RUPP Th17s cause Hypertension and Mitochondrial Dysfunction in the Kidney and Placenta during Pregnancy

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Abstract

BACKGROUND

Preeclampsia (PE), new-onset hypertension (HTN), and organ dysfunction during the second half of pregnancy, is associated with an increase in inflammatory immune cells, including T helper 17 (Th17) cells. Studies have demonstrated that mitochondrial (mt) dysfunction is important in the pathogenesis of PE though causative factors have yet to be fully identified. Although Th17 cells, natural killer (NK) cells, and mt dysfunction contribute to HTN in the reduced uterine perfusion pressure (RUPP) rat model, the role of Th17 cells or IL-17 in mt dysfunction is unknown. Therefore, we hypothesize that RUPP stimulated Th17 cells cause HTN and mt dysfunction, which is alleviated with the blockade of IL-17.

METHODS

On gestational day 12 (GD12), RUPP Th17 cells were transferred into normal pregnant (NP) Sprague Dawley rats. A subset of NP + RUPPTh17 rats received IL-17RC (100pg/day) on GD14-19. Blood pressure (MAP), NK cells, and mt function were measured on GD19 in all groups.

RESULTS

MAP increased in response to NP + RUPP Th17 compared to NP rats and was lowered with IL-17RC. Circulating and placental NK cells increased with NP + RUPP Th17 compared to NP and were lowered with IL-17RC. Renal mtROS increased in NP + RUPP Th17 compared to NP and was normalized with IL-17RC. Similar to PE women, placental mtROS decreased in NP + RUPP Th17 and was normalized with IL-17RC.

CONCLUSION

Our results indicate that IL-17RC inhibition normalizes HTN, NK cell activation, and multi-organ mt dysfunction caused by Th17 cells stimulated in response to placental ischemia.

Background

Preeclampsia (PE) is a multi-system disorder of pregnancy characterized by new-onset hypertension and organ dysfunction occurring after the 20th week of gestation (1–4). Preeclampsia is associated with oxidative stress, endothelial dysfunction, and fetal growth restriction (FGR) (1–3, 5–7). During a normal pregnancy, the placenta is as a hypoxic environment until spiral arteries are remodeled to provide increased blood flow to the maturing placenta, and antioxidant capacity is increased to compensate for oxidative stress (5, 8–10). However, in PE, spiral artery remodeling does not occur sufficiently to provide adequate blood flow to the placenta, which increases reactive oxygen species (ROS), leading to

inadequate antioxidant capacity, and an increase in oxidative stress and inflammatory cytokines (8–10). Multiple human studies have shown an association with placental mitochondrial (mt) dysfunction in PE, which we recently demonstrated to be associated with a decrease in mt ROS. (12) Moreover, we recently showed the decrease in mt respiration and ROS in the placentas of PE women, was worsened with severity of disease (13). Although mitochondrial function is compromised in PE patients, factors causing such changes in the PE placenta are not fully identified.

T helper 17 (Th17) cells are a subset of CD4 + T cells that release interleukin 17 (IL-17), resulting in the activation of Natural Killer (NK) cells, neutrophils, and CD8 + T cells. Th17 cells have been implicated in the pathology of many autoimmune disorders, including rheumatoid arthritis, asthma, multiple sclerosis, and irritable bowel syndrome (23–26). It has been shown that IL-17 signaling increases mean arterial pressure (MAP) and oxidative stress in pregnant rats (7, 10, 23, 27–29). There are also IL-17-associated increases in several pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α, all of which are increased in PE and have been shown to play a role in its pathophysiology (27, 30–32). IL-17 has also been shown to increase hypertension, fetal growth restriction, and placental tissue ROS (8, 9). IL-17 Receptor C (IL-17RC) is a receptor for IL-17 A-F, which is required for most IL-17 signaling pathways (23, 28, 33). Our group has previously shown that administration of soluble IL-17RC to reduced uterine perfusion pressure (RUPP) rats decreased Th17 cells, oxidative stress, angiotensin II type 1 receptor activating autoantibody (AT1-AA), activation of NK cells, and hypertension in this model of PE (8, 9, 23). Moreover, we have previously shown that the adoptive transfer of RUPP Th17 cells results in hypertension in normal pregnant (NP) rats, increased tissue ROS, and increased AT1-AA production (34). When recipients of RUPP Th17 cells were treated with tempol, both the blood pressure and AT1-AA were decreased, indicating the importance of ROS molecules as communicators among immune cells. Although oxidative stress molecules are increased in response to RUPP Th17 cells, the role for mitochondria as a source of ROS production has not been studied. Moreover, the role of IL-17 to cause mt ROS remains unknown.

Therefore, we sought to determine the importance of IL-17 as a mediator of mt ROS in response to Th17 cells stimulated by placental ischemia. We addressed this question by performing adoptive transfer of RUPP Th17 into normal pregnant rats with or without IL-17 blockade and measured mt function, mt ROS, and hypertension in recipient rats.

Methods

Animals

Timed-pregnant female Sprague Dawley (SD) rats (200–250 g) were purchased from Envigo (Indianapolis, IN, USA). Rats were housed in a temperature-controlled facility with a 12:12 hour light/dark cycle and were fed and watered ad libitum. All protocols and procedures for experiments were approved by the animal ethics committee of University of Mississippi Medical Center (UMMC) Institutional Animal Care and Use Committee (IACUC) (Animal Welfare Assurance Number D16-00174 (A3275-01); AAALAC accreditation (6/30/2021); protocol #1435B) according to the guidelines set forth by the National

Institutes of Health Guide for the Care of Animals. The study is reported in accordance with ARRIVE guidelines (The ARRIVE guidelines 2.0).

Reduction in uterine perfusion pressure

On gestational day (GD) 14, the RUPP surgery was performed by placing silver clips on the abdominal aorta (0.203 mm ID) above the iliac bifurcation and around the ovarian arteries (0.100 mm ID) on both sides under isoflurane anesthesia and carprofen (5mg/kg) was administered following the completion of the surgical procedures.

On GD 18, rats received in-dwelling carotid catheters, and carprofen (5 mg/kg) was administered. Mean arterial pressure (MAP) was measured on GD 19 on PowerLab setup (ADInstruments, Colorado Springs, CO, USA), where a 30-minute acclimation period followed by a 30-minute measurement period was recorded via LabChart software (ADInstruments, Colorado Springs, CO, USA) as previously described (37). Spleens were collected for isolation of Th17 cells used for adoptive transfer into normal pregnant (NP) rats. Our previous studies have shown that adoptive transfer of NP Th17 cells into NP rats had no effect on blood pressure or other features of PE. Therefore, this group was not included in the current study.

Isolation and Adoptive Transfer of Th17 cells

Lymphocytes were isolated from spleens of donor RUPP rats by centrifugation on a cushion of Ficoll-Hypaque (Lymphoprep; Accurate Chemical &Scientific, Westbury, NY, USA) according to the manufacturer's instructions (38). Anti-CD4 and Anti-CD25 antibodies (BD Biosciences, San Jose, CA) were biotinylated using the DSB-X Biotin protein labeling kit (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol. CD4+/CD25- T cells were isolated using FlowComp Dynabeads (Invitrogen, Oslo, Norway) according to the manufacturer's protocol. The CD4+/CD25- population of splenocytes was incubated on anti-CD3 (BD Biosciences Pharmingen, San Diego, CA, USA) and anti-CD28 magnetic beads (BD Biosciences Pharmingen, San Diego, CA, USA) for two days in T cell medium containing RPMI, HEPES (25 mM), and Pen/Strep (100 U/ml). After two days, cells were removed from magnetic beads and cultured in T-helper-specific media (RPMI, 10% FBS, 5% Pen-Strep, 1% HEPES, 20 ng/ml IL-6, 3 ng/ml TGFβ-1, and 20 ng/ml IL-23 (R&D Systems, Minneapolis, MN, USA)) at 5% CO2 at 37°C in a humidified atmosphere for five days to differentiate.

After differentiation, Th17 cells were collected and centrifuged at 300g for 10 mins at 4°C and purity was verified by flow cytometry. The collected cells were diluted in sterile saline at 1×10^6 cells/ml and injected intraperitoneally into NP rats (NP + RUPPTh17) on GD 12. Recombinant mouse IL-17 receptor (IL-17RC) (100 pg/day) (R&D Systems, Minneapolis, MN, USA) was infused on GD 14–19 via mini-osmotic pumps (model 2002, Alzet Scientific Corporation) in a subset of recipient pregnant rats (NP + RUPPTh17 + IL-17RC).

Determination of circulating and placental NK cell populations using flow cytometry

Circulating and placental populations of total and activated NK cells were quantified by flow cytometry of lymphocytes isolated from placental tissues and peripheral blood of GD19 NP, NP + RUPP Th17, and NP + RUPP Th17 + IL-17RC animals. At the time of harvest, one placenta from each rat was homogenized and filtered through a 70-μm cell strainer and resuspended in 10 mL of Roswell Park Memorial Institute medium (RPMI) (10% FBS). Whole blood was collected in an EDTA tube and diluted with 5 mL of RPMI. Peripheral blood mononuclear cells (PBMCs) and placental lymphocytes were isolated by centrifugation on a Ficoll-Hypaque cushion (Lymphoprep, Accurate Chemical & Scientific Corp., Westbury, NY, USA) according to the instructions of the manufacturer. Cells were incubated for 10 minutes at 4° C against rat Anti-Natural Killer Cell antibody (ANK44) (Abcam, Cambridge, MA, USA) as previously described (8, 23), and designated as activated natural killer cells.

Isolation of Intact Mitochondria

Mitochondria from rat placental or renal tissues were isolated by differential centrifugation (10). Tissues were homogenized using a Dounce homogenizer, and centrifuged at 4000 rpm for 3 minutes at 4°C. The supernatant was centrifuged at 10000 rpm for 10 minutes at 4°C. The pellet was suspended in Mito I buffer and centrifuged at 10000 rpm for 10 minutes at 4°C, was re-suspended in Mito II buffer and used for mitochondrial (mt) respiration and reactive oxygen species (ROS) assays(10).

Respiration in Isolated Mitochondria

Mitochondrial respiration was measured using the Oroboros Oxygraph-2k measuring state 3 respiration rate. Basal and state 3 respiration rates were measured by adding glutamate/malate and ADP. To measure non-mitochondrial respiration, rotenone and antimycin A were added and respiration recorded and subtracted from state 3 respiration. Data were analyzed and expressed as pmol of oxygen consumed per sec per mg of mitochondrial protein (10).

Mitochondrial ROS

Amplex red assay was used to measure mitochondrial hydrogen peroxide (H_2O_2) production. Briefly, mitochondria (0.4 mg/ml) were incubated in 96 well plates with respiration buffer, SOD, HRP, and succinate (10). Amplex red was added to the sample wells to begin the reaction. Appropriate controls (blank wells without mitochondrial protein or amplex red) were used. Hydrogen peroxide (H_2O_2) production was recorded in real-time using a plate reader at 555/581 nm excitation/emission for 30 min at 25°C (10). Results are plotted as an average of production over the 30 min read frame.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7.02 software (GraphPad Software, San Diego, CA, USA). Comparisons among the groups were analyzed using one-way ANOVA. Results were reported as means ± SEM and were considered statistically significant when $p < 0.05$.

Results

Effect of Th17 adoptive transfer and IL-17 inhibition on blood pressure

Mean arterial pressure was increased in recipients of RUPP Th17 cells (NP + RUPP Th17;112 ± 1.07 mmHg, *p < 0.05 vs NP; n = 12) compared to the normal pregnant rats (NP) (92 ± 3 mmHg, n = 12). Hypertension was attenuated by the administration of soluble IL-17RC (NP + RUPPTh17 + IL-17RC) (98 ± 2.0 mmHg #p < 0.05 vs. NP + RUPPTh17; n = 12) (Fig. 1**)**.

Effect of Th17 adoptive transfer and IL-17 inhibition on natural killer cell activation

Natural killer cell activation was increased in the NP + RUPPTh17 rat placental tissue (1.565 ± 0.51% Gated; *p < 0.05 vs NP; n = 10) in comparison to NP placental tissue (0.1 ± 0.08% Gated; n = 12) (Fig. 2a). Placental NK cell activation was normalized with the administration of IL-17RC (0.14 ± 0.08% Gated; #p < 0.05 vs. NP + RUPPTh17; n = 6) (Fig. 2a**).** Similarly, circulating natural killer cell activation was increased with the NP + RUPPTh17 (1.946 ± 0.59% Gated; *p < 0.05 vs NP; n = 12) compared to normal pregnant (0.11 ± 0.08% Gated; n = 12), and was normalized with IL-17RC (0.14 ± 0.08% Gate; #p < 0.05 vs. NP + RUPPTh17; n = 11) Fig. 2b.

Effect of Th17 adoptive transfer and IL-17 inhibition on mitochondrial function

Mitochondrial respiration in placental mitochondria was reduced, but not significantly, in NP + RUPPTh17 (22.4 ± 5.8 pmol/sec/mg; n = 9) compared to NP (40.4 ± 9 pmol/sec/mg; n = 5), and was unchanged with IL-17RC (18.3 ± 10.4 pmol/sec/mg; n = 4) (Fig. 3a**).** Oxygen consumption was elevated in the renal mitochondria with NP + RUPPTh17 (609 ± 153 pmol/sec/mg; n = 12; *p < 0.05 vs NP) in comparison to the normal pregnant (71.7 ± 16.9 pmol/sec/mg; n = 7) and was trending towards normalization with IL-17RC (129.5 ± 42.9 pmol/sec/mg; n = 4) (Fig. 3b).

The percent fold change over time in mitochondrial hydrogen peroxide production, was decreased in response to NP + RUPPTh17 in placental mitochondria (50.93 ± 2.63%-fold; *p < 0.05 vs. NP; n = 6) compared to NP placental mitochondria (100 ± 6.67%-fold; n = 6) and was improved with administration of IL-17RC (66.38 ± 4.36%-fold; #p < 0.05 vs. NP + RUPPTh17; n = 5) (Fig. 4a**)**. Renal mitochondrial reactive oxygen species production was increased in response to NP + RUPPTh17 (143.1 ± 3.33%-fold; *p < 0.05 vs NP; n = 11) compared to NP renal mitochondria (100 ± 2.59%-fold; n = 6), and was reduced with administration of IL-17RC (68.3 ± 3.8%-fold; #p < 0.05 vs. NP + RUPPTh17; n = 5) (Fig. 4b).

Discussion

We sought to identify the role that IL-17 plays in causing mitochondrial dysfunction in response to Th17 cells in the pathophysiology of PE. Th17 cells have been shown to cause hypertension, activate NK cells, and cause a pro-inflammatory shift in multiple cytokines in the RUPP rat model of placental ischemia (1, 23, 24, 30, 31, 34, 38–42). We demonstrate that the adoptive transfer of RUPP Th17 cells into NP recipient rats resulted in hypertension, NK cell activation, and renal and placental mitochondrial dysfunction. All of these endpoints were improved with IL-17 blockade, demonstrating that IL-17 cell signaling by Th 17 cells mediates multiple pathophysiological characteristics of PE. However, what was unknown is whether Th17 cells or IL-17 cause renal or placental mitochondrial dysfunction.

Similar to previous data published from our group, adoptive transfer of RUPP Th17 cells resulted in the activation of NK cells in both circulation and placenta (8, 10, 34, 38, 43). However, this study is the first to demonstrate an association between Th17 cell and IL-17 signaling with NK cell activation and mt dysfunction during pregnancy. Activation of cytolytic NK cells is associated with mitochondrial ROS production in RUPP rats, and is increased in recipients of RUPP CD4 + T cells, and AT1-AA infused hypertensive pregnant rats (11). In this study, IL-17 was shown to play a distinct role in mediating NK cell activation by administration of IL-17 RC which blocks IL-17 signaling and lowered NK cells in the placenta and circulation of RUPP Th17 recipient rats. Similarly, Travis et al. previously showed that infusion of IL-17 increased cytolytic NK cell activation and tissue ROS, resulting in significantly reduced vasorelaxation, further supporting a role for IL-17-mediated NK cell activation and multi-organ dysfunction during PE (9).

Similar to our recently published study in human PE patients, placental mitochondrial respiration and mt ROS production were significantly decreased in response to RUPP Th17 adoptive transfer compared to NP controls (71). ROS production was partially normalized with IL-17 inhibition. The decrease in mt respiration and mtROS indicates overall mitochondrial dysfunction seen in the placenta of recipients of RUPP Th17 cells, indicating a possible role for Th17 cells in the similar decrease in mt function in placentas of preeclamptic patients. One explanation for the decrease in mtROS could be that the overly hypoxic environment of the placenta is causing a decrease in the overall availability of oxygen and thereby oxidative stress. The lack of oxygen within the placenta, as a result of placental ischemia, would reduce the number of reactive oxygen species produced overall and decrease mitochondrial function over time. Placental decrease in mitochondrial reactive oxygen species could also be a result of uncoupling as a cytoprotective strategy in response to damage as seen in diabetes or ischemia-reperfusion injury (46–54).

An increase in mitochondrial ROS within the kidney has been shown to cause increases in blood pressure, lack of ion transport, and retention of sodium in many disease states, including hypertension (55–59), chronic kidney disease (50), and PE (34, 59–62). The combination of increased ROS and mitochondrial respiration in response to RUPP Th17 adoptive transfer indicates a mishandling of oxygen by the mitochondria within the kidney. Interestingly this was normalized with IL-17 inhibition, indicating the importance of IL-17 in mediating the multi-tissue dysfunction associated with PE. The mishandling of oxygen results in the creation of mtROS, which has been shown to occur in patients with asthma,

pulmonary hypertension, and preserved ejection fraction heart failure and is not uncommon for pathologic conditions, thus indicating the importance of better understanding causal factors that mediate these diseases and potential treatment modalities for possible intervention.

Mitochondria are an integral part of the pathogenesis of multiple renal diseases, including contributing to the development and progression of acute kidney injury, diabetic nephropathy, and chronic kidney disease (68–70). There is evidence that mitochondria are heterogeneously spread throughout the kidney, with the loop of Henle and proximal tubule being the locations of the most significant number of mitochondria. These sections are highly dependent on mitochondrial function, as a large portion of active sodium transport occurs here, thereby increasing oxygen demand as glomerular filtration rate and renal blood flow increase. Other portions of the nephron, such as the podocytes and endothelial cells, exhibit more glycolytic capacity than mitochondrial oxidative phosphorylation (43, 69). Inhibition of IL-17 with IL-17RC normalized the hypertensive response in the RUPP Th17 adoptive transfer animals, suggesting that Th17 and IL-17 signaling are important for causing renal dysfunction possibly via stimulation of NK cells and mt ROS to cause hypertension in PE.

Conclusions

Collectively our data indicate the importance IL-17 has in the pathophysiology of the kidney and placenta when Th17 cells are activated, leading to activation of NK cells. Moreover, Th17 cell-mediated activation of NK cells leads to renal and placental mt dysfunction which is normalized by IL-17 inhibition. This may be the essential mechanism whereby blood pressure is normalized in IL-17 RC treated recipient rats. Future studies are needed to investigate the mitochondrial dysfunction seen within these tissues so there is a better understanding of the metabolic reactions involved. Overall, our study suggests that Th17 cells and IL-17 signaling are essential for mediating chronic inflammation associated with mitochondrial oxidative stress and multiple tissue dysfunction associated with hypertension during PE.

Declarations

Ethics approval and consent to participate- All animal procedures in this study were approved by the ethics committee of University of Mississippi Medical Center (UMMC) Institutional Animal Care and Use Committee (IACUC), Jackson, Mississippi (Animal Welfare Assurance Number D16-00174 (A3275-01); protocol #1435B) in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication). The study is reported in accordance with ARRIVE guidelines (The ARRIVE guidelines 2.0).

Consent for publication- Not applicable.

Availability of data and materials- The datasets used and/or analyzed during the current study are available upon reasonable request from the corresponding author.

Competing interests- The authors declare that they have no known competing interests that could have appeared to influence the work reported in this paper.

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Authors' contributions- B.B.L designed the study, S.F., E.D., J.H., T.T., L.M.A., N.H, K.E., O.H., N.C., and T.I. carried out the experiments and immunoassays. B.B.L. and S.F. collected, analyzed, and interpreted the data. B.B.L., S.F., E.D., and D.C.C. drafted the manuscript. All authors read and approved the final draft.

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Figures

Figure 1

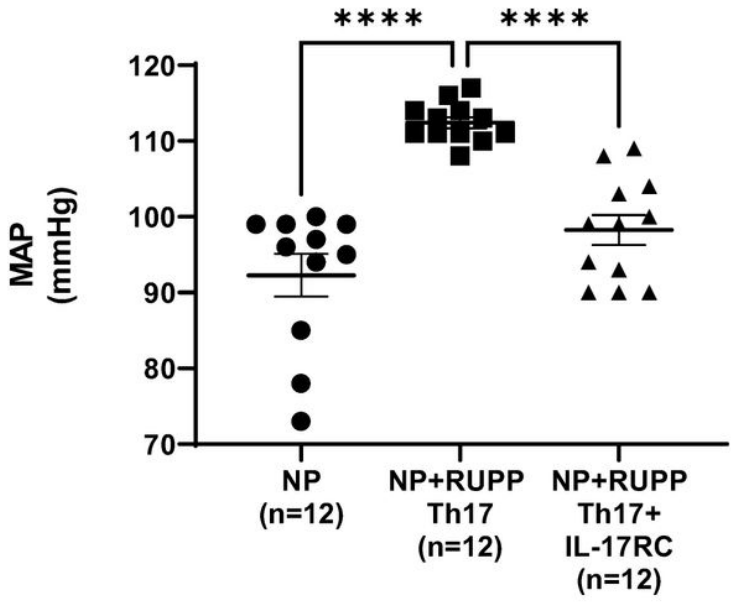

Figure 1

Mean arterial pressure is increased in response to RUPP Th17 cells (112± 1.07 mmHg, *p<0.05, n=12) compared to the normal pregnant rats (NP) (92 ± 3 mmHg, n=12). IL-17 inhibition lowered the mean arterial pressure in RUPPTh17 recipient rats (98 ± 2.0 mmHg #p<0.05; n=12). All data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons followed by Bonferroni post hoc correction. *P < 0.05 vs. NP. #P<0.05 vs. NP+RUPPTh17.

Figure 2

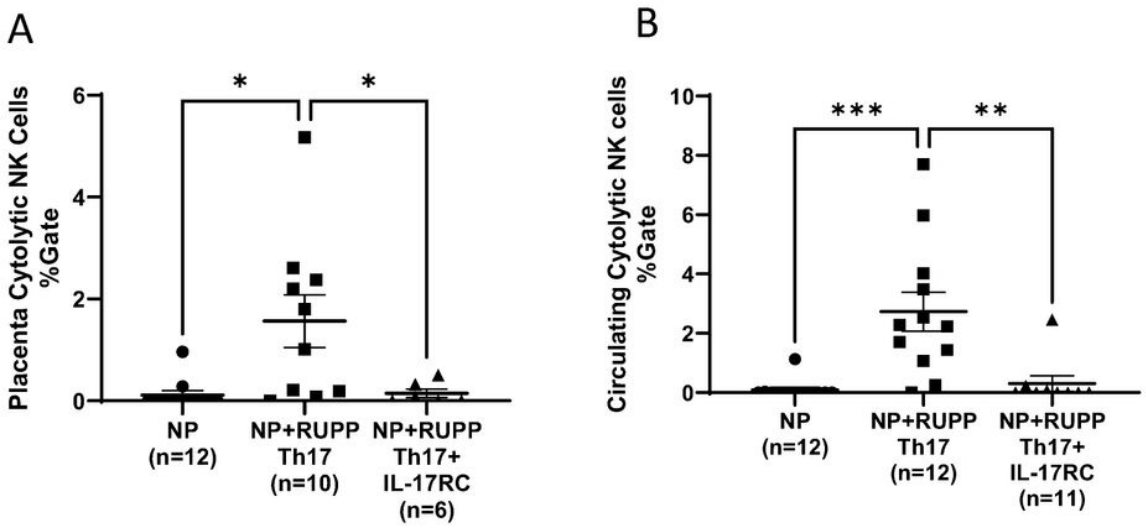

Figure 2

a) Cytolytic NK cells were increased in NP+RUPPTh17 rat placental tissue (1.565± 0.51% Gated; *p<0.05, n=10) in comparison to NP placental tissue (0.1± 0.08% Gated; n=12) (**Figure 2a**). and was normalized with the administration of IL-17RC (0.14± 0.08% Gated; #p<0.05; n=6). b) Circulating NK cell activation was increased in RUPPTh17 recipients (1.946± 0.59% Gated; *p<0.05; n=12) compared to normal pregnant rats (0.11± 0.08% Gated; n=12) and was normalized with RUPPTh17+IL-17RC (0.14± 0.08% Gate; #p<0.05; n=11). All data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons followed by Bonferroni post hoc correction. *P < 0.05 vs. NP. #P<0.05 vs. NP+RUPPTh17.

Figure 3

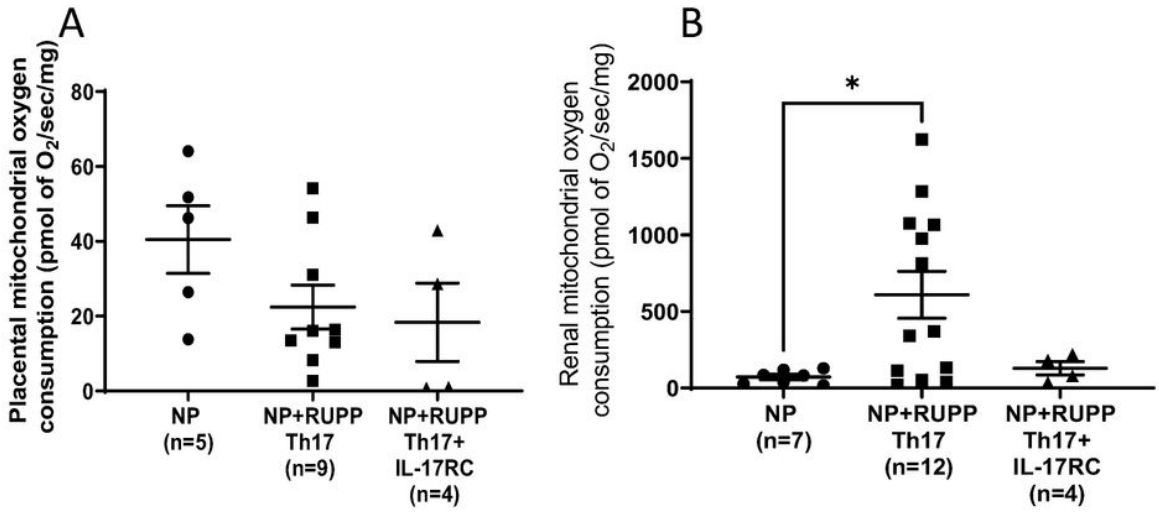

Figure 3

a) There were no significant changes in ATP production in response to RUPPTh17 adoptive transfer (22.4± 5.8 pmol/sec/mg; n=9) or treatment with IL-17RC treatment (18.3±10.4 pmol/sec/mg; n=4) in placental mitochondria compared to normal pregnant rats (40.4± 9 pmol/sec/mg; n=5). b) ATP production was elevated in the renal mitochondria with NP+RUPPTh17 (609± 153 pmol/sec/mg; n=12; p<0.05) in comparison to the normal pregnant (71.7± 16.9 pmol/sec/mg; n=7) and was normalized with IL-17RC (129.5± 42.9 pmol/sec/mg; n=4). All data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons followed by Bonferroni post hoc correction. *P < 0.05 vs. NP. #P<0.05 vs. NP+RUPPTh17.

Figure 4

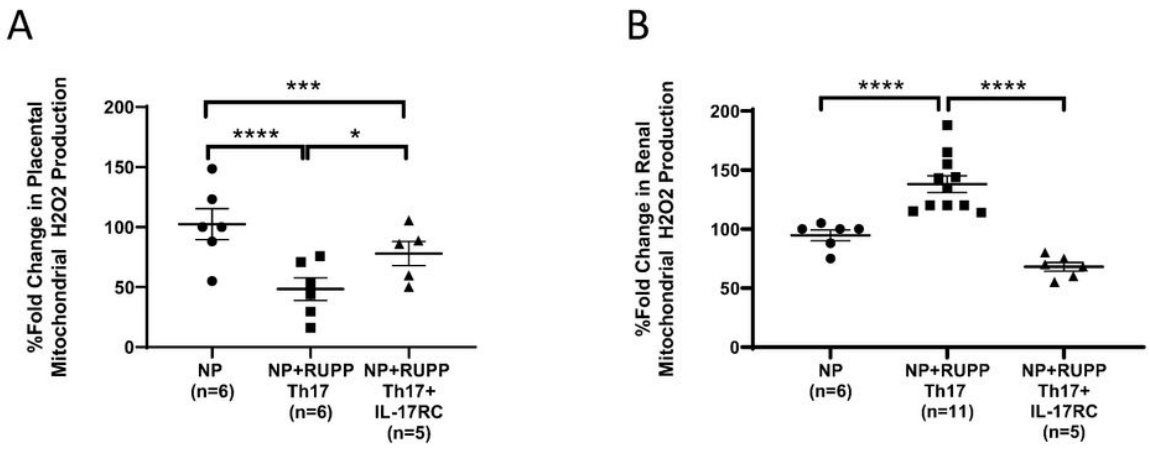

Figure 4

a) Placental mitochondrial ROS was decreased in response to RUPPTh17 adoptive transfer (50.93± 2.63%-fold; *p<0.05; n=6) and normalized with IL-17 inhibition (66.38± 4.36 %-fold; #p<0.05; n=5) compared to normal pregnant levels (100± 6.67 %-fold; n=6). b) Renal mitochondrial ROS was increased in response to RUPPTh17 adoptive transfer (143.1± 3.33%-fold; *p<0.05; n=11), and was normalized with IL-17 inhibition (68.3± 3.8 %-fold; #p<0.05; n=5) compared to normal pregnant (100± 2.59%-fold; n=6), All data are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with multiple comparisons followed by Bonferroni post hoc correction. *P < 0.05 vs. NP. #P<0.05 vs. NP+RUPPTh17.