Extracellular Adenosine Triphosphate Affects Systemic and Kidney Immune Cell Populations in Pregnant Rats

Floor Spaans[1], Barbro N. Melgert[1,2], Theo Borghuis[3], Pieter A. Klok[3], Paul de Vos[1], Winston W. Bakker[3,†], Harry van Goor[3], Marijke M. Faas[1]

[1]Division of Medical Biology, Department of Pathology and Medical Biology, University Medical Center Groningen and University of Groningen, Groningen, The Netherlands;
[2]Department of Pharmacokinetics, Toxicology and Targeting, University of Groningen, Groningen, The Netherlands;
[3]Division of Pathology, Department of Pathology and Medical Biology, University Medical Center Groningen and University of Groningen, Groningen, The Netherlands

Keywords
Extracellular ATP, kidney, leukocytes, preeclampsia, rat model

Correspondence
Floor Spaans, Division of Medical Biology, Department of Pathology and Medical Biology, University Medical Center Groningen, Hanzeplein 1, EA 11, 9713 GZ Groningen, The Netherlands.
E-mail: f.spaans@umcg.nl

†Deceased

Submission December 20, 2013;
accepted April 9, 2014.

Citation
Spaans F, Melgert BN, Borghuis T, Klok PA, de Vos P, Bakker WW, van Goor H, Faas MM. Extracellular adenosine triphosphate affects systemic and kidney immune cell populations in pregnant rats. Am J Reprod Immunol 2014; 72: 305–316

doi:10.1111/aji.12267

Problem
Changes in the systemic immune response are found in preeclampsia. This may be related to high extracellular adenosine triphosphate (ATP) levels. The question arose whether ATP could affect immune responses in pregnancy. Previously, we investigated whether ATP affected monocyte activation and subpopulations. Here, we investigated ATP-induced changes in other immune cell populations in pregnant rats, systemically and in the kidney, an affected organ in preeclampsia.

Method of study
Using flow cytometry or immunohistochemistry, blood and kidney leukocytes were studied in pregnant and non-pregnant rats at different intervals after ATP or saline infusion.

Results
Adenosine triphosphate (ATP) infusion induced increased peripheral blood non-classical monocytes and decreased T lymphocyte subsets in pregnant rats only, higher glomerular macrophage and T lymphocyte numbers in non-pregnant animals 1 day after infusion, and higher glomerular macrophage numbers in pregnant rats 6 days after infusion.

Conclusion
Adenosine triphosphate (ATP) infusion in pregnant rats induced a pregnancy-specific inflammatory response. Increased ATP levels could potentially contribute to development of the inflammatory response of preeclampsia.

Introduction

Preeclampsia is a major pregnancy complication characterized by hypertension and proteinuria in the second half of pregnancy. It affects 3–8% of the pregnancies.[1] The exact pathophysiology of preeclampsia remains unknown, but abnormal trophoblast invasion into the maternal spiral arteries in the first trimester is thought to be important in its pathogenesis.[2] This may give rise to placental ischemia and to the release of various factors, such as cytokines, sFlt-1, microparticles, or fetal DNA into the peripheral circulation in the second half of pregnancy.[2,3] This process eventually results in preeclampsia.[2,3] To date, the only cure for preeclampsia is delivery of the fetus and specifically of the placenta. Although the symptoms abate after delivery, long-term maternal effects of preeclampsia have

become accepted to be a major threat, as women with previous preeclampsia are more prone to develop cardiovascular or renal diseases later in life.[4,5]

The immune system plays an important role in the pathophysiology of preeclampsia. Many changes in the immune responses of preeclamptic women, both systemically and locally, have been observed.[6] In women with preeclampsia, the systemic innate immune system, that is, monocytes, granulocytes, and NK cells, is activated,[7,8] while various studies also observed a shift from a Th2-type immune response toward a Th1-type immune response and lower regulatory T lymphocyte numbers.[8–10] How these changes in maternal immune responses are induced remains to be determined, but circulating plasma factors produced by the ischemic placenta have been implicated.[11]

Adenosine triphosphate (ATP), mainly known as an intracellular energy source, can also be released from the cell upon cellular activation, stress, damage, or necrosis.[12] Extracellular ATP functions as a pro-inflammatory danger-associated molecular pattern (DAMP) molecule and can activate immune responses.[13] It is produced by activated and hypoxic cells. In healthy individuals, plasma ATP levels vary between 400 and 700 nM;[12] however, in various diseases, for instance preeclampsia, ATP levels are about five times higher.[14] The effects of ATP are conducted via their purinergic P2 receptors, which are widely expressed on immune cells,[13] the placenta[15], and other tissues.[16] Various studies have shown that ATP activates monocytes,[17,18] neutrophils[19], and T lymphocytes[20] and, as a chemotactic signal, stimulates recruitment of neutrophils and macrophages.[21,22]

We have previously shown that a single infusion of ATP in pregnant rats disturbs pregnancy, by inducing proteinuria, monocyte activation, and placental ischemia.[18,23] Such effects were not observed in non-pregnant rats, treated in a similar fashion.[18] In the present study, we hypothesized that ATP would also induce a pregnancy-specific response in granulocytes and T lymphocytes. In addition, as we have also shown proteinuria after ATP infusion specifically in pregnant rats,[23] we hypothesized that a pregnancy-specific inflammatory response will also be present in the kidney. Hence, in the present study, we investigated the effects of extracellular ATP on various immune cell populations in pregnant and non-pregnant rats in the peripheral circulation and in the kidney. We evaluated systemic granulocytes and lymphocyte subsets and activation at various intervals after ATP infusion. We also investigated glomerular leukocyte populations on days 15, 17, and 20 of pregnancy.

Materials and methods

Animals

The Institutional Animal Care and Use Committee of the University of Groningen approved all animal experiments. Female Wistar outbred rats (about 200 g) were kept in a temperature and light-controlled room (lights on from 7:30 AM till 7:30 PM) with free access to food and water. Until selection for experiments, vaginal smears were taken daily, and rats were rendered pregnant by housing them on pro-estrus with fertile males for one night.

When spermatozoa were detected in the smear the next day, this day was designated as day 0 of pregnancy. In both pregnant (on day 0 or 1 of pregnancy) and non-pregnant rats, a cannula was inserted into the right jugular vein under isoflurane/oxygen anesthesia for stress-free blood sampling and infusion of ATP or saline.[24]

Experimental Design

Pregnant rats were infused with 3000 μg/kg bw ATP in 2.0 mL saline ($n = 9$) or with 2.0 mL saline alone ($n = 7$) on day 14 of pregnancy.[24] Non-pregnant rats were infused with 3000 μg/kg bw ATP in 2.0 mL saline ($n = 6$) or saline alone ($n = 5$) on di-estrus. Ethylenediaminetetraacetic acid (EDTA) blood samples (0.4 mL) were taken from the jugular vein cannula on day 13 (1 day before), day 15 (1 day after), and day 17 (3 days after the infusion of ATP or saline) of pregnancy. On day 20, rats were killed and blood samples were taken during killing by aortic punction under anesthesia (isoflurane/oxygen). To study inflammation in the kidney at days 15 and 17, four additional groups of non-pregnant (NP) and pregnant (P) rats were infused with either saline or ATP and killed on day 15 (NP+saline: $n = 6$; NP+ATP: $n = 5$; P+saline: $n = 5$; P+ATP: $n = 7$) or day 17 (NP+saline: $n = 5$; NP+ATP: $n = 5$; P+saline: $n = 7$; P+ATP: $n = 6$). During killing on days 15, 17, and 20, kidneys were obtained and snap frozen for analysis of immune cells in the kidney using immunohistochemistry.

American Journal of Reproductive Immunology 72 (2014) 305–316

Rat Blood Leukocyte Counts

Twenty microliter of EDTA blood was diluted in 500 μL pOCH buffer (Sysmex Netherlands, Etten-Leur, the Netherlands) for leukocyte counts using a microcell counter (model Sysmex PocH 100i; Sysmex).

Staining of Rat Blood for Flow Cytometry

To identify monocytes and granulocytes, whole blood was washed with phosphate-buffered saline (PBS, pH = 7.2) and incubated with PE-conjugated anti-CD172a (Biolegend, Uithoorn, the Netherlands), AlexaFluor647-conjugated anti-CD43 (Biolegend), and Alexa Fluor 700-conjugated anti-CD11b (for granulocytes; AbD Serotec, Dusseldorf, Germany) antibodies or with isotype controls for 30 min in the dark.[18,25]

To identify lymphocyte subpopulations, whole blood was washed with PBS and incubated with biotin-conjugated anti-CD3 (eBioscience, Vienna, Austria), FITC-conjugated anti-CD4 (BD Biosciences, Breda, the Netherlands) and PE-conjugated anti-CD25 (BD Biosciences) for 30 min in the dark. Samples were washed with 0.5 mL fluorescence-activated cell sorter (FACS) buffer (PBS with 2% fetal calf serum and 1.3 mM EDTA) and incubated with PE/Cy7-labeled streptavidin for 15 min in the dark. Thereafter, samples were washed twice and incubated with BD FACS™ Lysing Solution (BD Biosciences) for 30 min. Then, the samples were washed with permeabilization buffer (Foxp3 staining kit; eBioscience) and incubated in permeabilization buffer with APC-conjugated anti-Foxp3 (eBioscience) or APC-conjugated isotype control antibodies (eBioscience). Cells were subsequently washed twice with permeabilization buffer, resuspended in FACS buffer, and kept in the dark at 4°C until FACS analysis (within 24 hr). All steps were performed at room temperature.

Flow Cytometry Data Collection and Evaluation

Data were collected (at least 200,000 events per sample) on a BD LSR II Flow Cytometer (BD Biosciences) and were analyzed using FlowJo software (Tree star, Inc., Ashland, OR, USA).

Peripheral blood monocyte subsets and granulocytes

Live leukocytes were selected from the forward/side scatter plot (Fig. 1a). From the leukocytes, a wide gate was drawn on the monocytes (rat monocytes are often not a distinct population) and copied to a CD43/CD172a plot (Fig. 1b,c). Here, the total $CD172a^+$ monocytes were selected, as well as the granulocyte population (Fig. 1c). The total monocyte population was copied to a new plot, in which the classical CD43lo and non-classical CD43hi monocyte subsets were selected (Fig. 1d). To analyze CD11b expression on granulocytes, samples stained with isotype controls were used to set a gate, so that each fluorescent label contained no more than 1% of the population. This gate was subsequently copied to the CD11b-stained samples, after which the mean fluorescent intensity (MFI) of CD11b expression on granulocytes was calculated.

Lymphocytes

Live leukocytes were selected from the forward/side scatter plot (Fig. 1e) and the $CD3^+$ population was selected, from which the $CD4^+$ and $CD4^-$ populations were selected in a CD4/CD3 plot (Fig. 1f,g). The $CD4^+$ population was copied in a CD25/Foxp3 plot, in which the $CD25^+$ $Foxp3^+$ (regulatory T lymphocyte) population and the $CD25^+$ $Foxp3^-$ (effector T lymphocyte) population were selected (Fig. 1h). Data were expressed as the percentage within the whole live leukocyte population (% of leukocytes).

Immunohistochemical Staining of Kidney Tissue

Rat cryostat kidney sections were stained for the presence of total macrophages (mouse-anti-rat CD68, 1:100 diluted, clone ED1; AbD Serotec), $iNOS^+$ macrophages (rabbit-anti-rat iNOS, 1:2000 diluted; Abcam, Cambridge, UK), $CD206^+$ macrophages (rabbit-anti-rat CD206, 1:1000 diluted; Abcam,), granulocytes (mouse-anti-rat His48, 1:50 diluted; BD Biosciences), and T lymphocytes (mouse-anti-rat CD3, 1:100 diluted; Abcam). All sections were fixed with ice-cold acetone (10 min). Only the iNOS stained sections were pre-incubated with a mixture of 0.5% saponin, 4% bovine serum albumin (BSA), 1% normal goat serum, and 10% normal rat serum in PBS (30 min) and washed with PBS afterward. Before incubation with primary antibodies [with 1% normal rat serum (60 min)], sections for CD68 were incubated with 10% normal goat serum (30 min) and sections for CD206, iNOS, His48, and CD3 with 2% BSA with 1% ELK in PBS (20 min). After washing with PBS, sections were blocked with 3% H_2O_2 in methanol and with a biotin blocking kit (Dako, Heverlee, Belgium). After washing with PBS, biotin-conjugated

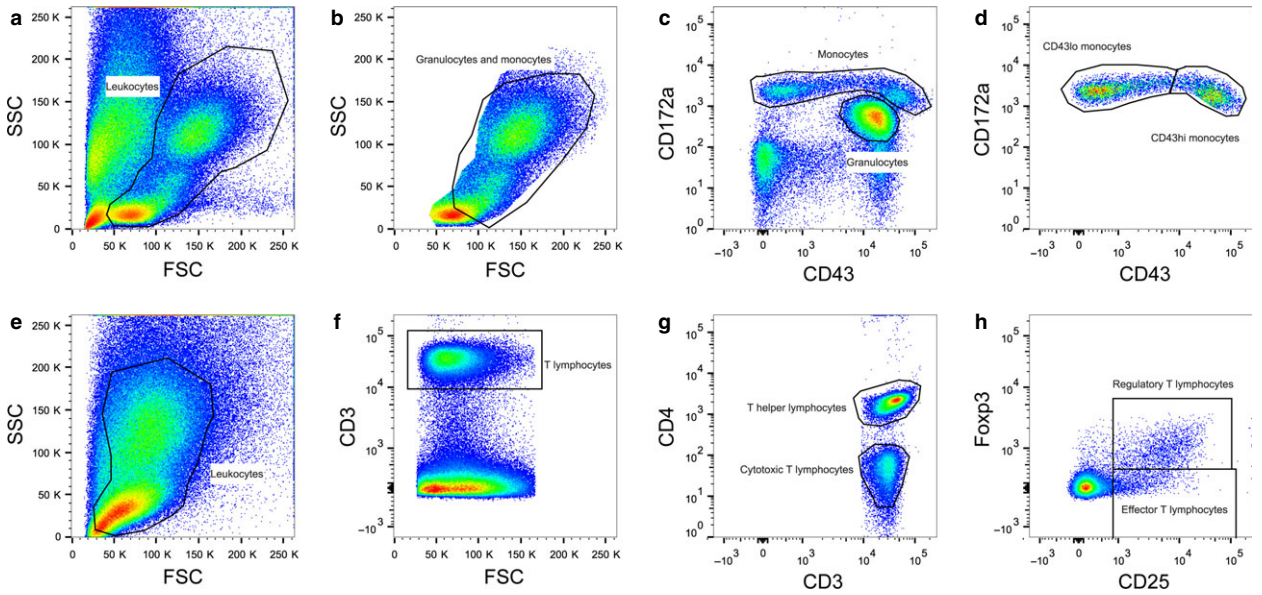

Fig. 1 Flow cytometric gating strategy for peripheral blood leukocyte populations. Flow cytometric staining of whole blood from a representative saline-infused pregnant rat. For the monocytes and granulocytes, leukocytes were selected first in a FSC/SSC plot (a), and after that, the monocytes and granulocytes were selected in a new FSC/SSC plot (b). This gate was copied to a CD43/CD172a plot and granulocytes and total monocytes were selected (c). Finally, the total monocytes were copied to a new CD43/CD172a plot and the two monocyte subsets ($CD172a^+/CD43^{low}$ and $CD172a^+/CD43^{high}$) were selected (d). For the lymphocytes, leukocytes were selected from the FSC/SSC plot (e) and copied to a CD3/FSC plot in which the $CD3^+$ lymphocytes (f) were selected. These were then copied to a CD4/CD3 plot where the $CD3^+$ CD4− cytotoxic T lymphocytes and $CD3^+$ $CD4^+$ T helper lymphocytes were selected (g). The latter were copied to a Foxp3/CD25 plot from which the $CD25^+$ Foxp3− effector T lymphocytes and the $CD25^+$ Foxp3+ regulatory T lymphocytes were selected (h).

goat-anti-mouse (for CD68, CD3 and His48; Southern Biotech, Birmingham, AL, USA) and biotin-conjugated goat-anti-rabbit (for iNOS and CD206; Dako) were added as a second step (30 min). After washing with PBS, peroxidase-conjugated streptavidin (Dako) was added (30 min) and the staining was subsequently visualized by 3-amino-9-ethyl-carbazole and hematoxylin. All of the incubation steps were carried out at room temperature.

Analysis of Kidney Sections

Kidney sections stained for total macrophages, $iNOS^+$ and $CD206^+$ macrophages, granulocytes, and lymphocytes were quantitatively scored in a double-blind manner by an independent observer. Positive cells were counted in all the glomeruli in the kidney section, and results are expressed as number of positive cells per glomerulus.

Statistical Analysis

The effect of pregnancy on leukocyte subsets and their activation status was evaluated by testing the differences between non-pregnant and pregnant saline-infused rats on the same experimental day using Mann–Whitney *U* tests. To evaluate the effect of ATP or saline infusion on peripheral blood leukocyte subsets and their activation status, Friedman repeated measures tests were performed followed by Dunn's post-tests comparing day 13 pre-infusion values with days 15, 17 or 20 post-infusion values.

To evaluate the effect of pregnancy on glomerular leukocyte populations, the saline-infused non-pregnant rats were compared with pregnant saline-infused rats using Mann–Whitney *U* tests. To evaluate the effect of ATP on leukocyte populations in the kidney, ATP and saline-infused rats were compared using Mann–Whitney *U* tests.

For all evaluations, differences were considered to be significant if $P < 0.05$ or a statistical trend if $P < 0.1$. Data are presented as medians with the interquartile range.

Results

No effects of ATP or saline infusion were found on leukocyte populations in non-pregnant rats.

Therefore, for clarity, data of the non-pregnant saline and ATP-infused rats are not shown in the tables and figures. Instead, the saline-infused non-pregnant group is depicted by a single bar showing the percentages on day 13 of the experiment (see Table II and Figs 2 and 3, indicated as NP).

No Effect of ATP on Peripheral Blood Leukocytes

No effects of pregnancy on total leukocyte counts were observed on days 13, 15, 17, or 20 of pregnancy (Table I). No effects of ATP infusion on blood leukocyte counts were observed in pregnant rats (Table I).

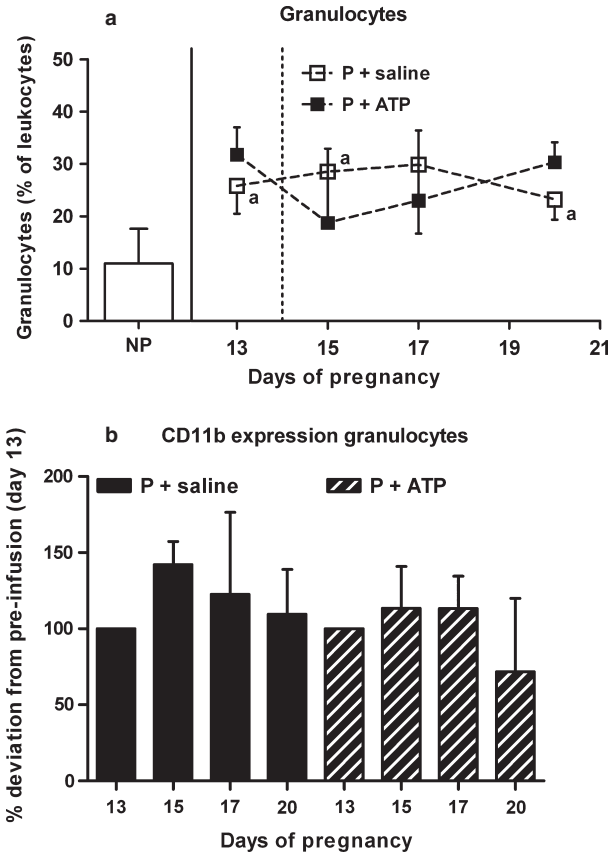

Fig. 2 Numbers of granulocytes after adenosine triphosphate (ATP) infusion in rat peripheral blood. (a) Circulating granulocytes in pregnant animals infused with saline (P+saline, open squares) or ATP (P+ATP, closed squares) on day 14 of pregnancy (dotted line) (a). Bars on the left side of the graph represent percentages for non-pregnant saline-infused animals on day 13 of pregnancy (NP). (b) CD11b expression on granulocytes in pregnant animals infused with saline (black solid bars) or ATP (striped bars) on day 14 of pregnancy. Medians with interquartile range are shown. $^{a}P < 0.05$ significantly different from non-pregnant saline-infused animals on the same experimental day, Mann–Whitney *U* test.

Peripheral Blood Non-classical Monocytes Increase After ATP Infusion in Pregnant Rats

To give a complete overview of the changes in immune cell populations after ATP infusion, we have included data here on monocyte subpopulations that have been published previously (see Table II).[18]

The total percentage of monocytes was higher in pregnant rats on days 15, 17, and 20 of pregnancy compared with non-pregnant rats on the same day (Table II). ATP did not affect total numbers of monocytes. In rats, monocyte subsets are identified by the markers CD43 and CD172a, rather than with CD14 and CD16.[25,26] Classical monocytes are defined as $CD172a^{+}$ and $CD43^{lo}$, while non-classical monocytes are defined as $CD172a^{+}$ and $CD43^{hi}$.[25,26] There was no effect of pregnancy or ATP infusion on the percentage of classical monocytes (Table II). However, the percentage of non-classical monocytes was higher on days 15, 17, and 20 in the pregnant rats compared with non-pregnant rats at the same time point (Table II). Furthermore, only ATP, but not saline infusion, enhanced the percentage of non-classical monocytes on days 17 and 20 of pregnancy compared with the pre-infusion day 13 value (Table II).

Peripheral Blood Granulocyte Numbers Increase during Pregnancy

Percentages of granulocytes were significantly higher in pregnant compared with non-pregnant rats on days 13, 15, and 20 (Fig. 2a). No changes in the percentages of granulocytes were observed compared with pre-infusion day 13 after both ATP and saline infusion in pregnant rats (Fig. 2a). No changes in CD11b expression were observed after saline or ATP infusion in pregnant animals (Fig. 2b).

Peripheral Blood Lymphocytes Decrease in Pregnancy and by ATP Infusion

In pregnant rats, percentages of $CD3^{+}$ T lymphocytes were lower on days 13 and 15 of pregnancy than in non-pregnant rats (Fig. 3a). ATP infusion during pregnancy decreased the percentage of T lymphocytes on day 20 of pregnancy as compared with the

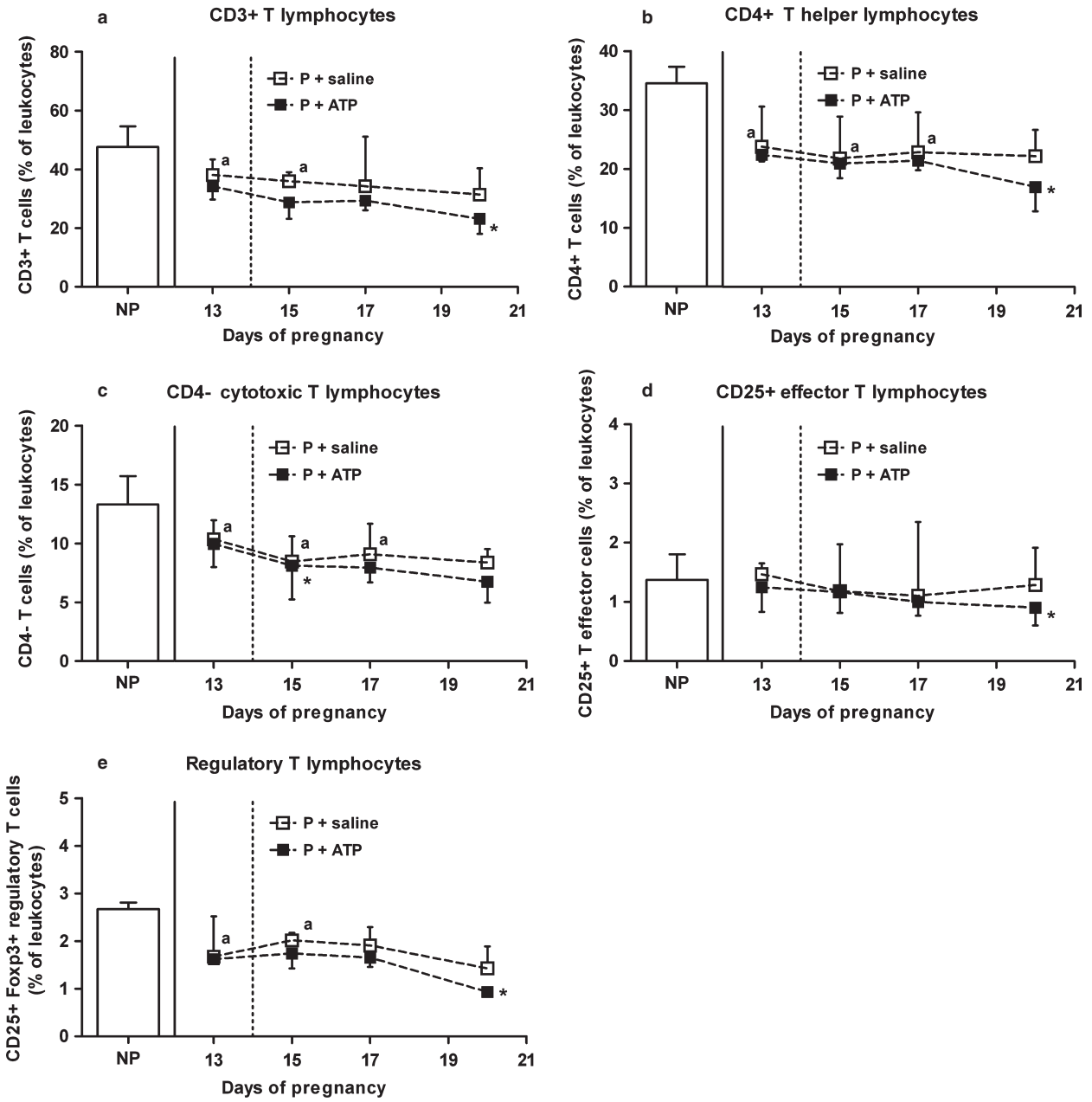

Fig. 3 Numbers of T lymphocytes after adenosine triphosphate (ATP) infusion in rat peripheral blood. Circulating $CD3^+$ (a), $CD4^+$ T helper (b), CD4– cytotoxic (c), $CD4^+$ $CD25^+$ T effector (d) and $CD4^+$ $CD25^+$ Foxp3+ T regulatory lymphocytes (e) in saline (P+saline, open squares) and ATP (P+ATP, closed squares) infused pregnant animals. Bars on the left side of the graph represent day 13 values of non-pregnant saline-infused animals (NP). Medians with interquartile range are shown. $^aP < 0.05$ significantly different from non-pregnant saline-infused animals on the same experimental day, Mann–Whitney *U* test; $*P < 0.05$ significantly decreased compared with pre-infusion (day 13) values in the same experimental group, Friedman followed by Dunns post-tests.

pre-infusion value, while no changes were observed in saline-infused pregnant animals (Fig. 3a).

In pregnant rats, both the percentages of T helper lymphocytes ($CD4^+$) and the percentage of cytotoxic T lymphocytes ($CD4^-$) were lower on days 13, 15, and 17 compared with non-pregnant rats (Fig. 3b,c). ATP infusion significantly reduced the percentage of T helper lymphocytes on day 20 (Fig. 3b) of

American Journal of Reproductive Immunology **72** (2014) 305–316

pregnancy compared with pre-infusion values. This was not observed in control pregnant rats. The percentage of cytotoxic T lymphocytes was decreased in ATP-infused rats on day 15 of pregnancy compared with pre-infusion values (Fig. 3c).

No changes in effector T lymphocytes ($CD4^+$ $CD25^+$ $Foxp3^-$ T lymphocytes) were observed in pregnant animals as compared to non-pregnant animals (Fig. 3d). However, a decreased percentage of effector T lymphocytes were found after ATP infusion on day 20 of pregnancy compared with the pre-infusion value, that is day 13 (Fig. 3d).

Percentages of regulatory T lymphocytes ($CD4^+$ $CD25^+$ $Foxp3^+$ T lymphocytes) were lower on days 13 and 15 in pregnant rats as compared to non-pregnant rats (Fig. 3e). In pregnant rats infused with ATP, lower percentages of regulatory T lymphocytes were observed on day 20 compared with pre-infusion values (Fig. 3e).

Table I Leukocyte Counts During Pregnancy and After Adenosine Triphosphate (ATP) Infusion

	Day of pregnancy	Leukocyte count ($\times 10^9$ cells/L)
Non-pregnant + saline	–	6.3 (4.1–9.2)
Pregnant + saline	13	7.2 (5.8–11)
	15	7.5 (5.8–9.5)
	17	7.4 (6–9.1)
	20	6 (3.7–7.7)
Pregnant + ATP	13	7.5 (5.4–9.7)
	15	8.1 (6.5–9.2)
	17	8.1 (6.5–10.1)
	20	6 (2.5–11)

Medians plus range are shown.

Effect of ATP on Leukocyte Populations in The Kidney

Macrophages

$CD68^+$ cells (total macrophages), $iNOS^+$ cells, and $CD206^+$ cells in the kidney were mainly located in the glomeruli and only occasionally in the interstitium at all time points. On days 15 and 20 of pregnancy, no differences in glomerular $CD68^+$, $iNOS^+$, and $CD206^+$ macrophages were observed between non-pregnant and pregnant rats (Fig. 4a–c). However, on day 17 of pregnancy, $iNOS^+$ cells tended to be higher and $CD206^+$ cells were significantly higher in pregnant as compared with non-pregnant animals (Fig. 4b,c), ATP infusion induced higher numbers of glomerular $CD68^+$ cells in non-pregnant rats on day 15, while in pregnant rats, ATP induced higher $CD68^+$ cell numbers on day 20 (Fig. 4a). ATP infusion had no effect on the number of $iNOS^+$ and $CD206^+$ cells in pregnant rats or in non-pregnant rats on days 15, 17, and 20 (Fig. 4b,c).

Granulocytes

On days 15, 17, and 20, $His48^+$ granulocytes in the kidney were located in the interstitial space as well

Table II Monocyte Subsets During Pregnancy and After Adenosine Triphosphate (ATP) Infusion

	Day of pregnancy	Total monocytes (% of leukocytes)	Classical monocytes (% of leukocytes)	Non-classical monocytes (% of leukocytes)
Non-pregnant + saline	–	5.5 (5.4–6.0)	4.1 (3.6–5.0)	1.7 (1.5–1.8)
Pregnant + saline	13	7.9 (5.8–10.1)	6.1 (4.3–6.9)	2.3 (2.0–2.5)
	15	9.4 (7.9–9.9)[a]	5.7 (4.9–6.6)	2.9 (2.8–3.9)[a]
	17	8.3 (7.0–8.5)[b]	2.1 (1.9–3.1)	2.5 (2.4–4.9)[b]
	20	9.5 (8.2–9.7)[a]	3.5 (2.9–4.3)	4.3 (3.9–4.9)[a]
Pregnant + ATP	13	8.3 (7.5–9.3)	6.1 (4.3–6.9)	2.3 (2.0–2.4)
	15	8.8 (8.2–9.6)	4.7 (4.1–5.3)	4.3 (3.7–4.3)
	17	7.2 (6.8–9.3)	3.1 (3.0–3.4)	4.4 (3.8–4.8)*
	20	9.9 (7.2–13.8)	3.7 (3.5–4.7)	6.2 (3.4–8.4)*

Medians plus range are shown.

[a]$P < 0.05$; [b]$P < 0.1$ significantly increased compared with non-pregnant saline-infused animals on the same experimental day, Mann–Whitney *U* test.

*$P < 0.05$ significantly increased compared with pre-infusion (day 13) values in the same experimental group, non-parametric Friedman followed by Dunns post-tests. This dataset has been previously published under a Creative Commons Attribution.[19]

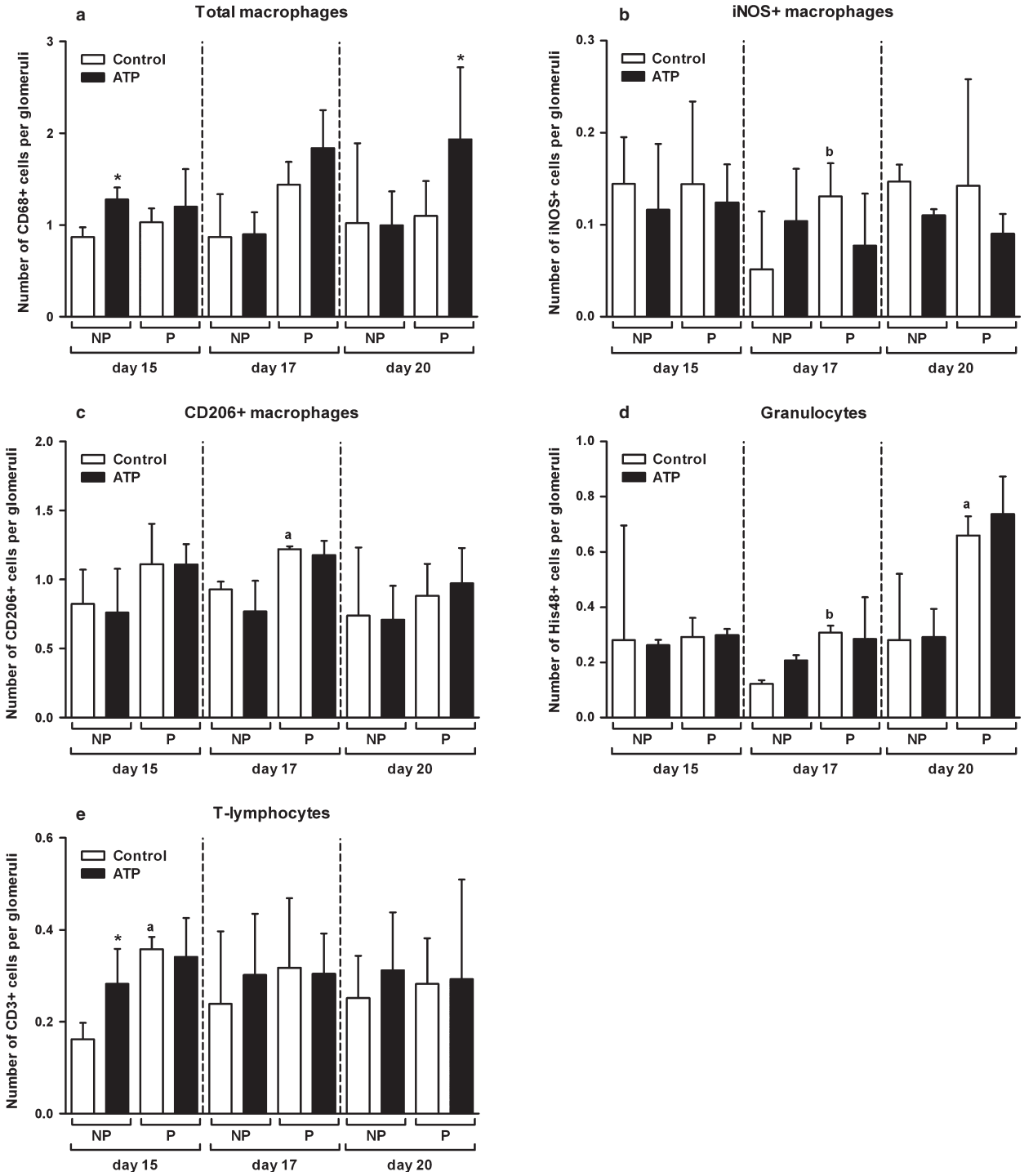

Fig. 4 Glomerular numbers of CD68, iNOS, CD206, His48 and CD3 positive cells. Glomerular numbers of (a) total macrophages (CD68), (b) iNOS$^+$ macrophages, (c) CD206$^+$ macrophages, (d) granulocytes (His48) and (e) T lymphocytes (CD3) on days 15, 17, or 20 of pregnancy, in pregnant (P) or non-pregnant (NP) animals infused with saline (open bars) or adenosine triphosphate (ATP) (black bars). Medians with interquartile range are shown. $^aP < 0.05$; $^bP < 0.10$ significantly different from non-pregnant saline-infused animals on the same experimental day; $*P < 0.05$ significantly increased compared with saline-infused animals, Mann–Whitney *U* test.

as in glomeruli, with most granulocytes being present within the glomeruli. Numbers of glomerular granulocytes tended to be higher on day 17 and were significantly higher on day 20 in pregnant compared with non-pregnant animals (Fig. 4d), while no significant effects of ATP infusion were observed in pregnant and non-pregnant rats on all time points.

T lymphocytes

$CD3^+$ lymphocytes in the kidney on days 15, 17 and 20 were found in the glomeruli, in most cases at the Bowman's capsule as well as in the interstitial space, often closely surrounding the glomeruli. On day 15, but not days 17 and 20 of pregnancy, increased numbers of $CD3^+$ lymphocytes were observed in pregnant compared with non-pregnant animals (Fig. 4e). In addition, ATP infusion increased the number of $CD3^+$ lymphocytes 1 day after infusion in non-pregnant animals, while no changes were found after ATP infusion in pregnant rats (Fig. 4e).

Discussion

Our findings demonstrated that extracellular ATP induced pregnancy-specific changes in peripheral and kidney leukocyte populations. Higher percentages of peripheral blood non-classical monocytes and lower percentages of various subsets of T lymphocytes characterized the pregnancy-specific peripheral response. In addition, a pregnancy-specific inflammatory response was also observed in the glomeruli after ATP infusion, in which we identified higher numbers of macrophages and T lymphocytes on day 15 in non-pregnant rats, while higher numbers of glomerular macrophages were found in ATP-infused pregnant rats on day 20.

Pregnancy in humans has been regarded a pro-inflammatory condition, characterized by increased numbers and activation of circulating monocytes and granulocytes, and decreased percentages of lymphocytes.[18,27–29] Our current data are in accordance with this suggestion, because we showed that pregnant rats have higher numbers of circulating monocytes (total monocytes as well as non-classical monocytes) and granulocytes, and lower numbers of circulating T lymphocytes compared with non-pregnant rats. We showed lower regulatory T lymphocyte numbers in pregnant compared with non-pregnant animals. This is in line with previous studies showing lower circulating regulatory T cells during pregnancy in humans[9,10] and coherent with the suggestion of Shima et al.[30] that regulatory T lymphocytes are not important for the maintenance of pregnancy. Locally in the glomeruli of the kidney, control pregnant animals showed increased T lymphocyte numbers on day 15, which is in line with our previous paper.[31] We also observed increased $CD206^+$ and $iNOS^+$ macrophages on day 17 and increased granulocyte numbers on days 17 and 20 of pregnancy. The exact mechanism of how numbers of certain leukocyte populations increase in glomeruli during pregnancy remains to be established, but they may be due to physiological changes in kidney function and blood pressure,[32] or may be the consequence of general activation of the immune system during pregnancy.[33]

The changes in immune responses in pregnancy may underlie the increased sensitivity of pregnant rats to the pro-inflammatory ATP. The present study showed that a single ATP infusion on day 14 of pregnancy induced prolonged effects on the immune response in pregnancy. This observation is in line with previous observations in our lab showing a persistent inflammatory response following another pro-inflammatory stimulus LPS in the pregnant rat.[31] The mechanism is unknown, but the prolonged effects cannot be due to long-lasting circulation of infused ATP, because ATP is degraded to adenosine in a few seconds by plasma and membrane bound enzymes, such as CD39, CD73, and alkaline phosphatase.[34] As a result, it cannot be found in the circulation after ATP infusion.[23] The increased sensitivity to ATP may be due to changes in P2 receptor expression during pregnancy, as has been shown for human uterine smooth muscle.[35] In addition, P2 receptors are also expressed by syncytiotrophoblast cells in the placenta.[36] Therefore, ATP may be able to activate syncytiotrophoblast cells,[36] which in their turn may activate and influence function of circulating immune cells.

We observed increased numbers of peripheral blood non-classical monocytes after ATP infusion in pregnant rats. Previously, we showed that only this specific subset was also activated following ATP infusion.[18] Various *in vitro* studies showed monocyte activation by ATP.[17,37,38] Only one study in bovine monocytes showed that responses to ATP seem to be subset specific, as classical and non-classical bovine monocytes produce different amounts of IL-1ß in response to ATP stimulation.[39] Non-classical monocytes are a more pro-inflammatory subset as

compared with classical monocytes as they produce higher amounts of TNF-α and IL-1ß after stimulation, exhibit lower phagocytic activity, and have higher antigen presenting capacity compared with classical monocytes.[40–42] The present results suggest that this more pro-inflammatory subset plays a role in the pathophysiology of this model and preeclampsia, as we have previously shown that also in human preeclampsia, the non-classical subset of monocytes was increased.[18] Interestingly, ATP infusion specifically increased numbers of monocytes, but not granulocytes, suggesting that monocytes may be important leukocytes in preeclampsia. The different response of granulocytes and monocytes might be due to differential P2 receptor expression patterns on these cells: $P2Y_{14}$ receptors are for instance expressed on granulocytes, but not expressed on monocytes, while $P2Y_{12}$ and $P2Y_{13}$ are expressed on monocytes, but not on granulocytes.[13,43,44] However, changes in expression patterns of P2 receptors have not been studied during pregnancy. Further insights into the role of the non-classical subset in preeclampsia as well as studies on sensitivity of granulocytes and classical and non-classical monocytes for ATP are warranted.

We showed decreased numbers of several peripheral blood T lymphocyte subpopulations after ATP infusion in pregnant rats. This could be due to a direct effect of ATP on these cells, because purinergic receptors are expressed on T lymphocytes.[44] Decreased numbers of circulating T lymphocytes may be due to ATP-induced migration of these cells into the mesometrial triangle, because we found increased numbers of T lymphocytes in this tissue. Alternatively, decreased T lymphocyte subpopulations could be due to an inhibition of production of these cells due to ATP. This has for instance been shown for regulatory T lymphocytes: ATP stimulation was shown to inhibit regulatory T lymphocyte formation *in vitro*.[45] Regulatory T lymphocytes are important in establishing pregnancy associated immune tolerance to the fetus,[46] and decreased numbers of these cells are associated with preeclampsia.[47] Our study suggests that the high ATP levels in preeclampsia[14] may be associated with decreased numbers of regulatory T lymphocytes in this condition.

Although ATP did not affect circulating leukocytes in non-pregnant rats, in the kidneys of non-pregnant rats, 1 day after the infusion of ATP, glomerular $CD68^+$ macrophages, and $CD3^+$ T lymphocytes increased. This suggests that ATP does have a short-term effect on immune responses of non-pregnant rats. The question may arise why non-pregnant rats do not develop proteinuria,[23] despite the increased presence of inflammatory cells? This may be due to the relatively low number of macrophages and T cells present in the glomeruli or to the fact that non-pregnant rats are less sensitive to damage inflicted by inflammatory cells.[48] The short-term effect of a pro-inflammatory stimulus on immune responses in non-pregnant rats, without inducing apparent damage, has been shown before in another model.[31,49]

Glomerular macrophage numbers were increased after ATP infusion in pregnant rats on day 20. However, ATP had no effect on the number of glomerular granulocytes and lymphocytes. This corroborates our previous findings.[23] The present study adds to the previous data in showing that at earlier intervals after the infusion of ATP in pregnant rats, no effect was observed on glomerular leukocyte numbers. Even though the number of macrophages was increased in response to ATP on day 20 of pregnancy, the numbers of glomerular $iNOS^+$ (M1) and $CD206^+$ (M2) macrophages were unchanged after ATP infusion. Therefore, at this time point, that is, 6 days after the infusion, ATP appeared not to influence macrophage phenotype. We have been limited to the use of iNOS and CD206 as potential markers of M1 and M2 phenotypes in our study, due to the unavailability of other markers for macrophage subsets in rats. Therefore, further studies on the effect of pregnancy on macrophage M1 and M2 markers are warranted.

It is tempting to speculate that increased glomerular macrophage numbers after ATP infusion in pregnant rats may be involved in the albuminuria as macrophage accumulation in the kidney has been shown to contribute to acute and chronic kidney injury,[50] and increased glomerular macrophage numbers in pregnant rats were shown to be correlated with the development of albuminuria.[23] The question remains as to the mechanism of increased macrophage numbers after ATP infusion. As these ATP-induced effects on glomerular macrophages were not observed until day 20 of pregnancy, it seems unlikely that it is a direct effect of the infusion on day 14, because, as described above, increased levels of ATP cannot be found after the infusion of ATP. However, the previously observed increase in ATP levels on day 20 in ATP-infused pregnant animals exclusively[23] may contribute to macrophage infiltration 6 days after the infusion.

American Journal of Reproductive Immunology **72** (2014) 305–316

Conclusions

Adenosine triphosphate (ATP) infusion in pregnant rats increased the numbers of peripheral blood non-classical monocytes and decreased T lymphocyte numbers compared with control pregnant rats. These changes coincided with infiltration of macrophages into the kidney, which may be involved in inducing proteinuria. Our data thus support our hypothesis that ATP infusion stimulates pregnancy-specific inflammatory responses that may result in proteinuria. These immune changes after ATP infusion in pregnant rats appear similar to changes found in human preeclampsia, although they are more subtle. The increased ATP levels in preeclamptic women may, therefore, potentially contribute to development of the pro-inflammatory condition in pre-eclampsia.

Acknowledgements

This study was funded by the Dutch Kidney Foundation (Grant No. C08.2266) and the Dutch Technology Foundation STW (Grant No. 10704). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors would like to acknowledge Dr. A. Smit-van Oosten, Dr. J.H. Zandvoort, and M. Weij for their surgical assistance.

References

1 Duley L: The global impact of pre-eclampsia and eclampsia. *Semin Perinatol* 2009; 33:130–137.
2 James JL, Whitley GS, Cartwright JE: Pre-eclampsia: fitting together the placental, immune and cardiovascular pieces. *J Pathol* 2010; 221:363–378.
3 Burton GJ, Yung HW, Cindrova-Davies T, Charnock-Jones DS: Placental endoplasmic reticulum stress and oxidative stress in the pathophysiology of unexplained intrauterine growth restriction and early onset preeclampsia. *Placenta* 2009; 30(Suppl. A):S43–S48.
4 Harskamp RE, Zeeman GG: Preeclampsia: at risk for remote cardiovascular disease. *Am J Med Sci* 2007; 334:291–295.
5 van der Graaf AM, Toering TJ, Faas MM, Lely AT: From preeclampsia to renal disease: a role of angiogenic factors and the renin-angiotensin aldosterone system? *Nephrol Dial Transplant* 2012; 27(Suppl. 3):iii51–iii57.
6 Sargent IL, Borzychowski AM, Redman CW: Immunoregulation in normal pregnancy and pre-eclampsia: an overview. *Reprod Biomed Online* 2007; 14, Spec No 1 :111–117.
7 Gervasi MT, Chaiworapongsa T, Pacora P, Naccasha N, Yoon BH, Maymon E, Romero R: Phenotypic and metabolic characteristics of monocytes and granulocytes in preeclampsia. *Am J Obstet Gynecol* 2001; 185:792–797.
8 Darmochwal-Kolarz D, Rolinski J, Leszczynska-Goarzelak B, Oleszczuk J: The expressions of intracellular cytokines in the lymphocytes of preeclamptic patients. *Am J Reprod Immunol* 2002; 48:381–386.
9 Mjosberg J, Svensson J, Johansson E, Hellstrom L, Casas R, Jenmalm MC, Boij R, Matthiesen L, Jonsson JI, Berg G, Ernerudh J: Systemic reduction of functionally suppressive $CD4^{dim}CD25^{high}Foxp3^{+}$ Tregs in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. *J Immunol* 2009; 183:759–769.
10 Tilburgs T, Roelen DL, van der Mast BJ, de Groot-Swings GM, Kleijburg C, Scherjon SA, Claas FH: Evidence for a selective migration of fetus-specific $CD4^{+}$ $CD25^{bright}$ regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol* 2008; 180:5737–5745.
11 Faas MM, van Pampus MG, Anninga ZA, Salomons J, Westra IM, Donker RB, Aarnoudse JG, de Vos P: Plasma from preeclamptic women activates endothelial cells via monocyte activation *in vitro*. *J Reprod Immunol* 2010; 87:28–38.
12 Burnstock G: Discovery of purinergic signalling, the initial resistance and current explosion of interest. *Br J Pharmacol* 2012; 167:238–255.
13 Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC: Adenosine 5′-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. *Pharmacol Ther* 2006; 112:358–404.
14 Bakker WW, Donker RB, Timmer A, van Pampus MG, van Son WJ, Aarnoudse JG, van Goor H, Niezen-Koning KE, Navis G, Borghuis T, Jongman RM, Faas MM: Plasma hemopexin activity in pregnancy and preeclampsia. *Hypertens Pregnancy* 2007; 26:227–239.
15 Roberts VH, Greenwood SL, Elliott AC, Sibley CP, Waters LH: Purinergic receptors in human placenta: evidence for functionally active P2X4, P2X7, P2Y2, and P2Y6. *Am J Physiol Regul Integr Comp Physiol* 2006; 290:R1374–R1386.
16 Burnstock G, Knight GE: Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 2004; 240:31–304.
17 Aga M, Johnson CJ, Hart AP, Guadarrama AG, Suresh M, Svaren J, Bertics PJ, Darien BJ: Modulation of monocyte signaling and pore formation in response to agonists of the nucleotide receptor P2X(7). *J Leukoc Biol* 2002; 72:222–232.
18 Melgert BN, Spaans F, Borghuis T, Klok PA, Groen B, Bolt A, de Vos P, van Pampus MG, Wong TY, van Goor H, Bakker WW, Faas MM: Pregnancy and preeclampsia affect monocyte subsets in humans and rats. *PLoS One* 2012; 7:e45229.
19 Suh BC, Kim JS, Namgung U, Ha H, Kim KT: P2X7 nucleotide receptor mediation of membrane pore formation and superoxide generation in human promyelocytes and neutrophils. *J Immunol* 2001; 166:6754–6763.
20 Langston HP, Ke Y, Gewirtz AT, Dombrowski KE, Kapp JA: Secretion of IL-2 and IFN-gamma, but not IL-4, by antigen-specific T cells requires extracellular ATP. *J Immunol* 2003; 170:2962–2970.
21 Kawamura H, Kawamura T, Kanda Y, Kobayashi T, Abo T: Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. *Immunology* 2012; 136:448–458.
22 Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, Nizet V, Insel PA, Junger WG: ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science* 2006; 314:1792–1795.
23 Faas MM, van der Schaaf G, Borghuis T, Jongman RM, van Pampus MG, de Vos P, van Goor H, Bakker WW: Extracellular ATP induces

albuminuria in pregnant rats. *Nephrol Dial Transplant* 2010; 25:2468–2478.
24 Faas MM, Broekema M, Moes H, van der Schaaf G, Heineman MJ, de Vos P: Altered monocyte function in experimental preeclampsia in the rat. *Am J Obstet Gynecol* 2004; 191:1192–1198.
25 Ahuja V, Miller SE, Howell DN: Identification of two subpopulations of rat monocytes expressing disparate molecular forms and quantities of CD43. *Cell Immunol* 1995; 163:59–69.
26 Gordon S, Taylor PR: Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; 5:953–964.
27 Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, Faas MM: Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle. *Fertil Steril* 2002; 77:1032–1037.
28 Valdimarsson H, Mulholland C, Fridriksdottir V, Coleman DV: A longitudinal study of leucocyte blood counts and lymphocyte responses in pregnancy: a marked early increase of monocyte-lymphocyte ratio. *Clin Exp Immunol* 1983; 53:437–443.
29 Luppi P, Haluszczak C, Trucco M, Deloia JA: Normal pregnancy is associated with peripheral leukocyte activation. *Am J Reprod Immunol* 2002; 47:72–81.
30 Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, Saito S: Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. *J Reprod Immunol* 2010; 85:121–129.
31 Faas MM, Schuiling GA, Baller JF, Bakker WW: Glomerular inflammation in pregnant rats after infusion of low dose endotoxin. An immunohistological study in experimental pre-eclampsia. *Am J Pathol* 1995; 147:1510–1518.
32 Cornelis T, Odutayo A, Keunen J, Hladunewich M: The kidney in normal pregnancy and preeclampsia. *Semin Nephrol* 2011; 31:4–14.
33 Svensson-Arvelund J, Ernerudh J, Buse E, Cline JM, Haeger JD, Dixon D, Markert UR, Pfarrer C, Vos PD, Faas MM: The placenta in toxicology. Part II: systemic and local immune adaptations in pregnancy. *Toxicol Pathol* 2013; 42:327–338.
34 Fitz JG: Regulation of cellular ATP release. *Trans Am Clin Climatol Assoc* 2007; 118:199–208.
35 Ziganshin AU, Zaitsev AP, Shamsutdinov AF: Pharmacological characteristics of P2 receptors in human uterus. *Bull Exp Biol Med* 2002; 133:255–257.
36 Roberts VH, Waters LH, Powell T: Purinergic receptor expression and activation in first trimester and term human placenta. *Placenta* 2007; 28:339–347.
37 Perregaux DG, McNiff P, Laliberte R, Conklyn M, Gabel CA: ATP acts as an agonist to promote stimulus-induced secretion of IL-1 beta and IL-18 in human blood. *J Immunol* 2000; 165: 4615–4623.
38 Kaufmann A, Musset B, Limberg SH, Renigunta V, Sus R, Dalpke AH, Heeg KM, Robaye B, Hanley PJ: "Host tissue damage" signal ATP promotes non-directional migration and negatively regulates toll-like receptor signaling in human monocytes. *J Biol Chem* 2005; 280:32459–32467.
39 Hussen J, Duvel A, Sandra O, Smith D, Sheldon IM, Zieger P, Schuberth HJ: Phenotypic and functional heterogeneity of bovine blood monocytes. *PLoS One* 2013; 8:e71502.
40 Ancuta P, Liu KY, Misra V, Wacleche VS, Gosselin A, Zhou X, Gabuzda D: Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16- monocyte subsets. *BMC Genomics* 2009; 10:403.
41 Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, Espevik T, Ziegler-Heitbrock L: The proinflammatory CD14+ CD16+ DR++ monocytes are a major source of TNF. *J Immunol* 2002; 168:3536–3542.
42 Frankenberger M, Sternsdorf T, Pechumer H, Pforte A, Ziegler-Heitbrock HW: Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood* 1996; 87:373–377.
43 Scrivens M, Dickenson JM: Functional expression of the P2Y14 receptor in human neutrophils. *Eur J Pharmacol* 2006; 543:166–173.
44 Wang L, Jacobsen SE, Bengtsson A, Erlinge D: P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunol* 2004; 5:16.
45 Schenk U, Frascoli M, Proietti M, Geffers R, Traggiai E, Buer J, Ricordi C, Westendorf AM, Grassi F: ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. *Sci Signal* 2011; 4:ra12.
46 Saito S: Th17 cells and regulatory T cells: new light on pathophysiology of preeclampsia. *Immunol Cell Biol* 2010; 88:615–617.
47 Toldi G, Saito S, Shima T, Halmos A, Veresh Z, Vasarhelyi B, Rigo J Jr, Molvarec A: The frequency of peripheral blood CD4+ CD25high FoxP3+ and CD4+ CD25- FoxP3+ regulatory T cells in normal pregnancy and pre-eclampsia. *Am J Reprod Immunol* 2012; 68:175–180.
48 Faas MM, Bakker WW, Baller JF, Schuiling GA: Pregnancy enhances the sensitivity of glomerular ecto-adenosine triphosphate-diphosphohydrolase to products of activated polymorphonuclear leukocytes. *Am J Obstet Gynecol* 1999; 180:112–113.
49 Faas MM, Schuiling GA, Baller JF, Visscher CA, Bakker WW: A new animal model for human preeclampsia: ultra-low-dose endotoxin infusion in pregnant rats. *Am J Obstet Gynecol* 1994; 171:158–164.
50 Mannon RB: Macrophages: contributors to allograft dysfunction, repair, or innocent bystanders? *Curr Opin Organ Transplant* 2012; 17:20–25.

American Journal of Reproductive Immunology **72** (2014) 305–316