Role of Tumor Necrosis Factor-α and Natural Killer Cells in Uterine Artery Function and Pregnancy Outcome in the Stroke-Prone Spontaneously Hypertensive Rat

Heather Yvonne Small, Ryszard Nosalski, Hannah Morgan, Elisabeth Beattie, Tomasz J. Guzik, Delyth Graham, Christian Delles

Abstract—Women with chronic hypertension are at increased risk of maternal and fetal morbidity and mortality. We have previously characterized the stroke-prone spontaneously hypertensive rat (SHRSP) as a model of deficient uterine artery function and adverse pregnancy outcome compared with the control Wistar–Kyoto. The activation of the immune system plays a role in hypertension and adverse pregnancy outcome. Therefore, we investigated the role of tumor necrosis factor-α in the SHRSP phenotype in an intervention study using etanercept (0.8 mg/kg SC) at gestational days 0, 6, 12, and 18 in pregnant SHRSP compared with vehicle-treated controls (n=6). Etanercept treatment significantly lowered systolic blood pressure after gestational day 12 and increased litter size in SHRSP. At gestational day 18, etanercept improved the function of uterine arteries from pregnant SHRSP normalizing the contractile response and increasing endothelium-dependent relaxation, resulting in increased pregnancy-dependent diastolic blood flow in the uterine arteries. We identified that the source of excess tumor necrosis factor-α in the SHRSP was a pregnancy-dependent increase in peripheral and placental CD3+ CD161+ natural killer cells. Etanercept treatment also had effects on placental CD161+ cells by reducing the expression of CD161 receptor, which was associated with a decrease in cytotoxic granzyme B expression. Etanercept treatment improves maternal blood pressure, pregnancy outcome, and uterine artery function in SHRSP by antagonizing signaling from excess tumor necrosis factor-α production and the reduction of granzyme B expression in CD161+ natural killer cells in SHRSP. (Hypertension. 2016;68:1298-1307. DOI: 10.1161/HYPERTENSIONAHA.116.07933.) • Online Data Supplement

Key Words: blood pressure determination • etanercept • hypertension • pregnancy outcome • uterine artery

Chronic hypertension in pregnancy is defined by the American College of Obstetricians and Gynaecologists as systolic blood pressure (SBP) >140 mm Hg and/or diastolic blood pressure >90 mm Hg, which has presented before pregnancy or before gestational week 20.1 Despite affecting a similar number of women as other hypertensive disorders of pregnancy, maternal chronic hypertension receives relatively less research attention.2 Women with chronic hypertension are at increased risk of maternal and fetal morbidity and mortality.3 The problem of chronic hypertension in pregnancy is growing in parallel to the prevalence of increased maternal age,4 obesity,5 and metabolic syndrome.6 There is no consensus on the treatment of pregnant women with chronic hypertension and no generally accepted target blood pressure at which the risk of adverse complications to both mother and fetus is substantially reduced.7 Research into the cardiovascular complications associated with pregnancy in women with pre-existing hypertension is therefore needed.

Vascular remodeling plays a central role in maternal adaptation to pregnancy to accommodate the increase in cardiac output and regulate maternal blood pressure.8 Specifically, the uterine vasculature must undergo extensive structural and functional changes to provide sufficient blood supply to the developing placenta and fetus. It is well established that deficient adaptation of the uterine vasculature leads to adverse pregnancy outcomes.9 We recently hypothesized that pre-existing maternal cardiovascular disease would negatively affect the ability of the vasculature to suitably adapt to pregnancy and tested this hypothesis in the stroke-prone spontaneously hypertensive rat (SHRSP). The SHRSP is a well-established rat model that mirrors many features of human hypertension-related...
cardiovascular complications and has been used in research for >30 years. Some aspects of pregnancy in the SHRSP have already been briefly characterized\(^{11,12}\); however, the vascular adaptation to pregnancy has not been studied systematically. We have shown that the SHRSP presents with reduced litter size and increased loss of glycogen storage from the placenta associated with spontaneous deficient uterine artery remodeling and blood flow relative to the contrasting strain, Wistar–Kyoto (WKY), at gestational day (GD) 18\(^{13}\).

Activation of the innate immune system has been reported in both chronic hypertension\(^{14}\) and adverse pregnancy outcome.\(^{15}\) Levels of the proinflammatory cytokine tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) have been found to be increased in hypertensive subjects\(^{16}\) and in women with severe hypertensive pregnancy complications.\(^{17}\) Furthermore, TNF-\(\alpha\) infusion induces vascular dysfunction in both humans and rodents.\(^{18,19}\) During pregnancy, TNF-\(\alpha\) can be produced from a plethora of cell types but predominantly by activated monocytes/macrophages, T lymphocytes, natural killer (NK) cells, and invasive fetal derived extravillous trophoblasts.\(^{20}\) Antagonism of TNF-\(\alpha\) signaling has not been used clinically to treat hypertension in pregnancy but has been shown to have therapeutic effects in preclinical models of pregnancy-induced hypertension.\(^{21,22}\)

Here, we hypothesize that TNF-\(\alpha\) provides a link between vascular dysfunction and adverse pregnancy outcome by playing a causative role in the abnormal uterine vascular function and reduced litter size in the SHRSP at GD 18. We performed an intervention study using etanercept in pregnant SHRSP and sought to identify the source of excess TNF-\(\alpha\).

**Methods**

Most of the methods have been previously described by our group including the implantation of radiotelemetry probes,\(^{23}\) Doppler ultrasound,\(^{13}\) myography of the uterine arteries,\(^{13,24}\) and periodic acid–Schiff stain of the placenta.\(^{13}\) Full details are found in the online-only Data Supplement.

**Animals**

Animals (SHRSP and WKY) were housed under controlled lighting (0700–1900 hours) and temperature (21±3 °C) and received a normal diet (rat and mouse no.1 maintenance diet; Special Diet Services, Grangemouth, United Kingdom) provided ad libitum. All animal procedures were approved by the Home Office according to the regulations on experiments with animals in the United Kingdom (Project License Number 60/4286). Females were time-mated at 12 weeks of age (±4 days). Non-pregnant animals were age-matched at 15 weeks±4 days (ie, 12 weeks of age+21 days of pregnancy). Day 0 of pregnancy was defined as the day that a coital plug was observed indicative of successful mating having taken place. The number of rats used for a given experiment is indicated in the associated figure legend.

**Etanercept Treatment**

Animals were treated with etanercept 0.8 mg/kg (Wyeth Pharmaceuticals, Maidenhead, United Kingdom) diluted in sterile phosphate-buffered saline (Thermo Fisher Scientific, Paisley, United Kingdom) on GD 0, 6, 12, and 18 of pregnancy via SC injection. Vehicle-treated animals were subject to the same procedure using sterile phosphate-buffered saline (Thermo Fisher Scientific, Paisley, United Kingdom).

**Data Analysis**

Analysis of flow cytometry data was performed using FlowJo v.10 where gates were applied according to fluorescence minus one control. For quantitative real-time PCR data, results were analyzed using the \(2^{-\Delta\Delta C_{T}}\) method. Statistical analysis was performed using GraphPad Prism v 4.0. Student \(t\) test and 1-way ANOVA followed by Tukey test were used to compare WKY and SHRSP and WKY, SHRSP, and SHRSP treated with etanercept. Radiotelemetry data were analyzed using 2-way ANOVA, and the difference in blood pressure between SHRSP and SHRSP treated with etanercept was analyzed by calculating the ASBP from GD 12–GD 21 followed by Student \(t\) test. \(P<0.05\) was considered to be statistically significant for all experiments.

**Results**

**TNF-\(\alpha\) Is Increased in Pregnant SHRSP Relative to WKY**

TNF-\(\alpha\) was increased in plasma and urine from pregnant (GD 18) SHRSP relative to pregnant WKY (Figure 1A and 1B). Increased secretion of TNF-\(\alpha\) was also detected in media taken from placental tissue explants from GD 18 SHRSP (Figure 1C). Etanercept treatment in SHRSP did not significantly alter TNF-\(\alpha\) levels from GD 18 plasma samples or placental explant media (Figure S1). Gene expression of the main proinflammatory TNF-\(\alpha\) receptor-1 (Tnfr1) was also significantly increased in the GD 18 uteroplacental unit from SHRSP relative to WKY (Figure 1D).

**Etanercept Treatment Reduces Systolic Blood Pressure in the Pregnant SHRSP**

Etanercept (0.8 mg/kg SC) was given at GD 0 of pregnancy and repeated at GD 6, 12, and 18, and the effect on blood pressure in the SHRSP relative to vehicle-treated controls was monitored using radiotelemetry. SHRSPs are hypertensive before and during pregnancy in contrast to the control WKY strain (Figure 2A and 2B). Etanercept had no effect on SHRSP blood pressure in early pregnancy (GD 0–12). After GD 12, there was a significant average decrease of 11.5 mm Hg in SBP in etanercept relative to vehicle-treated SHRSP (Figure 2A), which persisted until parturition. No significant difference between SHRSP and etanercept-treated SHRSP was observed in diastolic blood pressure (Figure 2B). Heart rate was not significantly different between groups (Figure S2A). Activity was significantly decreased in both SHRSP and SHRSP treated with etanercept relative to the WKY over the course of pregnancy (Figure S2B).

**Etanercept Improves Placental Abnormality and Litter Size in the SHRSP**

Placenta from SHRSP exhibited a significant decrease in glycogen storage at GD 18 relative to the WKY (Figure 2C through 2E). Etanercept treatment in the SHRSP restored periodic acid–Schiff–positive glycogen cells within the junctional zone of the placenta (Figure 2C through 2E). Litter size was significantly increased by etanercept treatment in SHRSP (Figure 2D). Concurrently, the number of dams, which presented with ≥1 resorptions, was decreased in SHRSP treated with etanercept (WKY, 30%; SHRSP, 66.7%; SHRSP treated with etanercept, 25%). Litter size was not related to the changes in blastocyst implantation as there was no difference in the number of implantation sites at GD 6 between the groups (Figure S3). Furthermore, there was no difference in fetal or placental weight between any of the groups (Figure S4).
Etanercept Improves Abnormal Uterine Artery Function and Blood Flow in the Pregnant SHRSP

Pressure myography was used to construct pressure–diameter relationships to assess uterine artery properties from GD 18 WKY, SHRSP, and SHRSP treated with etanercept. Etanercept treatment did not significantly alter the diameter or wall thickness of the SHRSP uterine arteries (Figure S5). Investigation of uterine artery vasomotor function using wire myography showed that uterine arteries from pregnant SHRSP had a significantly increased contractile response to noradrenaline and a blunted endothelium-dependent vasorelaxation relative to the WKY (Figure 3A and 3B). In contrast, uterine arteries from pregnant SHRSP treated with etanercept exhibited a marked reduction in contractile response that was not significantly different from WKY and a significant increase in vasorelaxation to carbachol (Figure 3A and 3B). Etanercept treatment did not have a significant effect on mesenteric artery function in SHRSP (Figure S6).

These changes in uterine artery vasomotor function translated into improvements in uterine artery blood flow were assessed by Doppler ultrasound. Etanercept treatment partially restored the physiological increase in diastolic volume over the course of pregnancy, which is present in the WKY but absent in the SHRSP (Figure 3C), resulting in significantly reduced uterine artery resistance index in etanercept-treated SHRSP relative to control SHRSP (Figure 3D).

NK Cells Are a Source of Excess TNF-α in the SHRSP

From the beneficial effects we observed in pregnancy outcome and uterine artery function in SHRSP treated with etanercept, we deduced that excess TNF-α signaling played a causal role in the pathology observed during pregnancy in this model. Although TNF-α can be produced in some quantity from almost all cell types, we focused on one of the major producers, the immune cells, to identify the source(s) of excess TNF-α in the SHRSP. We designed a flow cytometry panel to quantify immune cell populations in pregnant WKY and SHRSP in the maternal blood and placenta (Figure S7 and S8, full panel and Figure S9, gating strategy). Of the populations analyzed, NK cells (CD3+ CD161+) were the most markedly increased in the SHRSP relative to WKY in both maternal circulation (Figure 4A) and placenta (Figure 4B and 4C). Furthermore, we detected pregnancy-specific changes in peripheral CD3+ CD161+ cells in maternal blood. In WKY, these cells were significantly decreased from nonpregnant to pregnant (GD 18) WKY (Figure 4D). In contrast, there was a significant increase in peripheral NK cells in SHRSP from nonpregnant to GD 18 (Figure 4D).

The significant increase in NK cells in the maternal circulation and placenta of the SHRSP was a promising candidate as the source of excess TNF-α. Intracellular staining of CD161+ cells in both the maternal circulation (Figure 5A and 5B) and placenta (Figure 5C and 5D) showed that TNF-α production is significantly increased in the SHRSP relative to the WKY at GD 18. TNF-α production from CD3+ T cells or other CD45+ cells was not significantly different between the strains (Figure S10).

Etanercept Reduces Granzyme B Production From CD161+ Cells in the SHRSP Placenta

Etanercept treatment in the SHRSP significantly reduced the number of CD161+ NK cells present in the SHRSP placenta (Figure 6A) but not in the maternal circulation (Figure 6B). Within the placental CD161+ cells in SHRSP animals only, we identified a CD161 low (CD161_{low}) population and a CD161 positive (CD161+) population (Figure 6C). Reduction of CD161+ cells in the placenta from SHRSP treated with etanercept was associated with a significant increase in the CD161_{low} population (Figure 6D). The CD161+ population had a characteristically cytotoxic phenotype where the majority of these cells stained positively for intracellular granzyme B from placenta in both SHRSP and SHRSP treated with etanercept. In contrast, the CD161_{low} population had a significantly decreased proportion of granzyme B expression from placenta in both SHRSP and SHRSP treated with etanercept (Figure 6E and 6F). Therefore, etanercept treatment reduces CD161+ cells in the SHRSP placenta by...
reducing the expression of CD161 receptor, which is associated with a reduction in granzyme B production.

**Discussion**

Etanercept treatment has beneficial effects on the placenta and litter size of the SHRSP through the improvement of uterine artery function and uteroplacental blood flow. Etanercept antagonizes signaling from the excess TNF-α produced by a pregnancy-dependent increase in CD161+ NK cells found in the SHRSP and has direct effects on the placenta where it minimizes cytotoxic granzyme B from these CD161+ cells.

Etanercept has previously been shown to reduce blood pressure in a rat model of preeclampsia.\(^{21,25}\) In contrast, it had no effect on blood pressure in nonpregnant hypertensive
female rats. These differing results show that etanercept has context-dependent effects on blood pressure, perhaps related to the extent or localization of inflammation. The average SBP reduction of 11.5 mm Hg in pregnant SHRSP treated with etanercept is comparable to the reduction in blood pressure seen in other rat models. As etanercept does not completely normalize blood pressure in the SHRSP, there must be other as-yet-unknown mechanisms that contribute to the hypertension we observe in this model. In this study, the decrease in SBP is not present until after GD 12 in the SHRSP. This coincides with the development of the mature uteroplacental unit by GD 12, suggesting that the placenta may be the source of excess TNF-α. Reducing maternal blood pressure during pregnancy using antihypertensive agents has been related to fetal growth restriction. Importantly, in this study, the significant reduction in SBP in late pregnancy has no negative impact on fetal growth as no significant difference in fetal weight was observed between vehicle- and etanercept-treated SHRSP. In fact, pregnancy outcome is improved in SHRSP treated with etanercept where there is a significant increase in litter size and a decrease in dams presenting with resorptions at GD 18. Etanercept treatment has been previously shown to reduce spontaneous pregnancy loss in a model of abnormal maternal inflammation during pregnancy.

Etanercept treatment improves endothelium-dependent vasorelaxation in SHRSP uterine arteries. TNF-α has a well-defined role in endothelial dysfunction by diminishing the bioavailability of vasodilator nitric oxide through the down-regulation of the expression of the eNOS (endothelial NO synthase) pathway and through increased superoxide production. Etanercept also significantly reduces the uterine artery vasocontractile response to noradrenaline in our model.
Changes in the vascular response to noradrenaline in etanercept-treated SHRSP could indicate changes in sympathetic nervous system activity in these animals. As a crude measure of sympathetic drive, we analyzed heart rate variability that showed a significant reduction over pregnancy in etanercept-treated SHRSP (data not shown). However, these results are difficult to interpret taking into account the change in heart rate over pregnancy. TNF-α has been shown to induce the production of vasoconstrictor endothelin-1 and can alter calcium handling. In the context of the experiment presented here, etanercept may have a role in the downregulation or desensitization of contractile adrenoreceptors to blunt the response to noradrenaline. In vascular function studies using resistance vessels from ovariectomized female rats where systemic TNF-α is elevated, etanercept treatment reduces contractility and improves endothelium-dependent vasorelaxation. In keeping with the improvement in uterine artery function in the SHRSP, uteroplacental blood flow is partially restored with etanercept treatment. Correction of deficient uteroplacental blood flow during pregnancy using a TNF-α antagonist has not previously been shown in the literature. However, plasma TNF-α has been shown to have a positive correlation with abnormal uterine artery resistance index and the presence of notching in humans. We have previously hypothesized that deficient uteroplacental perfusion in SHRSP leads to the premature utilization of glycogen stores in the placenta. In agreement, improvement of uteroplacental blood flow in etanercept-treated SHRSP restores the presence of placental glycogen cells at GD 18.

In experiments into the source of excess TNF-α in pregnant SHRSP, we found that the number of CD3-CD161+ NK cells was increased in a pregnancy-dependent manner in the SHRSP but not in the control WKY. In keeping with the improvement in uterine artery function in the SHRSP, uteroplacental blood flow is partially restored with etanercept treatment. Correction of deficient uteroplacental blood flow during pregnancy using a TNF-α antagonist has

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**Figure 4.** CD161+ natural killer (NK) cells are significantly increased in pregnant stroke-prone spontaneously hypertensive rat (SHRSP). Flow cytometry was used to measure leukocyte populations in maternal blood (A) and placenta (B) from GD 18 Wistar–Kyoto (WKY) and SHRSP (n=5–6). CD3+ T cells and CD3-CD161+ NK cells were elevated in maternal blood from SHRSP relative to WKY (A; *P<0.05 vs WKY; **P<0.01 vs WKY). CD3-CD161+ NK cells were significantly increased in placenta from SHRSP relative to WKY (B). A representative dot plot showing the increase in CD3-CD161+ cells in placenta from SHRSP relative to WKY is shown in (C). NK cell numbers were assessed using flow cytometry in virgin (NP; n=4) and GD 18 (n=5-6) SHRSP and WKY (D; ††P<0.01 vs WKY nonpregnant [NP]; $P<0.01 vs SHRSP NP). Data were analyzed by 1-way ANOVA followed by post hoc Tukey test. Th indicates T helper cell; and Tc, cytotoxic T cell.
which plays an important role in remodeling the uterine spiral arteries in response to pregnancy.\(^{38}\) Changes in peripheral NK cells do not directly mirror the status of uterine-specific NK cells; therefore, the 2 populations should be considered separately.\(^{39}\) In humans, the number of peripheral NK cells and their cytotoxicity are increased relative to nonpregnant levels in the first trimester followed by a decline in late pregnancy.\(^{40}\) Alterations in peripheral NK cells have mostly been associated with recurrent pregnancy loss and infertility; however, these findings are based on relatively small, observational studies.\(^{39}\) Other small studies have also explored a proinflammatory shift in NK cells in preeclampsia.\(^{41}\) We identified that CD161\(^+\) cells from SHRSP produced excess TNF-\(\alpha\) relative to control WKY. Although we cannot conclusively prove that excess TNF-\(\alpha\) is not being produced by other cell types; intracellular staining for TNF-\(\alpha\) was not significantly different in CD3\(^+\) or other CD45\(^+\) cells. TNF-\(\alpha\) is also produced by invasive extravillous trophoblasts; however, at GD 18 of pregnancy, trophoblasts do not exhibit potent invasive behavior.\(^{42}\)

The presence of total CD161\(^+\) cells was increased in placenta from SHRSP and SHRSP treated with etanercept, whereas this was minimal in the WKY placenta. Therefore, a currently unknown TNF-\(\alpha\)-independent mechanism exists, which recruits NK cells to the placenta of SHRSP. Within this placental CD161\(^+\) cell population, we have shown that etanercept induces a significant reduction in CD161 expression, which is associated with a reduction in granzyme B production. Conversely, this infers that TNF-\(\alpha\) signaling is a critical step involved in CD161 upregulation and granzyme B production in NK cells. Previously, TNF-\(\alpha\) has been shown to work in concert with interferon-\(\gamma\) to promote the cytotoxic activity of NK cells.\(^{43}\) The CD161 antibody used in this study detects the NKRP1A and NKRP1B cell surface receptors selective for NK cells. The killer ability of NK cells is protected by a sophisticated mechanism of activating and inhibitory receptors, whereby the resulting action is determined by the balance of these signals. NKRP1A\(^{44}\) is an activating receptor and NKPR1B an inhibitory receptor in the rat.\(^{45}\) CD161 is conserved in mice where it is also selective for NK cells\(^{46}\) and in humans where it is only expressed on a subset of NK cells.\(^{47}\) Although we have shown that CD161\(^+\) NK cells are associated with a cytotoxic phenotype, future studies should examine the balance of receptors on the NK cell surface in hypertensive pregnancy to further define their role in pathology.

Pregnancies complicated by chronic hypertension are associated with an increased risk of developing serious pregnancy-related complications affecting both the mother and fetus.\(^{3}\) Treating women who are pregnant is challenging because of the potential effects on the fetus, and no cure exists for more severe hypertensive complications of pregnancy. Small amounts of etanercept have been shown to cross the placental barrier in humans; a ratio between 14:1 and 30:1 (maternal serum:cord blood) has been reported in the literature.\(^{48,49}\) Etanercept has been used during pregnancy...
in women with inflammatory bowel disease and rheumatoid arthritis, where small studies have reported no adverse pregnancy outcomes, and in pilot studies as a treatment in early pregnancy for women with recurrent spontaneous miscarriage where it has a promising therapeutic effect. More convincing evidence of the safety of anti–TNF-α therapy in pregnancy will come from the ongoing Organisation of Teratology Information Specialists Autoimmune Diseases in Pregnancy Project (NCT01086059) expected to be reported in early 2017.

Perspectives

Excess TNF-α associated with an increase in CD161+ NK cells plays a causative role in abnormal vascular adaptation and placental biology in this preclinical model of chronic hypertension in pregnancy. Etanercept ameliorates the adverse pregnancy outcomes associated with this model, including direct effects on the placenta where it reduces CD161 expression and granzyme B production from NK cells. Thus, etanercept should be considered in future studies as a promising therapeutic avenue for pregnancies complicated by hypertension.

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Disclosures

None.

References

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Novelty and Significance

What Is New?

- This is the first study to examine the effect of etanercept on the uterine vasculature during pregnancy.
- We demonstrated that a tumor necrosis factor-α–targeted strategy reduces blood pressure and increases litter size in the stroke-prone spontaneously hypertensive rat relative to the control Wistar–Kyoto rat.
- CD161+ natural killer cells which produce excess tumor necrosis factor-α accumulate in the placenta of the stroke-prone spontaneously hypertensive rat relative to the Wistar–Kyoto rat. Etanercept reduces the expression of cytotoxic granzyme B in natural killer cells found within the placenta of the stroke-prone spontaneously hypertensive rat.

What Is Relevant?

- Chronic hypertension during pregnancy is an increasing problem and confers significant risk for both maternal and fetal morbidity and mortality.
- The role of inflammatory cytokines and immune cells has been well characterized in the remodeling of the small spiral arteries of the uterus in response to pregnancy. Here we suggest they may also play a role in remodeling larger arteries.
- Etanercept, which is currently not contraindicated for use during pregnancy, presents itself as a promising drug for hypertensive complications of pregnancy in selected cases.

Summary

TNF-α associated with an increase in CD161+ NK cells plays a causative role in abnormal vascular adaptation and placental biology in this pre-clinical model of chronic hypertension in pregnancy. Treatment with etanercept ameliorates the adverse pregnancy outcomes associated with this model; including direct effects on the placenta where it reduces CD161 expression and granzyme B production from NK cells. Thus, etanercept should be considered in future studies as a promising therapeutic avenue for pregnancies complicated by hypertension.
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SUPPLEMENTAL MATERIAL: THE ROLE OF TNFα AND NATURAL KILLER CELLS IN UTERINE ARTERY FUNCTION AND PREGNANCY OUTCOME IN THE STROKE PRONE SPONTANEOUSLY HYPERTENSIVE RAT

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Supplemental Methods

Radiotelemetry

Systolic (SBP) and diastolic blood pressure (DBP), heart rate and activity were directly monitored using the Dataquest V telemetry system (Data Sciences International, Sheffield, UK) in WKY, SHRSP and SHRSP treated with etanercept (n=4 in each group). Prior to implantation, the radiotelemetry transmitter was checked for calibration to be accurate within ± 6 mmHg. Animals were allowed 2 weeks to recover before time mating.

Doppler Ultrasound

Doppler waveform recordings were used to assess uterine artery blood flow in WKY, SHRSP and SHRSP treated with etanercept (n=6 in each group). Rats were anaesthetized using an inhalational anaesthetic (1.5 % isoflurane/oxygen) throughout the procedure. Ultrasound gel was applied to exposed skin as an ultrasound coupling medium. Rats were imaged trans-abdominally using an Acuson Sequoia C256 imager fitted with a 15-MHz linear array transducer (Siemens, Surrey, UK). Peak systolic velocity (PSV) and end diastolic velocity (EDV) was measured from 6 consecutive cardiac cycles. Resistance index (RI) was calculated as (RI = [PSV - EDV]/PSV).

Wire Myography

Rings from the main uterine artery from pregnant (GD18) WKY, SHRSP and SHRSP treated with etanercept (n=6 in each group) were prepared consistently from arterial segments located closer to the vagina than the ovary from the most pregnant horn then harvested in calcium free physiological salt solution (PSS) (0.25 M NaCl, 0.001 M KCl, 2 mM MgSO4, 50 mM NaHCO3, 2 mM KH2PO4, 1 mM glucose, 0.5 ml of 23 mM EDTA). Uterine artery rings (1.8 -2.0 mm in length) were mounted on two stainless steel wires on a four channel small vessel myograph (AD Instruments, Oxford, UK) in PSS (0.25 M NaCl, 0.001 M KCl, 2 mM MgSO4, 50 mM NaHCO3, 2 mM KH2PO4, 1 mM glucose; 2.5 mM CaCl2). Vessels were normalized and subject to a wake-up procedure as described previously (5). To establish the vessel's contractile response, noradrenaline (Sigma-Aldrich, Dorset, UK) was added at the following increasing concentrations: 1x10^-9, 1x10^-8, 1x10^-7, 1x10^-6, 1x10^-5 and 3x10^-5 M. To determine the vessel's endothelium dependent relaxation response, vessels were pre-constricted with 1x10^-5 M noradrenaline followed by the addition of carbachol (Sigma-Aldrich, Dorset, UK) at the following increasing concentrations: 1x10^-8, 1x10^-7, 1x10^-6 and 1x10^-5 M.

Pressure Myography

Uterine artery rings from pregnant (GD18) WKY, SHRSP and SHRSP treated with etanercept (n=6 in each group) were mounted for pressure myography in calcium free PSS. The pressure myograph system (Danish Myo Technology, Aarhus N, Denmark) was set up and equilibrated according to manufacturer's instructions. Arteries were tied to two glass cannula using nylon thread. Any side branches of the artery were also tied closed with nylon thread. Vessels were maintained at 37 ºC
and 95 % O2 and 5 % CO2 throughout the experiment. After equilibration at 70 mmHg for one hour, the vessels were subject to increasing intraluminal pressure: 10, 20, 40, 60, 80, 100 and 110 mmHg. Measurements of internal and external diameter were taken after five minutes at each pressure. Wall thickness was calculated as [(external diameter - internal diameter) / 2]. Cross sectional area was calculated as [((π/4) x (external diameter² - internal diameter²)].

Identification of Implantation Sites

Rats were anaesthetized (2.5% isoflurane) throughout the procedure. 250 μl of 1% Evans' blue dye (Sigma-Aldrich, Dorset, UK) in PBS was injected into the exposed femoral vein. After 10 minutes, the animal was sacrificed under terminal anaesthesia and the uterine horn was excised. Implantation sites were determined by clear bands of positive blue staining.

Placental Tissue Explants

Placental tissue was harvested in ice cold PBS from WKY, SHRSP and SHRSP treated with etanercept (n=5 in each group). Under sterile conditions, the tissue was dissected into pieces <5mm in ice cold DMEM F-12 Media +0.5% FBS +1% penicillin/streptomycin (Thermo Fisher Scientific, Paisley, UK). The tissue pieces were split evenly between 2 wells of a 24 well plate (Thermo Fisher Scientific, Paisley, UK) in 1ml of media. After 1 hour, the explants were washed in PBS and replaced with a fresh 1ml of media. Explants were incubated for 20 hours at 37 °C after which the media was collected and stored at -80 °C.

ELISA

TNFα in plasma, urine and explant media from WKY, SHRSP and SHRSP treated with etanercept (n=6 in each group for plasma and urine measurements; n=5 in each group for placental explant media measurements) was measured using a commercially available kit (R&D Systems, Abingdon, UK) according to manufacturer’s instructions.

Leukocyte Isolation

Leukocytes were isolated on the day of collection. Tissue from 3 uteroplacental units from pregnant (GD18) WKY, SHRSP and SHRSP (n=6 in each group) treated with etanercept were dissected, perfused with PBS (Thermo Fisher Scientific, Paisley, UK) then harvested in ice-cold PBS (Thermo Fisher Scientific, Paisley, UK). The placental tissue was disrupted using scissors to produce a single cell suspension which was passed through a 70 μm cell strainer (BD Bioscience, Oxford, UK). Leukocytes were isolated from blood collected in heparinized tubes (BD Bioscience, Oxford, UK) from non-pregnant (n=4 in each group) and pregnant (GD18) (n=5-6 in each group) WKY and SHRSP by density gradient centrifugation with Histopaque® (Sigma-Aldrich, Dorset, UK). Cells were washed once (1300 rpm for 6 minutes) and resuspended in 3ml of 2% FBS/PBS (FACS buffer) and placed on ice. Cells were counted using a haemocytometer and 1 x 10⁶ leukocytes were subject to staining procedure. Cells were used for staining on the day of tissue collection.

Extracellular Flow Cytometry Panel
Isolated leukocytes (details in supplemental material) from WKY (NP: n=4, GD18: n=5), SHRSP (NP: n=4, GD18: n=5) and SHRSP treated with etanercept (n=6) were stained using Zombie® live/dead dye (Biolegend, London, UK) for 15 minutes at room temperature in the dark followed by staining for extracellular markers using fluorochrome conjugated primary antibodies for 20 minutes on ice in the dark. Two panels of antibodies were used. Panel 1: anti-CD45-PerCP/Cy5.5, anti-CD3-APC, anti-CD4-PE/Cy7, anti-CD8-FITC and anti-CD161-PE (Biolegend, London, UK). Panel 2: anti-CD45-PerCP/Cy5.5, anti-CD103-AlexaFluor®647, anti-CD4-PE/Cy7, anti-RT1B-FITC (Biolegend, London, UK) and anti-HIS36-PE (BD Bioscience, Oxford, UK). Cells were washed and resuspended in FACS buffer for immediate analysis using a BD FACSCanto II machine (BD Bioscience, Oxford, UK) with BD FACSDIVA™ software.

**Intracellular Staining**

Cells from pregnant (GD18) WKY, SHRSP and SHRSP treated with etanercept (n=4 in each group) were cultured in RPMI 1640 media +10% FBS +1% penicillin/streptomycin (Thermo Fisher Scientific, Paisley, UK) then stimulated using a cell stimulation cocktail (BD Bioscience, Oxford, UK) at 2 µl/ml media and incubated at 37 °C for 4 hours. Cells were harvested after this time and stained using Zombie® live/dead dye (Biolegend, London, UK) for 15 minutes at room temperature in the dark followed by staining for extracellular markers using fluorochrome conjugated primary antibodies for 20 minutes on ice in the dark (anti-CD45-PerCP/Cy5.5, anti-CD3-APC and anti-CD161-PE; Biolegend, London, UK). Cells were then fixed in intracellular fixation buffer (eBioscience, Hatfield, UK) for 45 minutes and washed in 1% permeabilisation buffer (eBioscience, Hatfield, UK) at 1100rpm for 6 minutes at 4 °C. Cells were then stained according to manufacturer’s instructions for intracellular TNFα (anti-TNFα-PE-Cy7; eBioscience, Hatfield, UK) and Granzyme-B (anti-granzyme-B-FITC antibody, Biolegend, London, UK) at room temperature for 30 minutes in the dark. Cells were washed and resuspended in FACS buffer for immediate analysis using a BD FACSCanto II machine (BD Bioscience, Oxford, UK) with BD FACSDIVA™ software where 10,000 CD45+ events was used as a cut-off in each sample.

**Histology**

Uteroplacental tissue from WKY, SHRSP and SHRSP treated with etanercept (n=6 in each group) was fixed for 24 hours in 10% formalin. Paraffin sections of 5 µm were used for staining. Immediately prior to staining, slides were deparaffinised and rehydrated through an ethanol gradient into distilled H2O. Periodic acid – Schiff (PAS) stain was used to quantify glycogen cell content in the placenta. Slides were incubated in 1% periodic acid/dH2O (Sigma-Aldrich, Dorset, UK) for 10 minutes followed by 1 minute under running tap water. Slides were then stained with Schiff’s reagent (Sigma-Aldrich, Dorset, UK) for 10 minutes. This was followed by 1 minute under running tap water and dehydration through an ethanol gradient and mounted using DPX (Sigma-Aldrich, Dorset, UK). Sections were viewed using light microscopy where 8-10 images were taken at 4x objective and laced together using Microsoft Image Composite Editor 2.0 Positive staining for histology was determined
using a threshold quantification method in Image J (National Institutes of Health, Bethesda, USA). Sections were analysed by an operator who was blinded to the identity of the slides. The % positive staining was determined as (the amount of pixels over the given threshold ratio/the total number of pixels) x 100.

**Gene Expression**

Uteroplacental tissue was snap frozen in liquid nitrogen and stored at -80 °C until use. RNA was extracted using the miRNeasy kit (Thermo Fisher Scientific, Paisley, UK) according to manufacturer’s instructions. RT-PCR was used to prepare complimentary DNA (cDNA). RT-PCR was performed using the Taqman® Reverse Transcription Kit (Thermo Fisher Scientific, Paisley, UK) according to manufacturer’s instructions with 1 µg RNA input. The reaction was run on a Multi Block System Satellite 0.2 Thermo Cooler (Thermo Fisher Scientific, Paisley, UK) on the following settings: 25 °C 10 min, 48 °C 30 min, 95 °C 5 min. qPCR was used to quantify gene expression. Reactions were set up using the following reagents: Taqman® Universal Mastermix (Thermo Fisher Scientific, Paisley, UK), nuclease free H₂O (Thermo Fisher Scientific, Paisley, UK) and relevant Taqman® probe (Thermo Fisher Scientific, Paisley, UK). The reaction was run on a QuantiStudio® 12K Flex (Thermo Fisher Scientific, Paisley, UK) at the following settings: 95 °C, 15 min; followed by 40 cycles of 95 °C, 15 s; 60 °C, 1 min. Ct values were analysed using the $2^{-\Delta\Delta Ct}$ method, with ΔCt indicating normalisation to the housekeeper β-actin (Actb). This experiment was run using the Taqman® system (Thermo Fisher, Paisley, UK) where Tnfr1 expression was assessed using the probe Rn01492348_m1 (Thermo Fisher, Paisley, UK) which was normalised to the housekeeper; Gapdh Rn01462661_g1 (Thermo Fisher, Paisley, UK).
**Supplemental Figures & Figure Legends**

**A**

**B**

**Supplementary Figure S1**: Etanercept does not significantly alter TNFα levels in maternal plasma or from placental explants at GD18. TNFα was measured in plasma (A) (n=6) and media from placental explants (B) (n=5) using ELISA at GD18 in WKY, SHRSP and SHRSP treated with etanercept. SHRSP and SHRSP treated with etanercept showed elevated levels of TNFα in both maternal plasma and in media from placental explants (* p>0.05 vs. GD18 WKY).
Supplementary Figure S2: Heart rate and activity before and during pregnancy in the WKY, SHRSP and SHRSP treated with Etanercept. Heart rate (A) was not significantly altered between the groups. Activity (B) was significantly decreased in SHRSP and SHRSP treated with Etanercept relative to WKY (* p<0.05 vs. WKY analysed by two-way ANOVA).
Supplementary Figure S3: Blastocyst implantation is not altered between WKY and SHRSP. Blastocyst implantation was determined at GD6 in untreated SHRSP and WKY (n=3-4) using Evans' blue dye infusion (A) under terminal anaesthesia. There was no significant difference between WKY and SHRSP in the number of implantation sites counted (B).

Supplementary Figure S4: Maternal, fetal and placental weight are unaffected by etanercept treatment in the SHRSP. Fetal (A) and placental (B) weight did not show any significant differences between strains at GD18 (n= 6-12). Maternal weight gain (B) during pregnancy was significantly decreased in both SHRSP and SHRSP treated with etanercept relative to WKY (data analysed by area under the curve followed by one way ANOVA; ** p<0.01 vs. WKY).
Supplementary Figure S5: Etanercept treatment does not alter uterine artery size in the SHRSP. Isolated uterine artery properties were assessed using pressure myography to measure external (A) and internal (B) diameter, cross-sectional area (C) and wall thickness (D) in etanercept treated SHRSP, vehicle treated SHRSP and untreated WKY (n=6) at GD18 of pregnancy. External and internal diameter as well as cross-sectional area was significantly decreased in both untreated and etanercept treated SHRSP (data analysed by area under the curve followed by one way ANOVA; * p<0.05 vs. WKY; ** p<0.01 vs. WKY).
Supplementary Figure S6: Etanercept treatment does not alter mesenteric artery function in the SHRSP. Isolated mesenteric artery function was assessed using wire myography in WKY (n=4), SHRSP and SHRSP treated with etanercept (n=6) at GD18 of pregnancy. There was a trend for etanercept to shift the contractile response to noradrenaline (A). Etanercept treatment did not significantly improve endothelium dependent vasorelaxation to carbachol (* p<0.05 vs. GD18 WKY) (B). Data analysed by calculating area under the curve followed by one way ANOVA and post-hoc Tukey test.
Supplementary Figure S7: Full flow cytometry panel in maternal blood from pregnant WKY, SHRSP and SHRSP treated with etanercept. A flow cytometry panel was used to quantify immune cell sub-types in the maternal blood from pregnant (GD18) WKY, SHRSP and SHRSP treated with etanercept (n=5-6). The percentage of T cells (A) in the maternal blood was marginally but significantly increased in SHRSP and SHRSP treated with etanercept relative to the WKY, however there was no significant difference between the balance of helper (B) and cytotoxic T cells (C). Both NK cells (D) and NK-T cells (E) were significantly increased in SHRSP and SHRSP treated with etanercept relative to the WKY. There were no significant alterations in the presence of dendritic cells. Data was analysed using one way ANOVA followed by a post-hoc Tukey’s test where * p<0.05, ** p<0.01, *** p<0.0005 vs. WKY GD18.
Supplementary Figure S8: Full flow cytometry panel in placenta from pregnant WKY, SHRSP and SHRSP treated with etanercept. A flow cytometry panel was used to quantify immune cell sub-types in placenta from pregnant (GD18) WKY, SHRSP and SHRSP treated with etanercept (n=5-6). T cells (A) and Th cells (B) were significantly increased in SHRSP treated with etanercept relative to WKY. NK cells (D) were significantly increased in the SHRSP relative to the WKY and SHRSP treated with etanercept. NK-T cells (E) were significantly increased in SHRSP and SHRSP treated with etanercept. The detection of dendritic cells (F) was variable and not measurable in all samples. There was a trend for etanercept to reduce the number of macrophages in the SHRSP (G). Data was analysed using one way ANOVA followed by a post-hoc Tukey’s test where * p<0.05, **p<0.01 vs. WKY GD18 and ## p<0.01 vs. SHRSP.
Supplementary Figure S9: Gating strategy for extracellular immune cell panel.

Supplementary Figure S10: TNFα production from CD3+ cells and other CD45+ cells is not significantly different between WKY and SHRSP. Intracellular staining was used to quantify TNFα production from immune cells in the maternal blood and placenta from WKY and SHRSP. In placenta (A-B) and maternal blood (C-D) there was no significant difference in TNFα production from CD3+ or other CD45+ cells, only in CD161+ cells (* p<0.05, ** p<0.01 vs. WKY GD18). Data analysed using Student’s t test.