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Extracellular ATP decreases trophoblast invasion, spiral artery remodeling and immune cells in the mesometrial triangle in pregnant rats

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ABSTRACT

Introduction: Preeclampsia is characterized by deficient trophoblast invasion and spiral artery remodeling, a process governed by inflammatory cells. High levels of the danger signal extracellular adenosine triphosphate (ATP) have been found in women with preeclampsia and infusion of ATP in pregnant rats induced preeclampsia-like symptoms such as albuminuria and placental ischemia. We hypothesized that ATP inhibits trophoblast invasion and spiral artery remodeling and affects macrophages and natural killer (NK) cells present in the rat mesometrial triangle.
Methods: Pregnant rats were infused with ATP or saline (control) on day 14 of pregnancy. Rats were sacrificed on day 15, 17 or 20 of pregnancy and placentas with mesometrial triangle were collected. Sections were stained for trophoblast cells, α-smooth muscle actin (spiral artery remodeling), NK cells and various macrophage populations. Expression of various cytokines in the mesometrial triangle was analyzed using real-time RT-PCR.
Results: ATP infusion decreased interstitial trophoblast invasion on day 17 and spiral artery remodeling on day 17 and 20, increased activated tartrate resistant acid phosphatase (TRAP)-positive macrophages on day 15, decreased NK cells on day 17 and 20, and decreased inducible nitric oxide synthase (iNOS)-positive and CD206-positive macrophages and TNF-α and IL-33 expression at the end of pregnancy (day 20).
Discussion: Interstitial trophoblast invasion and spiral artery remodeling in the rat mesometrial triangle were decreased by infusion of ATP. These ATP-induced modifications were preceded by an increase in activated TRAP-positive macrophages and coincided with NK cell numbers, suggesting that they are involved.
Conclusion: Trophoblast invasion and spiral artery remodeling may be inhibited by ATP-induced activated macrophages and decreased NK cells in the mesometrial triangle in rat pregnancy.

1. Introduction

Human and rodent pregnancies are characterized by hemochorial placentation. In both species, fetal trophoblast cells invade into the uterine wall and aid to placental development and function by transforming the maternal spiral arteries. This remodeling of the maternal spiral arteries is considered to be initiated by NK cells and macrophages present in the uterine wall [1]. These cells regulate trophoblast invasion and tissue remodeling by production of cytokines, chemokines and pro- and anti-angiogenic factors [2–5]. As an end result, the spiral arteries develop into high flow, low resistance vessels that facilitate sufficient blood flow to the placenta [6]. The timing of trophoblast invasion into the uterine wall differs between humans and rats:

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[1] The author Dr. Winston W. Bakker has recently passed away. However, he has contributed significantly to our current work and we thus want to acknowledge him as a co-author.

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while in humans trophoblast invasion is initiated early in pregnancy and is completed before week 20 of pregnancy [1], in rats trophoblast invasion into the mesometrial triangle (i.e. the equivalent of the placental bed) does not start until the last week, around day 12–13, of pregnancy [7].

Preeclampsia, a major pregnancy complication affecting 3–8% of the pregnancies [8], is characterized by hypertension and proteinuria in the second half of pregnancy. The exact pathophysiology of preeclampsia remains unknown, but poor placentation in the first trimester is thought to be important in its pathogenesis [9]. Poor placentation is characterized by shallow and aberrant invasion of trophoblast cells into the uterine wall and maternal spiral arteries [10]. This is associated with changes in the local environment, such as functional changes in NK cells and macrophages [11,12] and aberrant cytokine expression [13,14]. Thus spiral arteries from women with preeclampsia are reduced in diameter and higher in resistance compared to normal pregnancy [10]. This increases the velocity and turbulence of the blood flow in the placenta and may give rise to placental damage and oxidative stress in the second half of pregnancy [15]. The release of factors from the damaged and stressed placenta into the peripheral circulation, inducing an inflammatory response, and endothelial cell activation, eventually results in preeclampsia [9,16]. The exact cause of abnormal trophoblast invasion and spiral artery remodeling in this condition remains unknown.

We have previously found elevated plasma ATP levels in women with preeclampsia [17]. As extracellular ATP is a danger associated molecular pattern (DAMP), released upon cellular activation, stress, damage or necrosis to induce inflammation [18], it seems likely that ATP plays a role in the pathogenesis of preeclampsia. Indeed, ATP infusion in rats on day 14 of pregnancy induced preeclampsia-like symptoms such as proteinuria, changes in the systemic immune response and placental ischemia [19]. In the present study, we tested the hypothesis that ATP inhibited trophoblast invasion and spiral artery remodeling and affects macrophages and NK cells present in the mesometrial triangle. Therefore pregnant rats were infused with ATP on day 14 of pregnancy, at which time point trophoblast invasion has just started. At various time points after the infusion we evaluated trophoblast invasion, spiral artery remodeling, the presence and inflammatory status of macrophages and NK cells as well as cytokine production in the mesometrial triangle.

2. Methods

2.1. Experimental design

The effect of ATP infusion on trophoblast invasion, spiral artery remodeling and the numbers of NK cells and macrophages in the mesometrial triangle was investigated. Production of various cytokines in the mesometrial triangle was also studied. Pregnant rats with a permanent jugular vein cannula [20] (see Online Data Supplement) were infused with 3000 μg/kg bw ATP in 2.0 ml saline or with 2.0 ml saline alone on day 14 of pregnancy as described before [19]. Rats were sacrificed by aortic puncture under anesthesia (isoflurane/oxygen) on day 15 (saline: $n = 5$; ATP: $n = 7$), day 17 (saline: $n = 15$; ATP: $n = 12$) or day 20 (saline: $n = 15$; ATP: $n = 18$) of pregnancy. After sacrifice, the peritoneal cavity was opened and zinc buffer solution was sprinkled over both uterine horns. Placentas with mesometrial triangle were thereafter obtained as previously described [21] and snap frozen or fixed in zinc-buffer or 4% paraformaldehyde (PFA) solution for 24 h as described before [7], to evaluate trophoblast invasion, spiral artery remodeling and the presence of immune cells. The Institutional Animal Care and Use Committee of the University of Groningen approved all animal experiments.

2.2. Immunohistochemical staining for cytokeratin, α-smooth muscle actin (α-SMA), NK cells and macrophages

After 24 h fixation with zinc-buffer or 4% PFA, placental tissue was dehydrated and embedded in paraffin. 4 μm sections were cut. To be able to stain the same location within the mesometrial triangle, only sections containing the maternal channel (the large centrally located artery, see Fig. S1) were used for staining [7]. Sections of placentas with mesometrial triangle were stained for the presence of trophoblast cells (cytokeratin), α-SMA, NK cells (ANK61), total macrophages (CD68), iNOS-positive macrophages, CD206-positive macrophages and activated macrophages (TRAP) (see Online Data Supplement for extended methods).

2.3. Analysis of trophoblast invasion, spiral artery remodeling and presence of macrophages and NK cells

All analyses were performed with the Aperio Imagescope program (Aperio Vista, USA).

Trophoblast invasion: Trophoblast invasion was analyzed by calculating the surface area invaded by trophoblast cells and the total surface area of the mesometrial triangle; percentage of surface area of the mesometrial triangle invaded by trophoblast cells was calculated. To analyze the pattern of trophoblast invasion, the surface area invaded by trophoblast cells in the mesometrial triangle, the maximal distance into the width and depth (calculated from the center of the mesometrial triangle-decidual border) was measured and calculated as percentage of the total width and depth of the mesometrial triangle.

Spiral artery remodeling: Spiral artery remodeling was assessed in the total area of the mesometrial triangle as well as in each of three concentric depth zones separately (Fig. 1A). Spiral artery remodeling was assessed using the extent of α-SMA disappearance in the arterial walls of the spiral arteries. Therefore, spiral arteries were scored in four categories: category 1) unremodeled arteries with 0–3% α-SMA disappearance (Fig. 1B), category 2) slightly remodeled arteries with 3–33% α-SMA disappearance (Fig. 1C), category 3) moderately remodeled arteries with 33–67% α-SMA disappearance (Fig. 1D), and category 4) highly remodeled arteries with 67–100% α-SMA disappearance (Fig. 1E) in the arterial wall.

NK cells and macrophages: The amount of positively stained pixels as well as the total amount of pixels (reflecting the total amount of tissue) for ANK-61, CD68, iNOS, CD206 and TRAP staining in the mesometrial triangle were calculated using the 'Positive pixel count V9' algorithm and percentage positive area was calculated.

2.4. Cytokines in the mesometrial triangle

Expression of various pro- and anti-inflammatory cytokines in the isolated mesometrial triangle (laser dissection microscopy) was analyzed using real-time RT-PCR (see Online Data Supplement for extended methods).

2.5. Statistical analysis

Data are presented as medians with interquartile range. For statistical analysis of differences between saline and ATP-infused rats on each separate time point (day 15, 17 or 20) Mann Whitney U tests were used. Differences were considered to be significant if $p < 0.05$ and a statistical trend if $p < 0.1$.

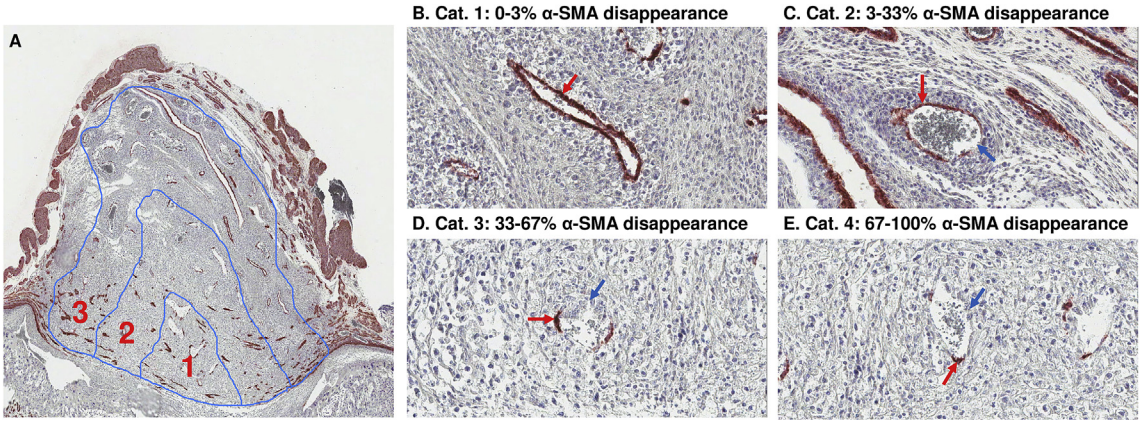

Fig. 1. Examples of spiral artery remodeling. A: Mesometrial triangle stained for α-SMA showing the three consecutive zones of equal width. B–E: Representative spiral arteries stained for the presence of α-SMA of the four categories of spiral artery remodeling: B: unremodeled arteries with 0–3% disappearance of α-SMA staining (cat.1), C: slightly remodeled arteries with 3–33% disappearance of α-SMA staining (cat.2), D: moderately remodeled arteries with 33–67% disappearance of α-SMA staining (cat.3) and E: highly remodeled arteries with 67–100% disappearance of α-SMA staining in the arterial wall (cat.4). Red arrows show positive α-SMA staining in the spiral arteries, while blue arrows demonstrate absence of α-SMA staining.

3. Results

3.1. No change in trophoblast cell surface area after ATP infusion

On day 15, 17 and 20 of pregnancy, the percentage of surface area invaded by trophoblast cells did not differ between ATP and control animals (Fig. 2A). Representative examples of placentas from saline and ATP-infused rats on days 15, 17 and 20 stained for cytokeratin are shown in Fig. 2B.

3.2. Decreased depth of trophoblast invasion after ATP infusion

Trophoblast invasion depth and width could not be appropriately analyzed on day 15 of pregnancy, since invasion was too shallow at this time-point. On day 17 of pregnancy, invasion depth was significantly lower in the ATP-infused rats compared to the control animals (Fig. 3B, $p < 0.05$; and Fig. 2B, middle panels). There was no effect of ATP infusion on the width of invasion (Fig. 3C). Consequently, the depth/width ratio was significantly decreased after ATP infusion on day 17 ($p < 0.05$, Fig. 3D).

3.3. ATP decreased spiral artery remodeling

In the total area of the mesometrial triangle, in control rats, most spiral arteries are unremodeled (category 1) at day 15 of pregnancy, while a low percentage of spiral arteries are remodeled (category 4) (Fig. 4A). The percentage of arteries in category 1 (unremodeled) decreased at day 17 and 20 while the percentage of category 4 (remodeled) arteries increased (Fig. 4E+I). ATP infusion did not affect spiral artery remodeling in the total area of the total area of the mesometrial triangle.

Since spiral artery remodeling starts at the decidual site of the mesometrial triangle (zone 1) and extends towards the myometrium (zone 3) [7], the effect of ATP may differ in the different zones.

Day 15: In control rats, in all zones, most of the spiral arteries were of category 1 (unremodeled) (Fig. 4B–D) with no effect of ATP infusion.

Day 17: As compared with day 15, the percentage of category 1 (unremodeled) spiral arteries decreased in all zones, while higher percentages of category 3 and 4 (remodeled) arteries were observed (Fig. 4F–H). In ATP infused rats, the percentage of category 1 (unremodeled) arteries was significantly higher than in control rats in zone 1 ($p < 0.05$, Fig. 4F). In zone 2, the percentage of category 3 (moderately remodeled) arteries significantly decreased in the ATP-infused rats compared with control rats ($p < 0.05$, Fig. 4G).

Day 20: In control rats, spiral artery remodeling was similar to day 17 (Fig. 4J–L), while infusion of ATP resulted in significantly increased percentages of category 1 (unremodeled) arteries in zone 1 ($p < 0.05$, Fig. 4J).

3.4. ATP decreased NK cell and macrophage numbers and increased activated macrophages

NK cells: In control and ATP-infused rats, on day 15 of pregnancy ANK-61-positive NK cells were located throughout the whole mesometrial triangle, mainly in zone 2 and 3, while on day 17 and 20 they decrease in number and are mainly found in zone 3. They are generally associated with unremodeled spiral arteries not yet surrounded by trophoblast cells. On days 17 and 20, the percentage of ANK-61-positive tissue was significantly lower in ATP-infused animals ($p < 0.05$, Fig. 5A and Supplemental Fig. S2).

Total macrophages: At all time points, CD68-positive macrophages in the mesometrial triangle were located throughout the interstitium and around the spiral arteries, with no apparent relation between their presence and the state of remodeling of the arteries. No changes in the location of CD68-positive macrophages or on the amount of CD68-positive tissue were observed after ATP infusion (Fig. 5B and Supplemental Fig. S3).

iNOS-positive macrophages: In control and ATP-infused rats, iNOS-positive cells were located in the proximity of remodeled spiral arteries in zone 1 and 2 but were also found throughout the mesometrial triangle and around the maternal channel. Only on day 20 of pregnancy, lower percentages of iNOS-positive tissue were found in ATP-infused rats as compared with control rats ($p < 0.05$; Fig. 5C and Supplemental Fig. S3).

CD206-positive macrophages: In all groups of rats, few CD206-positive cells were found. They were mainly located in the proximity of the arteries located in zone 3, around the NK cell cuff and sometimes in between the NK cells. Only on day 20 of pregnancy, percentages of CD206-positive tissue were significantly lower in ATP-infused rats as compared with control rats ($p < 0.05$; Fig. 5D and Supplemental Fig. S3).

Activated macrophages: TRAP-positive macrophages were typically located in zone 3, around the spiral arteries (independent of

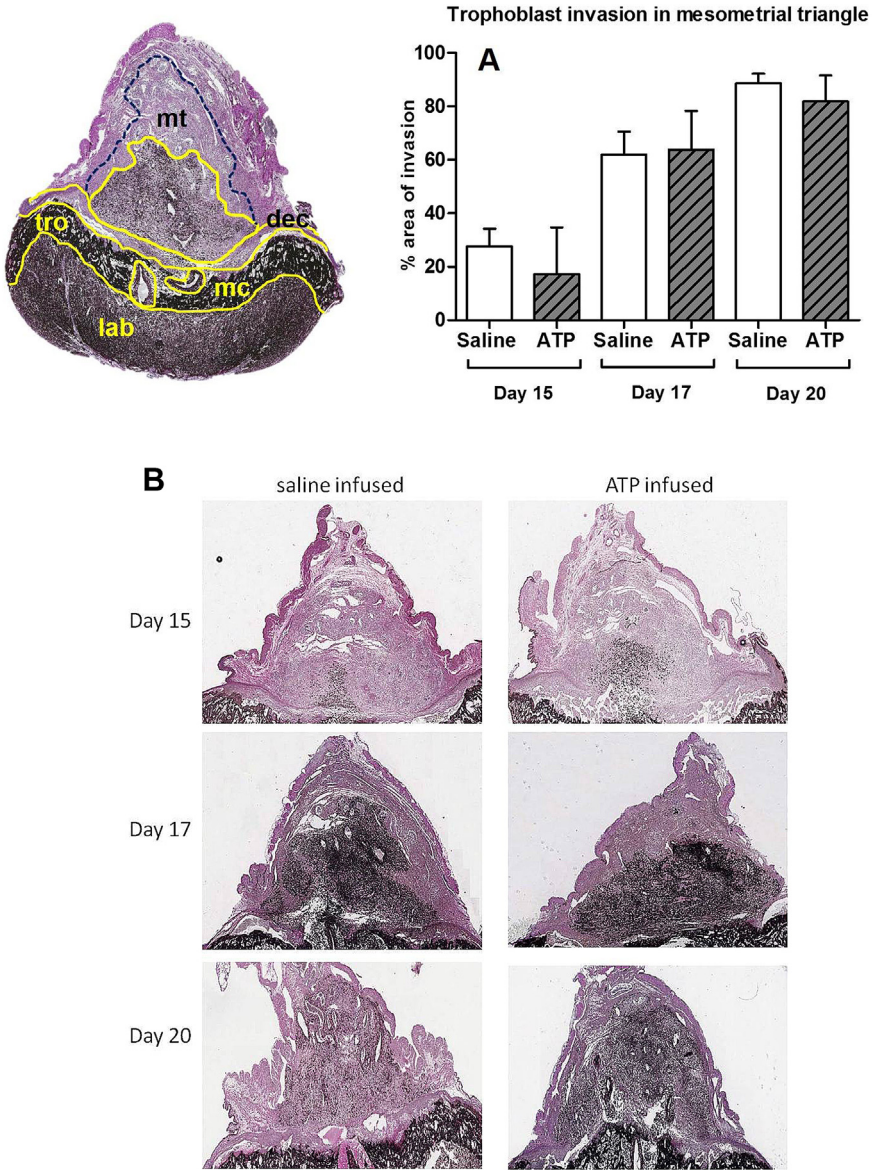

Fig. 2. Trophoblast invasion area in the mesometrial triangle. A: Left: mesometrial triangle of a pregnant saline-infused rat on day 17, showing mesometrial triangle (mt; blue dashed line) and surface of trophoblast invasion (yellow line in mt). (dec: decidua; mc: maternal channel; lab: labyrinth; tro: trophospongium). Right: Percentage (median with interquartile range) of cytokeratin-positive surface area on day 15, 17 and 20 of pregnancy in ATP (black bars) and saline (open bars) infused animals. B: Cross sections of representative mesometrial triangles of pregnant rats infused with saline (left photographs) or ATP (right photographs) on days 15 (top photographs), 17 (middle photographs) and 20 (bottom photographs) of pregnancy stained for cytokeratin (black staining).

arterial remodeling status), but were also present throughout the mesometrial triangle in both control and ATP-infused rats. On day 15 of pregnancy, higher percentages of TRAP-positive tissue were found in the mesometrial triangle of ATP-infused compared to control animals ($p < 0.05$; Fig. 5E), while on day 17 of pregnancy, ATP-infused animals displayed significantly lower percentages of TRAP-positive tissue ($p < 0.05$; Fig. 5E and Supplemental Fig. S3).

3.5. ATP decreased IL-33 and TNF-α expression

Expression of TGF-β, IL-6, IL-10 and IFN-γ in the mesometrial triangle was unchanged after ATP infusion compared to control animals at all time points (Fig. 6A, C, D and F). No differences in IL-33 and TNF-α mRNA expression were found on day 15 and 17 of pregnancy. However, on day 20 of pregnancy, IL-33 and TNF-α mRNA expression in the mesometrial triangle showed a trend towards lower mRNA expression in ATP-infused animals compared to control animals ($p < 0.1$; Fig. 6B+E). Expression of IL-4 or IL-17 was absent at all time points in all groups (data not shown).

4. Discussion

Our current findings demonstrated ATP-induced changes in trophoblast invasion, spiral artery remodeling and macrophage and NK cell numbers in the mesometrial triangle of pregnant rats. On day 17 of pregnancy interstitial trophoblast cell invasion was less deep and spiral artery remodeling was decreased after ATP infusion compared to control animals. These ATP-induced modifications were associated with higher percentages of TRAP-positive activated macrophages on day 15, lower percentages of NK cells on day 17 and 20, and lower percentages of iNOS-positive and CD206-positive macrophages on day 20 of pregnancy. Also, lower IL-33

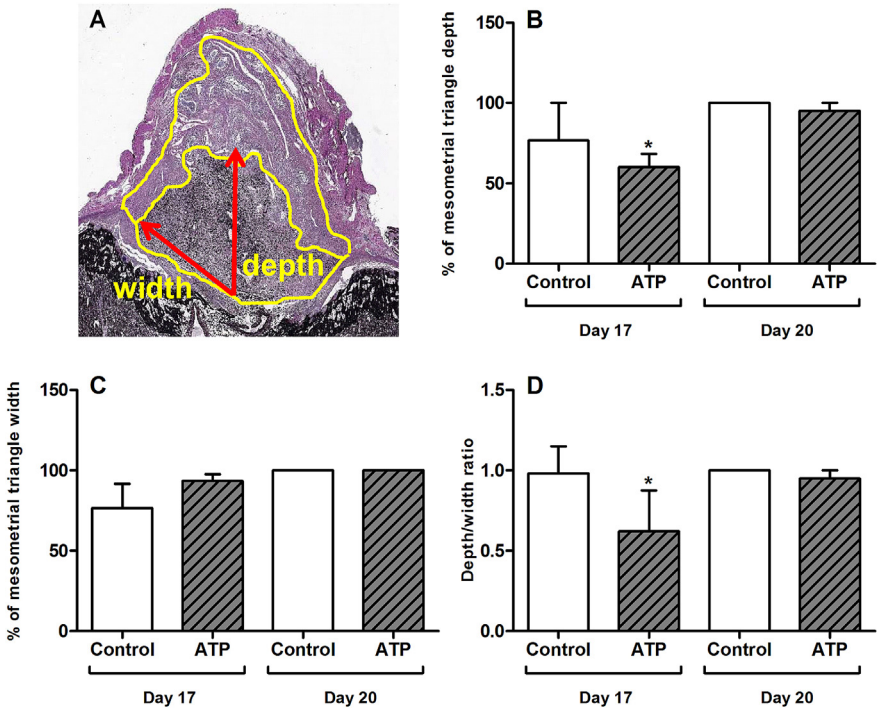

Fig. 3. Trophoblast invasion width and depth in the mesometrial triangle. A: Mesometrial triangle stained for cytokeratin, showing trophoblast invasion depth and width. Yellow delineation in mesometrial triangle shows the surface area invaded by trophoblast cells, red arrows demonstrate the directions of the depth and width measurements. B–D: the trophoblast invasion depth (B), width (C) and depth/width ratio (D) in control (open bars) and ATP-infused pregnant rats (black bars) on days 17 and 20 (medians with interquartile range). Trophoblast invasion pattern could not be analyzed on day 15 of pregnancy, since invasion was too shallow at this time-point. *: significantly decreased in ATP-infused rats compared with saline-infused rats at the same day, Mann Whitney *U* test, $p < 0.05$.

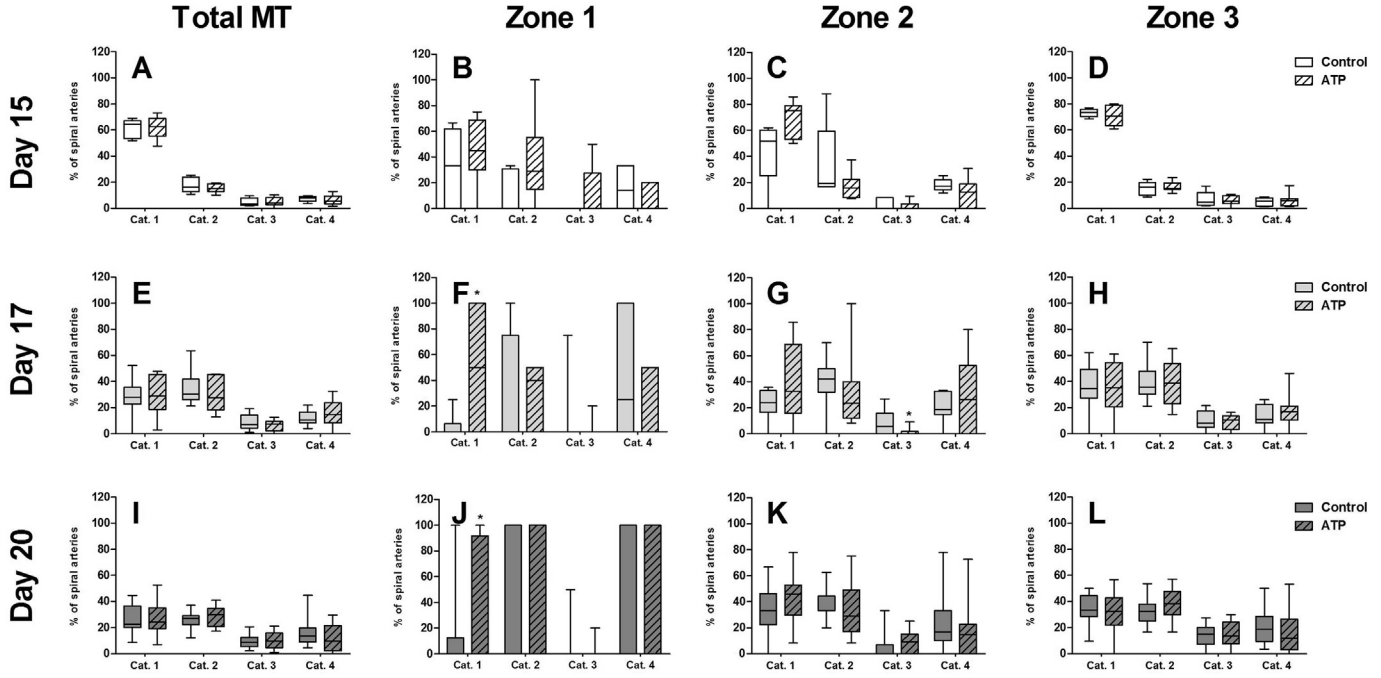

Fig. 4. Spiral artery remodeling in the mesometrial triangle. Spiral artery remodeling (median with minimum and maximum) on days 15A-D), 17 E-H) or 20, I-L) of pregnancy, in the total area of the mesometrial triangle (A + E + I), or in zone 1 (B + F + J), zone 2 (C + G + K) or zone 3 (D + H + L). of control pregnant rats (open bars) or ATP-infused pregnant rats (striped bars). Spiral arteries were scored in four categories of 0–3% (Cat.1), 3–33% (Cat.2), 33–67% (Cat.3) or 67–100% (Cat.4) disappearance of α-SMA staining. *: significantly different in ATP-infused rats compared with saline-infused rats at the same day, Mann Whitney *U* test, $p < 0.05$.

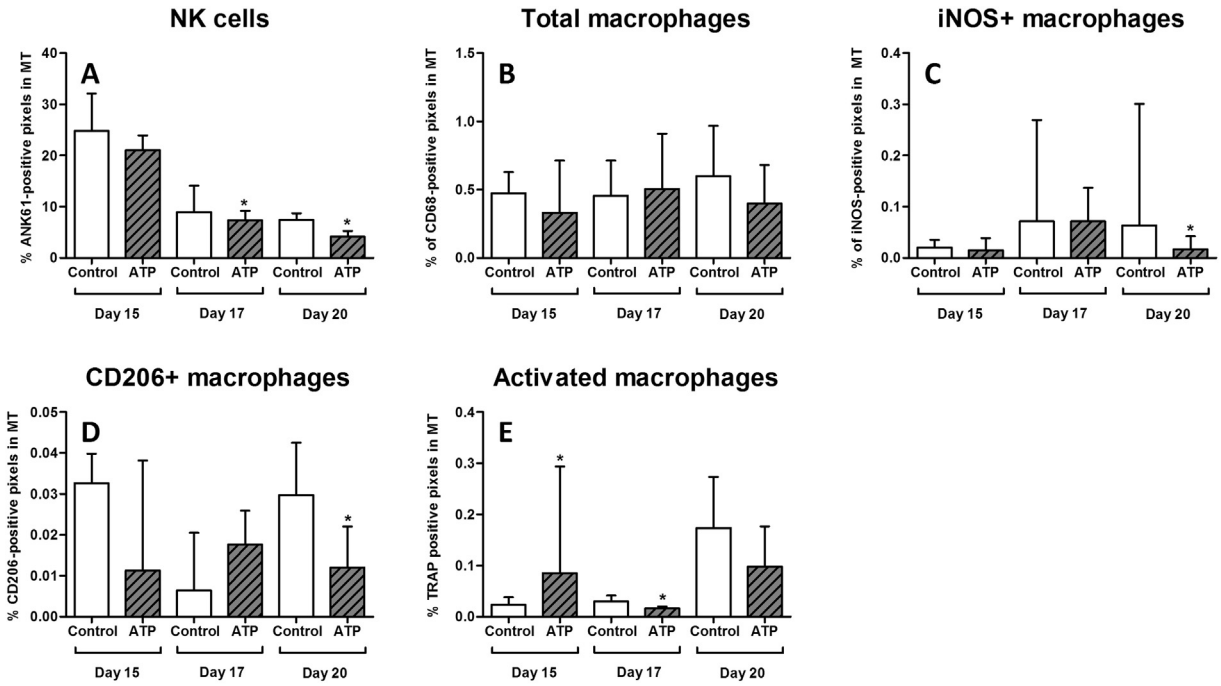

Fig. 5. NK cells and macrophage subpopulations in the mesometrial triangle. Percentages (medians with interquartile range) of ANK-61-positive (A, NK cells), CD68-positive (B, macrophages), iNOS-positive (C, M1 macrophages), CD206-positive (D, M2 macrophages) and TRAP-positive (E, activated macrophages) area on day 15, 17 and 20 of pregnancy in ATP (black bars) and saline (open bars) infused animals. *: significantly different in ATP-infused rats compared with saline-infused rats at the same day, Mann Whitney *U* test, $p < 0.05$.

and TNF-α expression in the mesometrial triangle on day 20 of pregnancy in ATP-infused versus control rats was observed.

ATP, as a danger signal, is released by stressed or damaged cells and binds to purinergic receptors that are expressed on many cell types, such as trophoblast cells [18,22]. ATP induced an aberrant pattern of trophoblast invasion in the mesometrial triangle on day 17 of pregnancy. This was the consequence of a decreased depth of interstitial trophoblast invasion. Interestingly, this decreased

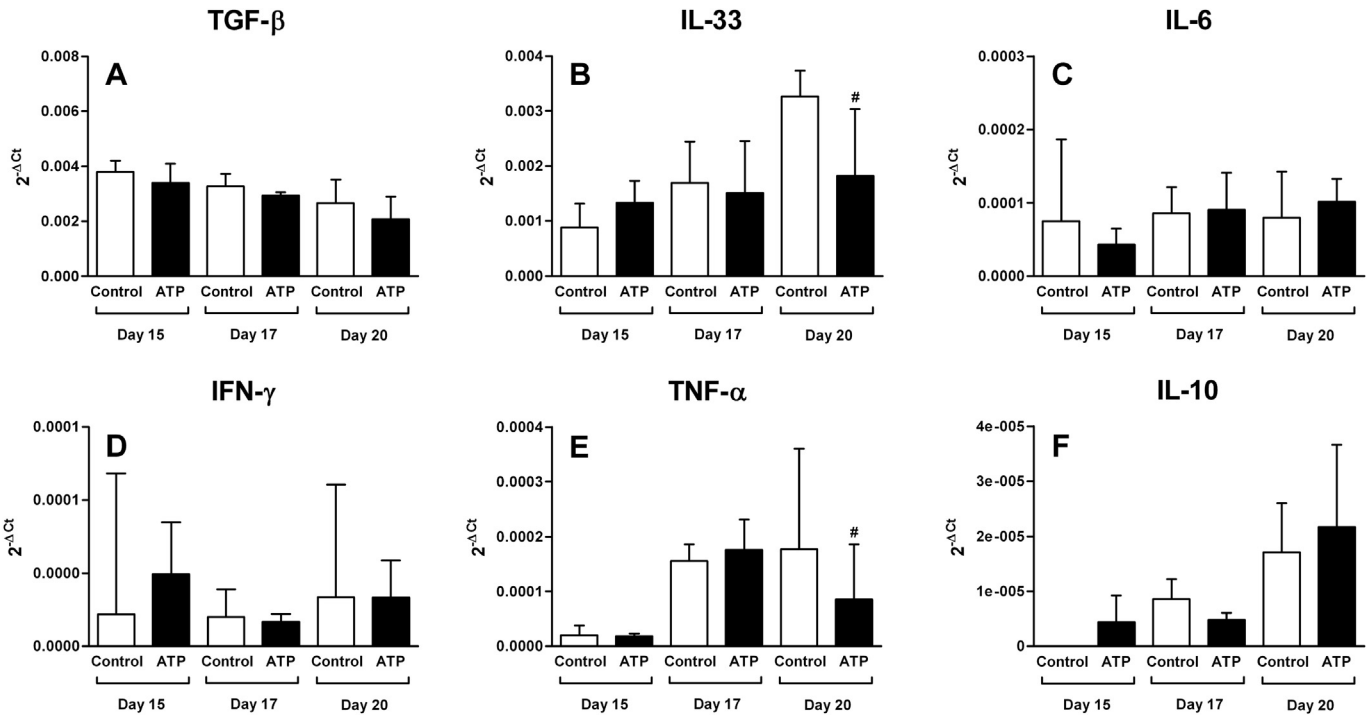

Fig. 6. Cytokine mRNA expression in the mesometrial triangle. mRNA expression (medians with interquartile range) of TGF-β (A), IL-33 (B), IL-6 (C), IFN-γ (D), TNF-α (E) and IL-10 (F) on days 15, 17 and 20 of pregnancy in ATP (black bars) and saline (open bars) infused animals. #: trend towards a decrease in ATP-infused rats compared with saline-infused rats at the same day, Mann Whitney *U* test, $p < 0.1$.

interstitial trophoblast invasion was not observed on day 20. The reason for this is unknown from the present study. It is however, tempting to speculate that after day 17, there is a compensational increase in trophoblast invasion, resulting in normal trophoblast invasion on day 20.

ATP also decreased spiral artery remodeling at days 17 and 20. As we have previously shown that ATP infusion induced a generalized maternal inflammatory response [23], our data appear to be in line with a recent study by Cotechini et al., who showed that induction of abnormal mild maternal inflammation by LPS resulted in decreased interstitial trophoblast invasion and spiral artery remodeling [24]. It may be speculated, therefore, that any compound that induces mild inflammation in pregnancy may affect trophoblast invasion. However, in the present study, reduced trophoblast invasion and spiral artery remodeling may also be a direct effect of ATP on trophoblast cell function. ATP can bind to their purinergic receptors, which has been shown to activate these cells in vitro [22,25]. As in preeclampsia decreased trophoblast invasion and decreased percentages of remodeled spiral arteries have been found [26], our study suggests that increased ATP levels may contribute to aberrant placentation in preeclampsia. The source of ATP during early placentation in preeclamptic women remains to be established. However, potential sources are activated immune cells [27] and endothelial cells [28], either in the peripheral circulation or locally at the implantation site [29]. Further studies are needed to evaluate the expression of purinergic receptors on (rat) trophoblasts and to elucidate a putative direct ATP effect on the trophoblast cells' invasive and remodeling capacity.

NK cells and macrophages play an important role in trophoblast invasion and spiral artery remodeling [30]. Since these cells also express purinergic receptors [18], ATP could also decrease trophoblast invasion via these cells. We used TRAP as a measure for activated, pro-inflammatory macrophages [31] as TRAP-positive macrophages have been shown to produce more reactive oxygen species [32,33]. We observed higher numbers of TRAP-positive macrophages on day 15 and lower numbers on day 17 of pregnancy in ATP-infused animals. Since the total number of macrophages did not increase in the mesometrial triangle after ATP infusion, this may suggest that ATP activates macrophages already present in the mesometrial triangle. Although the exact mechanism remains elusive from the present study, the localization of TRAP-positive cells around spiral arteries suggests that the increased percentage of TRAP-positive macrophages affect trophoblast invasion and spiral artery remodeling after ATP infusion. Although TRAP-positive cells were found in the human placenta [34], there are no reports on TRAP-positive macrophages in the placental bed.

The decreased trophoblast invasion and spiral artery remodeling may also be related to the decreased numbers of NK cells. We observed lower numbers of NK cells in the mesometrial triangle of rats infused with ATP on day 17 and day 20 of pregnancy. We mainly found NK cells in "cuffs" around unremodelled spiral arteries. This location is in line with a role for NK cells in spiral artery remodeling. Indeed, recent data from Chakrabotry et al., showed a pertinent role for NK cells in spiral artery remodeling in the rat [35]. However, it has been suggested that trophoblast and NK cells closely collaborate and influence each other in the remodeling of the spiral arteries [36]. Therefore, in the present study infusion of ATP may have affected either NK cells or interstitial trophoblast cells, which then may have affected each other resulting in decreased NK cell numbers and decreased trophoblast invasion as well as in decreased spiral artery remodeling.

Whether the decreased NK cell numbers are a direct or indirect effect of the ATP infusion thus remains to be established. A direct effect may be suggested, since ATP is able to decrease NK cell proliferation [37] and induce apoptosis or cell death in various cell types [38,39]. Also the activated macrophages on day 15 may have contributed to lower NK cell numbers on day 17 and 20, by for instance the release of ATP [40], or other factors [41] or mechanisms. In the present study, we only assessed NK cell numbers. Next to affecting the number of NK cells, it may also be possible that ATP-induced functional changes of NK cells, which may also play a role in the defective trophoblast invasion. This suggestion is in line with the finding that NK cells surrounding impaired remodeled vessels were functionally different compared with NK cells surrounding normal remodeled arteries in the first trimester of human pregnancy [42]. Although in preeclamptic placentae, the numbers of decidual NK cells were decreased as compared with normal pregnancy [11,43], it is uncertain whether NK cells play a role in the pathophysiology of preeclampsia, since a recent study showed no differences in NK cell numbers in early decidua (week 10–12) in women who later developed pregnancy-induced hypertension [44].

We used iNOS and CD206 antibodies as markers for M1 and M2 macrophages, respectively [45]. Throughout normal pregnancy in the rat, we found both subsets to be present in the mesometrial triangle. This is in line with human data in which it was also shown that in the human placental bed two subtypes of macrophages are present: one subset appeared to be more pro-inflammatory, while the other cell type appeared to have a more regulatory phenotype [46]. Our data suggest that defective trophoblast invasion per se decreased CD206 and iNOS-positive macrophages rather than the other way around, as in the ATP-infused rat the decreased numbers of CD206-positive and iNOS-positive cells followed decreased trophoblast invasion and spiral artery remodeling rather than preceding it. Alternatively, increased ATP levels may also have induced the decreased numbers of CD206 and iNOS-positive macrophages on day 20, as we have previously shown that in the ATP-infused rats ATP levels are increased at day 20 of pregnancy [19]. Our data are in line with data reported on human preeclampsia, in which lower macrophage numbers have been found in the decidua [11]. This also coincides with increased plasma ATP levels in these patients [17]. As increased decidual macrophage numbers have been associated with pre-term labor both in humans and rats, suggesting a role for decidual macrophages in labor initiation [47], our data suggest that ATP in our pregnant rats, by decreasing macrophage numbers, may delay parturition.

We measured the expression of various cytokines, which are known to be expressed in the decidua and involved in trophoblast invasion and spiral artery remodeling [48]. Two cytokines, i.e. IL-33 and TNF-α, appeared to be decreased in ATP-infused rats compared to control rats. These cytokines have opposing functions: while IL-33 is a danger signal with anti-inflammatory properties, stimulating Th2 type immune responses [49], TNF-α has pro-inflammatory properties and stimulates Th1 responses [50]. Both cytokines have been shown to be involved in early placentation [51,52]. However, the higher levels of these cytokines in the mesometrial triangle at the end of pregnancy in healthy rats, also suggests a role for these cytokines in parturition in the rat, which in healthy rats takes place in the night between day 21 and day 22. If so, the lower IL-33 and TNF-α expression in ATP-infused rats may suggest that parturition will be delayed in ATP-infused rats. This is subject of further study. The IL-33 and TNF-α may be produced by decidual macrophages [51,53] and via producing these cytokines macrophages may induce parturition [47] (see above). Indeed, it was shown that increased levels of TNF-α can contribute to LPS-induced pre-term labor [54].

ATP infusion led to decreased trophoblast invasion and spiral artery remodeling, while at the same time higher numbers of activated macrophages and lower numbers of NK cells were observed in the mesometrial triangle of the ATP-infused rat. This

supports our hypothesis that ATP decreased trophoblast invasion and spiral artery remodeling, and that activated macrophages and lower NK cell numbers may play a role in this.

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Conflict of interest statement

We have no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2014.05.013.

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