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Peripheral blood galectin-1-expressing T and natural killer cells in normal pregnancy and preeclampsia

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Abstract The purpose of this study was to determine whether the proportion of galectin-1-expressing peripheral blood T and NK cells is altered in normal pregnancy and preeclampsia (PE). We also examined whether circulating levels of galectin-1 and anti-galectin-1 autoantibodies are affected in PE. Seventy preeclamptic patients, 75 healthy pregnant and 21 healthy non-pregnant women were involved in this study. Serum galectin-1 and anti-galectin-1 autoantibody levels were measured by ELISA. Intracellular galectin-1 expression of lymphocytes was determined with flow cytometry. Serum galectin-1 and anti-galectin-1 IgG levels did not differ significantly between the healthy pregnant and the PE group. In healthy pregnant women, significantly higher percentage of T and NK cells expressed gal-1 in their cytoplasma than in healthy non-pregnant women. However, the proportion of galectin-1-expressing peripheral blood T and NK cells was markedly decreased in PE compared to normal pregnancy, which might contribute to the activation of innate and acquired immune cells.

Introduction

Preeclampsia, characterized by hypertension and proteinuria developing after midgestation in a previously normotensive woman, is a severe complication of human pregnancy with a worldwide incidence of 2–10% [1]. It is one of the leading causes of maternal, as well as perinatal morbidity and mortality, even in developed countries. Despite intensive research efforts, the etiology and pathogenesis of preeclampsia are not fully understood. There is an increasing body of evidence that the immunoregulatory system is down-

Abbreviations ANCOVA, analysis of covariance; gal-1, galectin-1; IUGR, intrauterine growth restriction; mAb, monoclonal antibody; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase-1

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regulated in preeclampsia leading to excessive immunostimulation. It has been recently reported that peripheral blood $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells decrease in preeclampsia [2–4]. As a result, both T and NK cells are activated and show a type 1 cytokine-predominant state [5,6]. The excessive production of type 1 cytokines along with other potent inflammatory mediators, such as proteases and free oxygen radicals, may trigger a generalized endothelial dysfunction characteristic of the maternal syndrome of preeclampsia [7].

Galectin-1 (gal-1), a member of a phylogenetically conserved family of β-galactoside-binding mammalian lectins, is a 14 kDa protein consisting of 135 amino acids. This protein is secreted as a non-covalent homodimer and interacts with poly-N-acetyl-lactosamine-containing cell surface glycoconjugates [8]. Within the immune system, it is expressed by activated T cells, B cells, NK cells and macrophages [9–12]. Galectin-1 exerts powerful immunoregulatory effects through various mechanisms. This protein inhibits T cell proliferation and induces apoptosis of activated, but not resting, $CD8^+$ T cells, Th1 and Th17 $CD4^+$ cells by binding to the cell surface glycoproteins on these cells [9,13,14]. Additionally, gal-1 promotes turnover of leukocytes without inducing apoptosis and negatively regulates T cell activation [15,16]. Galectin-1 has been demonstrated *in vitro* to inhibit T cell adhesion to the extracellular matrix and to abrogate the secretion of proinflammatory cytokines [17]. Furthermore, *in vivo* administration of gal-1 in experimental models of autoimmunity skewed the balance toward a Th2-dominant cytokine profile [18,19]. Recent data show that gal-1 promotes fetomaternal tolerance, since treatment with recombinant gal-1 prevented fetal loss in an abortion-prone model. Interestingly, the protective effect of gal-1 was abrogated in mice depleted on the Treg cell subset [20].

The purpose of this study was to determine whether the proportion of peripheral blood T and NK cells that express intracellular galectin-1 is altered in normal pregnancy and preeclampsia. We also measured circulating levels of galectin-1 and anti-galectin-1 autoantibodies in a larger number of preeclamptic patients and healthy pregnant women, and examined whether those are affected in preeclampsia and related to the proportions of galectin-1-expressing peripheral blood lymphocytes. Circulating angiogenic factors were also determined in normal pregnancy and preeclampsia and their relationship with circulating galectin-1 and anti-galectin-1 autoantibody levels, as well as with intracellular galectin-1 expression of peripheral blood lymphocytes, was assessed.

Materials and methods

Study patients

Our study was designed using a case-controlled approach. Seventy preeclamptic patients, 75 healthy pregnant women with uncomplicated pregnancies and 21 healthy non-pregnant women were involved in the study. The study participants were enrolled in the First Department of Obstetrics and Gynecology, at the Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The women were fasting, none of the pregnant women were in active labor, and none had rupture of membranes. The healthy non-pregnant women were in the early follicular phase of the menstrual cycle (between cycle days 3 and 7), and none of them received hormonal contraception.

Preeclampsia was defined by increased blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 occasions at least 6 h apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24 h or ≥1+ on dipstick in the absence of urinary tract infection). Blood pressure returned to normal by 12 weeks postpartum in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure ≥160 mmHg systolic or ≥110 mmHg diastolic, or proteinuria ≥5 g/24 h (or ≥3+ on dipstick). Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation (between 20 and 33 completed gestational weeks). Fetal growth restriction (IUGR) was diagnosed if the fetal birth weight was below the 10th percentile for gestational age and gender, based on Hungarian birth weight percentiles [21].

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University (IRB No. 188/2008), and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

Biological samples

Blood samples were obtained from an antecubital vein into plain and heparinized tubes. After centrifugation of native venous blood samples at room temperature with a relative centrifugal force of 3000 g for 10 min, the aliquots of serum were stored at −80 °C until the analyses. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by the standard Ficoll–Hypaque density–gradient centrifugation method. The aliquots of PBMC were stocked frozen in fetal calf serum (FCS) containing 10% dimethyl sulfoxide (DMSO) at −80 °C until the measurements.

Determination of circulating galectin-1 levels

Human gal-1 levels were measured with a specific sandwich ELISA protocol, as previously described [22]. Inmunolon 2 ELISA plates (Dynatech Laboratories, Chantilly, Virginia, USA) were covered with polyclonal anti-gal-1 antibodies (10 μg/ml; PeproTech, Rocky Hill, New Jersey, USA), and washed with washing buffer (0.05% Tween-20 in PBS). Plates were blocked with 1% BSA in PBS. Individual wells were incubated with serial dilutions of gal-1 or serum samples for 1 h at room temperature. Wells were washed and incubated with biotinylated polyclonal anti-gal-1 antibodies (0.3 μg/ml in PBS 0.1% BSA; Peprotech). Plates were washed 6 times and

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incubated with HRP-conjugated streptavidin (Zymed Laboratories, San Francisco, California, USA). After 6 additional washes, a colorimetric reaction was developed with TMB substrate (Pierce Biotechnology, Rockford, Illinois, USA). The reaction was stopped by adding one volume of 2 N H_2SO_4. Absorbance at 450 nm was recorded.

Determination of circulating anti-galectin-1 autoantibody levels

Human anti-gal-1 IgG levels were determined using a specific sandwich ELISA protocol employing recombinant human gal-1 as capture antibody (Sigma-Aldrich, St. Louis, Missouri, USA), anti-human gal-1 mAb as standard (Santa Cruz Biotechnology, Santa Cruz, California, USA), peroxidase-conjugated Goat anti-human IgG like as detection antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA), and TBS Gallati as substrate. Human gal-1 antibody standard was diluted to give a calibration curve within the range of expected anti-gal-1 expression. The serum samples were tested at 1/20 dilution in buffer. Fifty microliters of standard and samples were loaded manually into the wells, and the assay was performed using a Triturus automated ELISA analyzer (Grifols, Barcelona, Spain) at 490 nm.

Flow cytometry

After thawing, isolated mononuclear cells were washed twice with phosphate-buffered saline (PBS) and their viability was assessed by trypan blue exclusion (consistently >90%). Non-specific binding sites were blocked by incubation with 10% mouse serum for 10 min at room temperature. Cells were stained with fluorescein isothiocyanate (FITC)-labelled anti-human CD3 and phycoerythrin-cyanine 5 (PC5)-labelled anti-human CD56 or FITC-conjugated anti-human CD8 and PC5-conjugated anti-human CD4 mouse monoclonal antibodies (BD Pharmingen, San Diego, California, USA) for 15 min at room temperature in dark and then washed with washing buffer. Red blood cells were lysed by incubation with 1.5 ml of 1X fluorescence-activated cell sorter (FACS) Lysing Solution (BD Biosciences, San Jose, California, USA) for 10 min at room temperature in dark. Cells were centrifuged and the supernatant was removed. 500 μl of 1X FACS Permeabilizing Solution (BD Biosciences) was added and the mixture was incubated for 10 min at room temperature in the dark. After washing twice with washing buffer, the permeabilized cells were treated with in-house biotinylated (Biotin Labeling Kit-NH_2, Dojindo Laboratories, Tabaru, Kumamoto, Japan) mouse anti-human galectin-1 monoclonal antibody (Vector Laboratories, Burlingame, California, USA) for 30 min at room temperature in dark. In-house biotin-conjugated (Dojindo Laboratories), isotype-matched mouse immunoglobulin (Ig) G_1 (R&D Systems, Minneapolis, Minnesota, USA) was used as a control for detecting non-specific binding. Following washing off the unbound biotinylated antibodies, the cells were stained with phycoerythrin (PE)-labelled streptavidin (Immunotech, Marseille, France) for 15 min at room temperature in the dark. After washing twice with washing buffer, the cells were resuspended in 1% paraformaldehyde in PBS. Flow cytometric analysis was performed on a FACSCalibur flow cytometer and data were processed using CellQuest Pro software (BD Biosciences). A real-time gate was set around the viable lymphocytes based on their forward scatter/side scatter profile. Contaminating monocytes and necrotic cells were excluded from the analysis.

Determination of circulating angiogenic factors

Serum total soluble fms-like tyrosine kinase-1 (sFlt-1) and biologically active placental growth factor (PlGF) levels were measured by electrochemiluminescence immunoassay (Elecsys, Roche, Mannheim, Germany, Cat. No. 05109523 and 05144671, respectively) on a Cobas e 411 analyzer (Roche, Mannheim, Germany), as we described previously [23].

Statistical analysis

The normality of continuous variables was assessed using the Shapiro-Wilk's *W*-test. As the continuous variables were not normally distributed, nonparametric statistical methods were applied. To compare continuous variables between two groups, the Mann–Whitney *U*-test was used, whereas to compare them among multiple groups, the Kruskal–Wallis analysis of variance by ranks test was performed. Multiple comparisons of mean ranks for all groups were carried out as post-hoc tests. The Fisher exact and Pearson χ^2 tests were used to compare categorical variables between groups. The Spearman rank order correlation was applied to calculate correlation coefficients. As circulating galectin-1 and anti-galectin-1 autoantibody levels and the proportions of galectin-1-positive lymphocytes showed skewed distributions, we performed analysis of covariance (ANCOVA) with logarithmically transformed data.

Statistical analyses were carried out using the following software: STATISTICA (version 8.0; StatSoft, Inc., Tulsa, Oklahoma, USA) and Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS, Inc., Chicago, Illinois, USA). For all statistical analyses, a two-tailed $p<0.05$ was considered statistically significant.

In the article, data are reported as median (25–75 percentile) for continuous variables and as number (percentage) for categorical variables, if not otherwise specified.

Results

Patient characteristics

The clinical characteristics of the study participants are described in Table 1. There was no statistically significant difference in terms of age among the study groups. Furthermore, no significant differences were observed in gestational age at blood collection and the percentage of smokers between preeclamptic patients and healthy pregnant women. However, all of the other clinical features presented in Table 1 differed significantly among our study groups. The gestational age at delivery and the fetal birth weight were significantly lower in the preeclamptic group compared with the group of healthy pregnant women. Fetal growth restriction was absent in healthy pregnant women,

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Table 1 Clinical characteristics of healthy non-pregnant and pregnant women and preeclamptic patients.

	Healthy non-pregnant women (n=21)	Healthy pregnant women (n=75)	Preeclamptic patients (n=70)
Age (years)	29 (27–34)	31 (28–34)	31 (28–35)
BMI at blood collection (kg/m^2)	22.3 (20.8–24.5)	27.0 (24.5–31.6) [b]	29.3 (26.7–33.0) [b,c]
Smokers	6 (28.6%)	4 (5.3%) [a]	4 (5.7%) [a]
Primiparas	n.a.	26 (34.7%)	52 (74.3%) [d]
Systolic blood pressure (mmHg)	111 (108–120)	120 (110–125)	150 (145–165) [b,d]
Diastolic blood pressure (mmHg)	70 (70–80)	70 (70–80)	100 (90–101) [b,d]
Gestational age at blood collection (weeks)	n.a.	36 (32–38)	35.5 (32–37)
Gestational age at delivery (weeks)	n.a.	39 (38–40)	37 (32–38) [d]
Fetal birth weight (grams)	n.a.	3415 (3200–3820)	2575 (1540–3100) [d]
Fetal growth restriction	n.a.	0 (0%)	16 (22.9%) [d]

Data are presented as median (25–75 percentile) for continuous variables and as number (percentage) for categorical variables.
BMI: body mass index; n.a.: not applicable.
[a] $p<0.05$ versus healthy non-pregnant women.
[b] $p<0.001$ versus healthy non-pregnant women.
[c] $p<0.05$ preeclamptic patients versus healthy pregnant women.
[d] $p<0.001$ preeclamptic patients versus healthy pregnant women.

whereas the frequency of this condition was 22.9% in the preeclamptic group. Thirty-three women had severe preeclampsia and 29 patients experienced early onset of the disease.

Circulating gal-1 and anti-galectin-1 autoantibody levels of healthy pregnant women and preeclamptic patients

Serum gal-1 and anti-gal-1 IgG levels did not differ significantly between the healthy pregnant and the preeclamptic group (Table 2), even after adjustment for age, primiparity, BMI and gestational age at blood sampling in ANCOVA. In the group of preeclamptic patients, no statistically significant differences were observed in serum gal-1 and anti-gal-1 IgG levels between patients with mild and severe preeclampsia, between patients with late and early onset of the disease or between preeclamptic patients with and without fetal growth restriction (Table 2). In addition, clinical features of the study participants presented in Table 1 were not related to circulating levels of gal-1 and anti-gal-1 autoantibodies in either study group.

Intracellular gal-1 expression of unstimulated peripheral blood lymphocytes in healthy non-pregnant and pregnant women and preeclamptic patients

We determined intracellular galectin-1 expression of peripheral blood T (CD3$^+$), NK (CD56$^+$), helper (CD4$^+$) and cytotoxic (CD8$^+$) T lymphocytes in 21 healthy non-pregnant women, in 29 of our healthy pregnant women and in 23 of our preeclamptic patients by flow cytometric analysis. Representative examples are displayed in Fig. 1. Table 3 and Fig. 2 reveal the proportion of gal-1-expressing cells in relation to the parent populations in our study groups. In healthy pregnant women, significantly higher percentage of T (both CD4$^+$ helper and CD8$^+$ cytotoxic) and NK cells expressed gal-1 in their cytoplasma than in healthy non-pregnant women. However, as demonstrated in Table 3 and Fig. 2, the proportion of gal-1-positive peripheral blood T (both helper and cytotoxic) and NK cells was markedly decreased in preeclamptic patients as compared to healthy pregnant women. The differences in these variables between the two study groups remained significant even after adjustment for age, primiparity, BMI and gestational age at blood draw in ANCOVA (Table 3).

Table 2 Circulating galectin-1 and anti-galectin-1 autoantibody levels of the pregnant study groups.

	Serum galectin-1 level (ng/ml)	Serum anti-galectin-1 IgG level (mg/ml)
Healthy pregnant women (n=75)	50.3 (27.2–114.3)	2.0 (1.45–2.43)
Preeclamptic patients (n=70)	58.6 (44.3–69.6)	1.84 (1.43–2.27)
Mild preeclampsia (n=37)	59.0 (44.3–69.5)	1.77 (1.39–2.18)
Severe preeclampsia (n=33)	58.3 (45.6–69.6)	1.87 (1.53–2.27)
Late onset preeclampsia (n=41)	59.0 (44.3–69.6)	1.77 (1.53–2.21)
Early onset preeclampsia (n=29)	58.3 (45.6–69.5)	1.88 (1.34–2.34)
Preeclampsia without IUGR (n=54)	59.1 (43.7–69.6)	1.80 (1.45–2.34)
Preeclampsia with IUGR (n=16)	50.2 (48.6–69.6)	1.85 (1.13–2.12)

Data are presented as median (25–75 percentile).
IgG: Immunoglobulin G; IUGR: intrauterine growth restriction.

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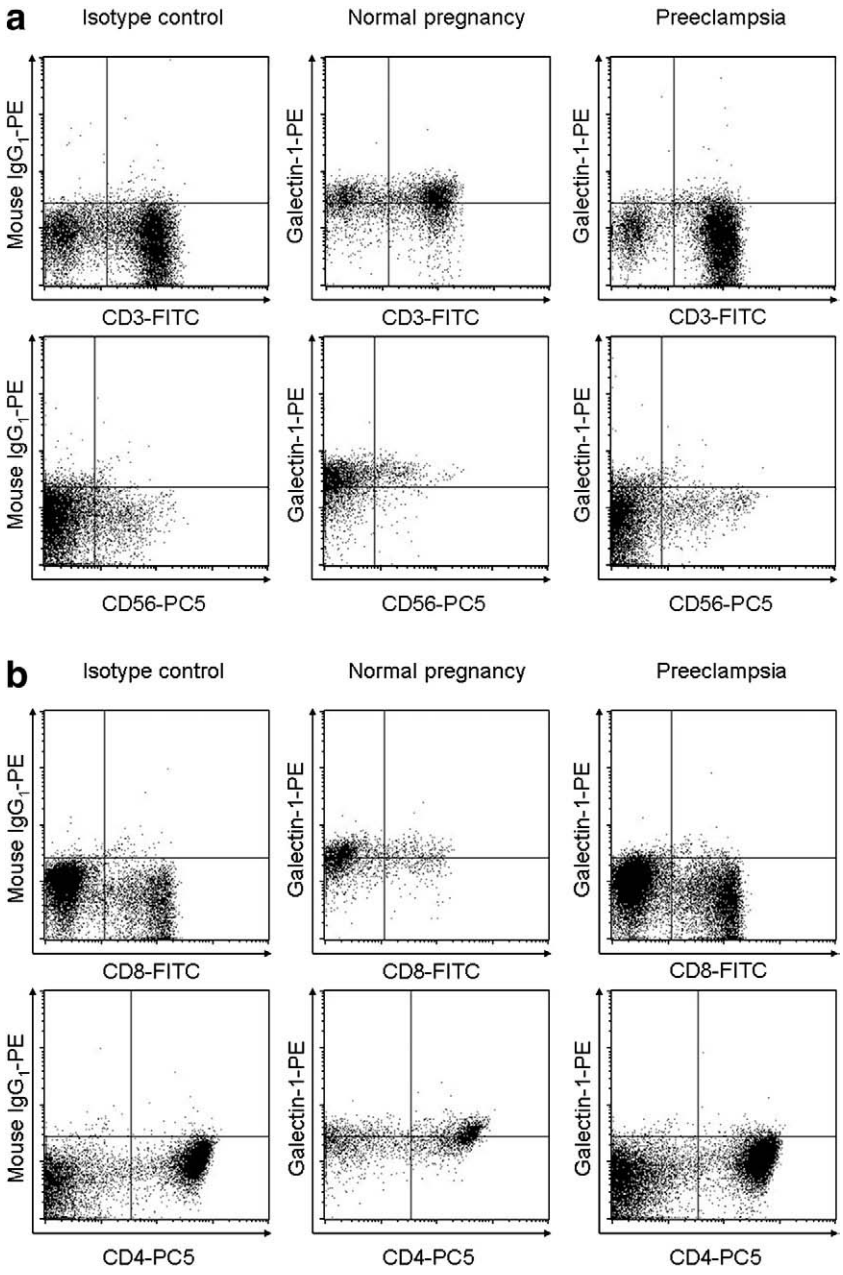

Fig. 1 Representative flow cytometric pattern demonstrating intracellular galectin-1 expression of peripheral blood $CD3^+$, $CD56^+$ (Fig. 1a), $CD4^+$ and $CD8^+$ (Fig. 1b) lymphocytes in a healthy pregnant woman and a preeclamptic patient. FITC: fluorescein isothiocyanate; PC-5: phycoerythrin-cyanine 5; PE: phycoerythrin.

In the group of preeclamptic patients, there were no significant differences in the frequency of gal-1-expressing lymphocyte populations between patients with mild and severe preeclampsia, between patients with late and early onset of the disease or between preeclamptic patients with and without fetal growth restriction (data not shown). Furthermore, the proportion of gal-1-positive cells did not differ between primiparas and multiparas, and did not correlate with clinical characteristics – including systolic and diastolic blood pressures – and circulating gal-1 and anti-gal-1 autoantibody levels of the study subjects in either study group.

Circulating angiogenic factors and their relationship with circulating gal-1 and anti-galectin-1 autoantibody levels and intracellular gal-1 expression of unstimulated peripheral blood lymphocytes in normal pregnancy and preeclampsia

We assessed circulating angiogenic factors in 29 of our healthy pregnant women and in 23 of our preeclamptic patients. Serum concentrations of sFlt-1 were significantly higher, while those of PlGF were significantly lower in preeclamptic patients than in healthy pregnant women

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Table 3 Proportion of peripheral blood galectin-1-positive lymphocyte populations in healthy non-pregnant and pregnant women and preeclamptic patients.

Galectin-1-positive cells/parent population	Healthy non-pregnant women (n=21)	Healthy pregnant women (n=29)	Preeclamptic patients (n=23)
$CD3^+$Galectin-$1^+/CD3^+$ (%)	8.4 (6.3–10.5)	59.0 (51.7–64.4)[a]	30.6 (21.3–34.4)[a,b]
Log ($CD3^+$Galectin-$1^+/CD3^+$ (%)) (adjusted mean ± SE)*	n.a.	1.75 ± 0.04	1.37 ± 0.04[b]
$CD4^+$Galectin-$1^+/CD4^+$ (%)	8.8 (7.5–11.8)	63.2 (55.0–70.5)[a,†]	33.1 (29.4–40.2)[a,b,†]
Log ($CD4^+$Galectin-$1^+/CD4^+$ (%)) (adjusted mean ± SE)*	n.a.	1.82 ± 0.04[†]	1.46 ± 0.04[b,†]
$CD8^+$Galectin-$1^+/CD8^+$ (%)	12.9 (10.5–15.2)	56.4 (52.6–58.7)[a,†]	30.5 (22.7–38.8)[a,b,†]
Log ($CD8^+$Galectin-$1^+/CD8^+$ (%)) (adjusted mean ± SE)*	n.a.	1.76 ± 0.04[†]	1.44 ± 0.04[b,†]
$CD56^+$Galectin-$1^+/CD56^+$ (%)	11.2 (8.0–17.1)	76.5 (70.3–82.9)[a]	42.9 (32.0–61.9)[a,b]
Log ($CD56^+$Galectin-$1^+/CD56^+$ (%)) (adjusted mean ± SE)*	n.a.	1.88 ± 0.04	1.55 ± 0.05[b]

Data are presented as median (25–75 percentile), if not otherwise specified.
SE: standard error; n.a.: not applicable.
* Adjustment was carried out for age, primiparity, BMI and gestational age at blood draw in analysis of covariance (ANCOVA).
[a] $p<0.001$ versus healthy non-pregnant women.
[b] $p<0.001$ preeclamptic patients versus healthy pregnant women.
[†] n=12.

(median (25–75 percentile), for sFlt-1: 10333 (6726–13538) pg/ml versus 2470 (1884–2998) pg/ml, $p<0.001$; for PlGF: 76.8 (44.4–88.3) pg/ml versus 217 (128–261) pg/ml, $p<0.001$). However, circulating levels of angiogenic factors did not show significant correlations with those of gal-1 and anti-galectin-1 autoantibodies or with intracellular gal-1 expression of unstimulated peripheral blood lymphocytes, either in healthy pregnant women or in preeclamptic patients (data not shown).

Discussion

In the present study, we measured circulating gal-1 and anti-gal-1 autoantibody levels, as well as intracellular gal-1 expression of unstimulated peripheral blood T and NK cells in normal pregnancy and preeclampsia. According to our findings, the majority of T (both $CD4^+$ helper and $CD8^+$ cytotoxic) and NK cells expressed gal-1 in their cytoplasma in healthy pregnant women, while only a small fraction of them did so in healthy non-pregnant women. In preeclampsia, the proportion of gal-1-expressing peripheral blood T (both helper and cytotoxic) and NK cells was markedly decreased as compared to normal pregnancy, suggesting decreased production of gal-1 by circulating T and NK cells in this pregnancy-specific disorder. However, circulating levels of gal-1 and anti-gal-1 autoantibodies were not altered in preeclamptic patients as compared to healthy pregnant women, nor were related to the proportions of gal-1-expressing peripheral blood lymphocytes determined by flow cytometry in either study group. In addition, changes in circulating angiogenic factors were not associated with intracellular gal-1 expression of peripheral blood lymphocytes in preeclampsia.

One of the most remarkable observations of our study is that significantly higher percentage of circulating T and NK cells expressed gal-1 in their cytoplasma in healthy pregnant than in non-pregnant women. Interestingly, estrogens and progesterone have been reported to up-regulate gal-1 expression in human endometrium and decidua, as well as in mouse uterine tissues [24,25]. A phylogenetically conserved estrogen responsive element in the 5' promoter region of the gal-1 gene could account for sexual steroid regulation of gal-1 expression [26]. Therefore, it is possible that estrogens and progesterone produced by the placenta are responsible – at least in part – for increased gal-1 expression of peripheral blood lymphocytes in the third trimester of normal pregnancy. Recent findings indicate a pivotal role for gal-1 in conferring fetomaternal tolerance [20]. Galectin-1 treatment skewed the cytokine balance toward a Th2-dominant profile at the fetomaternal interface in stress-challenged murine pregnancies. In addition, gal-1 induced the generation of tolerogenic, uterine dendritic cells, which in turn promoted the expansion of interleukin-10 (IL-10)-secreting regulatory T cells. Furthermore, a synergism has been demonstrated between gal-1 and progesterone in the maintenance of pregnancy. Secretion of gal-1 by decidual NK cells has recently been shown to induce T cell apoptosis at the maternal-fetal interface [22]. Our results that the majority of circulating T (helper and cytotoxic) and NK cells expressed gal-1 intracellularly in normal pregnancy are consistent with a central role of gal-1 in promoting maternal tolerance towards the semiallogeneic fetus. Galectin-1 production by peripheral blood lymphocytes might be involved in the establishment of a systemic Th2 environment and predominant regulatory T cell environment, which is essential for a successful pregnancy [27]. Expression of gal-1 by circulating T cells may represent an autocrine regulatory mechanism to inhibit antigen-induced T cell proliferation [9].

Another intriguing observation of our study is that the proportion of gal-1-positive T (both helper and cytotoxic) and NK cells in the peripheral blood is markedly decreased in preeclampsia compared to normal pregnancy. The reason for these findings is still unknown, but we hypothesize that down-regulation of gal-1 expression in peripheral blood lymphocytes is part of the generalized intravascular inflammatory reaction observed in preeclampsia, which is supposed to be triggered by placentally- and/or maternally-derived inflammatory stimuli [28]. It might be plausible that circulating T and NK cells, upon these stimuli, change their

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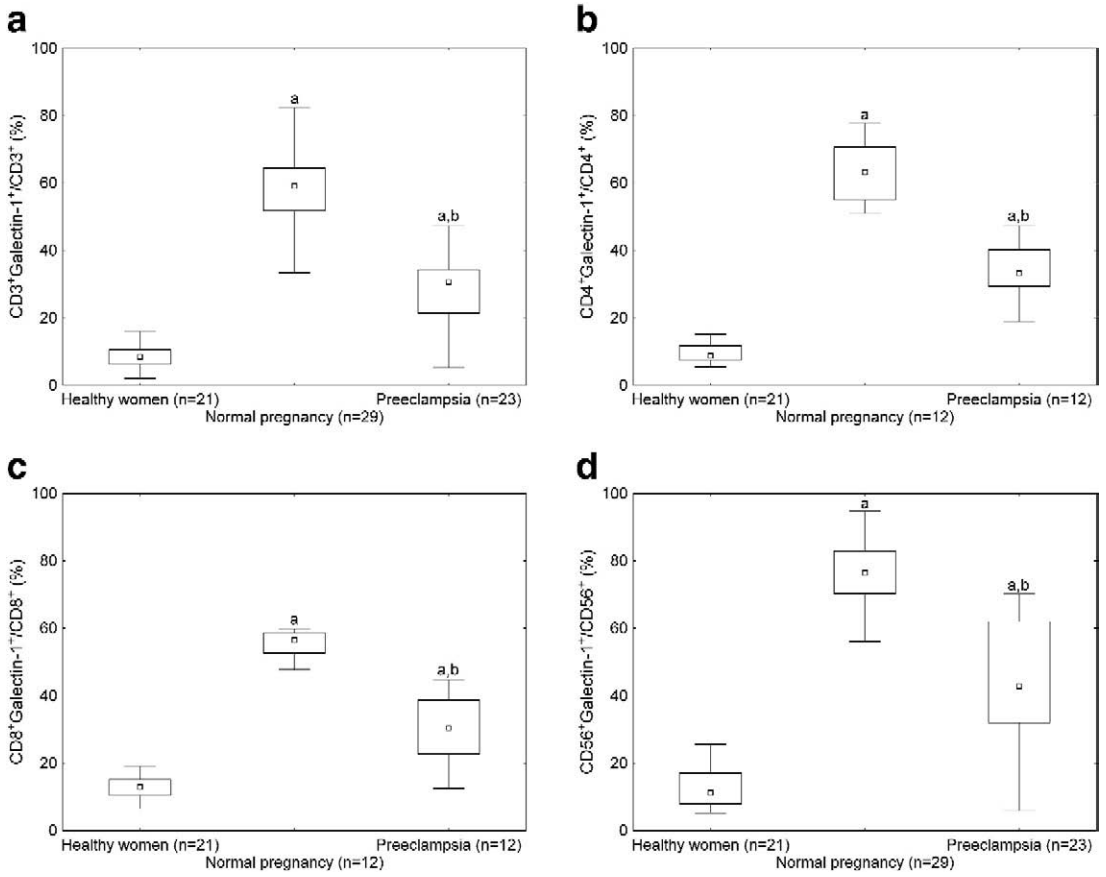

Fig. 2 Galectin-1-expressing $CD3^+$ (Fig. 2a), $CD4^+$ (Fig. 2b), $CD8^+$ (Fig. 2c) and $CD56^+$ (Fig. 2d) lymphocytes in proportion to the parent populations in healthy non-pregnant and pregnant women and preeclamptic patients. Middle line: median; Box: interquartile range (25–75 percentile); Whisker: range (excluding outliers). [a] $p<0.001$ versus healthy non-pregnant women. [b] $p<0.001$ preeclamptic patients versus healthy pregnant women.

phenotype and stop expressing gal-1 in favour of expressing inflammatory mediators. Preeclampsia is a multifactorial disorder with both genetic and environmental components [29]. Thus, sequence variations in the gene encoding gal-1 [30] might also have an effect on gal-1 expression of peripheral blood lymphocytes in preeclampsia. Further studies are warranted to explore the complex interaction between genetic and environmental factors in the regulation of gal-1 expression in different tissues, ethnic populations and pathological processes.

Although Th1 and Th17 cells are susceptible to gal-1-induced cell death, Th2 cells are protected from gal-1 through differential sialylation of their cell surface glycoproteins. Indeed, gal-1-deficient mice developed greater Th1 and Th17 responses [14]. Therefore, it is tempting to speculate that decreased production of gal-1 by circulating T and NK cells suggested by our results might contribute to the development of the pro-inflammatory Th1 and Th17 immune responses, which are characteristic features of the maternal syndrome of preeclampsia [4,6]. However, we did not examine gal-1 mRNA expression in or gal-1 secretion by circulating T and NK cells. An alternative explanation for our results might be that in preeclampsia, there is in fact more release of gal-1 from these cells in an attempt to control the exaggerated maternal systemic inflammation, which in turn leads to reduced intracellular gal-1 levels at the time of measurement. Additional studies are required to clarify these issues.

In this study, the similar pattern of intracellular gal-1 expression in peripheral blood T and NK cells in preeclampsia regardless of the severity, the time of onset of the disease or the presence of fetal growth restriction might be explained by its multifactorial etiology. Several genetic, behavioural and environmental factors need to interact to produce the complete picture of this pregnancy-specific disorder. Our research group reported various genetic and soluble factors that were associated with the severity or complications of preeclampsia, including HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome and fetal growth restriction [31–35]. Nevertheless, it is also possible that the relatively small sample size of this study prevented to detect an effect in the subgroup analyses.

Despite the decreased proportions of gal-1-expressing peripheral blood lymphocytes, serum gal-1 levels were not altered in preeclampsia. The mechanisms regulating circulating gal-1 levels are not completely understood at present, particularly in pregnancy, where the role of placental hormones needs also to be considered. Hemoconcentration is a common feature in preeclampsia, which might explain – at least partly – the observed controversy. Other sources of gal-1 than peripheral blood lymphocytes might also affect its circulating level. Indeed, gal-1 expression was up-regulated

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in preeclamptic placentas, which may compensate for the apoptotic effects of maternal immune cells [36,37]. Moreover, serum gal-1 levels were increased under stress conditions in a recent animal study, which seems to be regulated by the sympathetic nervous system [38]. Galectin-1 expression is also known to be up-regulated by hypoxia [39], which frequently affects the placenta and systemic organs of preeclamptic women. Interestingly, circulating gal-1 levels were not related to the proportions of gal-1-positive peripheral blood lymphocytes even in our healthy pregnant women. It is possible that rapid consumption of the protein at or near sites of its production did not allow us to detect changes at the systemic level in spite of its potent autocrine and paracrine effects.

A possible role of circulating autoantibodies produced against gal-1 was presumed in the pathogenesis of several autoimmune diseases. On the other hand, a number of autoantibodies have been implicated in the pathogenesis of preeclampsia. The most important of them appears to be an agonistic autoantibody against the angiotensin II type 1 receptor [40,41]. Given these observations, and that preeclampsia shares many features with autoimmune disorders [42], we also investigated whether anti-gal-1 autoantibody levels are elevated in the sera of preeclamptic compared to healthy pregnant women. However, in our study, no significant difference was found in serum anti-gal-1 autoantibody levels between the two groups. Accordingly, we suppose that humoral immunity against gal-1 does not play a remarkable role in the immune dysregulation observed in preeclampsia.

Recent findings indicate a central role of circulating angiogenic factors and their antagonists in the pathogenesis of preeclampsia [43,44]. In this study, we also measured serum sFlt-1 and PlGF concentrations in normal pregnancy and preeclampsia by electrochemiluminescence immunoassay. Nevertheless, increased sFlt-1 and decreased PlGF levels were not related to intracellular gal-1 expression of peripheral blood lymphocytes in preeclampsia, suggesting that alterations in angiogenic cytokine profile and gal-1 production by circulating T and NK cells are different mechanisms in the pathogenesis of this multifactorial disorder.

A limitation of our study is its case-control design. A prospective study should be undertaken to determine whether changes in intracellular gal-1 expression of circulating lymphocytes precede the development of preeclampsia, and thus can help to predict this serious complication in pregnancy. Additionally, we did not assess local gal-1 expression at the maternal–fetal interface. Further studies are needed to investigate gal-1 production by decidual T, NK and dendritic cells in normal pregnancy and preeclampsia.

In conclusion, the majority of circulating T (helper and cytotoxic) and NK cells shows intracellular gal-1 expression in the third trimester of normal pregnancy, while only a small fraction of them does so in non-pregnant women. In preeclampsia, the proportion of gal-1-expressing T (both helper and cytotoxic) and NK cells in the peripheral blood is markedly decreased compared to normal pregnancy, which might contribute to the development of the pro-inflammatory Th1 and Th17 immune responses characteristics of the maternal syndrome of the disease. However, circulating gal-1 and anti-gal-1 autoantibody levels are not affected in preeclampsia. The cause and clinical significance of these findings remain to be determined.

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