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PREECLAMPSIA, AUTOIMMUNITY AND THE AT1 RECEPTOR

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PREECLAMPSIA, AUTOIMMUNITY AND THE AT$_1$ RECEPTOR

A

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by

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DEDICATION

I would like to dedicate this thesis to my Grandmother, Professor Homai J. Moos.

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PREECLAMPSIA, AUTOIMMUNITY AND THE AT$_1$ RECEPTOR

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Preeclampsia (PE) is a disease of late pregnancy characterized by maternal hypertension and proteinuria. It is associated with preterm delivery and significant perinatal morbidity and mortality. Despite affecting ~7% of first pregnancies, there is no effective screening method to identify women at risk, nor is there a definitive treatment other than delivery of the baby and placenta. Though the pathogenesis of PE remains unclear, an imbalance in the renin-angiotensin and immune systems are thought to be major contributors. Bridging these two concepts, it has recently been shown that women with PE harbor specific autoantibodies: the angiotensin II type 1 receptor activating autoantibody (AT$_1$-AA). These autoantibodies act as angiotensin II and stimulate the ubiquitous AT$_1$ receptor.

To elucidate the role of AT$_1$-AA in the pathophysiology of PE, a model of adoptive transfer was generated wherein AT$_1$-AA isolated from human sera are injected into pregnant mice. This autoantibody incites the key features of the disease in pregnant mice: increased blood pressure, proteinuria, renal and placental abnormalities and increases in the anti-angiogenic factors soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin (sEng). These

experiments were also carried out in non-pregnant animals who did not share the same symptoms. This suggests that pregnancy is a requirement for the full spectrum of preeclamptic features. In addition, the placentas and fetuses of AT_1-AA-injected mice are reduced in size. The pups demonstrated intrauterine growth restriction (IUGR) and organ immaturity, especially in their kidneys and livers. To elucidate the mechanism by which these preeclamptic symptoms arise in the mouse model, the effects of increased inflammation were investigated. A multi-analyte screen indicated that the autoantibody induced inflammatory cytokines. TNF-alpha, a potent pro-apoptotic cytokine, known to be increased in both the sera and placentas of preeclamptic women, was most elevated, and was therefore the focus of further research. When incubated with AT_1-AA, human placental explants greatly increased their production of TNF-alpha. In addition, TNF-alpha-mediated apoptosis was increased due to AT_1-receptor activation in both the mouse placenta and human villous explants. Autoantibody-induced apoptosis and TNF-alpha production could be specifically reduced by co-treatment of AT_1-AA with an anti-TNF-alpha antibody, losartan (an AT_1 receptor blocker) or an antibody-neutralizing peptide, 7-aa. In order to test the pathophysiologic relationship between AT_1-AA and TNF-alpha *in vivo*, the established adoptive transfer mouse model was employed. Co-injection of AT_1-AA and an anti-TNF-alpha antibody reduced the features of PE in pregnant mice, implying an important pathogenic role for this cytokine.

Overall, when injected into pregnant mice, AT_1-AA induces the clinical features of PE and results in increased TNF-alpha production, placental apoptosis and fetal anomalies. Blockade of these features can be partially diminished by anti-TNF-alpha treatment, an AT_1 receptor blocking drug, or a peptide which specifically neutralizes autoantibody action. Significantly, these findings could lead to a screening tool for preeclampsia as well as a potential therapeutic strategy for this life-threatening disease of mother and child.

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LIST OF ABBREVIATIONS

(alphabetical)

7-aa, seven amino acid epitope peptide

ACE, angiotensin-converting enzyme

ANG II, angiotensin II

AT_1-AA, angiotensin II type I receptor agonistic autoantibody

CD, cluster of differentiation

ELISA, enzyme linked immunosorbent assay

ET_A, endothelin receptor A

HELLP, hemolysis, elevated liver enzymes and low platelets

hIgG, human immunoglobulin G

HIV, human immunodeficiency virus

HPV B19, human parvovirus B19

IgG, immunoglobulin G

IL, interleukin

IUGR, intrauterine growth restriction

NADPH oxidase, nicotinamide adenine dinucleotide phosphate-oxidase oxidase

NFAT, nuclear factor of activated T-cells

NT, normotensive

PAI-1, plasminogen activator inhibitor-1

PE, preeclampsia

PlGF, placental growth factor

RAS, renin-angiotensin system

ROS, reactive oxygen species

RUPP, reduction in uterine perfusion pressure

sEng, soluble endoglin

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

TF, tissue factor

TGF, transforming growth factor

TNF-α, tumor necrosis factor-alpha

VEGF, vascular endothelial growth factor

CHAPTER 1:

GENERAL INTRODUCTION

GENERAL INTRODUCTION

The classic circulating RAS pathway

Classically described in the kidney, the renin-angiotensin system, herein RAS, is a hormone signaling cascade which regulates blood pressure and systemic electrolyte and fluid balance. In response to decreased blood pressure and low circulating sodium chloride, angiotensinogen, an alpha-2-globulin protein produced constitutively by the liver, is cleaved by the enzyme renin, which is synthesized and released by juxtaglomerular cells of the afferent renal arterioles (Fig. 1). Renin is rapidly produced and released by the macula densa [1]. The cleavage of the 452 amino acid angiotensinogen by renin yields the ten amino acid long peptide, angiotensin I (ANG I), and is the rate limiting step of the cascade. The biologically inactive ANG I is then cleaved by angiotensin-converting enzyme (ACE), made primarily in lung endothelium, to the biologically functional angiotensin II (ANG II), the eight amino acid long effector molecule of the RAS.

ANG II exerts its effects through two major angiotensin receptors: AT_1 and AT_2. These highly conserved seven transmembrane G-protein-coupled receptors share a thirty-four percent sequence identity and have comparable affinities for ANG II [2]. The AT_1 receptor is the predominant angiotensin receptor and is responsible for the majority of ANG II signaling. Its expression is fairly ubiquitous, and it is found abundantly in the adult kidney and on the surface of many cell types including vascular smooth muscle cells, adrenal glands and syncytiotrophoblasts [3-5]. It is coupled to a G_q protein, whose stimulation results in increased intracellular calcium resulting in vasoconstriction, increased sympathetic activity and sodium and water retention. The minor angiotensin receptor, AT_2, is not highly expressed in the adult

but predominates during fetal development, with its expression decreasing throughout the neonatal period [6]. AT_1 is more abundant than AT_2 in the adult kidney [2]. Stimulation of the AT_2 receptor inhibits cell growth, increases apoptosis, causes vasodilation and regulates fetal tissue development [7].
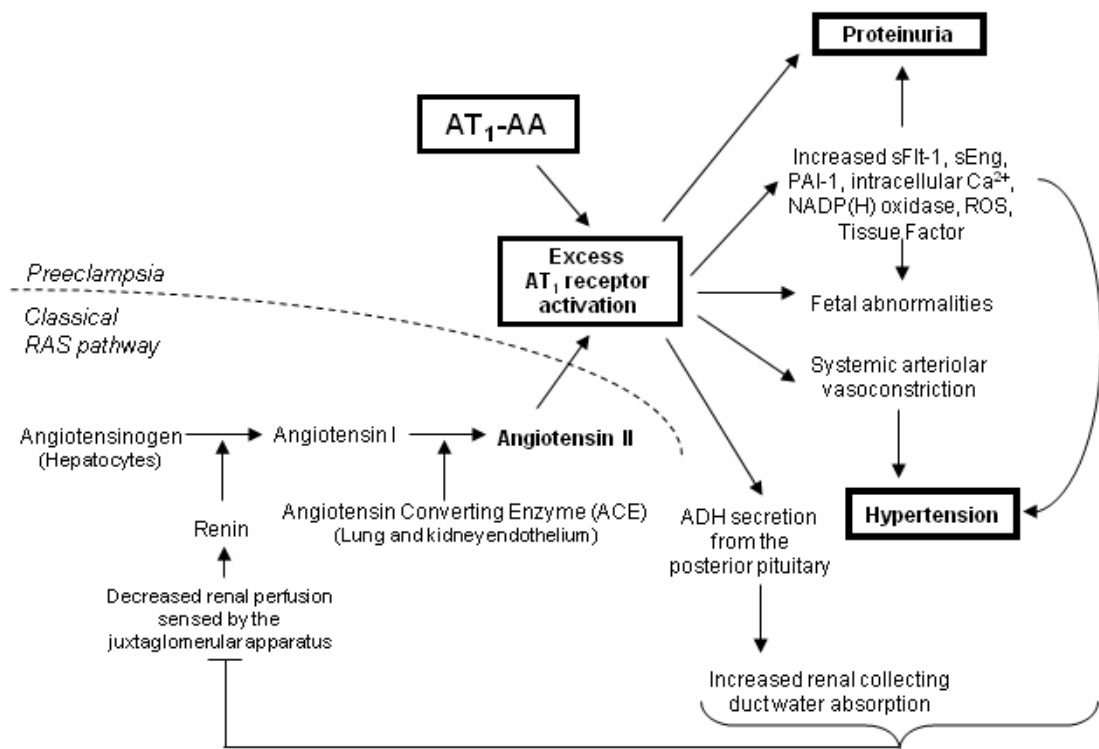
Figure 1. The renin-angiotensin system (RAS) cascade and PE. Though its end-effects are increased, ANG II, the key effector molecule of the RAS, is not upregulated in PE. The autoantibody, AT_1-AA, through AT_1 receptor activation, may lead to the maternal features, such as vasoconstriction and increased blood pressure, observed in the disease. ADH, antidiuretic hormone; PAI-1, plasminogen activator inhibitor-1; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase.

In addition to the classic circulating RAS, there is extensive evidence indicating that local renin-angiotensin systems are present in many organs, such as the heart, ovary, and placenta [8, 9]. Though these local systems may contribute to RAS functions, they are not the focus of this thesis, which will concentrate on the overall systemic effects of the RAS during pregnancy.

Uncomplicated pregnancies require strict regulation of the RAS

During an uncomplicated pregnancy, the RAS undergoes specific changes. The upregulation of renin is the first change to occur, mainly due to the extra-renal release locally by the ovaries and maternal deciduas [10]. As it grows, the placenta produces estrogen, a steroid hormone vital to sustain pregnancy. Estrogen also increases angiotensinogen synthesis by the liver, leading to increased serum ANG II [11]. The only RAS component that is reported to decrease during normal pregnancy is ACE [12-14]. Table 1 compares serum RAS component levels between non-pregnant women and pregnant women with no complications.

Many physiologic changes occur in the cardiac and renal systems during gestation to meet the expanding needs of blood supply and nutrients. Interestingly, during normal pregnancy, maternal blood pressure is often slightly decreased in the second trimester and returns to baseline by delivery [15]. This phenomenon is puzzling, as ANG II levels are elevated throughout gestation [16]. The historic study by Assali *et al.* revealed that healthy pregnant women are refractory to the vasopressor effects of ANG II [17]. In fact, pregnant women require twice as much ANG II by intravenous infusion as compared to their non-pregnant counterparts in order to achieve similar vasomotor responses [17, 18]. Some believe that this decreased ANG II sensitivity is explained by the presence of increased progesterone and prostacyclins during pregnancy which act a vasodilators [19]. In addition, AT_1 receptors are in a heterodimeric state in ANG II sensitive conditions, whereas during an uncomplicated pregnancy, they are monomeric and can be inactivated by reactive oxygen species (ROS) [20]. Taken together, these studies explain why a normotensive pregnant woman may be insensitive to ANG II stimulation.

A healthy placenta is a dynamic organ which undergoes many changes throughout gestation that are essential to maintain a normal pregnancy. Placental trophoblasts are AT_1 receptor-rich, making them responsive to changes in the RAS [4]. Several recent studies demonstrate that AT_1 receptor signaling regulates several genes responsible for normal trophoblast invasion (e.g., plasminogen activator inhibitor-1, PAI-1) [21-23] and angiogenesis (soluble fms-like tyrosine receptor-1, sFlt-1; soluble endoglin, sEng) [24-26]. In addition, AT_1 receptor stimulation also results in NF-kappa B (NFκB) and NADPH-oxidase synthesis by trophoblasts [27]. These RAS-related changes in the placenta are necessary in maintaining an uncomplicated pregnancy. Through the evidence provided in human studies, it is clear that the systemic and placenta-specific RAS undergo specific and necessary changes in order to sustain a healthy pregnancy.

Dysregulation of the RAS in preeclamptic women

Preeclampsia (PE) is a disorder of pregnancy characterized by new-onset maternal hypertension and proteinuria. This life-threatening condition affects approximately 7% of pregnancies and results in substantial maternal and neonatal morbidity and mortality [28]. In its severe form, the clinical symptoms of PE may include cerebral edema, renal failure and the HELLP (Hemolysis, Elevated Liver enzymes and Low Platelets) syndrome. It can be fatal and is the cause for approximately 18% of all pregnancy-related maternal deaths in the US each year [29-31]. This disturbing percentage of maternal mortality is due to the fact that treatment for PE is hampered by a paucity of screening or diagnostic tests. For centuries, the only cure for

Serum RAS Component	Normotensive Pregnancy	Preeclamptic Pregnancy	References
Renin	++	+	Hsueh [10] , Langer [16]
Angiotensin I	++	+	Merrill [12], Langer [16]
ACE	-	-	Merrill [12], Oats [13, 14], Langer [16]
Aldosterone	++	+	Brown [32], Langer [16]
ANG-(1-7)	++	-	Merrill [12]
Angiotensin II	++	+	Langer [16]
Angiotensin II sensitivity	Refractory	Sensitive	Gant [19], Abdul-Karim [18]
AT_1-AA presence	< 30%	> 90%	Wallukat [33], Siddiqui [34]
AT_1-AA bioactivity	Low	High	Siddiqui [34]
AT_1 receptor	+, homodimer	++, heterodimer	Herse [35], AbdAlla [20]
Molecules under partial AT_1r regulation			
sFlt-1	++	+++	Maynard [36], Levine [37], Zhou [25, 38]
sEng	++	+++	Venkatesha [39], Zhou [26, 38]
PAI-1	+	++	Estelles [40], Shaarawy [41], Bobst [21]
Tissue Factor	+	++	Estelles [40], Dechend [42]
NADPH oxidase, ROS	+	++	Hubel [43], Dechend [27]

Legend: **++**   Greatly increased over non-pregnant
        +   Slightly increased over non-pregnant
        -   Decreased compared to non-pregnant

Table 1: Comparison of circulating molecules in normotensive and preeclamptic pregnancies versus non-pregnant women. In general, circulating RAS molecules are increased in normal pregnancies versus the non-pregnant state. Though in most serum RAS components, there is a slight increase in preeclamptic women over the non-pregnant state, they are decreased as compared to normotensive pregnant women. Many of the other molecules known to be increased in PE are regulated by the AT_1 receptor. PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine kinase.

the disorder is delivery of the infant and placenta. The underlying pathogenic mechanisms of PE remain largely undetermined, however uteroplacental ischemia and the subsequent release of soluble factors, such as sFlt-1, from the placenta into the maternal circulation are thought to contribute to the systemic syndrome [44].

The regulation of the RAS in PE differs from that in healthy pregnancies. It is generally accepted that circulating RAS components increase in an uncomplicated pregnancy. Though elevated over the non-pregnant condition, preeclamptic women have *lower* circulating levels of RAS components than do their normotensive pregnant counterparts [16, 32, 45] (Table 1). Two exceptions to these decreases should be noted. First, ACE is reportedly lower in pregnant woman as compared to non-pregnant women, and Merrill *et al.* demonstrated that ACE levels are approximately equal in normotensive and preeclamptic women [12, 13]. Secondly, ANG-(1-7), a vasodilator produced by several tissues such as kidney, heart, hypothalamus and ovary, is significantly decreased in PE [12]. Its exact role in the RAS and the regulation of a healthy pregnancy remains undefined. Though it may act through its own receptor, ANG-(1-7) interacts primarily with AT_1 and AT_2 receptors [46, 47]. Furthermore, women experiencing an uncomplicated pregnancy demonstrate a relative vascular insensitivity to ANG II. Preeclamptic women, however, exhibit increased ANG II sensitivity in their adrenal cortex and vascular system [19, 48]. This can be explained by the heterodimerization of the AT_1 receptor in PE, whereas in healthy pregnancies, the receptors are monomeric and can be inactivated by ROS leading resulting in ANG II insensitivity [20]. In PE, the AT_1 receptor forms a heterodimer with the bradykinin receptor (B2) [20, 49] and the ROS-inactivation resistant AT_1/B2 heterodimers are hyperresponsive to ANG II [20, 50, 51]. Future investigation into the heterodimeric receptors as the symptoms of PE subside postpartum is necessary. Overall, these

findings indicate that the profile of RAS components in a preeclamptic woman differs greatly from that of a healthy pregnant woman.

A source of excess AT_1 receptor activation: the Angiotensin II type I receptor Agonistic Autoantibody (AT_1-AA)

Though the dysregulation of the RAS in PE is largely accepted, the causative factors leading to this imbalance remain unidentified. Though ANG II levels are reportedly decreased in preeclamptic women as compared to normotensive pregnant women (Table 1) [19, 48], these patients exhibit symptoms which could be attributed to excess AT_1 receptor activation, such as hypertension and renal dysfunction. The exact cause of this excess activation is unknown. One explanation to this puzzling feature is the recent discovery by Wallukat *et al.* that preeclamptic women harbor an autoantibody which stimulates the AT_1 receptor [33]. Through excess AT_1 receptor activation, the angiotensin II type I receptor agonistic autoantibody (AT_1-AA) could interfere with the normal function of the RAS in the pregnant woman and lead to preeclamptic symptoms. If true, this would suggest an important role of AT_1-AA in the pathogenesis of PE. Many recent studies have shown that by activating AT_1 receptors on a variety of cell types, these autoantibodies could increase certain factors which lead to preeclamptic pathophsyiology such as endothelial cell dysfunction and vascular damage [44, 52, 53]. Examples of the possible contributions of AT_1-AA in the pathogenesis of PE are reviewed in the subsequent section and summarized in Figure 2.
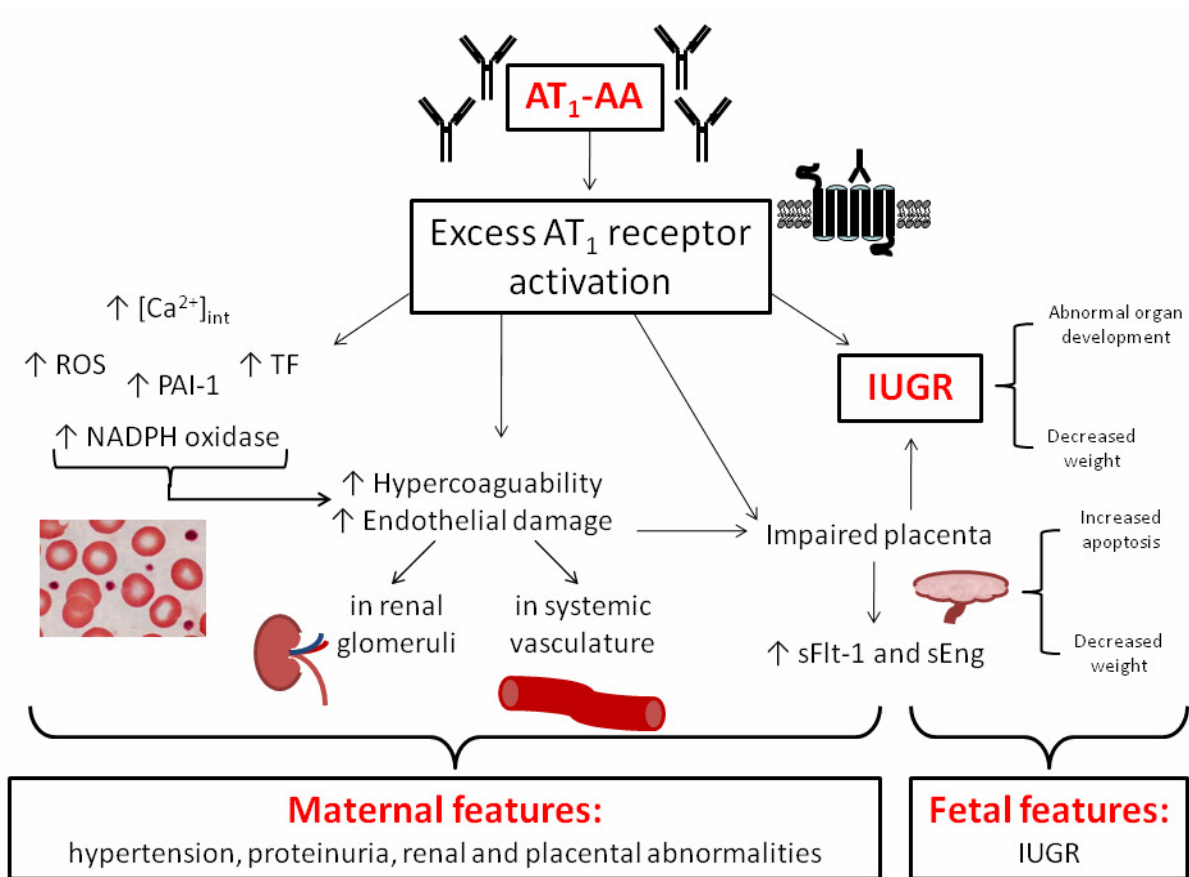
Figure 2. Possible roles of the autoantibody in the maternal and fetal features of PE. The autoantibody, AT_1-AA, through excess AT_1 receptor activation, may contribute to the maternal and fetal features observed in PE. AT_1–AA, angiotensin-II type I receptor agonistic autoantibody; IUGR, intrauterine growth restriction; PAI-1, plasminogen activator inhibitor-1; sEng, soluble endoglin; sFlt-1, soluble fms-like tyrosine; TF, tissue factor.

In vitro studies linking AT_1-AA to the maternal syndrome of PE

A growing body of evidence indicates that the autoantibody, through the upregulation of AT_1 receptor signaling, may contribute to the maternal symptoms of PE in a variety of ways. Wallukat *et al.* have shown that AT_1-AA can stimulate rat cardiomyocyte contraction rate [33]. In addition, several *in vitro* studies suggest that AT_1-AA may regulate the following molecules: sFlt-1, sEng, PAI-1, ROS, NADPH oxidase, intracellular calcium and tissue factor (TF).

a) Excess sFlt-1 secretion and impaired angiogenesis is induced by AT₁-AA.

Soluble fms-like tyrosine kinase-1 (sFlt-1) has recently been brought to the forefront of factors contributing to preeclamptic features [37, 54]. sFlt-1 is a splice variant of VEGFR-1 and is a secreted soluble form of the receptor which lacks the transmembrane and cytoplasmic domains. It binds to free vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) thereby inhibiting their angiogenic actions [55-57]. This soluble factor may contribute to the maternal symptoms of PE by impairing angiogenesis, leading to placental and renal dysfunction.

The soluble factor sFlt-1 is increased in both the circulaton and the placentas of preeclamptic women [36, 52, 58-60]. Circulating sFlt-1 is elevated in preeclamptic women as compared to normotensive pregnant women [37, 61], and is thought to be liberated primarily by the placenta. In the placenta itself, Roberts *et al.* report that sFlt-1 secretion is increased two- to five-fold in preeclamptic women over placentas from normotensive pregnancies [36, 58, 59]. The ratio of sFlt-1, VEGF and PlGF are important in the angiogenic balance of the placenta. Nagamatsu *et al.* have shown that placental hypoxia specifically induces sFlt-1, and not VEGF [62]. Khaliq *et al.* state that hypoxic BeWo choriocarcinoma cells have decreased PlGF expression [63]. Another study reports a diametric expression of VEGF and PlGF during normal gestation, and others demonstrate a cytotrophoblastic increase in VEGF and decrease in PlGF in PE [64, 65]. Total VEGF expression may increase during normal pregnancy, but when high sFlt-1 levels are apparent, the free VEGF decreases, which results in diminished angiogenic capability [66]. Therefore, excess placental sFlt-1 production leads to decreased free VEGF on a background of low PlGF. This results in an anti-angiogenic state overall in the placenta which could contribute to the small, hypoxic organs described in preeclamptic women.

The autoantibody, AT_1-AA, could contribute to the excess sFlt-1 in PE. The placenta produces sFlt-1 through AT_1 receptor stimulation of trophoblast cells via the calcineurin-NFAT pathway even during a normal gestation [25]. Therefore through the additional stimulation of the AT_1 receptor by the autoantibody, excessive sFlt-1 production may occur. Zhou *et al.* report that sFlt-1 secretion can be induced by autoantibodies derived from preeclamptic patient sera in human placental explants and a human trophoblast cell line [24, 25]. This suggests that AT_1-AA can additively contribute to the excess sFlt-1 secretion reported in preeclamptic patients. Excess sFlt-1 may not only cause placental damage, but may also lead to kidney abnormalities. Maynard *et al.* report that pregnant rats infused with sFlt-1 induces a preeclamptic-like state. These rats demonstrate increased blood pressure, proteinuria and renal histopathologic changes similar to those observed humans, such as glomerular endotheliosis [36]. Thus, through its induction of sFlt-1, AT_1-AA may contribute to the kidney dysfunction observed in PE. Taken together, these findings suggest that the autoantibody, through AT_1 receptor activation, can additively contribute to the excess sFlt-1 secretion reported in preeclamptic patients.

b) Soluble endoglin is induced by AT_1-AA

Endoglin (Eng, or CD-105) is a cell-surface co-receptor for transforming growth factor (TGF)-β1 and TGF-β3 which is highly expressed in endothelial cells and syncytiotrophoblasts [67, 68]. Soluble endoglin (sEng), a soluble form of the TGF-β receptor, is the second major anti-angiogenic factor recently implicated in the maternal syndrome of PE [69]. The balance of this TGF-β antagonist becomes undone in PE as sEng is secreted in excess by the placentas of preeclamptic women [61, 70, 71]. By impairing the ability of TGF-β1 to bind its receptors, downstream signaling, including the activation of eNOS and vasodilation, do not occur [39, 72]. In this way, sEng contributes to the disease features by dysregulating TGF-β signaling

pathways in the vasculature. Venkatesha *et al.* have definitively shown that sEng works additively with sFlt-1 to contribute to severe preeclamptic features in pregnant rats [39]. Moreover, Zhou *et al.* have recently shown that sEng can be induced by AT_1-AA in human villous explants [73]. Since this molecule has been proven to contribute to disease features in other experimental models, and some through AT_1 receptor activation, the autoantibody may induce this factor *in vivo* and lead to the symptoms of PE.

c) AT_1-AA induces excess PAI-1 secretion

Plasminogen activator inhibitor-1 (PAI-1) is another factor related to the pathogenesis of PE [40, 74]. PAI-1 is a serine protease which inhibits urokinase-like plasminogen activator (uPA) and decreases the conversion of plasminogen to plasmin resulting in decreased fibrinolysis and increased fibrosis. It also indirectly inhibits extracellular matrix breakdown via matrix metalloproteinases [75]. In the placenta, by decreasing fibrinolysis and extracellular matrix digestion, PAI-1 could lead to shallow trophoblast invasion, a hallmark of preeclamptic placentas. Studies have shown that by activating trophoblastic AT_1 receptors, AT_1-AA elevates PAI-1 levels [21, 76] and decreases trophoblast invasion *in vitro* [21, 22]. In the kidney, ANG II partially controls mesangial cell PAI-1 production [77, 78]. A series of *in vitro* experiments by Bobst *et al.* revealed that AT_1-AA, through AT_1 receptor activation on cultured human mesangial cells, increase PAI-1 secretion [21]. The accumulation of PAI-1 could result in decreased extracellular matrix degradation and subendothelial and subepithelial fibrin deposits, thereby contributing to the kidney damage observed in PE [79, 80]. Excess glomerular fibrin deposition decreases the kidney's filtration capability which contributes to proteinuria [81, 82]. Therefore, by overstimulating the RAS, AT_1-AA increase PAI-1 in both the placenta and kidney and lead to decreased fibrinolysis and extracellular matrix breakdown which could contribute to the organ damage and symptoms associated with the disorder.

d) ROS production is increased by AT_1-AA

Reactive oxygen species (ROS) are a physiologic by-product of aerobic respiration and regulate cellular functions through redox reactions [83]. When ROS aberrantly accumulate, the cell becomes burdened by an overload of non-specific damage to DNA, proteins and lipids. During pregnancy, this process of oxidative stress could directly lead directly to organ damage. In addition, ROS can act as teratogens and could harm the developing fetus, especially during organogenesis [84, 85]. In PE, the generation of ROS is increased and may contribute to end-organ damage [43]. Excess AT_1 receptor activation leads to increased NADPH oxidase thereby inducing intracellular ROS. Dechend *et al.* confirmed that NADPH oxidases are elevated in preeclamptic placentas and that the autoantibody increases ROS through this mechanism in vascular smooth muscle cells and placental trophoblasts [27]. In addition, this group demonstrated that AT_1-AA upregulated NFκB and confirmed elevated ROS production both in and around placental blood vessels. Therefore, the autoantibody, by activating NADPH oxidase, could lead to the increased ROS production observed in PE.

e) Intracellular calcium release is induced by AT_1-AA

Increased intracellular calcium levels are reported in the erythrocytes, lymphocytes and platelets of preeclamptic women [86-88]. Basal free intracellular calcium was shown by Haller *et al.* to be elevated in the platelets of PE women. The cation was also elevated in the both the lymphocytes and erythrocytes of these patients in comparison to normotensive pregnant women [87, 89]. Thway *et al.* investigated the possible role of AT_1-AA in the elevation of free intracellular Ca^{2+} [90]. This group found that IgG isolated from preeclamptic patients was capable of activating AT_1 receptors and consequently increasing intracellular calcium, whereas IgG derived from normotensive pregnant women could not. The increased intracellular Ca^{2+}

resulted in the activation of the NFAT transcription factor [90]. These studies suggest that the autoantibody may contribute to increased calcium in the cell, and could regulate the downstream signaling pathways activated in PE.

f) AT$_1$-AA may contribute to hypercoaguability

Tissue factor (TF), the initiating protein of the extrinsic path of coagulation, is elevated in PE [40, 74]. The overexpression of this transmembrane protein may induce vascular damage and could result in the state of hypercoaguability, and rarely disseminated intravascular coagulation (DIC) in some severely preeclamptic patients. AT$_1$ receptor activation, via AT$_1$-AA has been shown to increase TF expression in vascular smooth muscle cells [42] and monocytes [91]. In this way, AT$_1$-AA may play a role in the hypercoaguability associated with PE by upregulating TF expression in vascular smooth muscle cells and monocytes.

Overriding hypothesis

Taken together, these series of experiments lead to the overriding hypothesis of this dissertation: the autoantibody, AT$_1$-AA, contributes to the pathogenesis of the PE through excess AT$_1$ receptor activation. In order to prove this theory, in this thesis I will explore how biologically active autoantibodies from preeclamptic women can be purified and upon injection into pregnant mice, incite the maternal, placental and fetal features of this disease. Using both *in vitro* and *in vivo* systems, it will be illustrated how AT$_1$-AA induce several detrimental factors associated with PE, including increased inflammation. It will be shown how blockade of AT$_1$ receptor activation, specific autoantibody neutralization or blockade of TNF-α prevents disease features in adoptively transferred mice, human placental explants in culture and a

cultured human trophoblast cell line. Overall, these findings could have tremendous diagnostic

and therapeutic impact in the management of PE.

CHAPTER 2:

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

Patient consent and collection of samples

The human medical data and materials used in these experiments were acquired from patients admitted to Memorial Hermann Hospital which were identified by the Obstetrics and Gynecology faculty of the University of Texas Medical School at Houston. Based on the criteria defined by the National High Blood Pressure Education Program Working Group Report [92], some pregnant patients were diagnosed with severe PE. Table 2 reports the clinical characteristics of study participants. One of the two inclusion criteria was blood pressure readings of ≥160 mm Hg systolic or ≥110 mm Hg diastolic in a pregnant woman after 20 weeks gestation age (WGA). Qualifying blood pressure measurements must have been taken on two separate occasions, ≥6 hours apart. The second requirement for diagnosis was an absolute level of proteinuria measuring ≥300 mg protein in a urine specimen collected over 24 hours or ≥30 mg/dL (≥1+ reading on dipstick) in a random urine determination in the absence of a urinary tract infection. The preeclamptic women used in this work had no previous history of hypertension. The control patients used were normotensive pregnant women undergoing uncomplicated pregnancies and normal term deliveries or elective caesarean sections. Patients were approached for consent in the pre-partum or early intrapartum period. Blood samples were drawn from all participants before parturition, allowed to clot and then centrifuged at 18,000g at 4ºC for 20 minutes. The sera were collected, aliquoted and then stored at -80ºC. In some cases, cord blood was also collected from participants at the time of delivery and was handled in the same manner as the maternal blood. The research protocol, including the written informed consent form, was approved by the University of Texas Health Science Center at Houston Committee for the Protection of Human Subjects (CPHS), in Houston, Texas, USA.

		NT	PE
	n	49	54
Age (y)		27.7 ± 7.7	30.6 ± 6.2
Race (%)	Black	33	36
	White	19	14
	Hispanic	47	46
	Other	1	4
Gravity		2.1 ± 0.9	1.9 ± 1.1
BMI		31.2 ± 3.4	37.1 ± 6.7
Weeks gestational age		38.1 ± 2.4	31.3 ± 5.0
Systolic BP (mmHg)		121 ± 4	173 ± 16*
Diastolic BP (mmHg)		74 ± 7	103 ± 13*
Proteinuria (mg/24h)		<300	4572 ± 1326*
Mean AT_1-AA bioactivity[†] (fold induction over basal)		0.14 ± 0.04	5.17 ± 1.07*
Median AT_1-AA bioactivity[†] (fold induction over basal)		0.05	1.95*
AT_1-AA bioactivity range[†] (low, high; fold induction over basal)		0, 0.5	0.5, 11.9
Mean serum TNF-α[†] (pg/ml)		16.1 ± 2.9	48.0 ± 2.9*
Median serum TNF-α[†] (pg/ml)		23.6	44.2*
Serum TNF-α range[†] (low, high; pg/ml)		0, 25.9	32.8, 76.8

Table 2: Patient clinical characteristics. This table illustrates that the blood pressure, proteinuria and TNF-α levels are elevated in preeclamptic (PE) women, as compared to the normotensive (NT) pregnant women used as controls. The bioassay indicating AT_1 receptor activation due to the autoantibody (as measured by luciferase activity) is also increased in preeclamptic women. The category mean or median is indicated (± SEM, where applicable). * P<0.01 versus normotensive pregnant women. [†] For these variables: NT, n=16; PE, n=20.

Reagents

RPMI 1640 cell culture medium plus L-glutamine, antibiotics (penicillin-streptomycin 100X and geneticin G418, 50 mg/ml), bovine serum albumin (BSA), fetal bovine serum (FBS) were purchased from Invitrogen Life Technologies. Human angiotensin II (ANG II) was acquired from Sigma. Merck & Co., Inc. generously provided losartan (Cozaar). The seven amino acid peptide (7-aa) which corresponds to the second extracellular loop of the human AT_1 receptor (AFHYESQ) was purchased from Baylor College of Medicine Protein Chemistry Core Laboratory.

Purification of total immunoglobulin G from patient sera

Total IgG fractions were purified from pregnant patient sera as previously described [34, 38]. Briefly, 200µl GammaBind G Sepharose matrix beads (Amersham Biosciences) were loaded into a PolyPrep chromatography column (BioRad). Before applying any sera, the column was washed repeatedly with wash buffer (50mM tris-HCl, 0.02% NaN_3, ph 7.4) until the column flow-through was neutral. Then, 200µl of human sera was applied to the column, and after an incubation of 30 minutes, eluted using 600µl of eluent buffer (100mM glycine-HCl, pH 2.7) in accordance to the manufacturer's recommended protocol. The isolated total IgG was mixed with 40µl of neutralization buffer (1M tris-HCl, pH 9.0) to establish a neutral pH and was stored at -80ºC. Each fraction was then assessed for the presence of the autoantibody using a luciferase reporter assay described below. In the experiments reported here, IgG fractions from individual patients were used separately and were not pooled.

Affinity purification of AT$_1$-AA using total human IgG

Some experiments described here utilize AT$_1$-AA affinity-purified from total human IgG. 7-aa, the epitope peptide corresponding to a segment on the second extracellular loop of the AT$_1$ receptor, can block autoantibody-induced receptor activation [33] and therefore suggests a physical association between these autoantibodies and AT$_1$ receptors. This presumed physical association was the basis of an affinity purification strategy used in obtaining highly enriched preparations of AT$_1$-AA. A GST fusion protein containing a 27-aa peptide (GST-27mer) which encodes the entire second extracellular loop of the AT$_1$ receptor (accession code: NM_009585.2) was generated. The construction, expression and use of the GST-27mer:AT$_1$-receptor fusion protein in the affinity purification of AT$_1$-AA was carried out as previously described [38]. Briefly, BL21 DE3 *E. coli* cells (Stratagene) were transformed with the pGEX-4T-1 GST expression vector (Promega) containing the GST-27mer:AT$_1$-receptor fusion protein. After induction, the cells were collected and the GST-27mer-AT$_1$ receptor fusion protein was isolated using glutathione beads (Amersham Biosciences). The expression of the fusion protein was confirmed using western blot. The isolated GST-27mer:AT$_1$-receptor fusion protein was then linked according to the manufacturer's protocol to agarose beads using the microlink protein coupling kit (Pierce) which were then used in affinity chromatography columns. Total IgG was isolated from patient sera (as described above) and was loaded onto affinity chromatography columns. The total IgG incubated on the columns for 3h at room temperature. The IgG bound to the columns (AT$_1$-AA) was eluted by centrifugation after collection of the flow-through fraction. To confirm the presence of AT$_1$-AA, the eluted and flow-through fractions were tested for their ability to bind to the AT$_1$ receptor. Only the eluted fraction (the affinity-purified AT$_1$-AA) was capable of binding to AT$_1$ receptors transferred onto nitrocellulose membranes, whereas the flow-through fraction

could not bind to the AT_1 receptor and was not detectable by western blot. Furthermore, to confirm that the eluted fraction retained biologic activity, the fractions were tested for their ability to activate luciferase using an established bioactivity assay described below. Only the eluted fraction from total IgG from preeclamptic patients (containing AT_1-AA) could stimulate luciferase activity and the other fractions were unable to do so. This fraction, containing AT_1-AA, was used in subsequent experimentation.

Luciferase bioactivity assay for the presence of AT_1-AA

To assess for the presence and bioactivity of the autoantibody in an IgG fraction, an in-house luciferase bioassay was employed as previously described [34, 38]. Chinese hamster ovary cells which were stably transfected with the rat ANG II receptor type 1A (CHO.AT1A) were generously provided by Dr. Terry S. Elton of The Ohio State University in Columbus, OH. Cells were maintained and cultured in RPMI 1640 medium containing 5% FBS, 1% antibiotics, 8.75 g L-proline and 100 µg/ml gentamycin at $37^{o}C$ and 5% CO_2. Cells were then stably transfected with a nuclear factor of activated T cells (NFAT)-luciferase-hygromycin phosphotransferase construct containing 4X-NFAT binding elements driving the expression of the luciferase reporter gene. Stable transformants were maintained in the media described above enriched with 100 µg/ml hygromycin. $1x10^5$ CHO.AT1A cells stably integrated with copies of the rat AT_1 receptor:4X-NFAT-driven luciferase construct were evenly plated on 24-well plates overnight. The next morning the media was changed to serum-free media and the cells were treated with IgG (1:10 dilution). After 24 hours, the treated cells were lysed using 100µl of passive lysis buffer (Promega) for 30 minutes, shaking in the dark at room temperature. Luciferase activity was measured using a Luminometer (Pharmingen) as relative

light units (RLU) after mixing 20µl of lysate and 100µl of the Dual Luciferase system (Promega). The data is represented as either an increased or decreased ratio of change of RLU from the baseline (background luciferase activity of untreated cells).

Adoptive transfer: introduction of human IgG into mice

The adoptive transfer of AT_1-AA or purified IgG derived from normotensive pregnant women into mice was carried out as previously described [38]. Briefly, C57Bl/6J non-pregnant or pregnant mice (18-22 g; aged <8 weeks, Harlan) were used all mouse experiments. Mice anesthetized with sodium pentobarbital (50 mg/kg i.p.) were injected with 20µg affinity purified IgG or 100µl total IgG via retro-orbital sinus injection. Volumes less than 200µl represent less than 10% of mouse total blood volume and should not alter hemodynamics. If the experiment required injection into pregnant mice, this was done so on embryonic day (E) 13 and then again on E14. E13 was selected as this developmental stage in mouse pregnancy is comparable to the timeframe at which preeclamptic symptoms may occur in humans, and E13 is the earliest point at which mouse pregnancy is reliably confirmed. Also, when autoantibody injections were performed on E8-10, high rates of embryonic resorption were encountered. Some neutralization experiments required the simultaneous co-injection of the autoantibody with either losartan (8 mg/kg i.v.), or 7-aa, the epitope peptide, (50 mg/kg i.v.). Some dams were co-injected with purified human IgG and a goat polyclonal antibody raised against TNF-α (Abcam). They received 0.6µg/g body weight intraperitoneal shots of the anti-TNF-α antibody daily. This dosage was adapted from experiments previously described [93-96]. As controls, one group of mice was injected with the anti-TNF-α antibody (Abcam, 0.6µg/g body weight i.p. daily) alone, but with no accompanying purified human IgG and another was co-injected

with a goat IgG isotype (Abcam, 0.6μg/g body weight i.p. daily) and PE-IgG as described above in this section.

Blood pressure and proteinuria measurement in mice and fetal organ collection

The systolic blood pressure of all mice was measured at the same time daily (+/- 1h) by an automated carotid catheter-calibrated tail-cuff system (Kent Scientific). The mice were not anesthesized and once placed in the restrainer, were given a period of about fifteen minutes to acclimatize. Using a warming pad, their temperatures were monitored and controlled to be stable between 30-35°C (Kent Scientific). Twenty blood pressure cycles were measured daily using the automated system and then averaged. Proteinuria was determined by the ratio of urinary albumin to creatinine. Urine from each mouse was collected for analysis using metabolic cages (Nalgene) for 24h prior to sacrifice. Urinary albumin was quantified using an ELISA and creatinine was assessed using a picric acid colormetric kit (Exocell). If pregnant, the dams were sacrificed on E18, or if non-pregnant, they were sacrificed five days post the initial injection. At the time of sacrifice, the sera and organs of the female mice were collected. If pregnant, the placentas and fetal mouse organs including blood were also collected. Fetal blood was pooled from the all littermates, as the extractable blood volume is minimal. Pups born in litters of six to eight were analyzed in the fetal mouse experiments. The animal studies were approved of by the Animal Welfare Committee of the University of Texas Health Science Center at Houston, in Houston, Texas, USA.

ELISAs

For the screening of inflammatory markers, mouse serum was diluted 1:25 for use in the qualitative Multi-Analyte ELISArray Kit (SABiosciences). Human and mouse serum TNF-α, sFlt-1 and sEng were quantified using species-specific ELISA kits which are commercially available (R&D Systems). These ELISA procedures were carried out according to the manufacturers' protocol and the optical density was determined at 450 nm and corrected at 570 nm. All assays were performed in duplicate at minimum and the protein concentrations were derived from a standard curve generated from known amounts of the recombinant protein. Human IgG concentration was determined in maternal and fetal mouse circulation, by diluting sera 300-fold and quantified by a commercial ELISA kit (Pierce Biotechnology Inc.). For the standard curve experiment, either 0.0 (control), 0.5 or 5.0 µg/ml of anti-TNF-α (Abcam) was added to known concentrations of recombinant mouse TNF-α and the mixtures were assessed by ELISA for its ability to detect either bound or free cytokine (R&D Systems).

Protein extraction and quantification

Proteins for analysis by western blot were collected from cultured cells and from frozen tissues in the following manner. Cells were grown to ~80% confluence in 24-well plates (see below), media was removed and then cells were washed once with cold PBS. While the plate was on ice, 200µl of lysis buffer (Millipore) was added to the each well. After 2 minutes, the cells were scraped off the base of the wells using a plastic well scraper. The resultant mixture of cell lysate, fragments and buffer were collected into eppendorf tubes and placed on ice for 30 minutes. For frozen tissues, 500µl of lysis buffer (Millipore) and 3µl of a protease inhibitor cocktail (Sigma) was added to the tissue tubes upon removal from the liquid nitrogen storage

tank. While on ice, the tissues were sonicated for ~20 seconds at 20% amplitude. Once a homogenous mixture was obtained from either cell or tissue lysates, it was centrifuged for at least 15 minutes at 4^oC at 15,000 rpm. The pellet was discarded and the supernatant containing the protein was transferred to a new tube and stored at -80^oC. Before use, the protein concentration was checked by mixing 10μl of sample with 200μl of a protein assay reading solution (Bio-Rad) in duplicate in a 96-well plate. The mixture was allowed to sit at room temperature for 5 minutes and then the absorbance was read at 595 nm using a spectrophotometer. Protein concentrations were calculated using BSA standards of 0.1, 0.2, 0.4 and 0.5 mg/ml and generating a standard curve.

Western blot analysis

Western blot analysis was used to determine the presence of AT_1-AA in human sera as previously described [38]. Briefly, protein extracted from CHO.AT1A cells (CHO-NFAT cells which were stably transfected with the rat AT_1 receptor gene) was run on a 10% SDS-PAGE gel (30μg/well) and then transferred onto a nitrocellulose membrane. After the transfer, the membrane was cut to strips, blocked with 5% non-fat milk and then probed with purified human total IgG (1:10 dilution) and mouse anti-human IgG:horse peroxidase (HRP) (1:5000 dilution; Jackson ImmunoResearch Laboratories, Inc.), to determine if the isolated IgG had the capability of binding the AT_1 receptors transferred onto the nitrocellulose strips. As a positive control, one strip was probed with an anti-AT_1 receptor antibody (1:1000 dilution; Santa Cruz Biotechnology) and goat anti-rabbit IgG:HRP (1:5000 dilution; Jackson ImmunoReseach Laboratories, Inc.). Fetal mouse sera were also analyzed for circulating human IgG in a similar manner. Fetal mouse serum (12μl) was run on a 10% SDS-PAGE gel and transferred to a

nitrocellulose membrane which was then probed with a mouse anti-human IgG:HRP antibody, as above. Apoptotic markers were assessed by western blot analysis in mouse and human placentas. Rabbit anti-human or anti-mouse primary antibodies against Bax, a pro-apoptotic protein (23 kDa), and Bcl-2, an anti-apoptotic protein (26 kDa), were both used in a 1:200 dilution (Santa Cruz Biotechnology). β-actin, a housekeeping gene (43 kDa), was run as a loading control (Santa Cruz Biotechnology). All western blot signals were detected by ECL kit (Amersham Biosciences). The relative densiometry was assessed by the Storm 840 Phosphorimager and the associated ImageQuant TL analysis software (GE Healthcare).

Human placental explant culture

Placentas were obtained from normotensive pregnant women who experienced an uncomplicated pregnancy and underwent either an elective term cesarean section, or a normal term birth at Memorial Hermann Hospital in Houston, Texas. The culture system described here was adapted from Ahmad, *et al*. [97]. Upon delivery, the placentas were placed in a sterile container on ice and transferred to the laboratory where they were immediately submerged in and flushed with phenol red-free DMEM containing 0.2% BSA and 1% antibiotics. Villous explant fragments (~5-10mm^3) were dissected and transferred into 24-well culture plates for overnight equilibration at 37˚C and 5% carbon dioxide. All of the manipulations on the first day were performed within thirty minutes of delivery. The next morning, the dissected explants were incubated with either saline, ANG II (100nM) or IgG purified from preeclamptic or normotensive pregnant women (1:10 dilution). Some explants were co-incubated with human IgG and either losartan (5μM) or 7-aa (1μM). After 24h, the culture media was siphoned from the wells and stored at -80˚C. At this time, the placental explants were either lysed and their

protein was collected for western blot analysis or fixed for further histologic or immunohistochemical analysis.

Human trophoblast cell culture

An immortalized human trophoblast cell line, HTR-8/SVneo cells [98], were grown to 70-80% confluence using RPMI 1640 cell culture media with antibiotics, counted and then plated in Lab-Tek 8-well chamber slides (Nunc Inc) at $2\text{x}10^4$ cells per well overnight. The following day, serum-free media was added and cells were incubated with either ANG II (100nM) or IgG derived from either normotensive pregnant or preeclamptic women (1:10 dilution) and cultured for an additional 24 hours. Some experiments required the cells to be cultured with human IgG as well as either losartan (5μM) or the 7-aa, the seven amino acid epitope peptide (1μM). After the overnight incubation, cells were lysed and their lysates were collected for analysis of Caspase 3 activity or permanently fixed to the slide for TUNEL or routine immunohistochemistry staining.

Histologic analysis

The kidneys, livers and placentas of sacrificed pregnant mice, and the kidneys and livers of their pups were harvested, fixed and processed as previously described [38, 99]. Briefly, the fresh tissues were fixed in either a 4% formaldehyde solution (Fisher Scientific) or a zinc solution fixative solution (BD Biosciences) for 36-48h at room temperature. The fixed samples were then washed twice with PBS for 30 minutes, dehydrated, infiltrated and embedded in paraffin using standard techniques. Four micron serial sections were cut from

paraffin blocks and stained with Hematoxylin and Eosin (H&E), Periodic acid-Schiff (PAS) for glycogen, or Masson's Trichrome for collagen, all by standard techniques, or left unstained for further analysis.

The number of glomeruli or megakaryocytes in fetal mouse kidneys or liver, respectively, was assessed by blindly counting the number of glomeruli or megakaryocytes in 10 random high power microscopic fields per section and then averaging this number for the respective pup. For immunohistochemical detection of complement cascade factor C3 in both mouse kidneys and placentas, a primary rat anti-mouse C3 monoclonal antibody was used in a 1:50 dilution (Lifespan Biosciences) after antigen retrieval, using the BD Retriveagen A solution (BD Biosciences). The anti-C3 monoclonal antibody detects C3, C3b, C3d and iC3b. An anti-rat IgG horseradish peroxidase kit was used with DAB detection (BD Biosciences). CD-31 staining in mouse tissue was achieved using a rat anti-mouse CD-31 antibody (BD Pharmingen). Dissected tissues were incubated with a zinc fixative (BD Pharmingen) overnight at room temperature and then subsequently sectioned and prepared on slides. A 1:50 dilution of the primary antibody was used and an anti-rat IgG horseradish peroxidase kit was employed for detection (BD Pharmingen). The counterstain used was methyl green by standard techniques. Immunofluorescence for the presence of human IgG was assessed using rabbit anti-human IgG:FITC (1:30; Dako), visualized as green when excited at 515-565 nm. The sections were counterstained with five drops of a nuclear stain, 4',6-diamidino-2-phenylindole (DAPI) which is visualized as blue when excited at 360 nm (Abbott Molecular). Quantification of the immunohistochemical staining was achieved using the Image-Pro Plus 6.3 software (Media Cybernetics). The density of brown stain (positive CD-31) was measured. The average densities of 10 areas per placenta were averaged and the SEM was calculated. Four placentas were selected from each mouse and eight mice were used for each variable.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Four micron sections were cut from paraffin-embedded HTR-8/SVneo cells, mouse or human placental tissue collected and treated as described above. Using standard techniques, the sections were permanently fixed onto a glass slide, deparaffinized and re-hydrated through an alcohol gradient. To visualize apoptosis, tissues were permeabilized using cold, fresh 0.1% Triton X-100 in 0.1% sodium citrate and stained using a commercial kit by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to the recommended manufacturer's protocol (Roche Diagnostics). TUNEL-positive cells were identified by green staining under 515-565 nm fluorescent light and confirmed using cellular morphology (cell shrinkage, nuclear fragmentation, membrane blebbing). Negative controls were performed in parallel, where the terminal deoxynucleotidyl transferase enzyme was omitted from the protocol. Upon completion of TUNEL staining, 4',6-diamidino-2-phenylindole (DAPI) nuclear stain was added to identify cells with normal nuclear morphology. DAPI is visualized as blue when under 360 nm fluorescent light (Vector Laboratories). An apoptotic index was generated by the quantifying the number of apoptotic nuclei (TUNEL-positive cells) per total nuclei (DAPI-positive) x 100. These numbers were counted blindly for each sample in 10 random microscopic fields using Image Pro Plus 6.3 software (Media Cybernetics).

Transmission electron microscopy (TEM) of mouse kidneys

Upon sacrifice of the mice, their kidneys were immediately removed. Kidney tissue samples were dissected into 1mm^3 cubes and fixed overnight in 3% glutaraldehyde. The fragments were then rinsed, exposed to 1% osmium tetroxide and then dehydrated and embedded in an araldite-epon mixture. Semi-thin (0.6mm) tissue sections were prepared and

stained with uranyl acetate and lead citrate. The prepared mouse renal tissue samples were then examined with a JEOL 1210 transmission electron microscope (JEOL Corporation).

Caspase 3 activity

Caspase 3 activity was measured using a sensitive commercial assay (Millipore). HTR-8/SVneo cells were cultured (as described above), counted and then 0.5×10^6 cells were pelleted and lysed to obtain the cytosolic extract, upon which the Caspase 3 activity assay was performed according to the recommended manufacturer's protocol. The absorbance was measured at a wavelength of 405 nm using a spectrophotometer. The relative absorbance correlates to the Caspase 3 activity level.

Statistical analysis

All results are expressed as mean ± SEM, however, the median may also be reported and was indicated as such where applicable. The data were subjected to statistical analysis using GraphPad Prism 4 software (GraphPad Software). Student's *t* tests (paired or unpaired as appropriate) were applied in two-group analysis. Differences between means of multiple groups were analyzed by one-way analysis of variance (ANOVA) followed by a post-hoc Tukey's multiple comparisons test. To determine a statistical correlation between AT_1-AA bioactivity and serum TNF-α, Spearman's rank correlation was applied and an "r" coefficient value was obtained using the same software. A value of $P<0.05$ was considered significant and the threshold for rejection of the null hypothesis.

CHAPTER 3:

AUTOANTIBODIES PURIFIED FROM PREECLAMPTIC WOMEN

INDUCE DISEASE FEATURES IN PREGNANT MICE

BACKGROUND

Hypertensive diseases of pregnancy and their sequelae have troubled society since their recognition centuries ago. Since then, scientists have sought to understand the mechanisms which bring about maternal symptoms. Derangement of the renin-angiotensin system (RAS) has been implicated in the development of PE in pregnant women. As described earlier, many *in vitro* studies implicate that increased ANG II can provoke the many of the biologic responses pertinent to the disease via excessive AT_1 receptor activation. However, in order to fully understand the cellular interplay of this vascular disease and pathophysiologic consequences of RAS signaling, *in vivo* models must be employed. Animal systems can be used for this purpose, because the RAS of rodents and humans are remarkably similar. The mouse has two pharmacologically identical isotypes of the AT_1 receptor, AT_{1a} and AT_{1b} [100, 101]. Humans have a single AT_1 receptor isotype. In general, both humans and rodents demonstrate an upregulation of RAS components in an uncomplicated pregnancy [102]. There are no reports of the spontaneous development of PE in animals. Nevertheless, through genetic and experimental manipulation, animal models with altered RAS have been developed and proven useful in delineating its role in both normal and abnormal pregnancies. Examples of these models are reviewed here in brief.

In vivo studies of the RAS and hypertensive disorders of pregnancy

Both mouse and rat models have been employed to investigate the changes in the RAS during pregnancy. When transgenic female mice expressing human angiotensinogen were

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mated with male transgenics expressing the human renin gene, Takimoto *et al.* observed transient hypertension in the dams [103]. The hypertension was maximal in late pregnancy and resolved post-partum. These females also demonstrated other preeclamptic features, such as glomerular damage, proteinuria and placental abnormalities. The same group investigated the role of angiotensin receptors in mice during pregnancy. Female AT_{1a} receptor knockout mice expressing the human angiotensinogen gene were mated with male mice expressing the human renin gene. The dams remained at their baseline blood pressure throughout pregnancy, and demonstrated no preeclamptic-like symptoms, despite having intact AT_{1b} receptors [104]. These findings suggest the regulation of the RAS is imperative to a healthy pregnancy, and in the mouse, AT_{1a} receptors are important for the development of hypertension and other preeclamptic features during pregnancy in the setting of a dysregulated RAS. This group also used their transgenic mouse model to determine the timing of renin release in pregnancy [103]. They found that human renin expression increased late in gestation and was detectable both in chorionic trophoblasts and the maternal circulation of the pregnant transgenic mice. Several other mouse models [105-109] draw similar conclusions as they explore the effects AT_1 receptor signaling in the development of hypertension and end-organ damage in the heart, vasculature and kidney. Our group has also investigated the timing of renin gene expression during pregnancy using two different mouse strains, ICR and C57Bl/6. ICR mice exhibited high levels of renin expression at the maternal-fetal interface [102]. In C57Bl/6 pregnant mice, little placental expression of renin was observed, however the gene was upregulated in kidneys. Both ICR and C57Bl/6 mice demonstrated an increase in circulating maternal renin during gestation, however the sites of renin production differed. These animal models illustrate the importance of RAS regulation in order to sustain a healthy pregnancy. Taken together, the several animal models exploring RAS regulation in pregnancy suggest that these factors could

play an important role in the pathogenesis of gestational hypertensive disorders. While these animal models suggest that RAS dysregulation in pregnancy can lead to hypertension, they cannot adequately address the specific scenario of PE. In this disorder, while the end effects of AT_1 receptor signaling, such as vasoconstriction, appear to be increased, the components of the RAS, such as renin and ANG II, are not (Table 1). This puzzle implies that another factor must be responsible for the excessive AT_1 receptor activation observed in women with PE. The aforementioned autoantibody, AT_1-AA, which is found in the circulation of preeclamptic women, may be responsible for this stimulation. Though many *in vitro* studies imply that AT_1-AA can induce the many biologic responses relevant to PE, such as increased cardiomyocyte contractility and sFlt-1 secretion [33, 110], they cannot authoritatively label this autoantibody as a major pathogenic player. In order to definitively show this autoantibody is a causative agent of PE, an adoptive transfer experiment of AT_1-AA in pregnant mice must be performed.

Chapter overview

This Chapter will report that when AT_1-AA purified from preeclamptic women was injected into pregnant mice, they recapitulate the key maternal symptoms: hypertension, proteinuria and increased circulating sFlt-1 and sEng. Human IgG derived from healthy normotensive pregnant women did not induce preeclamptic features when injected into dams. In order to demonstrate autoantibody specificity, a short antibody-neutralizing epitope peptide, 7-aa, was co-injected in dams and decreased autoantibody-mediated effects. Losartan, an AT_1 receptor blocker, also attenuated AT_1-AA-induced features. The results of the adoptive transfer mouse model reveal that <u>the autoantibody found in preeclamptic women may contribute to the pathophysiology of maternal disease features through excessive AT_1-receptor activation</u>.

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RESULTS

Human IgG is detectable in the circulation of pregnant mice.

To evaluate the pathophysiologic consequences of AT_1–AA *in vivo*, purified total IgG from either normotensive (NT) or preeclamptic pregnant women was introduced into pregnant mice on embryonic day (E) 13 and E14 by retro-orbital injection. Western blot analysis indicated that human IgG (hIgG) was readily detected in the injected mice five days post-injection (Fig. 3a). To confirm these findings, an ELISA was employed to quantify the hIgG in mouse circulation. The ELISA results indicated that concentrations in injected pregnant mice were similar, whether injected with hIgG derived from NT pregnant women or preeclamptic patients (Fig. 3b). Finally, to determine if the injected IgG retained biologic activity, when mice were sacrificed on E18, IgG was purified from maternal mouse sera, and assayed for the ability to activate AT_1 receptors in a luciferase reporter cell line. These results (Fig. 3c) confirmed that hIgG from preeclamptic women retained their ability to
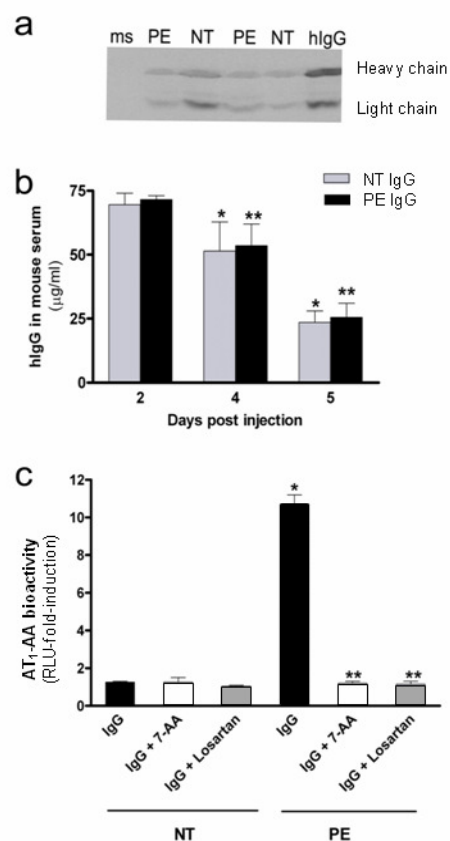
Figure 3: Human IgG is detectable in mouse sera. Human IgG (hIgG) injected into mice were detectable in their sera 5 days post-injection by (a) western blot, ms; mouse sera without injection (negative control), hIgG (positive control); (b) ELISA *$P<0.05$ vs 2 day NT-IgG, **$P<0.05$ vs 2 day PE-IgG, and (c) a luciferase-based bioassay reflecting AT_1-AA bioactivity on E18. *$P<0.01$ vs NT-IgG. **$P<0.05$ vs PE-IgG. Data displayed as the mean ± SEM, n=3 for each variable. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

activate AT$_1$ receptors at least five days post-retro-orbital injection into pregnant mice. This is contrast to the hIgG isolated from pregnant mice injected with IgG from NT pregnant women, which could not stimulate AT$_1$ receptor driven luciferase activity. These results verify that it is possible to adoptively transfer physiologically relevant concentrations of hIgG into pregnant mice, and that the biologically active injected human antibody persists for many days in the maternal mouse circulation.

The adoptive transfer of IgG from preeclamptic women into pregnant mice leads to hypertension and proteinuria through AT$_1$ receptor activation.
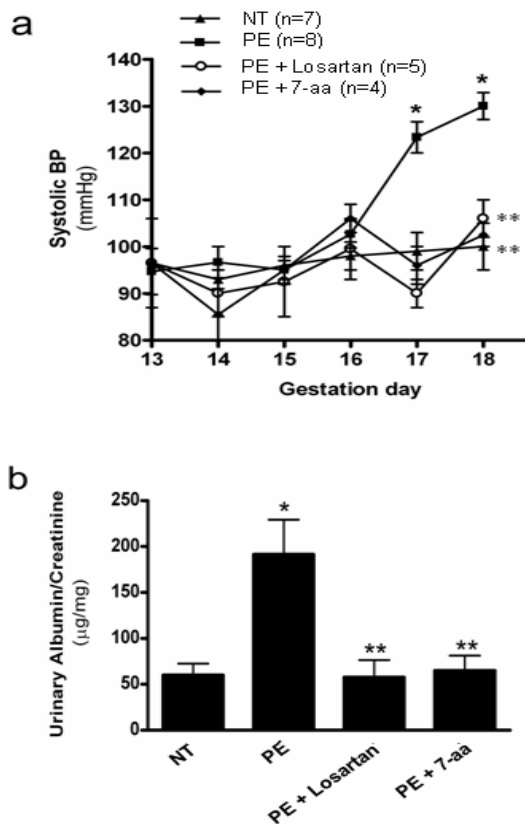
Figure 4: PE-IgG induce a preeclamptic-like state in pregnant mice. Injection of PE-IgG into pregnant mice increases (a) systolic blood pressure and (b) urinary protein. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG. Data displayed as the mean ± SEM. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

Hypertension and proteinuria are the two clinical characteristics defining the maternal syndrome of PE. To determine if AT$_1$-AA have the ability to contribute to gestational hypertension, systolic blood pressure of pregnant mice was measured daily following the retro-orbital injection of IgG purified from pregnant women. The introduction of IgG from preeclamptic patients (PE-IgG) but not IgG from normotensive pregnant women (NT-IgG) resulted in a significant increase in blood pressure that was evident four days post-injection (Fig. 2a). The increased blood pressure resulting from injection of PE-IgG was prevented by co-injection of losartan, an AT$_1$ receptor antagonist

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(Fig. 2a). This suggests that the antibody-induced increase in blood pressure required activation of the AT_1 receptor.

The antibody-injected mice were also analyzed for proteinuria. After a 24h urine collection using metabolic cages, the ratio of urinary albumin to creatinine on E18 was examined. The results (Fig. 2b) show that a significant increase in urinary protein occurred following injection of PE-IgG in contrast to injection of pregnant mice with NT-IgG. The autoantibody-induced proteinuria was prevented by co-injection with losartan, indicating that AT_1 receptor activation was essential for this process. Overall, these results show, for the first time, that IgG from preeclamptic women, in contrast to IgG derived from normotensive pregnant women, are capable of inducing hypertension and proteinuria in pregnant mice, and that these preeclamptic-like features required AT_1 receptor activation.

Autoantibody-induced features of PE are prevented by co-injection of 7-aa.

AT_1-AA interact with a specific seven amino acid sequence (AFHYESQ) present on the second extracellular loop of the AT_1 receptor [33]. Competition experiments indicated that this epitope peptide is sufficient to block autoantibody-induced AT_1 receptor activation [33, 111-115]. In this regard, a short peptide of this sequence was generated (7-aa), to be routinely employed as an autoantibody neutralizing agent. This is useful to determine if a particular biological response (e.g. hypertension or proteinuria) can be specifically attributed to the actions of AT_1-AA. Thus, on E13 and E14, mice were co-injected with PE-IgG and 7-aa, and then their blood pressure and proteinuria were monitored. These data (Fig. 2) show that co-injection with the 7-aa with PE-IgG successfully alleviated autoantibody-induced hypertension and proteinuria. These findings imply that the hypertension and proteinuria resulted from IgG

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derived from preeclamptic women which specifically binds to the second extracellular loop of the AT_1 receptor.

Affinity-purified AT_1-AA and total IgG induce similar features upon injection into pregnant mice.

To directly test the pathophysiological role of the autoantibody in PE, AT_1-AA were specifically isolated from total IgG derived from preeclamptic patients using an affinity-purification strategy. The ability of 7-aa to neutralize autoantibody-induced effects across multiple cellular systems and previous studies [23, 33, 115, 116] suggests a physical association between AT_1-AA and the specific sequence, AFHYESQ, on the second extracellular loop of AT_1 receptor. Briefly, the strategy employed is as follows: a GST-peptide fusion protein was generated containing the in frame insertion of a 27-amino acid DNA sequence corresponding to the second extracellular loop of human AT_1 receptor, herein named GST-27-aa. After overexpression in bacteria and its subsequent isolation using glutathione beads, the GST-27-aa was then coupled to affinity chromatography columns which were used to specifically isolate AT_1-AA from total IgG. The eluted fraction could bind to a 43kDa band corresponding to AT_1 receptors bound to a membrane by western blot analysis (Fig. 5a). In addition, using the previously mentioned luciferase-based bioassay, it was confirmed that the affinity-purified AT_1-AA were capable of recognizing and activating the AT_1 receptor (Fig. 5b). Only the eluted fraction (affinity-purified AT_1-AA) was capable of inducing expression of the luciferase reporter gene via AT_1 receptor activation. The flow-through component was unable to generate luciferase activity or recognize the 43 kDa band upon western blot analysis.

These two facts indicate that the IgG fraction eluted from the affinity chromatography columns contains IgG which bind to the second extracellular loop of the AT_1 receptor.

Upon the confirmation of retained biologic capability, the affinity-purified AT_1-AA were injected into pregnant mice. The introduction of the affinity-purified AT_1-AA resulted in a robust increase of blood pressure apparent within three days post-injection (Fig. 5c). These dams also developed significant proteinuria (Fig. 5d). Consistent with the findings of total IgG derived from preeclamptic women, hypertensive and proteinuria induced by affinity-purified AT_1-AA were diminished by losartan and 7-aa (Fig. 5c-d). None of the mice injected with control IgG displayed preeclamptic-like features.

Figure 5: Affinity-purified AT_1-AA induce a preeclamptic-like state in pregnant mice. Western blot analysis (a) depicts the ability of affinity purified fractions to detect AT_1 receptors (AT_1 r') at 43 kDa. Cell lysates from AT_1 r'-rich cells were bound to a membrane. A commericial anti-AT_1 r' antibody (lane 1) detected the 43 kDa band as did the eluted fraction (AT_1–AA; lane 3). The flow-through fraction (lane 2) could not. AT_1-AA bioactivity (b) was assessed using a luciferase assay. The eluted fraction (AT_1-AA) greatly stimulated luciferase activity. AT_1-AA purified from total PE-IgG injected into pregnant mice lead to increased (c) systolic blood pressure and (d) urinary protein. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

preeclamptic-like features. These results provide direct evidence that women with PE harbor autoantibodies which have an affinity for the second extracellular loop of the AT_1 receptor and

can be specifically isolated. Moreover, upon injection into pregnant mice activation of the AT_1 receptor induces the symptoms of PE. The results of the affinity-purified AT_1-AA also indicate no statistically significant differences between the hypertension and proteinuria measured when total PE-IgG was injected. This suggests that the total IgG houses a quantity of AT_1-AA sufficient to induce maximal blood pressure and proteinuria in pregnant mice.

Autoantibody-induced alterations in the mouse kidney.

Proteinuria is not the only renal dysfunction associated with PE. Characteristic alterations in renal histology are also commonly observed. To evaluate the potential role of AT_1-AA in the renal pathophysiology of PE, the kidneys of pregnant mice injected with human IgG derived from either NT or preeclamptic women were examined. On E18, five days post-injection, pregnant mice were sacrificed and their kidneys were isolated, fixed and sectioned. Analysis of H&E stained sections from PE-IgG-injected pregnant revealed extensive renal damage (Fig. 6a-b). The majority of their glomeruli were small and under high magnification, endothelial swelling, narrowing or obliteration and occlusion of the glomerular capillary spaces
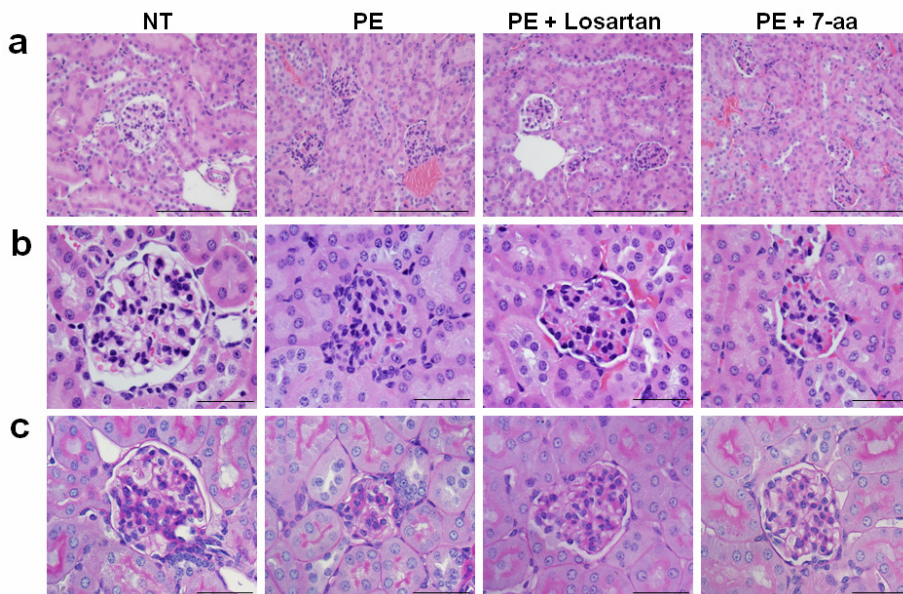
Figure 6: PE-IgG induced renal damage in pregnant mice. H&E staining (a & b) reveals small, contracted and consolidated glomeruli in PE-IgG injected mice. Scale bars (a) 200µm, 40X (b) 50 µm, 100X. PAS staining (panel c) is unremarkable. No PAS+ cells appear in the glomeruli of any treatments. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

were evident, but showed no evidence of PAS-positive materials in the cytoplasm (Fig. 6c). The affected glomeruli demonstrated the characteristic consolidated "bloodless" appearance, in contrast to the open glomerular tufts of the pregnant mice injected with NT-IgG (Fig. 6). To further confirm the AT_1-AA-induced renal damage, mouse kidneys were stained using Masson's Trichrome for excess collagen deposition, as well as for increased complement factor C3, by immunohistochemistry (Fig. 7). Elevated collagen (blue stain) was apparent in the tubules of PE-IgG injected dams, but not in NT-IgG injected mice, and greatly diminished in dams co-injected with PE-IgG and losartan or 7-aa (Fig 7b). The glomeruli of AT_1-AA-injected dams demonstrated increased positive complement C3 expression, whereas NT-IgG injected pregnant mice did not (Fig. 7). Those mice co-injected with PE-IgG and losartan or 7-aa had reduced C3 detected in their renal glomeruli. The increased deposition of collagen and complement activation observed in the pregnant mice further illustrates the damage due to autoantibody-mediated AT_1 receptor activation.
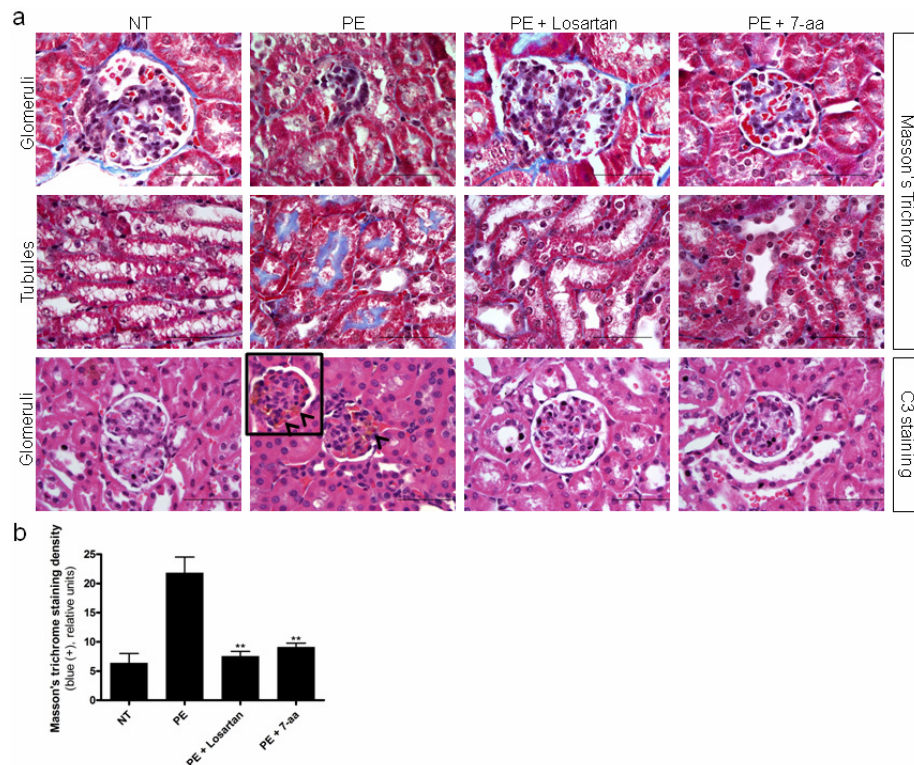
Figure 7: PE-IgG increase collagen and C3 deposition in pregnant mice. Masson's trichrome staining (a) reveals that increased collagen (blue) is deposited in the tubules of PE-IgG injected pregnant mice. Immunohistochemistry staining for C3 (a) indicates C3 deposition in the glomeruli of these mice (arrows). Inset is another glomeruli with C3 staining. Scale bars 50 µm, 100X. Quantification of Masson's Trichrome stain for collagen (b) indicates an increased blue density in the tubules of PE-IgG injected pregnant mice. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG.

Examination of these kidneys by transmission electron microscopy (TEM) confirmed the evidence of glomerular change by light microscopy. The endothelial cells of the affected glomeruli demonstrated the loss of the fenestrations and because of their swollen cytoplasm, lead to the narrowing or complete obliteration of capillary loop spaces (Fig 8). In addition, focal foot process effacement was observed in the podocytes, as were the occasional electron-dense materials in the mesangial and sub-endothelial areas. This collection of renal histopathology is pathognomonic for the type of damage seen in kidneys of preeclamptic patients. No significant mesangial cell proliferation or segmental glomerulosclerosis were seen on light microscopy or TEM. The pregnant mice injected with NT-IgG were unremarkable and did not show any of these histologic changes (Figs. 6-8). The histologic changes seen in the kidneys of PE-IgG were partially prevented in mice co-injected with losartan or 7-aa and PE-IgG (Figs. 6-8). Similar renal damage was consistently observed in pregnant mice injected with affinity-purified AT_1-AA. Taken together, these data imply that the autoantibody, through AT_1 receptor activation, can induce renal histopathological changes in pregnant mice which may lead to their renal impairment.
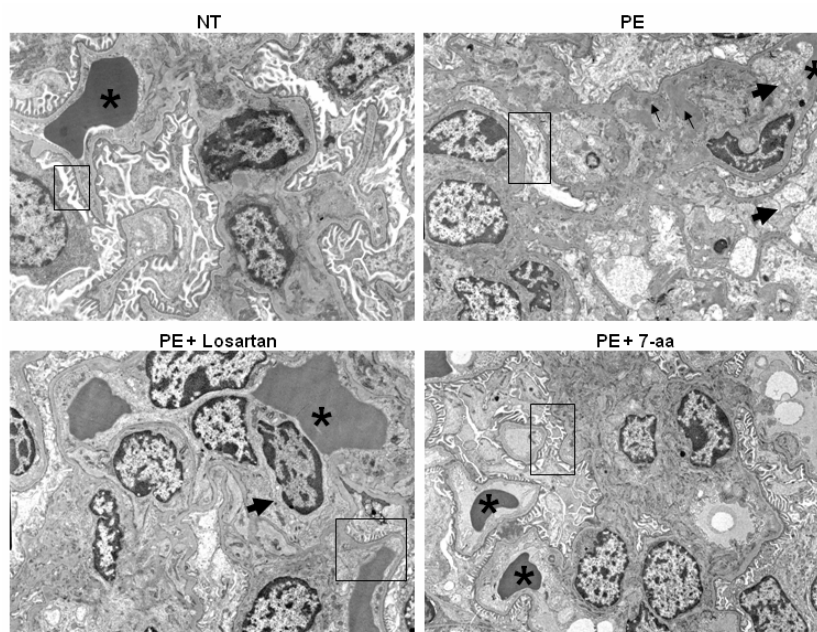
Figure 8: Ultrastructure of kidneys in PE-IgG injected mice. Features of glomerular endotheliosis are revealed by TEM analysis of PE-IgG injected mice. Swollen endothelial cells (thick arrows) occlude capillary spaces (*). Subendothelial deposits are evident (thin arrows). Capillary spaces appear open in NT-IgG injected animals. Boxes highlight podocytes, which remain unremarkable in all cases. 1500X. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

Autoantibody-mediated increases in placental-derived anti-angiogenic factors, sFlt-1 and sEng in pregnant mice.

Recently, sFlt-1 and sEng have been brought to the forefront in the study of PE. The anti-angiogenic factor sFlt-1 is a soluble form of the VEGF receptor that is elevated significantly in the circulation of preeclamptic women [37, 117]. During pregnancy, sFlt-1 is secreted by the placenta, and is thought to contribute to hypertension and proteinuria by interfering with VEGF signaling [118]. Similarly, soluble endoglin (sEng), a soluble form of the TGF-β receptor, is also secreted by the placenta and is elevated in preeclamptic women [39]. To evaluate the potential contribution of AT_1-AA to increased production of these two factors in PE, pregnant mice were injected with IgG from NT pregnant women or women with PE on E13 and E14, and the circulating levels of sFlt-1 and sEng were determined upon sacrifice on E18. The results (Fig. 9) reveal that both sFlt-1 and sEng levels were significantly greater in pregnant mice who received injections of PE-IgG in comparison to those who received NT-IgG. The autoantibody-mediated inductions of sFlt-1 and sEng in pregnant mice were inhibited by the co-injection of losartan or 7-aa. This indicates that the AT_1-AA-mediated liberation of the anti-angiogenic factors, sFlt-1 and sEng, required AT_1 receptor activation.

Figure 9: Increased circulating anti-angiogenic factors in PE-IgG injected pregnant mice. Both sFlt-1 and sEng are increased in the sera of pregnant mice secondary to AT_1 receptor activation by the autoantibody. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG.. n=6 for each variable.
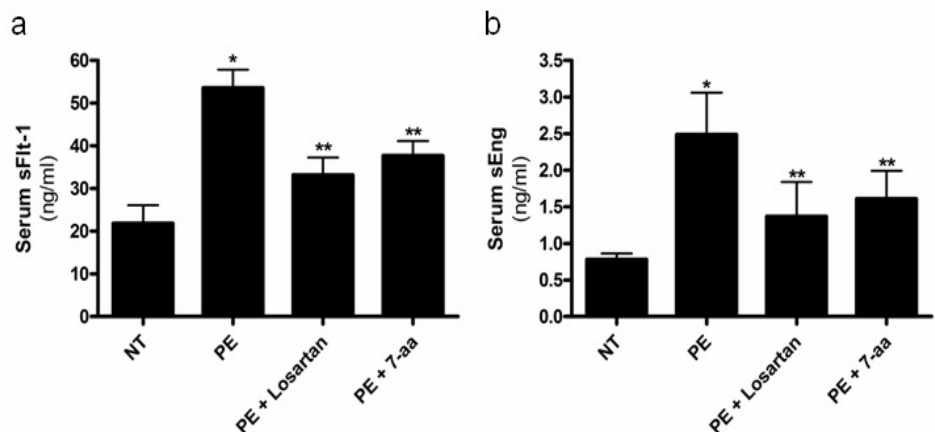
The effect of AT$_1$-AA in non-pregnant mice.

In the previous section, it was demonstrated that human IgG derived from preeclamptic patients and not normotensive pregnant women could induce sFlt-1 production upon injection into pregnant mice (Fig. 9), which could be specifically blocked by losartan or 7-aa. Thus, the autoantibody-induced hypertension and proteinuria observed in pregnant mice could be the result of excessive placenta-derived sFlt-1 action. However, it is possible that AT$_1$-AA could contribute to these features independent of excessive sFlt-1. To test this hypothesis, NT-IgG or PE-IgG were introduced into non-pregnant mice, and their blood pressure, renal function and sFlt-1 levels were monitored. In contrast to pregnant mice, the concentration of sFlt-1 in non-pregnant mice injected with NT-IgG remained very low, and most importantly, was not increased by the injection of IgG purified from preeclamptic women (Fig. 10). Therefore, sFlt-1 cannot be induced by IgG purified from preeclamptic patients in non-pregnant mice.

Figure 10: Non-pregnant mice do not liberate excess sFlt-1 when injected with PE-IgG. While sFlt-1 is increased in the circulation of pregnant mice due to AT$_1$ receptor activation, non-pregnant animals do not show elevated levels of the anti-angiogenic factor when injected with the autoantibody. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG. n=6 for each variable. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

The blood pressure of non-pregnant mice was then monitored. The results (Fig. 11a) show that PE-IgG, in contrast to NT-IgG, stimulated an increase in blood pressure. PE-IgG were capable of inducing a significant increase in blood pressure four days post-injection. Co-injection of either losartan or 7-aa specifically inhibited the autoantibody-mediated increase

(Fig. 11a). Additionally, there was no significant difference in the maximal AT_1-AA-induced hypertension observed in pregnant mice (Fig. 4) and non-pregnant mice (Fig. 11), suggesting that AT_1-AA-mediated hypertension is not entirely sFlt-1-dependent.
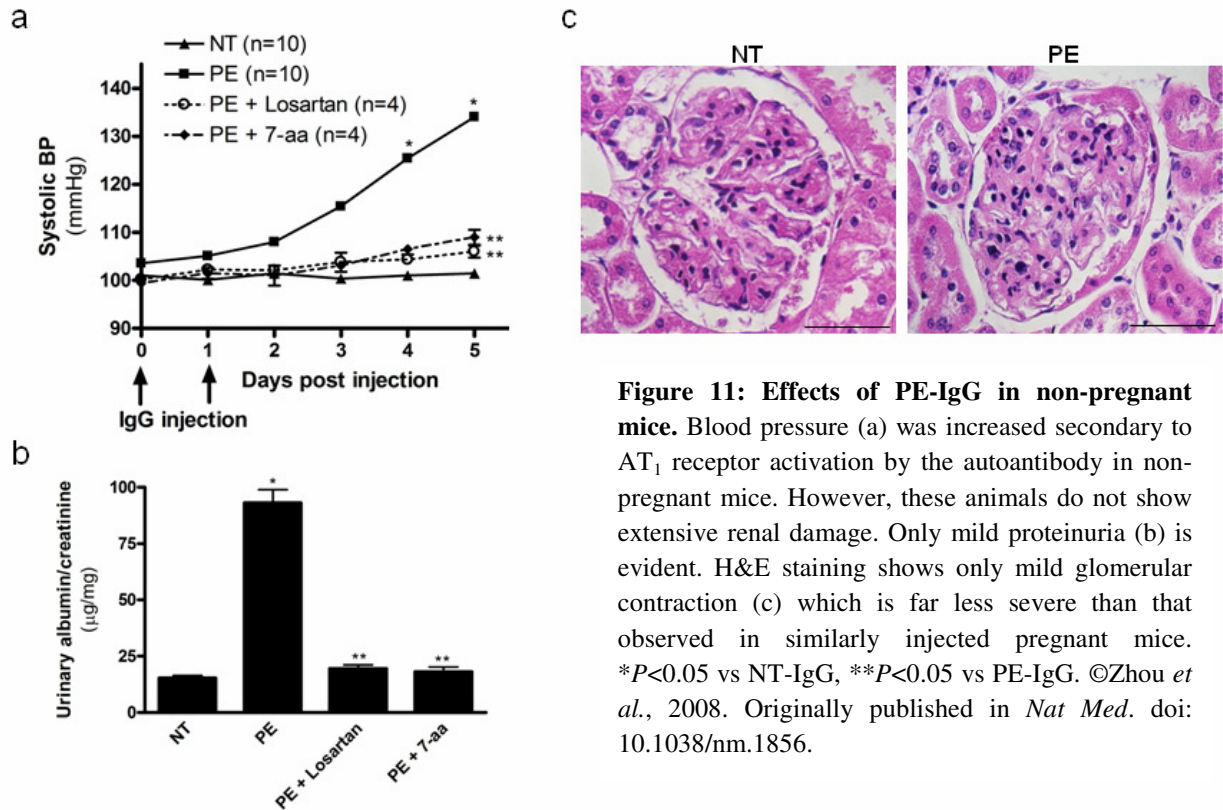
Figure 11: Effects of PE-IgG in non-pregnant mice. Blood pressure (a) was increased secondary to AT_1 receptor activation by the autoantibody in non-pregnant mice. However, these animals do not show extensive renal damage. Only mild proteinuria (b) is evident. H&E staining shows only mild glomerular contraction (c) which is far less severe than that observed in similarly injected pregnant mice. *$P<0.05$ vs NT-IgG, **$P<0.05$ vs PE-IgG. ©Zhou *et al.*, 2008. Originally published in *Nat Med*. doi: 10.1038/nm.1856.

The urinary protein of antibody-injected non-pregnant mice was then measured. A double injection of PE-IgG was required to induce a mild increase in urinary protein in non-pregnant mice (Fig. 11b). This slight autoantibody-induced proteinuria could be inhibited by co-injection of losartan or 7-aa, suggesting that the proteinuria observed in non-pregnant mice was mediated by AT_1 receptor activation. However, the amount of urinary protein achieved following autoantibody injection in pregnant mice was far greater, as compared to that achieved in non-pregnant mice. Finally, analysis of H&E stained sections from non-pregnant mice revealed only mild renal damage following injection of PE-IgG (Fig. 11c) in comparison to the

severe renal damage observed in similarly treated pregnant mice (Fig. 6). The glomeruli of mice injected with NT-IgG displayed no hypercellularity and were normal in size and shape (Fig. 11c). In the non-pregnant mice double-injected with IgG from preeclamptic women, though the majority of the glomeruli were normal to slightly reduced in size, the endothelial swelling evident in pregnant mice was absent. The mild decrease in glomerular size, likely due to mesangial cell contraction via AT_1 receptor activation, was not as pronounced as that observed in the pregnant animals. The glomeruli in the PE-IgG non-pregnant mice were not as consolidated as those of their pregnant counterparts and their capillary lumens and glomerular tufts appeared wide. Overall, non-pregnant mice injected with PE-IgG showed unsubstantial changes in renal histopathology.

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DISCUSSION

To formally examine the role of AT_1-AA in the pathophysiology of PE, an adoptive transfer experiment was performed. This autoantibody was injected into pregnant mice, who then recapitulated several key features of the disease: hypertension, proteinuria, renal morphologic changes, and the increase of the anti-angiogenic factors sFlt-1 and sEng. In each case the autoantibody-induced feature of PE could be prevented by the co-injection of losartan, an AT_1 receptor antagonist, or a 7-aa, autoantibody-neutralizing epitope peptide. Both total IgG and affinity-purified AT_1-AA induced similar symptoms in pregnant mice. Therefore, the human IgG, affinity purification and autoantibody neutralization experiments provide direct evidence supporting the claim that the features of PE observed in pregnant mice were induced by an autoantibody which binds to and activates the second extracellular loop of the AT_1 receptor. Though hypertension was detectable in non-pregnant animals injected with PE-IgG, the other parameters, such as proteinuria, renal histologic changes and sFlt-1 production, did not demonstrate clinically relevant increases. This implies that the observed autoantibody-induced features are pregnancy-dependent. <u>Overall, these findings confirm that AT_1-AA contributes to the pathophysiology of PE and raise the intriguing possibility that PE is an autoimmune disease whose symptoms arise secondary to autoantibody-induced AT_1 receptor activation.</u> Moreover, these *in vivo* studies offer direct evidence of the pathophysiological role of AT_1-AA in PE and provide an animal model to use as an investigative tool in the analysis of the underlying pathogenic mechanisms associated with the disorder.

The two antiangiogenic soluble factors sFlt-1 and sEng are likely contributors to the hypertension and maternal endothelial dysfunction associated with PE [37, 61, 69]. sFlt-1 is a

placental-derived soluble form of the VEGF receptor-1 (VEGFR-1) which binds to and forms a complex with free VEGF, thereby interfering with its signaling [118]. In the kidney, VEGF acts to sustain glomerular fenestrations required for adequate filtration [119]. Therefore, diminished VEGF signaling in glomeruli results in impaired renal function characterized by proteinuria and often glomerular endotheliosis [117, 118, 120]. sEng is a soluble form of the TGF-β receptor, endoglin, which complexes to free TGF-β and interferes with its signaling [39]. Both VEGF and TGF-β increase the production of endothelial-derived nitric oxide (NO), a potent vasodilator, resulting in vascular smooth muscle relaxation. In this way, sFlt-1 and sEng enhance vasoconstriction in the peripheral circulation which manifests in hypertension. These two factors are found to be elevated in the circulation of preeclamptic women, and cooperate together to produce preeclamptic-like symptoms in pregnant rats [39]. However, the factors accounting for increased production of sFlt-1 and sEng in preeclamptic women remain undefined. From the data presented here, the autoantibody derived from women with PE may be the instigating factor which stimulates sFlt-1 and sEng production through AT_1 receptor activation, and through this mechanism, may contribute the hypertension and proteinuria observed in the disease.

Both AT_1-AA and sFlt-1 can stimulate increased blood pressure. Therefore, it is possible that their effects may be additive. However, both non-pregnant (Fig. 11) and pregnant (Fig. 4) mice demonstrated similar maximal blood pressure readings of ~140 mmHg upon autoantibody injection, despite the latter group harboring a considerably higher concentration of circulating sFlt-1. A possible explanation for this is that the amount of AT_1-AA injected into mice (pregnant or non-pregnant) was sufficient to induce a maximal increase in blood pressure, and though present in pregnant animals, the additional sFlt-1 was unable to stimulate an additional increase. A similar phenomenon was recorded by Maynard *et al*. [118]. In

experimentally manipulated rats, a low dose (7.3 ng/ml) of sFlt-1 was capable of inducing a maximal blood pressure of ~120 mmHg. A 20-fold increase in the dose of sFlt-1 (>200 ng/ml) was unable to further increase their systolic pressure [118]. This suggests that the capability of sFlt-1 to elevate blood pressure is limited, and a maximal threshold for increase can be easily reached. Based on the findings reported here and those of Maynard *et al.*, AT_1-AA and sFlt-1 can independently and additively contribute to the hypertension associated with PE.

In addition to its well-recognized role in regulating blood pressure, ANG II also induces inflammation, vascular damage and proteinuria, through AT_1 receptor activation. Ray *et al.* have shown mesangial cell proliferation through this mechanism [121]. Others have shown ANG II stimulates the production of PAI-1 by cultured endothelial cells [122, 123]. AT_1 receptor activation has also been implicated in endothelial cell hypertrophy [124]. Excess ANG II can induce renal damage leading to proteinuria in both wild-type rats [124] and transgenic rats overexpressing human renin and angiotensinogen genes [125-128]. Since AT_1-AA acts in the place of ANG II, it could be expected to produce similar consequences. Previously, our group has shown that IgG derived from women with PE stimulated mesangial cell production of both PAI-1 and IL-6, a pro-inflammatory cytokine [113]. Therefore, AT_1-AA may contribute to renal histopathology by activating AT_1 receptors on glomerular endothelial and mesangial cells. It was shown here that introduction of AT_1-AA into pregnant and non-pregnant mice resulted in severe and mild renal dysfunction, respectively.

Renal damage was especially obvious in PE-IgG pregnant injected mice. Routine H&E staining revealed contracted glomeruli with a consolidated appearance, endothelial cell swelling and narrowed capillary lumens (Fig. 6). PAS staining indicated that there were no PAS-positive deposits throughout glomerular cytoplasm nor was there increased mesangial matrix observed in these mice (Fig. 6). When stained for excess collagen using Masson's

Trichrome, the PE-IgG injected dams showed elevated collagen in their tubules, but not glomeruli, whereas NT-IgG injected mice did not demonstrate excess collagenous deposition anywhere within their kidneys (Fig. 7). Though the extent of tubular damage in the human disease is not well-characterized, there are several varied reports of tubular atrophy, fibrosis, and collagen deposition, all of which could contribute to the proteinuria and renal dysfunction observed in these women [129-132]. Renal tubules are considered especially responsive to hypoxic changes and susceptible to injury, as the medulla has a lower oxygen content in comparison to the cortex [133]. Pedrycz *et al.* observed superimposed PE in an experimental model of nephrotic syndrome in rats who demonstrated considerable tubular dysmorphology [134]. Moreover, ANG II is capable of stimulating collagen synthesis in vascular smooth muscle cells [135] and glomerular mesangial cells [136], among others, which could contrible to tubular injury. Since co-injection of the autoantibody with losartan or 7-aa reduced renal tubular collagen deposition, AT_1 receptor activation by the autoantibody may be responsible for these changes in adoptively transferred mice, and could thereby contribute to similar features in the human disease.

Complement, the bridge between innate and acquired immunity, becomes activated to combat disease, or to remove immune complexes, ischemic or apoptotic cells [137, 138]. Activation of the complement cascade, in particular the converging component, C3, is a well-recognized contributor to proteinuric renal dysfunction [139-142]. Complement activation is reportedly increased in preeclamptic women, however, the factors leading to its induction remain unknown [143-146]. Interestingly, Shagdarsuren *et al.* have recently demonstrated using a double transgenic mouse model of RAS overexpression that ANG II mediates complement activation and subsequent renal damage [128]. There are other reports to support an AT_1 receptor-induced mechanism of complement activation [126, 147]. It is therefore

possible that the autoantibody, AT_1-AA, could induce a similar immunologic response. Here, it is shown that the glomeruli of PE-IgG injected pregnant mice demonstrate elevated C3 expression by immunohistochemistry (Fig. 7), whereas NT-IgG do not. Co-injection of losartan or 7-aa with the autoantibody reduced C3 activation. The evidence of complement activation in the kidneys of autoantibody injected pregnant mice is yet another clear indication that AT_1-AA may incite the renal damage associated with PE. The exact consequences of increased tubular collagen deposition and glomerular C3 activity in autoantibody-injected mice are the subjects of ongoing work in the lab.

Since non-pregnant animals did not display the same severity of renal damage (Fig. 11) as their pregnant counterparts, another factor may additively contribute to this feature: it could be that autoantibody-induced sFlt-1 in pregnant mice is the essential factor leading to the severe renal damage observed. The injection of exogenous sFlt-1 into a rat results in renal lesions similar to those observed in PE [118]. In pregnant mice, AT_1-AA can induce sFlt-1 production by the placenta, which could, therefore, be responsible for the renal changes recorded in these animals. Furthermore, the renal dysfunction and the level of urinary protein achieved in pregnant mice is much more severe than in non-pregnant mice, who demonstrate much lower levels of sFlt-1. It is likely that in pregnant mice, AT_1-AA-induced renal dysfunction may be mediated through both the action of sFlt-1 and the direct effects of autoantibody-induced AT_1 receptor activation on glomerular endothelial cells and/or mesangial cells. It could be that these effects are independent or additive. Future work will have to delineate whether the renal damage and proteinuria observed in the human disease is the result of sFlt-1 or AT_1-AA action. To test this, PE-IgG-injected pregnant mice could be co-injected with recombinant $VEGF_{121}$, a protein which blocks the anti-angiogenic action of sFlt-1 [148]. Certainly, from the evidence

presented here, it appears that both AT_1 receptor signaling and sFlt-1 are required to work in tandem to produce the severe renal damage in PE-IgG injected mice.

The results of the adoptive transfer experiments presented here provide strong support for the working hypothesis that <u>PE is an autoimmune disease specific to pregnancy in which AT_1 receptor agonistic autoantibodies contribute to the development of many disease features</u>. Losartan, an AT_1 receptor blocker, significantly reduces the key autoantibody-induced symptoms in PE-IgG injected pregnant mice. This fact implies that the activation of this receptor is specifically required as the mechanism of symptom progression. The biologic properties of AT_1-AA can also be attenuated by an autoantibody-neutralizing 7-aa peptide, corresponding to the specific epitope located on the second extracellular loop of the AT_1 receptor. This consistent control suggests a common immunologic origin for these autoantibodies in different women, and has substantial therapeutic implications. Currently there is no specific cure for PE, and severe cases often require the premature delivery of the infant. If maternal circulating AT_1-AA contributes to the pathophysiology of PE, as the adoptive transfer model suggests, the timely removal or inhibition of these autoantibodies from preeclamptic women may provide profound therapeutic benefit. Moreover, recent evidence suggests that AT_1-AA can be detected as early as 18 weeks, making it an early marker to identify women at risk for the disease [149]. If AT_1-AA play a significant role in the etiology of PE, as hypothesized, it may be possible to block autoantibody-mediated AT_1 receptor activation and in turn forestall or prevent the onset of the preeclamptic symptoms.

CHAPTER 4:

AT$_1$-AA-INDUCED FETAL AND PLACENTAL ABNORMALITIES IN AN

ADOPTIVE TRANSFER MOUSE MODEL OF PREECLAMPSIA

BACKGROUND

Two key features of PE were not discussed in the previous Chapter as they warrant their own discussion: placental abnormalities and fetal consequences. Both placental dysfunction and subsequent poor fetal outcomes are unfortunate features observed in the more severe cases of PE. In this Chapter, I will explore the effect of AT_1-AA on placentation and fetal outcome using both the aforementioned adoptive transfer mouse model and culture systems of human villous explants and a human trophoblast cell line. The data presented here will help broadly demonstrate the pathogenic role of the autoantibody in PE, in particular its effects on the placenta and fetus, and the essential need for improved therapeutics in the management of this disorder affecting both mother and developing child.

Placental function and development during normal pregnancy

Good placental health is essential for an uncomplicated pregnancy. This complex organ is the barrier between maternal and fetal cells, is a site of exchange for nutrients, oxygen and waste products and is a producer of hormones [150, 151]. But the placenta has another important function: it is an immunologic barrier. IgG may pass through the placenta from mother to fetus via transcytosis and confer passive immunity [152-154]. Though there is some controversy surrounding the rank of IgG subclasses which are actively transferred across the placenta [155-157], most consider the efficiency in the order of $IgG_1 > IgG_3 > IgG_4 > IgG_2$ [157, 158]. Of note, in a small study, Wallukat *et al.* report AT_1-AA as sublass IgG_3 [53].

Humans and mice share similar features of their uteroplacental units. They are both discoid chorioallantoic placentas and have hemochorial blood interfaces with three corresponding physiologic and anatomically distinct regions [159, 160]. In humans, on the maternal side, the placental bed is made up of the decidua basalis and the underlying myometrium. In mice, the corresponding area is the decidua basalis only, and does not include the myometrium [159]. The basal plate in humans and the junctional zone in the mouse are equivalent. They both produce hormones

Fig 12: Schematic of early mouse placental structures. By E10, the labyrinth zone is well-established.

and cell lineages are primarily cytotrophoblastic [159]. In mice, there are cell types which are not found in humans: spongiotrophoblasts (Mash2+), Trophoblast Giant Cells (TGCs; Hand1+, Stra1+) and glycogen cells [161]. Closest to the fetus, the human placenta and the murine labyrinth zone (LZ) are highly branched areas of maternal:fetal blood interface (Figs. 12-13) [162]. There are 3 layers of fetal cells on the chorionic villi which prevent the mixture of maternal and fetal blood, yet allowing for adequate exchange [162]. These zones are made up of syncytiotrophoblasts (Gcm-1+) [159, 161].
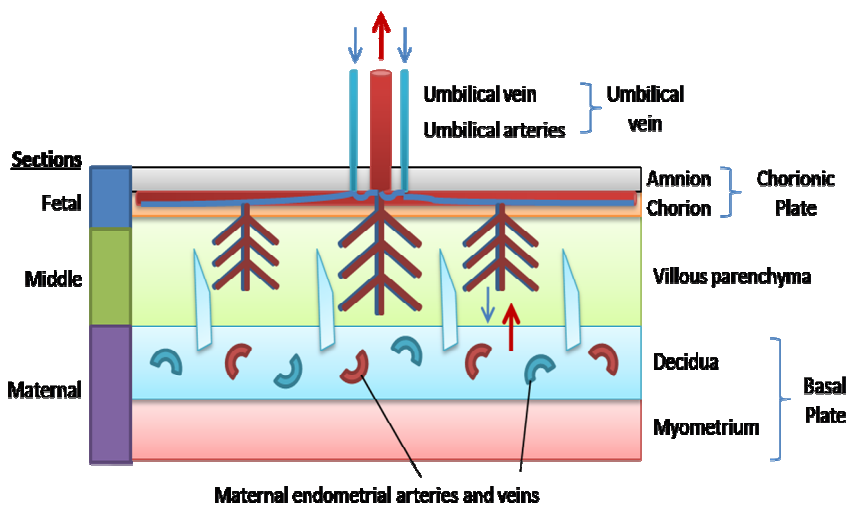
Fig 13: Schematic of human chorionic villous blood interface. In this diagram, the "middle section" is equivalent to the murine labyrinth zone.

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In humans, placental development begins towards the end of weeks gestational age 2 (WGA 2). By WGA 3, a primordial vascular network is apparent and a histiotropic-type nutrition predominates [163, 164]. This source of nutrition for the fetus is derived from the uterine glands which secrete nutrients. At this time, gas exchange relies entirely on diffusion [165] and is, therefore, considered a time of relative hypoxia [165-167]. In order to establish adequate blood-flow, endovascular trophoblasts invade the endothelial layer of the maternal spiral arteries, leading to a hemochorial blood interface. The endothelial cells of the maternal layer are entirely replaced by endovascular trophoblasts, resulting in the physiologic transformation of the high resistance, muscular spiral arteries into low resistance, flaccid, wide vessels [168, 169]. They become dilated 4-5 times to allow for increased uterine perfusion [168]. Maximal trophoblast invasion occurs between WGA 7-9. By WGA 22, all invasion into the placental bed is complete: all of the decidual and 75% of the myometrial spiral arteries are now physiologically transformed [170-172].

In mice, implantation occurs on embryonic day (E) 4.5 [173]. By E8.0 the ectoplacental cone is evident and there is abundant trophoblastic cell differentiation [173]. By E10.0, the LZ is established as the placenta matures (Fig. 12). After E12.5, it is rare to have further cell differentiation, but Gcm-1-mediated branching does occur in the LZ as the placenta increases in size [162]. It should be noted that prior to E13, the TGCs are the most invasive cell type, but >E13, glycogen cells take over that role [174, 175]. In the human, the analogous invasive cell is predominately the invasive interstitial trophoblast cell [176, 177].

Whether in mouse or human, correct placentation is required for a healthy pregnancy. Aberrant placental development may result in inadequate uteroplacental blood-flow and can lead to detrimental sequelae in both mother and fetus, such as in the case of PE.

Aberrant placental development in preeclampsia

As discussed above, the physiologic transformation brought about by the invasion of trophoblasts into the endothelial lining of maternal spiral arteries is essential for sufficient uterine blood-flow. However, in severe cases PE, placentas are often small, exhibit shallow trophoblast invasion into the decidua and inadequate spiral artery remodeling, as well as increased placental cell apoptosis [178, 179]. This insufficient placentation results in a substantial reduction in uteroplacental blood-flow. In PE, it is has been long hypothesized that it is this damaged placenta that liberates molecules, such as sFlt-1, which contribute to maternal vascular injury and inflammatory responses [180]. The initiating mechanism for this damage occurs has not been determined; though there is a mounting body of evidence implicating AT_1-AA, the autoantibody associated with PE.

AT_1-AA may impair trophoblast invasion in PE through the increase of both sFlt-1 and PAI-1. Zhou *et al.* have shown that AT_1-AA induces the secretion of the anti-angiogenic factor sFlt-1 in human placenta villous explants and human trophoblast cells [181], not to mention the data presented in Chapter 1 which demonstrated that PE-IgG injected pregnant mice also have elevated circulating sFlt-1 levels. This suggests that the autoantibody may contribute to the AT_1 receptor-mediated sFlt-1 oversecretion in the placenta, endothelial cell dysregulation and overly inhibited angiogenesis, which could result in impaired placentation. AT_1-AA may also induce the PAI-1-mediated placental damage observed in PE. By inhibiting urokinase-like plasminogen activator (uPA), PAI-1 activity results in decreased conversion of plasminogen to plasmin [75]. This leads to decreased fibrinolysis and extracellular matrix digestion, and shallow trophoblast invasion, the hallmark placental lesion in PE. AT_1-AA activates trophoblast cells' AT_1 receptors resulting in elevated PAI-1 levels [21, 23]. Excess PAI-1 has been shown *in vitro* to decreases trophoblast invasion using a matrigel assay [21, 22]. Thus,

AT_1-AA on human trophoblasts may contribute to increased PAI-1 production and lead to the placental damage observed in PE. Collectively, AT_1-AA-induced sFlt-1 and PAI-1 production may additively contribute to the histopathologic changes observed in the placentas of preeclamptic patients.

In PE, the changes in placental pathology are not without consequence. As previously mentioned, placental health is vital in order to sustain an uncomplicated pregnancy. In the case of PE, the small and underperfused placentas are thought to contribute to the poor fetal outcomes associated with the disease.

Intrauterine growth restriction (IUGR)

Intrauterine growth restriction (IUGR) is defined as fetal growth less than the 10th percentile for gestational age [182]. This complication of development affects ~15% of pregnancies in the US annually [183, 184]. There are, however, more sequelae to IUGR than simply being small; growth-restricted fetuses have a higher incidence of morbidity and mortality than fetuses of average growth. They are also at increased risk for future development of several metabolic disorders such as hypertension, dyslipidemia, coronary heart disease, obesity, type 2 diabetes mellitus, impaired glucose tolerance [185-188]. Most cases of IUGR are attributed to ischemic placental disease [184, 189]. Though the mechanisms leading to the placental distress resulting in IUGR remain largely unknown, they are essential to understand in order to prevent this very serious complication of pregnancy.

IUGR, PE and the dysregulation of the RAS

The effects on the fetus are often overlooked in the study of PE. Placental dysfunction leading to the premature births of babies who suffer from IGUR is commonly observed in this disorder [190, 191]. Exactly how the IUGR in PE occurs is largely undefined; however, the RAS regulates many components which could contribute to these problems. In fact, there are many examples of how the RAS may affect placentation and fetal development. For example, in the placenta, ANG II decreases system A amino acid transporter activity through AT_1 receptor activation. This decrease in amino acid supply is thought to contribute to IUGR [4]. Feto-placental gene expression and the changes in circulation which regulate fetal oxygenation, maturation and health are also regulated by the RAS [192]. A double AT_1 receptor knockout mouse model demonstrates that AT_1 receptors are essential to attain appropriate somatic growth and normal kidney structure [193]. Saito, *et al.* and Furuya *et al.* expanded on the work of Takimoto, *et al.* [103], and found that pups born to transgenic mice expressing human angiotensinogen who had mated with males expressing human renin suffered from IUGR [104, 194]. These newborns were small and their thoracic and visceral organs were undersized, suggesting that overexpression of RAS components may regulate fetal growth. These many examples illustrate how alterations in the RAS may contribute to IUGR in the setting of PE.

A role for AT_1-AA in preeclamptic IUGR

Based on the data presented in Chapter 3, where it was described that the introduction of AT_1-AA into pregnant mice resulted in the key maternal features of PE [38], I hypothesize that the autoantibody may contribute to the placental and fetal features consistent with the disease. Since the *in vivo* studies provided the first direct evidence of the pathophysiology

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induced by AT_1-AA in PE, this animal model was employed to address the exact role of AT_1-AA in IUGR and its underlying mechanisms.

Chapter overeiw

In this Chapter, it will be shown that AT_1-AA exist both in the cord blood of preeclamptic women and in the fetal circulation of pregnant mice injected with the autoantibody. The fetuses borne to autoantibody-injected mice are small and have impaired multiple organ development. These findings indicate that AT_1-AA enter fetal mouse circulation where they may impose a direct detrimental effect on fetal maturation. Additionally, AT_1-AA impair placental development by increasing apoptosis, resulting in smaller organs. The murine results were corroborated with similar findings in human placental explants and in cultured HTR-8 cells exposed to AT_1-AA. These studies demonstrate that abnormal placentation may be a secondary underlying mechanism for autoantibody-induced IUGR. Finally, AT_1-AA-mediated IUGR and placental damage were largely corrected by co-injection with either losartan or 7-aa, suggesting that autoantibody-induced AT_1 receptor activation was required. Overall, this work reveals the possible contribution of AT_1-AA to the development of IUGR, as well as two underlying mechanisms for this process. These novel findings suggest that exposure to AT_1-AA in the womb will have negative effects on a developing fetus and placenta, and identify these autoantibodies as potential therapeutic targets.

RESULTS

AT₁–AA can be physically and biologically detected in preeclamptic cord blood.
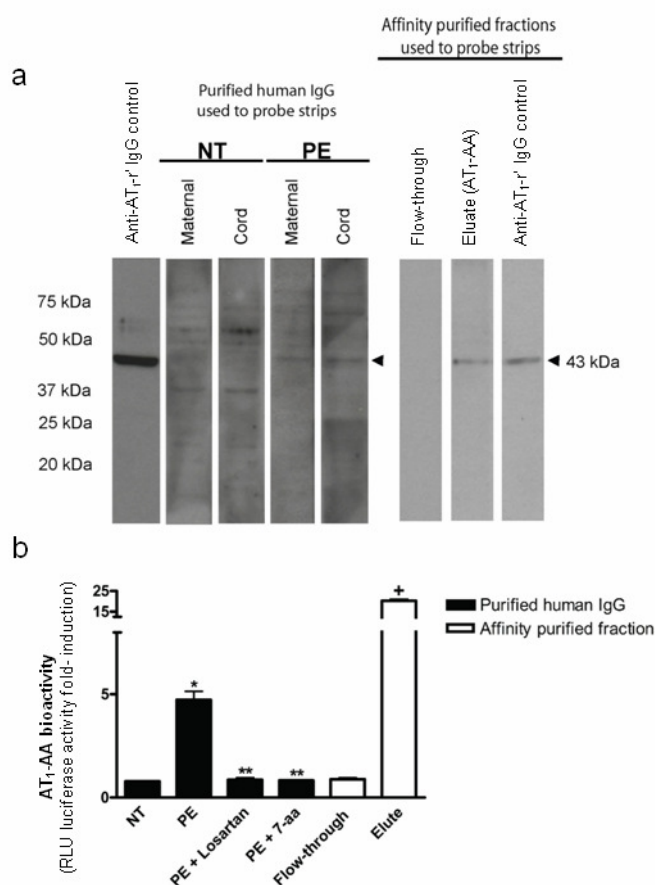
Figure 14: AT₁-AA passes through the human placenta and retains biologic activity. Celluar lysate of CHO.AT₁ cells was run on an SDS-PAGE gel then transferred to a nitrocellulose membrane. AT₁ r' rich lanes were cut into strips and probed with an anti-AT₁ r' control, or IgG purified from maternal sera or cord blood of NT (n=6) or PE (n=6) women. Only sera or cord blood-derived IgG from PE, but not NT, pregnant women can detect the AT₁ r' by western blot (a). The eluate fraction of affinity-purified total IgG from the cord blood of PE women could also detect the 43kDa band of the AT₁ r'. The luciferase-based AT₁-AA bioassay was employed to detect biologic activity (b). IgGs derived from the cord blood of PE women and the eluate fraction could stimulate AT₁ r' activated luciferase. n=10 in each category. Data are expressed as mean ± SEM. *$P<0.01$ vs NT-IgG. **$P<0.01$ vs PE-IgG. +$P<0.01$ vs flow-through fraction. ©Irani *et al.*, 2009. Originally published in *J Exp Med.* doi: 10.1084/jem.20090872

The placenta is a vascular organ which brings maternal and fetal blood into close proximity. This interaction facilitates the nutrient and gas exchange essential for appropriate fetal development [159]. Uterine spiral arteries supply maternal blood to the placenta and the fetus receives oxygenated blood from the umbilical vein housed in the umbilical cord. Therefore, cord blood represents an easily accessible source of fetal blood which can be obtained at the time of parturition. To determine if AT₁-AA cross from the maternal circulation into the fetus, maternal and cord blood from NT pregnant women and women with PE were obtained. Total IgG was isolated from their sera and examined for the presence of AT₁-AA. Western blot results (Fig. 14a) indicate that total IgG from maternal and

cord sera of PE women detected a band of 43 kD, which corresponds to the AT_1 receptor protein derived from cellular lysates enriched with the receptor which were transferred to a nitrocellulose membrane. In contrast, IgG from maternal and cord sera of NT pregnant women did not cross-react with the protein at this molecular weight (Fig. 14a). To confirm this result, an affinity chromatography was employed to specifically isolate AT_1-AA from the cord blood of fetuses from PE women. Only the eluted fraction (AT_1-AA) could detect a band at 43 kDa, corresponding to the AT_1 receptor, whereas the flow-through fraction did not. These findings suggest that specific IgG from PE women which bind to the AT_1 receptor cross the placenta and enter fetal circulation, where they can be physically detected.

To determine if the IgG which enter fetal circulation retain the biologic ability to activate AT_1 receptors, IgG isolated from cord blood were incubated with a reporter cell line in which activation of the AT_1 receptor results in increased luciferase reporter gene expression. The results showed that luciferase activity was increased only when cells were incubated with IgG isolated from preeclamptic cord blood (Fig. 14b). This activity was blocked by losartan (100nM), an AT_1 receptor antagonist, or the 7-aa peptide corresponding to the sequence on the second extracellular loop of the AT_1 receptor, and is the epitope of AT_1-AA. Similarly in using the fractions obtained in the affinity-purification experiment, only the fraction of IgG eluted from the cord blood of babies born to preeclamptic women increased luciferase activity, whereas the flow-through fraction from PE women or eluate from NT pregnant women could not. Taken together, these findings suggest that biologically active AT_1-AA from maternal circulation cross the placenta and enter fetal circulation where are capable of activating AT_1 receptors.

Human AT$_1$-AA cross the mouse placenta and enter fetal circulation.

To determine if human autoantibodies can cross the mouse placenta and enter into their fetal circulation, the autoantibody-injected model of PE was used. Briefly, pregnant mice were injected with total IgG from NT or PE pregnant women on E13 and E14. Upon sacrifice on E18, sera were obtained from the dams and fetuses and examined for the presence of human IgG by western blot and ELISA. The results (Fig. 15a-b) showed that human IgG was detectable in similar quantities in the sera of both the antibody-injected pregnant mice and their pups. Next, it was essential to determine if the human-derived PE-IgG retained its AT$_1$ receptor agonistic activity after crossing the mouse placenta and entering pup circulation. To do so, human IgG was isolated from mouse fetal circulation and assayed for its ability to activate AT$_1$ receptors using the AT$_1$ receptor activated luciferase reporter assay [34, 38]. The results indicate that IgG isolated from fetal blood of PE-IgG injected pregnant mice retained AT$_1$ receptor agonistic activity (Fig. 15c). This is in contrast to the fetuses of dams injected with NT-IgG, which harbored IgGs unable to stimulate luciferase activity. These data suggest that human AT$_1$-AA from women with PE can cross the mouse placenta, enter fetal mouse circulation and retain the biologic ability to activate AT$_1$ receptors.
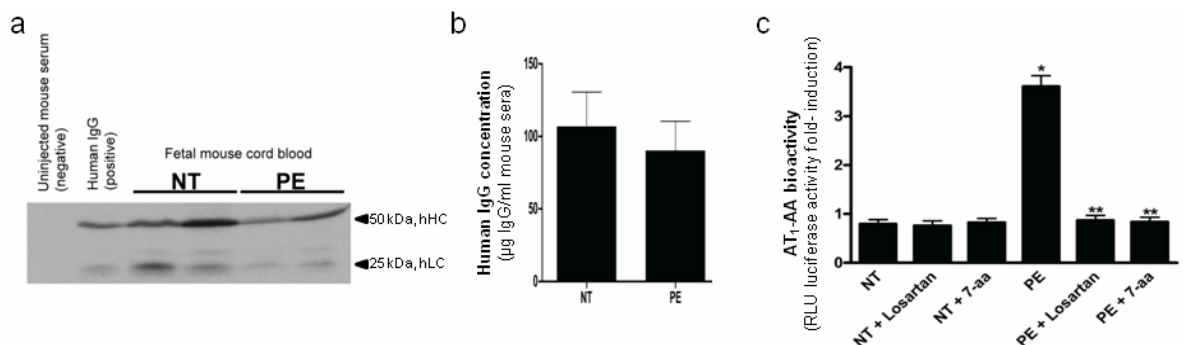
Figure 15: Human IgG passes through the mouse placenta and retains biologic activity. PE-IgG or NT-IgG were injected into pregnant mice. Upon sacrifice, fetal blood was collected. Human IgG was identified in pups of dams injected with either NT-IgG or PE-IgG, by (a) western blot, and (b) ELISA. The ELISA revealed no statistically significant difference of human IgG concentration between either cohort of animals (P=0.6139). IgG derived from the pups from PE-IgG injected dams were the only group which could stimulate AT$_1$ r' activated luciferase a bioassay. n=10 in each category. Data are expressed as mean ± SEM. *P<0.01 vs NT-IgG. **P<0.01 vs PE-IgG. hHC; human IgG heavy chain. hLC; human IgG light chain. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

Affinity-purified AT$_1$-AA injected into pregnant mice result in small fetuses with impaired organ development.

During the process of passive immunity, harmful autoantibodies may be conferred from mother to her developing child. In the case of autoimmune diseases, a potentially harmful autoantibody may enter fetal circulation and have a direct detrimental effect. This may be the case in PE, where it was just demonstrated that biologically active AT$_1$-AA cross the placenta and enter the fetal bloodstream. To investigate the profile of harm brought to the fetus by the autoantibody associated with PE, affinity-purified AT$_1$-AA were injected into pregnant mice on E13 and E14. The injected mice were examined on E18 for the clinical signs of PE and their fetuses were inspected for abnormalities of gestational growth. The results (Fig. 16) show that AT$_1$-AA-injected mice bore fetuses of reduced weight (1.01±0.02 g) compared to dams injected with NT-IgG (1.17±0.02 g). Co-injection of AT$_1$-AA with either losartan or 7-aa restored fetal weight to 1.119±0.01 g and 1.151±0.04 g, respectively.

	Normotensive	Preeclampsia
Fetal weight (g)	1.17 ± 0.02	1.01 ± 0.02*
Placental weight (g)	0.1039 ± 0.014	0.0939± 0.008*
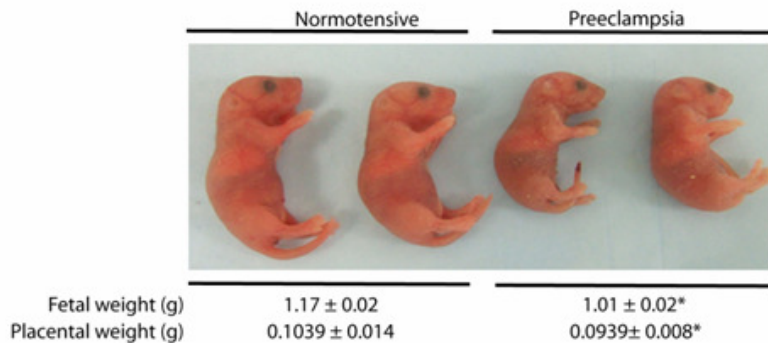
Figure 16: AT$_1$-AA reduces fetal weight. PE-IgG or NT-IgG were injected into pregnant mice. Their pups were weighed upon sacrifice. Pups of dams injected PE-IgG were smaller and weighed less than those born to NT-IgG injected animals. NT fetusues, n=80. PE fetusues, n=89. Data are expressed as mean ± SEM. *$P<0.01$ vs NT-IgG. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

In addition to their reduced weight, the fetuses born to AT$_1$-AA-injected mice exhibited delayed renal and hepatic maturation. Histologic analysis of fetal kidneys revealed a narrowed zone of nephrogenesis and a reduced number of glomeruli in the kidneys of fetuses born to

affinity-purified AT_1-AA-injected mice (Fig. 17a-c). These parameters are used to measure renal development [195], and their reductions suggest that of these kidneys was retarded.
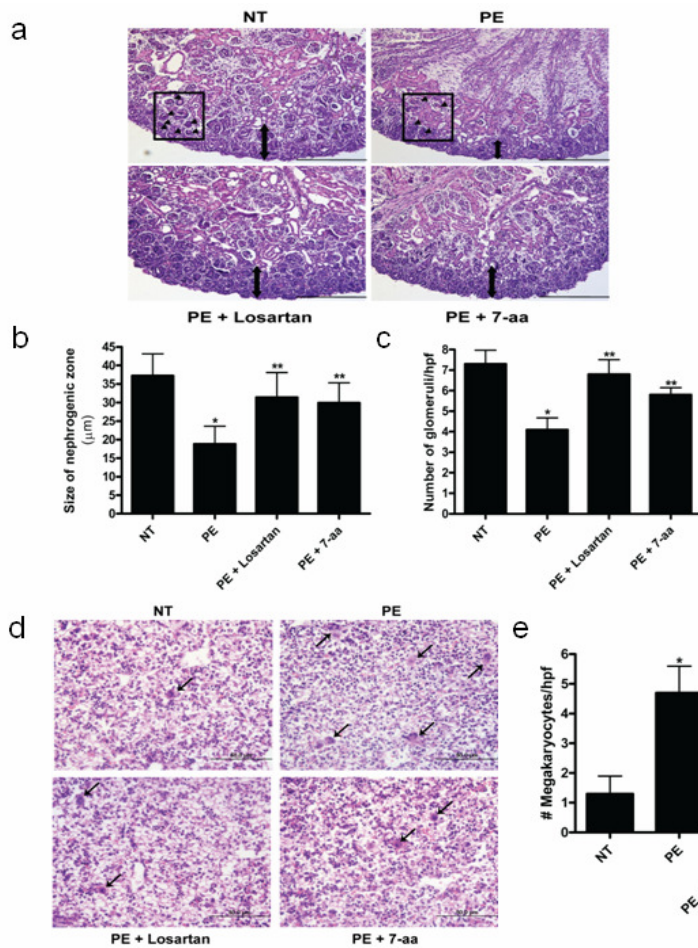
Figure 17: AT_1-AA impairs fetal organ development. The kidneys of pups born to PE-IgG injected mice display delayed maturation (a-c). The zone of nephrogensis (double-headed arrow) is narrowed (b) and the number of glomeruli (arrows) in these zones are reduced (c), as comapared to pups of NT-IgG injected mice. Livers of these pups also show developmental delay (d,e) as they retain elevated megakaryocytes (arrow). Scale bars, 50 µm. n=12 for each variable. Data are expressed as mean ± SEM. *$P<0.01$ vs NT-IgG. **$P<0.05$ vs PE-IgG. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

Similarly, the liver of pups born to AT_1-AA-injected dams showed developmental delay. During normal mouse gestation, the fetal liver is a major site of embryonic blood production. With advancing gestational age, the number of megakaryocyte progenitor cells begin to decrease, starting at E10 [196]. Our histologic analysis showed that the injection of affinity-purified AT_1-AA into pregnant mice is associated with an elevation of megakaryocytes in the fetal mouse liver (Fig. 17d-e). The persistence of megakaryocytes in the developing liver suggests a delay in typical organ maturation. Thus, the autoantibody-injection model of PE has provided evidence *in vivo* that AT_1-AA adversely affects fetal growth and organ development.

Notably, both human and murine studies indicate that biologically active AT_1-AA pass through the placenta and enter fetal circulation. These findings indicate a previously unrecognized underlying mechanism of the IUGR observed in PE: that AT_1-AA may have direct detrimental effects on fetal development.

AT_1-AA increases placental damage in mice and human villous explants.

Placental health is essential for normal fetal development. To determine if an impaired placenta is second potential underlying mechanism for AT_1-AA-induced IUGR, the weight and morphology of placentas in autoantibody-injected mice were analyzed. The placentas of AT_1-AA-injected pregnant mice were significantly smaller (0.0939 ± 0.008 g) than those of NT-IgG-injected mice (0.1039 ± 0.014 g), $P<0.05$. In addition, co-injection of AT_1-AA with either losartan or 7-aa restored placental weight to 0.0991 ± 0.009 g and 0.1050 ± 0.023 g, respectively.

To determine whether increased apoptosis is a potential cause of the small placentas observed in AT_1-AA-injected pregnant mice, histologic analysis and TUNEL staining for apoptotic cells were performed. Placental weight reduction was accompanied with increased apoptosis evident in the labyrinth zone of placentas from AT_1-AA-injected mice (Fig. 18a-b). In addition, western blot analysis of mouse placenta protein extracts indicated that Bax, a pro-apoptotic protein, was increased, and Bcl-2, an anti-apoptotic protein, was decreased (Fig. 18c-d). Co-injection of AT_1-AA with losartan or 7-aa significantly inhibited these features. Therefore, increased apoptosis could contribute to the reduction of placental size in AT_1-AA-injected pregnant mice. These findings also suggest that an impaired placenta may indirectly contribute to AT_1-AA-induced IUGR in this model.
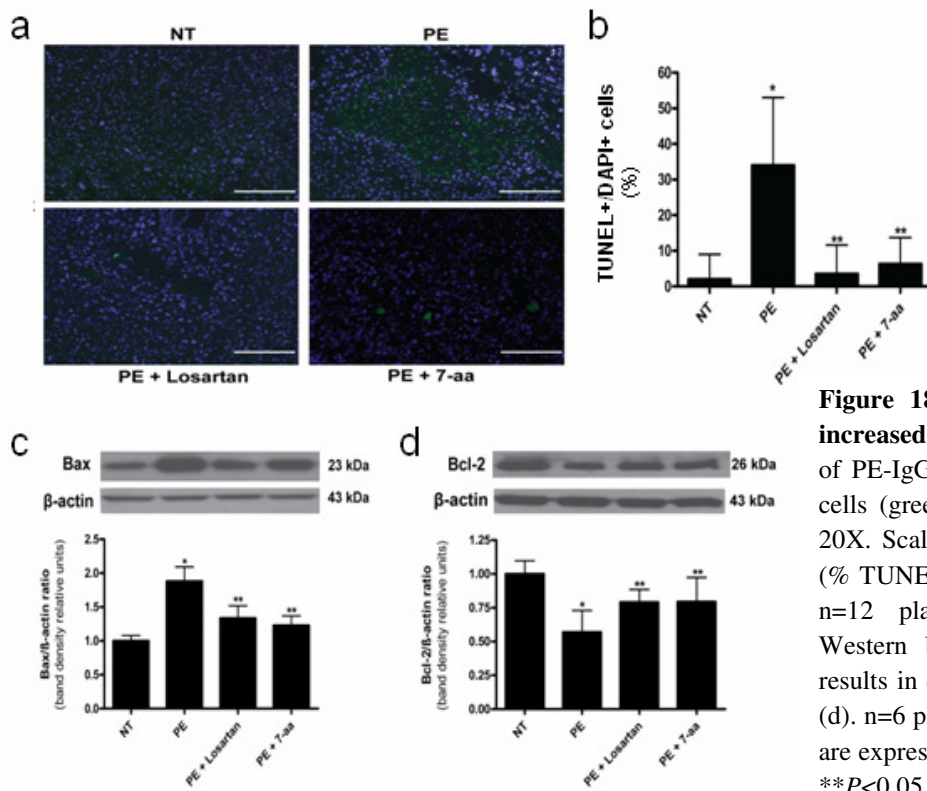
Figure 18: AT$_1$ receptor activation results in increased apoptosis in mouse placentas. Placentas of PE-IgG mice have elevated levels of TUNEL+ cells (green) in their LZ (a). DAPI+ cells (blue). 20X. Scale bar, 500µm. Quantified TUNEL index (% TUNEL+/DAPI+ cells) confirms the assay (b). n=12 placentas examined for each variable. Western blot analysis shows AT$_1$ r' activation results in elevated expression of Bax (c) and Bcl-2 (d). n=6 placentas examined for each variable. Data are expressed as mean ± SEM. *P<0.01 vs NT-IgG. **P<0.05 vs PE-IgG. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

Because apoptosis was increased in PE-IgG injected mice, the level of complement activity was also assessed. Complement factor C3 could be easily visualized in the placentas of PE-IgG injected mice (Fig 19, brown stain), especially in their junctional and labyrinth zones and around areas of calcification. This detectable level was qualitatively more evident in the
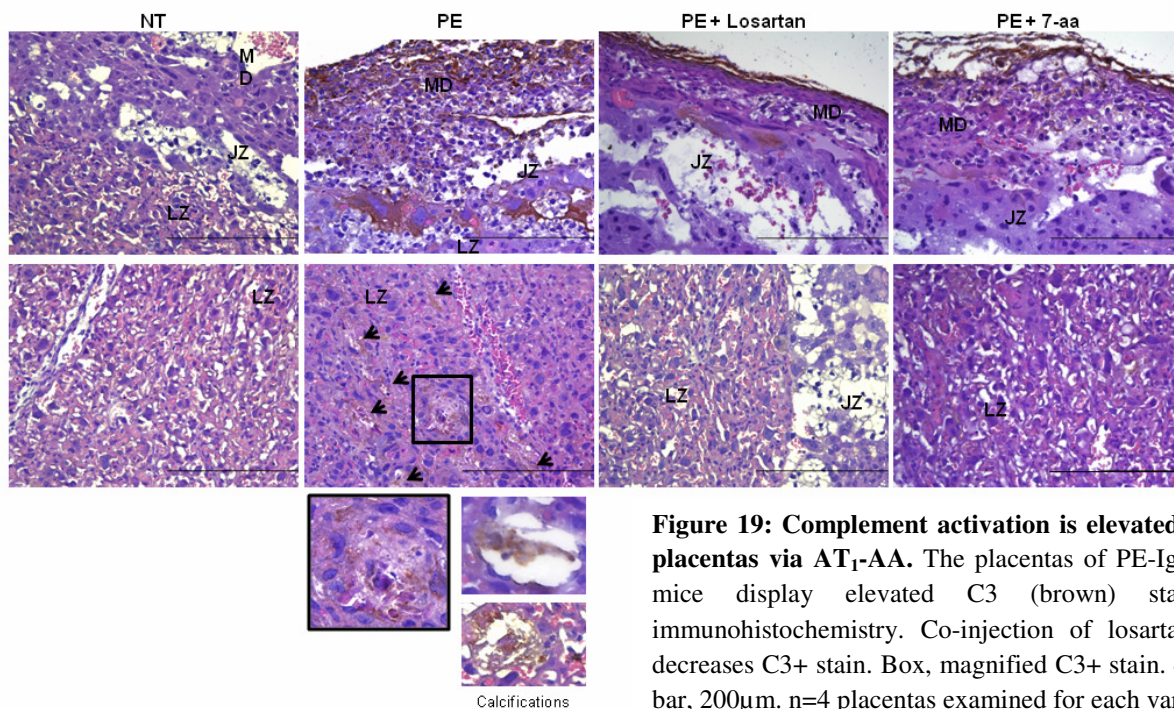
Figure 19: Complement activation is elevated in mouse placentas via AT$_1$-AA. The placentas of PE-IgG injected mice display elevated C3 (brown) staining by immunohistochemistry. Co-injection of losartan or 7-aa decreases C3+ stain. Box, magnified C3+ stain. 40X. Scale bar, 200µm. n=4 placentas examined for each variable.

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placentas of PE-IgG injected mice than in NT-IgG injected animals, and was reduced in those co-injected with the autoantibody and losartan or 7-aa. Then, to ascertain the level of angiogenesis in the placentas of adoptively-transferred dams, CD-31, an endothelial cell marker, was stained for by immunohistochemistry. The immunoreactivity of this marker was less prominent in the labyrinth zones of PE-IgG injected animals (Fig 20). Quantitative analysis of the immunostaining confirmed this observation (Fig. 20b). Co-injection of losartan or 7-aa with the autoantibody partially restored the level of detectable endothelial cells to that measurable in NT-IgG injected mouse placentas. Together, the histologic evidence suggests that AT_1-AA is capable of inducing significant placental damage when injected into pregnant mice.
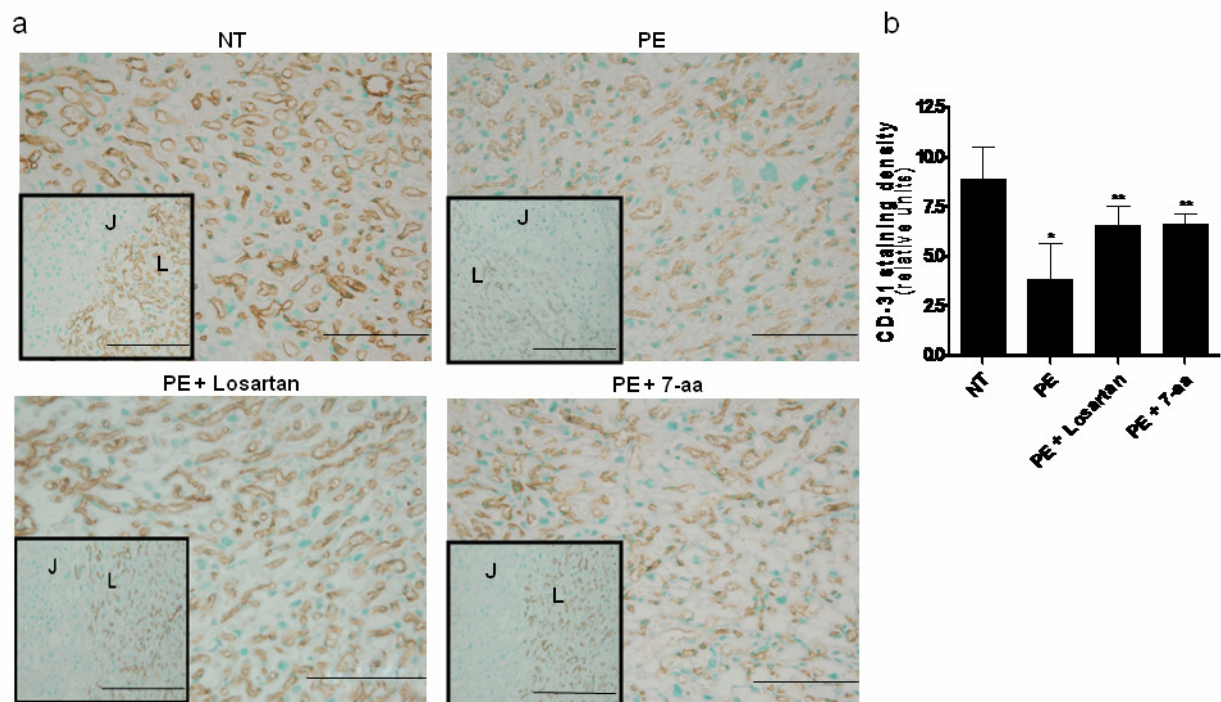
Figure 20: Angiogenesis is decreased in the placentas of PE-IgG injected mice. CD-31, an endothelial cell marker (brown), is less prominent in the labyrinth zone of the placentas of PE-IgG-injected mice in comparison to those injected with NT-IgG (a). Counterstain is methyl green. Inset box: Junctional (J) and Labyrinth (L) zone border. Blood vessels are not apparent in the junctional zone. n=4 placentas for each category. Scale bar: 50μm. Quantification of CD-31 confirms that the placentas of mice injected with PE-IgG are less densely stained than those injected with NT-IgG (b). Co-injection of losartan or 7-aa restores CD-31 positive staining. Mean scores are represented ± SEM. n=4 placentas for each category. * $P<0.01$ vs NT. **$P<0.05$ vs PE. ©Zhou *et al.*, 2000. Originally published in *Circulation*. doi: 10.1161/CIRCULATIONAHA.109.902890.

To evaluate the pathophysiologic significance AT_1-AA-induced placental damage in humans, human placental villous explants were obtained and cultured with NT-IgG or IgG derived from women with PE. Following incubation, the explants were embedded, sectioned and placed on slides in order to perform TUNEL staining. The results (Fig. 21) demonstrate that the presence of PE-IgG increased apoptosis in these explants. This autoantibody-induced increase in apoptosis was partially inhibited by co-incubation with losartan or 7-aa. The histologic evidence was corroborated with western blot analysis which indicated an increase in Bax and decrease in Bcl-2 proteins (Fig. 21c-d). Similarly, co-treatment with losartan or the autoantibody-neutralizing peptide partially abolished the PE-IgG-induced alterations in Bax and Bcl-2 proteins. These studies demonstrate that AT_1-AA are capable of increasing apoptosis in human placental explants through AT_1 receptor activation which may potentially contribute to IUGR associated with PE.
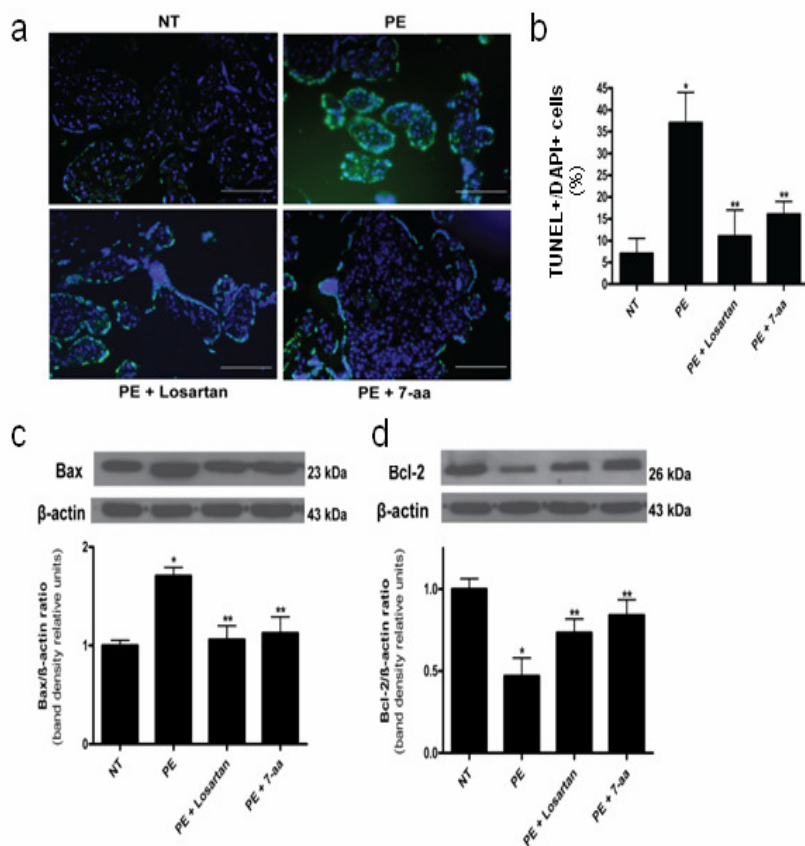
Figure 21: AT_1 receptor activation increased apoptosis in human villous explants. Cultured human placental explants incubated with PE-IgG demonstrate elevated levels of TUNEL+ cells (green), indicating increased apoptosis (a). DAPI+ cells (blue). 20X. Scale bar, 500µm. Quantification of a TUNEL index (% TUNEL+/DAPI+ cells) confirm the assay results (b). Western blot analysis reveals that AT_1 r' activation results in elevated expression of Bax (c) and Bcl-2 (d) in placental proteins. Co-incubation with losartan or 7-aa restores programmed cell death to a level similar to that observed in explants incubated with NT-IgG. Explants from 4 different patients were cultured, and each variable was examined 6 times per placenta, n=24. Data are expressed as mean ± SEM. *P<0.01 vs NT-IgG. **P<0.05 vs PE-IgG. ©Irani *et al.*, 2009. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

Apoptosis is induced in human trophoblasts via AT$_1$ receptor activation.

Human trophoblast cells maintain the full machinery for cell death by apoptosis [197-199] and they possess AT$_1$ receptors [3, 200]. To elucidate the mechanism of cell death which occurs in the placentas of preeclamptic women, the levels of programmed cell death in trophoblasts exposed to either PE or NT-IgG were monitored. IgG purified from preeclamptic patients induced apoptosis in an immortalized human trophoblast line, HTR-8/SVneo (Fig. 22). This is in contrast to NT-IgG, which was incapable of raising the level of apoptosis assessed by a TUNEL assay and an apoptotic index. These results suggest that excess AT$_1$ receptor activation can lead to cell death by apoptosis in human trophoblast cells. Consistent with the findings in the mouse placenta and in human placental villous explants, AT$_1$-AA-mediated increase in apoptosis in HTR-8/SVneo cells was inhibited by co-incubation with losartan or 7-aa.
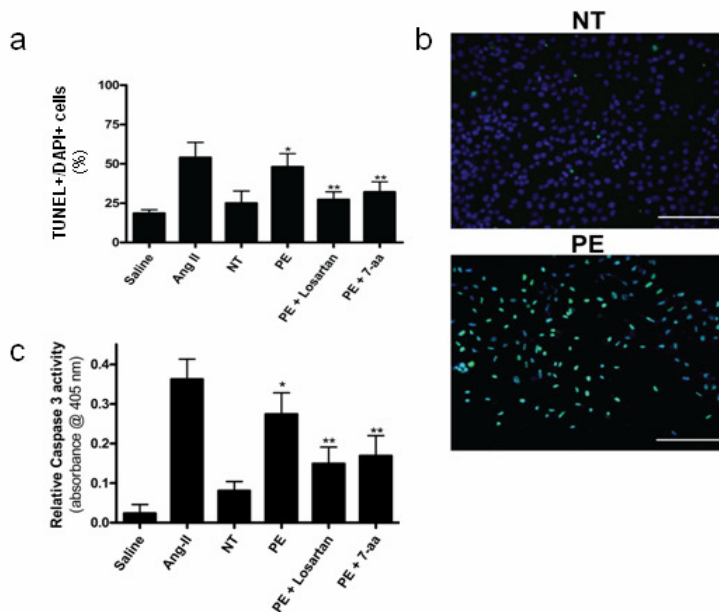
Figure 22: AT$_1$-AA induces trophoblast cell apoptosis. HTR-8/SVneo cells, cultured with PE-IgG demonstrate higher levels of TUNEL+ cells (green), indicating increased apoptosis (a-b) than do HTR-8 cells incubated with NT-IgG. DAPI+ cells (blue). 20X. Scale bar, 500µm. Caspase 3 activity is increased (c) in the PE-IgG treated cells. Co-incubation with losartan or 7-aa decreases programmed cell death and Caspase 3 activity to a level similar to that observed in cells exposed to NT-IgG. n=12 for each variable. Data are expressed as mean ± SEM. *$P<0.01$ vs NT-IgG. **$P<0.05$ vs PE-IgG. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

To corroborate the TUNEL assay results the activity of Caspase 3 was measured in cultured trophoblast cells exposed to AT$_1$-AA. Caspase 3 is a rapidly activated cysteine protease and an essential component of the apoptotic pathway. HTR-8/SVneo cells incubated with PE-IgG exhibited a higher Caspase 3 activity level over those incubated with NT-IgG

(Fig. 22c). Cultured trophoblast cells co-incubated with AT_1-AA and losartan or 7-aa significantly reduced Caspase 3 activity. This data supports the results obtained from the human placental explants experiments which demonstrated increased apoptosis upon exposure to the autoantibody. Overall, these findings provide strong *in vitro* evidence that the autoantibodies purified from the sera of preeclamptic women are capable inducing the programmed cell death of human trophoblast cells via excess AT_1 receptor activation.

Neutralization of AT_1-AA-mediated actions by losartan and 7-aa.

Lastly, to determine if AT_1-AA have a direct pathogenic role in the upregulation of the AT_1 receptor in PE-associated IUGR, losartan, a specific AT_1 receptor antagonist, was co-injected with purified AT_1-AA into pregnant mice on E13 and E14. Upon co-injection with the autoantibody, losartan was capable of attenuating the reduction in placental size and the AT_1-AA-induced placental apoptosis, as well as the increased expression of Bax and decreased expression of Bcl-2 (Fig. 18). This strategy to reduce AT_1 receptor activation by the autoantibody also ameliorated the reduction in fetal weight and poor maturation of the fetal kidneys and liver (Fig. 16). Taken together, these observations imply that the impaired fetal growth and increased placental apoptosis in this model were mediated by AT_1 receptor activation. Because the neutralizing peptide, 7-aa, blocked the apoptosis induced by AT_1-AA in cultured human placental explants (Fig. 21), this peptide could also be effective in blocking the placental apoptosis and IUGR observed in AT_1-AA-injected pregnant mice. Therefore, experiments were conducted in which pregnant mice were co-injected with AT_1-AA and excess 7-aa on E13 and E14, which would block the ability of the autoantibody to activate AT_1 receptors. On E18, the dams were sacrificed and their placentas and pups were collected. 7-aa

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decreased placental apoptosis and the alterations of apoptotic proteins, and improved the reduced fetal weights and impaired organ development (Figs. 16-20). The specific neutralizing effects of both losartan and 7-aa indicate that the autoantibody, through AT_1 receptor activation, contribute to the placental damage and IUGR associated with PE.

DISCUSSION

In this Chapter, I examine the capability of AT_1-AA to induce the placental damage and IUGR observed in PE. First, it was demonstrated that biologically active autoantibodies of preeclamptic women can cross the human placenta and enter into fetal circulation. Then, to examine the role of AT_1-AA in the fetal features of the disorder, the adoptive transfer mouse model of PE was employed. The fetuses of AT_1-AA-injected dams were small in size and showed delayed organ maturation, as compared to pups born to mice injected with IgG purified from normotensive pregnant women. The placentas of these mice were of reduced weight and demonstrated significant histopathologic changes, including apoptotic damage. Therefore, upon injection into pregnant mice, AT_1-AA may contribute to fetal growth restriction through two previously unrecognized mechanisms: (i) directly, via crossing the mouse placenta and entering fetal mouse circulation, and (ii) indirectly, via AT_1-AA-induced placental damage. Furthermore, autoantibody-induced fetal growth restriction and abnormal placentation were largely prevented by either losartan or an antibody-neutralizing epitope peptide, indicating that autoantibody-induced AT_1 receptor activation was required. These studies thereby reveal the detrimental role of AT_1-AA in PE-associated IUGR, offer two underlying mechanisms for this condition and suggest a novel preventative strategy. Though the significance of these findings must still be explored, this discovery highlights the important role of AT_1 receptor signaling in fetal development.

Antibodies generated by the mother can cross the placenta and enter into fetal circulation during the third trimester of pregnancy. The transfer of IgG from mother to her developing fetus is called passive immunity, and is a naturally occurring process. While usually

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of great benefit, passive immunity can put the growing fetus at risk if the mother harbors autoantibodies which are harmful. A well-recognized example of this is in Graves' disease. In this autoimmune disorder, maternal autoantibodies activate the thyroid stimulating hormone receptor and pass through the placenta and enter fetal circulation, resulting in hyperthyroidism of the fetus and newborn [201]. Other autoimmune diseases, such as antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE) and Sjogren's disease are also associated with passively transferred pathogenic autoantibodies which can result in major complications of pregnancy, like severe IUGR and fetal loss [202-205]. Because PE is also associated with deleterious maternal autoantibodies, it is possible that they may be transferred to the growing fetus and cause harm. Using the adoptive transfer model of PE, it is shown here that AT_1-AA are transported from maternal to fetal circulation where they can be physically and biologically detected. This implies that once the autoantibody reaches fetal circulation, it is free to bind directly with fetal cells possessing AT_1 receptors. In fact, the presence of the autoantibodies in fetal mouse circulation results in excess AT_1 receptor activation leading to smaller sized animals who suffer from renal and hepatic abnormalities. Thus, AT_1-AA may also have detrimental effects on the fetuses born to women suffering from PE.

An impaired placenta may be another underlying mechanism responsible for AT_1-AA-mediated IUGR in PE. When the autoantibody is injected into pregnant mice, their placentas are significantly smaller than normal and demonstrate vascular disorganization and calcifications, a sign of placental distress. Moreover, AT_1-AA induce apoptosis in a cultured human trophoblast cell line (HTR-8), cultured human villous explants, and the placentas of AT_1-AA-injected pregnant mice. Since AT_1 receptor stimulation by ANG II is capable of increasing cell death via pro-apoptotic pathways in both cardiac and renal systems [206, 207], and trophoblasts house AT_1 receptor as well as all apoptotic machinery [198, 208], it is not an

unexpected finding that AT_1-AA can induce placental cell death by excessive AT_1 receptor activation. Poor placental development is associated with a pro-apoptotic placental environment that includes the increased production of Bax and decreased production of Bcl-2 [209], features also observed in the placentas of AT_1-AA-injected pregnant mice. Taken together, these results indicate that this autoantibody can lead to significant placental damage.

As previously mentioned, complement activation is increased in preeclamptic women but the factors responsible for this induction remain unknown [143, 144]. Many groups have reported that ANG II-mediated AT_1 receptor stimulation leads to the activation of complement and organ damage [126, 128, 147]. In the placenta, highly active C3 is associated with the secretion of sFlt-1 and sEng, as well as the serious consequence of APS-induced fetal loss [203, 210]. Here, it is shown that the placentas of PE-IgG injected dams demonstrate elevated apoptotic activity, and therefore, it is not unreasonable that complement activity is also increased. Indeed, the placentas of these mice have elevated C3 activity in their junctional and labyrinth zones (Fig 19). NT-IgG injected mice did not demonstrate such an immunologic response in their placentas, and those mice injected with losartan or 7-aa displayed diminished C3 deposition. These facts indicate that AT_1 receptor activation by the autoantibody may contribute to placental damage through increased complement activity, in a similar manner to how it does in the kidney (see Chapter 3). The exact outcome of increased complement activity in the placentas of PE-IgG injected dams is the subject of ongoing work in the lab. It is, however, clear that the autoantibody induces heightened complement activation in the mouse placenta, implying that its blockade may prove beneficial in repairing AT_1-AA-induced placental damage. Furthermore, autoantibody-induced AT_1 receptor activation could contribute to placental damage through several other means, via decreased angiogenesis [24] and trophoblast invasion [76], or increased ROS production [211, 212] and thrombosis [21, 41]. To

determine if angiogenesis was decreased in the placentas of PE-IgG injected dams, CD-31, an endothelial cell marker, was stained for by immunohistochemistry. In these dams, the amount of CD-31 staining, representing the amount of placental endothelial cells, was reduced (Fig 20, brown stain). Dams co-injected with PE-IgG and losartan or 7-aa had CD-31 levels partially restored to that of the NT-IgG injected pregnant mice, suggesting the decrease in angiogenesis was mediated by AT_1-AA. Taken together, the autoantibody-induced increased cellular disorganization, calcification, apoptosis and complement activity, as well as decreased angiogenesis evident in the placentas of adoptively transferred mice indicate that AT_1-AA may contribute to similar features observed in the placentas of preeclamptic women.

These *in vitro* and *in vivo* studies support the findings of Takimoto *et al.* and Saito *et al.* who discovered that the placentas of transgenic female mice expressing human angiotensinogen which mated with males expressing human renin, are highly apoptotic and their pups suffer from severe IUGR [103, 104]. These dams have increased circulating ANG II and their placentas have increased renin levels. While these findings are significant, and have been paralleled to PE, the human disease is *not* associated with greatly increased ANG II levels [16] as in this double transgenic mouse model. However, human PE is associated with the presence of autoantibodies, AT_1-AA, which mimic the physiologic action of ANG II [33] and could explain this observation. Therefore, the added stimulation of the AT_1 receptor by the autoantibody may contribute to impaired placental development and the IUGR associated with PE. This hypothesis is supported by the work reported here and suggests that AT_1 receptor activation by the autoantibody is an underlying mechanism for the placental damage and the IUGR observed.

It is a widely recognized that the maternal features of PE are secondary to placental abnormalities, especially those stemming from placental ischemia [179, 213]. To investigate

this concept, Granger *et al.* developed a rat model of PE wherein placental ischemia is experimentally induced as a result of a surgical manipulation called reduced uterine perfusion pressure (RUPP) [214-216]. This group examined the placentas and factors in the maternal circulation of RUPP-treated rats with and found that sFlt-1 and inflammatory markers were elevated [217]. The RUPP-manipulated pregnant rats also developed preeclamptic-like syndrome including hypertension and proteinuria. Remarkably, these rats generated AT_1-AA [218], the same autoantibody found in the circulation of preeclamptic women. Consistent with the presence of AT_1-AA, when endothelial cells were cultured with the sera of RUPP-manipulated pregnant rats, endothelin-1 synthesis was increased by AT_1 receptor activation [216]. Endothelin-1 is a potent vasoconstrictor produced by endothelium which, in excess, can contribute to hypertension [219]. In that regard, Granger and colleagues treated RUPP-induced hypertension in two ways: using an antagonist to the AT_1 receptor [217], or to the ET_A endothelin receptor [220]. Both of these strategies attenuated the hypertension in the surgically manipulated pregnant rats, suggesting that RUPP-induced hypertension relies on AT_1-AA-mediated endothelin-1 synthesis. Collectively, these results indicate that autoantibody-induced factors liberated by an ischemic placenta play an important role in the maternal symptoms of PE [218].

The fetal abnormalities associated with PE most commonly occur in the severe early-onset form of the disease [221]. The placentas of these women are characteristically small and possess histopathologic evidence of ischemic change stemming from shallow trophoblast invasion and inadequate remodeling of the spiral arteries [169, 189]. A possible mechanism for the incomplete trophoblast invasion observed in PE is that a trophoblast population fails to migrate to the spiral arteries due to excessive programmed cell death [222, 223]. I illustrate here that autoantibodies present in the maternal circulation of women with PE can incite

apoptotic damage in mouse placentas, human villous explants and HTR-8 cells. Together, these results implicate a role for the autoantibody in the placental pathology associated with PE. This hypothesis is supported by the work of Walther *et al*., who also suggest that these pathogenic autoantibodies may contribute to the damage in the placentas of preeclamptic women when they occur in early pregnancy [149]. These investigators found that AT_1-AA were present by 18-22 weeks of gestation in women with impaired placental development as measured by abnormalities on Doppler ultrasound [224]. When followed to term, approximately 20% of these women developed PE, 20% developed IUGR without PE and 60% had an otherwise unremarkable pregnancy. AT_1-AA were not observed in women with a normal Doppler ultrasound. Therefore, AT_1-AA track with abnormal placental development, appear weeks before maternal symptoms appear, and could serve to identify women at risk for IUGR and/or PE. The authors of this study suggested, as our group had done earlier [23], that AT_1-AA may be responsible for the reduced trophoblast invasion and subsequent inadequate remodeling and dilation of spiral arteries resulting in impaired placental vascular development. This lack of vessel dilation could result in the hypoxic-ischemic damage detected in the placentas of preeclamptic women, which may contribute to the IUGR observed in their fetuses.

AT_1 receptor activation also regulates amino acid transport, whose reduction is another mechanism associated with the small placentas and IUGR of severe PE. Amino acids supply 20-40% of the energy needed for fetal growth [225]. If amino acid transportation in the placenta is impaired, the developing fetus may become nutritionally starved and at risk for growth defects. Many amino acid transport systems are Na^+-dependent and couple the movement of Na^+ into the cell with the uptake of amino acids. The Na^+-K^+-ATPase is an amino acid transporter which is highly abundant in most cell types, including the syncytiotrophoblasts of the placenta [226]. A syncytiotrophoblast maintains a low intracellular

Na^+ concentration by transporting the cation outside the cell. This creates a Na^+ gradient, which is the driving force for Na^+-dependent amino acid transport systems. Recent studies have shown that the Na^+-K^+-ATPase is downregulated in IUGR [227], and the inhibitory effect on Na^+-dependent amino acid transport systems is through AT_1 receptor activation. Another study by Shibata *et al.* reports that ANG II, through AT_1 receptor signaling, inhibits system A amino acid transporter activity in human placental villous fragments [4]. The system A amino acid transporter is a Na^+-dependent amino acid transporter which controls the movement of small neutral amino acids (alanine, serine, glutamine and glycine) in syncytiotrophoblasts. This work confirms that ANG II, via AT_1 receptor signaling, decreases system A activity by suppressing Na^+-K^+-ATPase activity in human placental villi. It is possible that other Na^+-dependent amino acid transport systems could also be inhibited by increased AT_1 receptor activation. Shibata *et al*. believe that their findings may account for the adverse affects of elevated AT_1 receptor activation on fetal growth. They specifically propose that one possible source of excess AT_1 receptor activation in IUGR associated with PE is the presence of maternal AT_1-AA. The work reported here adds support to their theory.

Other examples of IUGR involve the harmful effects of autoantibodies at the maternal-fetal interface, which often results in fetal loss. A well-characterized example of this is the antiphospholipid syndrome (APS). APS is a devastating disorder of pregnancy characterized by maternal thrombosis and recurrent fetal loss [228]. Recurrent fetal loss occurs in ~1% of pregnancies and ~20% of these women harbor anti-phospholipid antibodies (aPL) [229]. By injecting aPL into pregnant mice, Girardi *et al.* created a model of recurrent fetal demise. They show that these autoantibodies target the decidua at the maternal-fetal interface and instigate severe damage. Once bound to their epitope, aPL lead to the recruitment of neutrophils, complement activation and the enhanced production of TNF-α, several anti-angiogenic factors,

tissue factor, and ROS; all of which contribute to destruction of the decidua and fetal demise [230]. It is important to note that these same features can be induced by AT_1-AA in a variety of systems [24, 211, 212]. Thus, these two models of autoantibody adoptive transfer (AT_1-AA and aPL) induce IUGR accompanied by severe placental damage. They both demonstrate that immunologic factors, including those from innate and adaptive immunity, contribute to pregnancy loss and IUGR. In both scenarios, the sequence of events resulting in fetal complications is initiated by a deleterious maternal autoantibody. Together, AT_1-AA and aPL could additively account for one-third of IUGR and pregnancy loss cases [203]. In light of these findings, the role of autoantibodies in other cases of IUGR, impaired placental development and/or fetal loss warrants further investigation.

Autoantibody targeted therapies

In the work presented here, losartan and the 7-aa epitope peptide were used to assess the specificity of AT_1-AA-induced effects. Co-injection of the autoantibody with losartan, an AT_1 receptor blocker, resulted in diminished placental damage and fetal abnormalities. Consistent with the *in vivo* studies, apoptosis was reduced in human villous explants and trophoblast cell culture systems incubated with losartan and AT_1-AA. These results indicate that the observed effects were mediated via AT_1 receptor activation. Losartan is, however, contraindicated during the first trimester of pregnancy because of its fetotoxic effects [231, 232]. In lieu of using this AT_1 receptor blocker, drugs aimed at the specific neutralization of AT_1-AA would not be expected to cause harm to the developing fetus. When AT_1-AA was co-injected with the autoantibody neutralizaing epitope peptide, 7-aa, improvements in placental and fetal health were observed. This indicates that AT_1-AA is specifically responsible for the induction of the

AT_1 receptor. Taken together, the ability of losartan and 7-aa to reduce the harmful effects of AT_1-AA provides additional evidence that this autoantibody contributes to the IUGR and placental damage associated with PE. More importantly, the ability of 7-aa to neutralize AT_1-AA represents a potential preventative approach wherein blockade of autoantibody-mediated AT_1 receptor activation could reduce the incidence of IUGR associated with PE.

Impact and therapeutic implications

This study has great impact not only on the field of neonatology, but has far reaching implications to many other fields of medicine and science. Together, IUGR and SGA affect approximately 3% of all newborns and ~10% of those infants do not catch up in growth post-natally [233]. In general, those infants are at increased risk for future development of many metabolic disorders such as hypertension, dyslipidemia, Syndrome X, coronary heart disease, obesity, diabetes mellitus type II, impaired glucose tolerance [185-188, 234]. In the case of PE, many long-term follow-up studies report that the infants born to women suffering from the disease are at increased risk later in life for cardiovascular disease [235-237]. Therefore, this autoantibody-injected animal model with be very useful in pre-clinical trials to address neonatal issues, as well as the long-term study of the affected offspring. By identifying AT_1-AA a possible agent of IUGR and fetal maldevelopment, a wide range of therapeutic targets could be developed to improve fetal growth and possibly stave off future medical repercussions. By neutralizing the effects of the autoantibody-induced AT_1-receptor activation early on in the course of PE, physicians could not only improve the maternal symptoms, but also the detrimental effects on the fetus.

Conclusions

In conclusion, this study demonstrates that <u>AT$_1$-AA may contribute to IUGR in two ways: directly, by activating AT$_1$ receptors on multiple fetal organs, and indirectly, by inducing placental damage</u> (Fig. 23). Future work will have to delineate between the exact contributions of each mechanism. However, this study clearly identifies AT$_1$-AA as a detrimental factor which plays a role in IUGR. Furthermore, the blockade of excessive autoantibody-induced AT$_1$ receptor stimulation by losartan or 7-aa not only reduced the placental damaged observed in both mice and humans, but also the fetal abnormalities seen in AT$_1$-AA-injected mice. Thus,
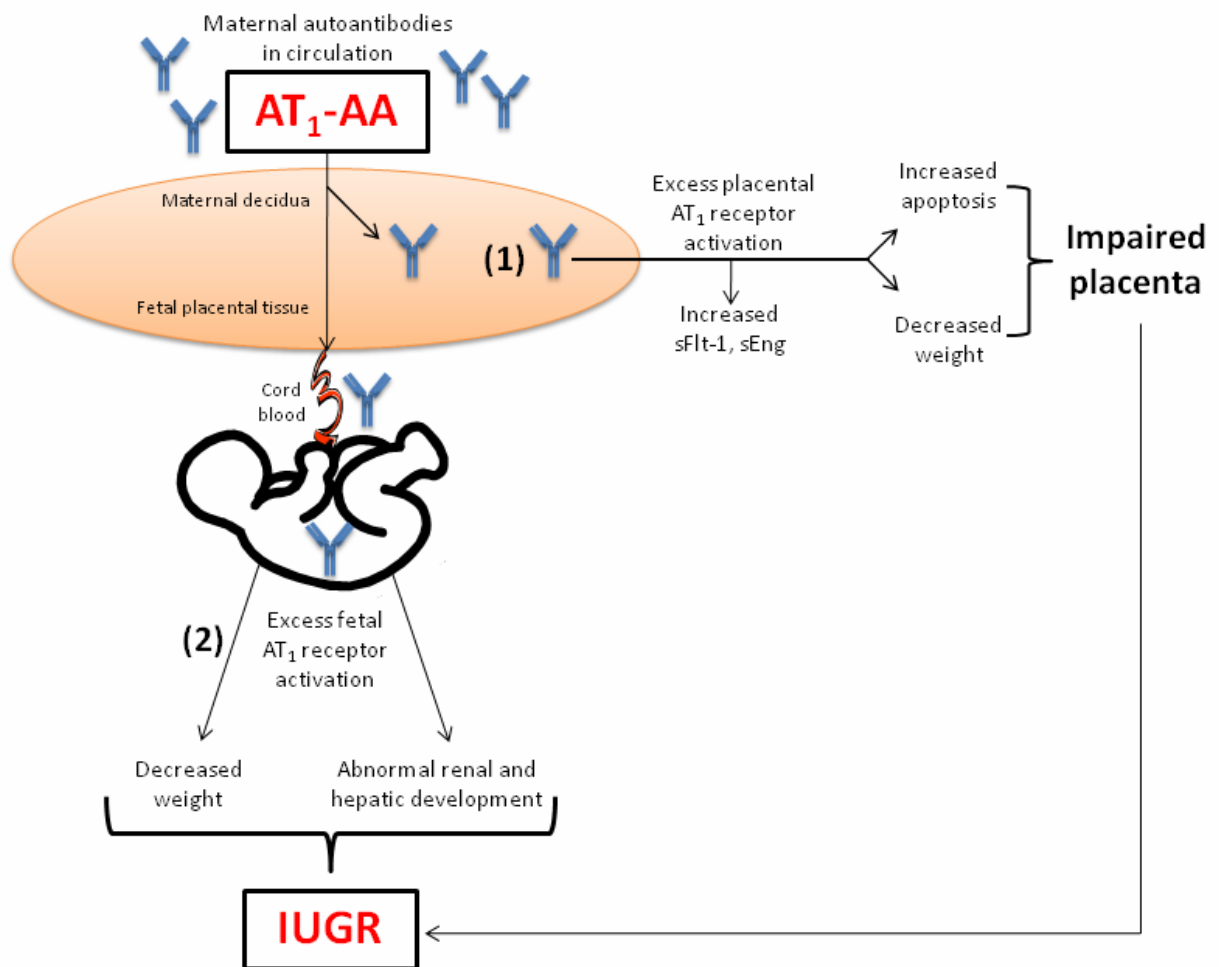
Figure 23: Working model of AT$_1$ receptor-mediated fetal and placental sequelae. AT$_1$-AA, found in maternal circulation, act (1) on the placenta to increase apoptosis and incite damage and (2) in the fetus itself, causing direct harm. ©Irani *et al.*, 2009. Originally published in *J Exp Med*. doi: 10.1084/jem.20090872.

this work identifies a detrimental role of AT_1-AA in PE-associated IUGR and reveals the underlying mechanisms for this process. Selective neutralization of the autoantibody would enhance physicians' ability to forestall IUGR and minimize fetotoxicity. Therefore, targeting AT_1-AA is a potentially important therapeutic strategy for the treatment of PE and its devastating fetal complications.

CHAPTER 5:

THE ESSENTIAL ROLE OF AT$_1$-AA-INDUCED TNF-α

IN A MOUSE MODEL OF PREECLAMPSIA

BACKGROUND

An increased maternal inflammatory response is associated with PE and has been speculated to contribute to the disease [238, 239]. A growing body of evidence supports the claims that there is an increase in pro-inflammatory cytokines and chemokines in the circulation of preeclamptic women [240, 241]. Some hypothesize that the activation of leukocytes and upregulation of certain cytokines propagate a state of chronic inflammation in some pregnant women which manifests in preeclamptic features [242, 243]. Increases in circulating tumor necrosis factor-α (TNF-α), interferon-γ (IFNγ) and interleukin-2 (IL-2) in preeclamptic women are well established [244-246]. In contrast, anti-inflammatory molecules, such as IL-10 and IL-4 are reportedly decreased in these patients [247, 248]. This kind of pro-inflammatory cytokine profile could contribute to the maternal features of PE in a variety of ways, such as inducing vascular and renal damage. However, what factors instigate this heightened inflammatory response in PE are unknown and the exact contribution of pro-inflammatory cytokines to symptom development remains undefined.

Of all the inflammatory cytokines, TNF-α has been widely implicated in the pathogenesis of PE. It is a potent cytokine with pleiotropic systemic effects [249] and is highly conserved between mice and humans [250]. It predominately binds to the constitutively expressed TNF-R1 in cells throughout the body in both species [251, 252]. In preeclamptic women TNF-α is increased in the circulation [253, 254] and placental tissues [40, 245, 255]. Schipper *et al.* have shown that TNF-α drastically elevated in PE with fetal involvement. These authors suggest that this increased production is related to impaired placentation [256]. Notably, when injected with low-dose exogenous TNF-α, pregnant rats develop the clinical

symptoms of PE, such as hypertension and proteinuria. This experiment implies a central role of this cytokine in the disease [217].

AT$_1$ receptor activation has been implicated in the increase of TNF-α and other inflammatory cytokines leading to organ damage in both cardiac and renal systems [206, 257-259]. Therefore, it is possible that through this mechanism, the autoantibody associated with PE, AT$_1$-AA, may contribute to the increased inflammation which characterizes the disease and instigate the vascular damage and systemic symptoms of preeclamptic women.

Chapter overview

Here, I explore if autoantibody-induced excess AT$_1$ receptor activation is the mechanism responsible for the increase in inflammation observed in preeclamptic women. I employ the AT$_1$-AA-injection pregnant mouse model, cultured human trophoblast cells and human villous explants to investigate the contributory role of the increased inflammatory response, specifically TNF-α, to disease symptoms and in the pathogenesis of PE. Moreover, I identify a potential therapeutic strategy to decrease maternal symptoms by the blockade of TNF-α action.

RESULTS

An increased inflammatory state is induced in AT$_1$-AA-injected pregnant mice.

A heightened inflammatory response is associated with PE and is hypothesized to contribute to disease pathophysiology [238, 260]. To determine the role of AT$_1$-AA in the increased inflammatory response seen in PE, IgG purified from normotensive (NT-IgG) or preeclamptic (PE-IgG) pregnant women was injected into pregnant mice at E13 and E14 as previously described [38]. Upon sacrifice on E18, the sera of antibody-injected pregnant mice were screened for the relative changes of a variety of cytokines using a sensitive multi-analyte inflammatory cytokine ELISA (Fig. 24a). Injection of PE-IgG, in contrast to that derived from normotensive pregnant women, resulted in a relative increase in pro-inflammatory cytokines (such as TNF-α, IFN-γ and IL-6) and a decrease in anti-inflammatory cytokines (such IL-10), which are similar to those changes reported in preeclamptic women [238, 241, 245, 261].

Circulating TNF-α is increased by AT$_1$ receptor activation in autoantibody-injected pregnant mice but not non-pregnant mice.

Among all the inflammatory molecules measured, TNF-α was the most elevated, and was therefore the cytokine pursued (Fig. 24a, box). The observed relative increase of TNF-α in autoantibody-injected pregnant mice was confirmed by quantifying its level using a commercially available ELISA (Fig. 24b). PE-IgG increased serum TNF-α in pregnant mice, as compared to NT-IgG (24.1±2.6 and 12.1±1.7 pg/ml, respectively). When PE-IgG was co-injected into pregnant mice with losartan, an AT$_1$ receptor blocker, or 7-aa, an autoantibody-neutralizing epitope peptide, the autoantibody-mediated induction of TNF-α was specifically
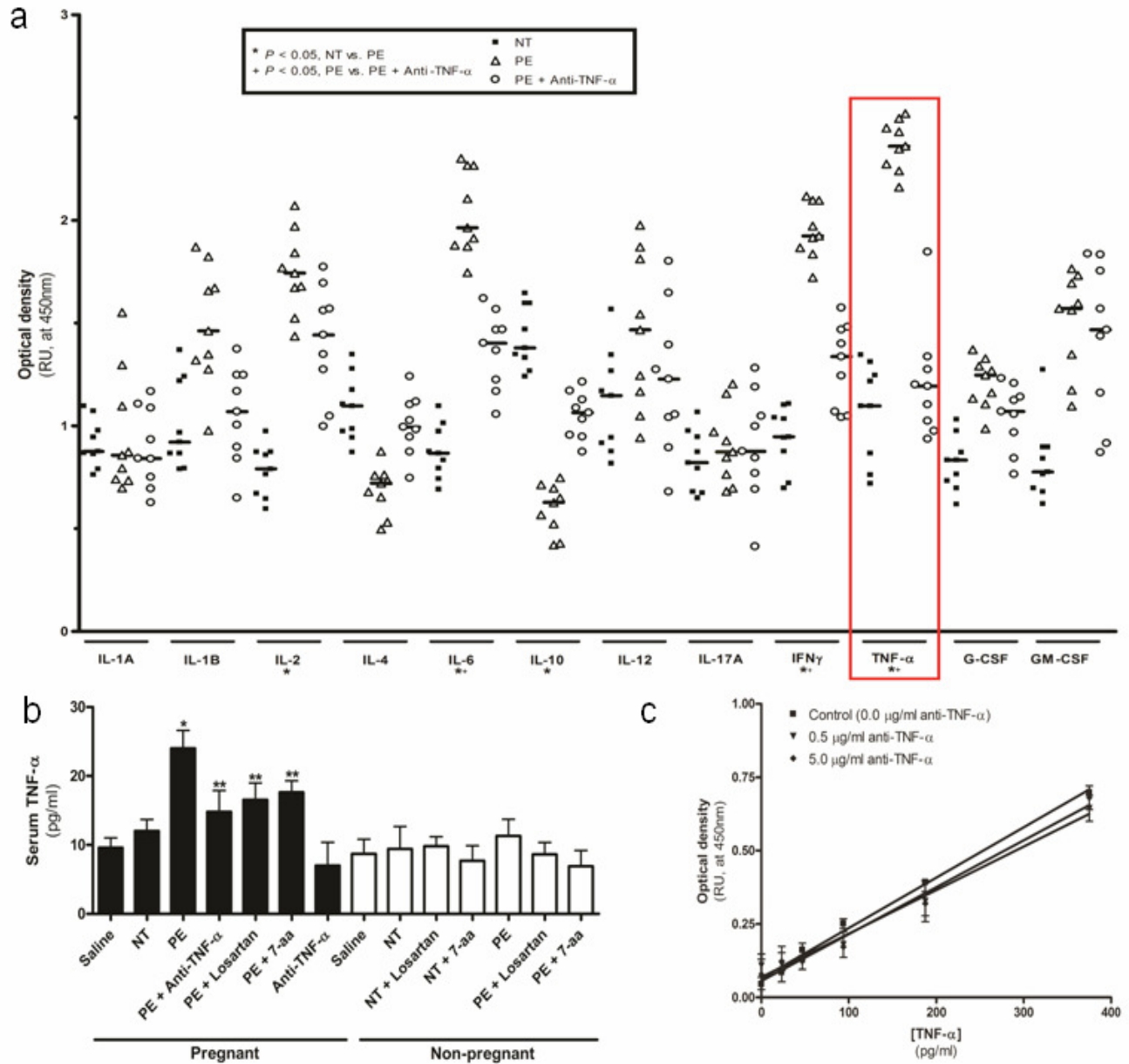
Figure 24: TNF-α regulaties the overall increased inflammatory response of AT₁-AA-injected pregnant mice.
Pregnant mice were injected with PE-IgG or NT-IgG on E13 and E14. Upon sacrifice on E18, their serum was screened for various cytokines using a sensitive assay. While NT-IgG injected mice have a predominantly anti-inflammatory profile, PE-IgG injected mice have a pro-inflammatory response (a). TNF-α was the most elevated of all the assayed molecules (box). Mice co-injected with AT₁-AA and a TNF-α neutralizing antibody demonstrated reductions in most pro-inflammatory cytokines. Further quantification (b) of serum TNF-α in these mice confirmed that the cytokine was elevated in PE-IgG injected pregnant mice and not in NT-IgG injected pregnant mice. Co-injection of losartan or 7-aa resulted in decreased serum TNF-α levels in PE-IgG injected pregnant mice. Non-pregnant animals injected with similarly purified human IgG fractions (white bars) did not demonstrate increased cytokine levels. n=9 for each variable. Using the ELISA employed to quantify mouse serum TNF-α, a standard curve was generated in the absence (control) or in the presence of two different doses of anti-TNF-α antibody (c). Using ANOVA and Tukey's post-hoc testing, there is no significant difference between the curves. *$P < 0.05$ vs NT. **$P < 0.05$ vs PE. Part b: ©Irani *et al.*, 2010. Originally published in *Hypertension*. doi: 10.1161/HYPERTENSIONAHA.110.150540.

inhibited. These results indicate AT_1-AA, through activation of the AT_1 receptor, could be responsible for the upregulation of TNF-α in pregnant mice.

To determine whether TNF-α induction by AT_1-AA *in vivo* is dependent upon pregnancy, PE-IgG or NT-IgG were injected into *non*-pregnant mice (Fig. 24b). Autoantibody-injected non-pregnant mice had lower levels of TNF-α than autoantibody-injected pregnant mice (11.3±2.4 and 24.1±2.6 pg/ml, respectively), and the level of TNF-α was not significantly higher in non-pregnant animals mice injected with either PE-IgG or NT-IgG (11.3±2.4 and 9.4±3.2 pg/ml, respectively). Thus, in contrast to what was observed in pregnant mice, circulating TNF-α did not increase in autoantibody-injected non-pregnant mice.

TNF-α blockade in autoantibody-injected pregnant mice reduces overall inflammatory response

To elucidate the critical role of TNF-α in the pathogenesis of PE, pregnant mice were co-injected with IgG derived from preeclamptic women and a TNF-α neutralizing antibody. At E18 the mice were sacrificed and their sera were collected for analysis using the multi-analyte ELISA array. This array revealed an unexpected finding: that the neutralization of TNF-α resulted in an increase in anti-inflammatory cytokines, such as IL-10, and a significant decrease of inflammatory cytokines including IL-6, IFN-γ and TNF-α itself in the autoantibody-injected pregnant mice (Fig. 24a). These results imply that TNF-α action is central to the recruitment of other inflammatory cytokines and that neutralization of its actions reduces this effect. Then, using a commercially available ELISA kit, it was quantitatively confirmed that the TNF-α neutralizing antibody attenuated the induction of the cytokine in the serum of autoantibody-injected pregnant mice (Fig. 24b).

Furthermore, to determine if the ELISA kit used measured only free, unbound TNF-α, or if it was capable of detecting the TNF-α bound to the anti-TNF-α antibody, a standard curve for the cytokine was generated in the absence or presence of varying amounts of the TNF-α blocker (0.0, 0.5 and 5.0 ng/ml) (Fig. 24c). The resultant curves showed no statistically significant differences. This finding suggests that any reductions of TNF-α observed using this ELISA are physiologic, and not due to interference of the neutralizing antibody.

Hypertension and proteinuria are reduced in AT$_1$-AA-injected pregnant mice due to TNF-α blockade

Because TNF-α is a key pro-inflammatory cytokine controlling the network of inflammatory cytokines in autoantibody-injected pregnant mice, it is possible that neutralizing TNF-α may also attenuate other maternal symptoms of PE observed in this model. The key diagnostic features, hypertension and proteinuria, were both partially attenuated in animals co-injected with PE-IgG and a TNF-α blocker as compared to pregnant mice injected with PE-IgG alone (Fig. 24). By E18, neutralization of TNF-α reduced hypertension from 132±4 to 110±4 mmHg and urinary protein 212±25 to 155±23 albumin (µg)/creatinine (mg) (both $P<0.05$). Pregnant mice injected with NT-IgG retained their baseline blood pressure and normal renal function, as did mice who were injected with the anti-TNF-α antibody alone. As a control, an isotype IgG was co-injected into pregnant mice along with PE-IgG. The isotype IgG was incapable of reducing the autoantibody-induced elevation in TNF-α. These findings provide direct evidence of the essential role of TNF-α in maternal key features of PE seen in autoantibody-injected pregnant mice.

Figure 25. TNF-α blockade reduces AT₁-AA induced preeclamptic-like features. To assess the pathophysiologic role of TNF-α in autoantibody-induced PE, hypertension and proteinuria were monitored in pregnant mice injected with PE-IgG, NT-IgG or were co-injected with PE-IgG and an anti-TNF-α antibody. The key features of PE, hypertension (a) and proteinuria (b), observed in the PE-IgG-injected pregnant mice were reduced with co-injection of the TNF-α blocker. In addition, sFlt-1 (c) and sEng (d) were also reduced by the TNF-α blocker. As a control, an isotype IgG to the TNF-α blocker was co-injected with PE-IgG and resulted in none of the changes observed. n=9 for each variable, except PE + Isotype, n=6. * $P<0.05$ vs NT **$P<0.05$ vs PE.
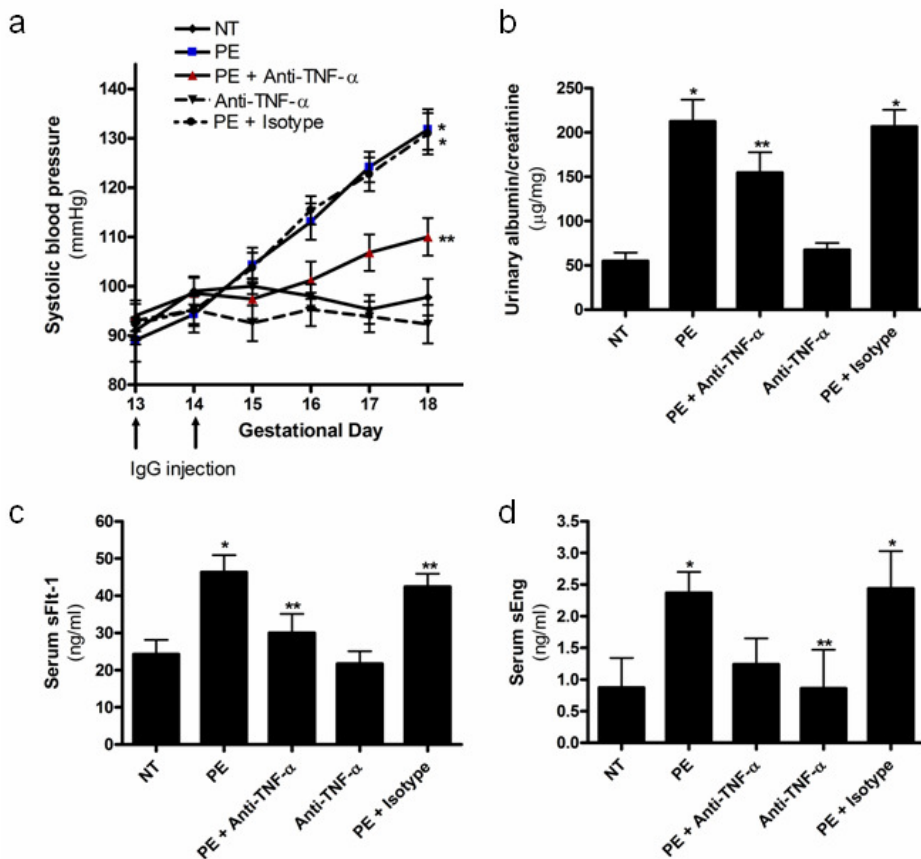
Increased TNF-α contributes to sFlt-1 and sEng induction in autoantibody-injected pregnant mice.

Two soluble factors, sFlt-1 and sEng, are elevated in preeclamptic women and are believed to contribute to hypertension and proteinuria [54, 61]. In Chapter 3, it was discussed how sFlt-1 is elevated in the autoantibody-injection model of PE in the pregnant mouse [38]. To determine if TNF-α contributes to autoantibody-induced sFlt-1 and sEng, the circulating concentration of both factors were assessed in antibody-injected animals. Injection of PE-IgG increased the serum levels of sFlt-1 and sEng, and co-injection of with an anti-TNF-α antibody significantly reduced the levels of sFlt-1 and sEng to values closer to those observed in mice injected with NT-IgG (Figs. 25c-d). An isotype IgG to the TNF-α blocker was incapable of achieving similar reductions, and when the anti-TNF-α antibody was injected into pregnant mice alone the serum concentrations of these two factors remain unaltered. Therefore, AT₁–

AA-induced TNF-α may contribute to the increased production of anti-angiogenic factors in this mouse model of PE, and that this induction may be attenuated by the presence of TNF-α blocking antibodies.

Autoantibody-induced TNF-α contributes to the renal abnormalities associated with PE.

Renal abnormalities are commonly associated with PE in the human disease [262-264] and these features are also seen in the antibody-injection model of PE in pregnant mice [38]. As such, I used the animal model to investigate the contribution of TNF-α to these kidney defects. Histologic evidence indicates that the glomeruli of PE-IgG-injected pregnant mice are smaller and hypercellular as compared to those of mice injected with IgG from normotensive women, whose glomeruli are open and easily distinguished (Fig. 26a). When AT_1-AA was co-injected with an antibody against TNF-α, the renal morphology was partially restored to normal. The
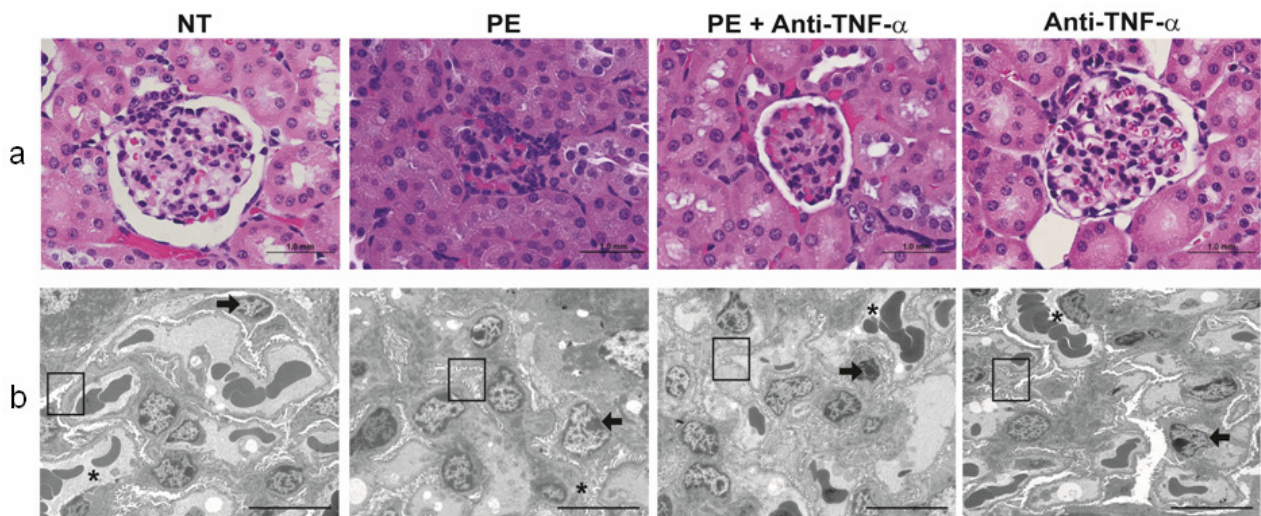
Figure 26. Autoantibody-induced renal damage is reduced by TNF-α blockade. Pregnant mice injected on E13 and E14 with NT-IgG, PE-IgG, or co-injected with PE-IgG and an anti-TNF-α antibody were sacrificed on E18. Their kidneys were harvested and fixed for either routine H&E staining (a) or transmission electron microscopy (TEM) (b). H&E staining demonstrates that the condensed, hypercellular glomeruli of the PE-IgG injected pregnant mouse are partially restored in when co-injected with the autoantibody and the TNF-α blocker. 100X. TEM demonstrates that the glomerular endotheliosis observed in the PE-IgG injected mice is reduced in the co-injected group. The kidneys of NT-IgG injected mice or mice injected with the TNF-α blocker alone are unremarkable. 1500X, scale bar=10 µm. n=6 for each variable. Box, intact podocytes. (*) capillary space. Thick arrow, endothelial cell nucleus. ©Irani *et al.*, 2010. Originally published in *Hypertension*. doi: 10.1161/HYPERTENSIONAHA.110.150540.

glomeruli of mice injected with NT-Ig did not display any renal morphologic changes. The light microscopy data was informative; however the hallmark renal lesion of PE, glomerular endotheliosis, can only be confirmed by transmission electron microscopy (TEM). TEM analysis indicates that this feature is present in autoantibody-injected mice (Fig. 26b). The glomeruli of these mice show endothelial swelling resulting in the occlusion of capillary loop spaces. Co-injection of autoantibody with an anti-TNF-α antibody partly restores the kidney phenotype resulting in capillary spaces which are partially opened as compared to the glomeruli of mice injected with NT-IgG, whose capillary spaces are wide and show no swelling or obstruction.

To confirm that the histologic changes in the kidneys of the autoantibody-injected mice were not secondary to the deposition of a human IgG complex, an immunofluorescence study using an antibody against human IgG was performed (Fig. 27). Renal sections from human kidney tissue involved by lupus nephritis, which are known to demonstrate IgG immune complex deposition [265], were used as positive controls. The glomeruli of these patients show granular staining for IgG in the glomerular basement membrane and the mesangium. Pregnant mice injected with IgG purified either from NT or PE pregnant women did not demonstrate staining consistent with immune complex deposition. This implies that the histologic changes observed in the autoantibody-injected mice were not due to antibody-antigen complex deposition in the kidney. Taken together, these findings illustrate that TNF-α is a downstream signaling molecule of the AT_1 receptor contributing to renal abnormalities associated with PE and that TNF-α blockade may decrease the maternal renal symptoms associated with AT_1-AA-induced PE.
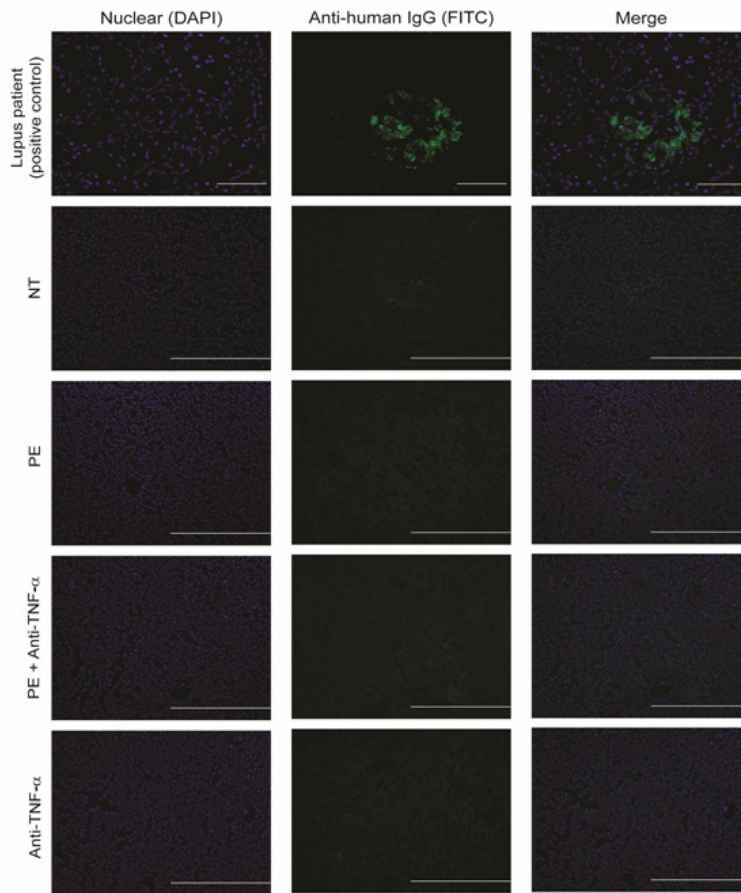
Figure 27: Injection of human IgG into pregnant mice does not result in renal immune complex deposition. Immunofluoresence using an anti-human IgG antibody shows no immune complex deposition (FITC, green) in the kidneys of pregnant mice injected with human antibody derived from either NT or PE pregnant women. Co-injection of human IgG with a TNF-α blocker, or the TNF-α blocker alone also demonstrated no deposits. A renal sections from human lupus patients known to have immune complex deposition were used positive controls, n=2. All other variables, n=4.

AT$_1$-AA-induced placental abnormalities in pregnant mice are reduced by TNF-α blockade.

Placental abnormalities are also associated with PE [266-268]. These features are also observed in autoantibody-injected pregnant mice [38], and so it is therefore a convenient model to evaluate the contribution of AT$_1$-AA-induced TNF-α on the placental abnormalities associated with PE. Pregnant mice were injected with PE-IgG or NT-IgG on E13 and E14 and the injected mice were sacrificed five days later, at which time their placentas were isolated and characterized. Routine H&E staining (Fig. 28a) demonstrated that the labyrinth zones of the placentas of mice injected with IgG derived from preeclamptic women had placental calcifications, a hallmark of placental distress, and centers of fibrinoid necrosis similar to that of acute atherosis, a feature observed in human placentas from women with PE [269, 270]. The

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placentas of mice injected with IgG from normotensive pregnant women had undamaged placentas free from calcifications and fibrinous centers. Notably, co-injection of pregnant mice with PE-IgG and an anti-TNF-α antibody reduced the histopathologic changes observed in the placentas of PE-IgG injected animals. These results indicate that AT_1 receptor activation can induce placental damage and that these changes can be reduced by blocking TNF-α action.
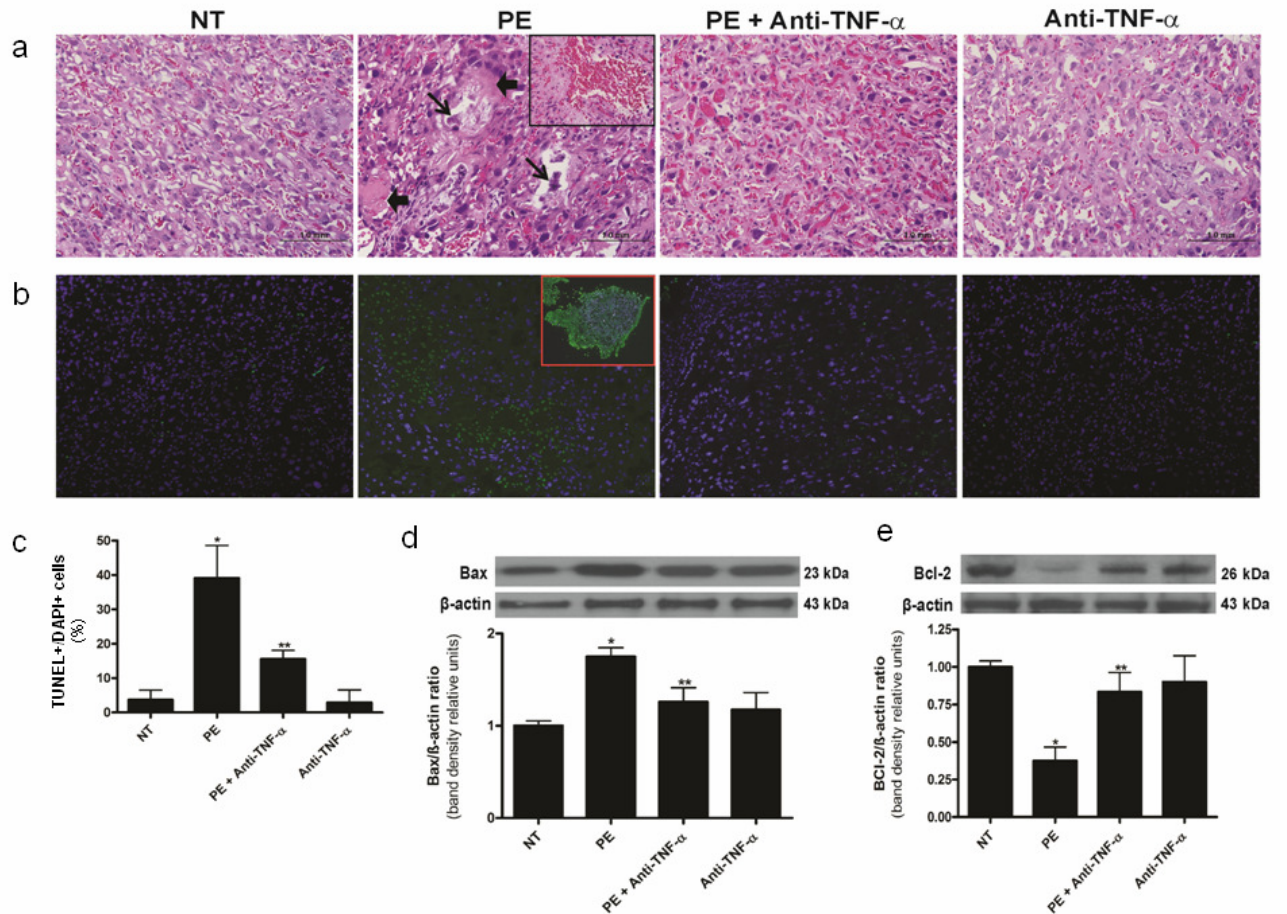
Figure 28. Autoantibody-induced placental damage can be reduced by TNF-α blockade. Pregnant mice were injected with NT-IgG, PE-IgG, or co-injected with PE-IgG and a TNF-α blocking antibody. Their placentas were assessed by H&E staining, 40X (a). PE-IgG injected mice had evidence of damaged placentas: calcifications (thin arrow) and fibrotic areas (thick arrow). Their labyrinth zones appear heterogeneous and have abnormal pools of blood (inset box). NT-IgG injected mice have unremarkable labyrinth zones and animals co-injected with PE-IgG and the TNF-α blocker have reduced placental damage. Placental apoptosis was assessed by TUNEL staining (b). 10X. Scale bar, 1mm. PE-IgG injected mice had increased apoptosis in their labyrinth zones as compared to NT-IgG injected animals. Quantification of the TUNEL assay (c) indicates a reduction in the TUNEL+ cells in pregnant mice injected with co-injected with PE-IgG and a TNF-α blocker as compared to the PE-IgG alone. Mice injected with the anti-TNF-α antibody alone had unremarkable placentas. n = 9 for each variable. Green; TUNEL+ cells. Blue; DAPI+ nuclei. Western blot densitometric analysis of placental protein extracts confirm the TUNEL findings. Bax (d) was increased and Bcl-2 (e) was decreased in PE-IgG injected mice and partially restored in those animals co-injected with the TNF-α blocker. n=6 for each variable. *$P<0.05$ vs NT. **$P<0.05$ vs PE. ©Irani *et al.*, 2010. Originally published in *Hypertension*. doi: 10.1161/HYPERTENSIONAHA.110.150540.

Small placentas and fetuses are often associated with PE [169, 182, 189, 190]. Therefore, we assessed both of these parameters. Placental weights of AT_1-AA-injected pregnant mice were smaller (0.0921±0.017 g) than placentas from mice injected with NT-IgG (0.1143±0.022 g). Co-injection of an anti-TNF-α antibody restored the autoantibody-induced placental weight reductions to 0.0991 ± 0.042 g. In addition, the weight of fetuses born in litters of 6-8 was analyzed. Autoantibody-injected mice bore fetuses of less weight (1.06±0.19 g) as compared to dams injected with NT-IgG (1.24±0.06 g). Co-injecting AT_1-AA with a TNF-α blocker restored fetal size to 1.11±0.43 g. As compared to the NT-IgG-injected animals, injection of the anti-TNF-α antibody alone had no statistically significant effect on placental or fetal weight (0.1157±0.048 g and 1.27±0.10 g, respectively). Fetal and placental pairs: PE, n=46; NT, n=53; PE + Anti-TNF-α, n=37; Anti-TNF-α alone, n=34. Overall, the autoantibody induced reductions in placental and fetal weights were restored by co-injection of a TNF-α blocker, implying an important role for this cytokine in the regulation of these effects.

Because TNF-α is a potent pro-apoptotic factor and increased placental apoptosis is associated with PE [245, 253, 271], I investigated the level of apoptosis in the placenta of autoantibody-injected pregnant mice. An increase in programmed cell death was observed in the labyrinth zone of placentas from AT_1-AA-injected mice as seen by quantified TUNEL staining (Figs. 28b-c). This was further confirmed western blot analysis of Bax and Bcl-2, two apoptotic regulatory proteins. An increase in Bax, a pro-apoptotic protein, and a decrease in Bcl-2, an anti-apoptotic protein, were observed (Figs. 28d-e). The degree of apoptosis was reduced in the placentas of mice co-injected with PE-IgG and the anti-TNF-α antibody. Mice injected with IgG from normotensive pregnant women, did not show increased apoptosis. This evidence confirms the fact that AT_1 receptor activation can increase mouse placental damage and TNF-α blockade can reduce these detrimental effects.

A cohort of preeclamptic patients has increased TNF-α levels correlating to AT$_1$-AA bioactivity.

From the mouse work presented here, it has been established that AT$_1$-AA contributes to the induction of TNF-α in pregnant mice. To determine if a relationship exists between the autoantibody and TNF-α in humans, serum levels of TNF-α was compared to AT$_1$–AA bioactivity in normotensive pregnant women (n=16) and women with PE (n=20). First, it was confirmed that the preeclamptic population had increased serum TNF-α. Similar to other reports [238, 241, 245, 253, 272], the results (Fig. 29a, Table 2) reflected that circulating TNF-α is increased in preeclamptic patients, and its mean concentration was higher than that found of normotensive pregnant women (48±3 and 16±3 pg/ml, respectively, $P<0.001$). Five of the sixteen normotensive pregnant patients and none of the twenty preeclamptic women had undetectable levels of the cytokine, which is also consistent with other studies [245, 272]. These findings confirm that serum TNF-α is increased in the cohort of preeclamptic women studied here.
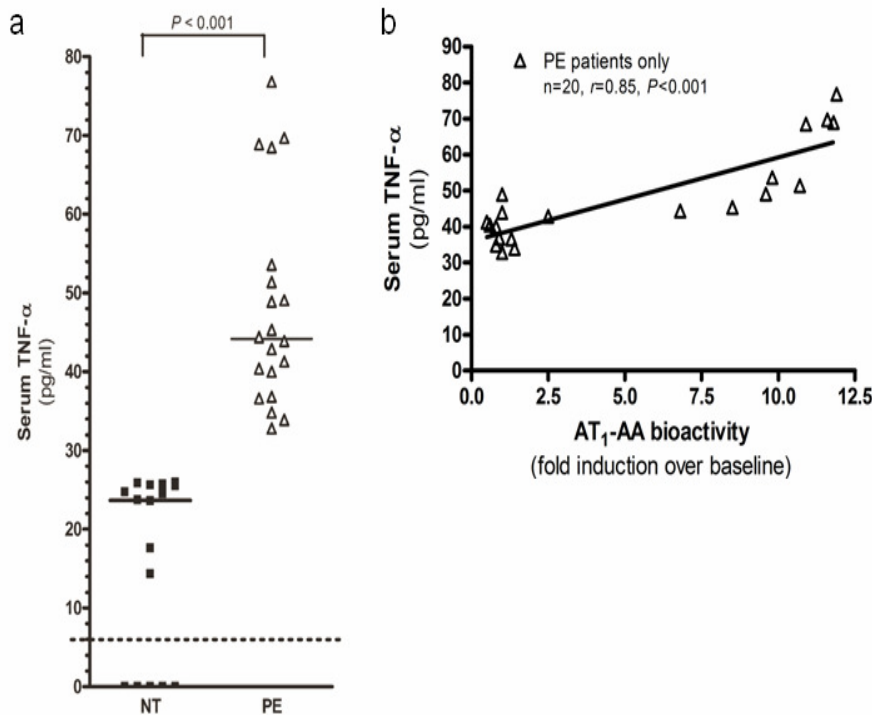
Figure 29: Circulating TNF-α positively correlates to AT$_1$-AA bioactivity in preeclamptic women. Preeclamptic patients (n=20) used in the study demonstrated an elevated level of serum TNF-α (a). Normotensive (NT) patients (n=16) did not demonstrate elevated cytokine levels, and 5 of 16 patients did not have detectable levels. Solid line indicates the median concentration. The dotted line indicates the lowest detectable threshold of the assay. A positive correlation (b) between AT$_1$-AA bioactivity, as assessed by an *in vitro* cell culture luciferase reporter assay, and serum TNF-α level in preeclamptic women was identified (r=0.85, n=20, $P<0.001$). ©Irani *et al.*, 2010. Originally published in *Hypertension*. doi: 10.1161/HYPERTENSIONAHA.110.150540.

Next, the bioactivity level of AT_1-AA in the sera of women with PE and NT pregnant women were determined by testing sera-derived purified IgGs for the ability to activate AT_1 receptors using an established luciferase reporter gene system [38]. The purified IgGs isolated from patient sera were incubated with a reporter cell line in which AT_1 receptor activation results in the stimulation of luciferase activity. Intriguingly, there was a positive correlation between AT_1-AA bioactivity and circulating TNF-α level in preeclamptic women (Fig. 29b, r=0.85, n=20, P<0.001). These data confirm earlier reports that preeclamptic patients harbor increased levels of AT_1-AA and show for the first time that AT_1-AA bioactivity is correlated to serum TNF-α in preeclamptic women. Thus, it is possible that AT_1-AA-mediated induction of TNF-α may significantly contribute to the pathophysiology of the disorder in humans.

AT_1 receptor-mediated TNF-α induction contributes to placental apoptosis and sFlt-1 and sEng secretion in human villous explants.

Because no elevation of the cytokine was observed in non-pregnant animals injected with the autoantibody, it is likely that the placenta contributes to the production of autoantibody-induced TNF-α. As such, human placental villous explants were used to assess the direct role of AT_1-AA in TNF-α production in human. Placental explants incubated with PE-IgG showed an increase in secreted TNF-α, whereas the cytokine was not induced in explants incubated with NT-IgG (913.1±62.3 and 250.6±21.6 pg/ml, respectively) (Fig. 30a). AT_1 receptor activation was required for TNF-α secretion, as co-incubation of PE-IgG with either losartan, an AT_1 receptor blocker, or a 7-aa epitope peptide which neutralizes autoantibody action, attenuated the induction of TNF-α levels (214.4±24.1 and 506.4±163.8

pg/ml, respectively). Thus, IgG purified from women with PE is capable of inducing TNF-α secretion via AT_1 receptor activation from human placental villous explants.

To elucidate the pathophysiologic consequences of AT_1-AA-induced TNF-α production by the placenta, human placental explants and the explant culture medium were examined for
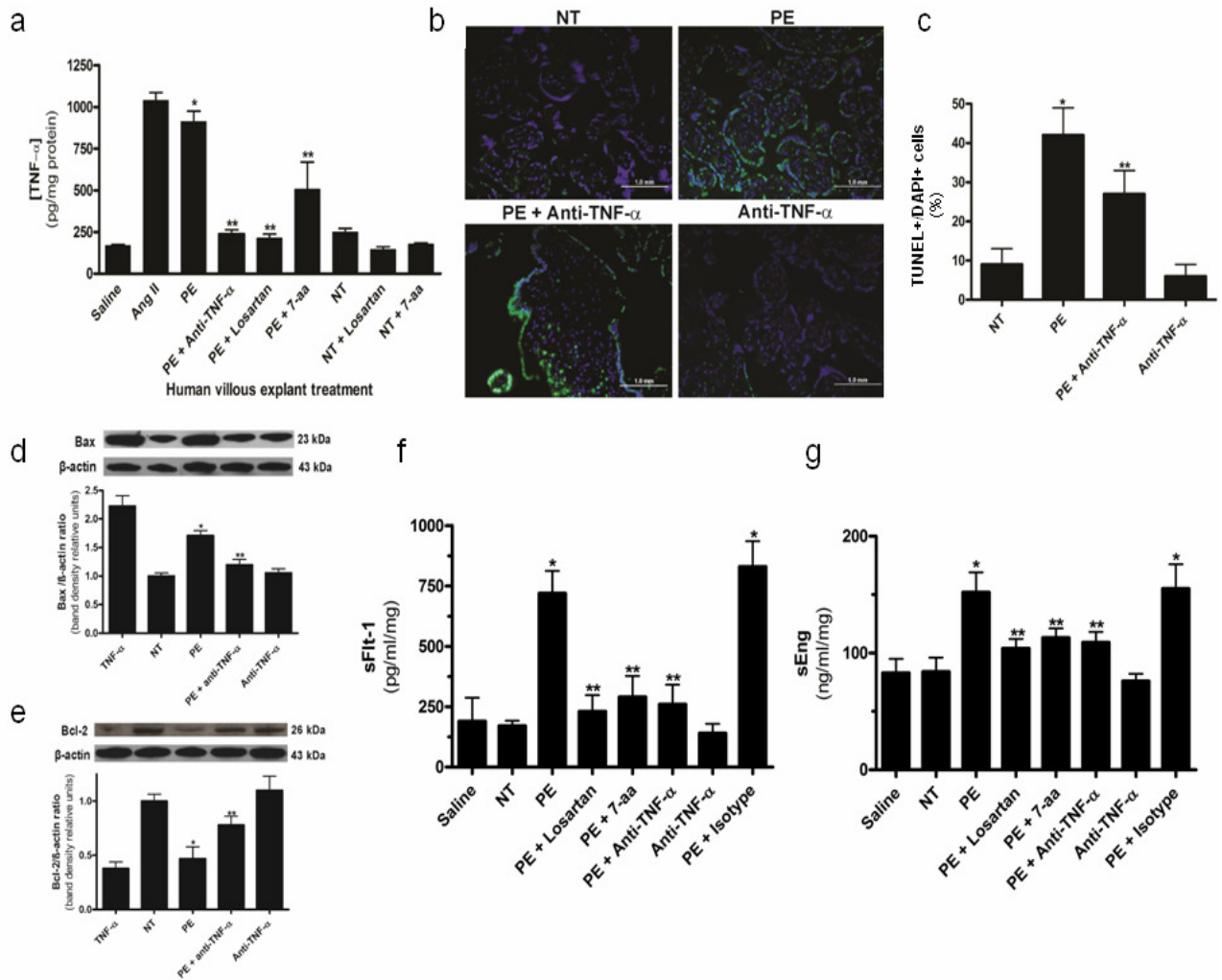
Figure 30: **TNF-α blockade reduces AT_1 receptor-mediated placental damage in human villous explants.** Culturing PE-IgG with human villous explants resulted in TNF-α secretion via AT_1 receptor activation (a). Co-culturing the explants with PE-IgG and losartan (5μM) or 7-aa (1μM) reduced the cytokine level, specifically indicating that autoantibody-induced AT_1-receptor activation was required. Incubation of the explants with NT-IgG did not increase TNF-α secretion. Apoptosis was increased in explants incubated with AT_1-AA and was partially diminished by blocking TNF-α activity (b, c). Explants cultured with NT-IgG demonstrated decreased cell death. TUNEL stained cultured human villous explants (b). Green; TUNEL+. Blue; DAPI+. 10X. Quantification of TUNEL staining (c) indicates that co-incubation with PE-IgG and an anti-TNF-α agent (5μg/ml) reduces the amount of apoptosis. Western blot densitometric analysis of explant proteins reflects an increase in Bax (d) and a decrease in Bcl-2 (e). In addition, sFlt-1 (f) and sEng (g) secretion were reduced by co-incubation of PE-IgG with an anti-TNF-α antibody. 6 different placentas were collected, and from each n=4, total n=24 for every variable. *$P<0.05$ vs NT. ** $P<0.05$ vs PE. ©Irani *et al.*, 2010. Originally published in *Hypertension*. doi: 10.1161/HYPERTENSIONAHA.110.150540.

pathological changes associated with PE. Placental explants were incubated with IgG purified from normotensive pregnant women and preeclamptic women in the presence or absence of a TNF-α neutralizing antibody. The level of apoptosis was determined by a TUNEL assay conducted on thin sections of fixed placental tissue. The results (Figs. 30b-c) show that explants exposed to PE-IgG demonstrated an increase in placental apoptosis which was blocked by the presence of a TNF-α blocking antibody. Placental fragments incubated with NT-IgG did not show significant apoptosis. This evidence was corroborated with western blot analysis indicating an increase in Bax and decrease in Bcl-2 proteins, resulting in a pro-apoptotic state in these explants (Figs. 30d-e). These results suggest that autoantibody-induced placental cell apoptosis is mediated through the action of TNF-α. Experiments were also conducted to determine the role of TNF-α in autoantibody-mediated induction of the placental derived anti-angiogenic factors, sFlt-1 and sEng. The results (Figs. 30f-g) show that the autoantibody-mediated increases in sFlt-1 and sEng were reduced by TNF-α blockade. These findings are consistent with those observed in the mouse model and suggest that AT_1-AA-induced TNF-α mediates sFlt-1 and sEng production.

DISCUSSION

In this study, it was identified for the first time that an elevated TNF-α level is correlated to AT_1-AA bioactivity in preeclamptic women and provided both *in vitro* human studies and *in vivo* mouse evidence that AT_1-AA is a novel candidate directly inducing TNF-α production via AT_1 receptor activation. Neutralizing AT_1-AA-mediated TNF-α induction attenuates the increased placenta apoptosis and sFlt-1 and sEng secretion by cultured human villous explants. Moreover, TNF-α blockade ameliorates all of the key features associated with PE seen in autoantibody-injected pregnant mice *in vivo*. Both the mouse and human studies reported here provide strong evidence that AT_1 receptor activation by the autoantibody induces TNF-α, and that the increased TNF-α production is an underlying mechanism contributing to pathogenesis of the disease. Overall, these studies have identified the essential role of AT_1-AA-induced TNF-α production in PE and demonstrated the importance of this cytokine in the pathogenesis of the disorder. These findings suggest a novel therapeutic option for the complicated management of this serious condition.

The increased maternal inflammatory response associated with PE is speculated to contribute to the pathogenesis in the disease [247, 273]. However, the direct cause of the increased inflammatory cytokine production is unknown and the pathogenic role of these inflammatory cytokines is undetermined. Multiple *in vitro* studies demonstrate that increased inflammatory cytokine production may lead to endothelial dysfunction, increased placenta apoptosis, decreased angiogenesis and kidney abnormalities that are relevant to the pathophysiology of the disease [242, 274, 275]. Because PE is a multisystem disorder, using animal models to understand the cellular interplay is an essential step towards deciphering the

specific signaling pathways involved. However, there are few animal models of PE available and none of them have delineated the cause of the increased inflammatory response and its pathogenic functions. Here, using a novel autoantibody-induced model of PE in pregnant mice, it was shown that autoantibody-mediated AT_1 receptor activation induces TNF-α, among other inflammatory cytokines, and that its production through this mechanism is pregnancy-dependent. The results of this screen imply that the autoantibodies from preeclamptic women are capable of inducing a pro-inflammatory response in pregnant mice. Since IgG purified from normotensive pregnant women did not elicit the same cytokine profile, the effect can be attributed to the autoantibody itself and not a non-specific immunologic response.

Next, it was demonstrated that TNF-α blockade attenuates autoantibody-induced preeclamptic features in AT_1-AA-injected pregnant mice, including hypertension and proteinuria as well as reducing circulating TNF-α itself. Using an antibody competition strategy, the evidence provided shows that the anti-TNF-α neutralizing antibody does not interfere with the measurement of TNF-α concentration by the ELISA used in the study. Thus, this finding indicates that anti-TNF-α antibody treatment decreases TNF-α induction in autoantibody-injected pregnant mice. Using a multi-analyte inflammatory cytokine ELISA, additional evidence shows that anti-TNF-α antibody treatment decreases other pro-inflammatory molecules observed in AT_1-AA-injected mice. So, without interference, TNF-α-induced cell damage and inflammation create a detrimental cycle, facilitating further cell damage and inflammation. However, in the presence of an anti-TNF-α antibody which neutralizes TNF-α effects, this damage is decreased, slowing the malicious cycle and results in blocking the heightened inflammatory network. Thus, it was revealed for the first time that AT_1-AA is a key mediator in inducing the inflammatory cascade in PE and that TNF-α blockade can attenuate this response.

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The direct role of TNF-α in disease mechanism

Although a potential role of TNF-α induction in hypertension and proteinuria seen in PE has been suggested, the pathogenic mechanisms underlying these effects have not been clearly identified. Earlier studies have shown that the pro-inflammatory TNF-α, a 51 kDa homotrimeric protein in its soluble form, is associated with both vascular damage and hypertension [276]. Jovinge *et al.* have shown that mice deficient in TNF-α have reduced atherosclerotic lesions, suggesting that the cytokine plays a key role in vascular injury [277]. Similarly, in salt-sensitive rats, TNF-α blockade has been successful in alleviating both the hypertension and renal damage observed in this model [278]. In pregnant rats, TNF-α enhances contraction and inhibits endothelial nitric oxide-cGMP-mediated relaxation in systemic vessels, which could contribute to hypertension [279]. Chronic infusion of TNF-α into pregnant rats to achieve a two-fold increase in concentration is sufficient to induce hypertension and increase endothelin production, which the authors believe contributes to the vascular damage associated with the maternal symptoms of PE [220]. These examples illustrate that the inflammatory properties of TNF-α contribute to vascular damage and high blood pressure and could therefore do the same in the situation of PE.

In addition, Muller *et al.* report a double transgenic rat model that has increased levels of circulating ANG II which exhibits hypertension, renal dysfunction as well as increased TNF-α [126]. In this model, the authors believe that increased TNF-α contributes to kidney injury via complement activation and that excess ANG II sensitizes the vasculature to the effects of the cytokine. The induction of TNF-α in the autoantibody-injection model of PE is accompanied with an autoantibody-mediated increases in sFlt-1 and sEng. Others have also shown that sFlt-1 and sEng are induced by TNF-α [97, 280]. Thus, in view of the known hypertensive and renal effects of sFlt-1 and sEng, it is possible that the hypertensive and renal effects of TNF-α are

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mediated through the increase of these soluble factors. The results of the animal model studies reported here provide evidence to support the novel concept that autoantibody-mediated AT_1 receptor activation induces TNF-α production which results in the maternal features of PE including hypertension and proteinuria.

Besides the direct detrimental effects of TNF-α on maternal features, TNF-α has long been implicated in the placental damage associated with PE [208, 209]. Though the nature of placental damage is well-established in PE, the exact role and specific source of TNF-α remain undefined. In this study, it was confirmed that TNF-α is increased in the serum of the preeclamptic patient cohort studied, but also revealed that these elevated levels of TNF-α are correlated to the AT_1-AA bioactivity in these preeclamptic individuals. Using non-pregnant mice it was demonstrated that AT_1-AA-mediated TNF-α induction is pregnancy-dependent, which implies that the placenta is a major source for increased TNF-α seen in circulation. Supporting this mouse study, it was shown here that AT_1-AA are also capable of inducing TNF-α in cultured human villous explants. More importantly, both mouse and human studies reveal the novel role of AT_1-AA-mediated TNF-α induction in placental abnormalities seen in PE including increased apoptosis, as well as sFlt-1 and sEng secretion. These findings are supported by earlier studies showing that TNF-α is not only increased in the serum of preeclamptic women, but also locally in the placenta where villous cytotrophoblasts express TNF-receptor 1 (TNF-R1) and house all the machinery necessary to carry out programmed cell death [198, 281]. Reister, *et al.* demonstrate that increased TNF-α generated by macrophages found in the placentas of preeclamptic women leads to apoptosis in extravillous trophoblasts [282]. More importantly, recent studies demonstrate that TNF-α directly induces the detrimental anti-angiogenic factors, sFlt-1 and sEng, in cultured human villous explants [97, 280]. Overall, these studies are in agreement with the current findings and suggest an essential

role of AT_1-AA-induced TNF-α in both the maternal symptoms and placental features observed in PE.

The indirect role of TNF-α in disease mechanisms

In other disease models it is unclear whether TNF-α has only a direct pathogenic role or whether it contributes to disease features indirectly via the induction of other mediators. For example, in rheumatoid arthritis, TNF-α induces the expression of other pro-inflammatory cytokines, such as IL-1 which initiates a potent inflammatory cascade [283, 284]. Therefore, in this situation, the blockade of TNF-α action decreases production of downstream mediators and arthritic features are indirectly abrogated. As demonstrated by the inflammatory cytokine array (Fig, 24), this may also be the case in PE.

It is clear through the evidence presented here that reducing TNF-α action significantly attenuates the key preeclamptic symptoms initiated by AT_1-AA in pregnant mice, indicating an essential role for this cytokine in PE, be it directly or indirectly. In the placenta, decreasing TNF-α production may directly reduce the amount of trophoblast apoptosis and result in a healthier organ (Figs. 28, 30). By limiting placental damage, reductions in TNF-α may also decrease the release of key anti-angiogenic factors, sFlt-1 and sEng (Fig. 30). With little increase in these factors, the subsequent maternal vascular and renal damage may be reduced thereby alleviating maternal symptoms. As described earlier, by decreasing circulating TNF-α, other inflammatory mediators, such as IFN-γ, are also reduced. This leads to less TNF-α-driven vascular injury via the initiation of inflammatory cascades. Should these pathways not be instigated, then the endothelial damage associated with PE may not be as severe, and the symptoms may be lessened. Together, these scenarios indicate that TNF-α may be directly or

indirectly contributing to preeclamptic features and regardless, its blockade can reduce their severity.

It should not be overlooked that AT_1-AA alone may contribute directly to certain features of PE which are independent of TNF-α. For example, the autoantibody can directly stimulate the AT_1 receptors of vascular smooth muscle cells and induce vasoconstriction [285, 286]. Likewise, the autoantibody could activate AT_1 receptors on endothelial cells resulting in the synthesis of endothelin-1, a powerful vasoconstrictive agent [219, 287]. The autoantibody may also directly bind to AT_1 receptors on renal mesangial cells to induce PAI-1 secretion [21]. Therefore, it is not surprising that TNF-α blockade only partially relieves autoantibody-induced features of PE, including the partial attenuation of hypertension and proteinuria observed in the pregnant mice co-injected with AT_1-AA and the anti-TNF-α antibody (Fig. 25).

TNF-α and hypertension

In general, increased inflammation has been implicated in the pathogenesis of cardiovascular injury and the development of hypertension. Immunosuppressed HIV+ patients display hypertension upon aggressive anti-retroviral therapy which restores their depleted cytokine-inducing CD4 T cell count [288]. Cancer patients infused with alloactivated T cells which mount an inflammatory response also develop hypertension [289]. Reduced systemic inflammation via thymectomy or pharmologic interventions decrease blood pressure in genetically modified hypertensive rats [290-292]. More specifically, elevations in the pro-inflammatory cytokine TNF-α have been reported in patients with severe atherosclerosis [293], congestive heart failure [294] and other forms of hypertension [295]. It has been proposed that this pro-inflammatory cytokine plays a role in both the initiation and amplification of

inflammatory cascades [296, 297]. The work reported here supports these claims, as it was shown that TNF-α blockade can significantly reduce the circulating levels of other inflammatory cytokines in PE-IgG pregnant mice (Fig. 24). There are many proposed mechanisms by which TNF-α brings about vascular injury: by induction of other cytokines, such as IL-6 [298], IL-1 [284]; increasing endothelin-1 [299, 300]; causing aberrant angiogenesis [301] and the inhibition of endothelial-dependent release of nitric oxide and vasodilation [302, 303]. All of these mechanisms reportedly contribute to systemic inflammation and the subsequent hypertension observed in patients. Interestingly, many of these same vascular injuries are observed in PE as well as other autoimmune diseases such as rheumatoid arthritis, as well as through AT_1 receptor activation [73, 304-307]. The mechanistic relationship between hypertension, autoimmunity and inflammation is a topic of great interest and will surely be the subject of exciting future work.

Several small-scale clinical trials have been performed to investigate the capability of TNF-α blockade in the treatment of cardiovascular disease. Hurlimann *et al.* have demonstrated that patients with rheumatoid arthritis receiving a TNF-α blocker have improved vascular function and decreased progression towards atherosclerotic disease and hypertension over those patients not receiving this drug [308]. Others have shown that anti-TNF-α treatment in rheumatoid patients improves various cardiovascular outcomes, whereas untreated groups suffer from a 5-fold increase in cardiovascular mortality [309, 310]. Fichtlscherer *et al.* observed improved cardiovascular functional status in patients suffering from chronic heart failure administered with a TNF-α blocker [311]. In addition, cardiovascular disease including the incidence of PE is elevated in the case of SLE and rheumatoid arthritis [312, 313], implying that autoimmune processes may play a role in the manifestations of disease symptoms.

Together, these reports suggest that TNF-α plays a pivotal role in the inflammation associated with hypertension, and reduction in its function may forestall symptom development.

Anti-TNF-α agents: a novel therapy for PE?

The current therapy for PE is extremely limited. There is no absolute cure for the disorder. While physicians use anti-seizure medications to stave off severe ecclamptic symptoms, delivery of the baby and placenta is the only way to abate progressing symptoms. There is a dire need to improve the current therapy of this hypertensive complication which is the indication for 15% of all preterm deliveries and 18% of all pregnancy-related deaths in the US per year [29, 30, 178].

Based on the findings reported here, TNF-α blockade may be a possible therapy to alleviate preeclamptic symptoms (Fig. 31). Similar to the effects of the anti-TNF-α antibody employed in our AT_1-AA-injected pregnant mice, a soluble TNF-α receptor also attenuates hypertension seen in pregnant rats generated by a reduced uterine placental perfusion (RUPP) [314]. Thus, both of these animal studies provide strong preclinical evidence to support the novel therapeutic possibility of targeting this deleterious cytokine which is associated with PE. Notably, both soluble TNF-α receptors and anti-TNF-α antibody therapies are used in many autoimmune diseases, such as rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis and inflammatory bowel disease [96, 315, 316]. Great success has been achieved by blocking TNF-α activity in these diseases and is the mainstay of their treatment. Though there are associated risks of infection with the available injectable proteins, the development of oral small-molecule inhibitors is on the pharmacological horizon [283]. Thus, these soluble TNF-α

receptor agents may prove to be safer, cheaper and require fewer doses, making them ideal for use in the developing world where PE is extremely prevalent and deadly.

Therapeutic side effects

The reported side effects and known teratogenicity of a drug are extremely important when considering its use in a pregnant woman. The US FDA currently classifies anti-TNF-α agents as having no documented human fetal toxicity (Category B) [283]. Animal studies, such as those of Giroir *et al.*, have shown no evidence of teratogenicity in the pups born to mice injected with an anti-TNF-α protein [317]. In addition, there is precedence for use of anti-TNF-α agents in pregnant women. Roux *et al.* in their comprehensive review report several studies where women being treated with anti-TNFα agents for various rheumatologic disorders have successful normal gestations and births [318, 319]. This evidence of drug safety supports the need for future work in employing anti-TNFα blockers in the management of PE.

While this dissertation did not follow the pups born to mice co-injected with AT_1-AA and the anti-TNF-α antibody, there were no adverse effects reported in the dams. Moreover, in the pregnant mice injected with the anti-TNF-α antibody alone, there were no obvious harmful consequences observed and these mice have blood pressure, renal function and placental morphology similar to that of control mice injected with IgG derived from normotensive pregnant women (Fig. 25). Based on these findings, the anti-TNF-α agent used do not cause harm to the pregnant mice used in the adoptive transfer model. However, future studies will have to be performed to ascertain any detrimental effects on the fetus.
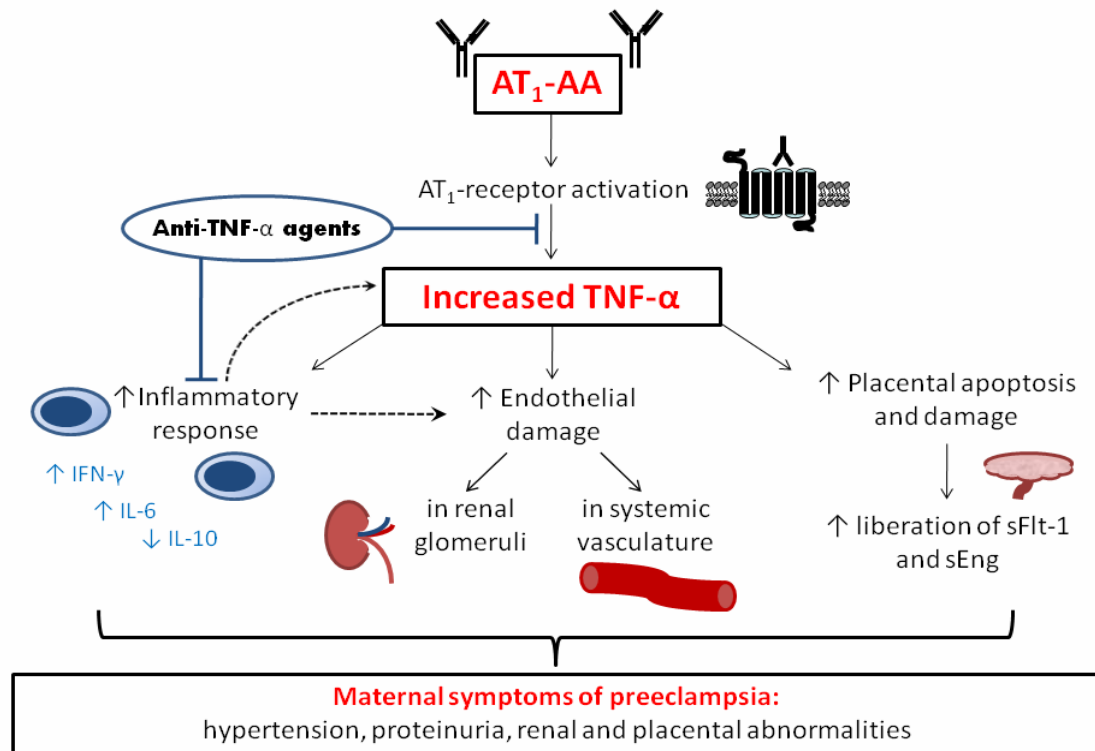
Figure 31: Schematic depicting the possible role of TNF-α in AT$_1$-AA-induced preeclamptic features. Autoantibody-mediated TNF-α signaling could generate the maternal symptoms associated with PE. This implies that blockade of both TNF-α and AT$_1$ receptor signaling may be potential therapeutic strategies in the management of this serious disorder of pregnancy.

Taken together, these studies identified AT$_1$-AA as a novel candidate responsible for the increased inflammatory response associated with PE by directly inducing TNF-α production via AT$_1$-receptor activation (Fig. 31). Both human and mouse studies demonstrate that this inflammatory cytokine plays an important role in the pathogenesis of this hypertensive condition. Of significant importance, its blockade reduces the maternal features of the disease in an adoptive transfer mouse model of PE. In addition, AT$_1$-AA-induced placental damage can be alleviated by preventing TNF-α action in human villous explants. These findings indicate a central role of TNF-α in placental damage and subsequent disease symptom development. The work reported here could be the foundation leading to future human trials and a possible therapy for PE, a life-threatening disorder of pregnancy for which the current treatment is extremely limited.

CHAPTER 6:

GENERAL CONCLUSIONS & SIGNIFICANCE

GENERAL CONCLUSIONS & SIGNIFICANCE

Overall conclusions

Taken together, the body of work presented here makes a strong case that the autoantibody, AT$_1$-AA, plays a major role in the pathogenesis of PE. Many groups, including our own, have shown that women with PE harbor increased levels of this autoantibody [33, 34]. In this dissertation, it was illustrated how biologically active AT$_1$-AA can be purified from the serum from preeclamptic patients and injected into pregnant mice. Upon injection with the autoantibody, these dams recapitulate preeclamptic disease features: hypertension, proteinuria and the liberation of detrimental anti-angiogenic factors [38]. Fetal and placental features were also examined. It was established that autoantibody-mediated AT$_1$ receptor activation contributes to IUGR and placental abnormalities [99]. All of the disease traits observed, both maternal and fetal, could be specifically reduced by the co-injection of an AT$_1$ receptor blocker, losartan, or an autoantibody-neutralizing peptide, 7-aa. Moreover, when human villous explants were cultured in the presence of AT$_1$-AA, increased liberation of sFlt-1, sEng and TNF-α were recorded, as was elevated placental explant apoptosis. For most of the studies described, parallel experiments were performed in non-pregnant mice in order to define the role of AT$_1$-AA independent of pregnancy. In general, the manifestation of autoantibody-mediated preeclamptic symptoms required the state of pregnancy. These experiments demonstrate that excess AT$_1$ receptor activation by the autoantibody results in a preeclamptic-like state in the pregnant mouse, and alongside the villous explant data, they demonstrate the pathogenic role of AT$_1$-AA in PE (Fig. 32).

To further examine the mechanism by which the autoantibody may cause these symptoms, the sera of PE-IgG-injected mice were examined for inflammatory cytokines. Excess AT_1 receptor activation resulted in the induction of several pro-inflammatory molecules, most notably, TNF-α, a factor known to be increased in PE. Therefore, I employed the adoptive transfer model to address the concept of TNF-α blockade in the disorder. Co-injection of AT_1-AA and an anti-TNF-α antibody was capable of reducing disease features in pregnant mice, including a heightened inflammatory response, and the liberation of detrimental factors in cultured human villous explants. Therefore, these results indicate that AT_1-AA-mediated induction of excess TNF-α may contribute to preeclamptic symptoms.
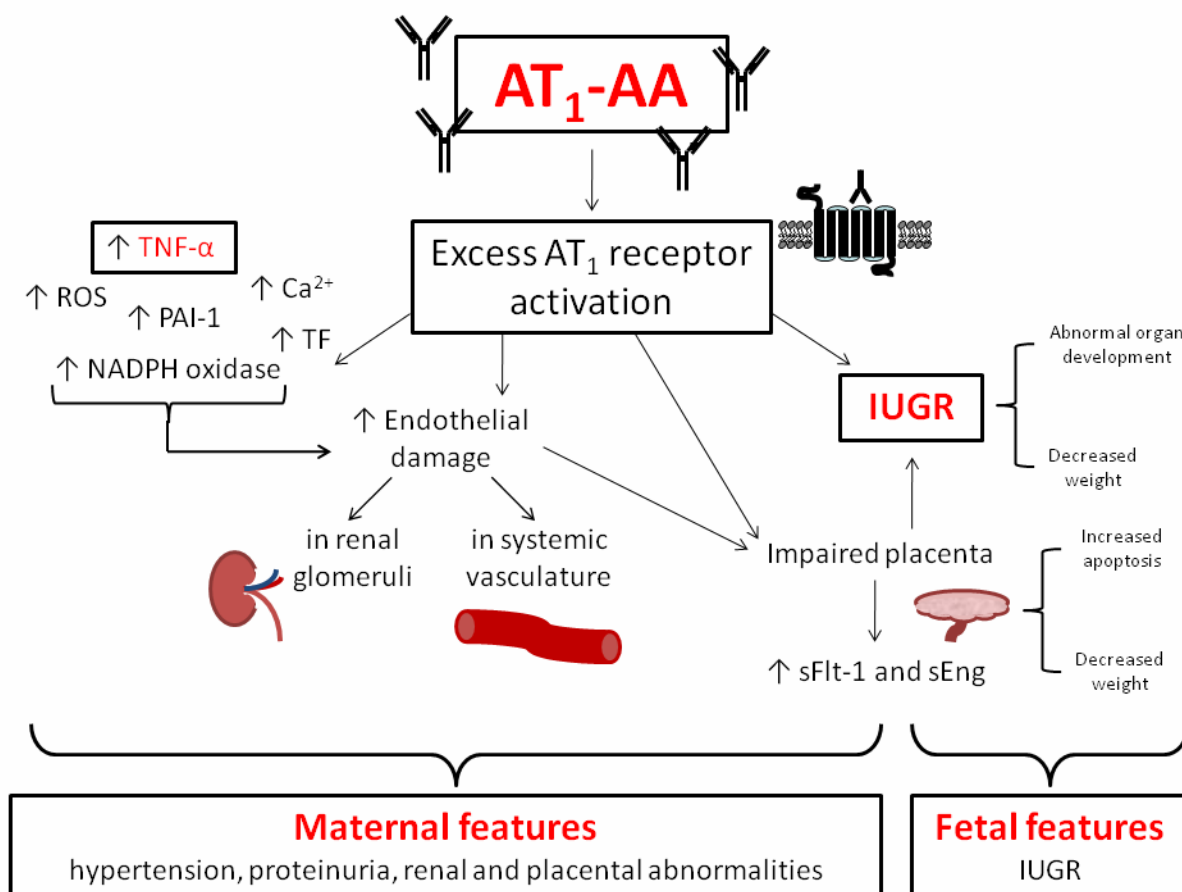
Figure 32: The role of AT_1-AA in preeclampsia. Excess AT_1 receptor activation may contribute to both the maternal and fetal features associated with PE.

Through the work presented here, <u>it can be concluded that the autoantibody, AT$_1$-AA,</u> <u>plays a pathogenic role in the hypertensive disease of pregnancy, preeclampsia</u>. Both *in vitro* and *in vivo* studies confirm that selective blockade of this autoantibody or its downstream targets, such as TNF-α, alleviates the features associated with the disease. These facts have immediate therapeutic implications which could improve upon the inadequate screening, diagnostic markers, and treatments available for this prevalent and deadly disorder of mother and child.

Other animal models elucidating the role of AT$_1$-AA and the maternal features of PE

Our group is not the only to investigate the role of AT$_1$-AA in the development of gestational hypertension. To explore this concept, Dechend *et al.* mated female rats expressing the human angiotensinogen gene with male transgenics expressing the human renin gene [320]. Towards the end of their pregnancy, these dams experienced hypertension and proteinuria which resolved post-partum. These females developed other preeclamptic-like features, such as glomerular fibrin deposition and placental vascular defects. Importantly, the same autoantibody circulating in preeclamptic women, AT$_1$-AA, was detectable in the serum of these pregnant transgenic rats [320]. The remarkable finding of AT$_1$-AA production in the setting of RAS dysregulation implies that these features have a close relationship in the pathophysiology of PE. Another group used a model of placental ischemia to elucidate the etiologic factors responsible for the maternal syndrome. Granger *et al.* performed a surgical manipulation called reduction uterine perfusion pressure (RUPP) in rats in order to determine if this reduced placental blood-flow could result in preeclamptic symptom manifestation [321]. Indeed, the RUPP-treated rats experienced preeclamptic-like features: hypertension, proteinuria, and increased sFlt-1, TNF-α,

endothelin production and endothelial dysfunction. Remarkably, AT_1-AA could be isolated from the manipulated rats, whereas un-manipulated pregnant rats did not produce the autoantibody [218]. The same group investigated the effect of TNF-α infusion during pregnancy. Again, hypertension developed and AT_1-AA was detectable in the circulation of pregnant rats infused with low-dose TNF-α throughout pregnancy [218]. Non-pregnant animals did not share similar features, implying that adequate balance of inflammatory molecules and placental perfusion are necessary for a healthy pregnancy, and that decreased perfusion may lead to an inflammatory response triggering autoantibody production. The development of AT_1-AA in genetically and surgically manipulated rats as well as those infused with low-dose TNF-α reveals the important relationship between RAS regulation and maternal health during gestation. The consistent development of AT_1-AA in these experimental animal models also suggests a common antigenic origin, which provides evidence for the concept that PE is an autoimmune disorder of pregnancy.

AT_1-AA: prevalence, persistence and the push forward

The exact etiology of self-recognizing antibodies in autoimmune diseases is difficult to discern. Many factors have been proposed which may lead to autoantibody production in general, including genetic predispositions, maladaptive immune responses and environmental triggers [322-324]. All of these mechanisms could contribute to the generation of the autoantibody associated with PE. It is currently unknown what triggers the production of AT_1-AA and when the autoantibody first arises in pregnancy. In their original paper, Wallukat *et al.* used affinity purification and peptide competition experiments to illustrate that the autoantibodies found circulating in preeclamptic women have a common epitope: a seven amino acid sequence on the second extracellular loop of the AT_1 receptor (AFHYESQ) [33]. In

another human study, we have shown >95% of 37 preeclamptic women harbored AT_1-AA and that the bioactivity of the autoantibody correlated significantly to disease severity, in particular proteinuria [34]. Normotensive pregnant women were also assessed for the presence of AT_1-AA. Less than 30% of these women had any detectable autoantibody levels, which were five-fold less than those observed in the preeclamptic group. The consistencies of these studies suggest a common immunologic origin of AT_1-AA in preeclamptic women, an area of exciting future work.

In the short term, preeclamptic symptoms usually abate within 48 hours postpartum and normal blood pressure is restored approximately 12 weeks after delivery. However, a definitive timeline of AT_1-AA persistence in preeclamptic women is currently unknown. In a small study, Hubel, *et al.* reported that 17.2% of 29 women with a previous history of PE harbored AT_1-AA 18 ± 9 months post-partum, versus 2.9% of 35 women without a previous history of the disorder [325]. Future work will have to build upon this study to determine exactly when autoantibody titers decrease post-partum in preeclamptic women.

The long-term cardiovascular and renal consequences of PE are areas of recent interest. Many groups have reported that having a previous history of PE puts a woman at increased risk for overall cardiovascular risk [326], stroke and chronic hypertension later in life [235, 327], ischemic heart disease [328] and death due to cardiovascular complications [329], as compared to women who have not suffered from the disorder. Renal complications may also persist in preeclamptic women. Glomerular endothlelial cell swelling with fibrin deposition [330], microalbuminuria and endothelial cell dysfunction [331, 332] have all been documented in preeclamptic women several months post-partum. It will be of particular interest to determine if AT_1-AA are present in the women with a history of PE who go on to suffer from cardiovascular and renal complications later in life.

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Taken together, the common immunologic features and long-term sequelae in these human studies provide mounting evidence that PE may in fact be an autoimmune disorder of pregnancy. Should this be the case, autoantibody-targeted therapies may be beneficial not only in the immediate treatment of this devastating disease, but also to abate future health risks. Further investigation into the natural history of these autoantibodies in preeclamptic women is necessary.

Molecular mimicry: Human parvovirus B19 and the AT$_1$ receptor

There are many theories as to how the process of autoimmunity occurs. Though none have proven unequivocally true in all cases, there is one prevailing theory which may apply to the generation of the autoantibody associated with PE: *molecular mimicry*. Molecular mimicry involves an infection-induced activation of self-reactive lymphocytes. The generation of autoreactive T and B cells results from sequence similarities between foreign peptides found on bacteria or viruses and self-peptides found naturally throughout the body. There are many examples of infectious agents causing autoimmune-induced pathology: β-hemolytic streptococci and cardiomyocyte antigen similarities resulting in rheumatic fever; coxsackieviruses leading to myocarditis; *Trypanosoma cruzi* infection followed by Chagas' disease; *Borrelia burgdorferi* infection and Lyme disease arthritis [333, 334]. Importantly in many cases, adoptive transfer experiments using animal models provide direct evidence that infection with a particular pathogen results in its associated autoimmune disease [335].

As discussed earlier, AT$_1$-AA, the autoantibody associated with PE, can be effectively neutralized by a peptide corresponding to a short sequence on the second extracellular loop of the AT$_1$ receptor, AFHYESQ. This consistent association implies that this sequence may be the

antigenic epitope to which the autoantibody is raised against. This fact begs the question, why would a sequence found on a receptor located on virtually every cell type in the body be autoreactive?

BLASTing AFHYESQ in the publically available protein sequence NCBI database reveals a striking homology between this 7-aa sequence and the two capsid proteins, VP1 and VP2, of the human parvovirus B19 (HPV B19). HPV B19 is a single-stranded DNA virus commonly associated with hydrops fetalis, fetal anemia and childhood erythema infectiosum [336]. 50-70% of adults are reportedly HPV B19 seropositive [337] and the persistence of myocardial HPV B19 is associated with cardiomyopathies and endothelial cell dysfunction [338, 339]. When comparing the sequences, there is 100% homology between that found in the human AT_1 receptor (accession number: EAW78905) and the VP1/VP2 capsid proteins of HPV B19 (accession number: ABX89697). *The conservation between the epitope of AT_1-AA and HPV B19 capsid proteins raises the possibility that molecular mimicry may underlie the autoimmune mechanism associated with PE.*

This finding has been observed by others who have performed small retroactive studies regarding remote HPV B19 infection, the autoantibody and the prevalence of PE. After the examination of normotensive pregnant and preeclamptic women for the presence of anti-HPV B19 antibodies and AT_1-AA, Stephan *et al.* found that there was no strict correlation between the factors [340]. Therefore, the authors propose that the generation of AT_1-AA was independent of epitope mimicry of HPV B19. This finding could be due to the confounding high seroprevalence in the population, and the requirement of a yet unidentified agent.

The fact remains that HPV B19 is a very common infection in most adults and only 8% of pregnancies are afflicted with the complication of PE [178]. It is possible that only a portion

of HPV B19 seropositive women develop autoantibodies against the conserved region on the AT$_1$ receptor; therefore implying that HPV B19 infection cannot be the only parameter resulting in this disease of pregnancy. Environment and a particular genetic background may also contribute to the generation of the autoantibody and preeclamptic pathophysiology. Immunization of mice with the VP1/VP2 capsid proteins may provide more direct evidence supporting (or disproving) this provocative hypothesis. Indeed, this intriguing scenario raises the possibility that the immunologic origin of AT$_1$-AA could be secondary to a parvovirus B19 infection: an exciting area requiring further investigation.

Autoantibody-targeted therapeutics

In the work presented here, 7-aa successfully attenuated the autoantibody-induced symptoms of PE in pregnant mice. Although these findings have tremendous implications, the evaluation of the therapeutic potential of autoantibody-neutralizing peptides was not a focus of this body of work. In the present study, the neutralization of AT$_1$-AA by the 7-aa epitope peptide primarily served as a control to identify that is was, in fact, this autoantibody which was responsible for the pathophysiology observed. Because the AT$_1$-AA derived from many different patients consistently interacted with the same epitope present on the second extracellular loop of the AT$_1$ receptor, it suggests a common immunologic origin of these autoantibodies. 7-aa was highly effective in reducing the actions of the injected AT$_1$-AA. It can therefore be inferred that they share a strong interaction and bind well to one another. Unfortunately, it is unlikely that this short peptide would remain stable in the circulation of humans or mice if it were not bound in complex with the autoantibody. Freely circulating peptidases would likely destroy it rapidly. Should 7-aa be used in preclinical studies and

potential clinical trials, the synthesis of stable derivatives will be necessary. Further investigation is warranted to fully exploit the therapeutic potential suggested by the results of these autoantibody-neutralization experiments. In a similar regard, the use of anti-idiotype antibodies directed at different antigen-binding regions of AT_1-AA could be employed therapeutically. However, it must be recognized that AT_1-AA stimulate the production of other detrimental molecules (e.g. sFlt-1, TNF-α, sEng, PAI-1, tissue factor, endothelin-1, ROS, NADPH oxidase), which can all contribute to the symptoms of PE. Blocking the autoantibody at the time of symptom-onset may not be sufficient to immediately lead to their abatement [149, 224]. Similar limitations would presumably apply to the potential use of plasmaphoresis, a strategy successfully employed in other autoimmune diseases, such as multiple sclerosis, which removes the harmful autoantibodies from circulation [341]. In the case of PE, once symptoms have arisen, many of the aforementioned factors would already be greatly elevated, and not be filtered from the blood during the process of plasmaphoresis. As they would remain in circulation, these factors would continue to incite damage and provoke preeclamptic features. Since the autoantibody likely appears several weeks prior to symptom development, it will be necessary to generate a screening method to detect it as early as possible. In this way, AT_1-AA-mediated inductions of other factors may be blocked before they are fully elevated and maximum therapeutic benefit could be achieved.

Autoimmune disease: a new classification of PE?

It would be remiss to avoid commenting on the fact that it is currently not the consensus of the field at-large that PE as an autoimmune disease specific to pregnancy; despite the growing body of work implicating the role of AT_1-AA.

Indeed, the as the *in vitro* and *in vivo* evidence mounts to support the case of AT_1-AA in the pathogenesis of PE, other human diseases have been linked to autoantibodies capable of activating G-protein-coupled receptors. As previously mentioned, Graves' disease, is an autoimmune condition wherein autoantibodies target thyroid cells and activate the thyroid stimulating hormone receptor, resulting in excessive production and secretion of thyroid hormones resulting in hyperthyroidism [201]. Some forms of hypoglycemia are associated with autoantibodies which agonize the insulin receptor [342]. Patients with dilated cardiomyopathy harbor autoantibodies directed against cardiac β1-adrenergic receptors, resulting in a positive chronotropic effect in cultured cardiomyocytes [343]. Using a rat model, Jahns *et al.* provided direct evidence for autoimmune-induced β1-adrenergic receptor activation as a cause of idiopathic dilated cardiomyopathy [344]. This group later showed that patients who possess these autoantibodies are at increased risk for future cardiovascular disease and mortality [345]. Autoantibodies which increase α1-adrenergic receptor activity are also associated with several forms of hypertension [346, 347]. Finally, autoantibodies which activate the muscarinic M2 receptor are also found in some patients with idiopathic dilated cardiomyopathy [33]. Thus agonistic autoantibodies which target and agonize G-protein-coupled receptors are commonly observed in many human conditions and considered a pathogenic factor leading to symptom development. AT_1-AA, which agonizes the G_q-coupled AT_1 receptor, may soon be recognized in that category. The findings presented here raise the intriguing possibility that PE may be an autoimmune disease associated with AT_1-AA.

Whether one believes PE is an autoimmune disease or not, it cannot disputed that AT_1-AA are found in the serum of preeclamptic patients and that they potentially contribute to the systemic symptoms. What initiates and perpetuates the generation of these autoantibodies are still areas of speculation which require further investigation. We believe that in the near future

PE will be considered a <u>pregnancy-induced autoimmune condition</u>, which could provide the field of immunology insight into how other conditions of autoimmunity arise and flourish.

Significance

Preeclampsia is encountered in 7% of pregnancies, responsible for 15% of all preterm births and 18% of all pregnancy-related maternal deaths in the US every year [29, 30, 178]. These are not merely statistics, but represent hundreds of thousands of families which are affected by this devastating disorder of pregnancy and billions of dollars spent in PE-related healthcare costs. Right now, delivery of the baby and placenta is our only option to "cure" PE. These deliveries, often premature, pose considerable immediate risk to both mom and baby, not to mention their increased risk for long-term health sequelae. Improvement upon the screening, diagnosis and treatment of this disorder are clearly required and essential to advancing the care not only in the US, but also in developing countries, where the prevalence of PE can be twice as high and the rate of death several fold higher as compared to developed nations [31, 348].

The research presented here expands our understanding of PE and the role that AT_1-AA may play in the manifestation of the disorder. Any insight into the disease mechanism and the identification of pathogenic players could improve upon the limited screening and therapeutic strategies available in its clinical management. It was shown that this autoantibody can induce preeclamptic-like symptoms in mice and that losartan, 7-aa and anti-TNF-α treatments can specifically reduce them. These facts have significant therapeutic implications: specific neutralization of the autoantibody in preeclamptic women could alleviate their symptoms. Moreover, should we develop an improved method of identifying the autoantibody, physicians

would be able to screen for AT_1-AA early in pregnancy and then block autoantibody-mediated AT_1 receptor activation or downstream molecules' actions. This blockade could forestall or prevent preeclamptic symptom, thereby reducing the short- and long-term risks to preeclamptic mothers and their unborn children.

CHAPTER 7:

FUTURE DIRECTIONS

FUTURE DIRECTIONS

Though the recent work reported by Dr. Xia's lab clearly establishes the pathophysiologic role of AT_1-AA in PE, there are still many mechanistic aspects to explore. In the near future, this group will attempt to: establish a timeline of autoantibody generation in pregnant women; determine the role of LIGHT, an emerging TNF-α superfamily member which contributes to apoptosis and inflammation, in AT_1-AA-mediated PE; and finally, to elucidate the relationship between the autoantibody and syncytiotrophoblastic microparticles (STBMs) and their role in preeclamptic symptom development.

Detection of AT_1-AA in CPEP clinical trial samples

The two most common, and arguably the most important, questions asked to our group and the other investigators studying AT_1-AA are: (i) when does the autoantibody appear during gestation, and (ii) does the generation of AT_1-AA precede preeclamptic symptom development. In order to answer these questions, one must have a collection of well-defined preeclamptic and control serum samples which span the course of gestation. In recent months, we have acquired just that. Based on our recent publication using the luciferase-based bioassay to detect AT_1-AA in pregnant women [34], we have begun what will surely be a fruitful collaboration with Dr. Richard Levine of the NIH.

To answer these two important clinical questions, we will employ the luciferase-based bioassay to detect AT_1-AA in the serum samples collected for the large-scale, nationwide Calcium for Preeclampsia Prevention (CPEP) Trial. These are the same valuable samples used in two landmark publications which clearly established the relationship between sFlt-1 and

sEng levels and the onset of PE [37, 61]. Our hypothesis is that the detection of AT_1-AA may precede overt preeclamptic symptoms in these patient samples.

In total, we will obtain at least 3 serum samples from various time-points throughout the pregnancies of: 120 normotensive women, 120 women who were normotensive and delivered SGA infants, 120 women with gestational hypertension, 72 women with preterm PE and 120 women with term PE. First, we will use our sensitive bioassay to detect AT_1-AA and correlate its presence with onset of PE symptoms, in particular, the induction of the two anti-angiogenic factors, sFlt-1 and sEng. Secondly, by establishing a timeline of autoantibody generation in this large bank of well-characterized samples, we hope to generate a means to identify women at risk for PE – a tool lacking in the clinical management of this disorder.

Preliminary data: AT_1-AA-CPEP Pilot Study

Recently, we conducted a pilot study to determine if our assay was capable of detecting the biologic activity of AT_1-AA isolated from these samples. Dr. Levine and his team randomly blinded us to 24 CPEP serum samples, and we ran them in our standard manner [34]. For each sample, we completed 4 luciferase readings and the average value of was computed to calculate a raw delta value and a fold-induction ratio for each sample by dividing the average value of the background controls in each batch (Fig. 33).

Though we received 24 samples, upon completion of the pilot study, we were informed that in fact there were only 12 patients in this pool, and that duplicates had been provided. Repeated measure analysis for ANOVA was applied to evaluate the within treatment effect (duplicate measurements for each patient) and between treatment effect (PE vs. NT). No significant difference was detected between the two duplicates in either raw delta or ratio values, P=0.3937. However, the PE group showed significantly higher deltas and ratios

compared to NT. Paired t-test and non-parametric Wilcoxon test were also used for additional testing on within and between treatment effects. The results were consistent with the repeated measure analysis.
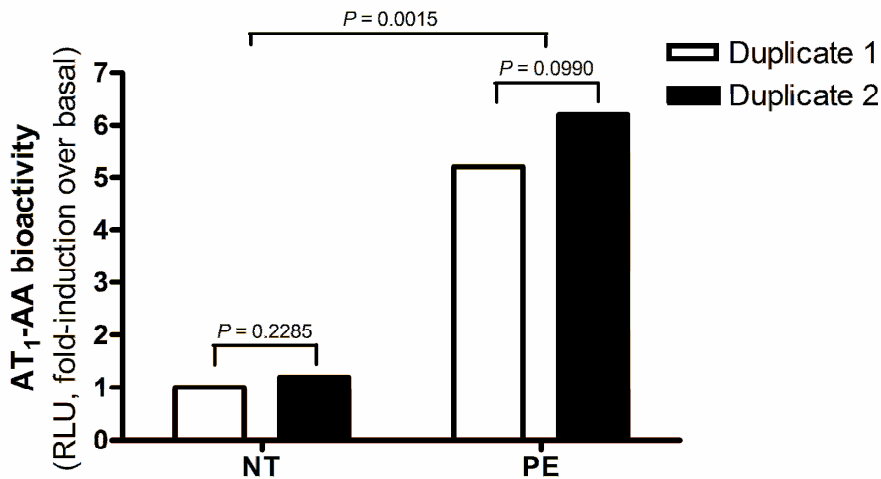
Figure 33: AT$_1$-AA bioactivity was successfully detected in CPEP serum samples. A total of 12 samples from the CPEP trial were randomized and measured in blinded duplicate by an NFAT-luciferase bioassay. Normotensive (NT), n=6; Preeclampsia (PE), n=6. There is a significant difference between NT and PE groups ($P<0.0015$), but no significant difference between duplicates.

The consistency of the bioassay between duplicates and statistically significant differences achieved between the two groups of interest in the AT$_1$-AA-CPEP Pilot Study results are highly encouraging. We are excited to proceed generating similar data in the near future with the remaining ~950 samples. Determining a clear and early biomarker for PE may provide a therapeutic window which could allow for the neutralization or removal of AT$_1$-AA, providing significant preventative measures to forestall the devastating symptoms of PE and subsequent fetal IUGR.

AT$_1$-AA and syncytiotrophoblastic microparticles

One feature of preeclamptic women which has puzzled investigators is the presence of increased syncytiotrophoblast microparticles (STBMs). STBMs are membrane-bound placental cell fragments produced by apoptotic activity or by vesicle secretion [349, 350]. In normal pregnancy, minimal STBMs are shed from the placenta into maternal circulation, in contrast to PE, where there is excessive shedding and increased circulating STBMs [351]. The cause for this excess shedding is undetermined. It may be due to the poor placental perfusion leading to hypoxic injury and/or excessive trophoblast apoptosis characteristic of preeclamptic placentas [352, 353]. Additionally, it is unknown what pathophysiology, if any, results secondary to these circulating microparticles. Redman *et al.*, believe that this STBM overload could be a stimulus for the maternal inflammatory response and the systemic endothelial cell damage observed in the disorder [354]. Others believe that since STBMs contain trophoblasts of fetal origin, which could be interpreted by maternal immune cells as foreign, a maternal immune response may be mounted [266]. Therefore, we will set out to answer a classic 'chicken or the egg' mystery, and will determine if STBMs represent a rich source of antigenic stimulation for the mother, resulting in the generation of AT$_1$-AA and the maternal syndrome of PE; or if the autoantibody precedes and is a driving force for the liberation of STBMs themselves.

In order to investigate the pathophysiologic consequences of STBMs, we must generate them. This can be achieved using several techniques: HTR-8 cells, mouse and human villous explants will be cultured under normoxic and hypoxic conditions as well as being treated with the apoptotic-inducing agents TNF-α and rotenone [355, 356]. STBM production will be quantified using flow cytometry using the trophoblastic marker, HLA-G, in a method similar to Orozco, *et al.* [357]. Then, STBMs will be analyzed by western blot for the presence of the AT$_1$ receptor, a possible antigenic source for AT$_1$-AA generation. Furthermore, the cultured STBMs

could be injected into pregnant mice which will be monitored for preeclamptic features and the generation of the autoantibody. We expect that STBM production will be increased under hypoxic conditions, and that TNF-α and rotenone will induce their production via increased apoptosis. Pregnant mice injected with excess STBMs may demonstrate a preeclamptic-like syndrome and if injected early enough, may also generate AT_1-AA.

Alternative to the timeline studies are those characterizing the cell-surface receptor profiles of STBMs throughout both uncomplicated and preeclamptic pregnancies. Appropriate integrin subunit switching is essential for a healthy pregnancy as it enables trophoblasts to fully infiltrate maternal decidual and endothelial linings [358, 359]. Cytotrophoblasts in the villous express epithelial-like integrins (α6β4). As they migrate towards the decidual endothelium, their integrins transform to a vascular-type (α1β3 or α5β3) [359]. This switching is aberrant in PE and results in shallow trophoblast invasion and inadequate endothelial cell infiltration [360]. Notably, this type of integrin switching is mediated by AT_1-receptor activation in other systems [361]. We plan to isolate STBMs from these two pregnant groups using established methods [351] and characterize their epithelial or endothelial-like cell surface markers using flow cytometry analysis. Since STBMs take on the composition of the cells from which they originate, we expect there to be a defined expression pattern of cell surface markers in PE versus NT STBMs (epithelial versus vascular) [169].

Overall, a woman's STBM marker profile could provide a novel screening tool as certain cell surface markers could indicate a risk factor for PE (e.g. integrin α6β4). Additionally, if STBMs prove to induce AT_1-AA production, these women would be at even higher risk for developing a hypertensive pregnancy. Any additional tools, such as an STBM cell surface marker profile, used for the screening of PE will be beneficial, as they allow for closer monitoring of high risk women and create a window for early intervention.

See the LIGHT and its role in PE

LIGHT [homologous to **L**ymphotoxin, exhibits **I**nducible expression, competes with HSV **G**lycoprotein D for **H**erpes virus entry mediator (HVEM), a receptor expressed by **T** lymphocytes] is a recently identified (and rather lengthily-named) type II transmembrane glycoprotein of the TNF ligand superfamily [362]. It has been recognized as a major contributor to the inflammation and increased apoptosis observed in several human diseases. By binding to HVEM, LIGHT co-stimulates T cells to accelerate their proliferation and cytokine production. In several tumor cell lines, LIGHT induces apoptosis and recent studies have suggested a potential role of LIGHT in inflammatory diseases including: atherosclerosis, arthritis, IgA nephropathy, hepatitis and inflammatory bowel disease [363-366]. Soluble LIGHT has been detected in these many *in vitro* system studies, however its presence and function in inflammatory diseases have not been explored *in vivo*. Moreover, the role of this inflammatory mediator has not been investigated *at all* in the field of PE. After reviewing the literature by which LIGHT produces pathology in other disorders, we decided that it may be a potential pathogenic player in PE, and will further investigate this novel target.

Before determining if the autoantibody and LIGHT share a pathophysiologic relationship, we must first establish if LIGHT is increased in the circulation of preeclamptic women. For a pilot study, we collected plasma samples from both PE and NT pregnant women. Once our patient samples were acquired, we ran an ELISA for LIGHT (R&D Systems) to determine its level in circulation. The data for this initial study is provided here (Fig. 34).

Preliminary data: Circulating LIGHT is increased in preeclamptic women

Figure 34: Circulating LIGHT is elevated in preeclamptic women. ELISA measurement of circulating LIGHT in preeclamptic (PE; n=34) and normotensive (NT; n=33) pregnant women reveals a significant increase in PE patients. Dotted line; lowest detectable level of assay.

The circulating LIGHT measured in patient plasma indicates preeclamptic women have a significantly increased level over their NT counterparts (Fig. 34). None of the NT patient samples had markedly increased serum levels of LIGHT. Of the PE patient samples, 9 of 34, or ~26% showed a significant increase in the circulating level of the glycoprotein. The means for NT and PE plasma LIGHT levels were 0.99 and 26.45 pg/ml respectively, P=0.0066. Though not every PE patient demonstrated increased LIGHT, a value of ~26% of patients with increased levels is considered clinically worthwhile to pursue. This percentage could also be explained if the ELISA is not an adequately sensitive detection method for this molecule. Certainly, further confirmation of elevated LIGHT in preeclamptic women is necessary. Therefore, the next step will be to collect the placentas from affected women with PE and measure both the transcript and protein level of LIGHT (by RT-PCR and western, respectively). Then, should the levels be increased and we are satisfied that this inflammatory agent is consistently increased in PE, we will move forward into *in vitro* and *in vivo* studies. We will culture human villous explants with ANG II or PE-IgG, and after a period of incubation, measure the amount of LIGHT liberated into the supernatant by the explants. At

this moment, there is no literature which delineates the mechanisms by which LIGHT is increased. Since many other TNF-related inflammatory molecules are increased by AT_1 receptor stimulation [206, 257, 259, 367], it is plausible that LIGHT may also be. Should this inflammatory mediator be increased by PE-IgG not by NT-IgG, then perhaps AT_1-AA induces LIGHT, which may play a causative role in the manifestation of PE. We could then pursue *in vivo* studies by injecting recombinant LIGHT into pregnant mice and then monitor them for disease symptoms, such as hypertension and renal dysfunction. We could also ascertain if AT_1-AA injected mice generate excess LIGHT and if so, whether co-injection of the autoantibody and losartan or 7-aa reduces this value. All of these exciting projects will help establish LIGHT as a novel inflammatory mediator in the pathogenesis of PE, and may potentially create a therapeutic opportunity in the years to come.

Overall goals of future work

The future work presented here seeks to further characterize AT_1-AA in the pathophysiology of PE. Currently, this prevalent disorder is not considered an autoimmune process; however, the growing body of work implicating AT_1-AA in its pathogenesis could lead to a paradigm shift in its screening, diagnosis and treatment. The results from the CPEP study could yield more defined dates for early screening of the autoantibody and STBM profiles prior to onset of preeclamptic symptoms. This could improve upon the limited clinical management available. In addition, if autoantibody-induced features could be specifically blocked by a drug or peptide targeting a novel molecule, such as LIGHT, perhaps the progression of this disease could be abated and the morbidity and mortality associated with this hypertensive disorder could be reduced.

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