Immunosuppression Improves Blood Pressure and Endothelial Function in a Rat Model of Pregnancy-Induced Hypertension

John H. Tinsley¹, Valorie L. Chiasson², Sanique South², Ashutosh Mahajan¹ and Brett M. Mitchell²

BACKGROUND
Hypertensive disorders of pregnancy, including preeclampsia (PE), affect ~7–10% of pregnancies in the US. Clinical and experimental studies strongly suggest that the maternal immune system plays a role in the development of these disorders; however, few therapeutic options exist aside from delivery.

METHODS
Using a deoxycorticosterone acetate (DOCA)/salt-low renin rat model, which exhibits hypertension, proteinuria, endothelial dysfunction, and intrauterine growth restriction (IUGR), we measured serum cytokine levels as an indication of immune system activation. In addition, we suppressed the immune system with either azathioprine (Aza) or mycophenolate mofetil (MMF) during the second half of pregnancy to determine whether these symptoms could be ameliorated.

RESULTS
Our results demonstrate that serum T helper-1 (Th1)-type inflammatory cytokines interleukin (IL)-2, IL-12, interferon-γ (IFNγ), and RANTES were significantly elevated in hypertensive pregnant rats while the Th2-type cytokine IL-4 was elevated in normal pregnant animals. Either Aza or MMF significantly attenuated the hypertension, proteinuria, and endothelial dysfunction as well as the increased proinflammatory Th1 cytokine profile in pregnant rats treated with DOCA/salt, and had no effect on these parameters in normal pregnant rats.

CONCLUSION
These data strongly suggest that maternal immune system activation plays a role in the development of pregnancy-induced hypertension (PIH).


Hypertensive disorders of pregnancy, including pregnancy-induced hypertension (PIH) and preeclampsia (PE), affect ~7–10% of pregnancies in the United States, and commonly lead to intrauterine growth restriction (IUGR) of the fetus as well as an increased risk of future ischemic heart disease in the mother. Despite numerous research efforts to understand these disorders, they remain one of the leading causes of premature births as well as fetal and maternal mortality, and the treatment has not improved significantly in the past 50 years.

To accommodate the needs of the growing uterus, placenta, and fetus, circulating volume and cardiac output increases in the pregnant female by 40–50% (refs. 1–3), which is offset by a decreased peripheral resistance thereby lowering blood pressure even beyond pre-pregnancy values during mid-gestation. Although the etiology of proteinuria and/or hypertension during pregnancy remains unknown, several mechanisms that play a role have been elucidated including inadequate placentation,⁴ endothelial dysfunction,⁵ angiogenic imbalance,⁶,⁷ abnormal activation of the uteroplacental renin-angiotensin system, placental hypoxia, and abnormal maternal immune activation.⁸–¹⁰ All of these lead to the clinical manifestation of a continuum of symptoms ranging from severe early-onset to mild–moderate late-onset.

Strong evidence exists for a role of the maternal immune system in the development of PIH/PE. In pregnancy, the mother is exposed to and must tolerate an antigen (the fetus) that is half foreign (paternal), in large part by modulating her immune system. It has been proposed that women who develop PIH and PE have abnormal immunological responses to the presence of a fetus and that proteinuria and/or hypertension are the clinical signs of a mild form of fetal rejection. Studies have reported increased serum levels of proinflammatory T helper-1 (Th1) cytokines and decreased levels of anti-inflammatory Th2 cytokines in women with PE compared to normal pregnancy.¹¹–¹³ However, pregnancy is also a state of inflammation and it appears that proinflammatory Th1 cytokines are necessary for successful pregnancies and fetal development.¹³,¹⁴ Other investigators have shown that the Th1/Th2 immune responses during normal pregnancy show a shift toward Th2-type immunity at the feto-maternal interface.¹⁵,¹⁶ Therefore, suppression of Th1 polarized T cells in the latter stages of pregnancy may keep inflammation and maternal immune responses to the fetus at “tolerable” levels and prevent PIH.

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It has been demonstrated that deoxycorticosterone acetate (DOCA)/saline treatment of rats increases circulating levels of the proinflammatory cytokine tumor necrosis factor (TNF)-α and inhibition of TNF-α can prevent the renal inflammatory effects of DOCA/saline. Additionally, mice lacking both T and B cells exhibit attenuated aortic superoxide production and blood pressure following DOCA/saline treatment. These data suggest that T-cell activation and proliferation (part of the adaptive immune response) contribute to the detrimental cardiovascular and renal effects of DOCA/saline. Thus, immunosuppression via prevention of T-cell proliferation may decrease hypertension induced by DOCA/saline as well as other forms of hypertension. Mycophenolate mofetil (MMF), as well as the structurally similar drug azathioprine (Aza), are purine synthesis inhibitors that prevent proliferation of cells, especially lymphocytes. Most cells of the body have more than one way to synthesize purines, with the exception of lymphocytes. By blocking a key enzyme involved in purine synthesis, MMF and Aza prevent proliferation of both T and B cells. Both drugs have been shown to be effective in kidney, liver, heart, and lung transplantation and have no detrimental effect on blood pressure. Studies have shown that MMF reduces blood pressure in various sodium-induced hypertensive rats.

Aza prevent proliferation of both T and B cells. Both drugs are synthesized that Th1 proinflammatory cytokine levels will be elevated in pregnant rats treated with DOCA/saline (PDS) while Th2 anti-inflammatory levels will be decreased in this group compared to normal pregnant (NP) rats, nonpregnant controls (Con), and nonpregnant rats treated with DOCA/saline (DS). Furthermore, if excessive maternal immune system activation contributes to the development of PIH, then immunosuppression with Aza or MMF during pregnancy should ameliorate the observed alterations in proinflammatory cytokine expression, blood pressure, endothelial function, and proteinuria.

**METHODS**

**Animals/treatments.** Male, for mating purposes only, and female Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 200–250 g were used in all experiments. Rats were on a 12:12 light/dark cycle and had free access to standard chow and water (NP and Con) or 0.9% saline (PDS and DS). Following 1 week of acclimatization, female PDS rats were placed hypertensive as described previously. Briefly, females received 12.5 mg of DOCA (intraperitoneal injection), were placed with males and given tap water to drink. DS rats were placed with males and given tap water to drink. DS rats received 12.5 mg of DOCA and placed on 0.9% saline. PDS and DS rats received one 6.25-mg DOCA injection during weeks 2 and 3 of gestation. Aza (2 mg/kg) or MMF (30 mg/kg) were administered daily by intraperitoneal injection beginning on day 10 of gestation. Rats were sacrificed on gestational day 20 or the corresponding day in nonpregnant animals. All procedures were approved by the Texas A&M Health Science Center/Scott & White Memorial Hospital Institutional Animal Care and Use Committee.

**Blood pressure.** Systolic arterial blood pressure was measured by tail-cuff plethysmography. Rats were trained for 3 days before data collection. Animals were warmed to 32 °C and measurements were taken using an IITC Model 59 amplifier (Woodland Hills, CA), a system that correlates well with telemetry measurements of systolic blood pressure.

**Proteinuria.** One day before euthanization, urine was collected with the animals housed individually in metabolic cages in the absence of food to eliminate contamination of urinary protein measurements by fallen food particles. Urine protein concentration was measured using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method; Sigma, St Louis, MO). Urinary creatinine was determined using a Nova electrolyte analyzer (Waltham, MA). Results are expressed as mg protein/mg creatinine as a percent of control.

**Determination of IUGR/fetal growth.** IUGR and fetal growth were assessed by weighing the intact uterus with pups and again after the pups were removed, giving total pup weight. Any fetal malformations or resorptions were observed by two investigators.

**Vasodilation/endothelial function.** Aortic rings were connected to an isometric force transducer in a custom-made 15-ml organ chamber filled with 37 °C physiological salt solution (119 mmol/l NaCl, 4.7 mmol/l KCl, 25 mmol/l NaHCO₃, 1.18 mmol/l KH₂PO₄, 1.17 mmol/l MgSO₄·7H₂O, 11.1 mmol/l dextrose, 2.5 mmol/l CaCl₂) with 95% O₂–5% CO₂. All experiments were performed in the presence of indomethacin (10 μmol/l) to inhibit prostacyclin production by cyclooxygenase. Passive tension on the vessels was set at 400 mg based on previously generated length–tension curves, and isometric force generation was recorded continuously with a PowerLab system (AD Instruments, Colorado Springs, CO). After a 60-min equilibration period, vessels were contracted with phenylephrine (0.1–1 μmol/l) and repeated until reproducible contractions were obtained. Acetylcholine (10 μmol/l) was administered to test the functional integrity of endothelium as measured by a relaxation response. Concentration–response curves were obtained in a half-log, cumulative fashion in response to acetylcholine (1 nmol/l to 100 μmol/l) following contraction to an EC₇₀ concentration of phenylephrine. Relaxation responses were expressed as percent relaxation from phenylephrine-induced contraction.
Serum cytokine levels. Serum Th1 and Th2 cytokine levels were measured by enzyme-linked immunosorbsent assay. Blood was collected at time of sacrifice in nonheparinized tubes and allowed to clot for 2 h followed by centrifugation at 2,000 g for 10 min. Collected sera was used for cytokine measurements with the following enzyme-linked immunosorbsent assay kits: interleukin (IL)-10, IL-6, TNF-α, and interferon-γ (IFN-γ) (Pierce, Rockford, IL); IL-12 and RANTES (Biosource, Carlsbad, CA); IL-2 (R&D Systems, Minneapolis, MN); and IL-4 (U-Cytech Bioscience, Utrecht, the Netherlands) according to the manufacturers’ protocols.

Statistics. For measures expressed as percent of control, values for each group including controls were compared to the mean of the control group, then mean and s.e.m. was determined. The Student’s t-test was used to compare variables between two groups. An analysis of variance was used for multiple comparisons followed by the Student–Newman–Keuls post hoc test when necessary. The significance level was 0.05.

RESULTS

The nonpregnant DS group did not exhibit significant differences in blood pressure, urinary protein, or endothelial function compared to either nonpregnant control (Con) or normal pregnant (NP) animals. This suggests that in the nonpregnant state the animal is able to handle this low concentration of DOCA and saline without raising arterial pressure. Furthermore, there were no significant increases in inflammatory cytokine levels with the exception of IL-2 in the DS rats compared to Con therefore these data are not shown.

Cytokine expression

Proinflammatory cytokine levels are elevated in women with hypertensive pregnancies; however, whether or not immune system activation occurs in PDS rats had not been examined. Therefore, we assayed serum levels of the proinflammatory Th1-type cytokines/chemokines IL-2, IL-6, IL-12, IFN-γ, TNF-α, and RANTES in our Con, NP, and PDS rats. All of these cytokines with the exception of IL-6 and TNF-α were significantly elevated in the PDS group compared to Con and/or NP animals (Figures 1–4). IL-6 and TNF-α were increased significantly in both NP and PDS groups compared to Con (data not shown). Immunosuppression with Aza or MMF normalized IL-2 and IFN-γ levels (Figures 1 and 3). With regard to IL-12, either Aza or MMF significantly decreased serum levels below Con levels while only MMF significantly decreased serum RANTES levels compared to Con (Figures 2 and 4).

According to clinical studies, anti-inflammatory cytokines generally increase in women undergoing normal pregnancies. In agreement with this, we found that serum levels of the Th2-type cytokine IL-4 were significantly elevated in NP rats compared to both PDS and Con animals (Figure 5). However, we found a significant increase in serum IL-10 levels in PDS rats compared to NP and Con (Figure 6). The upregulation of this anti-inflammatory cytokine likely represents a compensatory mechanism to help suppress inflammation.

As with the proinflammatory cytokines, immune suppression by Aza or MMF normalized serum IL-10 and IL-4 levels in NP and PDS rats to Con levels (Figures 5 and 6).

Endothelial function

Impaired endothelial function, specifically vasodilatory responses, is another hallmark symptom associated with PIH and PE. Aortic rings from PDS rats exhibited significantly diminished relaxation responses compared to Con and NP (Figure 7a). There were no differences in relaxation responses to the endothelium-independent dilator sodium nitroprusside between Con, NP, PDS, and DS rats (data not shown). In order to ascertain the possible effects of the adaptive immune system on endothelial-dependent relaxation responses, we also measured acetylcholine-induced relaxation responses in vessels from Aza- or MMF-treated rats. As shown in Figure 7b,c, both immunosuppressants restored vasodilatory responses in PDS animals to that of Con and NP levels. These data strongly...
Table 1 | Effects of immunosuppressive drugs on blood pressure, urinary protein, and fetal growth in rats

<table>
<thead>
<tr>
<th></th>
<th>NP (n = 8)</th>
<th>PDS (n = 8)</th>
<th>CON (n = 8)</th>
<th>AZA-NP (n = 9)</th>
<th>AZA-PDS (n = 9)</th>
<th>AZA-CON (n = 10)</th>
<th>MMF-NP (n = 8)</th>
<th>MMF-PDS (n = 8)</th>
<th>MMF-CON (n = 8)</th>
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<tr>
<td>SBP-day 0 (mm Hg)</td>
<td>113 ± 6</td>
<td>112 ± 4</td>
<td>120 ± 4</td>
<td>116 ± 5</td>
<td>118 ± 4</td>
<td>117 ± 3</td>
<td>117 ± 5</td>
<td>112 ± 5</td>
<td>113 ± 5</td>
</tr>
<tr>
<td>SBP-day 18 (mm Hg)</td>
<td>106 ± 4</td>
<td>133 ± 4**</td>
<td>116 ± 5</td>
<td>118 ± 5</td>
<td>122 ± 2</td>
<td>120 ± 2</td>
<td>111 ± 5</td>
<td>112 ± 6</td>
<td>114 ± 4</td>
</tr>
<tr>
<td>Urine prot/creat (mg/mg; % of CON)</td>
<td>91 ± 23</td>
<td>369 ± 47***</td>
<td>100 ± 48</td>
<td>116 ± 35</td>
<td>169 ± 36</td>
<td>100 ± 26</td>
<td>76 ± 12</td>
<td>121 ± 31</td>
<td>100 ± 23</td>
</tr>
<tr>
<td>Pups/litter (number)</td>
<td>14.0 ± 0.6</td>
<td>10.2 ± 0.7**</td>
<td>—</td>
<td>14.6 ± 0.8</td>
<td>13.2 ± 0.8</td>
<td>—</td>
<td>11.8 ± 1.1</td>
<td>17.1 ± 0.3**</td>
<td>—</td>
</tr>
<tr>
<td>Malform/litter (number)</td>
<td>0.0 ± 0.0</td>
<td>1.0 ± 0.3**</td>
<td>—</td>
<td>0.7 ± 0.4</td>
<td>3.9 ± 1.5</td>
<td>—</td>
<td>10.8 ± 1.6</td>
<td>17.1 ± 0.3**</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
AZA, azathioprine; CON, not pregnant; DOCA, deoxycorticosterone acetate; MMF, mycophenolate mofetil; NP, normal pregnant; PDS, pregnant + DOCA/saline; SBP, systolic blood pressure.
*P < 0.05 vs. CON. **P < 0.05 vs. NP.
suggest that the adaptive immune system contributes to endothelial dysfunction in PIH.

Blood pressure, urinary protein, and fetal growth

Based on elevated proinflammatory cytokine levels in women with PE, we predicted that suppressing the immune system during the latter half of gestation would attenuate the hypertension, proteinuria, and IUGR exhibited by PDS rats. The significant increase in systolic blood pressure in PDS compared to Con and NP rats was prevented by either Aza or MMF (Table 1). Furthermore, the nearly fourfold increase in urinary protein excretion in PDS rats was attenuated following treatment with either Aza or MMF (Table 1). Litters from PDS rats were significantly smaller in number than those from NP animals. Again, Aza and MMF prevented the reduction in litter size attributable to DOCA/salt treatment during pregnancy (Table 1). Although the number of pups per litter was not compromised by Aza and MMF, examination of the pups themselves revealed some detrimental effects. MMF is known to be teratogenic and ~90% of pups from NP animals and 100% of pups from PDS rats were resorbed. However, Aza had less of a detrimental effect on pup formation compared to MMF. The number of malformations in both NP and PDS rats tended to be higher compared to nontreated NP and PDS rats; however, this did not reach statistical significance. Litter weights were not statistically different between nontreated and Aza-treated rats; however, MMF-treated NP and PDS rats had significantly lower litter weights due to fetal resorption.

**DISCUSSION**

During pregnancy, physiological adaptations occur that not only allow the half-foreign fetus to escape maternal immune attack and rejection but also develop and grow. Placental hormones and cytokines are released from the feto-maternal interface into the maternal blood stream and signal the maternal immune system for these adaptations that are required...
for a successful pregnancy. Abnormal placental production of these factors and/or maternal responses may lead to pregnancy-related disorders such as PIH/PE and spontaneous abortion.

Although the maternal adaptive immune system has long been considered to play an important role in the development of PIH and PE, clinical data vary greatly with respect to differences in specific cytokine levels between normal pregnant women and hypertensive pregnant women. Jonsson et al. examined serum levels of 20 different cytokines and found significant differences in only two, IL-6 and IL-8, between hypertensive and normal pregnant women. Sharma et al. saw the same increases in serum IL-6 and IL-8 levels but also found both a significant increase in serum TNF-α and a decrease in IL-10 in hypertensive pregnant women compared to normal pregnant women. Additional studies have found increases in placental and/or peripheral blood levels of the inflammatory cytokines IL-2, IL-12, IL-15, IL-18, and IFN-γ in hypertensive pregnant women. In contrast, Freeman et al. found that IL-10 and TNF-α serum levels increased from the first to the third trimester in control pregnancies to nearly the same degree as in women with hypertensive pregnancies. These inconclusive and often contradictory findings reveal that our understanding of the factors contributing to hypertensive pregnancies is quite limited and may also represent differences in the etiology (i.e., excessive maternal immune system activation vs. placental insufficiency).

Our DOCA/salt rat model of PIH exhibits many symptoms including hypertension, proteinuria, endothelial dysfunction, and IUGR (Table 1 and Figure 7). Importantly, these symptoms do not manifest in nonpregnant females receiving this low concentration of DOCA/salt. We determined whether this experimental model exhibits cytokine profiles similar to those seen in women with hypertensive pregnancies. We found...
significantly increased serum levels of IL-2, IL-12, IFN-γ, and RANTES on gestational day 20 in PDS rats while only IL-2 was increased in nonpregnant rats treated with DOCA/saline. These findings support several clinical reports in which hypertensive pregnant women also demonstrated increased levels of these Th1 cytokines compared to normal pregnant women. Furthermore, levels of these Th1-type cytokines in our NP rats were not elevated above control, nonpregnant levels. However, we did find that both IL-6 and TNF-α levels at gestational day 20 were similarly increased in both NP and PDS rats supporting the notion that pregnancy is a proinflammatory state and that increased levels of certain proinflammatory cytokines aid in parturition. Interestingly, serum levels of IL-12 were drastically reduced to barely detectable levels in NP rats (Figure 2). This suggests that during the end of normal pregnancy the development of new Th1 cells is impaired as IL-12 along with IL-18 promotes the development of naive T (Th0) cells into proinflammatory T helper (Th1) cells that produce IL-2 and IFNγ, promoting cell-mediated immunity involving cytotoxic T cells, NK cells, and macrophages. Taken together, our data support a mechanism by which DOCA/salt provides a necessary signal (IL-2) that, only when coupled with normal pregnancy immunological responses, leads to the production of proinflammatory Th1 cytokines, endothelial dysfunction, and hypertension. The development of Th2 cells that secrete the cytokines IL-4 and IL-10 promotes antibody-mediated immunity but also suppresses Th1 cytokine production and end-organ damage. The inverse also occurs as Th1 cytokines such as IFN-γ are able to suppress Th2 responses. In normal pregnancy, this balance between the Th1 and Th2 arms of the adaptive immune response is altered by the presence of the placenta. The placenta is a Th2 cytokine–producing organ, releasing both IL-4 and progesterone and this activity stimulates a bias in favor of Th2 responses and inhibits the development of Th1 cells, supported by our finding of extremely low circulating levels of IL-12 in NP rats. This shift is thought to protect the fetus from maternal cell-mediated immune attack. In hypertensive disorders of pregnancy, the shift to Th2 does not occur properly; however, the underlying mechanisms responsible for this remain unknown. In agreement with this Th1 to Th2 switch, we found an approximate threefold increase in serum IL-4 levels in NP rats compared to Con (Figure 5). In addition, DOCA/salt treatment significantly attenuated this increase in IL-4 levels attributed to pregnancy. However, PDS rats exhibited an eightfold increase in serum IL-10 levels. IL-10 is generally considered to have anti-inflammatory properties and this dramatic increase in circulating levels may be playing a compensatory role in PDS rats to prolong fetal tolerance. Elevations in IL-10 levels have been reported to induce tolerance or prolong allograft survival, but other studies have reported that levels of IL-10 are increased, decreased, or not changed in hypertensive pregnant women compared to normal pregnant women. The role of IL-10 and IL-4 in normal and hypertensive pregnancies is currently under investigation in our laboratory.

We hypothesized that if PIH is mediated by a maternal immune response, then immunosuppression during pregnancy should ameliorate the hypertension and endothelial dysfunction in this experimental model of PIH. In this study, we used clinically relevant doses of the immunosuppressants Aza and MMF, both of which decrease IL-2 mediated T-cell proliferation and cell-mediated immune responses. As expected, Aza and MMF blocked the increases in IL-2, IFN-γ, and RANTES elicited by DOCA/salt treatment of pregnant female rats while having no effect on these Th1 cytokine levels in NP rats (Figures 1, 3, and 4, respectively). Additionally, both Aza and MMF significantly lowered IL-12 levels in PDS rats to that of NP levels (Figure 2). We next determined whether this reduction in proinflammatory cytokines translated to improvements in cardiovascular function. Either Aza or MMF blocked the DOCA/salt–mediated hypertension, proteinuria, and endothelial dysfunction in PDS animals (Table 1, Figure 7), while having no effect on these variables in NP rats. These data support previous studies demonstrating the blood pressure lowering effects of MMF in animals with salt-sensitive hypertension.

The use of MMF as a hypertension therapeutic is gaining traction both due to its immunosuppressive and antioxidant properties. However, due to the known teratogenic effects of MMF women of reproductive age are switched to Aza, which is considered safer to the fetus. In this study, we utilized both as proof of concept; MMF caused fetal resorption but was able to prevent the development of hypertension, proteinuria, and endothelial dysfunction. The beneficial cardiovascular effects of MMF in PDS rats were not likely mediated by the fetal resorption as placentas were still present and blood pressure did not change in MMF-treated NP rats compared to nontreated NP rats. Furthermore, we suppressed the immune system during the second half of pregnancy only which allowed for placentation to occur.

The overall findings of this study suggest that DOCA/salt treatment during pregnancy is sufficient to activate the maternal adaptive immune system, which leads to the development of hypertension, proteinuria, and endothelial dysfunction. Additionally, the development of immunosuppressive drugs that do not cross the placenta may be beneficial in women with PIH and those at a high risk of developing PIH.

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