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)-l 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

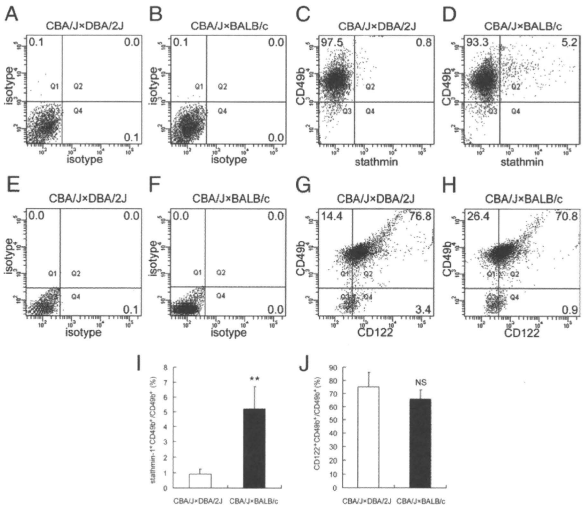


Figure 1. Flow cytometric analysis of stathmin-1$^+$ and CD122$^+$ cells in uNK cells. **A–H:** Representative results derived from cells purified using microbead-conjugated anti-CD49b and a Mini MACS. **A, B, E, F:** Isotype controls established using cells stained with FITC- and PE-conjugated isotype 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).

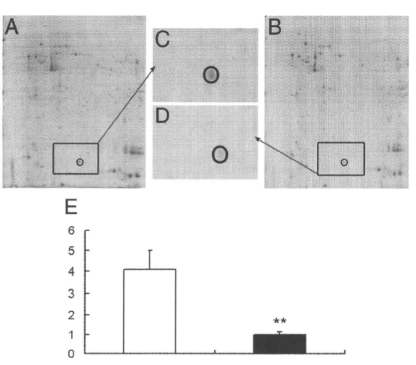
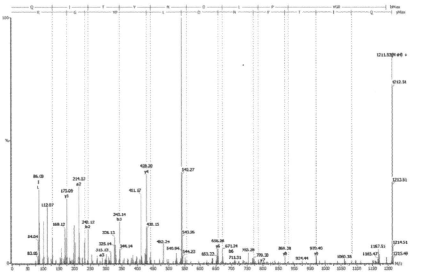


Figure 2. Stathmin-1 expression determined using 2-DE. **A–D:** Map of uterine lymphocyte lysates from CBA/J×BALB/c mice (**A** and **C**) or CBA/J×DBA/2J mice (**B** and **D**). **C** and **D** indicate the original magnification of the stathmin-1 protein spot from CBA/J×BALB/c and CBA/J×DBA/2J mice, 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.



1 MASGHAQIGK SAPDFTATAV VDGAFKEIKL SDYRGKYVVL FFYPLDFTFV
51 CPTEIIAFSD HAEDFRKLGC EVLGVSVDSQ FTHLAWINTP RKEGGLGPLN
101 IPLLADVTKS LSQNYGVLKN DEGIAYRGLF IIDAKGVLRQ ITVNDLPVGR
151 SVDEALRLVQ AFQYTDEHGE VCPAGWKPGS DTIKPNVDDS KEYFSKHN

Figure 3. Mass spectrometry of the stathmin-1 spot. MS/MS signals were derived from the parent ion, for which the amino acid sequence, QITVNDLPVGR, 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.

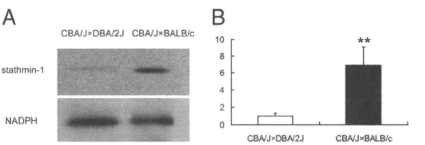
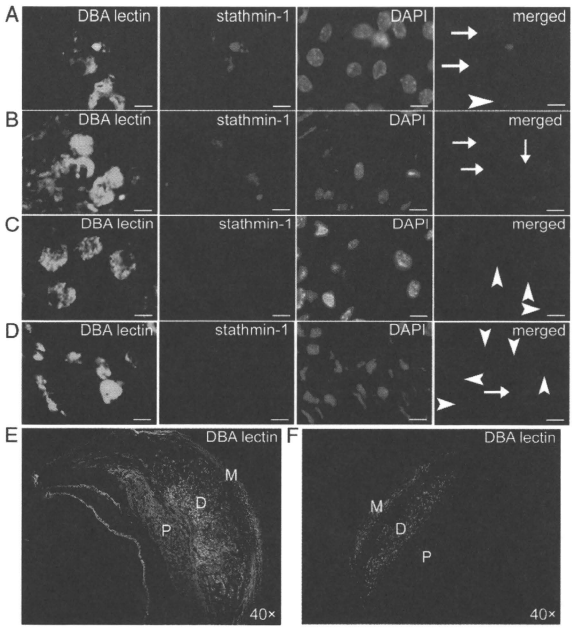


Figure 4. Western blot analysis of stathmin-1 expression in CBA/J×DBA/2J and CBA/J×BALB/c matings. **A:** Stathmin-1 expression is significantly down-regulated in CBA/J×DBA/2J mice compared with CBA/J×BALB/c mice. NADPH was used as an internal loading control. **B:** Histogram showing the relative expression 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.



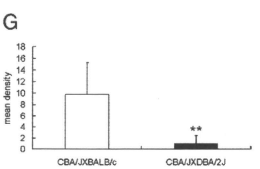


Figure 5. The distribution pattern of stathmin-1$^+$DBA-lectin$^+$ cells in murine tissue. In multivision IHC, uNK cells were stained green with FITC-conjugated DBA-lectin, stathmin-1$^+$ cells were indirectly stained red with rabbit anti–stathmin-1 and PE-conjugated antirabbit IgG, and nuclei were stained 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

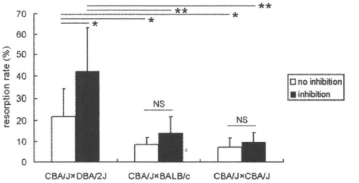


Figure 7. Effect of anti–stathmin-1 antibody treatment on the embryo resorption rate. No inhibition, multiple injections with isotype control antibody; inhibition, multiple injections with neutralizing antibody. In the no-inhibition CBA/J×DBA/2J group, the percentage of embryo loss was significantly 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.

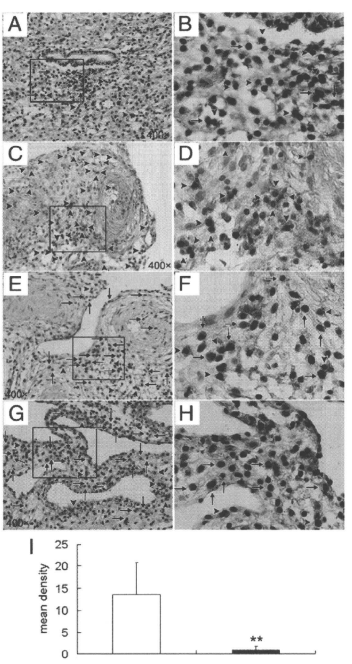


Figure 6. The distribution pattern of stathmin-1$^+$CD56$^+$ cells in human decidua. The distribution pattern of stathmin-1$^+$CD56$^+$ cells was examined by means of double-staining IHC in decidual tissue from RSA patients (**A–D**) and subjects undergoing healthy elective pregnancy termination (**E–H**). 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, co-localization 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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Th1/Th2/Th17 and Regulatory T-Cell Paradigm in Pregnancy

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Keywords
Abortion, cytokine network, implantation, preterm labor, reproduction

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T-helper (Th) cells play a central role in modulating immune responses. The Th1/Th2 paradigm has now developed into the new Th1/Th2/Th17 paradigm. In addition to effector cells, Th cells are regulated by regulatory T (Treg) cells. Their capacity to produce cytokines is suppressed by immunoregulatory cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10 or by cell-to-cell interaction. Here, we will review the immunological environment in normal pregnancy and complicated pregnancy, such as implantation failure, abortion, preterm labor, and preeclampsia from the viewpoint of the new Th1/Th2/Th17 and Treg paradigms.

Introduction

T cells play a central role in immunoregulation and immunostimulation. T-helper (Th) cells can be classified into Th1 cells, which produce interleukin (IL)-2 and interferon (IFN) γ and are involved in cellular immunity, and Th2 cells, which produce IL-4, IL-5 and IL-13 and are involved in humoral immunity.[1] In the 1980s to 1990s, maternal tolerance toward fetal alloantigens was explained by the predominant Th2-type immunity during pregnancy, which overrules Th1-type immunity, therefore protecting the fetus from maternal Th1-cell attack.[2] Indeed, predominant Th1-type immunity has been observed in recurrent spontaneous abortion[3,4] and in preeclampsia.[5] However, Th2-dominant immunity was also reported in recurrent abortion cases,[6,7] and therefore, the Th1/Th2 paradigm is now insufficient to explain the mechanism of why the fetus is not rejected by maternal immune cells. Now, the Th1/Th2 paradigm has been expanded into the Th1/Th2/Th17 and regulatory T (Treg) cells paradigm.[8] Th17 cells, which produce the proinflammatory cytokine, IL-17, play important roles for the induction of inflammation.[8,9] They have been proposed as a pathogenetic mechanism in autoimmune diseases and acute transplant rejection. In contrast, Treg cells play central roles for immunoregulation and induction of tolerance. Treg cells are now known to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T cells, immunoglobulin production by B cells, cytotoxic activity of natural killer (NK) cells, and maturation of dendritic cells (DCs), resulting in induction of tolerance.[10,11]

This review aims to reexamine the Th1/Th2/Th17 and Treg paradigms in normal pregnancy and complicated cases such as implantation failure, recurrent pregnancy loss, and preterm labor.

Reciprocal developmental pathways between Th1/Th17 subsets and between Th17/Treg subsets

Th1 cells are characterized by the transcription factor T-bet and signal transducer and activator of

transcription (STAT) 4, and the production of IL-2, IFN-γ, and tumor necrosis factor (TNF) β. They are involved in the cellular immunity and rejection process. In contrast, mediators of humoral immunity, Th2 cells develop into IL-4-, IL-5-, and IL-13-producing cells by the transcription factor GATA-3 and STAT6. A new lineage of Th cells that selectively produce IL-17 has been proposed. This population, which has been termed Th17, plays a critical role for the induction of inflammation and plays a critical role in the pathogenesis of autoimmune diseases and rejection.[8,9] Th17 cells are characterized by unique signaling pathways, receptor-related orphan receptor (ROR) C2 or ROR-α. Th17 cells are involved in the host defense against bacteria, fungi, and viruses. But evidence for the pathogenic role of Th17 in rheumatoid arthritis (RA), psoriasis, multiple sclerosis, and inflammatory bowel disease has been reported.[8,9]

The function of effector T cells, such as Th1, Th2 and Th17 cells, is regulated by $CD4^+$ $CD25^+$ regulatory T (Treg) cells. $CD4^+$ $CD25^+$ Treg cells are important cells for the maintenance of peripheral tolerance.[10,11] The master gene for the differentiation to Treg cells is transcription factor forkhead box P3 (Foxp3).

Interestingly, recent data show the reciprocal development pathways between Th17/Th1 subset and Th17/Treg subsets[8] (Fig. 1). The progenitor cells differentiate to Th17/Treg intermediate cells, which express both RORC and Foxp3. The differentiation of both Th17 and Treg cells requires transforming growth factor (TGF)-β. When DCs are activated by microorganisms that induce production of pro-inflammatory cytokine IL-6 or IL-1, TGF-β induced differentiation of native T cells diverted away from the induced Treg cells pathway and toward the Th17 cells pathway. The differentiation of human Th17 cells is inhibited by high TGF-β concentrations but requires IL-1β and IL-6.[8,9] Prostaglandin E_2 (PGE_2), which is a mediator of tissue inflammation, induces up-regulation of IL-23R and IL-1R and promotes the differentiation and expansion of Th17 cells. The plasticity of Th17/Th1 lineage has also been reported.[8] The existence of $IFN\gamma^+$ $IL\text{-}17^+$-double positive T cells has been reported. Stimulation by IL-12 down-regulates IL-17 production and induces IFNγ production, suggesting Th1 polarization (Fig. 1), but interplay between the Th1 and Th17 lineages has not been fully clarified.

Interestingly, the conversion of Treg cells to Th17 cells has been reported in humans and mice. IL-6

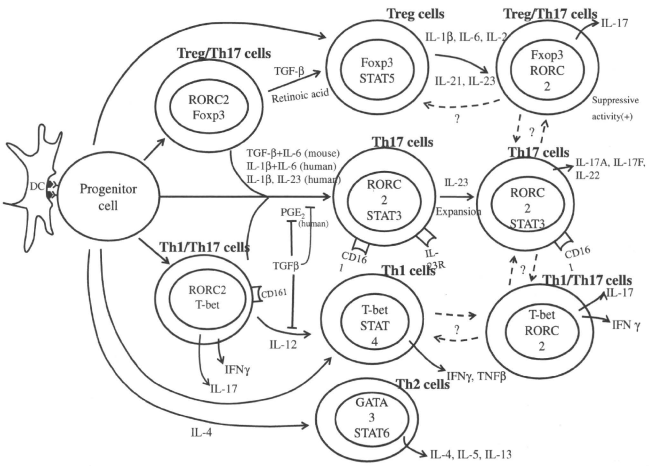

Fig. 1 Th1, Th2, Th17, and Treg cells development from $CD4^+$ progenitors. Plasticity of T-cell phenotype between Th1 cells and Th17 cells or Treg cells and Th17 cells is reported.

plays an important role in this process in mice, and IL-1β and IL-2 are the key cytokines in this process in humans.

These data show that Th1/Th2/Th17 and Treg lineages are associated with each other, and they are able to convert to other lineages.

Th1/Th2/Th17 and Treg paradigms in implantation

Studies in humans and mice have shown that leukemia inhibitory factor (LIF), IL-1, IL-6, IL-11, vascular endothelial growth factor, placenta growth factor, fibroblast growth factor-2, heparin-binding epidermal growth factor (HB-EGF), and TGF-β play important roles for successful implantation by modulating angiogenesis processes, trophoblast differentiation, or the immune system.[12] Recent data show that uterine DCs play a central role for successful implantation.[13,14] The number of uterine DCs increases at the implantation period, and depletion of DCs results in severe implantation failure.[13,14] DC depletion impairs uterine NK cell maturation, tissue remodeling, and angiogenesis.[13,14] Surprisingly, depletion of DCs also causes embryo resorption in syngeneic and T-cell-deficient pregnancy, suggesting that DCs appear to govern uterine receptivity independent of the immunological tolerance.[14] Although tolerogenic DCs take a part in inducing maternofetal tolerance, DCs may play a principal role in implantation. But depletion of Treg cell by anti-CD25 monoclonal antibody on the day or 2.5 days after mating results in severe impairment of implantation in allogeneic mice, but this was not observed in syngeneic mice, suggesting that Treg cells are essential for inducing immunological tolerance.[15,16] Treg cells are already increased in the lymph nodes draining the uterus 2 or 3 days after mating, suggesting that Treg cells accumulate into the lymph nodes draining the uterus before the implantation.[13,14] Robertson et al. reported seminal plasma, but not sperm, plays an essential role for the induction of paternal antigen-specific tolerance.[19] The maternal immune system prepares for the semiallograftic embryo to come into the uterus. Alvihare et al. reported that expansion of Treg cells in the lymph nodes draining uterus was also observed in allogeneic mice and syngeneic mice.[17] They proposed that pregnancy hormones such as estrogen might induce Treg cells numbers and Arruvito et al.[18] showed that Treg cells increased during the follicular phase of the menstrual cycle, suggesting that estrogen plays a part for the expansion of Treg. But our recent data showed paternal antigen-specific Treg cells proliferate in the lymph nodes draining the uterus 3 days after coitus. When BALB/c female mice were mated to DBA/2 male mice, fetuses express DBA/2-derived Mls Ia antigen on the cell surface. As Mls Ia antigen is recognized by T-cell receptor Vβ6, $CD4^+$ $CD25^+$ $Foxp3^+$ $V\beta6^+$ cells are paternal antigen-specific Treg cells. Ki67 is a marker for cell-proliferation; therefore, $Ki67^+$ $CD4^+$ $CD25^+$ - $Foxp3^+$ $V\beta6^+$ cells are proliferating fetal antigen-specific Treg cells. Interestingly, $Ki67^+$ $V\beta6^+$ Treg cells increase in the uterine draining lymph node on 3.5 days post-coitus in BALB/c x DBA/2 mating but not in BALB/c x BALB/c mating, suggesting that paternal antigen-specific Treg cells proliferate in the uterine draining lymph node before the implantation.[19] After implantation, on day 5.5 post-coitus, $Ki67^+$ paternal antigen-specific Treg cells increase in the uterus in BALB/c × DBA/2 mating.[20] These findings demonstrate that paternal antigen-specific Treg cell in draining lymph nodes quickly move to the pregnant uterus and proliferate in the uterus, resulting in the induction of paternal antigen-specific tolerance at the early stage of pregnancy (Fig. 2).[20] The ligands such as CCR2, CCR4, CCR5, CCR6, and CD62L are known as molecules for Treg cell migration. CCL2, CCL22, CCL17, CCL4, CCL20, or L-selection in the uterus might selectively accumulate Treg cells from peripheral tissues.[21] Recent data demonstrate that some Treg cells express LH/CG receptor, and human chorionic gonadotropin produced by human trophoblast has an ability to attract Treg cells to the uterus.[22] Further studies are needed to clarify whether paternal antigen-specific Treg cells express these chemokine receptors or LH/CG receptors on their surface. Th1/Th2/Th17 balance at implantation in such Treg cell-deficient or depleted mice has not been reported, and further studies are needed.

In humans, augmented Th1-type immunity or suppressed Th1-type immunity in endometrium is observed in repeated implantation failure.[23] Deceased number or function of Treg cells might be a cause of augmented Th1-type immunity in such cases. Jasper et al.[24] reported that primary unexplained infertility is associated with reduced expression of Foxp3 mRNA in endometrial tissue. It can be speculated that decreased Treg cells might induce implantation failure, resulting in unexplained infertility.

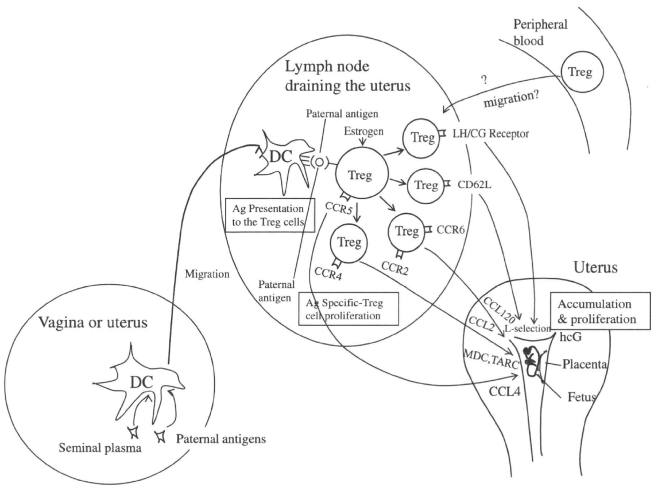

Fig. 2 A model for the paternal antigens-specific Treg cells expansion, proliferation, and mobilization from vagina to pregnant uterus. As a first step, DCs uptake paternal antigen, and these DCs migrate to lymph nodes draining the uterus. DCs present paternal antigens to Treg cells, and Treg cells proliferate before the implantation. These paternal antigen-specific Treg cells migrate to the pregnant uterus by chemokine and hcG-induced chemoattractant mechanisms.

Th1/Th2/Th17 and Treg paradigms in normal pregnancy

Many studies have reported a predominant Th2-type immunity and suppressed Th1-type immunity during pregnancy.[2,3,25] This tendency is more clear at the feto–maternal interface (Table 1). Both Th2 cells and T cytotoxic (Tc) 2 cells accumulate in the decidua basalis,[26,27] and uterine DC cells can differentiate naïve T cells to Th2 cells.[28] Therefore, both Th2 cell migration and Th2 cell differentiation induce Th2-type immunity at the feto–maternal interface. But the systemic immune system does not change so much.[25,26] Moreover, IL-4, IL-5, IL-9, and IL-13 knockout mice show normal pregnancy in allogeneic pregnancy,[28] suggesting that predominant Th2-type immunity might not be essential for successful pregnancy. Administration of excess amount of Th1-type cytokine such as IL-2 or IFNγ induces abortion in mice, and stimulation of toll-like receptor (TLR) induces Th1-type cytokine production, resulting in abortion.[29] But IFNγ also plays an important role for vascular remodeling at the early stage of murine pregnancy.[30] Thus, Th1-type immunity is well controlled to avoid overstimulation of Th1-type immunity. Treg cells might play a part in this process.

IL-17 plays an important role for the pathophysiology in RA.[8,9] The symptoms of RA usually improve during pregnancy,[2] suggesting that Th17 cells might be decreased during pregnancy (Table 1).

Two recent articles show that the frequency of circulating Th17 cells to $CD4^+$ T cells is very low (0.64–1.4%) in healthy subjects.[31,32] In our study, the frequency of Th17 cells to $CD4^+$ T cells during all stages of pregnancy period was similar to that in non-pregnant women,[32] but Santner-Nanan et al.[33] reported that the frequency of Th17 cells in the third trimester of pregnancy was significantly lower compared to that of non-pregnant women. Further studies are needed to determine circulating Th17 cell levels during pregnancy. The main producer of IL-17 in both peripheral blood and deciduas is $CD4^+$ T cells, and IL-17-expressing $CD8^+$ T cells are rare (approximately 0.1%).[32] $CD14^+$ monocytes,

$CD56^{dim}$ NK cells, and $CD56^{bright}$ NK cells do not express IL-17.[34]

Previous studies demonstrate that elevation of IL-17 is observed in acute renal rejection,[35] suggesting that increased Th17 cells in pregnancy decidua might be disadvantageous for the maintenance of pregnancy.[33]

Unexpectedly, the frequency of Th17 cells in the decidua is significantly higher compared to that in peripheral blood. The uterine cavity is not completely sterile, and therefore, Th17 cells might play a role to induce protective immune response against extracellular microbes. Recent reports show that IL-17 increases progesterone secretion by JEG-3 human choriocarcinoma cells and induces the invasive capacity of JEG cells.[36] These data suggest IL-17 may be useful for a successful pregnancy.

Inflammation is necessary for successful implantation, but excessive inflammation can cause embryo resorption. Treg cells might regulate excessive inflammation in the uterus at the implantation period. Indeed, endometrial (decidual) Treg cells increase in mice and humans,[16,18,37–39] and Treg cell number in allogeneic pregnant mice is much higher compared to that in syngeneic pregnancy (Table 1).[39] And these Treg cells in the decidua selectively inhibit the mixed lymphocytes reaction (MLR) to umbilical mononuclear cells, suggesting selective migration of fetal antigen-specific Treg cells to the decidua.[40] In humans, extravillous trophoblasts express polymorphic histocompatibility antigen, HLA-C, which can elicit an allogeneic T-cell-response. Tilburgs et al.[41] recently reported that pregnancy with a HLA-C-mismatched child induces an increased percentage of activated T cells in decidual tissue. Interestingly, HLA-C-mismatched pregnancies exhibit significantly increased suppressive capacity in one-way MLR reaction to umbilical mononuclear cells, suggesting decidual Treg cells inhibit HLA-C-recognized-T-cell attack. These findings show that Treg cells also play an important role for regulating fetal rejection by maternal immune cells in human pregnancy.

Th1/Th2/Th17 and Treg paradigms in abortion

Predominant Th1-type immunity is observed in abortion, but predominant Th2-type immunity is also reported in recurrent pregnancy loss.[2–4,6,7] Therefore, adequate balance for Th1/Th2 immunity, i.e., slightly shifted to Th2-type immunity, may be suitable for the maintenance of pregnancy. Overstimulation of Th1 immunity or Th2 immunity might be harmful for successful pregnancy. A pro-inflammatory cytokine IL-17 induces the expression of many mediators of inflammation. Recent data show an increased prevalence of Th17 cells in peripheral blood and decidua in unexplained recurrent spontaneous abortion patients.[42,43] The master transcription factor for Th17 cells, RORc and IL-23, which play a crucial role in Th17 cells expansion, is also increased in the decidual tissue in recurrent spontaneous abortion cases.[43] An inverse relationship between the numbers of Th17 cells and Treg cells is observed in peripheral blood and decidua of unexplained recurrent spontaneous abortion cases.[43] IL-6 is a key cytokine that blocks the development of Treg cells and induces the differentiation of Th17 cells. The serum IL-6 and soluble IL-6 receptor (sIL-6R) levels are increased. On the other hand, the trans-signaling inhibitor, soluble gp130 (sgp130) level, is decreased in recurrent spontaneous cases.[44] After paternal lymphocytes alloimmunization, sIL-6R levels are decreased and sgp130 levels are increased, and Treg cell number is increased.[44] These findings suggest IL-6 trans-signaling plays a very important role for regulating Th17/Treg balance.

We have already reported that Th17 cells increased in decidua of inevitable abortion, i.e., progression stage of abortion.[42] And the number of $IL-17^{+}$ cells is well correlated with that of neutrophils, suggesting that IL-17 plays an important role in neutrophil infiltration. But Th17 cells did not change in the decidua of missed abortion cases that did not show vaginal bleeding, uterine cramping or cervical deletion.[42] Importantly, all the abortion cases in Wang's study were inevitable abortion.[43] These findings suggest that increased Th17 cells are a consequence of fetal loss, but not a cause of fetal loss. After embryonic death, increased IL-1 or IL-6 production and decreased TGF-β production might cause increased Th17 cells and decreased Treg cells in the uterus.

Our group first reported the decreased numbers of peripheral and decidual Treg cells in spontaneous abortion cases.[37] Yang et al.[45] reported that the proportion of Treg cells in both decidua and peripheral blood in unexplained recurrent spontaneous abortion was significantly lower than those in control women. And the immunosuppressive activity of Treg cells in repeated miscarriage cases have reduced

Table I Th1/Th2/Th17 and Treg Cells in Normal Pregnancy and Abortion

	Normal pregnancy		Abortion		
	Peripheral blood	Uterus	Peripheral blood	Uterus	Depletion of Th1, Th2,Th17 or Treg cells
Th1 cells	↘	↓	↗ →	↑ →	Abortion is not observed.
Th2 cells	↗	↑	→	↓ → ↑(conflict data)	Abortion is not observed.
Th17 cells	→ ↘	↗	→ ↗	→ (missed abortion) ↑ (inevitable abortion) ↑ (recurrent abortion: inevitable abortion)	There is no data, but IL-17 null mice are fertile.
Treg cells	↑	↑↑	→	→	Abortion and implantation failure are observed in allogeneic pregnancy.

→ : no change, ↗: slightly elevate, ↑: elevate, ↑↑: markedly elevate, ↘: slightly decrease, ↓: decrease.

suppressive capacity compared with normal fertile women.[18] Persistent TLR stimulation or IL-6 suppression of Treg function have been reported,[46,47] and therefore these reduced Treg functions in recurrent spontaneous abortion might be caused by chronic inflammation. In a mouse model, when BALB/c $CD25^-$ lymphocytes are injected into BALB/c nu/nu T-cell-deficient mice, they did not have $CD4^+$ $CD25^+$ Treg cells. These Treg-deficient mice showed abortion in allogeneic pregnancy, but this was not observed in syngeneic pregnancy.[17] But it has not been clarified how greatly Treg cells induce abortion in allogeneic pregnancy. We have injected various amounts of anti-CD25 monoclonal antibody on 4.5 and 7.5 days after mating and found that an over 60% decrease of Treg cells could induce abortion in allogeneic pregnancy.[48] CBA/J female mice mated with DBA/2J male mice is a good model for abortion. Interestingly, Zenclussens et al.[16] reported fewer Treg cells associated with elevated Th1-type immunity in CBA/J × DBA/2J mating. Adoptive transfer of $CD4^+$ $CD25^+$ cells from normal pregnant mice prevented fetal loss, but adoptive transfer of $CD4^+$ $CD25^+$ T cells from non-pregnant mice had no effect for protecting fetal loss. Transfer of $CD4^+$ $CD25^+$ T cells from pregnant mice on day 4 of pregnancy did not prevent abortion.[16] Interestingly, transfer of $CD4^+$ $CD25^+$ Treg cells to abortion-prone mice induced the expression of LIF and TGF-β, but the expression of Th1-type cytokines such as IFNγ and TNFαwas unchanged, and Th1/Th2 ratio did not change.[49] These findings show that the protective effect against abortion by Treg cells might not be caused by the correction of abnormal Th1/Th2 balance.

Th1/Th2/Th17 and Treg paradigms in preterm labor

Preterm labor is associated with subclinical infection, and intrauterine inflammatory response causes preterm labor and delivery. Therefore, the secretion of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNFα is increased in amniotic fluid, decidual tissue, and chronic tissue. Although the secretion of Th1-type cytokines, i.e., IL-2, IFNγ, and TNFα, which mediate inflammatory reactions, is up-regulated in preterm labor,[50] the secretion of Th2-type cytokines, i.e., IL-4 and IL-6, is also elevated.[51]

TGF-β might be a key cytokine that regulates Th17/Treg lineage. As IL-17 is a key cytokine that induces inflammation, IL-17 might have some roles in pathophysiology of preterm labor. Our groups recently found that Th17 cell number is increased in the chorioamniotic membrane of preterm delivery cases with chorioamnionitis (CAM).[34] Amniotic fluid IL-17 levels in severe CAM (stage III) preterm delivery cases were significantly higher than those in CAM-negative preterm delivery cases. Amniotic fluid IL-17 levels were positively correlated with IL-8 levels. Interestingly, although IL-17 did not enhance IL-8 secretion by amniotic mesenchymal cells, TNFα-induced IL-8 secretion was enhanced by IL-17 in a dose-dependent manner. Amniotic mesenchymal cells express IL-17 receptor, and therefore, IL-17 signaling pathway might up-regulate TNFα-induced IL-8 secretion by amniotic mesenchymal cells. Indeed, the IKK inhibitor and MAPK inhibitor significantly inhibited IL-17 with TNFα-induced IL-8 secretion in amniotic mesenchymal cells.[34] These findings show that Th17 cells promote inflammation at the feto–maternal interface in preterm delivery.

American Journal of Reproductive Immunology 63 (2010) 601–610

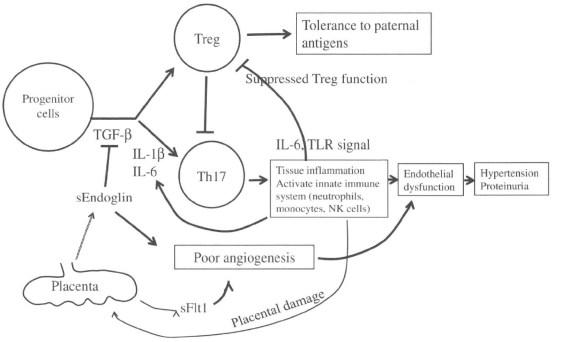

Fig. 3 The interrelationship between inflammation, poor angiogenesis, endothelial damage, and imbalance of Th17 and Treg differentiation.

Th1/Th2/Th17 and Treg paradigms in preeclampsia

In the early days of reproductive immunology when the Th1/Th2 paradigm was first proposed, Th1/Th2 balance in preeclampsia was examined by measuring cytokines or detecting intracellular cytokines by flow cytometry.[5] It has been clarified that predominant Th1-type immunity is present in preeclampsia.[5] A later study demonstrated that the production of Th1-type cytokine, IFN-γ, is also enhanced in NK cells.[52] Secreted IL-12p70 and IL-18, which differentiate Th1 cells from the progenitor cells, are increased in preeclampsia.[5,52] Predominant Th1-type immunity may suppress the tolerance system, resulting in preeclampsia.

As a next stage, our interest shifted to the Treg cells in preeclampsia, because Treg cells were proposed as major contributors to the maintenance of tolerance during pregnancy.[17,37,38] Recent reports described decreased numbers of Treg cells in preeclampsia,[53,54] although two other articles found stable Treg cells in preeclampsia.[55,56] However, the sample number was too small in one study,[56] and the other study[55] evaluated only total $CD4^+$ $CD25^+$ as Treg cells. The frequency of a $CD4^+$ $CD25^{high}$ cell population was not described. In humans, Treg cells are $CD4^+$ $CD25^{high}$ cells, and $CD4^+$ $CD25^{dim}$ cells have no ability for immunoregulation.

Recently, Santner-Nanan et al.[33] reported very interesting findings. They studied the frequency of $CD4^+$ $CD25^{high}$, $CD4^+$ $CD127^{low}CD25^+$, and $CD4^+$ $Foxp3^+$ cells in preeclampsia. Treg cells are involved in all of these three populations. The frequencies of the three populations in preeclampsia were significantly lower than those in normal pregnancy, and the immunosuppressive function of Treg cells did not change in preeclampsia. These findings strongly support that inadequate tolerance because of small numbers of Treg cells may be present in preeclampsia.

Recently, the developmental and functional links between induced Treg (iTreg) cells and Th17 cells have been reported.[8,9] Th17 cells and iTreg cells share a requirement for TGF-β, and high TGF-β concentrations induce Treg cells. Terminal differentiation of Th17 cells requires IL-1β and TGF-β.[57] An imbalance between Treg cells and Th17 cells has been proposed as a pathogenic mechanism in several human diseases. Santner-Nanan et al. recently reported an increased population of peripheral blood Treg cells and a decreased population of peripheral Th17 cells in normal pregnancy. In contrast, a decreased population of Treg cells and an increased population of Th17 cells compared to non-pregnancy levels in preeclampsia have been reported.[33]

Preeclampsia is associated with exaggerated systemic inflammatory changes[58] and poor angiogenesis because of increased levels of soluble Flt-1 and soluble endoglin[59] (Fig. 3). Very interestingly, soluble endoglin is an inhibitor of TGF-β; therefore, TGF-β signaling is suppressed in preeclampsia. Furthermore, IL-1β and IL-6, which induce Th17 cell differentiation, are produced by monocytes in preeclampsia. These factors may easily differentiate Th17 cells, which induce exaggerated inflammation, resulting decreased numbers of Treg cells. Increased Th17 cells

may induce exaggerated systemic inflammatory changes and vascular endothelial dysfunction. Furthermore, chronic inflammation may impair Treg function.[47] The chronic inflammation theory, endothelial dysfunction theory, poor angiogenesis theory, and immune maladaption theory are interrelated, and the imbalanced differentiation of Treg cells and Th17 cells may explain the pathophysiology of preeclampsia.

Conclusions

The new paradigm, i.e., the Th1/Th2/Th17 and Treg paradigms, has been proposed, and emerging literature provides many new findings in reproductive biology and immunology. The balance and correlation between Th1 cells, Th2 cells, Th17 cells, and Treg cells should be discussed to understand reproductive immunology, and it is important to find new therapies for implantation failure, recurrent pregnancy loss, preterm labor, and preeclampsia.

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Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice

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Tolerance

ABSTRACT

Maternal T cells acquire a transient state of tolerance specific for paternal alloantigens during pregnancy. $CD4^+CD25^+$ regulatory T (Treg) cells play a central role in induction and maintenance of tolerance. We have studied the role of Treg cells for the maintenance of allogeneic pregnancy during the implantation period, early pregnancy period and late pregnancy period. We performed depletion of Treg cells using treatment with anti-CD25 monoclonal antibody (mAb) in allogeneic or syngeneic pregnant mice. BALB/c or C57BL/6 female mice were mated with BALB/c or C57BL/6 male mice, and anti-CD25 mAb was injected intraperitoneally on day 2.5 post-coitum (pc), or days 4.5 and 7.5 pc, or days 10.5 and 13.5 pc. Administration of 0.5 mg of anti-CD25 mAb induced depletion of $CD4^+CD25^+Foxp3^+$ Treg cells in both allogeneic and syngeneic pregnancy. The extent of depletion of $CD4^+CD25^+$ Treg cells in spleen cells was 82.7%. This mAb treatment on day 2.5 pc of pregnancy induced implantation failure in allogeneic pregnant mice, but not in syngeneic pregnant mice. In addition, anti-CD25 mAb treatment on days 4.5 and 7.5 pc significantly increased resorption rates in allogeneic pregnant mice, but not in syngeneic pregnant mice. Interestingly, anti-CD25mAb treatment on days 10.5 and 13.5 pc reduced Treg cell numbers, but this treatment did not induce any abnormal pregnancy parameters such as intrauterine growth restriction, hypertension, or proteinuria. These findings suggest that $CD4^+CD25^+Foxp3^+$ Treg cells are important to mediate maternal tolerance to the allogeneic fetus in the implantation phase and early stage of pregnancy, but Treg cells might not be necessary for maintenance of the late stage of allogeneic pregnancy.

1. Introduction

During pregnancy, the fetal 'allograft' is not rejected by the maternal host. It has been recognized that the systemic maternal immune system is altered in normal pregnancy. Maternal T cells acquire a transient state of tolerance specific for paternal alloantigens during pregnancy. After delivery, tumor grafts that express paternal antigens are rejected, suggesting that the tolerance specific to paternal alloantigens is restricted to the pregnancy period (Tafuri et al., 1995). Jiang et al., reported that the number of T cells that recognize fetal antigens decreased in an antigen-specific manner during pregnancy, consistent with peripheral clonal deletion in the maternal immune system (Jiang and Vacchio, 1998).

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Recent findings demonstrate that alloantigen-specific immunoregulatory T cells control immune responsiveness, and this activity is enhanced in a population of $CD4^+CD25^+$ T cells (Sakaguchi et al., 1995). Accumulating evidence from both experimental and clinical studies indicates that a balance between regulation and deletion of responder T cells is an effective strategy to control immune responsiveness after organ or cell transplantation (Wood and Sakaguchi, 2003). $CD4^+CD25^+$ regulatory T (Treg) cells have been shown to have potent regulatory properties in both the induction and maintenance phases of *in vivo* tolerance to alloantigens in mice (Sakaguchi et al., 1995; Taylor et al., 2001; Karim et al., 2002; Hoffmann et al., 2002) and humans (Baecher-Allan et al., 2001; Jonuleit et al., 2001; Levings et al., 2001; Ng et al., 2001; Stephens et al., 2001).

Recent studies suggest that circulating and decidual (uterine) $CD4^+CD25^+$ Treg cells increase during pregnancy in humans (Sasaki et al., 2004; Heikkinen et al., 2004; Somerset et al., 2004; Saito et al., 2005) and in mice (Aluvihare et al., 2004; Zenclussen et al., 2005; Zenclussen, 2005; Zhu et al., 2005; Robertson et al., 2009). Also, these maternal Treg cells suppress responses to paternal alloantigens. Decreased Treg cells have been reported in human miscarriage cases (Sasaki et al., 2004; Yang et al., 2008) and abortion-prone mice (Zenclussen et al., 2005; Zhu et al., 2005). Decreased expression of Foxp3 mRNA in the endometrium of women experiencing primary unexplained infertility was also reported (Jasper et al., 2006).

Aluvihare et al. (2004) injected BALB/c nu/nu mice with 2×10^7 lymphocytes with or without prior depletion of $CD25^+$ cells. All recipient BALB/c nu/nu females were mated with C57BL/6 male mice or BALB/c male mice on the day after adoptive transfer. When total lymphocytes were injected, they observed viable fetuses in allogeneic pregnancy. However, when $CD25^-$ lymphocytes were injected, all fetuses were resorbed in allogeneic pregnancy, while they observed viable fetuses in syngeneic pregnancy. These findings strongly suggest that Treg cells mediate maternal tolerance to the fetus. However, these immune conditions were similar to those in scurfy mice or immune dysregulation, polyendocrinopathy, and X-linked syndrome (IPEX) in humans in whom Treg are absent because of the *FOXP3* mutation. These immune conditions cause systemic autoimmune disease, and may affect pregnancy outcome. To exclude this confounding effect, anti-CD25 monoclonal antibody (mAb) treatment has been performed in experiments to deplete $CD4^+CD25^+$ Treg cells. Anti-CD25 mAb treatment reduces $CD4^+CD25^+$ T cells maximally for 3 days, and $CD4^+CD25^+$ T cells fully recover 7 days after administration (Onizuka et al., 1999). Anti-CD25 mAb treatment on the day of mating has been shown to induce the activation of $CD4^+$ T cells and $CD8^+$ T cells resulting in a small number of allogeneic fetuses that survive to term (Darrasse-Jèze et al., 2006). However, it has not been clarified as to what levels of Treg cell-depletion induce abortion, and when during gestation Treg cells are most critical for maintenance of the fetal allograft. To investigate these points, we injected various amounts of anti-CD25 mAb into allogeneic or syngeneic pregnant mice in the implantation period, early pregnancy period and late pregnancy period.

2. Materials and methods

2.1. Mice

Mice were purchased from Charles River (Shizuoka, Japan) and maintained in an animal facility with a 12 h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines of Toyama Medical and Pharmaceutical University.

BALB/c or C57BL/6 female mice (8–10 weeks old) were mated with C57BL/6 or BALB/c male mice. We checked for vaginal plugs and sperm in vaginal fluid the next morning and separated them from the males if mated. The day of the vaginal plug was considered to be day 0.5 post-coitum (pc).

2.2. Anti-CD25 mAb treatment

The anti-CD25 mAb produced by hybridoma PC61 (Lowenthal et al., 1985) is a rat IgG1 antibody. Onizuka et al. reported that $CD4^+CD25^+$ T cells were reduced maximally after 3–4 days and fully recovered by 7 days after a single *in vivo* administration of anti-CD25 mAb (PC61) (Onizuka et al., 1999). Therefore, we injected anti-CD25 mAb into pregnant mice on day 2.5 pc (study 1) (Fig. 1), on days 4.5 and 7.5 pc (study 2) or days 10.5 and 13.5 pc (study 3). Control groups of pregnant mice were injected intraperitoneally with normal rat IgG (0.5 mg/mouse). On day 7.5 pc (study 1), day 11.5 pc (study 2) or day 18.5 pc (study 3) of pregnancy, pregnant mice were sacrificed, the uteri were removed and implantation sites were documented.

2.3. Blood pressure

The animals were restrained for 10 min in a plastic holder designed for mice in a temperature control chamber (35 °C). Systolic blood pressure was determined by the tail-cuff method using a programmed sphygmomanometer (MMK-1100, Muromachi Kikai, Tokyo, Japan). Each estimation was the average of three recordings taken at 1 min intervals.

2.4. Proteinuria

Urine samples were analyzed for proteinuria using a qualitative method (Uristics, Siemens, Tokyo, Japan). The test allows distinction among values from 0, 30, 100, 300 to 1000 mg/dl.

2.5. Flow cytometry

We isolated mononuclear cells from the spleen, draining lymph nodes and peripheral blood by density gradient (Lympholyte M, Cat No: CL5030, Cedarlane Lab, Ontario, Canada). Mononuclear cells (1×10^6) were stained with anti-mouse CD4-FITC (Cat No: 553729) or CD4-PerCP-Cy5.5 (Cat No: 550954, both Becton Dickinson, Mountain View, CA, USA) and anti-mouse CD25-PE (Cat No: 553075, clone 3C7, Becton Dickinson). Intracellular detection of Foxp3 with anti-mouse/rat Foxp3-PE or Foxp3-APC (Cat No: 17-5773-82, clone FJK-16s, e-Bioscience, San Diego,