Regulatory T Cells Ameliorate Intrauterine Growth Retardation in a Transgenic Rat Model For Preeclampsia

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Abstract—Preeclampsia is a multisystemic syndrome during pregnancy that is often associated with intrauterine growth retardation. Immunologic dysregulation, involving T cells, is implicated in the pathogenesis. The aim of this study was to evaluate the effect of upregulating regulatory T cells in an established transgenic rat model for preeclampsia. Application of superagonistic monoclonal antibody for CD28 has been shown to effectively upregulate regulatory T cells. In the first protocol (treatment protocol), we applied 1 mg of CD28 superagonist or control antibody on days 11 and 15 of pregnancy. In the second protocol (prevention protocol), the superagonist or control antibody was applied on days 1, 5, and 9. Superagonist increased regulatory T cells in circulation and placenta from 8.49±0.09% of CD4-positive T cells to 23.50±3.05% and from 3.85±1.45% to 23.27±7.64%, respectively. Blood pressure and albuminuria (30.6±15.1 versus 14.6±5.5 mg/dl) were similar in the superagonist or control antibody–treated preeclamptic group for both protocols. Rats treated with CD28 superagonist showed increased pup weights in the prevention protocol (2.66±0.03 versus 2.37±0.05 g) and in the treatment protocol (3.04±0.04 versus 2.54±0.1 g). Intrauterine growth retardation, calculated by brain:liver weight ratio, was also decreased by the superagonist in both protocols. Further analysis of brain development revealed a 20% increase in brain volume by the superagonist. Induction of regulatory T cells in the circulation and the uteroplacental unit in an established preeclamptic rat model had no influence on maternal hypertension and proteinuria. However, it substantially improved fetal outcome by ameliorating intrauterine growth retardation. (Hypertension. 2015;65:00-00. DOI: 10.1161/HYPERTENSIONAHA.114.04892.)

Key Words: fetal growth retardation ■ preeclampsia ■ pregnancy ■ T lymphocytes, regulatory

Preeclampsia is characterized by new onset of maternal hypertension after 20th week of gestation and proteinuria or in association with thrombocytopenia, impaired liver function, the new development of renal insufficiency, pulmonary edema, or new-onset cerebral or visual disturbances.1 It is a leading cause of maternal and perinatal morbidity and mortality worldwide, with a global incidence of 3% to 5% of all pregnancies. Preeclampsia originates in the placenta, but the underlying cause is complex and probably heterogeneous in origin.2 Preeclampsia causes variable maternal and fetal problems, and intrauterine growth retardation (IUGR) is a common sequel of preeclampsia.3,4 Newborns with IUGR have structural and metabolic abnormalities that compromise their immediate development and also adversely affect their long-term cardiovascular and metabolic outcomes.3

Recently, dysregulation of immune mechanisms have been implicated in preeclampsia and IUGR, leading to a pathologial maternal immune recognition of the trophoblast, resulting in abnormal placentation and an imbalance between factors produced by the placenta and maternal adaptation to them.3 A genetically foreign fetus (presented as extravillous trophoblast cells in the maternal uterine wall) challenges the maternal immune system, and both innate and adaptive immune cells are necessary for several important processes during pregnancy for normal maternal physiology leading to a robust fetal development.3,5

One CD4+ lymphocyte subset, CD4+CD25+FoxP3+ regulatory T cells (Tregs) play an important role in maintaining immunologic tolerance. They are induced by tolerogenic dendritic cells and home to the uterus before implantation.6 Tregs are necessary for implantation and maintenance of early pregnancy. An association between the number of Tregs and implantation failure or recurrent spontaneous miscarriage in humans and mouse models has been demonstrated.7,8

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Several authors have proposed that defective control of effector T cells by Tregs leads to an increased T helper 17 (Th17) and increased Th1/Th2 balance, causing maternal hypertension, associated clinical manifestations, and poor placentation with IUGR. Reduced numbers of Tregs in pre-eclamptic patients have been reported by several groups. However, experimental evidence is scarce. In a previous study, adoptive transfer of pregnancy-induced CD4+ CD25+ Tregs reversed the increase in the abortion rate caused by interleukin 17 in a mouse model. We reasoned that Treg upregulation might ameliorate the preeclamptic phenotype in an established animal model of preeclampsia and possibly favorably influence IUGR.

A rodent model for preeclampsia by mating female rats transgenic for human angiotensinogen with rats transgenic for human renin has been established. Dams exhibit an increase in blood pressure from 100/80 mm Hg to 180/140 mm Hg, develop proteinuria, a pathological trophoblast invasion, and display uteroplacental vascular remodeling. Fetuses develop IUGR with an increased brain:live ratio compared with normal Sprague-Dawley rats. We have shown previously that Tregs ameliorate angiotensin II–induced target-organ damage; CD28 is a costimulatory receptor required for activation of T cells. We used a well-established rat specific monoclonal superagonistic antibody for CD28 (JJ316), which has been shown in various experimental models to expand and upregulate Tregs and their function in vivo. We have tested the hypothesis that application of the superagonistic antibody for CD28 will induce regulatory T cells during pregnancy and ameliorate hypertension and proteinuria, as well as IUGR in our preeclamptic rat model.

**Methods**

**Animals and Protocol**

Female rats transgenic for human angiotensinogen were mated with male rats transgenic for human renin. Pregnant dams developed hypertension on day 13 of pregnancy (plug-recognition day is assigned as day 1) and albuminuria. We also used Sprague Dawley rats to compare with pregnant dams. Both transgenic rats are of Sprague Dawley background. Local authorities approved the studies, which were performed according to American Physiological Society guidelines. During telemetry implantation, tail-cuff measurements were performed on days 8, 13, 15, and 18 of pregnancy. In selected rats as indicated, radiotelemetry was used to follow blood pressure. Rats were transferred to metabolic cage for 24 hours at days 8 and 17.

All experimental animals were euthanized on day 21. Fetuses were weighted and their brains were removed from the skulls for analysis. Maternal and fetal organs were collected and weighed. Urinary rat albumin was measured with a commercially available ELISA (CellTrend, Germany). Creatinine was determined in serum by an automated clinical method.

Expanded methods are described in the online-only Data Supplement.

**Results**

**Fewer Tregs in Preeclamptic Placentas**

We first investigated whether T or B cells are dysregulated in the placenta of our transgenic preeclamptic rat model, compared with controls (Figure 1A). CD4+ cells (Figure 1B) and CD8+ T cells (Figure 1C) were not significantly different on day 15 of pregnancy between the 2 groups. However, Tregs were markedly reduced in placentas (25.37±7.60% versus 3.85±1.45% of CD4+ T cells; *P<0.05) of preeclamptic rats compared with control rats (Figure 1D). B cells (CD45R+ were not different in the placenta from the 2 groups (data not shown).

A representative flow cytometry analysis is shown in Figure 2A, gated for FoxP3 and CD25 from a preeclamptic placenta derived from a dam treated with control antibody (vehicle; left) or with CD28 superagonist (JJ316; right). Tregs were increased from 3.85±1.45% to 23.27±7.64% of CD4+ T cells measured on day 15 and from 8.52±1.93% to 23.93±7.88% on day 21 in preeclamptic placenta (Figure 2B). These levels corresponded to Treg frequencies from control pregnant rats, so that JJ316 treatment normalized placental Treg levels during the last trimester of pregnancy. We found a similar upregulation of Tregs in blood (Figure 2C) and spleen (Figure 2D) on performing all treatments using monoclonal superagonistic antibody specific to rat CD28 (clone JJ316) that has been previously described and can induce Tregs without the involvement of T-cell receptor. We used the same isotype monoclonal antibodies (MOPC-21) as control (vehicle).

For the treatment protocol, pregnant rats were transferred to a metabolic cage on days 10 and 17 for 24 hours each. On days 11 and 15, 1 mg of JJ316 was given by intraperitoneal injection. For the prevention protocol, rats were injected on days 1, 5, and 9 with 1 mg of JJ316. To investigate blood pressure, tail-cuff measurements were performed on days 8, 13, 15, and 18 of pregnancy. In selected rats as indicated, radiotelemetry was used to follow blood pressure. Rats were transferred to metabolic cage for 24 hours at days 8 and 17.

![Figure 1. T-cell repertoire in the placenta of healthy and preeclamptic pregnant rats on day 15. A. Frequencies of total CD3+ T cells were not different in healthy pregnant rats compared with preeclamptic. B and C. Levels of CD4+ and CD8+ T cells were presented as percentages of T-cell population and showed no difference in the 2 groups. D. Frequencies of double positive (CD25+FoxP3+) Tregs are presented as percentages of CD4+ T cell population and were significantly reduced in preeclamptic placentas. Data are shown as mean±SEM. Mann–Whitney U test was used. *P<0.05. ns indicates nonsignificant; PE, preeclamptic rats; and SD, Sprague Dawley.](image)
days 15 and 21 after JJ316 application, compared with vehicle in preeclamptic rats. We additionally analyzed other subsets of T cells (Figure S3). Memory T cells (CD3+CD4+CD45R−), naive T cells (CD3+CD4+CD45R+), general cytotoxic T cells (CD3+CD8+CD45R−), and their subpopulation suppressor effector T cells (CD3+CD8+CD45R+) were not imbalanced in spleen, blood, and placenta at days 15 and 21 of pregnancy.

No Influence on Hypertension and Proteinuria

To investigate the effect of JJ316 on the preeclamptic phenotype, we used 2 different strategies (Figure S1). In the treatment protocol, we applied JJ316 or vehicle when blood pressure increased at day 11. For the prevention strategy, JJ316 or vehicle was given after mating so that Tregs were upregulated during implantation and placental development. For the treatment strategy (Figure 3A), we observed no effects on blood pressure over the course of pregnancy, as measured by radiotelemetry (Figure 3A, left), no improvement in albuminuria (Figure 3A, right top) or effects on serum creatinine (Figure 3A, right bottom). Maternal organs from treated rats showed similar weights compared with control, except spleen, which was heavier (0.45±0.06 versus 0.66±0.04 g; data not shown) and corresponds to the findings of previous studies and is because of the increase in CD4 T-cell numbers.20 Previous application of JJ316 (Figure 3B) did not alter the results, which we observed with the treatment protocol. Early upregulation of Tregs did not alter blood pressure (Figure 3B, left), albuminuria (Figure 3B, right top), or creatinine (Figure 3B, right bottom) in preeclamptic rats.

Upregulation of Tregs Is Associated With Improved IUGR

We recorded increased fetal weights (2.54±0.10 versus 3.04±0.04 g, P<0.005) in preeclamptic rats treated with JJ316 compared with control antibody–treated preeclamptic rats (Figure 4A). Interestingly, the effect on mean fetal weight was mediated by reducing the numbers of markedly underdeveloped pups (<10th percentile). The effect on fetuses <the 10th percentile (Figure 4B and 4C) showed that Treg upregulation was predominantly successful in the smaller pups. The IUGR ratio is calculated by dividing the weight of fetal brains by the weight of fetal livers. This ratio (Figure 4D) was improved by 30% in the JJ316-treated group (1.05±0.07 in controls versus 0.72±0.02; P<0.0001). Brain is the paramount organ in fetal development. Brain weights (Figure 4E) in the treatment group were heavier (0.14±0.00 in controls versus 0.16±0.00 g; P<0.05). Liver weights (Figure 4F) were substantially increased in the treatment group (0.155±0.01 in controls versus 0.22±0.01 g; P<0.0001), indicating that Treg upregulation could overcome the brain sparing effect of IUGR in our preeclamptic rat model. Similar effects were observed when JJ316 was applied early during placental development (prevention protocol). Fetal weights (Figure 4G) were significantly reduced with a dominant effect on fetuses below the 10th percentile (Figure 4H and 4I). IUGR was significantly reduced (Figure 4J), showing a mild increase in brain weight (Figure 4K) but significant upregulation of liver weight (Figure 4L).
Further analysis of brain development revealed that JJ316 induced a 20% increase in brain volume (23.21±0.66 versus 29.36±2.46 mm³; \( P = 0.04 \); Figure 5A). Cellular density was determined in 4 regions of interest: cortex, hippocampus, thalamus, and striatum. Representative brain sections are shown in Figure 5B. In all 4 brain regions, treatment with JJ316 induced a higher cellular density compared with controls (Figure 5C). These effects were also present in the late treatment group, however, to a lesser degree (Figure S4).

Notably, analysis of the different neural cell types revealed a profound increase of mature neurons (neuronal nuclei positive) in the developing cortex of animals treated with JJ316 (0.87±0.06 versus 1.27±0.05; \( P = 0.0005 \)), whereas neither immature neurons (doublecortin positive) nor microglia (ionized calcium-binding adapter molecule 1 positive), and oligodendrocytes (Oligodendrocyte transcription factor positive) showed obvious differences in staining intensities. Furthermore, reactive astrogliosis (glial fibrillary acidic protein positive) was downregulated in cortical (0.77±0.6 versus 0.69±0.03) and hippocampal areas (0.88±0.07 versus 0.67±0.04; \( P = 0.03 \); Figure S6).

**Placental Development**

We found no influence on angiogenesis profile and trophoblast invasion by upregulation of Tregs in the placenta. We also investigated whether the angiogenesis profile of the placenta (fetal tissue) or mesometrial triangle (maternal tissue with fetal cells invading the area) was altered. However, mRNA expression of placental growth factor and soluble fms-like tyrosine kinase-1 was not altered in
the placenta or mesometrial triangle after JJ316 (Figure S5A). We next investigated whether Treg upregulation had an effect on uteroplacental morphology and trophoblast cell invasion. JJ316 treatment did not change placental area (Figure S5B) and did not influence the relationship between labyrinth and trophospongium area (data not shown). We also found no difference in the area of the mesometrial triangle (Figure S5C) and the area of interstitial trophoblast cell invasion into the mesometrial triangle (Figure S5D).
Autoantibodies Against the Angiotensin II Receptor Type 1 Receptor

Because this preeclampsia model features autoantibodies directed to angiotensin II receptor type 1 autoantibodies (AT1-AA), we next investigated whether Treg upregulation could influence AT1-AA. Both early and late application of JJ316 reduced the presence of AT1-AA (Figure 6A and 6B).

Discussion

We have shown that Tregs are reduced in the uteroplacental unit in our preeclamptic rat model. We were successful in restoring Tregs in preeclamptic placentas of our animals, as well as in maternal circulation and spleen. However, increasing Tregs did not ameliorate the maternal phenotype. Importantly, we improved IUGR, reversed the brain sparing effect, showed improved brain size with an increase in mature neurons, and reduced reactive astrogliosis in the cortex, indicating an improved fetal development. Interestingly, we reduced the number of severely retarded pups (<10th percentile). The improvement of fetal outcome was independent of altering the antiangiogenic balance and of altering trophoblast cell invasion in the uteroplacental unit, although we reduced AT1-AA. We think that the improvement of fetal outcome, independent of reducing hypertension, deserves further attention.

Tregs play an important role in regulating immune tolerance in pregnancy. The invading fetal trophoblast cells are

Figure 5. Administration of JJ316 improves brain development of the fetus. Brain volume was increased after early treatment with JJ316 compared with control (A). A representative fetal brain is shown in B. Cell density is measured in 4 regions of interest: cortex (Cx), hippocampus (HC), thalamus (Tha), and striatum (Str) and revealed a significant increase after JJ316 treatment (C). ns indicates nonsignificant; and ROI, region of interest. *P<0.05, **P<0.01.

Figure 6. Treatment with JJ316 decreases the concentration of angiotensin II receptor type 1 autoantibodies (AT1-AA) in serum of pregnant preeclamptic rat model. Isolated rat neonatal cardiomyocytes were treated in vitro with IgG from vehicle or JJ316-treated rats. Data are presented as an increase of beating rate of cardiomyocytes after treatment with IgG from prevention (A) and treatment (B) protocols compared with baseline. Administration of AT1 receptor blocker (Losartan) was used to show that the effect is mediated via the AT1 receptor. Mean of 6 view fields per treatment was used in analysis. Data are shown as mean±SEM. Mann–Whitney U test was used. *P<0.05.
recognized by the maternal immune system, and Tregs suppress reactive cells so that a transient state of tolerance of specific paternal alloantigens is achieved. Presentation of paternal antigens on invading fetal trophoblasts and their interaction with Tregs besides uterine natural killer cells are responsible not only for suppression of antifetal cytotoxic T cell responses but also for long-term immunologic memory. Tregs are expanded already before implantation.

In the setting of preeclampsia, little is known about the role of Tregs. Sasaki et al reported reduced Tregs in circulation and in placental bed biopsies of preeclamptic patients compared with those in normal pregnancy. These findings suggest that a decreased number of Tregs might break the maternal tolerance to the fetus. However, others found no differences in Treg numbers between healthy and preeclamptic pregnancies. The reason why Tregs are reduced in preeclampsia is unknown. Inflammation at the decidua might impair the immunoregulatory function of Tregs. Tregs suppress systemic and mucosal activation to control inflammation. Therefore, decreased Tregs might augment systemic inflammation in preeclampsia, so that a vicious circle is induced.

Tregs are needed for a successful allogeneic pregnancy in mice. These Tregs expand during pregnancy, induced by paternal antigens, and prevent rejection of the fetus. Zenclussen et al showed that allogeneic fetuses are rejected when CD4+CD25+ Tregs are absent and adoptive transfer of CD4+CD25+ Tregs improved the phenotype. Treatment with anti-CD25 monoclonal antibodies during implantation period induced implantation failure in allogenic but not in syngeneic pregnant mice. Anti-CD25 monoclonal antibodies treatment in the late pregnancy phase reduced the Tregs in a similar manner to treatment during the implantation period, but interestingly, this treatment did not induce preeclampsia symptoms, such as hypertension and proteinuria. Thus, although Tregs seem to be necessary for implantation and the early phase of pregnancy, they may not have a critical role in the maintenance of pregnancy in the late gestation phase. Our data also show a more pronounced effect on brain development by early upregulation of Tregs.

We expended the effects of preeclampsia on IUGR on investigating the fetal brain around time of delivery (21st embryonic day) in our experimental model. In addition to poor pup somatic growth, the present experiments revealed an obvious abnormality on brain development with reduced cellular density in cortex, hippocampus, thalamus, and striatum. Increasing Tregs by injection of JJ316 restored brain growth by increased number of mature cortical neurons and decreased astrogliosis. Moreover, it increased fetal weight and reduced the number of severely retarded offsprings. In other experimental models of IUGR, induced by unilateral ligation of the uterine artery, significant delays in oligodendrocyte differentiation and myelination that resolved at adult age have been observed. Whether reduction of brain growth shown in our model is a temporary phenomenon needs to be investigated in postnatal studies up to adult age where myelination and synaptic formation can be assessed.

JJ316 causes preferential expansion of Tregs compared with conventional T cells and enhances their suppressive activity. Tregs expand numerically, are functionally hyperactive, and migrate to inflamed tissues in rats after JJ316 treatment. Accordingly, CD28 superagonists have been used effectively in the treatment of rodent models of autoimmunity, such as experimental autoimmune neuritis and encephalitis, arthritis, tumor necrosis factor–induced bone destruction, and unwanted T-cell responses in transplantation of both solid organs and hematopoietic stem cells. CD28 superagonist has also been used in rodent models with cardiovascular endorgan damage, such as cardiac remodeling after myocardial infarction, diabetes mellitus, and glomerulonephritis. We do not know the mechanisms by which increased numbers of Tregs ameliorated IUGR in our model. We observed neither a change in the local angiogenic profile nor an improvement in trophoblast invasion. The rat model itself does not show all key features of human preeclamptic placental pathology, namely the deeper trophoblast invasion. Nevertheless, several other features are in concordance to the human situation. Vessels which had been invaded by endovascular trophoblasts show a higher presence of vascular smooth muscle cells, indicating that the trophoblasts are remodeling the vessels to a lesser extent, although they migrate deeper. In 2 intervention studies, we already could show that the trophoblast function can be altered. However, we observed a reduction in AT1-receptor autoantibodies. Previous studies have shown that increased T cell differentiation into a proinflammatory phenotype leads to an increased number of inflammatory T helper cells and possibly B cell stimulation for AT1-AA production. Adoptive transfer of CD4+ T cells from preeclamptic rats (reduced uterine perfusion pressure model) induced a preeclamptic phenotype and generation of AT1-AA. Administration of Abatacept, a fusion molecule of cytotoxic T-lymphocyte–associated protein 4, reduces T cells and hypertension in reduced uterine perfusion pressure rats, as well as AT1-AA production.

Our data elucidate novel aspects on AT1-AA in preeclampsia. Although we did not aim at elucidating the precise molecular mechanism of Treg-induced improvement of fetal brain development, our data are in line with the concept that Tregs control autoantibody-induced inflammation and antibody production. In this study, we have not considered the interaction between Tregs and regulatory B cells induced by the CD28 superagonist. Regulatory B cells restrain excessive inflammatory responses that occur during autoimmune diseases and might be involved in reducing AT1-AA. Tacke et al showed that B cells are activated in spleen and lymph nodes after 3 and 7 days in response of superagonist application. The effect is mediated via T-cells because there is no upregulation of B cells in response to superagonist application. We know from human studies that AT1-AA are present in the fetal circulation of preeclamptic women. Recently, Cipolla et al could show that plasma from preeclamptic women induce hyperexcitability in the brain through activation of microglia in the maternal brain. In a follow-up study, they could show that neuroinflammation and activation of microglia are present in reduced uterine perfusion pressure rats, a different rodent model for preeclampsia with established pathophysiological role for AT1-AA. Girardi et al could show in a recent study that autoantibodies can be detected in vivo in placenta and fetal brain by a magnetic resonance imaging–based method. They could show that autoantibodies induce IUGR and fetal
brain retardation with cortical axonal cytoarchitecture disruption and increased neurodegeneration.

This study does not necessarily question the role of AT1-AA in preeclampsia or vascular pathology. In general, Treg induction led to significantly lower AT1-AA levels; nevertheless, they were still present and above the cut-off. Future studies will have to determine whether a critical cut-off level might be important. In a previous study, we had shown that AT1-AA alone were not capable of inducing hypertension but only proteinuria in pregnant rats. Although together with a subthreshold old angiotensin II level, they induced an increase in blood pressure. Others have shown that passive transfer of AT1-AA induces preeclampsia-like symptoms in mice and rats. We learn that AT1-AA are complex and their mode of action is only poorly understood, a phenomenon which AT1-AA share with many other autoantibodies in rheumatology, nephrology, and endocrinology.

Novel drugs to improve maternal and fetal health in preeclampsia have not been developed in the past decade. Nonspecific interventions, especially antihypertensive therapy, failed to improve the clinical manifestations and prolong pregnancy. A large meta-analysis reported a relationship between treatment-induced reduction of blood pressure and impaired fetal growth. Recently, Thadhani et al. presented preliminary data that extracorporeal apheresis might prolong pregnancy.

The concept of developing effective and safe treatment application that promotes generation of tolerogenic dendritic cells and differentiation and maintenance of Treg phenotype is appealing. One possible dietary approach was introduced by Park et al. They showed that short-chain fatty acids, an important microbial metabolite, are able to upregulate effector and Tregs. The authors found that the cytokine milieu and immunologic context were important for the effect. Tregs were increased by short-chain fatty acids under the steady conditions in vivo, whereas effector T cells, such as Th17, were increased only during active immune responses. Further studies will have to determine whether dietary interventions are able to upregulate Tregs during pregnancy and whether this leads to improved fetal health.

Perspectives

Other than optimizing nutrition, few strategies are available for ameliorating IUGR. Our findings suggest a role for Tregs. We speculate that CD28 superagonist strategy or alternative approaches to mobilize Tregs might help offspring in which IUGR is anticipated. Importantly, after a dramatic failure in the First-in-human (FIH) trial in 2006, in which a life-threatening cytokine release syndrome (CRS) was observed, human CD28-SA treatment has re-entered clinical development and effectively activates Tregs if appropriately dosed.

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Disclosures

None.

References


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REGULATORY T CELLS AMELIORATE INTRAUTERINE GROWTH RETARDATION IN A TRANSGENIC RAT MODEL FOR PREECLAMPSIA.

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Supplementary Materials and Methods

Flow cytometry

Organs were kept on ice in Phosphate-buffered saline (PBS) containing 0.1% fetal calf serum (FCS) solution which was then used for all washing and incubation steps. Organs were minced on a 100µm sieve. To reduce the remnants of maternal tissue and blood from the placenta, we discarded the trophospongium that is filled with maternal blood and outer membranes from the Labyrinth zone prior to mincing. All cells suspensions and blood were subjected to erythrocyte lysis in lysis buffer for 10 min in 37 °C. Cells were sieved again through a 70 µm sieve and centrifuged down, counted and 10⁶ cells were used for FACS staining including CD3 (APC), CD4 (FITC), CD8 (Pacific Blue), CD25 (PE), CD45R (PE) (all from BD Biosciences) and FoxP3 (Alexa647) (BioLegend). Gating strategy is shown and described in the supplement (Fig. S2 online supplement).

mRNA isolation and real-time RT-PCR

All organs of interest were homogenized by ceramic balls and total mRNA was isolated using QIAzol lysis reagent and Qiagen RNeasy mini kit (Qiagen) according to the manufacturer’s protocol. Reverse Transcription (RT) and real-time PCR were performed as described previously. Primers and probes were designed with PrimerExpress 3.0 (Applied Biosystems), synthetized by Biotez Germany) and are shown in the Supplementary Table 1. Expression was normalized 18s expression.

Fetal brain volumetric and densitometric analysis

Fetal rat brains (embryonic day ED 21) were fixed in 4% formalin and embedded in paraffin. To determine brain volumes 10 µm paraffin sections (every 160 µm between +1.54 and-4.08 mm from bregma according to Watson & Paxinos, The Rat Brain, 3rd Edition) were stained with hematoxilin/eosin. Hemispheres were visualized on an Axioplan (Zeiss, Germany) with a CCD-camera (Microfire, AVT Horn, Germany) with a 2.5x objective and converted to 8bit greyscale images. Hemispheres of each section were analyzed using NIH ImageJ software and volumetric analysis was performed by integration of the areas. Cellular density was assessed on images with higher magnification (20x objective) by densitometric analysis in specific brain regions (striatum, thalamus, cortex and hippocampus).

Immunohistochemistry

Rat uteroplacental units were fixed in buffer according to Beckstead J.H., truncated from two lateral placental parts and embedded in paraffin. Consecutive sections were stained for a trophoblast cell marker (cytokeratin; DAKO, Germany) and smooth muscle cell marker (α-actin; DAKO, Germany) and visualized on microscope Axio Imager M2 with AxioCam HRc (all Zeiss, Germany) using 10x magnification. On cross sections of the most central part of the uteroplacental unit, areas (placental area, mesometrial triangle, and interstitial trophoblast cell invasion into mesometrial triangle) were calculated using AxioVision software (Zeiss, Germany). Actin staining was used to set the border between mesometrial
triangle and myometrium. Interstitial trophoblast cell invasion into mesometrial triangle was calculated as the area of trophoblast cells in the mesometrial triangle. Brain sections were incubated with primary antibodies for immature neurons (Doublecortin X (Dcx), Cell Signaling, Germany), mature neurons (NeuN, Millipore, Germany), astrocytes (GFAP, Genetex, Germany), microglia (Iba1, Wako, Japan), and oligodendrocytes (Olig2, Millipore, Germany) followed by AlexaFluor488 secondary antibody incubation (Invitrogen, Germany). Nuclei were counterstained with DAPI (Invitrogen, Germany). Cortical, hippocampal and thalamic regions were visualized on an Axioplan (Zeiss, Germany) with a CCD-camera (Axiocam Icc1, Zeiss, Germany) using 20x objective. Mean intensity profiles of three different images per region were assessed with ZEN software (Zeiss, Germany) and neural cell type mean intensities were normalized to DAPI.

**Cardiomyocyte beating rate assay**

Immunoglobulin was isolated from 200 µL of rat serum by protein G sepharose on bioline protein purification system (Knauer, Germany). This IgG fraction was used in the bioassay. The AT1-AA activity was measured using spontaneously beating neonatal rat cardiomyocytes and characterized and antagonized specifically using AT1 receptor antagonists as described by Wallukat et al.³

**Statistics**

We used parametric statistics, analysis of variance where indicated, on normally distributed data (mean±SEM). Otherwise, when not normally distributed, the Mann Whitney test was applied to the data. A limit of p<0.05 was considered significant.
Figure S1. Experimental design of “treatment” (A) and “prevention” (B) protocols. d1 is considered as vaginal plug day recognition. Arrows indicate which day 1mg of JJ316 or vehicle was injected into pregnant rats.
Figure S2. Gating strategy for blood, spleen and placenta regulatory T cell FACS analysis. 1 x 10^5 events were recorded in every analysis. All gates were drawn according to isotype controls. Within CD4-positive population, CD25 and FoxP3 positivity was assessed with negative gating (black box).
Figure S3. JJ316 did not show a significantly effect on different T-cell subsets in spleen, blood and placenta of treated PE-rats analyzed on day 15 and 21 of pregnancies. PE, preeclamptic rats.
Figure S4. JJ316 did not show such profound effect in treatment compared to prevention protocol in respect of brain volume (A) and four different brain compartments: cortex (Cx), hippocampus (HC), thalamus (Tha) and striatum (Str) (B) between JJ316 and vehicle treated groups. *p<0.05; ns indicates nonsignificant.
Figure S5. JJ316 did not alter the angiogenesis profile or trophoblast invasion. PlGF and sFlt1 expressions in placenta and mesometrial triangle of JJ316 treated rats were similar compared to controls (A). JJ316 treatment did not change placental area (B) or the area of the mesometrial triangle (C). Interstitial trophoblast cell invasion into the mesometrial triangle (MT) was similar between the two groups (D). ns indicates nonsignificant.
**Figure S6.** JJ316 application improves neuronal maturation. Immunohistochemical stainings revealed an increased cortical neuronal differentiation in JJ316 treated animals (NeuN) and a decrease in reactive astrogliosis (GFAP). No marked alterations in immature neurons (Dcx), microglia (Iba1) or oligodendrocytes (Olig2) were detected. Representative images of neural cell types, Scale bar 100 µm, nuclei were counterstained with DAPI (blue). Data are shown as mean ±SEM. No staining detectable (n.d.). *p<0.05, ***p<0.001

**References:**


**Supplementary table**

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*Table S1. Primer sequences for quantitative real-time RT-PCR.*