mice. IL-1β and IL-6, but not TGF-β, are essential for the differentiation of human Th17 cells.[24]

McClain et al. reported that mice immunized with neuroantigen during pregnancy showed a reduced incidence of experimental autoimmune encephalomyelitis as well as reduced clinical severity.[25] They also showed that immunized pregnant mice produced less IL-17 and TNF-α, suggesting that Th17 cells might decrease in number during pregnancy. Ostojic et al. reported that IL-17$^+$ cells were localized in the glands and in the basal proliferative stroma at days 6.5, 8.5, 9.5, and 10.5 in mice.[26] By day 12.5, the decidua was totally negative for IL-17 staining. This study suggests that the number of Th17 cells is decreased after day 12.5 at the feto–maternal interface in mice. However, our results showed that the ratio of IL-17$^+$ cells was increased in decidual lymphocytes in the first trimester, compared with that in peripheral blood lymphocytes. We did not obtain exactly the same results in humans as that found in mice; i.e. the population of peripheral blood Th17 cells did not change during pregnancy. Serum levels of IL-17 do not change in normal pregnancy or pre-eclampsia.[27] Th17 levels might not change in pre-eclampsia, although Th1 type immunity is predominantly observed in pre-eclampsia.[6,7] Additionally, as shown in Fig. 2, we observed that the proportions of CD4$^+$ T cells that were IL-17$^+$ remained stable between PBL and decidual lymphocytes in four cases of 12 paired samples. Unknown immunologic factors may affect the population of Th17 cells in humans.

Th17 cells play a pivotal role in the induction of the neutrophil-mediated protective immune response against extracellular bacteria and fungal pathogens.[10,13] To prevent these infections, Th17 levels must be kept stable during pregnancy. However, we have recently found that the amniotic fluid IL-17 levels in chorioamnionitis complicated preterm labor cases were significantly higher than those in pregnancies without chorioamnionitis.[28] Therefore, the number of IL-17 cells might be increased in cases of chorioamniotic infection, and IL-17 might participate in host defense.

Choriocarcinoma-derived JEG-3 cell culture supernatant reduces IL-17 and IFN-γ production in mixed-lymphocytes reactions.[29] On the contrary, immunostaining for IL-17 was observed in term placental trophoblasts,[30] and IL-17 was found to induce an increased invasive capacity in JEG cells.[31] However, in our study, we did not observe IL-17 staining in early or term placental trophoblasts. Further studies are needed to elucidate the effects of IL-17 on successful and complicated pregnancies in future.

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Biomarkers, Genomics, Proteomics, and Gene Regulation

Reduced Stathmin-1 Expression in Natural Killer Cells Associated with Spontaneous Abortion

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Female CBA/J mice impregnated by male DBA/2J mice (CBA/J×DBA/2J matings) are prone to spontaneous abortion, although the reason for this is unclear. In this study, the stathmin-1 expression pattern was evaluated in uterine natural killer (uNK) cells purified from CBA/J×DBA/2J matings. Results were compared with those in a CBA/J×BALB/c control group that yields successful pregnancies. The mean ± SD percentage of stathmin-1$^+$ cells in the CD49b$^+$ uNK cell population was lower in CBA/J×DBA/2J mice (0.7% ± 0.4%) than in control CBA/J×BALB/c mice (4.9% ± 1.5%, $P < 0.01$) using flow cytometry, and the intracellular stathmin-1 level in uNK cells was lower in CBA/J×DBA/2J mice than in control mice using Western blot analysis. Co-localization of lectin from Dolichos biflorus agglutinin (DBA-lectin) and stathmin-1 was confirmed using multivision immunohistochemical analysis. The frequency of stathmin-1$^+$DBA-lectin$^+$ cells was lower in CBA/J×DBA/2J mice than in CBA/J×BALB/c mice. A similar trend in the frequency of stathmin-1$^+$CD56$^+$ cells was seen in patients with unexplained spontaneous abortion compared with normal early pregnancy. A neutralizing antibody against stathmin-1 further increased the percentage of embryo loss in CBA/J×DBA/2J matings. These results provide evidence that stathmin-1 expression in uNK cells at the maternal-fetal interface may help modulate uNK cell function and may be beneficial for a successful pregnancy. (Am J Pathol 2011, 178:506–514; DOI: 10.1016/j.ajpath.2010.10.005)

Stathmin-1 is a small (19-kDa) regulatory phosphoprotein that integrates diverse intracellular signaling pathways. It is highly conserved among vertebrates and is associated with tubulin binding and microtubule destabilization.[1,2] Stathmin-1 has a complex phosphorylation pattern in response to various extracellular signals, in particular growth and differentiation factors.[3] Moreover, stathmin-1 phosphorylation varies during the cell cycle.[4] It has thus been thought that stathmin-1 can act as a relay integrating the activation of diverse intracellular signaling pathways and mediating the control of cell proliferation, differentiation, and other functions.[5]

Stathmin-1 protein and mRNA were previously shown to be expressed in the pregnant uterus and decidualizing endometrial stromal cells in human and murine models.[6–8] Furthermore, stathmin-1 is up-regulated in rodent uteri at the site of embryo implantation and is highly

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expressed in the decidual zone during the decidualization process.[7,8] These results suggest that stathmin-1 may participate in the modulation of embryo implantation and decidualization.

Female CBA/J mice impregnated by male DBA/2J mice (CBA/J×DBA/2J matings) are prone to abortion, in contrast to the major histocompatibility complex–identical CBA/J×BALB/c matings, which are resistant to abortion.[9] The underlying mechanisms for these observations are unclear. Clark and colleagues[9] suggested that endothelium is the primary effector cell population, and this was supported by a recent work using CBA/J×DBA/2J matings.[10] Notably, inhibition of natural killer (NK) cells using anti-asialo GM1 antiserum significantly decreased the resorption rate of embryos in CBA/J×DBA/2J matings.[9] In the present study, uterine NK (uNK) cells were purified from CBA/J×DBA/2J and CBA/J×BALB/c allogeneic pregnant models using magnetic affinity cell sorting (MACS). The percentage of stathmin-1$^+$ cells in the uNK cell population was determined using flow cytometry, and the stathmin-1 protein expression level in uNK cells was determined using two-dimensional gel electrophoresis (2-DE), mass spectrometry (MS), and Western blot analysis. Multivision immunohistochemical analysis (IHC) was used to examine the distribution patterns of stathmin-1$^+$ cells in the uteri of pregnant female mice and in first-trimester human decidual tissue. In addition, inhibition of stathmin-1 was performed in CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J mice. From these data, the possible role of stathmin-1 in allogeneic pregnancy tolerance was investigated.

Materials and Methods

Pregnant Models of CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J Matings

Female CBA/J mice and male CBA/J, DBA/2J, and BALB/c mice (8 to 12 weeks old) were purchased from the Model Animal Center of Nanjing University (Nanjing, China) and were housed under specific pathogen-free conditions. Pregnant models of CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J matings were established by co-caging female CBA/J mice with DBA/2J, BALB/c, and CBA/J males, respectively. Detection of a vaginal plug was chosen to indicate day 0.5 of gestation (E0.5).[11,12] Embryonic day E12.5 was chosen as the gestational time to collect uNK cells because the uNK cells are at peak density on day E10 and have not yet begun to decrease in density through apoptosis (which begins on day E13 or E14).[13] Furthermore, we expected that it would be easier to distinguish healthy embryos from resorbing ones on day E12.5 than at an earlier time point. All animal procedures followed the national animal care guidelines, and associated data were approved for publication by the institutional review board of Shanghai Jiaotong University.

Purification of uNK Cells

Cell purification was performed by means of MACS.[11,12] Briefly, hysterolaparotomy was performed on day E12.5 to collect embryo-depleted placentas from CBA/J×DBA/2J and CBA/J×BALB/c matings. The uterine horns were opened longitudinally, and the fetoplacental unit was separated easily from the uterine implantation sites. The whole placental and decidual unit was separated individually from the respective embryo. The pooled placentas and decidua basalis (ie, decidual tissue in implantation sites) were collected into a dish and carefully cut into small pieces, collected in 0.9% NaCl solution, and subsequently filtered through a nylon mesh (50-μm pore size) to obtain a single cell suspension. Mononuclear cells were obtained by centrifuging of the single cell suspension using a Ficoll-Hipaque density column. Any red blood cells that contaminated the single cell suspension were eliminated by incubation with red blood cell lysis buffer (eBioscience Inc., San Diego, CA) two times at 37°C. Subsequently, NK cells were isolated using magnetic bead–conjugated antimouse CD49b monoclonal antibody, and CD49b$^+$ cells were purified by means of Mini MACS columns (Miltenyi Biotec Inc., Auburn, CA),[11,12] where CD49b was used as a common marker for murine NK cells.[14] The purity of the MACS-isolated NK cells routinely exceeded 95% as determined using flow cytometry.[12,15]

Flow Cytometry

Uterine NK cells were stained with phosphatidylethanolamine (PE)-conjugated antimouse CD49b (BioLegend, San Diego, CA) and rabbit anti–stathmin-1 (catalog number ab52906; Abcam, Cambridge, England) antibodies, followed by fluorescein isothiocyanate (FITC)–conjugated antirabbit IgG (Molecular Probes Inc., Eugene, OR). The percentage of stathmin-1$^+$ cells in the CD49b$^+$ NK cell population was determined by using a flow cytometer (FACSAria; BD Biosciences, Franklin Lakes, NJ).[11] Cells were stained with PE-conjugated antimouse CD49b and FITC-conjugated antimouse CD122 antibodies (both from BioLegend) to determine the percentage of CD122$^+$ cells in the CD49b$^+$ population. Isotype controls were established by using isotype control antibodies (BioLegend) to exclude false-positive cells. All the experiments were independently performed six times.[16–18]

Preparation of uNK Cell Lysates

Uterine NK cells were suspended in a modified radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 0.66 μg/ml of aprotinin, 0.5 μg/ml of leupeptin, 1 μg/ml of pepstatin, 1 mmol/L Na_3VO_4, and 1 mmol/L NaF] and were sonicated three times for 5 seconds each. The cell lysates were centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatants were collected, and their protein concen-

trations were measured by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA).[19,20]

Two-Dimensional Gel Electrophoresis

The 2-DE was performed according to the manufacturer's instructions. Samples were loaded onto linear immobilized pH gradient (IPG) strips (IPGstrip, pH 4–7 L, 180 × 3 × 0.5 mm; Amersham Biosciences, Piscataway, NJ). Briefly, 1-mg protein samples were diluted to 350 μL with a rehydration solution [7 mol/L urea, 2 mol/L thiourea, 2% 3-([3-Cholamidopropyl]dimethylammonio)-I propanesulfonate, 18 mmol/L dithiothreitol, 0.5% (v/v) pH 4–7 IPG buffer, and trace bromophenol blue] and were applied to the IPG strips with 14 hours of rehydration at 30 V. The proteins were successively focused for 1 hour at 500 V, 1 hour at 1000 V, and 5 hours at 8000 V for a total of 41,920 V hours on an IPGphor (Amersham Biosciences). The focused IPG strips were equilibrated for 15 minutes in solution (6 mol/L urea, 2% SDS, 30% glycerol, 50 mmol/L Tris-HCl, pH 8.8, and 1% dithiothreitol) and then for an additional 15 minutes in the same solution containing 2.5% iodoacetamide instead of dithiothreitol. After equilibration, SDS–polyacrylamide gel electrophoresis was performed at 10°C on 10% SDS slab gels using the Ettan DALT II system (Amersham Biosciences) with the IPG strips sealed on the top of the gels with 0.5% agarose. An SDS–polyacrylamide gel electrophoresis was performed at a constant power of 2W/gel for 30 minutes and then switched to 12 W/gel until the bromophenol blue marker reached the bottom of the gel. Finally, the blue silver staining method (a modified Neuhoff's colloidal Coomassie Blue G-250 stain) was used to visualize the protein spots in the 2-DE gels.[17,20]

2-DE Image Data Analysis

Stained 2-DE gels were scanned using LabScan software and ImageScanner (Amersham Biosciences) at a solution of 300 dpi. Spot-intensity calibration, spot detection, matching, 1-D calibration, and establishment of an average gel were performed using the PDQuest System (Bio-Rad Laboratories). The theoretical molecular weight and pI value of the identified protein spots were calculated according to algorithms included in the PDQuest analysis software package. Significant differences in the protein expression levels were determined using the *t*-test, with significance defined at $P < 0.05$.[17,20]

Preparation of Protein Spots

Protein spots were excised from the preparative gels and were placed into a 96-well microtiter plate. Proteins were digested in gel as previously described.[17,20] The gel spots were destained using destaining solution [200 mmol/L NH_4HCO_3 and 100% acetonitrile (1:1)] for 20 minutes at room temperature. Gel spots were washed twice with deionized water, shrunk by dehydration in acetonitrile solution, and dried in a vacuum centrifuge. Samples were then swollen in a digestion buffer (20 mmol/L ammonium bicarbonate and 12.5 ng/μL of trypsin) (Sigma-Aldrich, St. Louis, MO) for 30 minutes at 4°C. The gels were then digested for 12 hours at 37°C. Tryptic peptides were extracted twice from the gel slices by sonication for 15 minutes in a 0.1% trifluoroacetic acid/50% acetonitrile solution. The supernatants were collected and dried to a pellet in a high-purity nitrogen flow. Peptides were eluted with 0.7 μL of α-cyano-4-hydroxycinnamic acid matrix solution and were loaded onto a stainless steel target with 192 wells (Applied Biosystems, Framingham, MA).[17,20]

Mass Spectrometry

Samples were air-dried and then were analyzed by using the Voyager System 4700 matrix-assisted laser desorption/ionization–time of flight–time of flight mass spectrometer (Applied Biosystems).[17,20]

Protein Identification

Known contaminating peaks (eg, keratin and autoproteolysis peaks) were removed before the database search. Spectra were processed and analyzed using a GPS Explorer (Applied Biosystems). Mascot software (Matrix Science, London, England) was used to search for peptide mass fingerprints and MS/MS data in the NCBInr database. Protein scores by Mascot search analysis that were >63 were considered significant ($P < 0.05$).[17,20]

Western Blotting Analysis

Tissue aliquots were homogenized to powder using liquid nitrogen and then were dissolved in lysis buffer [150 mmol/L NaCl, 50 mmol/L Tris-Cl, pH 8.0, 0.1% Nonidet P-40 (Caledon Laboratories Ltd., Georgetown, Ontario, Canada), 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/ml of aprotinin, and 25 μg/ml of leupeptin], vortexed, and incubated at room temperature for 2 hours. The mixture was centrifuged at 20,644 × g for 30 minutes at 4°C, and the supernatant was used as the total protein solution. The lysate concentration was assayed using the Bradford assay.

Western blotting analysis was performed as previously described.[17,20] Briefly, 100 μg of total protein was separated on a 12% SDS–polyacrylamide gel electrophoresis gel before being transferred onto a nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline/0.2% Tween 20 for 1 hour at room temperature, the membrane was incubated with rabbit antimouse stathmin-1 antibody (1:100 dilution) (Abcam) for 1 hour at room temperature, followed by incubation with horseradish peroxidase–conjugated goat antirabbit IgG secondary antibody (1:10,000 dilution; Amersham Biosciences) for 1 hour at room temperature. Detection of NADPH was used as a loading control. Reactions were visualized using an enhanced chemiluminescence detection system (ECL; Amersham Biosciences). Signals on the blots were visualized using autography.

Multivision IHC of Placental Tissue in Murine Models

Placentas together with decidua basalis harvested on day E10.5 from CBA/J×DBA/2J and CBA/J×BALB/c matings (none of which were analyzed by means of 2-DE) were used to measure the distribution of stathmin-1 protein in lectin from Dolichos biflorus agglutinin–positive (DBA-lectin$^+$) cells using a multivision IHC procedure. Paraffin-embedded tissue blocks were cut into 4-μm-thick sections, which were then deparaffinized in xylene and rehydrated in graded alcohol concentrations. Nonspecific binding was further blocked by preincubation with blocking solution for 5 minutes, followed by incubation for 1 hour at 4°C with rabbit antimouse stathmin-1 (1:200 dilution) (Cell Signaling Technology Inc., Beverly, MA). Meanwhile, FITC-conjugated DBA-lectin (1:200 dilution) (Sigma-Aldrich) was added onto the section in the dark for 1 hour. The sections were then washed three times with PBS for 5 minutes each and incubated with PE-conjugated antirabbit IgG (1:200 dilution) (Alpha Diagnostic International, San Antonio, TX) for 30 minutes at room temperature in the dark. Then, 4′,6-diamidino-2-phenylindole (Invitrogen, San Diego, CA) was used to stain nuclei for 10 minutes in the dark. Negative controls were established using rabbit Ig of the isotype identical to the rabbit antimouse primary antibody in place of the specific primary antibody (Cell Signaling Technology Inc.).[21]

Multivision IHC of Human Decidual Tissue

First-trimester human decidual tissue was obtained from five normal pregnancies (free of spontaneous abortion history; mean ± SD age, 27.5 ± 2.2 years; mean ± SD gestational age at sampling, 8.2 ± 1.1 weeks, terminated for nonmedical reasons) and five miscarriages [maternal history of more than three unexplained recurrent spontaneous abortions (RSAs); mean ± SD age, 32.4 ± 3.9 years; mean ± SD gestational age at sampling, 8.5 ± 2.8 weeks], which were classified as unexplained after the exclusion of maternal anatomical or hormonal abnormalities and paternal or maternal chromosomal abnormalities. All the samples were obtained from Renji Hospital, Shanghai Jiaotong University, with written informed consent from the patients and permission from the research ethics committee of Shanghai Jiaotong University.

To confirm the existence and define the distribution pattern of CD56$^+$stathmin-1$^-$ and CD56$^+$stathmin-1$^+$ cells in human decidual tissue, paraffin sections were stained with rabbit antihuman stathmin-1 (Abcam) and mouse antihuman CD56 (Lab Vision/NeoMarkers, Fremont, CA) monoclonal antibodies, followed by staining with multivision antirabbit/horseradish peroxidase (horseradish peroxidase/diaminobenzidine) plus antimouse/alkaline phosphatase polymers (Biolab Science, Beijing, China), according to the manufacturers' instructions. Using this multivision polymer detection system, stathmin-1$^+$ cells were stained brown, CD56$^+$ cells were stained red, and double-positive cells were double colored. Nuclei were lightly stained with hematoxylin.

Inhibition of Stathmin-1 in Vivo

Inhibition of stathmin-1 was performed in CBA/J×DBA/2J, CBA/J×BALB/c, and CBA/J×CBA/J matings by i.p. injection of anti–stathmin-1 antibody (GenScript USA Inc., Piscataway, NJ) on days E4.5, E5.5, and E6.5 (20 μg in 0.2 ml of PBS) once a day. Mice injected with the same volume of rabbit IgG isotype control antibody were used as controls for each group. The percentage of embryo resorption was detected on day E12.5 by using the method described previously herein (n = 8 per group).

Statistical Analysis

Flow cytometry data were analyzed by using Quad statistics.[16] The resorption rate was compared using the χ^2 test, and the cell percentage was compared using the independent-samples t-test. Cell percentage results are presented as mean ± SD.[17,18] Significance was defined at $P < 0.05$.

Results

The Percentage of Embryo Loss Is Increased in CBA/J×DBA/2J Matings

The percentage of spontaneously resorbed embryos on day E12.5 was 22.6% (35 of 155; n = 16) in CBA/J×DBA/2J matings, 7.6% (13 of 170; n = 18) in CBA/J×BALB/c matings, and 7.2% (8 of 111; n = 12) in CBA/J×CBA/J matings. There was no significant difference between CBA/J×BALB/c and CBA/J×CBA/J matings in the percentage of embryo loss, whereas the percentage of embryo resorption in CBA/J×DBA/2J matings was significantly higher than that in CBA/J×BALB/c and CBA/J×CBA/J matings ($P < 0.01$ for both). The increased resorption rate of CBA/J×DBA/2J matings supports the hypothesis that these mice are prone to spontaneous embryo loss.

Flow Cytometric Analysis of MACS-Purified CD49b$^+$ Cells

The mean ± SD percentage of stathmin-1$^+$ cells in the CD49b$^+$ NK cell population was approximately six-fold higher in CBA/J×BALB/c matings (5.2% ± 1.5%) than in CBA/J×DBA/2J matings (0.9% ± 0.4%) ($P < 0.01$), as indicated by flow cytometry (Figure 1).

Two-color flow cytometry revealed that most CD49b$^+$ cells also expressed CD122 molecules in both matings. The mean ± SD percentage of CD122$^+$CD49b$^+$ cells in the CD49b$^+$ population was 74.9% ± 11.4% in CBA/J×DBA/2J matings, which was not significantly different from that in CBA/J×BALB/c matings (65.6% ± 8.8%).

Reduced Stathmin-1 Production in uNK Cells in CBA/J×DBA/2J Matings in 2-DE

Proteins from two sets of pooled (12 pregnant mice per group) uNK cell lysates isolated from CBA/J×DBA/2J

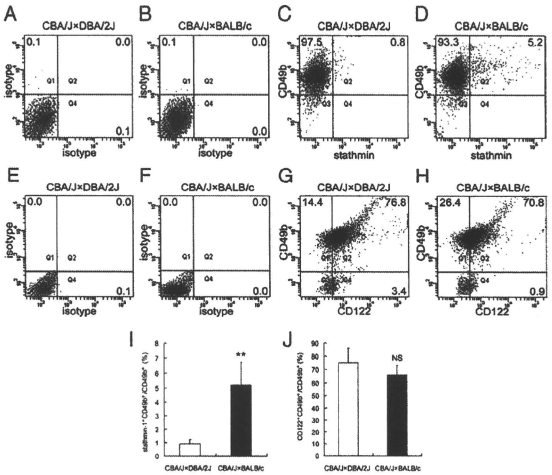

Figure 1. Flow cytometric analysis of stathmin-1$^+$ and CD122$^+$ cells in uNK cells. **A–H:** Representative results derived from cells purified using microbead-conjugated anti-CD49b and a Mini MACS. **A, B, E, F:** Isotype controls established using cells stained with FITC- and PE-conjugated isotype antibodies. **C, D:** Analysis of stathmin-1$^+$ cells in the CD49b$^+$ cell population. The cell percentage is indicated. **G, H:** Analysis of CD122$^+$ cells in the CD49b$^+$ cell population. **I, J:** Data summary of flow cytometry. Experiments were independently repeated six times in each group. The mean ± SD percentage of stathmin-1$^+$CD49b$^+$ cells was lower in CBA/J×DBA/2J mice than in CBA/J×BALB/c mice (0.9% ± 0.4% versus 5.2% ± 1.5%, $P < 0.01$). A strong CD122 signal was detected in both mating combinations. The mean ± SD percentage of CD122$^+$CD49b$^+$ cells in the CD49b$^+$ population was 74.9% ± 11.4% in CBA/J×DBA/2J mice and 65.6% ± 8.8% in CBA/J×BALB/c mice, suggesting that most of the MACS-purified CD49b$^+$ cells also express CD122. Error bars represent SD. $^{**}P < 0.01$.

matings and age-matched CBA/J×BALB/c matings on day E12.5 were resolved using 2-DE. These experiments were repeated four times under identical experimental conditions and parameters to confirm reproducibility. Well-resolved and reproducible Coomassie Brilliant Blue–stained 2-DE maps from CBA/J×DBA/2J and CBA/J×BALB/c matings were obtained. The intensity of the stathmin-1 protein spot derived from CBA/J×BALB/c matings was approximately four-fold higher than that in CBA/J×DBA/2J matings (mean ± SD, 4.1 ± 0.9–fold; $P < 0.01$) (Figure 2).

Identification and Quantification of Stathmin-1 Protein Using Matrix-Assisted Laser Desorption/Ionization–Time of Flight–MS

In the 2-DE analysis described previously herein, differentially expressed protein spots were excised from Coomassie Brilliant Blue–stained gels and were subjected to in-gel digestion with trypsin. An aliquot of the supernatant containing tryptic peptides was analyzed by means of matrix-assisted laser desorption/ionization–time of flight–time of flight–MS/MS, and the Mascot search program software package was used to identify the analyzed protein spots. Proteins receiving a significant score >63 ($P < 0.05$) in the Mascot database were ranked as the best hits. Stathmin-1 was one protein that was differentially expressed between the two sets of samples. The protein spot shown in Figure 2 was analyzed using MS and was identified as stathmin-1 (Figure 3). Stathmin-1 received a protein score of 280, a molecular weight of 17,264 Da, a pI of 5.76, and accession number gi 9789995.

The differential expression of stathmin-1 was further confirmed by immunoblots using a stathmin-1–specific antibody. The intensity of the stathmin-1 spot in CBA/J×BALB/c matings was a mean ± SD 6.9 ± 2.2–fold higher than that in CBA/J×DBA/2J matings ($^{**}P < 0.01$) (Figure 4).

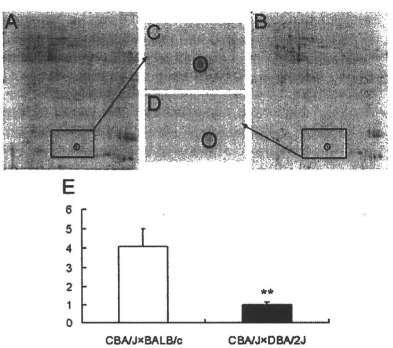

Figure 2. Stathmin-1 expression determined using 2-DE. **A–D:** Map of uterine lymphocyte lysates from CBA/J×BALB/c mice (**A** and **C**) or CBA/J×DBA/2J mice (**B** and **D**). **C** and **D** indicate the original magnification of the stathmin-1 protein spot from CBA/J×BALB/c and CBA/J×DBA/2J mice, respectively. The density of the stathmin-1 protein spot from CBA/J×BALB/c mice was higher than that from CBA/J×DBA/2J mice as observed by the naked eye. **E:** Mean density as determined using Image-Pro Plus 6.0. The mean density of stathmin-1 spots in CBA/J×BALB/c mice was a mean ± SD 4.1 ± 0.9–fold higher than that in CBA/J×DBA/2J mice ($^{**}P < 0.01$). Experiments were independently performed four times. Error bars represent SD.

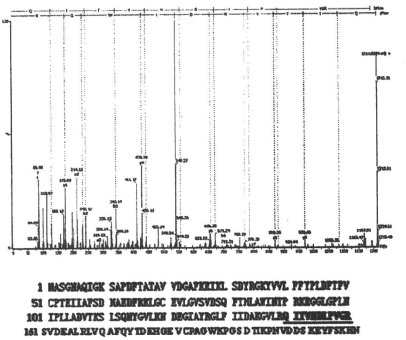

Figure 3. Mass spectrometry of the stathmin-1 spot. MS/MS signals were derived from the parent ion, for which the amino acid sequence, QIT-VNDLPVGR, was deduced based upon these ions in tandem MS spectrum. MS/MS spectrum of stathmin-1 is shown. Peptide was identified by matrix-assisted laser desorption/ionization time of flight/time of flight MS/MS and matched with residues 140-150 of stathmin-1. Protein sequence of stathmin-1 is shown, and matched MS/MS fragmentation is underlined. The peptide mass fingerprinting and the MS/MS maps of stathmin-1 were combined to confirm the MS results.

Disparate Abundance of Stathmin-1$^+$DBA-Lectin$^+$ Cells between CBA/J×BALB/c and CBA/J×DBA/2J Matings

As shown in Figure 5, the presence of stathmin-1$^+$DBA-lectin$^+$ cells was confirmed in CBA/J×BALB/c and CBA/J×DBA/2J matings using multivision IHC. The DBA-lectin$^+$ cells were stained green, stathmin-1$^+$ cells were stained red, and double-positive cells were yellow in the merged images. Nuclei were stained blue by 4′,6-diamidino-2-phenylindole.

However, the frequency of stathmin-1$^+$DBA-lectin$^+$ cells was significantly higher in CBA/J×BALB/c matings, whereas DBA-lectin$^+$stathmin-1$^+$ cells were rare in CBA/J×DBA/2J matings. As indicated in Figure 5, A-D, DBA-lectin$^+$ cells (green) are more dominant than stathmin-1$^+$ cells in both mating combinations. However, red (stathmin-1) is robust in CBA/J×BALB/c matings but either absent or faint in CBA/J×DBA/2J matings. In the merged images, the relative number of stathmin-1$^+$DBA-lectin$^+$ cells (yellow) is higher in CBA/J×BALB/c matings (Figure 5, A and B) than in CBA/J×DBA/2J matings (Figure 5, C and D).

The murine cells positive for DBA-lectin were scattered intensively in decidual basalis tissue and in the mesometrial lymphoid aggregate of pregnancy in both matings, whereas no DBA-lectin$^+$ cells were observed in placenta tissue (region P) (Figure 5, E and F).[22]

The mean density of stathmin-1$^+$DBA-lectin$^+$ cells was determined using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, MD). The mean ± SD density of the double-positive cells was significantly higher in CBA/J×BALB/c matings than in CBA/J×DBA/2J matings (9.6 ± 5.5 versus 1.0 ± 0.9; $P < 0.01$) (Figure 5G).

The Distribution Pattern of Stathmin-1$^+$ Cells in the Human CD56$^+$ Cell Population

The distribution pattern of stathmin-1$^-$CD56$^+$ and stathmin-1$^+$CD56$^+$ cells was evaluated by means of double-vision IHC. Stathmin-1$^+$CD56$^+$ cells were observed in human decidua from RSA patients (Figure 6, A–D) and those with normal early pregnancy (Figure 6, E–H) but were more frequently detected in the latter. Stathmin-1$^-$CD56$^+$ and stathmin-1$^+$CD56$^+$ cells were mainly detected in tissues near blood vessels, whereas stathmin-1$^+$CD56$^+$ cells were seldom found in tissues where there were almost no blood vessels. The mean density of stathmin-1$^+$CD56$^+$ cells was determined using Image-Pro Plus 6.0. The mean ± SD density of the double-positive cells was significantly higher in normal early pregnancy than in RSA patients (13.6 ± 7.2 versus 1.0 ± 0.7; $P < 0.01$) (Figure 6I).

Effects of Anti–Stathmin-1 Antibody Treatment on Embryo Loss

The mean ± SD percentage of embryo resorption was higher in CBA/J×DBA/2J matings when stathmin-1 protein was inhibited using a neutralizing antibody (with inhibition: 42.1% ± 21.2%, 32 of 76; control: 21.8% ± 12.4%, 17 of 78; $P < 0.05$) (Figure 7). The change in the percentage of embryos lost in the CBA/J×BALB/c and CBA/J×CBA/J matings treated with the stathmin-1 neutralizing antibody did not reach statistical significance (CBA/J×BALB/c, with inhibition: 14.1% ± 10.1%, 11 of 78; CBA/J×BALB/c, control: 8.5% ± 5.4%, 6 of 71; CBA/J×CBA/J, with inhibition: 9.9% ± 6.5%, 8 of 75; CBA/J×CBA/J, control: 7.3% ± 6.5%; 6 of 79) (Figure 7).

Discussion

Stathmin is reportedly expressed in the glandular epithelium and stromal cells of human endometrial tissue by cytotrophoblasts and extravillous trophoblasts but not by syncytiotrophoblasts.[6] When stromal cells isolated from normal endometrial tissues were previously cultured and stimulated to decidualize by progesterone plus estrogen or cAMP, their total and phosphorylated stathmin levels decreased.

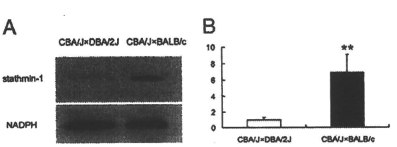

Figure 4. Western blot analysis of stathmin-1 expression in CBA/J×DBA/2J and CBA/J×BALB/c matings. **A:** Stathmin-1 expression is significantly down-regulated in CBA/J×DBA/2J mice compared with CBA/J×BALB/c mice. NADPH was used as an internal loading control. **B:** Histogram showing the relative expression level of stathmin-1 protein in CBA/J×BALB/c and CBA/J×DBA/2J mice as determined using Image-Pro Plus 6.0. The stathmin-1 protein level is a mean ± SD 6.9 ± 2.2–fold higher in CBA/J×BALB/c mice than in CBA/J×DBA/2J mice as determined using densitometric analysis (**$P < 0.01$). Experiments were independently repeated four times for each group. Error bars represent SD.

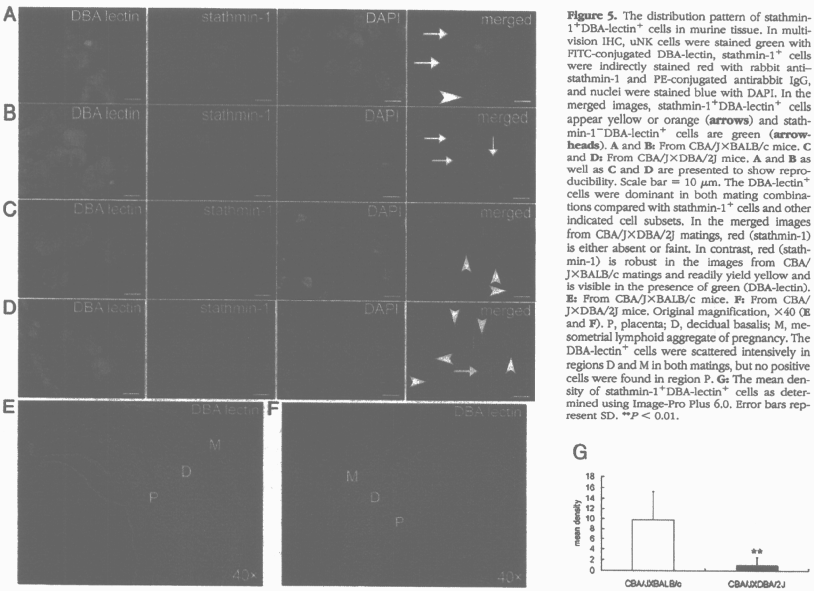

Figure 5. The distribution pattern of stathmin-1$^+$DBA-lectin$^+$ cells in murine tissue. In multivision IHC, uNK cells were stained green with FITC-conjugated DBA-lectin, stathmin-1$^+$ cells were indirectly stained red with rabbit anti–stathmin-1 and PE-conjugated antirabbit IgG, and nuclei were stained blue with DAPI. In the merged images, stathmin-1$^+$DBA-lectin$^+$ cells appear yellow or orange (**arrows**) and stathmin-1$^-$DBA-lectin$^+$ cells are green (**arrowheads**). **A** and **B**: From CBA/J×BALB/c mice. **C** and **D**: From CBA/J×DBA/2J mice. **A** and **B** as well as **C** and **D** are presented to show reproducibility. Scale bar = 10 μm. The DBA-lectin$^+$ cells were dominant in both mating combinations compared with stathmin-1$^+$ cells and other indicated cell subsets. In the merged images from CBA/J×DBA/2J matings, red (stathmin-1) is either absent or faint. In contrast, red (stathmin-1) is robust in the images from CBA/J×BALB/c matings and readily yield yellow and is visible in the presence of green (DBA-lectin). **E**: From CBA/J×BALB/c mice. **F**: From CBA/J×DBA/2J mice. Original magnification, ×40 (**E** and **F**). P, placenta; D, decidual basalis; M, mesometrial lymphoid aggregate of pregnancy. The DBA-lectin$^+$ cells were scattered intensively in regions D and M in both matings, but no positive cells were found in region P. **G**: The mean density of stathmin-1$^+$DBA-lectin$^+$ cells as determined using Image-Pro Plus 6.0. Error bars represent SD. $^{**}P < 0.01$.

Stathmin silencing in primary stromal cells using small interfering RNA before the cells are exposed to decidualizing agents also markedly suppresses decidualization, suggesting that stathmin may play a key role in decidualization.[6] Stathmin overexpression favors microtubule destabilization, whereas decreased stathmin expression favors elongated, bundled microtubules and an increased ratio of polymerized to soluble tubulin.[23] Immunohistochemical analyses using a rat model previously revealed that stathmin-1 is exclusively localized in decidual cells, especially in the primary decidual zone surrounding the embryo with markedly more intense staining on day E9.5 than on day E7.5. On day E14.5, when the endometrial stromal cells have completely differentiated into decidual cells, the staining of decidual cells is faint.[7] An experiment in the delayed implantation pregnant rat model revealed low uterine stathmin expression that was increased after implantation, which was induced by 17β-estradiol administration to progesterone-primed animals. Furthermore, decidualization in the pseudopregnant rat, induced by intrauterine oil infusion, stimulates stathmin expression. Stathmin expression clearly increases in the uterus when stimulated by embryo implantation and decidualization and is believed to play a role in the early stages of pregnancy.[8]

Herein, we demonstrated by using two-color flow cytometry that stathmin-1 is expressed intensively in uNK cells isolated from mouse models. The uNK cells were previously purified by means of MACS and were confirmed to be CD3$^-$CD49b$^+$ cells.[11] In multivision IHC using decidual samples from humans and CD56 as a pan marker for human NK cells, the distribution pattern of stathmin-1$^+$CD56$^+$ cells was consistent with the reported roles of NK cells in the pregnant uterus. Under physiologic conditions, NK cells are the dominant cell population up to mid-gestation in the pregnant uterus and are believed to participate in blood vessel remodeling.[24–26] The origin of these NK cells is unclear. A previous study demonstrated that NK cells from extrauterine tissue migrate into the pregnant uterus.[26] Thus, we conclude that a fraction of the stathmin-1$^+$ cells are actually uNK cells, which immigrate into the pregnant uterus at the early stages of pregnancy and are involved in the modulation of implantation and decidualization.

The percentage of stathmin-1$^+$ cells in the uNK cell population was significantly higher in CBA/J×BALB/c matings than in abortion-prone CBA/J×DBA/2J matings. In addition, the intensity of stathmin-1 expression was stronger in CBA/J×BALB/c matings than in CBA/J×DBA/2J matings, as indicated by 2-DE and Western blot analysis. This differential stathmin-1 expression correlated with the difference in the embryo loss rate, which was lower in CBA/J×BALB/c matings than in CBA/J×DBA/2J matings. Thus, the reduced stathmin-1 production in uNK cells correlates with the increased failure of healthy embryos by CBA/J×DBA/2J matings. Furthermore, stathmin-1 inhibition with a neutralizing

antibody increased the percentage of embryo loss in CBA/J×DBA/2J matings, but no such trend was observed in CBA/J×BALB/c, CBA/J×CBA/J, or syngeneic CBA/J×CBA/J matings. These results suggest that stathmin-1 may be a key regulator in the maintenance of allogeneic pregnancy tolerance, at least in CBA/J×DBA/2J matings. In CBA/J×BALB/c matings, the increase in embryo resorption after stathmin-1 inhibition did not reach statistical significance. Although the reason for this is unclear, it may be explained by the difference in the uNK cell functional status between CBA/J×DBA/2J and CBA/J×BALB/c matings, indicating the presence of a fragile system of stathmin-1–mediated modulation in CBA/J×DBA/2J matings.

In multivision IHC, colocalization of stathmin-1 and DBA-lectin was confirmed in murine decidual tissue from both mating combinations. However, the frequency of

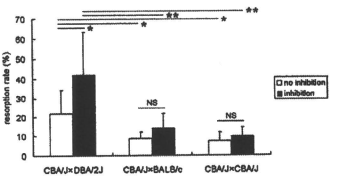

Figure 7. Effect of anti-stathmin-1 antibody treatment on the embryo resorption rate. No inhibition, multiple injections with isotype control antibody; inhibition, multiple injections with neutralizing antibody. In the no-inhibition CBA/J×DBA/2J group, the percentage of embryo loss was significantly higher than that in the no-inhibition CBA/J×BALB/c and CBA/J×CBA/J groups, confirming that CBA/J×DBA/2J mice are prone to abortion. The percentage of embryo loss was increased after stathmin-1 inhibition in CBA/J×DBA/2J mice ($^{*}P < 0.05$, $^{**}P < 0.01$), but the trend of embryo loss increase did not reach statistical significance in CBA/J×BALB/c or CBA/J×CBA/J mice. $n = 8$ per group. Error bars represent SD.

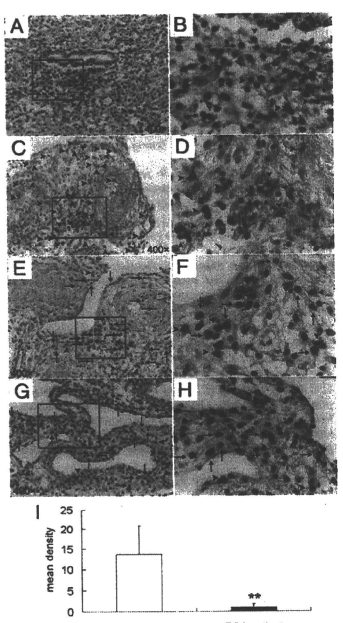

Figure 6. The distribution pattern of stathmin-1^{+}CD56^{+} cells in human decidua. The distribution pattern of stathmin-1^{+}CD56^{+} cells was examined by means of double-staining IHC in decidual tissue from RSA patients (**A–D**) and subjects undergoing healthy elective pregnancy termination (**E–H**). Single CD56^{+} cells are stained red (**arrowheads**), stathmin-1^{+}CD56^{+} cells are stained brown (**arrows**), and nuclei are stained blue with hematoxylin. Original magnification is marked. **B**, **D**, **F**, and **H** are local magnifications of the regions enclosed with boxes on **A**, **C**, **E**, and **G**, respectively. **I** Mean density of stathmin-1^{+}CD56^{+} cells as determined using Image-Pro Plus 6.0. Error bars represent SD. $^{**}P < 0.01$.

stathmin-1^{+}DBA-lectin^{+} cells was significantly higher in CBA/J×BALB/c mice than in CBA/J×DBA/2J mice (Figure 5). Stathmin-1^{+}DBA-lectin^{+} cells were found mainly scattered in the decidua basalis and mesometrial lymphoid aggregate of pregnancy in the murine pregnant uteri. Because the decidua basalis and mesometrial lymphoid aggregate of pregnancy, to some extent, represent the maternal-fetal interface and because more immunopotent cells can infiltrate into these tissues than into other parts of murine placental and decidual tissues, these results suggest that stathmin-1^{+}DBA-lectin^{+} NK cells may be important in the modulation of maternal-fetal cross talk.[25,26] Similar results were obtained in human decidual tissue. Using CD56 as a pan-NK cell marker for human uNK cells, we found that the frequency of stathmin-1^{+}CD56^{+} cells was significantly higher in decidual tissues from normal early pregnancy than in those from spontaneous abortion patients (Figure 6). This suggests that some cases of unexplained spontaneous miscarriage may be attributable to reduced function of uNK cells, including reduced production of stathmin-1 protein in uNK cells. In addition, stathmin-1^{+}CD56^{+} cells were found mainly scattered in tissues near blood vessels, suggesting that they may participate in establishment, remodeling, or other functions of the blood vessel system. Stathmin-1^{+}CD56^{+} cells were less frequently detected in RSA samples, consistent with the results obtained using mouse two-color flow cytometry. Taken together, the results of the present study suggest that a fraction of uNK cells express stathmin-1 molecules and that insufficient stathmin-1 expression in uNK cells may be related to increased embryo loss in abortion-prone mice and some patients with RSA.

In addition to murine uNK cells, CD49b is also expressed by a small fraction of other cell types.[13] To our knowledge, there is not an ideal marker for murine uNK cells. A cell purification strategy using DBA-lectin and CD122 marker may be more specific for mouse uNK cell purification.[22] However, microbead-conjugated antibodies for DBA-lectin or CD122 are not commercially available. At present, microbead-conjugated anti-CD49b is used in MACS to purify uNK cells.[26] In future studies, it

would be helpful to exclude T cells and other cells by negative selection during uNK cell purification using microbead-conjugated CD3 and other antibodies specific for non-uNK cells that also express CD49b. Because B cells are virtually absent from the pregnant uterus, the possibility of B-cell contamination is small.[27] A recent report suggested that CD122 is a good marker for uNK cells.[22] To define the percentage of $CD122^+$ cells in the $CD49b^+$ population, we performed two-color flow cytometry using MACS-purified $CD49b^+$ cells stained with FITC-conjugated anti-CD122 and PE-conjugated anti-CD49b antibodies, which showed that most $CD49b^+$ cells were also positive for CD122 in CBA/J×DBA/2J and CBA/J×BALB/c mice.

Flow cytometry is quantitative by nature, allowing thousands of cells to be counted and objectively analyzed within minutes. Using two-color flow cytometry with cells stained by FITC-conjugated anti–stathmin-1 and PE-conjugated anti-CD49b, we confirmed that there are double-positive cells that express stathmin-1 and CD49b and determined the constitutional ratio of these cells. Stathmin-1 expression in $CD49b^+$ cells was also confirmed using MACS-purified $CD49b^+$ cells and proteomic assays in the present study (Figures 2 and 3).

In summary, decreased stathmin-1 expression in a murine abortion-prone model was confirmed using flow cytometry, 2-DE, MS, and Western blot analysis compared with normal fertile controls. In multivision IHC, colocalization of stathmin-1 and DBA-lectin was confirmed in both matings, but the frequency of stathmin-1^+DBA-$lectin^+$ cells was significantly lower in abortion-prone matings. Inhibition of stathmin-1 significantly boosted embryo resorption rates in mouse models. In patients who experience unexplained spontaneous abortion, the frequency of stathmin-1^+CD56^+ cells was also significantly lower than in normal pregnancy. These results suggest that adequate stathmin-1 expression in uNK cells may be critical to pregnancy success. In contrast, insufficient stathmin-1 expression may be correlated with pregnancy failure.

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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.

1. 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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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

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.

2.4. 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.

2.5. 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.

2.6. 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].

2.7. 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.

3. Results

3.1. 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,

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 pre-eclampsia and 29 patients experienced early onset of the disease.

3.2. 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.

3.3. 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.

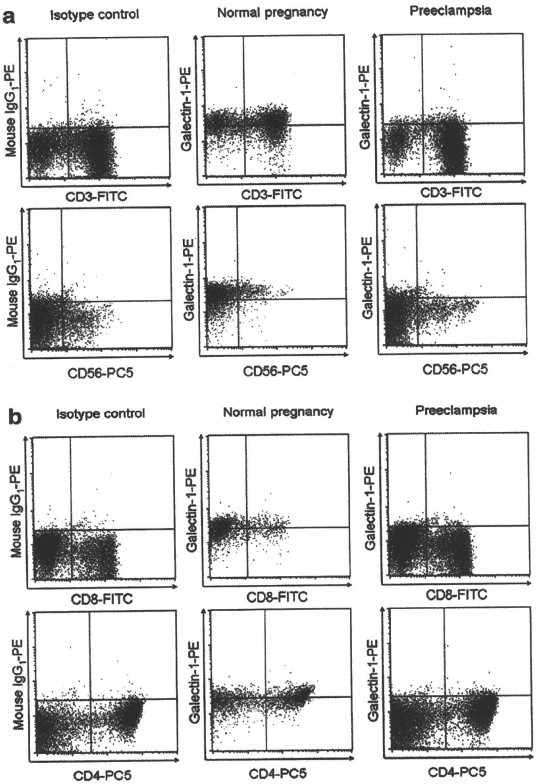

Figure 1 Representative flow cytometric pattern demonstrating intracellular galectin-1 expression of peripheral blood $CD3^+$, $CD56^+$ (Fig. 1a), $CD4^+$ and $CD8^+$ (Fig. 1b) lymphocytes in a healthy pregnant woman and a preeclamptic patient. FITC: fluorescein isothiocyanate; PC-5: phycoerythrin-cyanine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

3.4. 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

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)
$CD3^+Galectin\text{-}1^+/CD3^+$ (%)	8.4 (6.3–10.5)	59.0 (51.7–64.4)[a]	30.6 (21.3–34.4)[a,b]
Log ($CD3^+Galectin\text{-}1^+/CD3^+$ (%)) (adjusted mean±SE)*	n.a.	1.75±0.04	1.37±0.04[b]
$CD4^+Galectin\text{-}1^+/CD4^+$ (%)	8.8 (7.5–11.8)	63.2 (55.0–70.5)[a,†]	33.1 (29.4–40.2)[a,b,†]
Log ($CD4^+Galectin\text{-}1^+/CD4^+$ (%)) (adjusted mean±SE)*	n.a.	1.82±0.04[†]	1.46±0.04[b,†]
$CD8^+Galectin\text{-}1^+/CD8^+$ (%)	12.9 (10.5–15.2)	56.4 (52.6–58.7)[a,†]	30.5 (22.7–38.8)[a,b,†]
Log ($CD8^+Galectin\text{-}1^+/CD8^+$ (%)) (adjusted mean±SE)*	n.a.	1.76±0.04[†]	1.44±0.04[b,†]
$CD56^+Galectin\text{-}1^+/CD56^+$ (%)	11.2 (8.0–17.1)	76.5 (70.3–82.9)[a]	42.9 (32.0–61.9)[a,b]
Log ($CD56^+Galectin\text{-}1^+/CD56^+$ (%)) (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).

4. 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

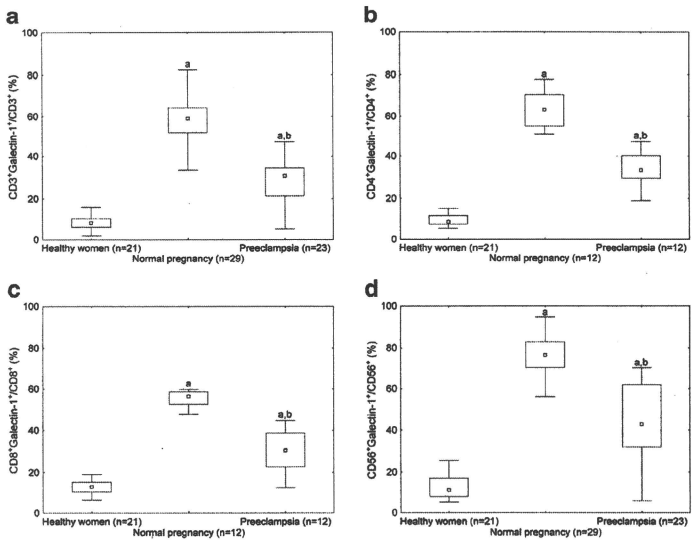

Figure 2 Galectin-1-expressing $CD3^+$ (Fig. 2a), $CD4^+$ (Fig. 2b), $CD8^+$ (Fig. 2c) and $CD56^+$ (Fig. 2d) lymphocytes in proportion to the parent populations in healthy non-pregnant and pregnant women and preeclamptic patients. Middle line: median; Box: interquartile range (25–75 percentile); Whisker: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

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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