Role of Inflammation and the Angiotensin Type 2 Receptor in the Regulation of Arterial Pressure During Pregnancy in Mice

Katrina M. Mirabito, Lucinda M. Hilliard, Zihui Wei, Chris Tikellis, Robert E. Widdop, Antony Vinh,* Kate M. Denton*

Abstract—During normal pregnancy the renin–angiotensin system is activated, yet pregnant women are resistant to the pressor effects of angiotensin II. Our aim was to determine the role of the angiotensin type 2 receptor (AT$_2$R) in the regulation of arterial pressure, natriuresis, and immune cell infiltration during pregnancy. Mean arterial pressure was measured via telemetry, and flow cytometry was used to enumerate immune cell infiltration in 14-week-old wild-type and AT$_2$R knockout mice during gestation. In wild-type mice, mean arterial pressure decreased during gestation, reaching a nadir at gestational day 9 ($-6\pm2$ mm Hg) and returned to near preconception levels during late gestation. In AT$_2$R-deficient mice, the midgestational decrease in mean arterial pressure was absent. Furthermore, mean arterial pressure was significantly increased during late gestation compared with wild-type mice ($10$ mm Hg). As expected, circulating immune cell activation was suppressed during pregnancy. However, this response was absent in AT$_2$R-deficient mice. While renal immune cell infiltration was similar between the genotypes, there was a significant T cell phenotypic switch toward a proinflammatory T-helper 1 phenotype in AT$_2$R-deficient mice. These data indicate that the AT$_2$R plays an important role in arterial pressure regulation and may modulate T cell activation and renal cytokine production during pregnancy. Therefore, deficits in AT$_2$R expression may contribute to pregnancy-induced hypertension and thus represents a potential therapeutic target. (Hypertension. 2014;64:626-631.) • Online Data Supplement

Key Words: arterial pressure ■ inflammation ■ pregnancy ■ renal

During a normotensive pregnancy circulating angiotensinogen, renin activity, and angiotensin II (AngII) are increased, yet pregnant women are resistant to the pressor effects of AngII.$^{1,2}$ Recently, we have gained strong evidence that the angiotensin type 2 receptor (AT$_2$R) blunts pressor responsiveness, sodium retention, tubuloglomerular feedback, and renal vasoconstrictor responses to AngII in female rodents.$^3$ The renal responses to AngII are also different in healthy men and women,$^6$ and it has been suggested that the explanation for these disparities might also lie with differences in AT$_2$R function.$^7$ Given that estrogen enhances AT$_2$R expression,$^15$ the AT$_2$R may contribute to the cardiovascular and renal adaptations that occur during pregnancy.

While the immune system is suppressed during normotensive pregnancy, the pathophysiology of preeclampsia is characterized by immune system activation.$^{10}$ It has been demonstrated that T cells, part of the adaptive immune system, are essential for the development of AngII-induced hypertension.$^{11}$ Within the kidney, the angiotensin type 1 receptor (AT$_1$R) increases production of the proinflammatory T-helper (Th) 1 cytokine, interferon-$\gamma$ (IFN-$\gamma$), and decreases production of the anti-inflammatory Th2 cytokine interleukin-4.$^{12}$ Skewing of Th cells toward a Th1 phenotype is well established in several inflammatory disorders including hypertension and preeclampsia.$^{12,13}$ Recent studies indicate that AT$_2$R agonism with compound 21 elicits anti-inflammatory actions, skewing the Th1:Th2 balance toward the Th2 phenotype.$^{14,15}$ Thus, the AT$_2$R may contribute to the increase in Th2 cytokines and subsequent suppression of Th1-mediated immune responses during normotensive pregnancies, whereas AT$_2$R deficiency may promote an increase in the Th1:Th2 ratio and pregnancy-induced hypertension.

We hypothesized that during pregnancy the AT$_2$R modulates natriuresis and contributes to the suppression of the immune system, which facilitates the decrease in arterial pressure during pregnancy. Our aims were to examine mean arterial pressure (MAP) throughout pregnancy and the renal and placental immune responses on gestational day (Gd) 8 and Gd16 and renal excretory function at Gd18 in wild-type (WT) and AT$_2$R-knockout (AT$_2$R-KO) mice.

Methods

Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. MAP was measured via radiotelemetry at baseline, during gestation and 2 weeks postpartum in WT (FVB/N)
and AT2R-KO mice. In additional cohorts, immune system activation, infiltration, and the proportion of ex vivo–stimulated T cells producing Th1 (IFN-γ, tumor necrosis factor α, interleukin-17) and Th2 (interleukin-4) cytokines were determined using flow cytometry, renal excretory function was measured via 24-hour urine collection, and renal and placental gene expression of renin angiotensin system (RAS) components (AT1aR, AT1bR, AT2R, angiotensin-converting enzyme 2 [ACE2], and mas receptor [MasR]) were determined using real-time reverse transcription-polymerase chain reaction. See the online-only Data Supplement for full methods.

**Results**

**Arterial Pressure**

Preconception MAP was similar in WT and AT2R-KO mice (94±1 versus 92±1 mm Hg, respectively, P=0.2). During pregnancy, MAP decreased in WT mice. This gestational decrease in MAP reached a nadir of –6±2 mm Hg at Gd9 (P<0.01 as compared with baseline; Figure 1). Compared with WT, the midgestational decrease in MAP was abolished in AT2R-KO mice (P=0.04; Figure 1). Near term, MAP returned to baseline levels in WT mice; however, during late gestation MAP was augmented in AT2R-KO mice (2±3 versus 13±7 mm Hg at Gd20, P<0.05). In mice with radiotelemetry probes that continued to record MAP for the full 2-week postpartum period, it was observed that MAP returned to preconception levels in both WT (88±2 mm Hg; n=6) and AT2R-KO (93±3 mm Hg; n=4) mice.

**Renal Excretory Function**

Basal body weight was similar between the genotypes and increased during pregnancy (P<0.001; Table S1 in the online-only Data Supplement). Water intake and urine flow were increased at Gd18 (both P<0.05), but the increases were not different between the genotypes (Table S1). Sodium intake was increased at Gd18 as compared with baseline (P=0.004; Figure 2A). In contrast, there was no significant difference in sodium excretion between the genotypes or during pregnancy (Figure 2B). Determination of sodium balance (sodium intake minus excretion) demonstrated that during pregnancy there was net sodium retention (P=0.008). Moreover, this effect was enhanced in AT2R-KO mice (P=0.05; Figure 2C). Basal albuminuria was not significantly different between the genotypes. During pregnancy albuminuria decreased 38±6% in the WT and increased 57±35% in the AT2R-KO mice (P<0.001; Table S1). There were no significant differences in fetal number, fetal weight, placental weight, or placental efficiency between the genotypes (Table S2).

**Immune System Activation**

**Circulating**

There was no significant difference in the proportion of circulating T cells (CD3+) between the genotypes at baseline or during gestation (P=0.4; Figure S4A). The proportion of the T cell subpopulations characterized by CD4+ (Th cells), FoxP3+ (T regulatory cells), and CD8+ (cytotoxic T cells) expression was similar between the genotypes at baseline and unchanged during pregnancy (Figure S4B–S4D). Analysis of circulating Th cells demonstrated that at baseline Th activation was significantly lower in AT2R-KO as compared with WT mice (Figure 3A and 3B). Circulating Th activation was suppressed during pregnancy in WT but unaffected in AT2R-KO mice (Figure 3A and 3B). Cytotoxic T cell activation was also suppressed during pregnancy; however, this was not significantly different between the genotypes (data not shown).
The proportion of IFN-γ- and interleukin-4-producing T cells (Figure 3C and 3D) and the Th1:Th2 ratio were similar between the genotypes at baseline (Figure 3E). During gestation, the proportion of T cells producing the IFN-γ at Gd8 and Gd16 decreased to a similar extent in WT and AT2R-KO mice (P<0.05; P<0.0001; Figure 3C). Conversely, the proportion of T cells producing interleukin-4 was significantly increased at Gd16 in both genotypes (P<0.0001; P<0.001; Figure 3D). Consequently, at Gd16 the circulating Th1:Th2 ratio was similarly decreased from basal between the genotypes (P<0.005; P<0.05; Figure 3E). There were no significant differences in circulating tumor necrosis factor-α- and interleukin-17-producing T cells between the genotypes at baseline or during gestation (Figure S5A and S5D).

Renal
Basal renal leukocyte and T cell infiltration were similar in WT and AT,R-KO females, and there were no significant differences in renal cytokine expression or the Th1:Th2 ratio (Figure 4). During pregnancy, renal T cell infiltration was similar between the genotypes (P=0.84; P=0.87; Figure 4B).

However, compared with WT counterparts, the proportion of T cells producing IFN-γ was increased in AT,R-KO females during pregnancy, representing a phenotypic change in the T cells infiltrating the kidney at Gd8 (4.5±1.5 and 9.2±4.2% CD3+ cells, respectively) and Gd16 (4.9±1.7 and 7.3±0.9% CD3+ cells, respectively; P<0.05; P<0.005; Figure 4D). There were no significant differences in the proportion of T cells producing tumor necrosis factor-α and interleukin-17 between the genotypes at baseline or during gestation (Figure S5B and S5E). Comparison of the renal Th1:Th2 ratios demonstrated a trend at Gd8, with AT2R-KO mice having a higher Th1:Th2 ratio than WT mice (P=0.06; Figure 4F). At Gd16, the renal Th1:Th2 ratio was significantly increased in AT2R-KO mice compared with baseline (P<0.04; Figure 4F). The number of macrophages infiltrating the kidney was also greater in AT,R-KO than WT mice at baseline, and this difference was maintained during gestation (P=0.004; P=0.06; Figure 4C).

Placenta
The expected decrease in placental Th1:Th2 ratio was observed at Gd16 as compared with Gd8 in both genotypes (Figure S6F). There were no significant differences in T cell infiltration or cytokine production between the genotypes (Figures S5C, S5F, S6B, S6D, and S6E).
Renal and Placental Gene Expression

Compared with WT mice, $\text{AT}_2\text{R-KO}$ mice had lower basal renal $\text{AT}_1\text{R}$ and $\text{AT}_2\text{R}$ gene expression (Figure S7A and S7B, both $P<0.05$). Basal renal ACE2 and MasR gene expression were not significantly different between the genotypes. During pregnancy there was no change in $\text{AT}_1\text{R}$, $\text{AT}_2\text{R}$, or MasR gene expression (Figure S7). In WT mice, renal $\text{AT}_1\text{R}$ gene expression was reduced at Gd18 as compared with basal ($P=0.02$). In pregnant $\text{AT}_2\text{R-KO}$ mice, renal ACE2 expression was significantly higher at Gd8 versus Gd16 ($P=0.04$). Pregnancy altered the expression of components of the RAS in the placenta with marked increases in $\text{AT}_1\text{R}$, ACE2, and MasR. However, there was no significant impact of $\text{AT}_2\text{R}$ deficiency (see Results in the online-only Data Supplement and Figure S8).

Discussion

There were 3 major findings in the present study. Firstly, the $\text{AT}_2\text{R}$ mediates the normal midgestational decrease in arterial pressure and contributes to arterial pressure regulation during late gestation. These findings extend previous observations of the arterial pressure-lowering effects of the $\text{AT}_2\text{R}$ during pregnancy.16–18 Secondly, in $\text{AT}_2\text{R-KO}$ mice there was a phenotypic switch in the T cells infiltrating the kidney toward a proinflammatory phenotype, resulting in an increase in the renal Th1:Th2 ratio at Gd16. Moreover, the increase in the Th1:Th2 ratio was observed only in the kidneys of pregnant $\text{AT}_2\text{R-KO}$ mice, which markedly contrasted the reduced Th1:Th2 ratios in the circulation and placentae as per regular pregnancies. Finally, as expected, pregnant mice were in positive sodium balance. However, sodium retention was enhanced in pregnant $\text{AT}_2\text{R-KO}$ mice, and this may have contributed to the increase in arterial pressure in late gestation. Therefore, the $\text{AT}_2\text{R}$ plays an important role in arterial pressure regulation and may modulate renal immune cell phenotype as well as pressure-natriuresis during pregnancy. Our studies demonstrate that the $\text{AT}_2\text{R}$ contributes to the normal cardiovascular and renal adaptations that facilitate the gestational decrease in arterial pressure. A corollary of this is that deficits in $\text{AT}_2\text{R}$ expression may contribute to pregnancy-induced hypertension.

Pregnancy in mammals, including humans19 and mice,20 is characterized by a midgestational fall in arterial pressure, with MAP returning to preconception levels during the peripartum period. In the present study, we have documented for the first time the full impact of the $\text{AT}_2\text{R}$ on arterial pressure during pregnancy in mice. It was demonstrated that during gestation, the $\text{AT}_2\text{R}$ mediates the normal midgestational decrease in arterial pressure, because the fall was absent during pregnancy in $\text{AT}_2\text{R-KO}$ mice. Furthermore, $\text{AT}_2\text{R}$ deficiency was associated with an elevation in arterial pressure during late gestation. Previously, Takeda-Matsubara et al18 observed a significant increase in systolic arterial pressure during late gestation as compared with prepregnancy levels in $\text{AT}_2\text{R-KO}$ mice. However, this study did not observe the normal midgestational decrease in arterial pressure in WT (C57BL/6) mice and consequently did not report an effect of $\text{AT}_2\text{R}$ deletion on arterial pressure during midgestation. Conversely, Chen et al16 and Carey et al17 have demonstrated that pharmacological and genetic $\text{AT}_2\text{R}$ deficiency abolishes the normal midgestational decrease in arterial pressure. However, these studies observed no effect of $\text{AT}_2\text{R}$ deficiency on arterial pressure during late gestation. The discord between these findings and those of the current study most likely reflects methodological differences. In our study, MAP was measured using radiotelemetry, which is the gold standard method to measure arterial pressure. The earlier studies used tail-cuff plethysmography to measure arterial pressure, which may mask subtle differences in arterial pressure during pregnancy because of the inherent stress involved with restraint and frequent handling.21 Thus, our data unequivocally demonstrate that the normal midgestational decrease observed in the arterial pressure profile of pregnant WT mice is absent in $\text{AT}_2\text{R-KO}$ mice and, importantly, that $\text{AT}_2\text{R}$ deficiency leads to higher arterial pressure during late gestation.

To protect the feto-maternal unit during pregnancy, the immune system is normally suppressed.19 Previous studies investigating Th1:Th2 ratio during pregnancy have measured IFN-$\gamma$ and interleukin-4 levels and shown a distinct shift toward the Th2 phenotype, both systemically and within placenta.22–24 IFN-$\gamma$ and interleukin-4 are the principal cytokines that promote Th1 and Th2 differentiation, respectively.25,26 In the present study we have shown a similar pattern, with a decrease in the Th1:Th2 ratio observed in the circulation and placentae of pregnant WT mice. Surprisingly, $\text{AT}_2\text{R}$ deficiency in mice did not alter the Th1:Th2 ratio within the circulation or placenta as hypothesized. Thus, the findings of the present study suggest that $\text{AT}_2\text{R}$ deficiency does not affect the systemic immune response. Furthermore, because placental $\text{AT}_2\text{R}$ expression decreased during gestation in WT mice and there was no change in the placental Th1:Th2 ratio or number of live fetuses between the genotypes, it is not likely that $\text{AT}_2\text{R}$ deficiency is associated with poorer fetal outcomes. This is consistent with a previous report suggesting that the $\text{AT}_2\text{R}$ does not play a pivotal role in the placenta.27 Conversely, placental ACE2 and MasR gene expression was increased during late gestation in both genotypes. This finding is consistent with previous studies which have demonstrated that uteroplacental levels of Ang(1–7) and ACE2 are elevated during pregnancy.28,29 Thus the ACE2/Ang(1–7)/MasR pathway may play an important role in uteroplacental blood flow and fetal growth.

The most remarkable finding of the present study was that although there was no difference in the number of T cells infiltrating the kidney between pregnant WT and $\text{AT}_2\text{R-KO}$ mice, $\text{AT}_2\text{R}$ deficiency promoted a phenotypic shift of renal T cells and resulting in an increase in the renal Th1:Th2 ratio at Gd16. Moreover, the increase in the Th1:Th2 ratio was observed only in the kidneys of pregnant $\text{AT}_2\text{R-KO}$ mice, which markedly contrasted the reduced Th1:Th2 ratios in the circulation and placentae as per regular pregnancies. Thus, the AT2R mediates the normal midgestational decrease in arterial pressure, because the fall was absent during pregnancy because of the inherent stress involved with restraint and frequent handling.21 Thus, our data unequivocally demonstrate that the normal midgestational decrease observed in the arterial pressure profile of pregnant WT mice is absent in $\text{AT}_2\text{R-KO}$ mice and, importantly, that $\text{AT}_2\text{R}$ deficiency leads to higher arterial pressure during late gestation.
nuclear factor-xB in the kidney. Collectively these findings suggest that the AT\(_R\) suppresses AT\(_R\)-mediated immune system activation within the kidney during pregnancy. Because the increase in the renal Th1:Th2 ratio preceded the rise in arterial pressure during late gestation in AT\(_R\)-KO mice, this suggests that renal inflammation may contribute to pregnancy-induced hypertension.

During pregnancy there was an increase in sodium intake; however, sodium excretion did not change. Thus, pregnant mice were in a state of positive sodium balance. Significantly, the sodium retention was greater in pregnant AT\(_R\)-KO than WT mice. Renal adaptations to pregnancy promote sodium retention, which facilitates the marked increase in plasma volume. Moreover, sodium retention during pregnancy occurs while both natriuretic and antinatriuretic signals are activated. However, previous studies in pregnant rats have demonstrated that the pressure-natriuresis relationship is blunted during late gestation. In the present study, arterial pressure was significantly elevated at Gd18 above prepregnancy levels in AT\(_R\)-KO mice. This finding coupled with the similar natriuretic response at Gd18 between WT and AT\(_R\)-KO mice indicates a rightward shift of the pressure-natriuresis relationship in AT\(_R\)-KO mice during late gestation. These results suggest that the blunted pressure-natriuresis relationship observed in normotensive pregnancy is, at least in part, AT\(_R\) mediated. This is consistent with previous findings, which have demonstrated that the AT\(_R\) modulates pressure-natriuresis in nonpregnant rodents.

Consequently, deficits in AT\(_R\) expression seem to alter the normal renal adaptations to pregnancy. Indeed, albuminuria was increased in pregnant AT\(_R\)-KO mice compared with WT mice.

Contrary to our hypothesis, renal AT\(_R\) expression during pregnancy did not increase in WT females, nor did other components (ACE2 and MasR) of the protective arm of the RAS. However, we, and others, have previously demonstrated that AT\(_R\), ACE2, and MasR gene expression is greater in female than in male rodents. Furthermore, here we demonstrate that these high levels of expression persist during pregnancy. In contrast, AT\(_R\) expression was significantly increased in the aorta, renal artery, and kidneys of pregnant normotensive rats at Gd14 as compared with nulliparous females, and this time point correlated with the peak increase in renal function. In another study in pregnant ewes, AT\(_R\) expression was increased in the uterine artery and unchanged in the aorta. In these studies, AT\(_R\) expression was unchanged between virgin and pregnant females. Therefore, females have high expression of the protective components of the RAS, which are maintained or increased in a regional and temporal manner during pregnancy, in various animals. Furthermore, as AngII binds with 15-fold greater affinity to the AT\(_R\) as compared with the AT\(_R\), this may underlie the reduced pressor responsiveness to AngII during pregnancy. Alternatively, the facts that plasma levels of Ang(1–7) increase 16-fold during normotensive pregnancy in women and Ang(1–7) is able to elicit its actions via the AT\(_R\), as well as its own receptor, MasR, suggest that Ang(1–7) may play a pivotal role in counterstressing the pressor actions of AngII during normal pregnancy. Future studies are needed to further characterize the role of the depressor RAS pathways in the normal regulation of arterial pressure during pregnancy.

Perspectives

Given that women with a history of preeclampsia are at an increased risk for developing cardiovascular disease in later life, understanding the mechanisms regulating arterial pressure during pregnancy are of importance. Our study demonstrates a key role for the AT\(_R\) in the normal regulation of arterial pressure during pregnancy. Therefore, the enhanced AT\(_R\)-AT\(_R\) ratio observed in females may underlie reduced pressor responsiveness to AngII during normal pregnancy. Conversely, increased sensitivity to AngII during preeclampsia is associated with a decrease in the AT\(_R\)-AT\(_R\) ratio. Thus, deficits in the depressor RAS pathways may be a predisposing factor to the development of pregnancy-induced hypertension and future cardiovascular risk.

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Disclosures

None.

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ROLE OF INFLAMMATION AND THE ANGIOTENSIN TYPE 2 RECEPTOR IN THE REGULATION OF ARTERIAL PRESSURE DURING PREGNANCY IN MICE

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Short title: AT₄R regulates arterial pressure during pregnancy

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Methods
Experiments were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Monash University School of Biomedical Sciences Animal Ethics Committee. Nulliparous 12-week old FVB/N WT and AT$_2$R-KO mice, initially established by Hein et al.,$^1$ were obtained from Monash Animal Services and randomly assigned to one of the experimental protocols described below. Animals were housed individually in an experimental room with temperature maintained at 24–26°C and a 12 h light-dark cycle. Mice had ad libitum access to normal salt diet (0.26% (wt/wt) NaCl; AIN93G Specialty Feeds, Australia) and water.

Experimental protocols

Mean arterial pressure during pregnancy

WT (n=7) and AT$_2$R-KO (n=8) females were anaesthetized (2.2-2.6 % isoflurane in 40% O$_2$-60% N$_2$, Rhodia Australia) for implantation of a radiotelemetry transmitter (TA11PA-C10, DSI, MN, USA) into the left carotid artery, as described previously.$^2$ After 10 days recovery, basal mean arterial pressure (MAP) was measured over 3 days. Mice were then time-mated with a male of the same genotype and the presence of a vaginal plug was designated as gestation day (Gd) 0.5. MAP was measured throughout gestation and 2 weeks postpartum. Data were collected for 10 s every 10 min using Dataquest ART data acquisition system (DSI, MN, USA) and were analyzed as 24 h averages.

Renal excretory function

In an additional cohort of WT (n=8) and AT$_2$R-KO (n=6) mice, metabolic studies consisting of a 24 h urine collection were performed to establish basal renal excretory function. Food and water consumption and urine and fecal production were recorded, and urine samples were collected and frozen (−20°C) for subsequent analysis of Na$^+$ (RAPIDChem 744 Electrolyte Analyser, Siemens Healthcare Diagnostics Inc, Deerfield, IL, USA), osmolality (Advanced Osmometer 2020, Advanced Instruments, Needham Heights, MA, USA) and albumin (Albuwell M ELISA, Exocell Inc, Philadelphia, PA, USA) concentrations. Mice were then time mated as described above. On Gd17-18 mice underwent a second 24 h urine collection. At the conclusion of the second metabolic study, mice were killed by isoflurane overdose to determine fetal number.

Circulating T-cell activation and inflammatory cell infiltration

Virgin, Gd8 and Gd16 WT and AT$_2$R-KO females (n=6-12 per group) were humanely killed via CO$_2$ asphyxiation and a 1ml blood sample was collected via cardiac puncture. Mice were then intracardially perfused with phosphate buffered saline (PBS; NaCl 137mmol/L, KCl, 2.7mmol/L, Na$_2$HPO$_4$ 10mmol/L, KH$_2$PO$_4$ 2mmol/L), and kidneys and placentae were removed. Blood samples were mixed with red blood cell (RBC) lysis buffer (NH$_4$Cl 155mmol/L, KHCO$_3$ 10mmol/L, EDTA 0.01mmol/L) to remove all erythrocytes, and were then washed in PBS and cells were counted using a Countess® Automated Cell Counter (Life Technologies). To isolate kidney mononuclear cells, kidneys were minced and enzymatically digested using collagenase type IX (125 U/mL), hyaluronidase (60 U/mL) and collagenase type I-S (450 U/mL; all enzymes from Sigma Aldrich) dissolved in PBS buffer containing calcium and magnesium for 45 min at 37°C, as previously described.$^3$ Samples were then passed through a 70μm cell strainer (BD Biosciences) to yield a
single cell suspension. After washing with PBS, cells were centrifuged at 1200 RPM for 10 min at 4°C. Samples were then resuspended in 40% isotonic percoll solution (GE Healthcare), and 60% isotonic percoll was gently underlaid beneath the sample for density centrifugation. Density gradients were then spun at 2700 RPM for 20 min at room temperature with the brake off. Mononuclear cells were isolated from the interface of the percoll layers, and washed with PBS. Placentae were digested as described for the kidney excluding the percoll density centrifugation. Blood, kidney mononuclear cells and placentae were stained with an aqua live/dead viability stain (Life Technologies) for 15 min at 4°C. After washing with FACS buffer (PBS with 0.5% bovine serum albumin) cells were simultaneously stained with fluorochrome-conjugated antibodies (all purchased from BD Biosciences or Biolegend) for surface markers including CD45 (leukocytes; APC-Cy7; 30-F11), CD3 (T cells; APC; 145-2C11), CD4 (T-helper cells; BV605; RM4-5), CD8 (cytotoxic T cells; PerCP-Cy5.5; 53-6.7), CD69 (early activation marker; PE; H1.2F3), CD44^Hi (effector T cell marker; V450; IM7) and F4/80 (macrophages; PE-Cy7; BM8). Cells were then fixed and permeabilised (FoxP3 staining kit; eBiosciences) for intracellular staining of the T regulatory (Treg) cell marker, FoxP3 (FITC; FJK-16s).

Multi-colour flow cytometry using an LSR II flow cytometer (BD Biosciences) was employed to enumerate T cell populations and tissue-infiltrating T cells, as previously described.\(^3\) For blood T cells, 20,000 CD3+ positive events was set as the stopping gate using BD FACSDIVA Software. For kidney and placenta samples, Countbright counting beads (50 μL; Life Technologies) were added to samples prior to analysis on the LSR II, and 10,000 bead events recorded. FlowJo Software Version 10.0.6 (Tree Star) was used gate and analyze all flow cytometric data. Separate gating strategies were used for blood compared to kidney and placenta (Figures S1-3). For blood samples, T cells were expressed as a percentage of lymphocytes and T cell subsets were expressed as a percentage of CD3+ cells. Expression of T cell activation markers, CD69 and CD44^Hi, was expressed as proportion of the T cell subset (CD4+ and CD8+). For kidney and placenta samples, total cell counts for each total leukocytes, T cells and subsets and also macrophages were calculated using the counting beads according to manufacturers instructions. In brief, 50 μL of counting beads solution was added to each kidney and placenta sample. Based on the lot number, each 50 μL contained ~50,000 counting beads. Since we recorded 10,000 counting beads on the flow cytometer, we also recorded how many cells were counted per 10,000 beads. Thus, we were able to calculate the total cell count per sample, since the total amount of beads added was 50,000, based on the equation below:

\[
\frac{50,000}{\text{Beads counted}} \times \text{Specific cell count} = \text{Exact cell count per tube}
\]

**Intracellular Cytokine Analysis**

Blood, kidney and placenta samples were prepared as described above. Following preparation of mononuclear cells and digestions, cells were resuspended in complete RPMI1640 media (fetal bovine serum, 10%; streptomycin/penicillin, 100 U/mL; HEPES, 25 mmol/L; 2-mercaptoethanol, 2μM) and seeded onto a 96-well plate. Blood mononuclear cells were seeded at 10^5 cells per well. Kidney and placenta samples were resuspended in 400 μL of complete media and only 100 μL
of each sample was seeded for stimulation. Cells were then stimulated with phorbol-12-myristate (PMA; 50ng/mL) and ionomycin (500ng/mL) in the presence of golgi transport inhibitors brefeldin A and monensin (BD Biosciences) for 6 hours at 37°C with 95% O₂ and 5% CO₂. Following stimulation cells were washed and stained with a viability stain and surface markers (CD45, CD3, CD4 and CD8) as described above. Cells were then fixed and permeabilised (FoxP3 staining kit; eBiosciences) for staining of intracellular cytokines IFN-γ (AlexaFluor700; XMG1.2), TNF-α (PE; TN3-19.12), IL-4 (PE-Cy7; 11B11) and IL-17 (FITC; TC11-18H10.1). Cells were then analyzed using an LSR II flow cytometer (BD Biosciences) and identical settings for recording events were used as described above. Gating for all cytokines were defined by fluorescence minus one controls. FlowJo Software Version 10.0.6 (Tree Star) was used to analyze all flow cytometric data. Separate gating strategies were used for blood compared to kidney and placenta (Figures S2-3). As kidney and placenta samples do not have abundant resident/infiltrating T cells under healthy physiological conditions compared to blood samples, Th cytokines were expressed as a percentage of all ex vivo stimulated T cells (CD3+), as this provided more robust analyses with greater cell number. Analysis of only the Th cell (CD4+) cytokine production demonstrated a similar pattern as cytokines expressed as a percentage of CD3+.

**AT_{1a}R, AT_{1b}R, AT_{2}R and ACE2 gene expression**

Total RNA was extracted from frozen kidney (virgin, Gd 8, Gd 16 and Gd 18) and placenta (Gd8, Gd16 and Gd18) tissue collected from WT and AT_{2}R-KO females (n=6-8 per group) using the RNeasy Mini kit (Qiagen, Doncaster, Victoria, Australia), with 1 μg of extracted RNA converted to cDNA (5x iSCRIPT supermix, BioRad, Life Sciences, Australia). AT_{1a}R, AT_{1b}R, AT_{2}R, ACE2 and MasR gene expressions were analyzed using real-time quantitative RT-PCR with TaqMan gene expression assays (Applied Biosystems, Life technologies, Australia). Real-time PCR was performed using Realplex software with the Applied Biosystems 7900HT Fast RT-PCR system (Applied Biosystems, Life technologies, Australia). Reactions were duplexed with the internal housekeeping gene 18s with samples run in triplicate on a 384-well PCR plate using an automated liquid handler (CAS-1200 liquid handler, Qiagen, Australia). Each 10μl reaction contained 6μl of the PCR reaction mix (2x TaqMan universal PCR master mix and appropriate TaqMan gene expression assays; Applied Biosystems, Life technologies, Australia) as per manufactures instructions, and 4μl of cDNA in RNase free water, equivalent to 50ng of cDNA. Relative expression was calculated using a comparative cycle of threshold fluorescence (2^{-ΔΔCT}), as described previously.4

**Statistical analyses**

Data are presented as mean ± SEM. Preconception MAP was analyzed using an unpaired t-test. The change in MAP during pregnancy compared to baseline and renal excretory function data were analyzed using repeated-measures analysis of variance (ANOVA) with the factors genotype (P_{g}), time (P_{t}) and their interaction. Immune system activation data and AT_{1a}R, AT_{1b}R, ACE2 and MasR gene expression data were analyzed using an ANOVA with the factors genotype (P_{g}), time (P_{t}) and their interaction. AT_{2}R gene expression was analyzed using a one-way ANOVA. Tukey post-hoc tests were performed where appropriate. P≤0.05 was considered statistically significant.
Results

Placental RAS gene expression

Placental AT1aR, AT1bR, AT2R, ACE2 and MasR gene expression were examined at Gd8, Gd16 and Gd18 (Figure S7). Within the placentae of WT and AT2R-KO mice, expression of the pro-inflammatory AT1aR was ~8-fold higher at Gd16 and ~30-fold higher at Gd18 versus Gd8, respectively. In contrast, placental expression of the anti-inflammatory AT2R was significantly lower in WT mice during late gestation (Gd16 and Gd18 versus Gd8, \( P=0.001 \) respectively). Both WT and AT2R-KO mice had higher placental ACE2 expression at Gd16 (~100-fold respectively) and Gd18 (620-fold, \( P<0.0001 \) and 429-fold, \( P=0.04 \), respectively) versus Gd8. Furthermore, MasR was ~2-fold higher at Gd16 (\( P=0.2 \)) and Gd18 (\( P=0.1 \)) versus Gd8 in WT mice, whilst in AT2R-KO mice, MasR gene expression was 4-fold (\( P=0.002 \)) and 2.5-fold greater at Gd18 (\( P=0.06 \)) greater at Gd16 versus Gd8 and 2.5-fold greater at Gd18 (\( P=0.06 \)) versus Gd8. AT1bR expression was lower during late versus early gestation in both genotypes.

References

Table S1. Body weight and 24 h renal excretory data at baseline and gestational day (Gd) 18 in WT (n=8) and AT2R-KO (n=6) mice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>AT2R KO</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Gd18</td>
<td>Basal</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>22±1</td>
<td>37±1</td>
<td>23±1</td>
</tr>
<tr>
<td>Water intake (ml)</td>
<td>2.7±0.2</td>
<td>3.3±0.3</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>Urine flow (ml)</td>
<td>0.7±0.1</td>
<td>0.9±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>3.2±0.2</td>
<td>4.0±0.4</td>
<td>3.3±0.4</td>
</tr>
<tr>
<td>Osmolar excretion (µmol)</td>
<td>2.4±0.1</td>
<td>2.1±0.3</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td>Albuminuria (µg)</td>
<td>17±2</td>
<td>11±2</td>
<td>10±2</td>
</tr>
</tbody>
</table>

Data were analyzed using repeated-measures ANOVA with the factors genotype (P_g) and time (P_t). Data are presented as mean ± SEM. P<0.05 was considered statistically significant.

Table S2. Fetal characteristics of eighteen-day gestation in WT (n=8) and AT2R-KO (n=6) mice.

<table>
<thead>
<tr>
<th>Fetal Characteristic</th>
<th>WT</th>
<th>AT2R KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litter size (fetuses/pregnancy)</td>
<td>9±1</td>
<td>8±1</td>
</tr>
<tr>
<td>Fetal weight (g)</td>
<td>0.88±0.07</td>
<td>1.03±0.10</td>
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<tr>
<td>Placental weight (g)</td>
<td>0.0527±0.002</td>
<td>0.0577±0.002</td>
</tr>
<tr>
<td>Placental efficiency (fetal weight/placental weight)</td>
<td>16.6±0.2</td>
<td>16.8±0.3</td>
</tr>
<tr>
<td>Resorption (pups resorbed/mother)</td>
<td>0.9±0.3</td>
<td>2.0±0.9</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Data were analyzed using an unpaired t-test. P<0.05 was considered statistically significant.
Figure S1. Gating strategy for circulating (blood) T cell activation. From left, lymphocytes were firstly gated based on forward and side scatter properties. From this population, dead cells were excluded and T cells (CD3+) were gated. CD8+ and CD4+ T cells subsets were subsequently gated from which activation markers CD69, CD44^Hi, CD25 and T regulatory cells (CD25+FoxP3+) were gated.
Figure S2. Gating strategy for blood T cell cytokine production and representative FACS plots of WT and AT2R-KO blood T cell cytokine producing populations. From left, lymphocytes were firstly gated based on forward and side scatter properties. From this population, dead cells were excluded and T cells (CD3+) were gated. Gates were then constructed for IFN-γ- and IL-4-producing T cells using FMO controls, which were then applied to all WT and AT2R-KO blood samples.
**Figure S3.** Gating strategy for kidney T cell cytokine production and representative FACS plots of WT and AT$_2$R-KO kidney T cell cytokine producing populations. From left, counting beads were firstly gated based on forward and side scatter properties. Live cells and subsequently leukocytes (CD45+) were gated. T cells (CD3+) were then isolated from this population from which gates were then constructed for IFN-γ- and IL-4-producing T cells using fluorescence minus one (FMO) controls, which were then applied to all WT and AT$_2$R-KO kidney samples.
Figure S4 Circulating (A) T cells, (B) T helper cells, (C) T regulatory cells and (D) cytotoxic T cells at baseline, gestation day (Gd) 8 and Gd16 in WT (●; n=4-12 per group) and AT₂R-KO (■; n=4-12 per group) mice. Data are presented as mean ± sem. Data were analyzed using an ANOVA with the factors genotype (P₀), time (P₁) and their interaction. Tukey post-hoc tests were performed where appropriate. *P<0.05 versus respective basal group. #P<0.05 versus respective Gd8 group.
Figure S5 Circulating, renal and placental TNF-α (A, B and C, respectively) and IL-17 (D, E and F, respectively) producing ex vivo stimulated T cells in WT (■; n=4-12 per group) and AT2R-KO (◼; n=4-12 per group) mice at baseline, gestation day (Gd) 8 and Gd16. Data are presented as mean ± sem. Data were analyzed using an ANOVA with the factors genotype (P_g) and time (P_t) and their interaction. Tukey post-hoc tests were performed where appropriate. *P<0.05 versus respective basal group. #P<0.05 versus respective Gd8 group.
Figure S6. Placental immune cell infiltration and cytokine production in WT (■; n=4-12 per group) and AT2R-KO (▲; n=4-12 per group) mice at gestation day (Gd) 8 and Gd16. (A) total leukocyte infiltration, (B) T cell infiltration, (C) macrophage infiltration, the proportion of (D) IFN-γ and (E) IL-4 producing ex vivo stimulated T cells and the (F) Th1:Th2 ratio in. Data are presented as mean ± sem. Data were analyzed using an ANOVA with the factors genotype (P g) and time (P t) and their interaction. Tukey post-hoc tests were performed where appropriate. †P<0.05 versus respective WT group. #P<0.05 versus respective Gd8 group.
Figure S7. Renal (A) $AT_1aR$, (B) $AT_1bR$, (C) $AT_2R$, (D) ACE2 and (E) MasR gene expression in WT (■; n=6-8 per group) and $AT_2R$-KO (■; n=6-8 per group) mice at baseline, gestation day (Gd) 8, Gd16 and Gd18. Data are expressed relative to the basal WT group and are presented as mean ± sem. Data were analyzed using an ANOVA with the factors genotype ($P_g$), time ($P_t$) and their interaction. $AT_2R$ gene expression was analyzed using a one-way ANOVA. Tukey post-hoc tests were performed where appropriate. $P<0.05$ was considered significant.
Figure S8. Placental (A) AT₁₆R, (B) AT₁₇R, (C) AT₂R, (D) ACE2 and (E) MasR gene expression in WT (◼; n=6-8 per group) and AT₂R-KO (■; n=6-8 per group) mice at gestation day (Gd) 8, Gd16 and Gd18. Data are expressed relative to the basal WT group and are presented as mean ± sem. Data were analyzed using an ANOVA with the factors genotype (Pᵍ), time (Pᵗ) and their interaction. AT₂R gene expression was analyzed using a one-way ANOVA. Tukey post-hoc tests were performed where appropriate. †P<0.05 versus respective WT group. *P<0.05 versus respective basal group. #P<0.05 versus respective Gd8 group. §P<0.05 versus respective Gd16 group.