Introducing a mouse model for pre-eclampsia: adoptive transfer of activated Th1 cells leads to pre-eclampsia-like symptoms exclusively in pregnant mice

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Pre-eclampsia (PE) is the most severe pregnancy-related disease, leading to high maternal and fetal morbidity/mortality. Immunological imbalances associated with endothelial cell dysfunction have been hypothesized as a cause for the onset and perpetuation of PE. Valid and reliable animal models are urgently required to test this hypothesis and to better understand the mechanisms underlying PE. We developed a novel PE-model by adoptively transferring activated BALB/c Th1-like splenocytes into allogeneically pregnant BALB/c female mice during late gestation; the model mimicked the symptoms of PE, *i.e.* increased blood pressure and glomerulonephritis accompanied by proteinuria. Interestingly, these PE-like symptoms were not detectable in non-pregnant recipients of activated Th1-like cells. Adoptive cell transfer adversely affected the outcome of pregnancy by increasing fetal rejection, with uterine immune cells showing an inflammatory profile. In conclusion, we have established a valid and reliable PE mouse model, which opens vast opportunities for therapeutic interventions.

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1 Introduction

The survival of the fetus in the maternal uterus still remains an immunological paradox, since maternal immune cells and "allogeneic" fetal cells are in direct contact, which should result in the rejection of the fetus. Fortunately, most pregnancies end in the birth of healthy offspring, on the basis of a successful symbiosis of the maternal immune system with fetal alloantigens. Nevertheless, several complications of pregnancies are known, based on an inappropriate maternal immune response, e.g. pre-eclampsia (PE), which affects about 10% of late-pregnancies [1]. The clinical symptoms of PE include elevated maternal blood pressure, proteinuria and abnormal fluid-retention [1].

The etiology of PE still remains controversial. Some data indicate an abnormally shallow and therefore inadequate invasion of trophoblast cells during the second physiological wave of trophoblasts invasion [2, 3], which represents a direct cause for endothelial cell dysfunction, thus increasing maternal blood pressure and leading to abnormal kidney function [4]. The so-called "inflammatory theory" proposes that an excessive maternal inflammatory response leads to generalized endothelial cell dysfunction, finally resulting in PE [4]. A wealth of data propose that the placenta itself as well as some of its metabolites, e.g. TNF-α, are the stimuli leading to such an immune activation [4–9]. A systemic response of activated maternal inflammatory cells (including elevated numbers of granulocytes and monocytes) has been reported in patients suffering from PE [9–11], accompanied by increased levels of pro-inflammatory Th1-type cytokines, mainly TNF-α [8, 12, 13].

Considering the severe impact of PE on maternal and fetal health, a valid and reliable animal model is urgently required to help create effective therapeutic approaches. To date, some PE animal models have been proposed in the rabbit, rat and mouse [14–19]. The experimental design of such models was primarily based on mechanisms involved in the regulation of vasoconstriction/

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Abbreviations: PE: Pre-eclampsia **rm:** Recombinant murine

vasodilation, *i.e.* administration of nitric oxide synthase inhibitors (such as L-NAME) [14], transfecting animals with genes of the human renin–angiotensin system [15], stimulating the sympathetic nerve [16], or administrating endothelin-1 during late pregnancy [17]. Other, rather immunologically based models, included the administration of low doses of endotoxin, which led to PE-like symptoms exclusively in pregnant rats [6]. Further, the transfer of lymphocytes that had been incubated in IL-4 and IL-12 into pregnant mice, but not of lymphocytes that had been polyclonally or monoclonally activated, has been described [18]; this led to an increase in blood pressure in pregnant mice compared with pregnant animals given mock-treated cells. However, data delineating the pregnancy-specificity of these symptoms were not provided. Lastly, another approach was to employ genetically hypertensive mouse strains [19].

Taken together, in the various currently available PE models, PE-like symptoms were clearly present; however, most of these approaches are limited by the fact that renal changes and hypertension — the prototypic PE-symptoms — could also be observed in non-pregnant animals. Thus, since PE is a pregnancy-specific disease, none of these approaches would represent an adequate animal model.

On the basis that the immune system is important in the course of pregnancy and that it possibly participates in the etiology of PE, we decided to venture an immunological approach to develop a reliable animal PE model. Thus, we focused on adoptively transferring activated immune cells, mainly of a Th1 phenotype, into pregnant mice in an attempt to mimic the PE-like symptoms observed in humans. Activated immune cells with ability to secrete Th1-type cytokines are nowadays being suc-

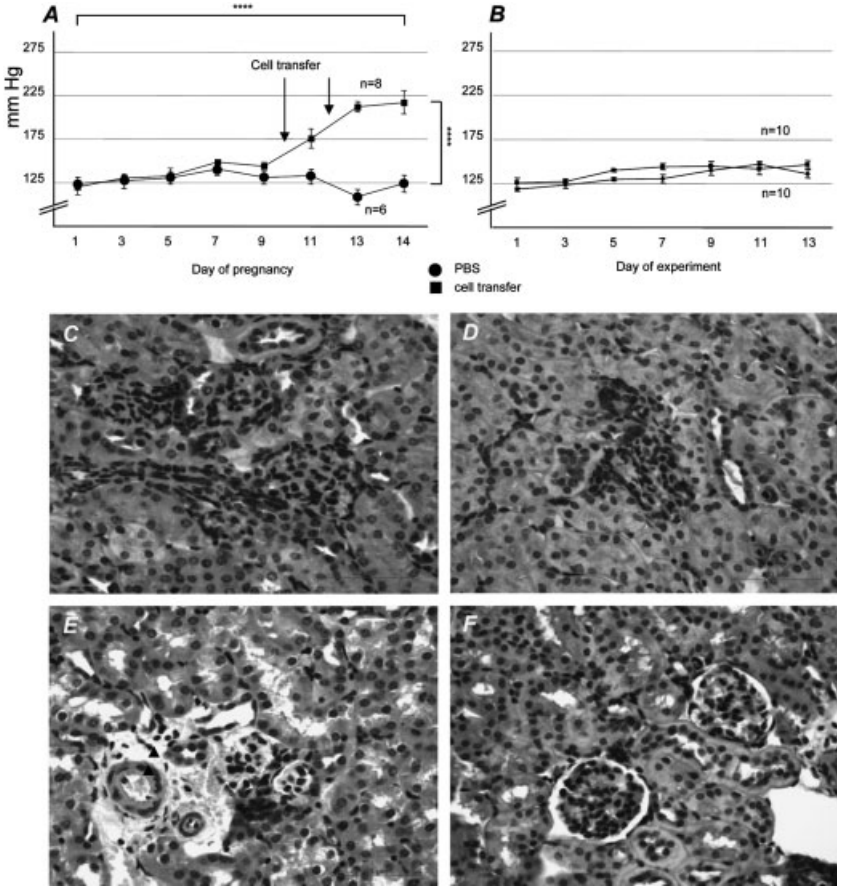

Fig. 1. Th1-activated cell transfer increases blood pressure and provokes kidney abnormalities exclusively in pregnant recipients. (A, B) Blood pressure curves, showing mm Hg *versus* gestational age or experiment day from pregnant (Fig. 1A) and non-pregnant (Fig. 1B) mice throughout the experiments. Mice received i.v. either PBS (full circles) or activated Th1 cells (full squares). ****, $p<0.00001$, as analyzed by the Mann-Whitney or Friedman test. (C–F) Hematoxylin and eosin staining of paraffin-embedded kidneys sections: (C, D) representative examples of abnormal cell distribution as well as cell clusters in the glomerulum of pregnant cell recipients; in (E), the arrowhead points to infiltrating cells (PMN and lymphocytes) in the glomerulum of a pregnant cell-recipient; (F) two glomeruli without pathological histology from a non-pregnant mice that received activated Th1-like cells i.v.

cessfully used in the treatment of malignant tumors [20, 21] and are further known to mediate acute graft rejection [21–23]. In mammalian pregnancy, uterine and systemic Th1 cells are known to be directly involved in fetal rejection [23–27]. Recently, the activation of maternal cytotoxic T cells was shown to provoke inflammatory responses leading to complement activation and placental thrombosis, which then resulted in fetal rejection in a murine model [28]. Here, we show that the transfer of activated Th1-like cells lead to PE-like symptoms (hypertension, proteinuria and kidney pathology) exclusively in pregnant mice, pointing out the validity of our mouse model.

2 Results

2.1 Adoptive transfer of activated Th1-like cells induced PE-like symptoms exclusively in pregnant mice

To address the question of whether activated Th1 cells would suffice to mimic PE-like symptoms on mice, we injected pregnant mice with activated Th1-like cells i.v. twice during late gestation (on days 10 and 12 of pregnancy). The cell transfer abruptly induced high blood pressure levels (from 124.13±9.26 mm Hg to 217.13±12.54 mm Hg, $p<0.001$) exclusively in pregnant recipients (Fig. 1A). Before the onset of pregnancy the females displayed normal values of blood pressure (data not shown), as they did during the early stages of pregnancy before cell transfer (Fig. 1A). Control, pregnant mice injected with PBS i.v. presented a mild decrease of blood pressure at the end of pregnancy, which nicely supported previously published data on murine pregnancies [29]. To probe whether hypertension triggered by adoptive cell transfer would exclusively affect pregnant mice, non-pregnant control mice were exposed to the same procedures (injection of either PBS or activated Th1-like cells). Most interestingly, after adoptive transfer of activated Th1-cells, no changes in the blood pressure values could be registered in non-pregnant mice compared to PBS-treated non-pregnant (Fig. 1B), which clearly points out the pregnancy-specificity of the treatment.

Inoculation of activated Th1-like cells into pregnant mice further resulted in another typical PE-symptom — increased urinary protein excretion (Table 1). PBS-treated pregnant mice as well as non-pregnant mice, injected i.v. with PBS or activated Th1-like cells did not show any signs of proteinuria, which again point towards the pregnancy restriction of the experimentally induced PE-like symptoms (Table 1). Proteinuria in pregnant recipients of Th1-like activated cells was further associated with histopathological characteristics in the kidneys. We observed enlarged glomeruli, with increased glomerular cell density (Fig. 1C, D), and the presence of infiltrating immune cells, especially PMN and monocytes (Fig. 1E). Further, samples derived from mice with PE-like symptoms showed glomerular fibrosis (Fig. 1C), deposition of acidophilic material (data not shown) and increased vessel wall density (Fig. 1E). A representative field of normal kidney histology (*i.e.* non-pregnant recipients of Th1-like cells) is shown in Fig. 1F.

2.2 Mice with PE-like symptoms showed an abnormal pregnancy outcome and uterine histopathology

The transfer of Th1-like activated cells provoked an increased fetal rejection rate, although this increase was not statistically significant (Fig. 2A), which supports our hypothesis of a PE model, since PE in humans does not necessarily imply fetal death. Further, we observed preterm labor and signs of pain in addition to the presence of inflamed uteri. No macroscopic differences between PBS- and cell-treated animals were observed with respect to the size or characteristics of the pups or placentas. Interestingly, the transfer of Th2-like cells in C57BL/6-mated BALB/c mice did not lead to an augmentation of the rejection rate (data not shown).

Placental samples of Th1-cell-treated mice presented fibrosis in spongiotrophoblasts, extensive hemorrhagic sites in the labyrinthic and spongiotrophoblast zones, and a massive infiltration with immune cells (Fig. 2B) when compared with placenta samples from PBS-treated pregnant mice (Fig. 2C). The infiltrating cells were not $CD8^+$ cells, as we confirmed by immunohistochemis-

Table 1. Transfer of activated Th1-like cells lead to proteinuria only in pregnant animals[a)]

Protein excretion	Non-pregnant + PBS	Non-pregnat + cell transfer	Pregnant + PBS	Pregnant + cell transfer
$<$ 1 g/l	$n = 10$	$n = 10$	$n = 5$	$n = 1$
$>$ 1 g/l	$n = 0$	$n = 0$	$n = 0$	$n = 5$****

[a)] This table represents protein excretion in urine from all groups. ****, $p \leq 0.0001$ as analyzed by the Chi-square test.

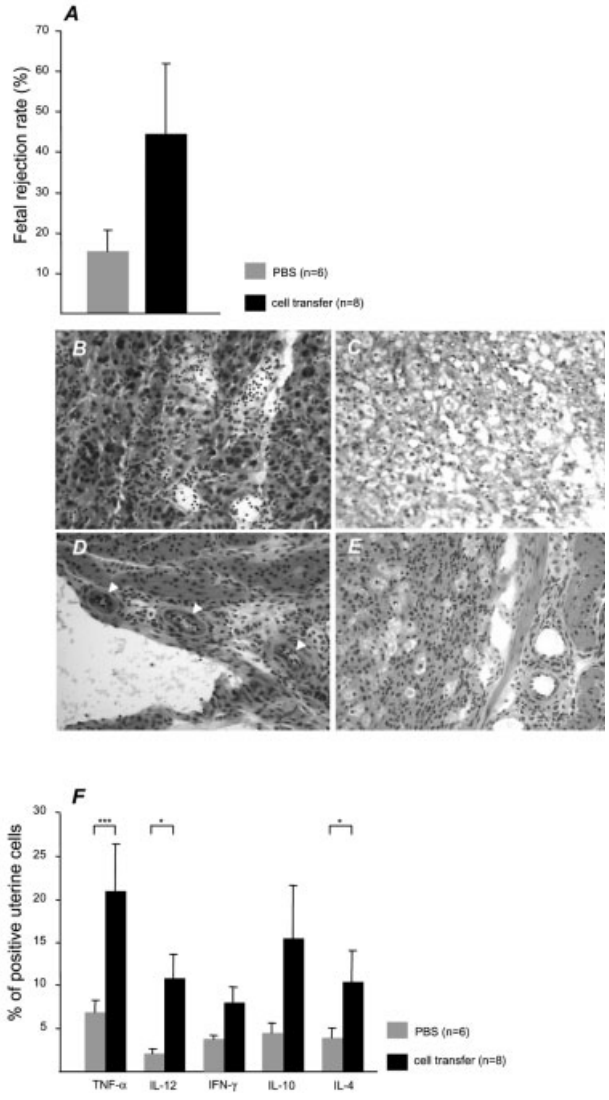

Fig. 2. Recipients of Th1 cells present an abnormal pregnancy outcome and signs of inflammation at the feto–-maternal interface. (A) The fetal rejection rate in pregnant mice after PBS treatment (n=6) or transfer of activated Th1-like cells (n=8), as calculated from the ratio of rejected fetuses to total implantation sites. (B, D) Decidual histopathological signs in mice that previously received activated Th1-like cells i.v: (B) shows a representative field of placental samples from mice having PE-like symptoms, showing fibrosis in the spongiotrophoblast area as well as massive lymphocyte infiltration; (D) shows abnormally thick uterine blood vessel walls, as indicated by the arrowheads. (C, E) Placental and decidual samples from PBS-treated pregnant mice, without pathological findings. (F) An illustration of the percentage of uterine lymphocytes positive for TNF-α, IL-12, IFN-γ, IL-10 and IL-4 in pregnant mice that received PBS (n=6) or activated Th1-like cells (n=8), as analyzed by flow cytometry. *, $p<0.05$, and ***, $p<0.001$, as analyzed by the Mann-Whitney non-parametric test.

try (data not shown). In decidua, mice with PE-like symptoms presented much thicker decidual vessel walls (Fig. 2D) than normal pregnant mice (Fig. 2E).

2.3 Adoptive transfer of Th1-like cells induced an increased cytokine production in uterine lymphocytes

Uterine lymphocytes from pregnant mice produced significantly more Th1 cytokines (TNF-α and IL-12) (Fig. 2F) after Th1 cell transfer in comparison with PBS-treated pregnant mice. A mild, although not statistically significant, augmentation in the percentage of IFN-γ^+ uterus cells was also observed in Th1-cell-treated mice (Fig. 2F). The Th1-type cytokines were produced by $CD2^+$ lymphocytes, only a proportion of which were $CD8^+$ cells (data not shown). Additionally, the production of Th2 cytokines IL-10 and IL-4 by uterine lymphocytes was augmented in mice with PE-like symptoms, but only for IL-4 was this difference statistically significant (Fig. 2F). $CD8^+$ cells produced very low levels of Th2 cytokines (data not shown).

The total number of $CD8^+$ T cells was significantly enhanced in decidua from pregnant mice receiving activated Th1-like cells, compared with PBS-treated mice. Further, the number of activated uterine lymphocytes (double-positive $CD2^+CD25^+$ cells) was drastically augmented in the decidua from cell-recipient pregnant females compared with the PBS group (data not shown).

2.4 CCR5 expression is augmented in blood and uterine lymphocytes from mice showing PE-like symptoms

Next, we wished to look deeper into the mechanisms by which Th1-type, activated cells would cause PE-like symptoms only in the pregnant mice. Since PE has previously been described as a “Th1 disease”, we opted to investigate the role of CCR5-expressing cells. CCR5 was reported to be a reliable Th1 marker and also a Th1 trafficking marker, allowing the chemokine-mediated contact with endothelial cells that is necessary for extravasation into tissues. Thus, it is likely that CCR5 enables the migration of cells into target tissues, *i.e.* the uterus, during PE.

Transfer of Th1 cells, which included a high number of $CCR5^+$ cells (approximately 2×10^6 $CCR5^+$ cells/injection) did not provoke a variation in the total number of $CCR5^+$ blood cells in non-pregnant mice compared to PBS treatment (Fig. 3A). Interestingly, PBS-treated pregnant mice showed significantly lower levels of $CCR5^+$ cells in

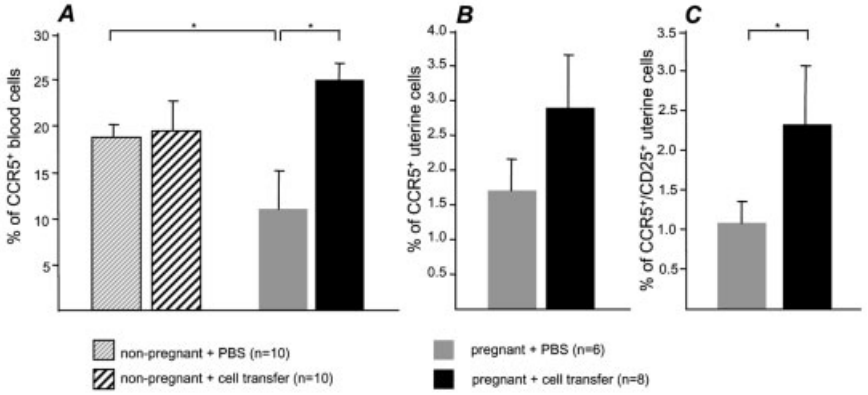

Fig. 3. CCR5 expression in the blood and uterus is augmented in mice with PE-like symptoms. (A) CCR5 expression on blood lymphocytes from pregnant and non-pregnant mice that received PBS or activated Th1-like cells i.v. (B) The percentage of CCR5$^+$ uterine cells of PBS-treated mice compared to cell-treated mice. (C) The proportion of double-positive CCR5$^+$CD25$^+$ cells in the uterus from PBS- or cell-treated pregnant mice. *, $p<0.05$, as analyzed by the Mann-Whitney non-parametric test.

the blood compared with non-pregnant mice (Fig. 3A). When pregnant mice received activated Th1-like cells, a significant increase in the percentage of CCR5$^+$ blood cells was detected (Fig. 3A). Levels of uterine lymphocytes positive for CCR5 were also augmented in mice with PE-like symptoms compared with PBS-treated mice (Fig. 3B). The co-expression of both the Th1 and trafficking marker CCR5 and the activation marker CD25 (double-positive cells, CCR5$^+$CD25$^+$) was also significantly enhanced (Fig. 3C).

2.5 Organ distribution of CFSE-labeled Th1-like cells after adoptive transfer

We next assessed the target organs where adoptively transferred Th1-like cells would home, by employing adoptive transfer of CFSE-labeled fluorescent cells in pregnant and non-pregnant animals. This further allowed us to identify whether the infiltrating cells at the feto–-maternal interface and in the kidneys were donor or host cells. We confirmed the presence of CFSE$^+$ fluorescent cells in the lung (Fig. 4A), liver (Fig. 4B) and kidneys (Fig. 4C) of pregnant and non-pregnant recipient animals and at the feto–maternal interface in pregnant mice (Fig. 4D). We could not find any fluorescent cells in the intestine (data not shown). We further quantified the number of CFSE$^+$ cells in decidua and kidneys by flow cytometry and found no significant differences in the total number of CFSE$^+$ cells in kidneys between pregnant and non-pregnant animals (Fig. 4E, F). We observed that only a limited number of the infiltrating cells in kidneys and uterus were CFSE$^+$CCR5$^+$ double-positive cells, *i.e.* from the donor (Fig. 4E, F as an example for flow cytometry; Fig. 4G, H for means of all groups). Significantly more CCR5$^+$ lymphocytes migrated into the kidneys of pregnant cell recipients, compared with non-pregnant cell recipients. Interestingly, most of the CCR5$^+$ lymphocytes in the uterus and in the kidneys were not positive

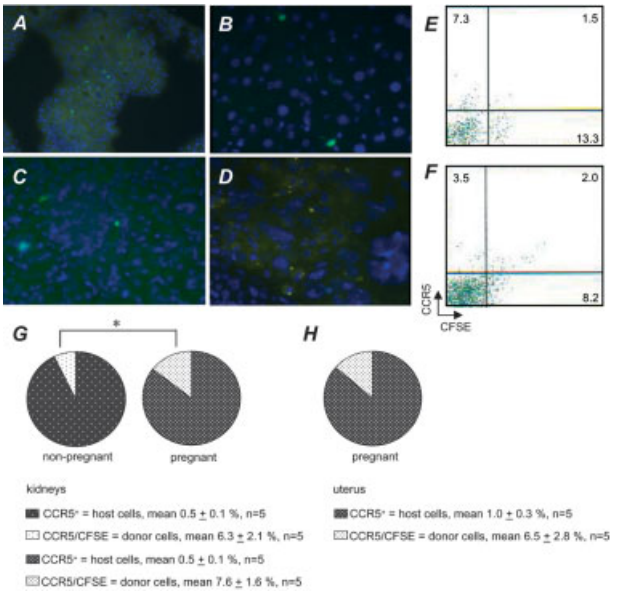

Fig. 4. Transfer of activated Th1-like cells labeled with a fluorescent membrane protein (CFSE). The presence of the transferred cells in lung (A), liver (B), kidney (C) and at the feto–maternal interface (D), as analyzed under a fluorescent microscope. (E) Depicts a representative flow cytometry plot of kidney cells (F) depicts decidual cells. (G) Represents the ratio of double-positive CCR5$^+$CFSE$^+$ cells (=donor cells), shown using a mainly white background, to CCR5$^+$ cells (=host cells), shown using a mainly gray background, in kidneys of non-pregnant and pregnant cell recipients, whereas (H) gives the same ratio (CCR5$^+$CFSE$^+$ to CCR5$^+$ cells) for the uterus cells of pregnant recipients. *, $p<0.05$, as analyzed by the Mann-Whitney non-parametric test.

for CSFE, which indicates a selective migration of host cells into the target tissues — decidua and kidneys (Fig. 4G, H).

3 Discussion

Over the past decade, various mammalian models to investigate the pathophysiology of PE have been suggested; however, their validity was hampered by the fact that in most of them, the non-pregnant control animals also developed PE-like symptoms [14–19]. Here, we introduce a new model in which activated Th1 cells are transferred into pregnant or non-pregnant recipients, which leads to PE-like symptoms exclusively in pregnant mice.

Previous data had suggested that transferred, activated immune cells secreting Th1 cytokines or transferred Th1 cell clones in mice can be involved in the development of lupus or delayed-type hypersensitivity [30, 31]. Transfer of activated immune cells has also been shown to effectively cure malignant tumors in mice [21, 32]. Since Th1 cells play a detrimental role during mammalian pregnancy [24–27] and taking into account the close relationship between activated Th1 immune cells and PE in humans [6, 9–13], we hypothesize that activated Th1 cells will negatively affect late allogeneic, rather than syngeneic, murine gestation and induce PE-like symptoms.

Our data clearly show that the adoptive transfer of activated Th1-like cells but not of Th2-like cells leads to elevated blood pressure, proteinuria and kidney damage exclusively in pregnant recipients. These symptoms undoubtedly resemble the pathophysiology of PE. It should be noted though that the "immunosuppressive" state of the host could be a prerequisite for the tissue lesions observed in PE. However, on the basis of published evidence indicating that Th1 cytokines can directly damage organs and destroy vessels [33], we believe that the vessel damage was induced by direct interaction of immune cells (secreting Th1 cytokines) with the vessels. These Th1 cells are not necessarily the cells inoculated by the adoptive transfer, and host cells primed to paternal antigens and stimulated by the cell injections may secrete additional Th1 cytokines.

The main question that arises from these observations is how the transfer of activated Th1-like cells provokes these physiological abnormalities exclusively in pregnant animals. We believe that, since the transferred cells produced predominantly Th1-type cytokines, they expand and stimulate host cells primed to paternal antigens toward the secretion of inflammatory cytokines, as demonstrated *in vitro* [32]. Increased secretion of Th1 cytokines by activated *host* cells or, in other words, an inflammatory host response, was in fact proposed to be the main cause leading to PE in humans [2, 6, 34]. This concept is further supported by our experiments with the transfer of labeled cells, in which we could show that most of the $CCR5^+$ Th1-like infiltrating cells at the feto–-maternal interface where host cells and not transferred cells.

It is tempting to suggest that the transfer of Th1-like cells is acting like a nonspecific stimulator of the "pregnancy-modified" maternal immune system, since we did not observe PE-like symptoms in virgin cell recipients. This "modification" of the immune system during pregnancy, which may be called "immunosuppression" or "tolerance" and allows the survival of the fetus within the maternal uterus, was proposed some years ago [25, 35]. The activated immune system of the pregnant mice, as triggered by adoptive cell transfer, may act specifically at the feto–maternal interface, reinforcing the natural but scarce maternal immunity due to the presence of the allogeneic fetus [36, 37]. Additionally, it is possible that the Th1 cytokines secreted by transferred immune cells induce the further infiltration of macrophages or other antigen-presenting cells, which prime cytotoxic T cells to paternal antigens that are expressed by fetal cells [37].

To try to further answer questions regarding the specificity of the treatment for pregnant recipients, we would like to refer to our observations regarding CCR5 expression, since analyzing trafficking pathways might give us some clues to understand how the activated immune cells (mostly from the host but even the transferred cells) migrate from the circulation to target organs — the feto-maternal interface or the kidneys — only in pregnant mice. We observed infiltration of immune cell in kidneys from cell-treated pregnant animals, but not in non-pregnant animals. Additionally, immune cell infiltrates at the feto–maternal interface were present in cell-treated pregnant animals, compared with PBS-treated animals.

The chemokine receptor CCR5, a reliable Th1-marker [38] and an important mediator in the lymphocyte trafficking cascade, plays a crucial role in the contact between Th1 cells and endothelial cells, which is necessary for lymphocyte extravasation into inflamed tissue [39]. In non-pregnant mice, CCR5 basal blood levels showed no modification after the transfer of Th1 cells. Normal pregnant mice showed lower basal CCR5 levels than non-pregnant mice, supporting previous data that CCR5 up-regulation may be deleterious for pregnancy outcome ([27]; Fest and Arck, unpublished observations). CCR5 levels increased significantly in the blood after the transfer of activated immune cells to pregnant

mice, contrasting with the observations in non-pregnant mice. CCR5 up-regulation could be further observed in decidual immune cells after cell transfer, reinforcing our proposal of host cell expansion and migration. These $CCR5^+$ cells were additionally activated — they expressed CD25. A mild augmentation in $CCR5^+$ cell number after cell transfer was also observed in the kidneys of pregnant animals compared with non-pregnant animals.

Since we confirmed, by means of the CFSE-labeling experiments, that most of the $CCR5^+$ cells were activated host cells and not transferred cells, CCR5 seems to contribute to the selective migration of Th1 host cells into the feto–maternal interface and into the kidneys to provoke the main symptoms of PE exclusively in pregnant animals. In the light of our data, we suppose that after nonspecific activation, host activated cells from pregnant animals are able to migrate to the feto–-maternal interface, using CCR5-dependent pathways. Interestingly, up-regulation of additional adhesion molecules in peripheral circulation and the placental bed has been described in human PE [3, 40].

We observed immune cell infiltration as well as fibrosis at the feto–maternal interface from pregnant mice that received activated Th1-like cells, especially in the spongy (junctional) zone of the placenta. These placental alterations could be explained by the increased levels of pro-inflammatory cytokines in this area, since Th1 cytokines have widely been shown to cause local rejection reactions [24–27]. *In vitro* studies confirm the ability of activated immune cells, mainly of a Th1-profile, to attack trophoblast cells expressing "foreign" paternal antigens [41]. Many authors have pointed out TNF-α as a key player in the etiology of PE [4, 12, 42]; this is supported by our data on very high amounts of TNF-α at the feto–-maternal interface of mice with PE-like symptoms.

The inoculation of activated cells led to cellular infiltrates (mainly PMN and lymphocytes) in the kidneys from pregnant animals, together with other pathologic characteristics like increased number of cells/glomeruli, sclerosis (from mild to severe) and collapsed glomerular capillaries, which were exclusively present in pregnant recipients. The infiltrating cells in the kidneys were predominantly host Th1 cells, as suggested by the experiments employing CFSE-labeled cells. It is known that Th1-cytokines, especially TNF-α, up-regulate mediators of the renin-angiotensin system, which may then mediate kidney damage [43]. TNF-α was also demonstrated to have a direct and not apoptosis-mediated effect on kidney damage, leading to diminished glomerular filtration rate with subsequent proteinuria and renal failure [44]. Here, we could not find signs of increased apoptosis using the TUNEL technique in kidney samples from mice with PE-like symptoms (data not shown), suggesting direct damage and not an apoptosis-mediated effect of TNF-α on kidney cells.

We further observed vessel injury in kidneys and the uterus from pregnant recipients of Th1-cells. Over the last five years, much research has focused on understanding the mechanisms of inflammatory conditions leading to vessel injury [33]. The direct interaction between activated immune cells and endothelial cells is gaining more scientific attention, and indications are that activated T cells or the cytokines produced by these cells can mount inflammatory reactions that remodel or even destroy blood vessels [33, 45]. Taking into account the time-point of the cell inoculations (days 10 and 12 of pregnancy), we believe that the vessel damage was induced by direct interaction between the vessels and immune cells that secreted Th1 cytokines. Indeed, Th1-cytokines are sufficient for destruction of tumor vasculature in animal models [46]. In our model, activated cells as well as the augmented secretion of TNF-α, IFN-γ or IL-12 may contribute to the vessel alterations observed at the feto–maternal interface.

Interestingly, on the basis of *in vivo* experiments in mice, Croy et al. suggest that uNK-cell-derived IFN-γ modifies the expression of genes in the uterine vasculature and stroma [47, 48]. Since implantation sites in uNK cell deficient mice and mice with disrupted IFN-γ signaling display anomalies in decidua and its spiral arteries, IFN-γ appears to initiate vessel instability and, thus, facilitates pregnancy-induced remodeling of decidual arteries, suggesting a pregnancy-protective role of IFN-γ [48]. However, positive correlations between augmented blood pressure and concentrations of the Th1 type cytokines IL-2 and IFN-γ were described in PE patients [8].

In conclusion, in our mouse model, the main characteristics of PE could be mimicked by adoptively transferring activated Th1-like immune cells exclusively in pregnant mice. We conclude that the new model presented in this paper will be extremely useful in fostering research on the pathophysiology of PE and opens possibilities for new therapy strategies.

4 Materials and methods

4.1 Mice

All mice were obtained from BgVV (Berlin, Germany) and maintained in a barrier animal facility. Animal care and experimental procedures were approved by the German ministry and followed according to institutional guidelines (LaGetSi,

Berlin, Germany; Reg 0112/02). Two-month-old BALB/c females were housed with three-month-old C57BL/6 males and checked for vaginal plugs every morning. The day of the plug was considered day 0 of pregnancy; plugged females were further removed from breeding cages and randomized. Non-pregnant animals (n=20) were also randomized. Pregnant (n=8) and non-pregnant (n=10) females received two doses of 10^7 activated Th1-switched splenocytes (100 μl) i.v., between 9 and 12 AM on days 12 and 14 of gestation or experiment. A second injection of activated cells was given to boost the effect on initiating PE-like symptoms. Control groups from pregnant (n=6) or non-pregnant (n=10) females received 100 μl sterile PBS (PAA Laboratories GmbH, Linz, Austria) instead of cells i.v. At day 14 of pregnancy or experiment, the mice were killed and analysis carried out. In some mice, pre-term labor and pain could be observed on gestation day 14, whereby pre-term labor was diagnosed by the presence of blood at the area of the vagina of the animals or the presence of blood in the cage bedding. Signs of pain were defined by obvious distress, *e.g.* uncoordinated "shaking" movements of the animals and aggressive behavior.

4.2 Measurement of blood pressure

We first conditioned non-pregnant 6- to 8-week-old BALB/c females in the tail-cuff blood pressure apparatus every other day for about 20 min between 9 and 11 AM for 10 days. Pregnant and non-pregnant mice were evaluated for blood pressure at days 1, 3, 5, 7, 9, 11, 13 and 14 of pregnancy or experiment. For measuring blood pressure, animals were placed onto a warm plate (the temperature was set at 37°C). A 17 mm tail cuff was applied to the tail base and a pulse transmitter was applied to the tail. The apparatus (TSE BP-Systems, Bad Homburg, Germany) was calibrated to insufflate from 90 mm Hg to 300 mm Hg. A rest period of insufflation of 1 s was allowed between each measurement. We recorded 10 tracings without movement artifacts, which were averaged and considered as the systolic blood pressure value for each mouse.

4.3 Cell harvesting

Briefly, spleens from female BALB/c mice were crushed in culture dishes with RPMI medium (Biochrom AG, Berlin, Germany) containing 10% FCS (Seromed, Berlin, Germany) and filtered through a 100 μm cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Mononuclear cells were isolated by density gradient (Lympholyte-M Solution, Cedarlane, Ontario, Canada) and activated by incubation with anti-CD3 mAb (BD Biosciences, Heidelberg, Germany) at 3 μg/ml; the mAb was applied directly onto the cell pellet for 20 min [21]. The cells (1.5×10^6 cells/ml) were placed in culture media and incubated for an additional 30 h in an atmosphere containing 5% CO_2 at 37°C. The culture media consisted of RPMI 1640 containing Hepes (25 mmol/l) and glutamine (2 mmol/l) (Biochrom AG), supplemented with antibiotic mix (Gibco, Karlsruhe, Germany) and (where appropriate) 1.022 ng recombinant murine (rm) IL-2 and 4 ng rm IL-12 /ml (R & D Systems, Wiesbaden-Nordenstadt, Germany). Cell viability and the fold increase in cell number were determined by trypan-blue exclusion and light microscopy. The characteristics of the cells are described in Table 2 and noticeably indicate a Th1 predominance in activated splenocytes upon IL-2 and IL-12 stimulation. After being washed twice with PBS solution, cells were adjusted to a concentration of 10^7 cells /100 μl with sterile PBS and i.v. injected into the recipients.

4.4 Sample collection

At day 14 of pregnancy or experiment, blood samples were obtained by retro-orbital puncture, collected in tubes containing heparin and conserved at 4°C. Urine samples were obtained and conserved at 4°C. The animals were then killed and the uteri removed. Fetal rejection sites were identified by their small size and necrotic hemorrhagic appearance compared with normal embryos. We isolated uterine samples, which were cut in small pieces and conserved in HBSS at 4°C until use (average time 1 h) for flow cytometry studies. For immunohistochemical studies, the feto–

Table 2. Activation of lymphocytes with anti-CD3, rm IL-2 and rm IL-12 leads to a Th1 profile[a)]

Cells	% of positive cells						
	IFN-γ^+	TNF-α^+	IL-12^+	IL-4^+	IL-10^+	CCR5^5	CD2^+CD25^+
Untreated splenocytes	0.64	0.55	0.16	0.29	0.26	0.23	3.14
Activated splenocytes	3.37	0.58	0.38	0.20	0.26	5.25	15.58
Activated splenocytes +IL-2 + IL-12	4.71	8.29	2.68	0.65	0.85	20.21	39.25

[a)] This table shows the cytokine production and CCR5/CD25 surface expression after culture of various BALB/c splenocyte populations, as analyzed by flow cytometry: untreated splenocytes, anti-CD3-activated splenocytes, and anti-CD3-activated splenocytes incubated with 1.022 ng/ml rm IL-2 and 4.0 ng/ml rm IL-12. These splenocytes were used for cell transfer. Flow cytometry was performed after 30 h of culture at 37°C in an atmosphere with 5% CO_2.

maternal unit and one kidney were isolated, embedded in freezing medium (Jung, Nussloch, Germany), snap frozen in liquid nitrogen and kept at –80°C. Cryostat sections were cut at 8 μm, air-dried, fixed in acetone for 10 min and stored at –20°C. The remaining kidney was fixed in 10% neutral buffered formalin, embedded in paraffin and cut at 5 μm.

4.5 Histological analysis

Samples (uterus and kidneys) were stained with hematoxylin and eosin (Roth, Germany) and were evaluated for pathological characteristics by two investigators.

4.6 Flow cytometry analysis

Tubes with pieces of uteri were filled with HBSS containing 1 mM dithiothreitol (Sigma, Germany) and incubated under agitation and rotation for 20 min at 37°C. The cells were passed tube trough a 100 μm net (Beckton Dickinson) and washed with RPMI containing 10% FCS. White blood cells were obtained from whole blood after lysis of erythrocytes. Blood and uterine cells were incubated for 3 h with Brefeldin A [10^6 cells /ml medium containing 1 μl/ml of Golgi Plug™ (BD Pharmingen, Heidelberg, Germany)] at 37°C with 5% CO_2. Cells were washed, incubated with surface antibodies and fixed (Fix™ solution, Becton Dickinson, Erembodegem, Belgium). The cells were then permeabilized (FACS™ Permeabilizing Solution, Becton Dickinson) and incubated with the intracellular antibody. The cells were analyzed in a FACS Calibur (Becton Dickinson). Phycoerythrin-labeled antibodies against IFN-γ, TNF-α, IL-12, IL-4, IL-10 or CD25, the FITC-conjugated antibodies against CD2 and CD8 and the PerCP-Biotin-labeled antibody against CCR5 as well as the respective isotype controls were purchased from BD Bioscience.

4.7 Proteinuria

Urine samples were analyzed for proteinuria using a qualitative method (Combur Test, Roche, Mannheim, Germany). The test allows one to differentiate between values from 0, 0.3, 1.0 and 5.0 g/l. Proteinuria was considered positive when levels were >1.0 g/l.

4.8 Tracing of labeled cells, after adoptive transfer, by fluorescent microscopy and flow cytometry

We performed another set of experiments to investigate the target organ of the migrating transferred cells. On days 10 and 12 of pregnancy or experiment, C57/BL6-mated BALB/c (n=5) and non-pregnant BALB/c (n=5) female mice were given fluorescent Th1-like cells i.v. The Th1-like cells were obtained as previously described, washed three times in PBS and resuspended in 5 μM CFSE solution (10^7 cells/ml, Molecular Probes, Eugene, OR) for between 3 and 4 min at room temperature. The reaction was stopped by washing the cells four times with culture medium containing 10% FCS and three times with PBS. Before cell transfer, cell viability and count were checked. Thirty-five hours after the second cell transfer, the animals [49] were killed and samples of uterus as well as kidney were processed for flow cytometry for this study. Samples of uterus were obtained and processed as described previously, whereas one kidney was removed and processed as described elsewhere [49]. Immune cells from the uterus and kidney were labeled and analyzed using flow cytometry for CCR5, as previously described. For histological analysis of CFSE-labeled cell distribution, organs (kidneys, spleen, liver, lungs, intestine and placenta plus decidua) were frozen in Tissue-Tek medium (Jung), cut in 5-μm sections, mounted on slides and dried at room temperature in the dark. For further analysis, we counterstained our slides (using DAPI) and analyzed them under a fluorescent microscope.

4.9 Statistical analysis

All data, except blood pressure curves and proteinuria, were analyzed by the non-parametric Mann-Whitney U test. $p<0.05$ was considered as a significant difference. When analyzing blood pressure curves, differences between the groups over the period of gestation were analyzed with the non-parametric Kruskall Wallis H test, whereas the variation within the same group (*i.e.* at the beginning and at the end of the experiment) was analyzed using the Friedmann test. Proteinuria values were analyzed with the Chi-square method, using as a limiting value of 1 g/l as proteinuria.

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References

1 **Robillard, P. Y.,** Interest in preeclampsia for researchers in reproduction. *J. Reprod. Immunol.* 2002. **53:** 279–287.

2 **Pijnenborg, R., Luyten, C., Vercruysse, L. and Van Assche, F. A.,** Attachment and differentiation in vitro of trophoblast from normal and preeclamptic human placentas. *Am. J. Obstet. Gynecol.* 1996. **175:** 30–36.

3 **Zhou, Y., Damsky, C. and Fisher, S.,** Pre-eclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. *J. Clin. Invest.* 1999. **99:** 2152–2164.

4 **Redman, C. W. G., Sacks, G. P. and Sargent, I. L.,** Preeclampsia: An excessive maternal inflammatory response to pregnancy. *Am. J. Obstet. Gynecol*. 1999. **180:** 499–506.

5 **Roberts, J. M., Taylor, R. N. and Goldfien, A.,** Clinical and biochemical evidence of endothelial cell dysfunction in the pregnancy syndrome preeclampsia. *Am. J. Hypertens.* 1991. **8:** 700–708.

6 **Faas, M. M., Schuiling, G., Baller, J. F. W., Visscher, C. A. and Bakker, W. W.,** A new animal model for human preeclampsia: ultralow dose endotoxin infusion in pregnant rats. *Am. J. Obstet. Gynecol.* 1994. **171:** 158–164.

7 **Faas, M. M. and Schuiling, G. A.,** Pre-eclampsia and the inflammatory response. *Eur. J. Obstet. and Gynecol. and Reprod. Biol.* 2001. **95:** 213–217.

8 **Saito, S., Umekage, H., Sakamoto, Y., Sakai, M., Tanebe, K., Sasaki, Y. and Morikawa, H.,** Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with pre-eclampsia. *Am. J. Reprod. Immunol.* 1999. **5:** 297–306.

9 **Sacks, G. P., Studena, K., Sargent, I. L. and Redman, C. W.,** CD11b expression on circulating neutrophils in pre-eclampsia. *Clin. Sci.* 1997. **93:** 187–189.

10 **Oian, P., Omsjo, I., Maltau, J. M. and Osterud, B.,** Increased sensitivity to thromboplastin synthesis in blood monocytes from pre-eclamptic patients. *Br. J. Obstet. Gynaecol.* 1985. **92:** 511–517.

11 **Greer, I. A., Haddad, N. G., Dawes, J., Johnstone, F. D. and Calder, A. A.,** Neutrophil activation in pregnancy-induced hypertension. *Br. J. Obstet. Gynaecol.* 1989. **96:** 978–982.

12 **Darmochwal-Kolarz, D., Leszczynska-Gorzelak, B., Rolinski, I. and Oleszczuk, J.,** T helper 1- and T helper 2-type cytokine imbalance in pregnant women with pre-eclampsia. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 1999. **86:** 165–170.

13 **Stark, J. M.,** Pre-eclampsia and cytokine induced oxidative stress. *Br. J. Obstet. Gynaecol.* 1993. **100:** 105–109.

14 **Ma, C., Zhuang, Y. and Xu, Y.,** The effect of inhibition of nitric oxide synthesis on vasoregulatory factors in pregnant rats. *Zhonghua Fu Chan Ke Za Zhi.* 1999. **34:** 521–524.

15 **Takimoto, E., Ishida, J., Sugiyama, F., Horiguchi, H., Murakami, K. and Fukamizu, A.,** Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science* 1996. **274:** 995–998.

16 **Kanayama, N., Tsujimura, R., She, L., Maehara, K. and Terao, T.,** Cold-induced stress stimulates the sympathetic nervous system, causing hypertension and proteinuria in rats. *J. Hypertens.* 1997. **15:** 383–389.

17 **Halim, A., Kanayama, N., Maehara, K., Takahashi, M. and Terao, T.,** HELLP syndrome-like biochemical parameters obtained with endothelin-1 injections in rabbits. *Gynecol. Obstet. Invest*. 1993. **35:** 193–198.

18 **Hayakawa, S., Fujikawa, T., Fukuoka, H., Chisima, F., Karasaki-Suzuki, M., Ohkoshi, E., Ohi, T., Kiyoshi Fujii, M., Tochigi, K., Satoh, T. et al.,** Murine fetal resorption and experimental pre-eclampsia are induced by both excessive Th1 and Th2 activation. *J. Reprod. Immunol.* 2000. **47:** 121–138.

19 **Davisson, R. L., Hoffmann, D. S., Butz, G. M., Aldape, G., Schlager, G., Merrill, D. C., Sethi, S., Weiss, R. M. and Bates, J. N.,** Discovery of a spontaneous genetic mouse model of pre-eclampsia. *Hypertension* 2002. **39:** 337–342.

20 **Anderson, P. M., Blazar, B. R., Bach, F. H. and Ochoa, A. C.,** Anti-CD3 + IL-2-stimulated murine killer cells. In vitro generation and in vivo antitumor activity. *J. Immunol.* 1989. **142:** 1383–1394.

21 **Saxton, M. L., Longo, D. L., Wetzel, H. E., Tribble, H., Alvord, W. G., Kwak, L. W., Leonard, A. S., Ullmann, C. D., Curti, B. D. and Ochoa, A. C.,** Adoptive transfer of anti-CD3-activated CD4+ T cells plus cyclophosphamide and liposome-encapsulated interleukin-2 cure murine MC-38 and 3LL tumors and establish tumor-specific immunity. *Blood* 1997. **89:** 2529–2536.

22 **Onodera, K., Chandraker, A., Volk, H. D., Lehmann, M., Korom, S., Stadlbauer, T. H., Kato, K., Kasai, S., Sayegh, M. H. and Kupiec-Weglinski, J. W.,** Role of regulatory T cells in the "infectious" tolerance pathway in transplant recipients. *Transplant. Proc.* 1998. **30:** 13–15.

23 **Schmidt, H., Filantenkov, A., Reutzel-Selke, A., Seifert, M., Doebis, C., Jurisch, A., Pratschke, J., Neuhaus, P., Tullius, S. G. and Volk, H. D.,** Adoptive transfer of regulatory T cells following chronic allograft rejection induces tolerance for secondary allografts. *Transplant. Proc.* 2002. **34:** 2893–2894.

24 **Lin, H., Mossmann, T. R., Guilbert, L., Tuntipopipat, S. and Wegmann, T. G.,** Synthesis of T helper 2-type cytokines at the feto-maternal interface. *J. Immunol.* 1993. **151:** 4562–4573.

25 **Wegmann, T. G., Lin, H., Guilbert, L. and Mossmann, T.,** Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomen? *Immunol. Today*. 1993. **14:** 353–356.

26 **Raghupathy, R.,** Th1-type immunity is incompatible with successful pregnancy. *Immunol. Today*. 1997. **10:** 434–437.

27 **Zenclussen, A. C., Fest, S., Sehmsdorf, U.-S., Hagen, E., Klapp, B. F. and Arck, P. C.,** Up-regulation of decidual P-selectin is associated with increased number of Th1 producing cells populations in patients with spontaneous abortion. *Cell. Immunol.* 2001. **213:** 94–103.

28 **Mellor, A. L., Sivakumar, J., Chandler, P., Smith, K., Molina, H., Mao, D. and Munn, D. H.,** Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. *Nat. Immunol.* 2001. **2:** 64–68.

29 **Hefler, L. A., Tempfer, C. B., Moreno, R. M., O'Brien, W. E. and Gregg, A. R.,** Endothelial-derived nitric oxide and angiotensinogen: blood pressure and metabolism during mouse pregnancy. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001. **280:** 174–182.

30 **Nakajima, A., Hirose, S., Yagita, H. and Okumura, K.,** Roles of IL-4 and IL-12 in the development of lupus in NZB/W F1 mice. *J. Immunol*. 1997. **158:** 1466–1472.

31 **Torii, I., Morikawa, S. and Harada, T.,** MD41, a novel T helper 0 clone, mediates mast-cell dependent delayed-type hypersensitivity in mice. *Immunology* 2002. **107:** 426–434.

32 **Oelke, M., Moehrle, U., Chen, J. L., Behringer, D., Cerundolo, V., Lindemann, A. and Mackensen, A.,** Generation and purification of CD8+ melan-A-specific cytotoxic T lymphocytes for adoptive transfer in tumor immunotherapy. *Clin. Cancer Res.* 2000. **6:** 1997–2005.

33 **Pober, J. S.,** Activation and injury of endothelial cells by cytokines. *Pathol. Biol. (Paris)* 1998. **46:** 159–163.

34 **von Dadelzen, P. and Magee, L.,** Could an infectious trigger explain the differential maternal response to the shared placental pathology of preeclampsia and normotensive intrauterine growth restriction? *Acta Obstet. Gynecol. Scand.* 2002. **81:** 642–648.

35 **Medawar, P. B.,** Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symp. Soc. Exp. Biol.* 1953. **44:** 320–338.

36 **Sargent, I. L.,** Maternal and fetal immune responses during pregnancy. Exp. *Clin. Immunogenet.* 1993. **10:** 85–102.

37 **Mellor, A. L. and Munn, D. H.,** Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? *Immunol. Today* 1999. **20:** 469–473.

38 **Annunziato, F., Galli, G., Romagnani, P., Cosmi, L., Manetti, R., Maggi, E. and Romagnani, S.,** Chemokine receptors and other surface molecules preferentially associated with human Th1 or Th2 cells. *Microbes Infect.* 1999. **1:** 103–106.

39 **Weber, C., Weber, K., Klier, C., Gu, S., Wank, R., Horuk, R. and Nelson, P. J.,** Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and Th1-like/CD45RO+ T cells. *Blood* 2001. **97:** 1144–1146.

40 **Clausen, T., Djurovic, S., Brosstad, F. R., Berg, K. and Henriksen, T.,** Altered circulating levels of adhesion molecules at 18 weeks' gestation among women with eventual preeclampsia: indicators of disturbed placentation in absence of evidence of endothelial dysfunction? *Am. J. Obstet. Gynecol.* 2000. **182:** 321–325.

41 **Hill, J. A., Haimovici, F. and Anderson, D. J.,** Products of activated lymphocytes and macrophages inhibit mouse embryo development in vitro. *J. Immunol.* 1987. **139:** 2250–2254.

42 **Todt, J. C., Yang, Y., Lei, J., Lauria, M. R., Sorokin, Y., Cotton, D. B. and Yelian, F. D.,** Effects of tumor necrosis factor-alpha on human trophoblast cell adhesion and motility. *Am. J. Reprod. Immunol.* 1996. **36:** 65–71.

43 **Guo, G., Morrissey, J., Mc Cracken, R., Tolley, T., Liapis, H. and Klahr, S.,** Contributions of angiotensin II and tumor necrosis factor-alpha to the development of renal fibrosis. *Am. J. Physiol. Renal Physiol.* 2001. **280:** 777–785.

44 **Knotek, M., Rogachev, B., Wang, W., Ecder, T., Melnikov, V., Gengaro, P. E., Esson, M., Edelstein, C. L., Dinarello, C. A. and Schrier, R. W.,** Endotoxemic renal failure in mice: Role of tumor necrosis factor independent of inducible nitric oxide synthase. *Kidney Int*. 2001. **59:** 2243–2249.

45 **Bochner, B. S., Klunk, D. A., Sterbinsky, S. A., Coffman, R. L. and Schleimer, R. P.,** IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J. Immunol.* 1995. **154:** 799–803.

46 **de Kossodo, S., Moore, R., Gschmeissner, S., East, N., Upton, C. and Balkwill, F. R.,** Changes in endogenous cytokines, adhesion molecules and platelets during cytokine-induced tumour necrosis. *Br. J. Cancer* 1995. **72:** 1165–1172.

47 **Croy, B. A., Ashkar, A. A., Minhas, K. and Greenwood, J. D.,** Can murine uterine natural killer cells give insights into the pathogenesis of preeclampsia? *J. Soc. Gynecol. Investig.* 2000. **7:** 12–20.

48 **Ashkar, A. A., Di Santo, J. P. and Croy, B. A.,** Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J. Exp. Med.* 2000.**192:** 259–270.

49 **Hammer, M. H., Zhai, Y., Katori, M., Ritter, T., Volk, H. D., Coito, A. J. and Kupiec-Weglinski, J. W.,** Homing of in vitro-generated donor antigen-reactive CD4+ T lymphocytes to renal allografts is alpha 4 beta 1 but not alpha L beta 2 integrin dependent. *J. Immunol.* 2001. **166:** 596–601.

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