50 mM Tris (pH7.4), 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF supplemented with protease inhibitor cocktail. For tissue lysates, RIPA with 0.5% sodium-deoxycholate was used. Lysates were centrifuged (13000 x g for 10 min at 4^oC) and insoluble debris was discarded. Protein concentration in supernatants was quantified using BCA reagent. Equal amounts of protein were electrophoretically separated on 7.5%, 10% or 12.5% SDS polyacrylamide gel, transferred to PVDF membranes and probed with desired primary antibodies overnight at 4^oC. Membranes were then washed with PBST and incubated with anti-mouse IgG (1:5000) or anti-rabbit IgG (1:2000) horseradish peroxidase conjugated antibodies as indicated. Blots were developed with the enhanced chemiluminescence system. To compare and quantify levels of

proteins, the density of each band was measured using Image J software. Equal loading for total cell or tissue lysates was determined by GAPDH western blot.

2.18 Immunoprecipitation

Immunoprecipitation for Gcm-1 was carried out in a total volume of 500µl containing 200 µg of cell lysate was diluted in RIPA buffer. Lysates were incubated with 1µg of anti-Gcm-1 antibody for 4 hours at 4^{o}C while rotation. 20 µl of protein A/G agarose beads were then added and incubated further for overnight at 4^{o}C while rotation. Immunoprecipitates conjugated to beads were collected by centrifugation at 12000 x g for 30s. They were then washed with 1 ml RIPA buffer each for three times at 3000 x g for 5 minutes each. Immunoprecipitates were eluted from the beads by addition of 1X Laemelli's buffer and boiling the samples at 95^{o}C for 10 minutes. Beads were separated by centrifugation and immunoprecipitates were analyzed by immunoblotting.

2.19 Measurement of ATP release

Citrate anticoagulated whole mice blood was centrifuged as described above to obtain PRP. The plasma was then recalcified and treated with MP. Thrombin was used as a positive control. The plasma was then centrifuged as described above to obtain PPP. Cell-titer Glo reagent containing Ultra-Glo™ Recombinant Luciferase was added to PPP (containing the released ATP) and incubated for 15 minutes resulting in ATP dependent luciferase reaction. The luminescence obtained as a result of the luciferase reaction was quantified using luminometer.

2.20 Thromboelastometry

ROTEM™ (Rotational thromboelastometry) analyses with citrated human and mouse blood was conducted to determine the impact of MP on whole blood clotting. Star-tem reagent containing $CaCl_2$ in HEPES buffer was added for re-calcification of blood which was then treated with either ex-tem reagent (recombinant tissue factor and phospholipids) as a positive control or with MP. Star-tem reagent alone (only re-calcification) was used as a negative control. The kinetics of hemostasis was studied and indices of blood clotting such as clot time (CT), clot formation time (CFT), α-angle (indicator of coagulation speed), and amplitude of clot firmness after 10 minutes were recorded.

2.21 Statistical analysis

The data are summarized as the mean ± SEM (standard error of the mean). Statistical analyses were performed with Student's t-test, chi square test, Spearman's correlation, Mann-whitney U

test, or ANOVA, as appropriate. Posthoc comparisons of ANOVA were corrected with the method of Tukey. The Kolmogorov-Smirnov (KS) test or D'Agostino-Pearson normality-test was used to determine whether the data are consistent with a Gaussian distribution. Prism 5 (www.graphpad.com) software was used for statistical analyses. Statistical significance was accepted at p-values of <0.05.

2.22 Study approval

All animal experiments were conducted following standards and procedures approved by the local Animal Care and Use Committee (Landesverwaltungsamt Halle, Germany). Human placenta samples were collected in accordance with the guidelines and with the approval of the local ethics committee and after obtaining informed consent of patients.

3.0 Results

3.1 MP cause a PE-like phenotype in pregnant mice

To evaluate the potential pathogenicity of MP in pregnancy we injected pregnant mice with procoagulant MP and evaluated their impact on pregnancy outcome, renal function and pathology, blood pressure and plasma sFlt-1 levels. Platelet or endothelial-derived MP injected at day 10.5 and 11.5p.c. impaired embryonic survival at day 12.5p.c. (Fig. 3.1). This shows that a potential pathogenetic function of MP during pregnancy and that they can lead to an impaired pregnancy outcome.

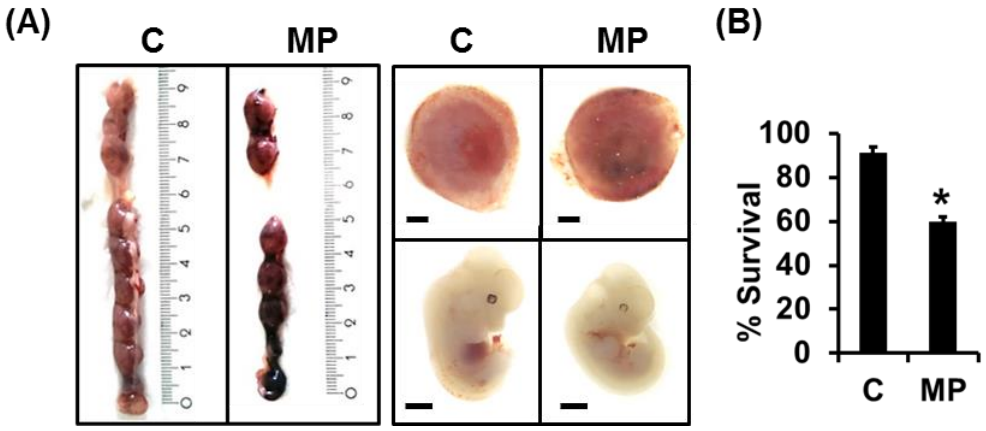


Fig. 3.1: MP impair pregnancy outcome (A-B) Impaired pregnancy outcome in C57BL/6 mice at day 12.5p.c. following i.v. injection of mouse endothelial cell derived MP at day 10.5p.c. and 11.5p.c. Representative images (A) of uterus (top left), placenta (top right), and embryo (bottom right) and bar graphs quantifying embryonic survival (B). Control mice (C) were injected with the supernatant obtained after the last PBS-wash during MP isolation. Data shown represent mean±SEM of at least 5 pregnant females per group; *$p<0.05$ (t-test).

We further studied the effect on embryonic and placental development in MP injected pregnant mice. Morphologically, surviving embryos were smaller and showed reduced placental diameter (Fig. 3.2A,B), which was indicative of impaired embryonic and placental development. According to Theiler staging criteria for mouse embryo development, the embryos show retinal pigmentation and sign of fingers between days 10.5 and 12.5 p.c. [87-89]. In parallel, the labyrinth layer of mouse placenta comprising of syncytiotrophoblast invades into the giant trophoblast and spongiotrophoblast and further expands till birth [95-97]. We observed that MP injected pregnant females possessed embryos with impaired fore-limb and reduced retinal pigmentation indicating developmental delay (Fig. 3.2C,D). Concomitantly, MP enhanced giant trophoblast (PL-II) and spongiotrophoblast (Tpbpa) while reducing syncytiotrophoblast (Gcm-1)

marker expression, corroborating impaired placental development (Fig. 3.2E). These placental changes were associated with reduced embryonic but increased maternal placental vascularization, reflecting placental malperfusion (Fig. 3.2F,G).

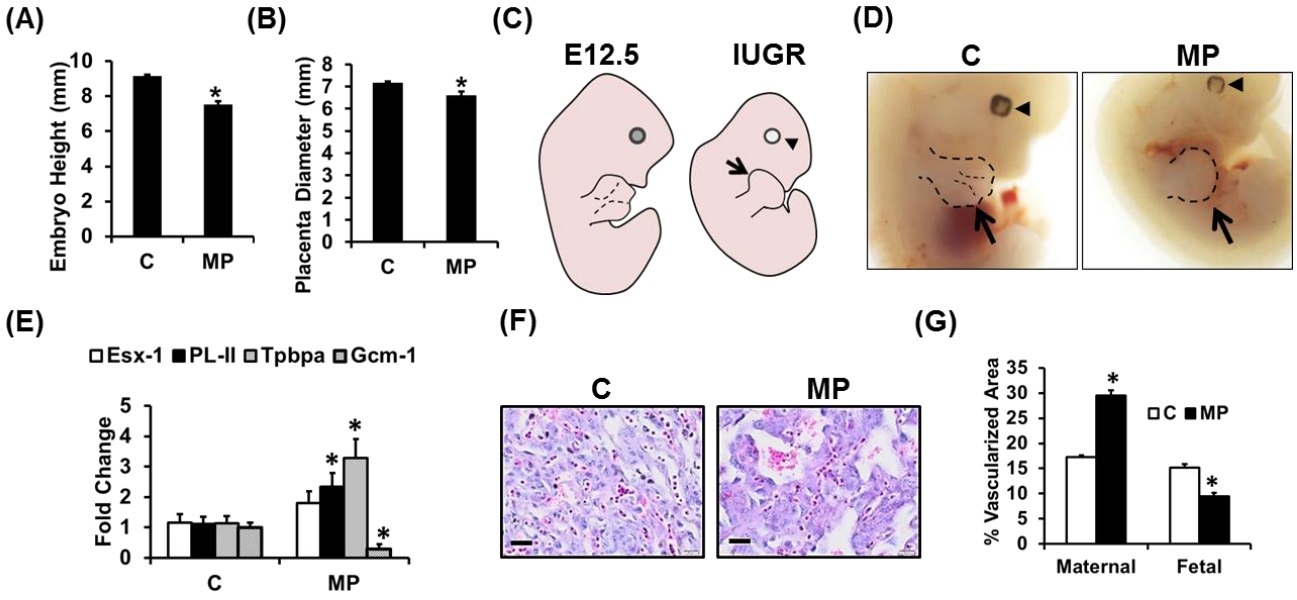


Fig. 3.2: MP impair embryonic and placental development. (A-B) Bar graphs quantifying embryonic height (A) and placental diameter (B). (C) Schematic representation of an embryo at E12.5 (left) and a growth retarded embryo with impaired fore-limb development (arrow) and retinal pigmentation (arrow head) as typically observed following MP injection. (D) High magnification images of embryos (shown in Fig. 3.1) showing impaired fore-limb development (arrow) and retinal pigmentation (arrow head) after MP treatment. (E-G) Altered placental morphology after MP injections. Bar graph (E) summarizing results from qRT-PCR analyses for trophoblast differentiation marker genes showing elevated expression of spongiotrophoblast marker (Tpbpa) and giant trophoblast marker (PL-II) along with decreased expression of syncytiotrophoblast marker (Gcm-1) in MP injected pregnant mice. The labyrinth marker Esx-1 did not differ between groups. Representative images (F) of placental histology (H&E staining) showing enhanced maternal vascularization (blood lacunae; enucleated erythrocytes) and reduced fetal vascularization (nucleated erythrocytes) after MP injections. Bar graph (G) summarizing quantification of total vascularized area; analyses performed at day 12.5p.c. Data shown represent mean±SEM of at least 8 placentae or embryos analyzed from at least 3 different litters of each group. Control mice (C) were injected with the supernatant obtained after the last PBS-wash during MP isolation; *$p<0.05$ (a,b,g: t-test; e: ANOVA).

Additionally, MP-injected pregnant mice developed characteristic hallmarks of PE defined by renal dysfunction (enlarged glomeruli, thickened glomerular basement membrane, podocyte effacement, and proteinuria), elevated blood pressure, and increased plasma sFlt-1 levels (Fig. 3.3). Importantly, MP had no impact on renal dysfunction or blood pressure in non-pregnant mice (Fig. 3.3). Hence, MP induce a PE-like phenotype characterized by impaired pregnancy success, hypertension and renal pathology specifically in pregnant mice through an unknown mechanism.

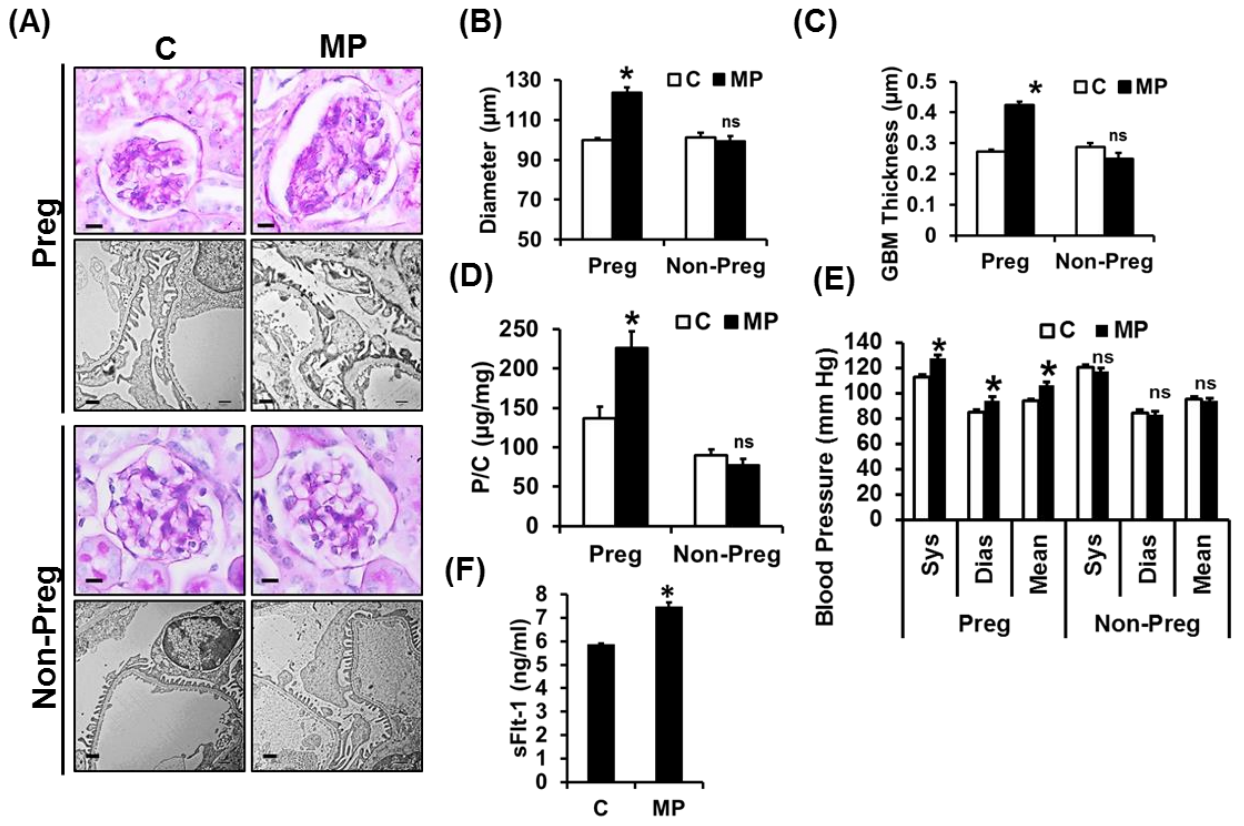
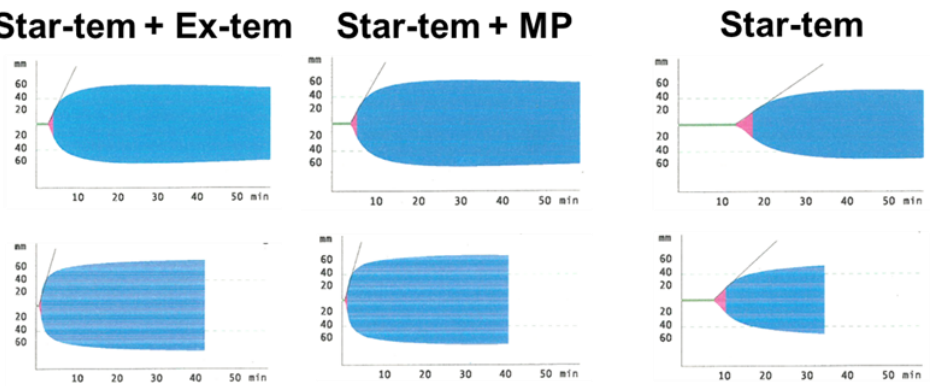


Fig. 3.3: MP cause a PE-like phenotype in mice Characterization of renal pathology in pregnant (Preg) and non-pregnant (Non-Preg) mice following MP-injection. (A) Representative images showing enhanced renal pathology in pregnant mice after MP injections, characterized by enlarged glomeruli (A, PAS staining, top; B, bar graph reflecting quantification) and podocyte effacement and thickened glomerular basement membrane (A, transmission electron microscopy, n=3, bottom; C, bar graph reflecting glomerular basement membrane thickness). (D) Proteinuria is increased in MP-injected pregnant mothers at day 12.5 p.c. (D, bar graph summarizing data of protein creatinine ratio, P/C). These features of renal dysfunction are not observed in MP injected non-pregnant females. Size bar represents 15µm for PAS staining and 1µm for TEM images, respectively. (E) Elevated blood pressure in MP-injected pregnant mice at day 12.5p.c. MP have no impact on blood pressure in non-pregnant mice; bar graph summarizing results. (F) sFlt-1 plasma levels. The pre-eclampsia marker sFlt-1 is increased in blood samples obtained from MP-injected pregnant mice as compared to controls (bar graph summarizing results). Data shown represent mean±SEM of 5 pregnant females per group. *p<0.05, **p<0.01, ns: not significant (b-f: t-test).

3.2 MP cause activation and accumulation of maternal platelets within the placenta

Quantitative and qualitative changes of MP are associated with coagulation and platelet-activation in women with PE [98]. Using a whole blood clotting assay (ROTEM) we established that the MP used within this study efficiently induced blood clotting (Fig. 3.4).

(A)



(B)

	CT (s)	CFT (s)	α⁰	A10 (mm)
Murine Blood				
Ex-tem (TF)	52**	36**	82**	63**
Star-tem + MP	62**	42**	81**	62**
Star-tem	486	171	58	41
Human Blood				
Ex-tem (TF)	188**	72**	75**	56**
Star-tem + MP	289**	95**	72**	56**
Star-tem	836	241	49	39

Fig. 3.4: MP cause whole blood clotting: (A,B) ROTEM analysis of citrated blood showing that *ex vivo* treatment of whole citrate blood with MP induces blood clotting. As a positive control, Ex-tem reagent was used along with star-tem reagent containing calcium (left). MP along with star-tem (containing Ca2+ and but no other coagulation initiator) were added to whole blood (middle). As a negative control, only star-tem was used (A, graphic illustration of clot formation; B, parameters of blood clot formation). Data represents mean±SEM of 3 mice or humans. **p<0.01; t-test. CT: clotting time, CFT: clot formation time, α: alpha angle, A10: amplitude 10 min after CT.

Detailed morphological analyses of placental H&E stained sections obtained from control or MP injected pregnant mice did not reveal an increase of blood clots in MP-injected pregnant mice (Fig. 3.5). The absence of increased placental thrombosis was confirmed by immunohistochemical staining of fibrin(ogen) (Fig. 3.5). The absence of increased placental thrombosis in MP-injected pregnant mice is congruent with observations made in pregnant mice with increased coagulation activation and with histopathological studies in humans [74, 78, 99].

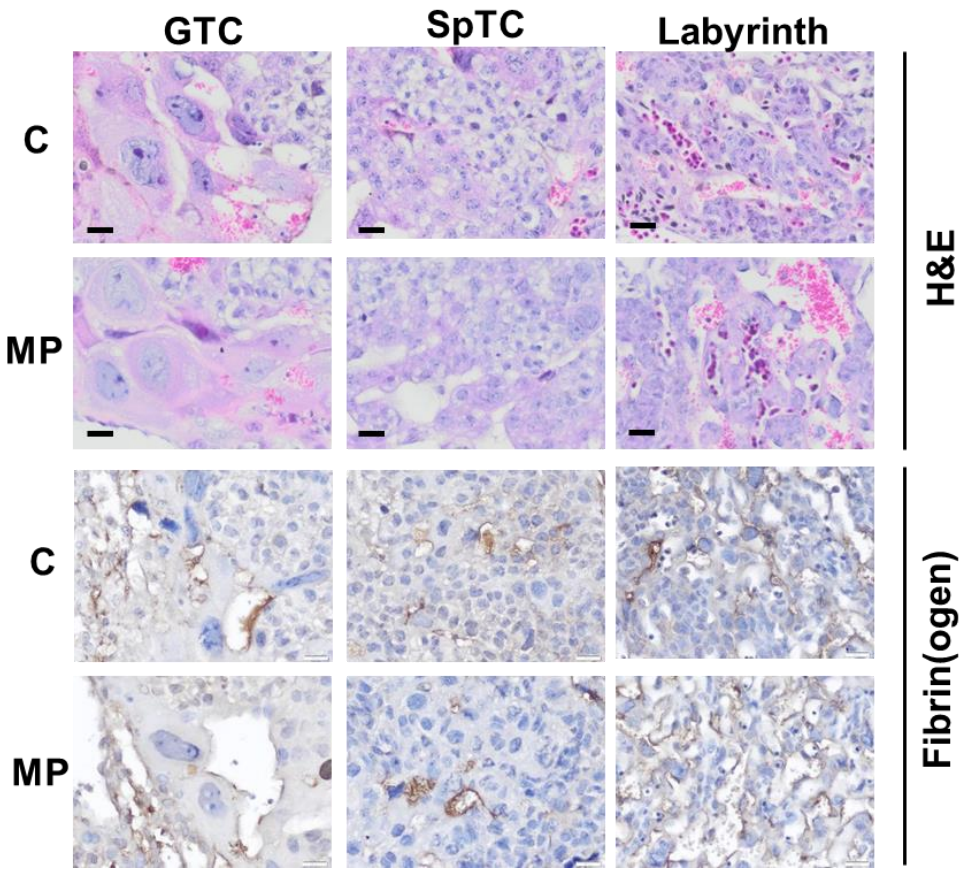


Fig. 3.5: Absence of placental blood clots in MP-injected mice (A) High magnification images for placenta (upper panel; H&E Staining, lower panel; Fibrin(ogen) staining); obvious blood clots are not apparent in different layers of placentae obtained from both control or MP-injected pregnant mice. Representative H&E stained images; size bar represents 20µm.

While we failed to detect an increased frequency of blood clots or enhanced fibrin(ogen) staining (Fig. 3.5) we readily detected activated (P-selectin-positive) platelets within the placenta of MP-injected, but not of control pregnant mice (Fig. 3.6). Activated platelets lined the maternal blood space and were in direct contact with embryonic trophoblast cells in placentae obtained from MP-injected pregnant mice. Strikingly, accumulation of activated platelets was only observed in the placenta, but not in other organs such as the kidney (Fig. 3.6). The presence of activated platelets within the placentae of MP-injected pregnant mice suggests that – despite the absence of blood clots – the local accumulation of activated platelets is mechanistically linked with the evolution of the PE, a disease caused by a placental defect.

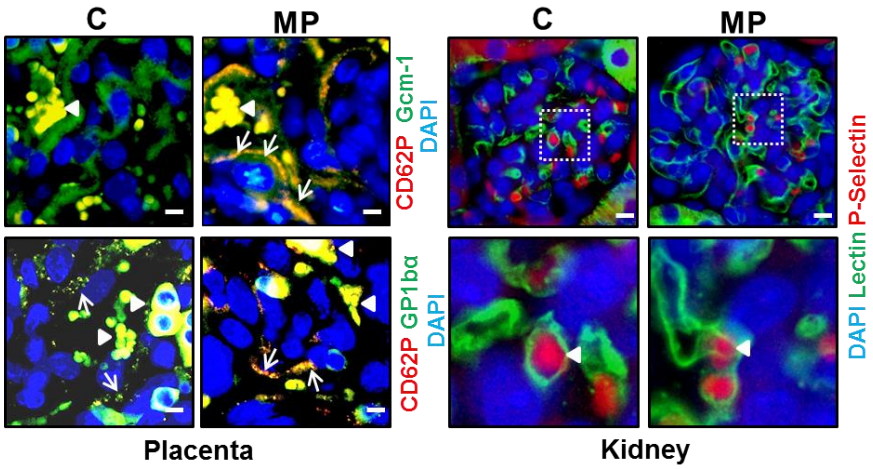


Fig. 3.6: MP injection induce accumulation of activated platelets in the placenta. Double immunofluorescence staining showing activated platelets (CD62P, P-selectin; red) in the placenta, but not in the kidney, after injections of mouse endothelial cell derived MP. Activated platelets are in direct contact with syncytiotrophoblast (Gcm-1; green) and line placental blood spaces; yellow (placenta-upper panel) indicates co-localization of activated platelets and Gcm-1 positive syncytiotrophoblast (arrows). Activated platelet marker CD62P (red) colocalizes (yellow) with platelet marker GP1bα (green) indicating endogenous platelet specificity (placenta-lower panel); conventional immunofluorescence analyses; arrow heads: autofluorescence of erythrocytes. Size bar represents 80µm for placenta and 10µm for kidney.

To evaluate whether maternal platelets contribute to MP-induced PE *in vivo*, we used several complimentary approaches to inhibit platelets (Fig. 3.7). First, we depleted maternal platelets using an antibody against mouse GPI1bα which is expressed on platelets. Immune-depletion of maternal platelets with anti-mouse GP1bα antibodies protected pregnant WT mice from MP-induced PE. We then treated MP-injected pregnant mice with Aspirin. Likewise, aspirin treatment improved pregnancy outcome in MP-injected pregnant mice.

Mice lacking the transcription factor p45 NF-E2 lack functional platelets and Gαq-/- mice have severely impaired platelet-activation[58]. Importantly, while p45 NF-E2 itself is required for normal placental development and embryonic growth, the p45 NF-E2-dependent phenotype becomes apparent only after day 14.5 p.c., is independent of platelets, and depends on embryonic p45 NF-E2 deficiency [62]. Pregnant p45NFE2-/- or Gαq-/- mice mated with WT male yielding pregnant mice with non-activatable platelets and embryos heterozygous for p45NFE2 or Gαq. Following the injection of pregnant Gαq-/- mice using the same protocol as above these mice were protected from MP-induced pregnancy loss, IUGR, placental dysfunction, and did not develop hallmarks of PE (Fig. 3.7). The absence of obvious blood clots or increased fibrin(ogen) accumulation suggest that MP and platelets induce PE independent of blood clot formation.

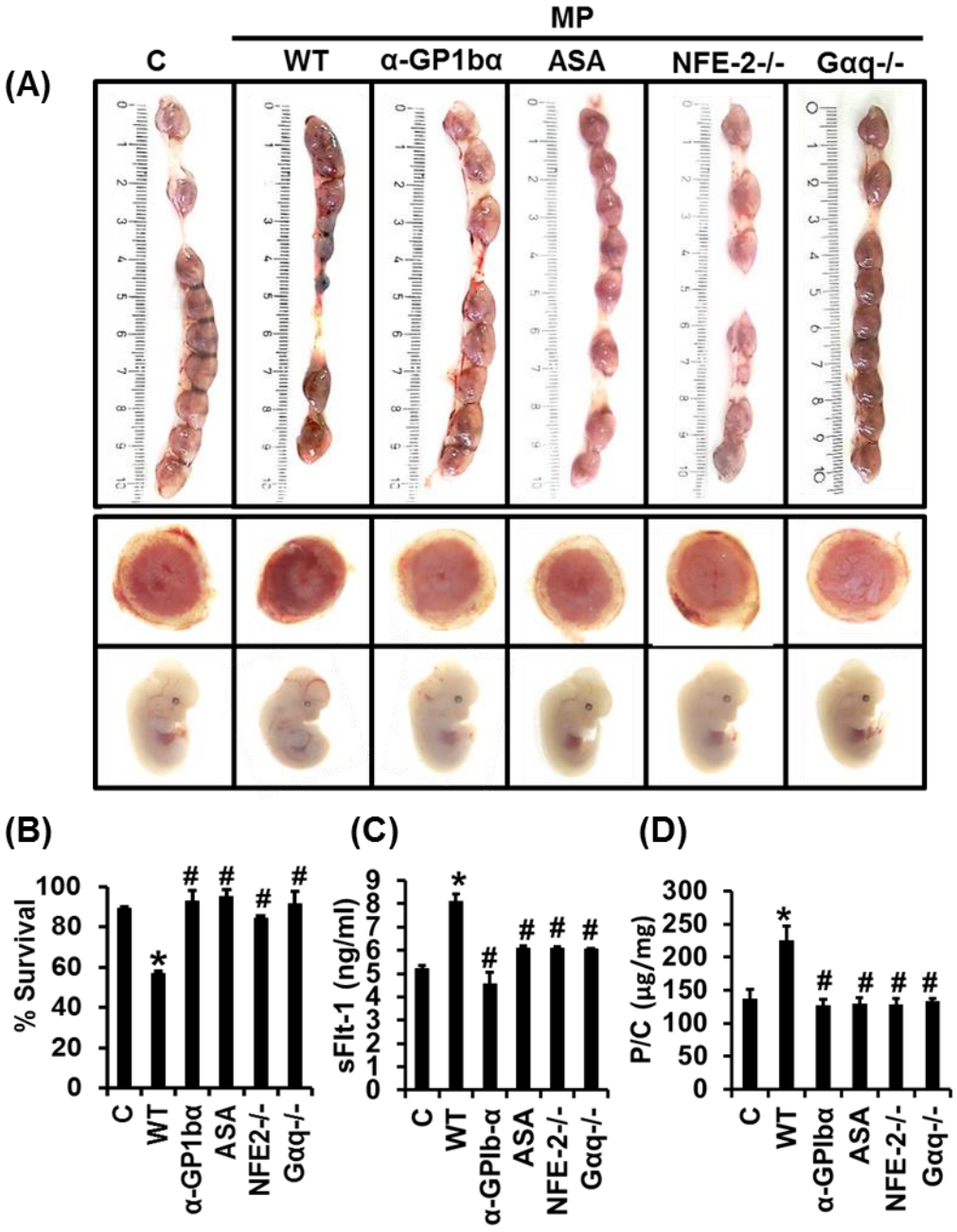


Fig. 3.7: Maternal platelets mediate MP-induced PE-like phenotype in mice (A, B) Depletion of maternal platelets using anti-GP1bα antibody, inhibition of maternal platelets using Aspirin, genetic platelet deficiency (p45 NF-E2-/- mice), or genetically superimposed impaired platelet activation (Gαq deficiency) protects pregnant female mice from MP induced pregnancy impairment. Pregnancy outcome at day 12.5p.c. after MP-injection (mouse endothelial cell derived MP) at day 10.5p.c. and 11.5p.c. into pregnant females mated to WT males. Representative images (A) of uterus (top), placentae (middle), and embryos (bottom) along with bar graphs quantifying embryonic survival (B). (C-E) Plasma sFlt-1 levels (C) and proteinuria (D) are normal despite MP treatment in platelet depleted, Aspirin treated, NFE-2-/-, or Gαq-/- pregnant female mice. Size bar represents 1mm for embryo and placenta (B). Data represents mean ± SEM of at least 8 placentae or embryos analyzed from at least 3 different litters of each group or 5 pregnant females per group. Control mice (C) were injected with the supernatant obtained after the last PBS-wash during MP isolation. (B-D: t-test).

3.3 Normal pregnancy in NLRP3 or caspase-1 deficient mice despite MP-injection

In addition to blood clotting platelets regulate inflammatory responses, in part through the modulation of the inflammasome [100]. Intriguingly, one characteristic feature of PE is the elevation of inflammatory cytokines, including IL-1β, the pivotal cytokine reflecting inflammasome activation [101]. To determine whether the MP- and platelet-induced PE-like phenotype in pregnant WT mice is associated with inflammasome activation we analyzed inflammasome markers (cleaved caspase-1 and IL-1β and NLRP3 expression) in placentae obtained from control and MP-injected pregnant mice. MP induced these markers in placenta tissue obtained from MP-injected mice, establishing inflammasome activation by MPs (Fig. 3.8).

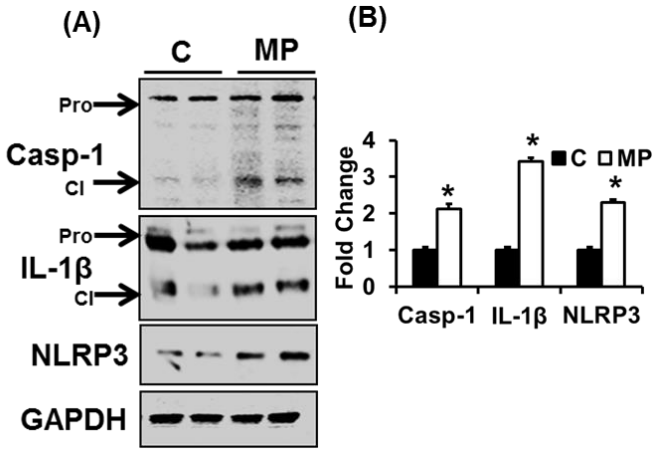


Fig 3.8: MP cause inflammasome activation in placenta (A,B) Inflammasome activation in murine placentae after MP injections (mouse endothelial cell derived MP). Immunoblots showing increased cleaved caspase-1 and cleaved IL-1β and increased NLRP3 expression in murine placentae after MP injections, analyzed at day 12.5p.c. (A, representative immunoblots; B, bar graph summarizing results). Arrows indicate inactive (pre-form, top arrow) and active (cleaved-form, bottom arrow) caspase-1 (Casp-1) or IL-1β respectively (A). Only the expression of active forms was quantified (B). Data shown represent mean±SEM of at least 8 placentae or embryos analyzed from at least 3 different litters of each group. *$p<0.05$ (t-test).

To ascertain whether the MP- and platelet-induced PE-like phenotype is dependent on inflammasome activation we next injected NLRP3-/- or Casp-1-/- female mice mated with NLRP3-/- or Casp-1-/- male mice, respectively, with MP. NLRP3 or caspase-1 deficiency prevented MP-induced embryonic death (Fig. 3.9, 3.10). Concomitantly, embryonic and placental growth and development were normalized in NLRP3 or caspase-1 deficient mice despite MP injection (Fig. 3.9, 3.10). Furthermore, NLRP3 or caspase-1 deficient pregnant mice did not present hallmarks of PE, such as proteinuria, renal pathology, hypertension, or increased plasma sFlt-1 levels (Fig. 3.11). Hence, inflammasome activation is required for MP-induced PE in mice. Intriguingly, activated platelets were still detectable within the placenta of MP-injected pregnant

mice (Fig. 3.12). This raises the question as to whether inflammasome activation in platelets or a different cell-type conveys the PE-like phenotype.

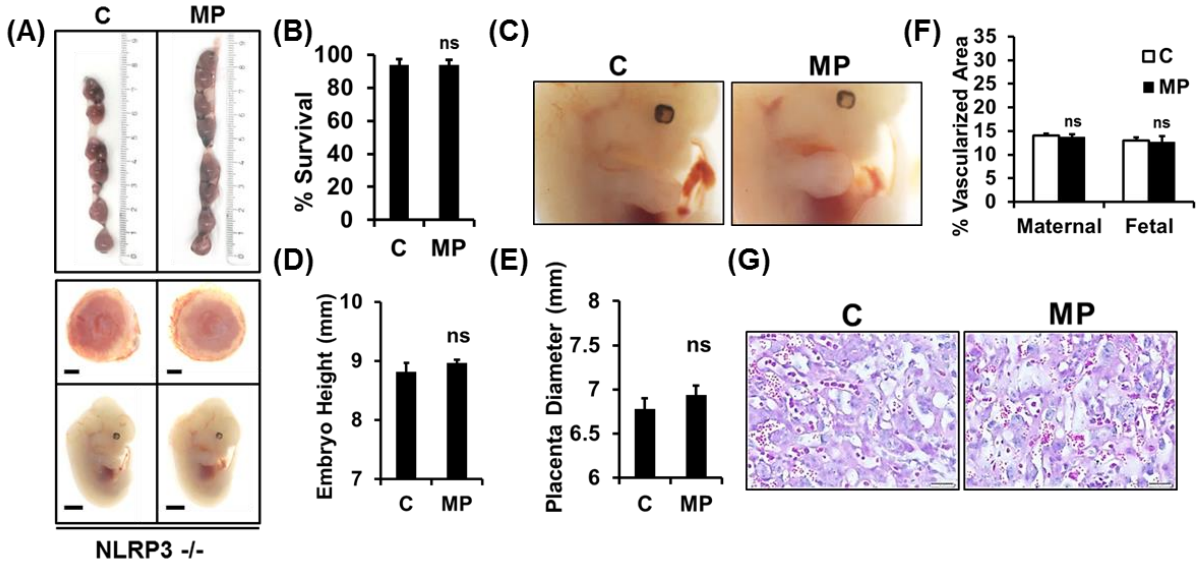


Fig. 3.9: Normal placentation and embryonic development in NLRP3 deficient mice despite MP-injection (A) Representative images of uterus (top), placentae (middle), and embryos (bottom) along with quantification of embryonic survival (B) showing protection from MP-induced pregnancy complications in NLRP3-/- mice. At least 8 placentae or embryos analyzed from 3 different litters of each group; size bar: 1 mm. (C) High magnification images of embryos (as in A) showing normal fore-limb development and retinal pigmentation after MP treatment. (D-E) Bar graphs quantifying embryonic height (D) and placental diameter (E). (F-G) Placental histology (F, bar graph summarizing results; G, representative images, H&E staining) showing normal maternal vascularization (enucleated erythrocytes) and fetal vascularization (nucleated erythrocytes) after MP treatment in NLRP3-/- placenta. Size bar represents 20µm. Data represents mean±SEM of at least 8 placentae or embryos analyzed from at least 3 different litters of each group. ns=not significant (B, D-F: t-test).

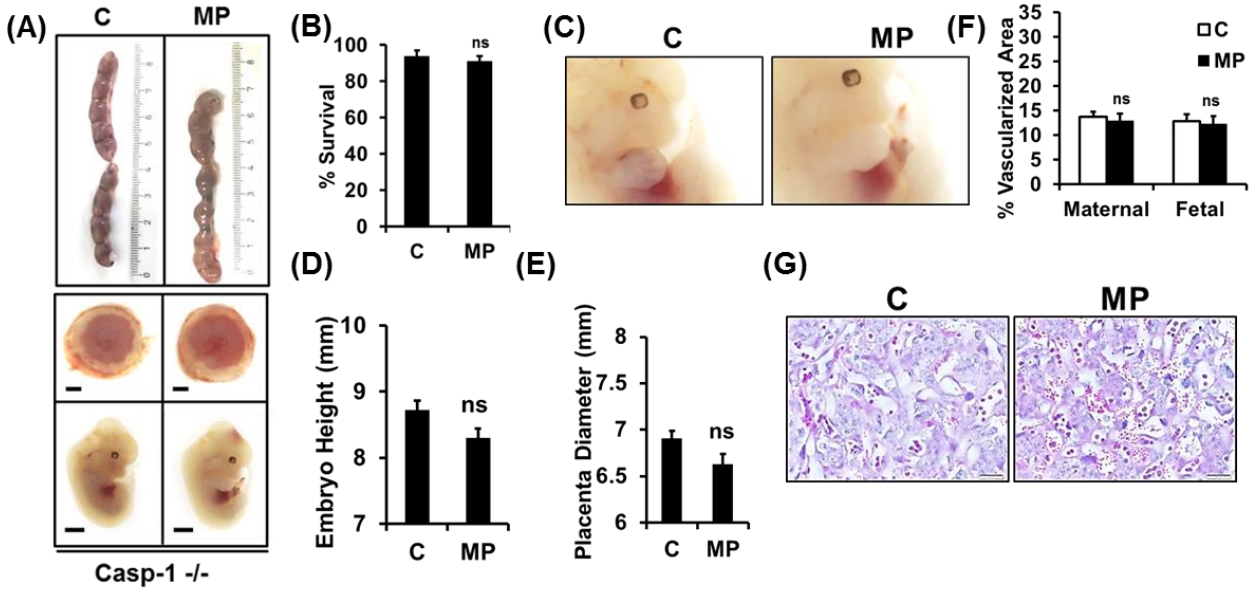


Fig. 3.10: Normal placentation and embryonic development in Caspase-1 deficient mice despite MP-injection (A) Representative images of uterus (top), placentae (middle), and embryos (bottom) along with quantification of

embryonic survival (B) showing protection from MP-induced pregnancy complications in Casp-1-/- mice. At least 8 placentae or embryos analyzed from 3 different litters of each group; size bar: 1 mm. (C) High magnification images of embryos (as in A) showing normal fore-limb development and retinal pigmentation after MP treatment. (D-E) Bar graphs quantifying embryonic height (D) and placental diameter (E). (F-G) Placental histology (F, bar graph summarizing results; G, representative images, H&E staining) showing normal maternal vascularization (enucleated erythrocytes) and fetal vascularization (nucleated erythrocytes) after MP treatment in Casp-1-/- placenta. Size bar represents 20µm. Data represents mean±SEM of at least 8 placentae or embryos analyzed from at least 3 different litters of each group. ns=not significant (B, D-F: t-test).

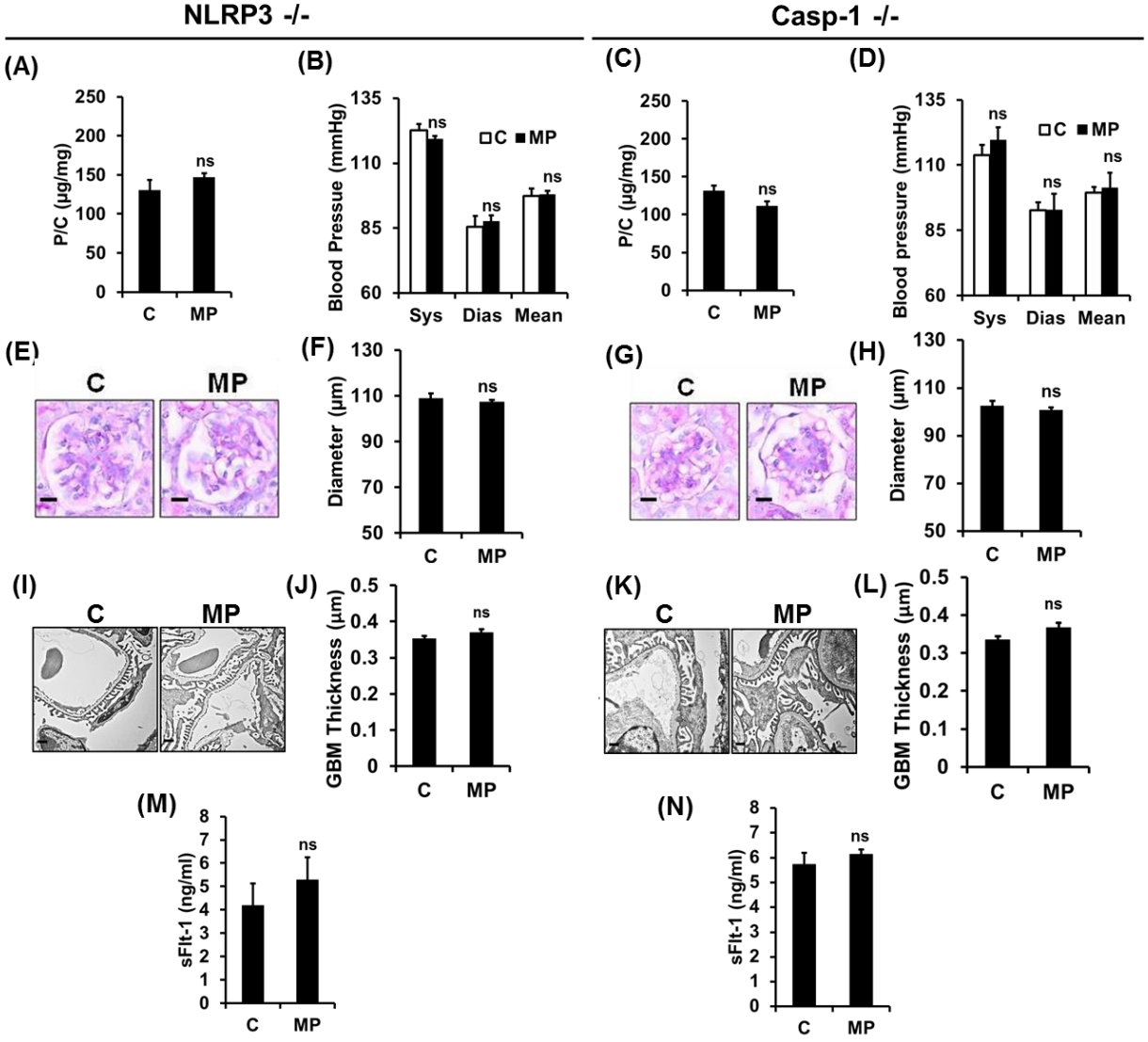


Fig. 3.11: NLRP3 and caspase-1 deficiency protects from the MP-induced PE-like phenotype (A-D) Bar graphs showing that NLRP3-/- (A, B) and caspase-1-/- (C, D) pregnant mice are protected from MP induced proteinuria (A,C) and hypertension (B, D). (E-H) Representative images of kidney (E, G, PAS staining) showing normal glomerular diameter (F, H, bar graph summarizing data) after MP treatment in NLRP3-/- (E, F) and caspase-1-/- (G, H) mice. (I-L) Representative images of kidney (I, K, TEM) showing normal glomerular basement membrane (J, L, bar graph quantifying GBM thickness) after MP treatment in NLRP3-/- (I, J) and caspase-1-/- (K, L) mice. (M-N) Plasma sFlt-1 levels (M, N) after MP treatment in NLRP3-/- (M) and Casp-1-/- (N) pregnant female mice showing

protection from MP induced preeclampsia. Data shown represent mean+SEM obtained from at least 5 pregnant females of each group. Size bar represents 15µm (C,D). ns=non-significant; t-test.

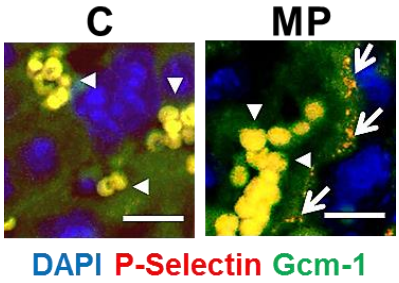


Fig. 3.12: Activated platelets in NLRP3-/- placenta. Double immunofluorescence staining showing activated platelets (P-selectin; red) in the placenta after MP injection in pregnant NLRP3-/- females mated with NLRP3-/- males. Activated platelets are in direct contact with syncytiotrophoblast (Gcm-1; green); yellow indicates co-localization of activated platelets and Gcm-1 positive syncytiotrophoblast (arrows); conventional immunofluorescence analyses; arrow heads: autofluorescence of erythrocytes. Scale bar: 20µm.

3.4 MP and platelets cause inflammasome activation in trophoblast cells

To identify the cells in which MP induce inflammasome activation we conducted immunohistochemical analyses. Following MP-injection a marked induction of NLRP3 expression and of cleaved caspase-1 was readily detectable in trophoblast cells reflecting inflammasome activation (Fig. 3.13A).

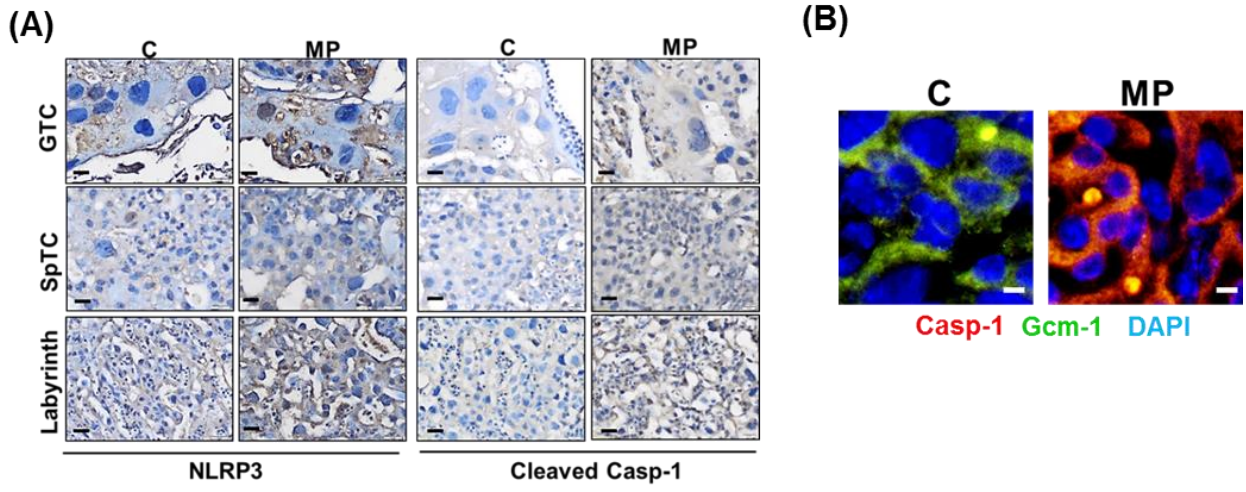


Fig. 3.13: Inflammasome activation occurs in the trophoblast. (A) Representative images showing increased expression of NLRP3 and cleaved caspase-1 in trophoblast cells detected by immunohistochemistry of murine placenta after injections of endothelial cell derived MP. Size bar represents 20µm. (B) Representative images (double immunofluorescence staining) showing co-localization (yellow) of cleaved caspase-1 (red) and Gcm1 (green, syncytiotrophoblast marker) indicating inflammasome activation in trophoblasts after MP injections. Size bar represents 80µm.

Induction of inflammasome activation in embryonic trophoblast cells was confirmed by immunoflourescent analyses, demonstrating co-localization of cleaved caspase-1 with the syncytiotrophoblast cell marker Gcm-1 (Fig. 3.13B). These data demonstrate that inflammasome activation is induced in embryonic trophoblast cells, which are directly exposed to maternal blood and maternal derived MP and platelets.

To gain mechanistic insight we next analyzed MP-dependent inflammasome activation in trophoblast cells *in vitro*. MP dose-dependently increased caspase-1 and IL-1β activation and NLRP3 expression in murine trophoblast cells *in vitro* (Fig. 3.14A.B). Akin to the *in vivo* situation MP treatment cell-autonomously altered differentiation of murine trophoblast cells (Fig. 3.14C), demonstrating that inflammasome activation within trophoblast cells directly alters their differentiation.

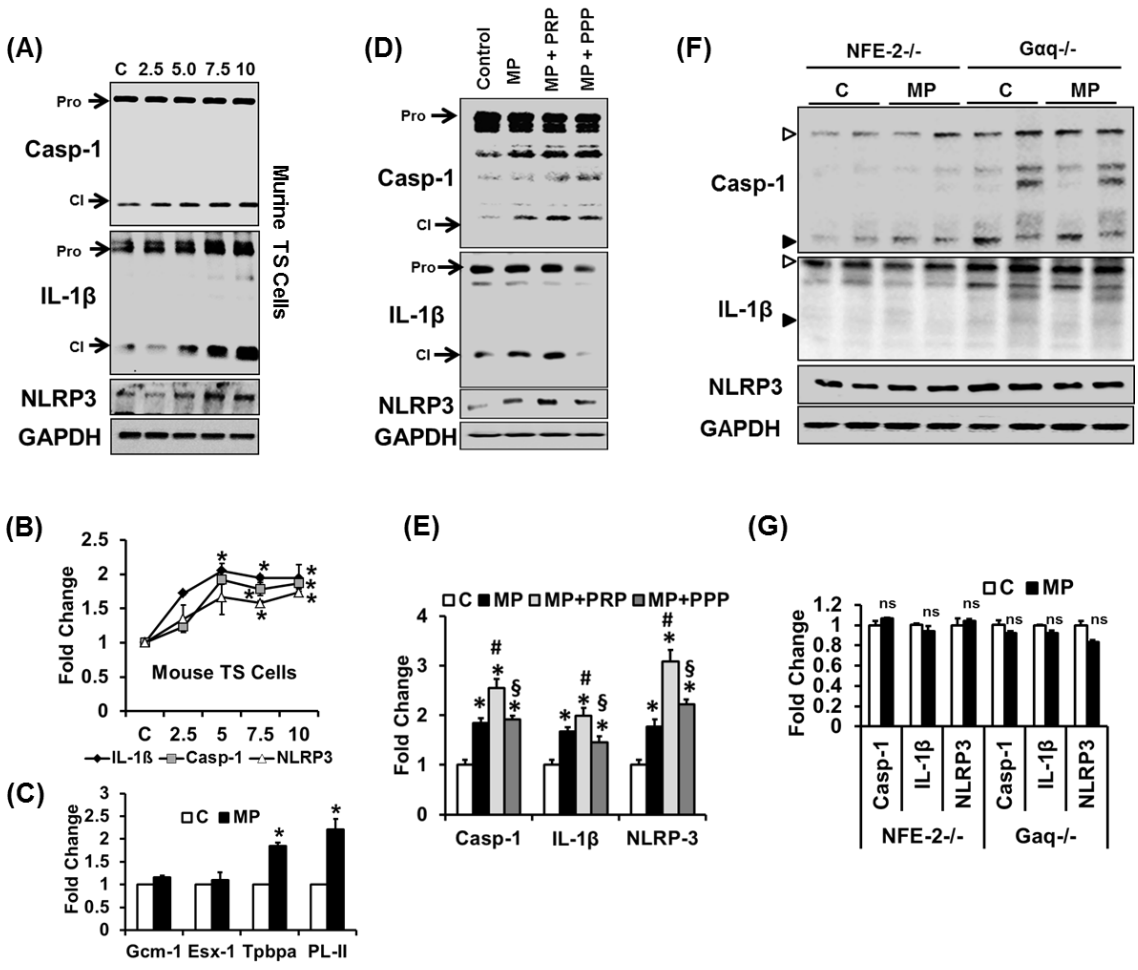


Fig. 3.14: MP cause platelet dependent inflammasome activation in trophoblast cells (A, B) Mouse endothelial cell derived MP dose-dependently cause inflammasome activation *in vitro* in murine trophoblast cells (A, immunoblot, B, bar graph summarizing results). (C) *In vitro* mRNA expression analysis of trophoblast differentiation marker genes after exposure of murine trophoblast cells to MPs. Compared to control cells Tpbpa and PL-II

expression is increased. (D, E) Platelets enhance MP-mediated (mouse endothelial cell derived MP) inflammasome activation in murine trophoblast cells. Representative immunoblots showing enhanced MP-mediated inflammasome activation in trophoblast cells in the presence of platelet rich plasma (MP+PRP) compared to MP only (MP) or MP with platelet poor plasma (MP+PPP). Control cells were exposed to the supernatant obtained after the last PBS-wash during MP isolation (D, representative immunblots; E, bar graph summarizing results). (F, G) Immunoblots showing no effect on inflammasome activation in NFE-2+/- and Gαq+/- placenta after MP treatment in NFE-2-/- and Gαq-/- pregnant female mice respectively (F, representative immunoblots; G, bar graph summarizing results). Data obtained from at least 8 placentae from 3 different litters of each group or from five independent experiments. Arrows indicate inactive (pre-form, top arrow) and active (cleaved-form, bottom arrow) caspase-1 (Casp-1) or IL-1β respectively (A, D, F). Only the expression of cleaved forms was quantified (B, E, G). Data shown represent mean±SEM of three independent experiments performed in triplicates or at least 8 placentae analyzed from at least 3 different litters of each group. *$p<0.05$ (relative to control, C); #$p<0.05$ (relative to MP); §$p<0.05$ (relative to MP+PRP); ns: not significant (B, E, G: t-test; C:ANOVA).

We next assessed the role of platelets for MP-dependent inflammasome activation within the trophoblast cells. Exposure of murine trophoblast cells to MP along with platelet rich plasma (PRP) enhanced levels of cleaved caspase-1 and IL-1β and increased NLRP3 expression in comparison to trophoblast cells exposed to MP only or to MP in the presence of platelet poor plasma (PPP, Fig. 3.14D,E). This demonstrates that platelets augment MP-induced inflammasome activation in trophoblast cells *in vitro*. To determine the relevance of platelets for MP induced inflammasome activation *in vivo* we analyzed placenta tissues obtained from pregnant Gαq-/- mice mated with WT mice and injected MP or the supernatant from the last MP-washing step (controls). In pregnant NFE-2-/- or Gαq-/- mice, carrying platelets with a severe activation defect, MP failed to induce placental inflammasome activation (Fig. 3.14F,G). This establishes a crucial role of maternal platelets for MP-dependent inflammasome activation in trophoblast cells. Taken together, maternal MP and platelets induce sterile inflammation *via* inflammasome activation within embryonic trophoblast cells, establishing a thrombo-inflammatory cross-talk at the maternal-embryonic interface.

3.5 Placental inflammasome activation causes pre-eclampsia

The above findings suggest that inflammasome activation within trophoblast cells may be at the core of PE, a disease originating from the placenta. To evaluate the mechanistic relevance of trophoblast specific inflammasome activation in PE we bred NLRP3+/- females with NLRP3-/- males, which is expected to yield an equal ratio of NLRP3-/- and NLRP3+/- embryos (having the same genotype in their trophoblast cells). MP injection selectively induced IUGR and death of NLRP3-expressing embryos (NLRP3+/-, Fig. 3.15A-D). The placentae of surviving NLRP3-

expressing embryos displayed the same morphological aberrations as described above in MP-injected wild-type mice (Fig. 3.15E-H).

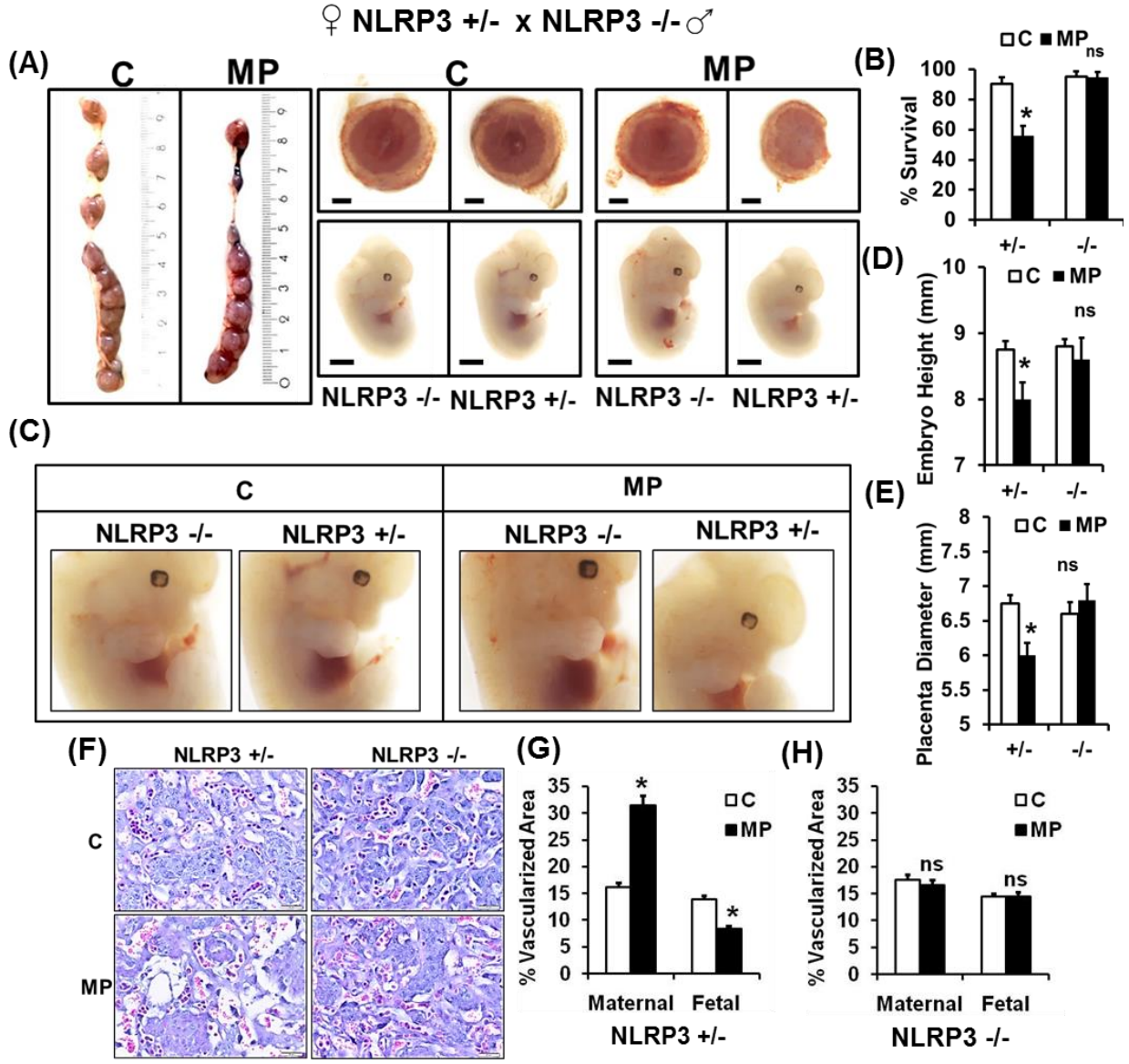


Fig. 3.15: Pregnancy outcome at day 12.5p.c. after i.v. MP (mouse endothelial cell derived) injection at day 10.5p.c. and 11.5p.c. into pregnant NLRP3+/- females mated to NLRP3-/- males. Representative images (A) of uterus (left panel), placenta (right-top panel), and embryo (right-bottom panel) and bar graph (B) quantifying embryonic survival. Size bar represents 1mm. (C) High magnification images of embryos (as in A) showing impaired fore-limb development and retinal pigmentation after MP treatment in NLRP3+/- embryos after MP treatment in NLRP3 +/- pregnant female mice. These characteristic features reflecting IUGR are normal in NLRP3-/- littermate embyros. (D-E) Bar graphs quantifying embryonic height (D) and placental diameter (E) of NLRP3+/- and NLRP3-/- embryos in NLRP3+/- pregnant female mice after MP treatment. (F-H) Representative images (F) of placenta histology (H&E staining; G, H, bar graphs summarizing data) showing enhanced maternal vascularization and reduced fetal vascularization in NLRP3+/- placentae after MP treatment in NLRP3+/- pregnant female mice. NLRP3-/- littermate placentae are protected from this vascularization defect. Size bar represents 20µm. Data shown represents mean ± SEM from at least 8 placentae or embryos analyzed from 3 different litters. *p<0.05 (relative to control, C); ns: not significant (B, D, E: ANOVA; G, H: t-test).

Conversely, despite maternal NLRP3 expression the survival rate of NLRP3-/- embryos was comparable to that observed in control pregnant mice and NLRP3-/- embryos were of normal size and developmental stage (Fig. 3.15A-D). As expected, pregnant NLRP3+/- mice carrying both NLRP3-expressing and deficient embryos developed all signs of preeclampsia (Fig. 3.16). The normal survival and phenotype of NLRP3-/- embryos despite maternal NLRP3 expression in the mothers establishes that maternal NLRP3 (including NLRP3 in maternal platelets) does not impair placental and embryonic development. Furthermore, despite the maternal PE-like phenotype the survival and development of NLRP3-/- embryos were normal, demonstrating that impaired embryonic development is a consequence of placental inflammasome activation and not of the maternal disease.

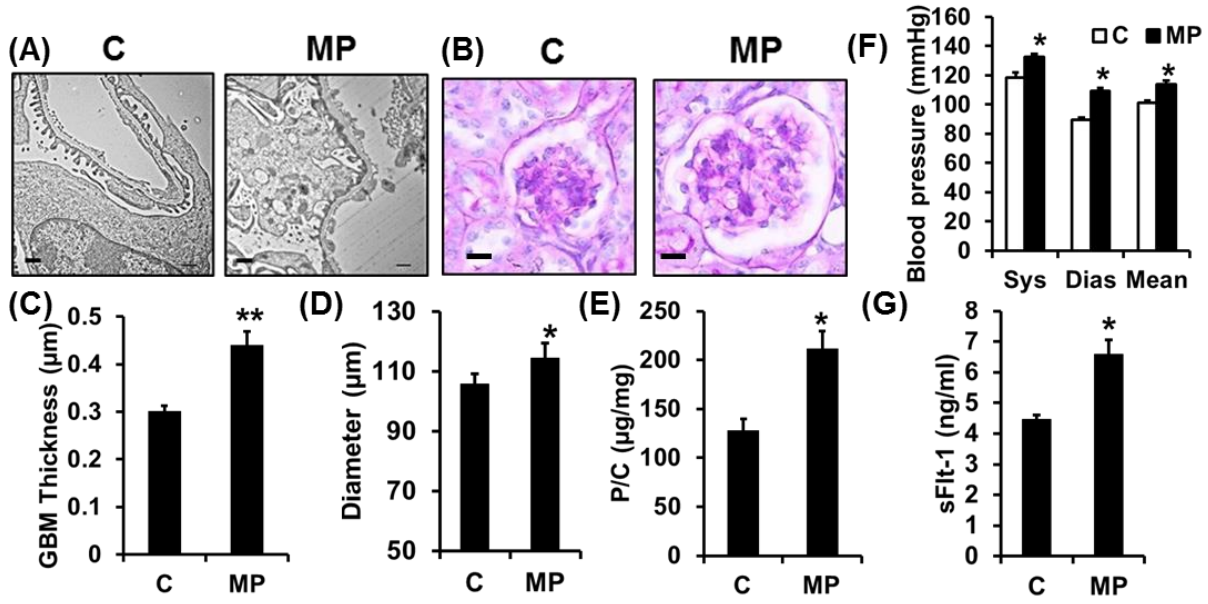


Fig. 3.16: Embryonic NLRP3 expression causes MP-dependent PE-like phenotype in mice. Representative images showing enhanced renal pathology in pregnant NLRP3+/- females mated to NLRP3-/- males after MP injections, characterized by thickened glomerular basement membrane (A, TEM; C, bar graph reflecting quantification) and enlarged glomeruli (B, PAS staining; D, bar graph reflecting quantification). (E) Proteinuria (protein-creatinine ratio, P/C) is increased in MP-injected pregnant female mice at day 12.5p.c. (E, bar-graph summarizing data). Elevated Blood pressure (F) and sFlt-1 plasma levels (G) in MP-injected pregnant mice following mating NLRP3+/- females with NLRP3-/- males; bar graphs summarizing results. Size bar represents 1µm for TEM (A) and 15µm for PAS (B). Data represents mean±SEM of atleast 5 pregnant females per group *p<0.05, **p<0.01(F-G: t-test)

To ascertain whether placental inflammasome activation is sufficient for the MP-induced PE-like phenotype we mated NLRP3-/- females with WT males resulting in NLRP3+/- embryos and trophoblast cells. Despite maternal NLRP3 deficiency MP reduced embryonic survival, impaired the development of surviving NLRP3+/- embryos and caused placental malperfusion (Fig. 3.17).

Hence, embryonic NLRP3 expression and hence NLRP3 expression within trophoblast cells is sufficient to impair placental and embryonic development in pregnant mice injected with MP.

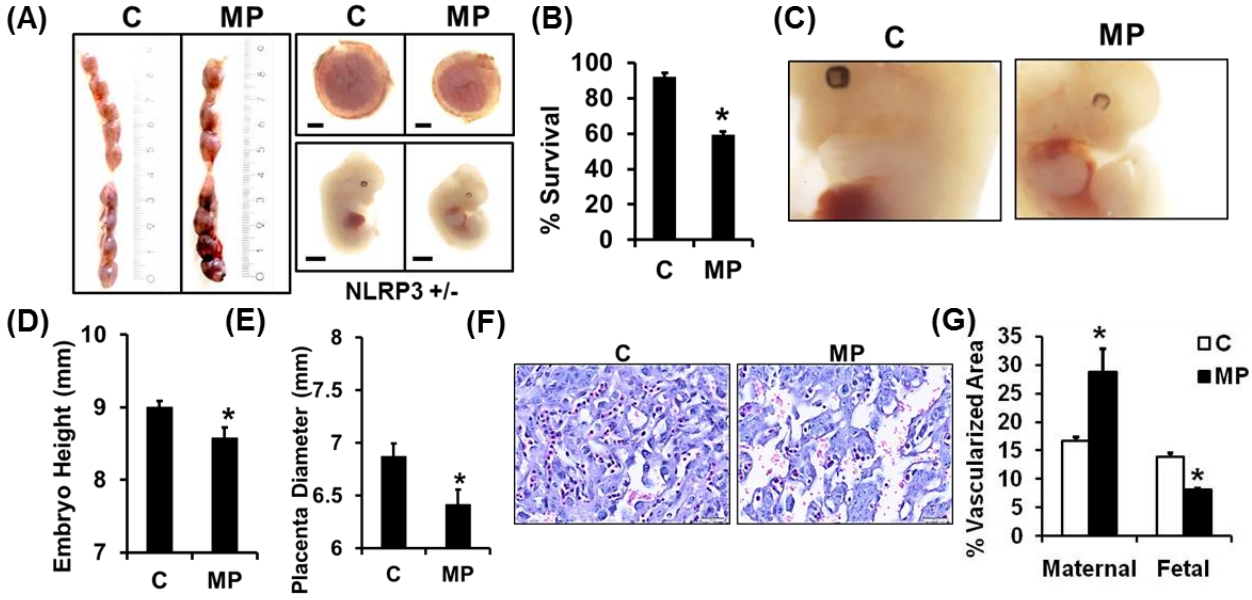


Fig. 3.17: Pregnancy outcome at day 12.5p.c. after i.v. MP injection at day 10.5p.c. and 11.5p.c. in NLRP3-/- females mated to NLRP3+/+ males. (A-C) Representative images (A) of uterus (left panel), placenta (right-top panel) and embryos (right-bottom panel) along with bar graph (B) quantifying embryonic survival. Size bar represents 1mm. (C) High magnification images of embryos (as in A) showing impaired fore-limb development and retinal pigmentation after MP treatment in NLRP3+/- embryos after MP treatment in NLRP3 -/- pregnant mice. (D-E) Bar graphs quantifying embryonic height (D) and placenta diameter (E) of NLRP3+/- embryos in NLRP3-/- pregnant female mice after MP treatment. (F-G) Representative images (F) of placenta histology (H&E staining; G, bar graph summarizing data) showing enhanced maternal vascularization and reduced fetal vascularization in NLRP3+/- placenta after MP treatment in NLRP3-/- pregnant female mice. Size bar represents 20µm. Data shown represents mean ± SEM from at least 8 placentae or embryos analyzed from 3 different litters *p<0.05 (B, D, E, G: t-test).

Importantly, following MP injection of pregnant NLRP3-/- mice carrying NLRP3-expressing embryos (NLRP3+/- embryos, obtained through mating with male NLRP3+/+ mice) these pregnant mice developed all signs of PE, e.g. proteinuria, altered kidney morphology, increased plasma levels of sFlt-1, and increased blood pressure (Fig. 3.18). This establishes a central pathogenetic function of the placental inflammasome for the induction of the PE-like phenotype, placental dysfunction, and embryonic demise as in these pregnant mice NLRP3 was only expressed in the placenta, but not in maternal tissues (including the platelets).

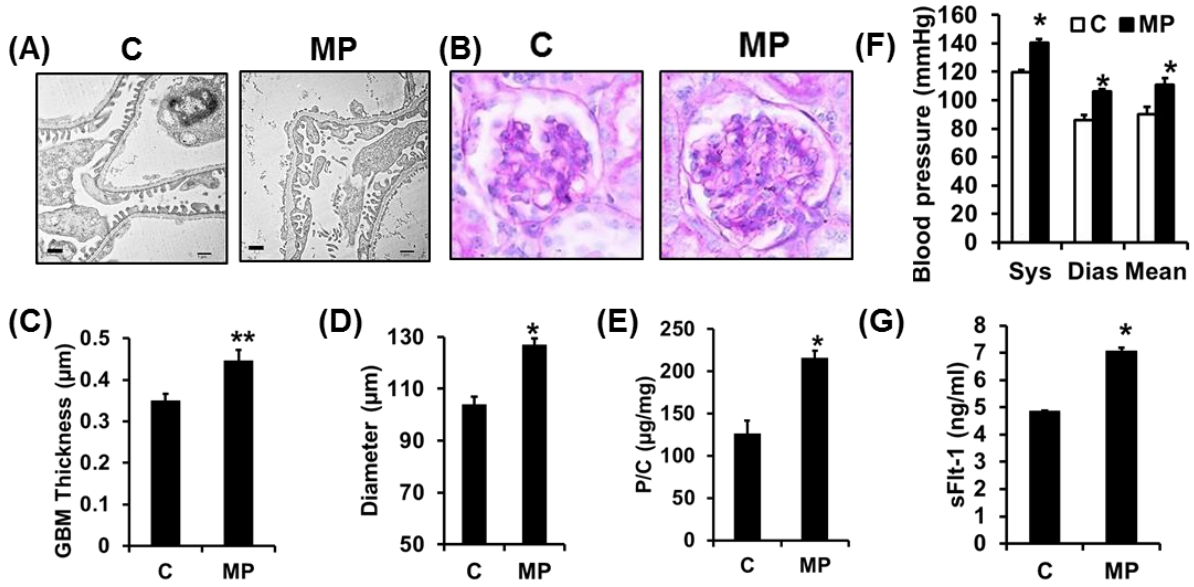


Fig. 3.18: Embryonic NLRP3 expression is sufficient to cause MP-dependent PE-like phenotype in mice. Representative images showing enhanced renal pathology in pregnant NLRP3-/- females mated to NLRP3+/+ males after MP injections, characterized by thickened glomerular basement membrane (A, TEM; C, bar graph reflecting quantification) and enlarged glomeruli (B, PAS staining; D, bar graph reflecting quantification). (E) Proteinuria (protein-creatinine ratio, P/C) is increased in MP-injected pregnant female mice at day 12.5p.c. (E, bar-graph summarizing data). Elevated Blood pressure (F) and sFlt-1 plasma levels (G) in MP-injected pregnant mice following mating NLRP3-/- females with NLRP3+/+ males; bar graphs summarizing results. Size bar represents 1µm for TEM (A) and 15µm for PAS (B). Data represents mean±SEM of 5 pregnant females per group. *p<0.05, **p<0.01 (F-G: t-test)

3.6 Purinergic and inflammasome signaling induces pre-eclampsia

The above data establish that inflammasome activation in trophoblast cells induces a PE-like phenotype in mice. Strikingly, maternal platelets induce inflammasome activation within the placenta, while the maternal inflammasome, including that in platelets, is dispensable for the PE-like phenotype. To decipher the mechanism underlying this maternal-embryonic thrombo-inflammatory cross-talk we considered a potential role of platelet-derived ATP. Upon activation platelets release ATP, and ATP induces inflammasome activation *via* purinergic receptors [85]. Purinergic receptors, in turn, are expressed by trophoblast cells [102]. First we ascertained that MP are capable to induce ATP-release from platelets (Fig. 3.19A). To determine the mechanistic relevance of platelets derived ATP and the involvement of purinergic receptors we exposed trophoblast cells to MP and platelets (PRP) in the absence (MP only) or presence of apyrase (an ATPase) or oATP (purinergic receptor antagonist). Exposure of trophoblast cells to MP and platelets resulted in a strong inflammasome activation, which was inhibited by treatment with apyrase or oATP (Fig. 3.19B,C).

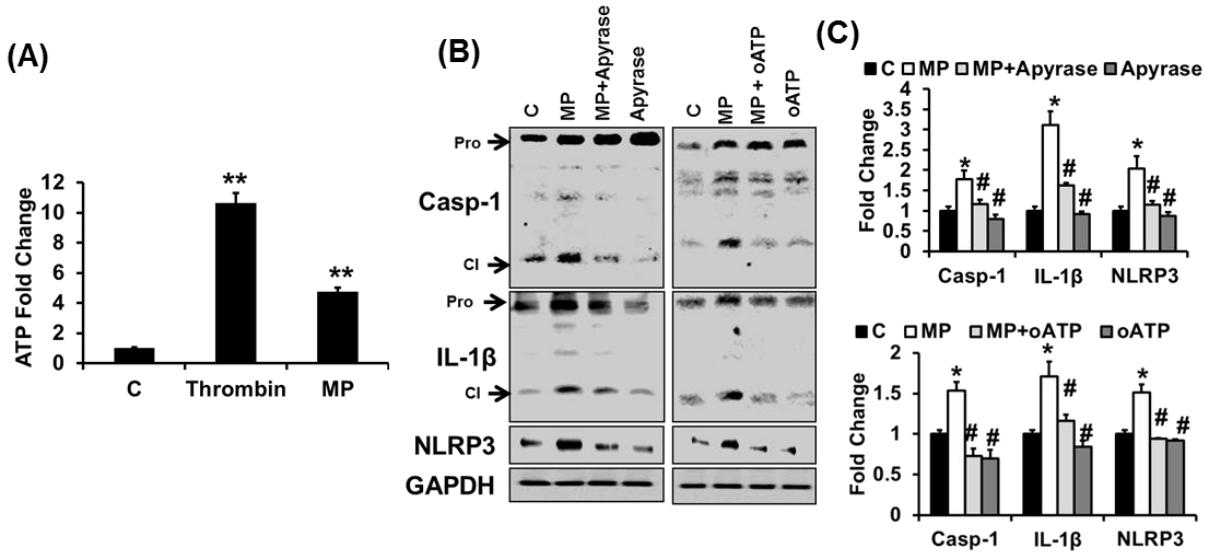


Fig. 3.19: MP cause ATP mediated inflammasome activation *in vitro*. (A) Mouse Endothelial MP induce ATP release from platelet rich plasma (PRP) after recalcification. Negative control (C): recalification only; Thrombin was used as a positive control. (B-C) Inhibition of MP induced platelet mediated inflammasome activation in murine TS cells using apyrase or oATP (B, representative immunoblots; C, bar graphs summarizing results). Cells were treated with MP (MP only) or MP and apyrase (MP+Apyrase) or MP and oATP (MP+oATP) along with PRP. Arrows in each blot indicate inactive pro (top arrow) and active cleaved (bottom arrow) forms of caspase-1 or IL-1β respectively. Expression of only cleaved forms was quantified. Data shown represents mean ± SEM from 5 independent experiments; *p<0.05 (relative to C); #p<0.05 (relative to MP) (C: ANOVA).

To evaluate the mechanistic relevance of ATP induced purinergic inflammasome activation i*n vivo* we treated pregnant mice with apyrase (an ATPase), oATP (purinergic receptor antagonist), or anakinra (IL-1R antagonist). Treatment of MP-injected pregnant mice with either agent markedly improved pregnancy outcome, as reflected by normal embryonic survival, size and development, and normal placental size and vascularization (Fig 3.20). Concomitantly, signs of PE were prevented (Fig. 3.20). Apyrase or oATP treatment furthermore reduced NLRP3 expression and caspase-1 or IL-1β cleavage in the placenta tissues (Fig. 3.21). Hence, ATP-induced purinergic signaling causes placental inflammasome activation and a PE-like phenotype. Inhibition of purinergic or IL-1β signaling protects pregnant mice from the MP and platelet dependent induction of PE. These results establish that purinergic signaling mediates the MP and platelets dependent PE-like phenotype. Furthermore, these results suggest that targeting purinergic inflammasome activation might by a new therapeutic approach to PE in humans.

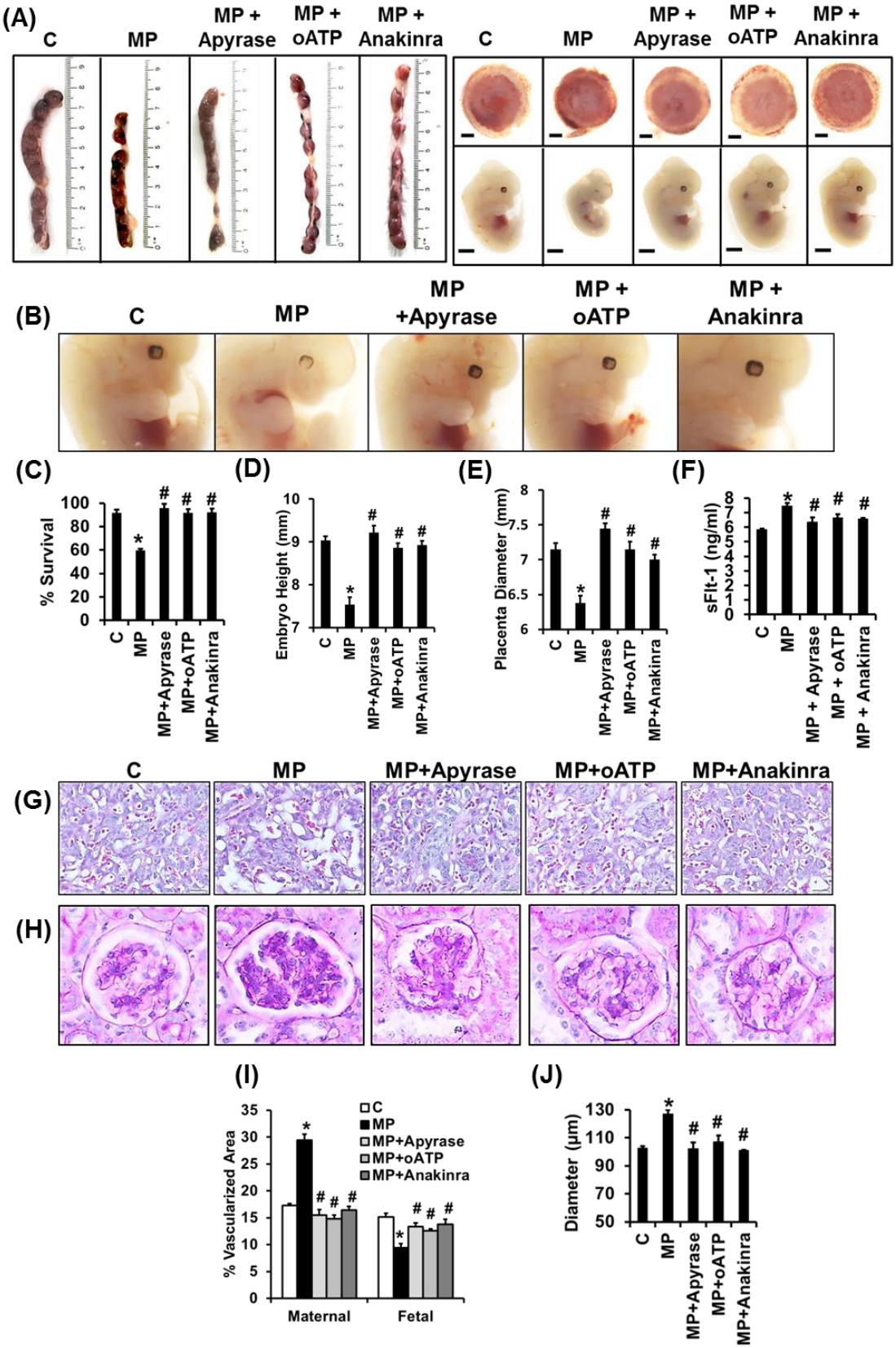


Fig. 3.20: Inflammasome inhibition using apyrase, oATP, or anakinra protects mice from MP induced placental and fetal impairment. (A) Representative images showing uterus (left), placentae (top right), or embryos

(bottom right) obtained from control (C) or MP-injected (MP) pregnant mice without or with treatment (apyrase, oATP, or anakinra). size bar represents 1mm. (B) High magnification images of embryos (as in A) in control (C) or MP-injected pregnant mice without or with treatment using the inflammasome inhibitors apyrase, oATP, or anakinra. Fore-limb development and retinal pigmentation are normal after inflammasome inhibition using apyrase, oATP or anakinra despite MP injection. (B-D) Bar graphs quantifying embryonic survival (C), embryonic height (D) and placenta diameter (E). (F) Injections with apyrase, oATP, or anakinra reduces plasma sFlt-1 levels in MP injected pregnant mice; bar graph summarizing results. (G-J) Representative images of placenta (G, H&E staining; I, bar graph summarizing results) and kidney histology (H, PAS staining; J, bar graph summarizing results) in control (C) or MP-injected pregnant mice without or with treatment using the inflammasome inhibitors apyrase, oATP, or anakinra. Maternal and fetal vascularization (I) as well as glomerular diameter (J) are normal after inflammasome inhibition using apyrase, oATP or anakinra. Size bar represents 20µm. Data shown represent at least 8 placentae or embryos analyzed from 3 different litters or 5 pregnant females of each group; mean ± SEM; *p<0.05 (relative to C); #p<0.05 (relative to MP) (C-D, H, I: ANOVA).

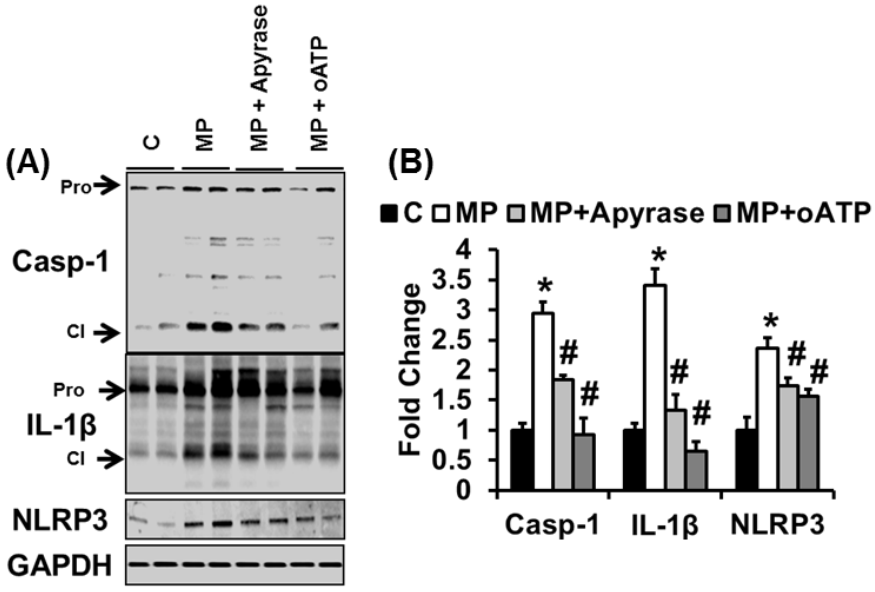


Fig. 3.21: Apyrase or oATP prevent inflammasome activation in mouse placenta. Immunoblots showing reduced cleaved caspase-1 and IL-1β and NLRP3 expression indicating inhibition of MP-induced inflammasome activation in murine placentae after apyrase or oATP treatment. (A, representative immunoblots; B, bar graph summarizing results). Arrows in each blot indicate inactive pro (top arrow) and active cleaved (bottom arrow) forms of caspase-1 or IL-1β respectively. Expression of only cleaved forms was quantified. Data shown represents mean ± SEM of at least 8 placentae analyzed from 3 different litters. *p<0.05 (relative to C); #p<0.05 (relative to MP) (B: ANOVA).

3.7 Placenta specific inflammasome activation in human PE patients

In order to corroborate the findings obtained in mice with human pathophysiology and to ascertain the translational relevance of the above findings we first investigated MP-mediated inflammasome activation in human trophoblast cells. Exposure of BeWo cells to MP and platelets (PRP) enhanced inflammasome activation (increased caspase-1 and IL-1β cleavage and NLRP3 expression) in comparison to trophoblast cells exposed to MP only or to MP in the

presence of PPP (PPP; Fig. 3.22A,B). Exposure of trophoblast cells to apyrase or oATP inhibited the MP and platelet (PRP) induced inflammasome activation (Fig. 3.22A,C,D). Importantly, platelets obtained from healthy donors who had taken aspirin for 3 consecutive days were less potent in inducing inflammasome compared to untreated donors (Fig. 3.22A,E).

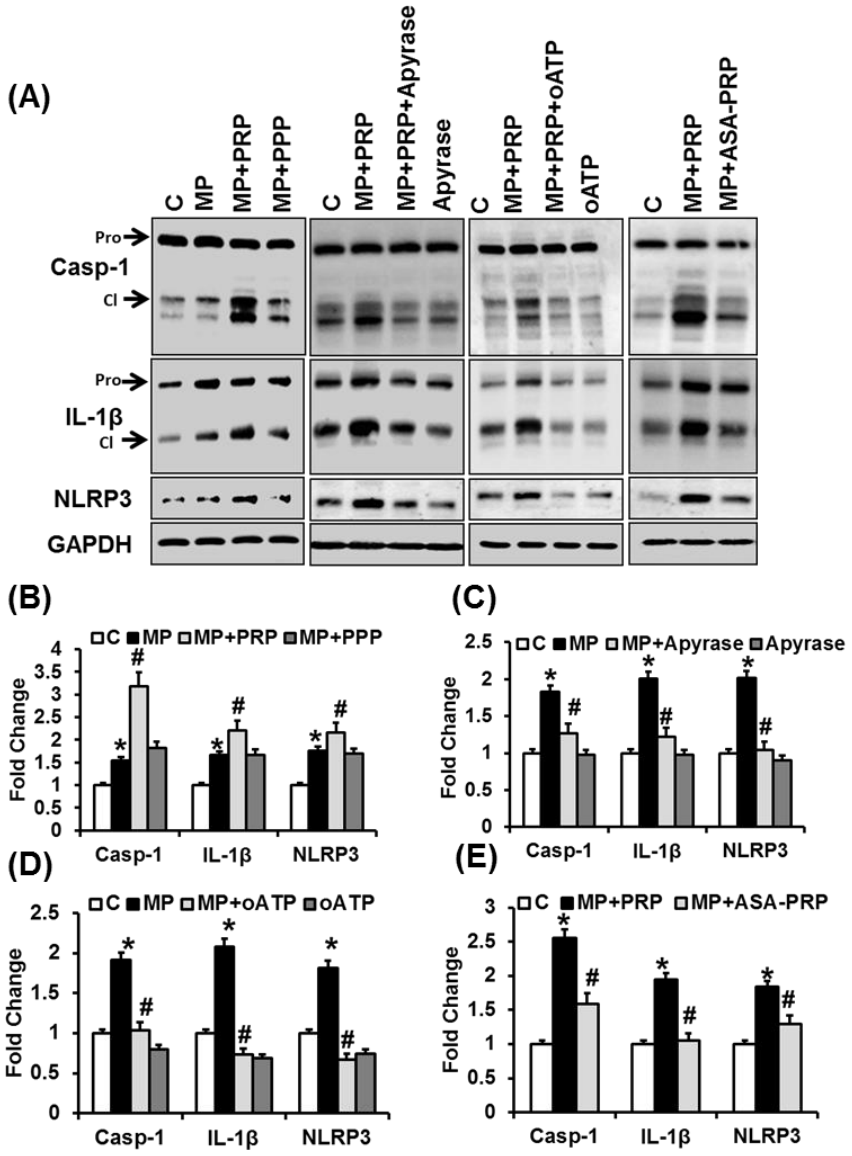


Fig. 3.22: Inflammasome activation in human trophoblast cells. (A-E) Platelets enhance ATP dependent MP-mediated (HUVEC-derived MPs) inflammasome activation in human trophoblast cells. (BeWo cells; A, representative immunoblots; B-E, bar graph summarizing results). MP-mediated inflammasome activation in BeWo cells is enhanced in the presence of platelet rich plasma (MP+PRP) compared to MP only (MP) or MP with platelet poor plasma (MP+PPP). MP induced platelet mediated inflammasome activation (MP+PRP) in BeWo cells is reduced using apyrase (MP+PRP+Apyrase), oATP (MP+PRP+oATP) or when using platelets from donors receiving aspirin (MP+ASA-PRP). Data shown represents mean±SEM from five independent experiments (B-E) *$p<0.05$ (B-E: t-test)

We next determined whether placental inflammasome activation occurs in human patients with PE. In placenta tissue samples obtained from women with PE levels of cleaved caspase-1 and cleaved IL-1β were increased in comparison to placenta tissues obtained from healthy pregnant women (Table 1, Fig. 3.23A,B). As in mice, NLRP3 expression was readily detectable at baseline in human placentae (Fig. 3.23B), but NLRP3 expression did not increase in women with PE (Fig. 3.23B). However, dimerization of NLRP3 and ASC, reflecting inflammasome activation, was only detectable in placentae obtained from women with PE, but not in control samples (Fig. 3.23C,D).

	Controls (n =15)		**PE (n=15)**		
	Mean	**SD**	**Mean**	**SD**	p value
Age (years)	33	4	31	5	0.32
BMI (kg/m2)	28	4	28	6	0.81
Parity					
Primipara	7 (46.67%)	-	10 (66.67%)	-	0.46
Multipara	8 (53.33%)	-	5 (33.33%)	-	
Systolic blood pressure (mmHg)	123.00	8.52	167.67	8.54	<0.001
Diastolic blood pressure (mmHg)	75.67	2.20	103.33	2.20	<0.001
Proteinuria (g/24h)	0	0	4.80	5.52	<0.001
Platelet counts (10^9/L)	235	46	190	44	0.03
sFlt-1/PIGF	9.87	3.92	185.7	118.20	<0.01
Gestational diabetes	0	0	0		
Gestational age at birth (weeks)	39	1	36	4	0.01
Relative birth weight (g)	3489	325	2420	252	0.001
Method of delivery					
Vaginal	0	-	0	-	-
C-section	15	-	15	-	-
Contractions	0	-	0	-	-
Sex of child					
Boy	8 (53.33%)	-	7 (46.67%)	-	1
Girl	7 (46.67%)	-	8 (53.33%)	-	

Table-1: Clinical Patient Characteristics

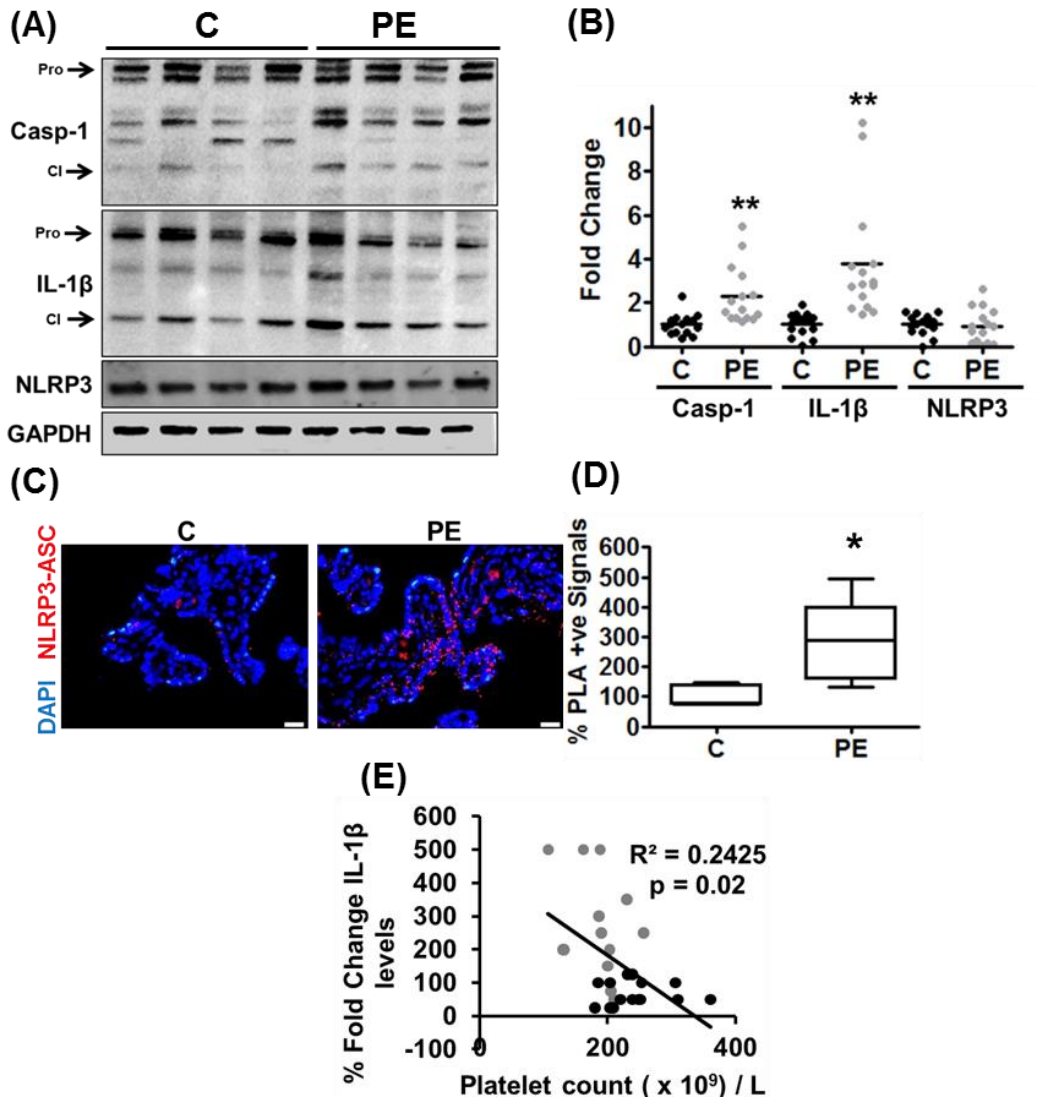


Fig. 3.23: Inflammasome activation in human placenta. (A-D) Inflammasome activation in human placentae obtained from women without pregnancy complications (controls; C) or with preeclampsia (PE). Representative immunoblot analyses (A) shows increased cleavage of caspase-1 and IL-1β in PE vs. controls, while NLRP3 expression does not change; dot-plot summarizing results (B). Arrows indicate inactive (pre-form, top arrow) and active (cleaved-form, bottom arrow) caspase-1 (Casp-1) or IL-1β, respectively. Only the expression of cleaved forms was quantified. Representative images (C) for PLA assay (D, Box-plot summarizing results) showing an increased frequency of NLRP3-ASC complexes in PE placentae compared to controls. Size bar represents 20µm. (E) Graph showing an inverse correlation between platelet counts and cleaved IL-1β in human placentae obtained from women without pregnancy complications (controls; C; black) or with preeclampsia (PE; gray). Data shown represent mean±SEM from 15 (B) or 5 (D) different placentae per group; *p<0.05, **p<0.0005 (B,D: non-parametric Mann–Whitney U test, E: Spearmen's correlation).

Platelet counts in women with PE were lower compared to controls, compatible with platelet activation and consumption in women with PE (Table 1). Furthermore, platelets counts were inversely correlated with placental cleaved IL-1β levels (Fig. 3.23E), corroborating a mechanistic interaction of platelet activation and placental inflammasome activation in humans. Taken

together, MP induces inflammasome activation in human trophoblast cells and in placentae tissues samples obtained from women with PE. These findings substantiate a translational relevance of the PE-inducing mechanism identified within this study. Taken together, these results identify a new thrombo-inflammatory mechanism at the feto-placental interface, which impedes placental and embryonic development. Importantly, this mechanism depends on enhanced activation of maternal platelets. Thus, unlike embryonic platelets maternal platelets can interfere with placental function.

3.8 p45 NF-E2 plays a role in IUGR complicated human placenta

Mice lacking the transcription factor p45 NF-E2 have been successfully used to demonstrate that embryonic platelets are dispensable during development. However, independent of embryonic platelets absence of p45 NF-E2 in mouse placenta or murine trophoblast cells impairs placental vascularization and results in IUGR [62]. In order to ascertain the relevance of p45 NF-E2 in syncytiotrophoblast formation and placental dysfunction in humans and establish translational relevance of our previous findings in murine models, we analyzed human placentae from 30 women complicated with normotensive IUGR pregnancies along with control placentae from 30 women with normal pregnancies. Histological assessment by H&E staining revealed excess syncytial knot formation in placentae obtained from pregnancies complicated by IUGR (Fig. 3.24).

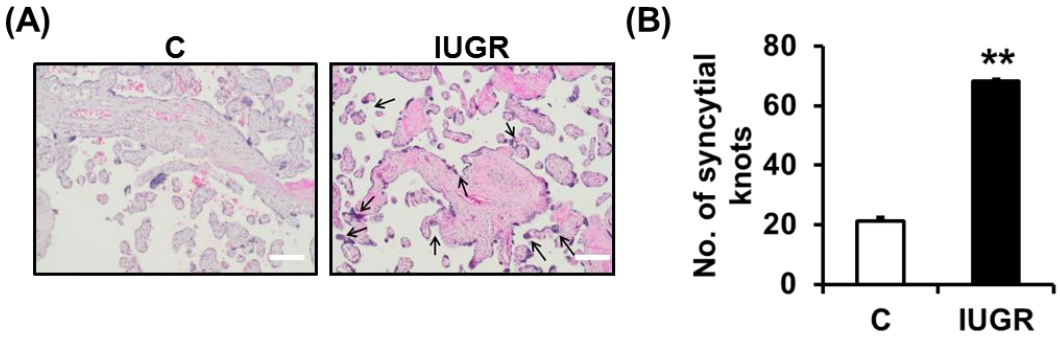


Fig. 3.24: Syncytial knots in IUGR placentae. (A-B) Histological assessment (A, representative images; B, bar-graph summarizing results) of placentae obtained from women with pregnancies complicated with IUGR compared to controls showing increased syncytial knot formation in IUGR placentae. Data shown represents mean±SEM from 30 placentae per group. **p<0.01(B: t-test).

Immunoblotting and immunofluorescence analysis of these placentae revealed reduced p45 NF-E2 expression in placentae from pregnancies complicated by IUGR compared to controls (Fig. 3.25A-C). Thus, IUGR and excess syncytium formation, as reflected by an increase of syncytial knots, are associated with reduced p45 NF-E2 expression in humans, suggesting that the observations made in mice and the mechanistic deduction may also be relevant in human pregnancy complications.

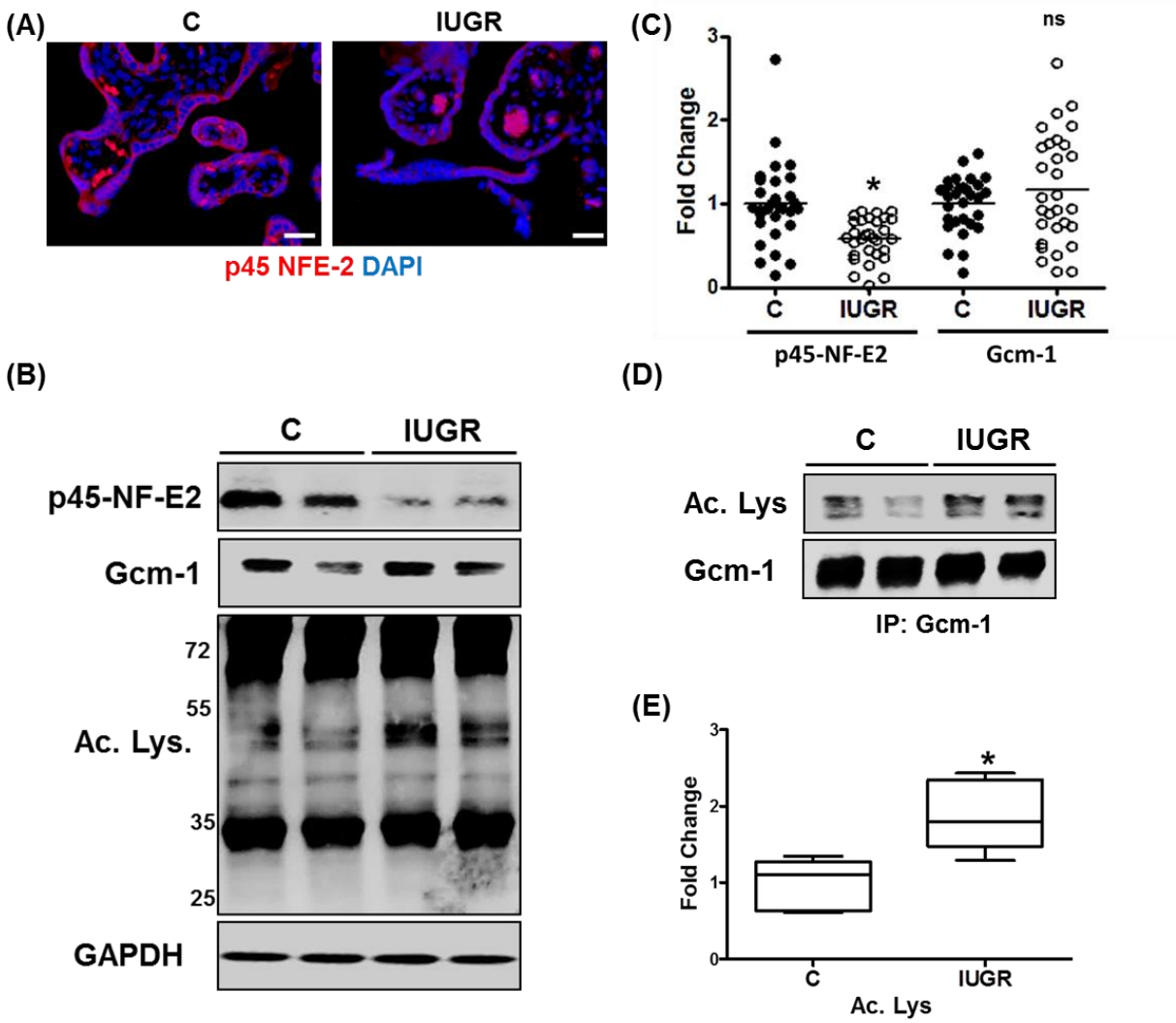


Fig. 3.25: p45 NF-E2 plays a role in human IUGR placentae through Gcm-1 acetylation (A) Representative immunofluorescence images (red: p45 NF-E2; blue: DAPI nuclear staining) showing reduced trophoblast expression of p45 NF-E2 in placentae obtained from women with pregnancies complicated with IUGR compared to controls. (B-C) Immunoblot analysis (B, representative immunoblots; C, dot-plot summarizing results) showing reduced expression of p45 NF-E2 and enhanced acetylation in placenta tissue lysates obtained from women with pregnancies complicated with IUGR compared to controls. Gcm-1 expression did not differ significantly between the two cohorts. (D-E) Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (D, representative immunoblots; E, box-plot summarizing results)showing enhanced expression of acetylated Gcm-1 in placenta tissue

lysates obtained from women with pregnancies complicated with IUGR compared to controls. Data shown represents mean±SEM from 30 placentae per group. *p<0.05 (C, E: t-test)

However, in contrast to the observations made in the murine model the expression of Gcm-1 did not differ between human placentae obtained from pregnancies complicated by IUGR and healthy control placentae (Fig. 3.24B,C). Of note, not only expression, but also acetylation of Gcm-1 was altered in p45 NF-E2-/- murine placentae [62]. Additionally, post-translational modifications of Gcm-1 such as acetylation are known to control the Gcm-1 activity and thus trophoblast cell fusion [68]. This raises the question as to whether activity of Gcm-1 in human placentae may be primarily regulated by posttranslational modifications. Given our and others previous results [62, 68] we next analyzed protein acetylation in general and specifically that of Gcm1 in human placental tissues. Analyses of protein acetylation using an antibody against acetylated lysine revealed increased acetylation of some proteins, including proteins matching the size of Gcm-1 (49 kDa; Fig. 3.25B). Increased acetylation of Gcm-1 in placentae from pregnancies complicated by IUGR was confirmed by immunoprecipitation (Fig. 3.25D,E).

Taken together, these findings demonstrate that increased Gcm-1 acetylation is readily detectable in human placentae from pregnancies complicated by IUGR, while on average Gcm-1 expression is not changed. This raises the question as to whether p45 NF-E2 may regulate Gcm-1 acetylation in human trophoblast cells.

3.9 p45 NF-E2 regulates syncytiotrophoblast formation in human trophoblast cells

In order to ascertain the mechanism through which p45 NF-E2 controls syncytium formation in human trophoblast cells we first established a suitable *in vitro* model using the trophoblast-derived choriocarcinoma cell line BeWo. BeWo cells were treated with 8-Br-cAMP to induce syncytiotrophoblast formation [103]. Treatment of BeWo cells with 8-Br-cAMP resulted in formation of multi-nucleate syncytia, as determined by immunostaining for E-Cadherin and nuclear DAPI counterstain (Fig. 3.26A,B). Trophoblast syncytium formation was confirmed by increased expression of the syncytiotrophoblast markers hCG-β and Syn-1 (Fig. 3.26C,D). Intriguingly, in this *in vitro* model Gcm-1 expression was likewise increased, consistent with increased syncytiotrophoblast formation. Importantly, increased syncytiotrophoblast formation was paralleled by reduced p45 NF-E2 protein expression (Fig. 3.26C,D).

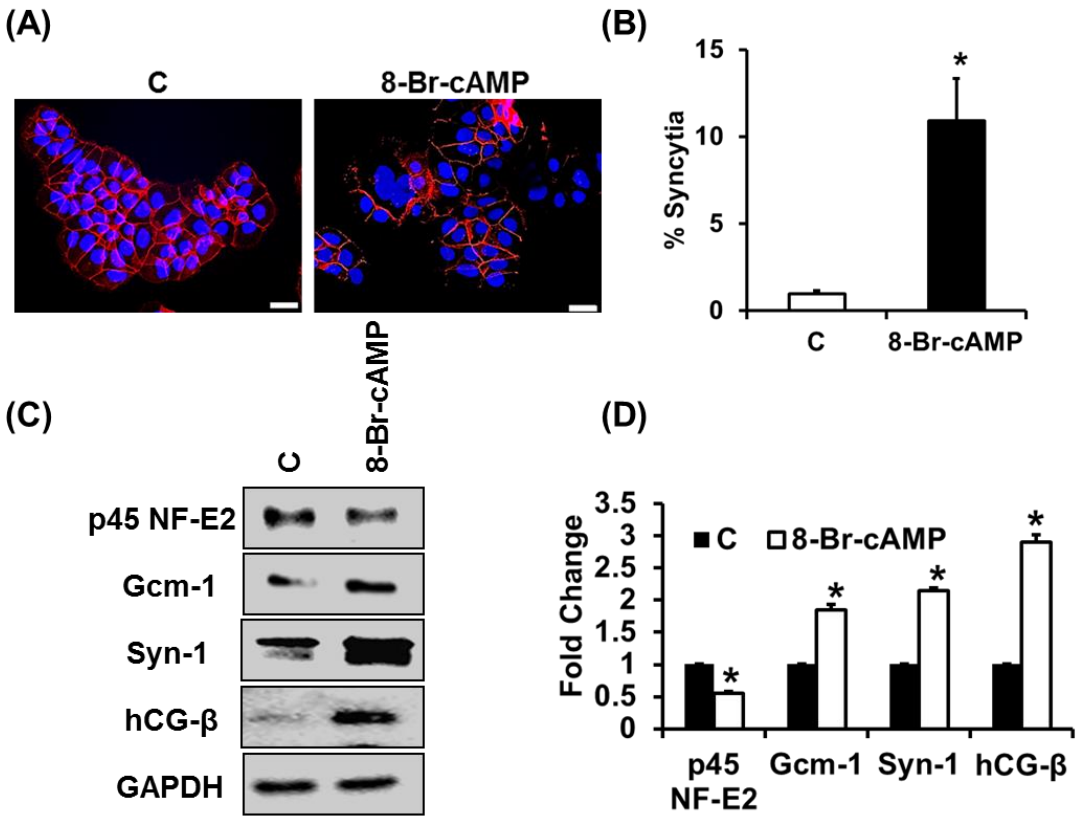


Fig. 3.26: p45 NF-E2 regulates *in vitro* syncytiotrophoblast formation in human trophoblast cells. Treatment of BeWo cells with 8-Br-cAMP induces syncytia formation and downregulation of p45 NF-E2. (A-B) Immunofluorescence staining (A, representative images; B, bar graph summarizing results) of BeWo cells using E-Cadherin showing syncytia formation after 8-Br-cAMP treatment (red: E-cadherin; blue: DAPI nuclear staining). (C-D) Immunoblotting analysis (A, representative immunoblots; B, bar graph summarizing results) showing downregulation of p45 NF-E2 expression after 8-Br-cAMP treatment in BeWo cells accompanied by upregulation of Gcm-1, Syn-1 and hCG-β compared to control indicating syncytiotrophoblast formation. Data represents mean±SEM of five independent experiments. *p<0.05 (t-test)

In parallel, 8-Br-cAMP treatment also enhanced Gcm-1 acetylation in BeWo cells (Fig. 3.27) These results demonstrate that excess syncytiotrophoblast formation in human trophoblast cells is associated with a reduced expression of p45 NF-E2, consistent with the proposed role of p45 NF-E2 as a negative regulator of syncytia formation in human trophoblast cells. Furthermore, these results suggest that human BeWo cells are a suitable *in vitro* model for mechanistic studies evaluating the role of p45 NF-E2 for syncytiotrophoblast formation.

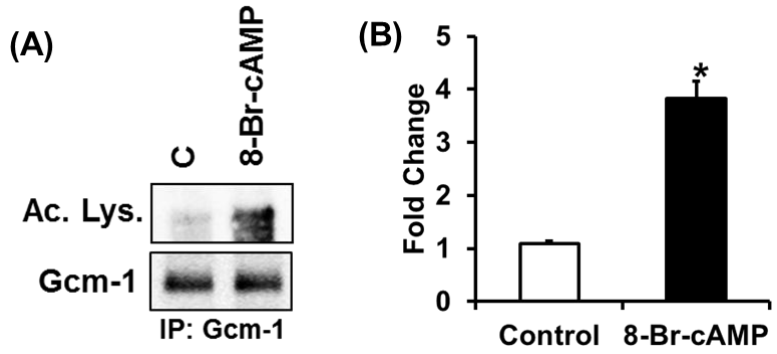


Fig. 3.27: 8-Br-cAMP induces acetylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (A, representative immunoblots; B, bar graph summarizing results) showing an increase in the expression of acetylated Gcm-1 after treatment of BeWo cells with 8-Br-cAMP indicating enhanced Gcm-1 acetylation. Data shown represent mean±SEM from 30 placentae per group. *p<0.05 (B: t-test).

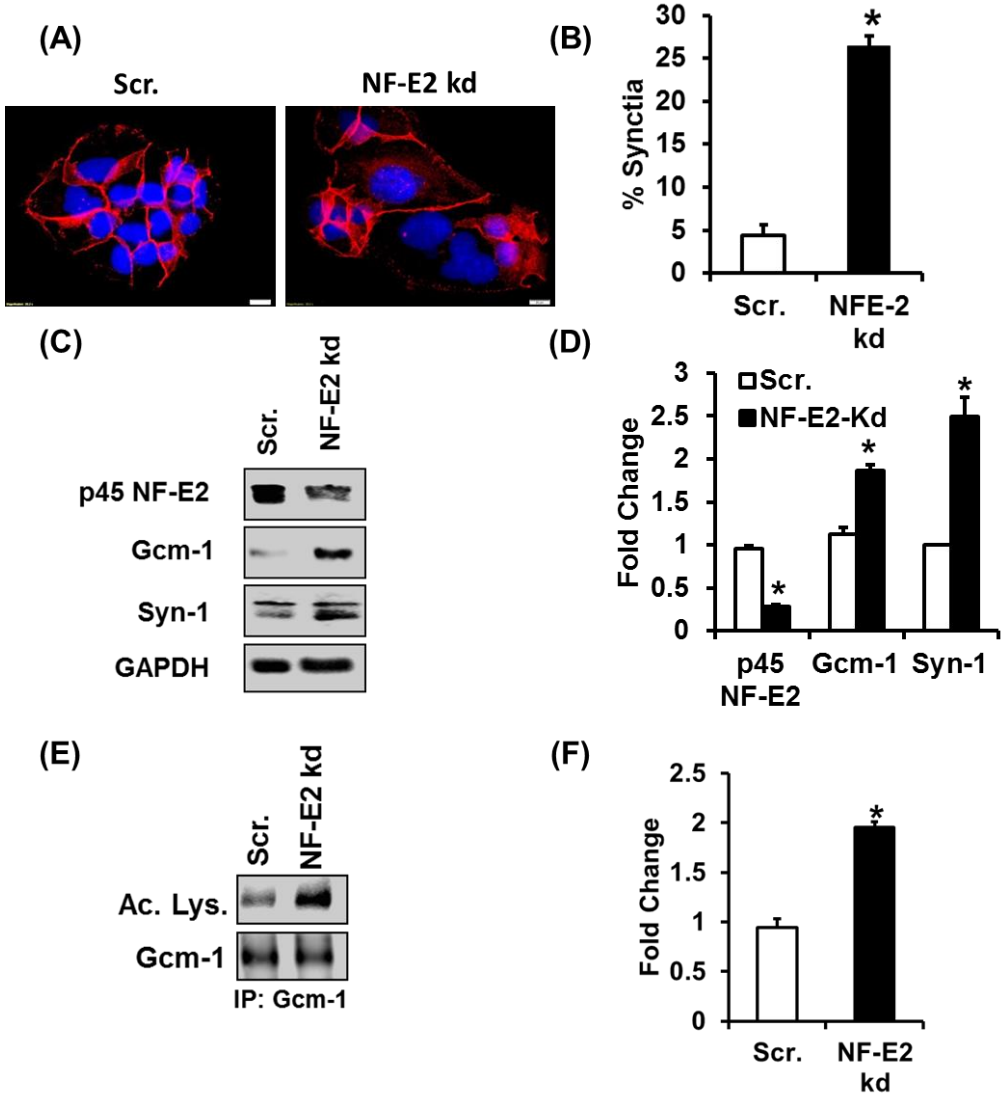


Fig. 3.28: p45 NF-E2 alters syncytiotrophoblast formation in human trophoblast cells. (A-B) Immunofluorescence staining (A, representative images; B, bar graph summarizing results) of BeWo cells using E-

Cadherin showing syncytia formation after shRNA mediated knockdown of p45 NF-E2 (red: E-cadherin; blue: DAPI nuclear staining). (C-D) Immunoblotting analysis (C, representative immunoblots; D, bar graph summarizing results) showing knockdown of p45NF-E2 expression after electroporation of BeWo cells with NF-E2 shRNA. The expression of Gcm-1 after p45NF-E2 knockdown is increased indicating syncytiotrophoblast formation due to shRNA mediated knockdown. (E-F) p45 NF-E2 knockdown induces acetylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (E, representative immunoblots; F, bar graph summarizing results) showing an increase in the expression of acetylated Gcm-1 indicating enhanced Gcm-1 acetylation after shRNA mediated knockdown of p45 NF-E2 in BeWo cells. Data shown represents mean+SEM from 5 independent experiments. *$p<0.05$, **$p<0.01$ (B, D, F: t-test).

In order to evaluate whether syncytium formation in human trophoblast cells depends on p45 NF-E2 we next reduced p45 NF-E2 expression in BeWo cells via shRNA knockdown (Fig. 3.28). Knockdown of p45 NF-E2 expression in these cells resulted in increased syncytiotrophoblast formation, as indicated by increased multi-nucleate syncytium determined by immunostaining for E-cadherin combined with nuclear DAPI stain (Fig. 3.28A,B). Increased syncytiotrophoblast formation was confirmed by an increased expression of Syn-1 and Gcm-1 expression (Fig. 3.28C,D). Importantly, not only Gcm-1 expression, but also Gcm-1 acetylation was increased in p45 NF-E2 deficient trophoblast cells (Fig. 3.28E,F).

In order to ascertain the causal relevance of p45 NF-E2 in regulating Gcm-1 acetylation in human trophoblast cells we next increased p45 NF-E2 expression using a CMV-driven expression construct. Forced expression of p45 NF-E2 was sufficient to repress Gcm-1 expression (Fig. 3.29A,B) and Gcm-1 acetylation (Fig. 3.29C,D). Intriguingly, 8-Br-cAMP failed to induce Gcm-1 expression, Gcm-1 acetylation, and syncytiotrophoblast formation in BeWo cells overexpressing p45 NF-E2 (Fig. 3.29). These observations suggest that p45 NF-E2 is a dominant negative regulator syncytiotrophoblast formation in human trophoblast cells, at least in the employed model.

Taken together, these results establish that p45 NF-E2 regulates Gcm-1 expression, Gcm-1 acetylation, and syncytiotrophoblast formation in human trophoblast cells. However, the mechanism through which the transcription factor p45 NF-E2 regulates Gcm-1 acetylation and syncytiotrophoblast formation remains unknown.

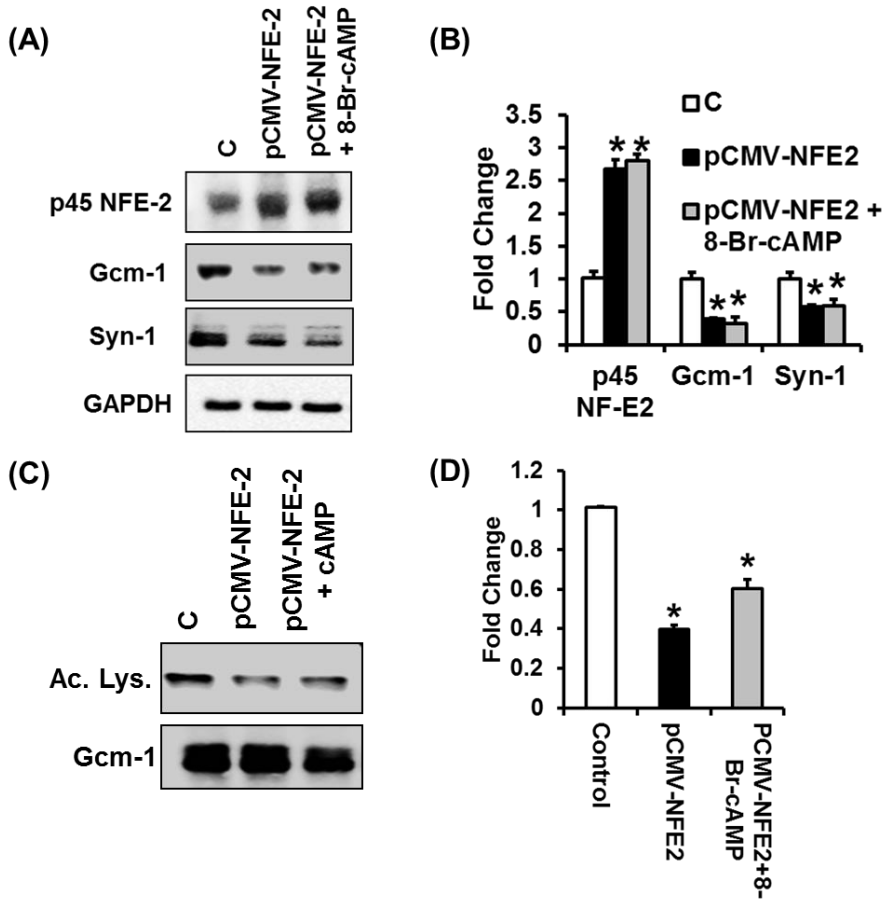


Fig. 3.29: Overexpression of p45 NF-E2 prevents syncytiotrophoblast formation in human trophoblast cells (A-B) Immunoblot analysis (E, representative immunoblots; F, bar graph summarizing results) showing that overexpression of p45 NF-E2 after electroporation of BeWo cells with pCMV-NF-E2 prevents 8-Br-cAMP induced syncytiotrophoblast formation (reduced Gcm-1, Syn-1 expression). (C-D) Overexpression of p45 NF-E2 prevents 8-Br-cAMP induced acetylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (G, representative immunoblots; H, bar graph summarizing results) showing reduced expression of acetylated Gcm-1 after overexpression of p45 NF-E2. Treatment of BeWo cells overexpressing p45 NF-E2 with 8-Br-cAMP did not increase Gcm-1 acetylation. Data shown represents mean±SEM from 5 independent experiments. *$p<0.05$ (B, D: t-test)

3.10 p45 NF-E2 regulates syncytiotrophoblast formation by CBP mediated acetylation of Gcm-1

The transcriptional activity stability of Gcm-1 is regulated at least in part by CBP [68]. We therefore investigated whether p45 NF-E2 regulates Gcm-1 acetylation and expression levels via CBP in human trophoblast cells. Using the above *in vitro* syncytiotrophoblast formation model we first analyzed the impact of syncytiotrophoblast formation on the interaction of CBP with Gcm-1 and p45 NF-E2. In untreated control cells a strong interaction of CBP with p45 NF-E2 was observed, while that of CBP with Gcm-1 was negligible (Fig. 3.30A,B). Following induction

of syncytiotrophoblast formation using 8-Br-cAMP the interaction of CBP with p45 NF-E2 markedly decreased, while that with Gcm-1 markedly increased (Fig. 3.30A,B).

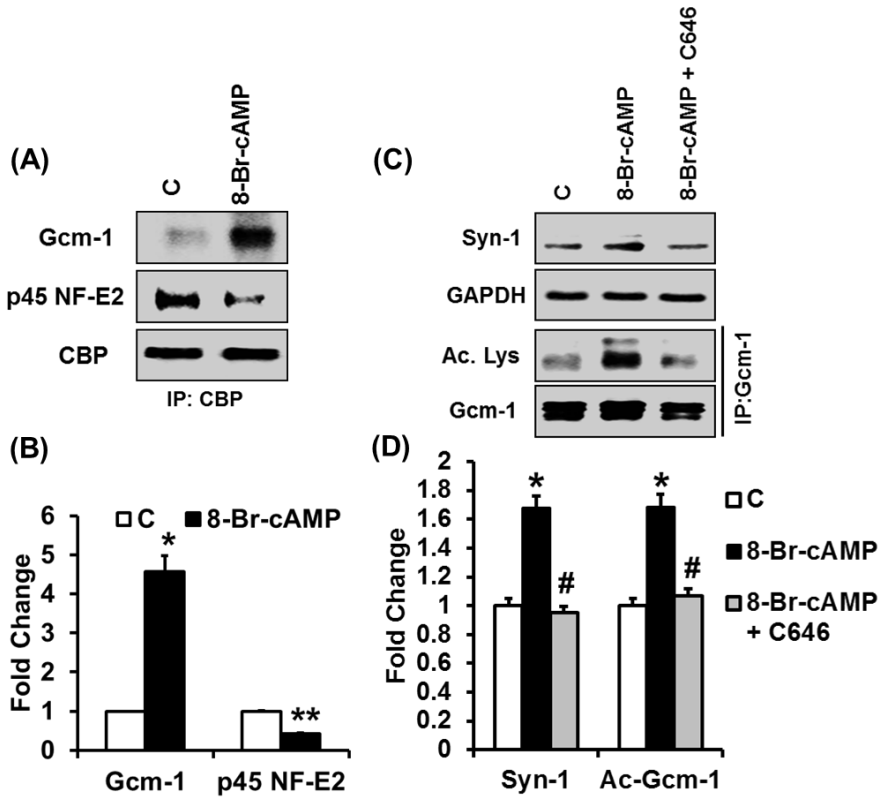


Fig. 3.30: p45 NF-E2 regulates syncytiotrophoblast formation by CBP mediated Gcm-1 acetylation in human trophoblast cells. (A-B) p45 NF-E2 inhibits the interaction of CBP with Gcm-1. Immunoprecipitation using anti-CBP antibody followed by immunoblotting (A, representative immunoblots; B, bar graph summarizing results) showing that a reduction in the interaction between CBP and p45 NF-E2 but an increase in the interaction between CBP and Gcm-1 after treatment of BeWo cells with 8-Br-cAMP. (C-D) Immunoblot analysis (C, representative immunoblots; D, bar graph summarizing results) showing inhibition of CBP acetyltransferase activity by C646 reduces expression of Syn-1 and prevents Gcm-1 acetylation (analyzed by immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting). Data shown represents mean±SEM from 5 independent experiments. *p<0.05 (B, D: t-test).

This interaction pattern of CBP with p45 NF-E2 and Gcm-1 indicates that p45 NF-E2 may reduce Gcm-1 acetylation by inhibiting its interaction with CBP, which possesses acetyltransferase activity. Indeed, inhibiting the acetyltransferase activity of CBP using C646 normalized Gcm-1 acetylation and Syn-1 expression in the 8-Br-cAMP model (Fig. 3.30C,D). Based on these data

we propose that p45 NF-E2 inhibits Gcm-1 acetylation by preventing the interaction of Gcm-1 with CBP.

3.11 p45 NF-E2 regulates syncytiotrophoblast formation by SENP mediated desumoylation of Gcm-1

While Gcm-1 stability is regulated by its acetylation, DNA-binding activity is regulated by SENP1 mediated desumoylation [104, 105]. As shown above we observed increased syncytin-1 expression and syncytiotrophoblast formation in p45 deficient trophoblast cells, indicating increased DNA-binding activity of Gcm-1 and hence desumoylation.

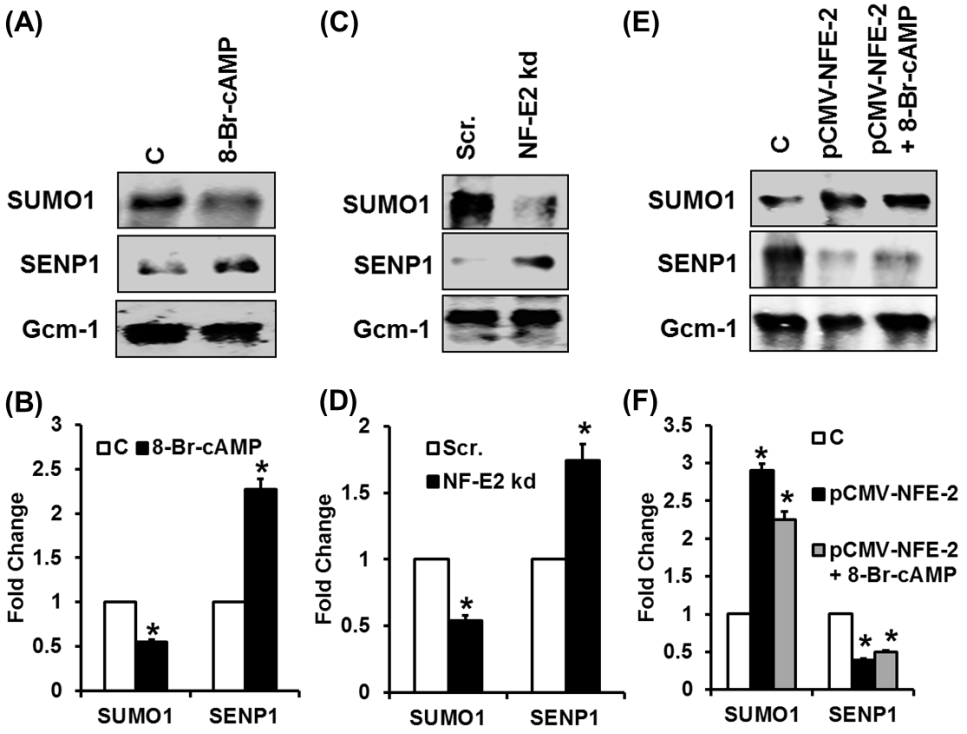


Fig. 3.31: p45 NF-E2 regulates syncytiotrophoblast formation by SENP1 mediated Gcm-1 desumoylation in human trophoblast cells. (A-B) 8-Br-cAMP causes desumoylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (C, representative immunoblots; D, bar graph summarizing results) showing a decrease in sumoylation of Gcm-1 and enhanced Gcm-1-SENP1 interaction after treatment of BeWo cells with 8-Br-cAMP indicating enhanced Gcm-1 acetylation. (C-D) p45 NF-E2 knockdown causes desumoylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (C, representative immunoblots; D, bar graph summarizing results) showing a decrease in sumoylation of Gcm-1 and enhanced Gcm-1-SENP1 interaction indicating Gcm-1 desumoylation after shRNA mediated knockdown of p45 NF-E2 in BeWo cells. (E-F) Overexpression of p45 NF-E2 prevents 8-Br-cAMP induced desumoylation of Gcm-1. Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (E, representative immunoblots; F, bar graph summarizing results) showing enhanced expression of SUMO1 and reduced expression of SENP1 after overexpression of p45 NF-E2. Treatment of BeWo cells overexpressing p45 NF-E2 with 8-Br-cAMP did not reduce Gcm-1 sumoylation. Data shown represents mean±SEM from 5 independent experiments. *$p<0.05$ (B, D, F: t-test)

First, we ascertained that Gcm-1 interacts with SENP1, resulting in desumoylation of Gcm-1 in the 8-Br-cAMP syncytiotrophoblast formation model. Indeed, following exposure of BeWo cells to 8-Br-cAMP we observed an increased interaction of Gcm-1 with SENP1, which was associated with a reduced sumoylation of Gcm-1 (Fig. 3.31A,B). To determine whether p45 NF-E2 regulates the interaction of Gcm-1 with SENP1 and Gcm-1 desumoylation we next evaluated p45 NF-E2 deficient BeWo cells. A reduced expression of p45 NF-E2 enhanced the interaction of Gcm-1 and SENP1, which was associated with Gcm-1 desumoylation (Fig. 3.31C,D). Conversely, in BeWo cells with increased p45 NF-E2 expression Gcm-1-SENP1 interaction was reduced, while sumoylation of Gcm-1 was increased (Fig. 3.31E,F). This effect was sustained even after treatment with 8-Br-cAMP indicating that p45 NF-E2 has a dominant effect in this model (Fig. 3.31E,F).

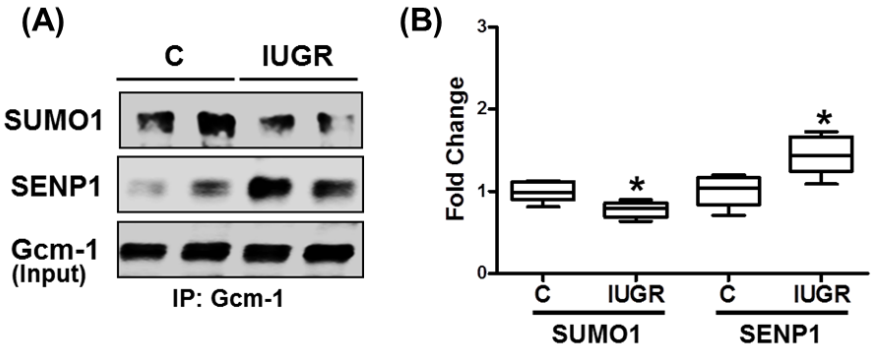


Fig. 3.32: Gcm-1 sumoylation in IUGR placentae. (A-B) Immunoprecipitation using anti-Gcm-1 antibody followed by immunoblotting (A, representative immunoblots; B, box-plot summarizing results)showing reduced sumoylation of Gcm-1 and enhanced SENP1-Gcm-1 interaction in placenta tissue lysates obtained from women with pregnancies complicated with IUGR compared to controls. Data shown represent mean±SEM from 30 placentae per group. *p<0.05 (t-test).

These *in vitro* studies suggest that p45 NF-E2 does not only regulate Gcm-1 acetylation, but also Gcm-1 desumoylation, which is an important determinant of Gcm-1 DNA-binding activity [104]. To evaluate the potential *in vivo* relevance of these findings we next analyzed the human placentae obtained from pregnancies complicated by IUGR and healthy controls. Indeed, reduced p45 NF-E2 expression was not only associated with increased Gcm-1 acetylation (Fig. 3.32), but also with desumoylation of Gcm-1 in placentae obtained from pregnancies complicated by IUGR (Fig. 3.32). Desumoylation of Gcm-1 was associated with an increased interaction of SENP1 with Gcm-1 in human placentae obtained from pregnancies complicated by IUGR (Fig. 3.32). These results demonstrate a negative interaction between acetylation and sumoylation of Gcm-1

in IUGR placentae, which – based on the *in vitro* studies – appears to be regulated by p45 NF-E2 expression. These data suggest that in human placentae disease Gcm-1 is primarily regulated by posttranslational modifications and that p45 NF-E2 negatively regulates Gcm-1 activity and syncytiotrophoblast formation through a coordinated regulation of Gcm-1 acetylation and desumoylation. These results corroborate a function of p45 NF-E2 for placental development and function independent of embryonic platelets and establish the mechanistic relevance of the p45 NF-E2 dependent Gcm-1 regulation in human trophoblast cells and placenta. In addition, these studies provide new mechanistic insight into the regulation of Gcm-1 by p45 NF-E2.

4.0 Discussion

Placental insufficiency, PE and IUGR remain understudied and unresolved complications of pregnancy. Due to the lack of sufficient pathophysiologic insights efficient therapies for these are lacking. The only "remedy" for PE is the delivery of the placenta, which exemplifies that the disease is of placental origin. Unreliable and insufficient biomarkers for IUGR make it difficult to identify the disease and could lead to severe post-natal complications. A disturbance in the hypercoagulable state of pregnancy can lead to serious complications during pregnancy affecting placental function and causing pregnancy loss. The role of platelets in this context is important but has been undermined. The current study identifies a new patho-mechanism for PE and IUGR localized to the placenta.

Through several *in vitro* and *in vivo* complimentary approaches, we show a pathogenic association between microparticles, maternal platelets and placental inflammasome. Microparticles, which are known to be associated with PE, cause accumulation of activated maternal platelets specifically in the placental vascular bed, inducing *via* ATP-release and purinergic signaling inflammasome activation in embryonic trophoblast cells. This identifies a novel thrombo-inflammatory pathway at the maternal-embryonic interface. Importantly, this pathogenetic maternal-embryonic cross-talk can be therapeutically targeted through inhibition of purinergic or inflammasome signaling or by preventing platelet activation. Concurrently, we demonstrate inflammasome activation in trophoblast cells of women with PE, corroborating the disease relevance and the potential translational relevance of the current results.

The relevance of platelets as inflammatory regulators has been increasingly recognized in recent years. Platelet activation has been linked with activation of the complement system or the contact and kallikrein system [80]. In addition, platelets contain a functional NLRP3 inflammasome, contributing to sterile and infection-associated inflammation [106]. Here we uncover a novel platelet related thrombo-inflammatory mechanism at the maternal-embryonic interface. MP induce accumulation of activated platelets specifically within the placental vascular bed, resulting in inflammasome activation in trophoblast cells. Importantly, platelets induce sterile inflammation in embryonic trophoblast cells in the absence of blood clots or increased fibrin(ogen) accumulation, demonstrating that platelets specifically modulate the innate immune response within the placenta [107]. Concurrently, quantitative or qualitative platelet deficiency

prevents this pathological sterile inflammation in the placenta underlying the harmful fate of platelet activation in pregnancy.

PE is associated with placental inflammatory dysregulation and higher levels of inflammatory cytokine IL-1β which is a key cytokine released during inflammasome activation. Studies focusing on the role of sterile inflammation involving inflammasome activation during pregnancy are lacking. Moreover, the origin of such inflammatory responses is not known. In this study, we show that the deficiency of NLRP3 in the embryo and therefore in trophoblast cells prevents the MP-induced systemic PE like phenotype, characterized by hypertension, proteinuria, and renal pathology. Thus, MP-induced and platelet mediated inflammasome activation in trophoblast cells is required for the induction of the PE-like phenotype. Intriguingly, trophoblast specific inflammasome activation also impaired trophoblast differentiation (both *in vitro* and *in vivo*) and compromised placental development, a typical finding in patients with PE. This suggests that the activated inflammasome does not only cause a maternal inflammatory response but in addition directly impedes placental development. The pathomechanism identified within this study is entirely consistent with a disease promoting placental focus. We hypothesize that inflammasome activation within the placenta causes systemic inflammation and endothelial dysfunction and ultimately all systemic manifestations of PE through the release of free or exosomes-bound IL-1β and potentially other disease promoting mediators, which have been previously linked with PE (e.g. soluble endoglin, sFlt-1) [108, 109].

The identified mechanism may initiate a self-perpetuating disease process. Activated platelets release ATP along with dense granules and platelet derived MPs. These activated platelets can further activate more platelets and also lead to endothelial dysfunction and release of endothelial cells derived MPs. Both, human and mice placenta is in close contact with maternal platelets and hence the surplus of ATP is sufficient to induce inflammasome activation in the trophoblast cells. ATP has been shown to act as a danger signal during pregnancy resulting in endothelial dysfunction and PE. Overexpression of CD39, a dominant ecto-nucleotidase, in placental trophoblastic tissue protects from preeclampsia and modulates ATP dependent trophoblast functions in mice.

Activated platelets, which accumulated within the placenta, will promote IL-1β release from trophoblast cells. IL-1β, in turn, can stimulate platelets [110], resulting in more ATP-release and

inflammasome activation. This may promote further endothelial damage and release of trophoblast derived MPs, thus amplifying the disease process. Furthermore, ATP can induce the generation of procoagulant MP *via* inflammasome signaling [111], further promoting the disease process. Platelet activation, therefore, can trigger a viscous cycle of MP and ATP release which is amplified at every stage. Thrombin induces splicing of IL-1β pre-mRNA in platelets [82, 110], which may further accelerate the proposed pathomechanism. Importantly, IL-1β mediated platelet activation and ATP-induced MP formation can be therapeutically targeted by inhibiting inflammasome signaling (using anakinra or a caspase-1 inhibitor, respectively) [111] (this study), establishing that this self-perpetuating disease process can be pharmaceutically targeted. Inflammasome inhibition will allow targeting this platelet dependent disease mechanism without compromising the hemostatic function of platelets – an important aspect in pregnant women.

Anticoagulants and antiplatelet agents are used to reduce this risk associated with thrombophilia and PE. In regard to thrombophilia the evidence remains weak at best and careful consideration of the expected benefit and side-effects is required. Anticoagulants such as heparin are associated with a risk of heparin induced thrombocytopenia or osteoporosis [112]. Considering the above-described role of platelets during placentation antiplatelet agents may be an interesting alternative for PE. According to a Cochrane review that looked at 39 trials involving >30.000 women, treatment with antiplatelet agents significantly reduces the risk of preeclampsia by 15% [113, 114]. Intriguingly, the risk of hypertension (without PE) in pregnancy was not affected, implying a specific role of platelets in PE [113, 114]. Use of antiplatelet agents was also associated with a small, but significant, reduction in the risk of delivery before 37 weeks (-8%) or infant death (-14%). In regard to the risk reduction of PE, aspirin, the most commonly used antiplatelet agent, showed a beneficial effect. Reliable suggestions for the best dosage regimen cannot be made and the suggestion to use 75 mg or less simply reflects the fact that most studies used such low dosages of aspirin [113-115]. Other platelet inhibitors, such as TXA2 synthase inhibitors, TXA2 receptor antagonists, 5-hydroxytryptamine receptor type 2 blockers, have been proposed, but experience in women with HPD is limited. Clopidogrel has been found to be effective with animal models with no fetal toxicity, but data from human patients is limited [116, 117]. Prasugrel and Ticagrelor are two other recent antiplatelet agents that have been investigated, but to date only in animal studies [118]. Anticoagulants such as hirudin, have been likewise considered, but at face-value heparins, which do not cross the placenta, appear to be a safer

alternative [119]. Endothelin antagonists target specific endothelial dysfunction in PE and have also shown protective effects in animal models [120]. By increasing endothelial nitrite oxide endothelin antagonists would also restrict platelet activation. Evaluating the efficacy of antiplatelet therapies for pregnancy-associated complications such as PE has been limited hitherto reflecting the difficulties to conduct randomized controlled trials in pregnant women. It remains a major challenge to test agents which might be potential relevance and benefit for affected pregnant women. Given the current findings, reports of the safe use of IL-1R antagonists in pregnant women, and development of new drugs targeting the pathomechanisms identified here we propose that targeting purinergic or inflammasome signaling may constitute an efficacious and possibly safe approach in women with PE [121-123].

The thrombo-inflammatory mechanism identified here provides a new conceptual framework linking MP, platelet-activation, thrombophilia, sterile inflammation, and impaired pregnancy outcome with PE-like signs in pregnant mice that are compatible with human PE (Fig. 4.1). As MP are not only associated with PE, but also with recurrent miscarriage, intrauterine growth restriction, and preterm delivery [124-126] the current insights may have implications beyond PE. In addition, MP-generation and platelet activation are induced in various infectious diseases and hence the mechanism identified here may induce abortion if maternal health is jeopardized. Intriguingly, as in other cell-types inflammasome regulators are already expressed in trophoblast cells at baseline [127] (this study). This may allow prompt activation of a placental inflammatory process and hence abortion while limiting the risk for the mother in the presence of danger. MP are, however, heterogeneous and the question remains whether only certain subtypes of MP, e.g. those providing sufficient procoagulant activity [128], will impair placental function. Characterization of the disease promoting MP may provide a tool for early diagnosis of PE and potentially other complications during pregnancy. Carefully planned pre-clinical studies and meticulous analyses of observational clinical studies are needed to address the open questions and to evaluate the therapeutic potential of the pathway described here for pregnancy associated complications.

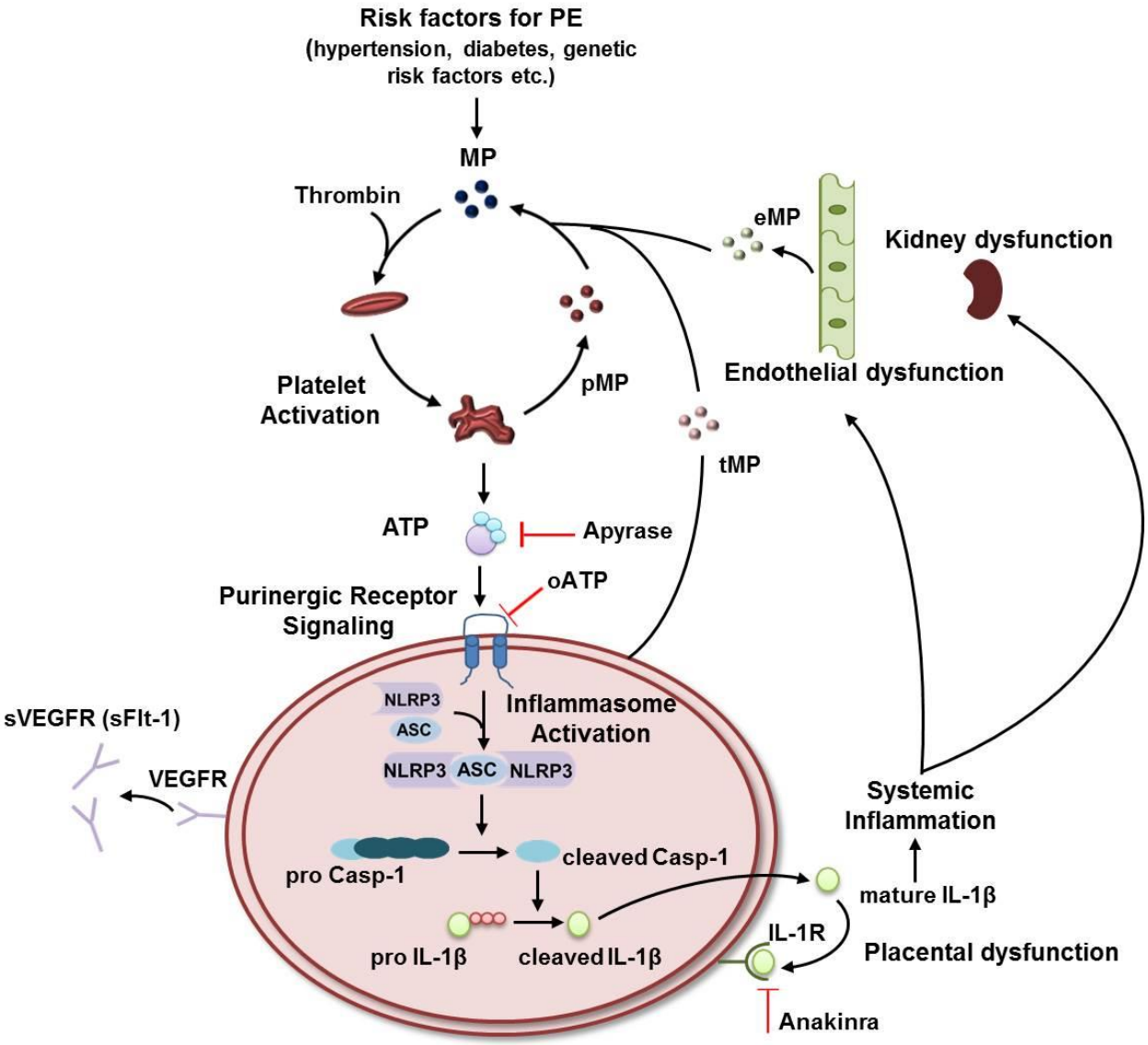


Fig. 4.1: Scheme reflecting the proposed role of MP for placental dysfunction and pregnancy complications Procoagulant MP promote platelet activation and hence ATP release causing purinergic receptor mediated inflammasome activation in the placenta and preeclampsia which is accompanied by increased plasma sFlt-1 levels, hypertension, endothelial dysfunction and kidney dysfunction. Inflammasome activation in trophoblast cells triggers further MP release from trophoblast cells (tMP) potentially triggering a vicious cycle of MP generation and endothelial dysfunction. Inhibition of inflammasome using apyrase, oATP, or anakinra is protective.

Another aspect of the study identifies the role of transcription factor p45 NF-E2 in human placenta. It has been previously shown that embryonic platelets are dispensable for development. The deficiency of transcription factor p45 NF-E2 in the placenta (but not platelet deficiency) results in impaired placental vascularization and IUGR establishing that the placental defect and IUGR observed in p45 NF-E2-/- embryos is independent of hemostasis [62, 65]. This conclusion

has been supported by several evidences and multiple approaches in human trophoblast cells and *ex vivo* analyses of human placenta tissue. Earlier studies have shown that stimulation of cAMP signaling pathway by Forskolin or 8-Br-cAMP results in Gcm-1 acetylation and desumoylation promoting placental cell fusion [68, 105]. This study identifies a mechanism which may negatively regulate these processes independent of the cAMP agonists in human trophoblast cells (Fig. 4.2). Importantly, our study also shows a pathological relevance of these p45 NF-E2 dependent processes and provides a candidate gene for studying placental pregnancy complications.

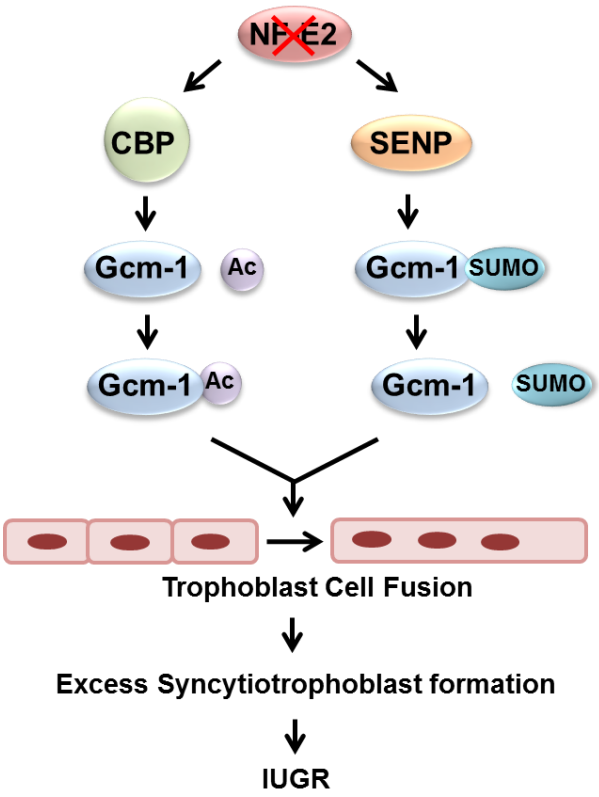


Fig. 4.2: Scheme reflecting the proposed role of p45 NF-E2 in IUGR. Absence of p45 NF-E2 in the placenta results in CBP mediated Gcm-1 acetylation and SENP mediated Gcm-1 desumoylation, both resulting in trophoblast cell fusion and enhanced syncytiotrophoblast formation which is a reason for placental malperfusion resulting in IUGR.

Protein acetylation and sumoylation are two important post-translational modifications regulating protein function. We show that p45 NF-E2 regulates placental fusion by modulating acetylation and sumoylation of Gcm-1. In accordance with this study, we and others have shown that protein

acetylation may play an important role in regulating developmental processes [62, 129]. This highlights the relevance of identifying protein function beyond its expression. Identification of such post-translational modifications of proteins through proteomics approaches could therefore help in identification of disease targets which may be otherwise ignored during expression profiling for target gene identifications. A role of Gcm-1 in development has been certainly established. However, its translational relevance in pregnancy complications has been ambiguous. There are studies showing both downregulation and upregulation of Gcm-1 contribute to placenta-associated pathologies [130-132]. Accordingly, in our study we observed difference at the protein-expression levels only in a subset of our cohort. This further highlights that not just protein expression levels, but rather their post-translational modifications and the functional relevance of these need to be characterized in order to identify new biomarkers and mechanisms of placental dysfunction.

5.0 Future Outlook

While providing a rationale for the placental disease origin, the current results raise a number of questions. The anti-inflammatory effect of ADP-depletion is well established and the placenta expresses several proteins with Ecto-ADPase activity [133]. Why and under what conditions these systems fail in the placenta remains to be uncovered. Furthermore, we observed accumulation of activated platelets within the placental maternal vascular bed (which is lined by trophoblast cells), but not elsewhere in the maternal vascular system (which is lined by endothelial cells). A direct interaction of platelets with trophoblast cells and spiral arteries has been documented [6], but the mechanism mediating accumulation of activated platelets predominately within the placental vascular bed remains unknown.

Heparins are the most common therapy during thromboembolic events of pregnancy, reflecting the good clinical experience in using these anticoagulants during pregnancy and the fact that they do not cross the placenta [119]. While heparins are established for the treatment of thromboembolic events in pregnancy and have been frequently proposed to convey a therapeutic benefit in thrombophilia associated placental dysfunction, the role of anticoagulants such as heparins during PE remains unclear. We show that platelet-activation by MP triggers results in placental sterile inflammation. We hypothesize that the activation of platelets by MP is thrombin-dependent and therefore studying this in detail would help in identification of factors from the coagulation cascade involved in this process and further provide us with relevant anti-coagulants which could provide better efficacy.

Thrombophilia has been linked with pregnancy-associated complications such as PE, but given the lack of occluding placental blood clots and poorly designed and partially contradictory studies its mechanistic and clinical relevance remains unknown [77]. Thrombophilia may boost platelet-activation and / or MP-generation in pregnant women, putting these women at an increased risk of trophoblast specific inflammasome activation and PE. However, additional risk-factors predisposing pregnant women to MP-generation, such as hypertension or diabetes mellitus, or impairing placental ecto-ADPase activity may be required for the manifestation of placental dysfunction. The interaction of thrombophilia with other risk factors and the relation of these with the propose mechanism – generation of procoagulant MP – remains to be studied.

We further describe the role of p45 NF-E2 in syncytiotrophoblast formation and IUGR in humans. We show that p45 NF-E2 negatively regulates post-translational modifications of Gcm-1 thereby preventing IUGR. However, it remains to be evaluated whether p45 NF-E2 is also involved in other hypertensive disorders of pregnancy such as PE. Since the role of Gcm-1 has been shown to be ambiguous in these disorders, it would be interesting to study whether p45 NF-E2 has different functions in these disorders.

We expect that answering these questions will provide novel pathogenetic insights into and potential translational targets for placental dysfunction.

6.0 Conclusion

This study evaluates novel aspects of platelet function in placental dysfunction. Although platelets have been shown to be dispensable for normal development, we highlight the pathological role of maternal platelet-activation in PE. We show that maternal platelets together with microparticles cause sterile inflammation of the placenta which is independent of its primary role in hemostasis identifying a thrombo-inflammatory process at the feto-maternal interface. Furthermore, we demonstrate a role of p45 NF-E2 transcription factor in the human placenta independent of platelets. We show that p45 NF-E2 negatively regulates syncytiotrophoblast formation in the human placenta by modulating acetylation and sumoylation of Gcm-1. This highlights an important role of post-translational modifications in regulating protein function in placental development, which may lay ground for new therapies of placental related pregnancy complications. By studying these mechanisms we identify disease targets and pharmacological therapeutics which could be effective in ameliorating the disease progression.

7.0 References

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8.0 List of publications

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10. Shahzad K, Bock F, Dong W, Wang H, Kopf S, **Kohli S**, Al-Dabet MM, Ranjan S, Wolter J, Wacker C, Biemann R, Stoyanov S, Reymann K, Söderkvist P, Groß O, Schwenger V, Pahernik S, Nawroth PP, Gröne HJ, Madhusudhan T, Isermann B. 2015. Nlrp3-inflammasome activation in non-myeloid-derived cells aggravates diabetic nephropathy. *Kidney Int*. 87: 74–84. **Impact factor: 8.9**
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9.0 Curriculum Vitae

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Nationality: Indian

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Education and degrees	
Dec 2012 – April 2017	PhD – Natural Sciences (Biology), Otto-von-Guericke Univeristy Magdeburg, Germany.
July 2006 - June 2011	Bachelor of Technology & Master of Technology (Dual degree) in Biotechnology from Jaypee Institute of Information Technology, NOIDA, India with a CGPA of 8.9 (equivalent to 89%) on a scale of 10.
June 2005	Completed Senior Secondary School Education (New Delhi, India) with aggregate of 81.8%

Scientific Career	
Dec 2012 – Feb 2017	Doctoral Scientist at Medical Faculty, Otto-von-Guericke Univeristy Magdeburg, Germany – Role of Platelets in placental dysfunction.
Oct 2011 - Sep 2012	MyoGrad Internship at Max Delbruck centre for Molecular Medicine, Berlin, Germany: Worked on Role of Caveolins in Statin induced Myopathy under Project 10 (Regulation and Dysregulation of Skeletal Muscle Growth) of Clinical Research Group 192.
July 2010 - May 2011	**Master Thesis:** Effect of Curcumin on Extra Cellular Matrix

	Proteins in Norepinephrine induced Cardiac Hypertrophy: The effect on Collagens and Matrix metalloproteinases was studied
June 2010 – July 2010	**Research Project:** Effect of Curcumin on Matrix Metalloproteases in Cardiac Hypertrophy
July 2009 – May 2010	**Bachelor Thesis:** Reversal of Norepinephrine induced Cardiac Hypertrophy by curcumin- Cardioprotective properties of curcumin were studied on H9c2 Cell line under norepinephrine induced hypetrophic conditions.

Achievements

- 2017 - Health Campus Immunology, Infectiology and Inflammation (GC-I^3) Paper of the year.
- 2017 - Young Scientist Award, "Nachwuchsförderpreis" for Thrombosis and Vascular Medicine Research (2017) by the Society of Thrombosis and Haemostatsis (GTH).
- 2017 - Conference Travel grant, "Kongress- und Vortragsreiseprogramm" by German Academic Exchange Service (DAAD).
- 2016 - Y.W. (Charlie) Loke Award for Early Career Researchers from the International Federation of Placenta Associations (IFPA).
- 2014, 2016 - "Reisestipendium" (Travel Grant) for the German Congress of Laboratory Medicine (DKLM) in Mannheim.
- 2011 - Qualified CSIR/UGC-National Eligibility Test for Junior Research Fellowship/Lectureship in India.
- 2010-2011 - Recepient of Teaching Assistantship (Biochemistry, Molecular Biology and Basic Biosciences) at JIIT.

Selected Oral/Poster Presentations

- **Shrey Kohli,** Juliane Hoffman, Moh'd Mohanad Al-Dabet, Khurrum Shahzad, Anat Aharon, Benjamin Brenner, Berend Isermann. 2017. p45-NF-E22 regulates syncytiotrophoblast formation in human placenta by modulating Gcm-1 post translational

modifications. Women’s Health Issues in Thrombosis and Haemostasis, Barcelona, Spain. (Poster)

- **Shrey Kohli,** Moh'd Mohanad Al-Dabet, Satish Ranjan, Fabian Bock, Khurrum Shahzad, Anat Aharon, Benjamin Brenner, Berend Isermann. 2017. Microparticles cause preeclampsia and embryonic growth restriction by platelet-mediated inflammasome activation in the embryonic trophoblast. Women’s Health Issues in Thrombosis and Haemostasis, Barcelona, Spain. (Poster)
- **Shrey Kohli**, Moh'd Mohanad Al-Dabet, Satish Ranjan, Fabian Bock, Khurrum Shahzad, Berend Isermann. 2016. Microparticles cause preeclampsia and embryonic growth restriction by platelet-mediated inflammasome activation in the embryonic trophoblast. Deutschen Kongress der Laboratoriumsmedizin, Manheim, Germany. (Oral)
- **Shrey Kohli**, Juliane Hoffmann, Moh'd Mohanad Al-Dabet, Satish Ranjan, Khurrum Shahzad, Berend Isermann. 2016. Role of p45-NF-E2 in regulating syncytiotrophoblast formation in human placenta. Deutschen Kongress der Laboratoriumsmedizin, Manheim, Germany. (Poster)
- **Shrey Kohli**, Moh'd Mohanad Al-Dabet, Satish Ranjan, Fabian Bock, Khurrum Shahzad, Berend Isermann. 2016. Microparticles cause preeclampsia and embryonic growth restriction by platelet-mediated inflammasome activation in the embryonic trophoblast. The International Federation of Placenta Associations (IFPA), Portland, USA. (Poster)
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- **Shrey Kohli**, Suchit Ahuja. 2009. Production of a modified and effective Recombinant Erythropoietin. Techkriti '09, Indian Institute of Technology, Kanpur, India. (Poster)
- **Shrey Kohli**. 2009. Insights on RNA Interference. Biofest '09 at School of Biosciences, IMS Ghaziabad, India. (Oral)

Other Activties

- Member of the International Society for Thrombosis and Haemostasis (ISTH).

- Member of the International Federation of Placenta Association (IFPA).
- Member of the American Society of Nephrology (ASN).
- Attended International Proteomics Summer School, Griefswald (2013).
- Pursued a Mouse Course at Max Delbruck centre for Molecular Medicine, Berlin, Germany.
- MyoGrad Berlin Summer School for Basic Muscle Sciences (2012).

Shrey Kohli

10.0 Declaration

Hiermit erkläre ich, dass ich die von mir zur Promotion eingereichte Dissertation mit dem Titel

Role of platelets in placental dysfunction

im Institut für Klinische Chemie und Pathobiochemie

mit Unterstützung durch Prof. Dr. med. Berend Isermann

selbständig verfasst und ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 31.08.2016

Shrey Kohli

11.0 Acknowledgement

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