Table 2. Association of aPL with pregnancy outcome.

	Poor outcome	Good outcome	*P* value
Lupus Anticoaglulant	9/9 (100%)	2/6 (33%)	0.041*
dRVVT	6/9 (67%)	2/6 (33%)	0.315
aPTT (Staclot LA)	9/9 (100%)	2/5 (40%)	0.047*
IgG aβ2GPI	8/9 (89%)	1/6 (17%)	0.011*
aCL	8/9 (89%)	6/6 (100%)	1.0
IgG	8/9 (89%)	6/6 (100%)	1.0
IgM	3/9 (33%)	0/6	0.338
IgG aPS/PT	5/5 (100%)	0/3	0.072
IgG aPE	0/8 (0%)	0/3	1.0

*Significant ($P < 0.05$).

although they did not have high anticoagulant activity (data not shown). IgG aβ2GP1 was positive only in one case (#5) but with low titer. Neither IgG aPS/PT nor IgG aPE was detected in any of the patients.

Characteristics of cases with poor outcome (n = 9)

The mean age at diagnosis was 32.1 years (range: 25–37 years). Four cases had history of thrombosis. In terms of autoimmune diseases, seven cases were complicated with SLE; however, SLE activity in all cases was under control (all had a SLEDAI score [15] of less than 3).

Intrauterine growth retardation (IUGR) was suggested by severely low birth weight compared to gestational period. Pregnancy had to be terminated due to adverse obstetrical outcomes in two cases (#6 and #9 in Table 1). The other cases were successfully treated to achieve live birth. However, most of the cases had delivery before 34 weeks of gestation (#7, #8, #8', #10, #12, and #13). IUGR occurred in three cases (#11, #12, and #13). Two cases (#8 and #8') were complicated with pregnancy-induced hypertension.

LA was positive in all cases, while IgG aβ2GP1 and IgG aCL were low or negative in some cases. IgG aPS/PT presented high titer in all cases. IgG aPE was not detected in any of the patients.

Low-dose corticosteroids, intravenous immunoglobulin (IVIg), or plasma exchange (PE) was used for additional treatment.

Comparison of aPL profiles between both groups

The aPL profile was compared between the good and poor outcome groups (Table 2). LA, IgG aβ2GPI, and IgG aPS/PT were more frequently found in the poor outcome group compared to that of good outcome.

Discussion

In this study, we have demonstrated the correlation of aPL profile with pregnancy outcome in APS patients; LA was strongly associated with poor outcomes, while IgG aPS/PT, a marker strongly associated with LA [13], was generally positive in the poor outcome group. Meanwhile, aPE, a marker significantly related to recurrent pregnancy loss [8], did not accurately indicate the severity of the condition. As we could not examine IgG aPS/PT and aPE in all of the poor outcome cases, further study is necessary to clarify these correlations.

In both groups, there were a number of cases in which obstetric complications were difficult to control with only LDA and heparin therapy. In addition to being LA positive and having a higher frequency of IgG aβ2GPI and IgG aPS/PT, most of the cases with poor outcome appeared to have all of LA, aCL-β2GPI and aPS/PT, but not aPE. Regarding the titer of each aPL in our study – although there is no previous report which revealed the relation between aPL titer and pregnancy outcome – LA, aCL-β2GPI and aPS/PT, but not aPE, had a tendency to show higher titer or higher activity in poor outcome cases than the good ones. The titer of aPE did not correlate with the outcome. A point to note is that LA was not always positive in both dRVVT and Staclot LA tests even in the cases with poor outcome. This suggested that more than one test is necessary for detecting LA, a key marker for diagnosing APS and predicting the outcome. We found that IgG aPS/PT was helpful for identifying LA, while IgG aPS/PT was useful for assessing LA activity during heparin treatment.

None of the patients in our study presented IgG aPE. This was likely to be associated with recurrent early pregnancy losses [8], suggesting that recurrent early pregnancy losses may have a different pathogenesis from severe obstetric APS.

Because the average age was younger in the group with poor outcome than the other group, we propose that age is not a major determinant of outcome among patients in this study.

Prior thrombosis was observed in one (20%) in the group with good outcome and four (50%) in the group with poor outcome, presumably affecting the severity of APS pregnancy. In fact, one patient (#6 in Table 1) presented cerebral infarction as soon as she became pregnant, while another (#13) presented spleen infarct during puerperal period after delivery. These experiences indicated that careful management is crucial in preventing thrombotic events throughout pregnancy and perinatal period.

Seven cases had SLE (two in the good outcome group and five in the other), one in the good outcome group had Grave's disease, while one in poor outcome group had autoimmune hepatitis. As patients with autoimmune diseases were found in both groups, we propose that pregnancy outcome was not dependent on the type of underlying autoimmune condition.

While optimal pharmacologic treatment is essential to achieve a successful outcome in APS pregnancy, such treatment by itself is not sufficient. Careful obstetric monitoring, proper delivery timing and skillful neonatal care are all necessary factors for a more desirable outcome. Meticulous management by a cooperative team constituting of rheumatologists, obstetricians, and neonatologists is important in minimizing potential adverse consequences associated with premature delivery.

One limitation of this study was the small number of patients enrolled. Despite that, our investigation suggested the importance of aPL profile in accessing pregnancy outcome in APS patients. Considering the low prevalence of severe cases, multi-center large-scale studies should be designed to confirm the significance of aPL profile in the obstetric APS.

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Conflicts of interest

None.

References

1. Ruiz-Irastorza G, Crowther M, Branch W, Khamashta MA. Antiphospholipid syndrome. Lancet. 2010;376(9751):1498–509.
2. Wilson WA, Gharavi AE, Koike T, Lockshin MD, Branch DW, Piette JC, et al. International consensus statement on preliminary classification criteria for definite antiphospholipid syndrome: report of an international workshop. Arthritis Rheum. 1999; 42(7):1309.
3. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). J Thromb Haemost. 2006;4:295–306.

4. American College of Obstetricians and Gynecologists Committee on Practice Bulletins-Obstetrics. ACOG Practice Bulletin No. 118: Antiphospholipid syndrome. Obstet Gynecol. 2011;117:192.
5. Infante-Rivard C, David M, Gauthier R, Rivard GE. Lupus anticoagulants, anticardiolipin antibodies, and fetal loss. A case-control study. N Engl J Med 1991;325:1063
6. Oshiro BT, Silver RM, Scott JR, Yu H, Branch DW. Antiphospholipid antibodies and fetal death. Obstet Gynecol. 1996;87:489.
7. Roqué H, Paidas MJ, Funai EF, et al. Maternal thrombophilias are not associated with early pregnancy loss. Thromb Haemost. 2004;91:290.
8. Sugi T, Katsunuma J, Izumi S, McIntyre JA, Makino T. Prevalence and heterogeneity of antiphosphatidylethanolamine antibodies in patients with recurrent early pregnancy losses. Fertil Steril. 1999; 71(6):1060–5.
9. Mak A, Cheung MW, Cheak AA, Ho RC. Combination of heparin and aspirin is superior to aspirin alone in enhancing live births in patients with recurrent pregnancy loss and positive anti-phospholipid antibodies: a meta-analysis of randomized controlled trials and meta-regression. Rheumatology (Oxford). 2010;49:281.
10. Pengo V, Tripodi A, Reber G, Rand JH, Ortel TL, Galli M, et al. Up- date of the guidelines for lupus anticoagulant detection. J Thromb Haemost. 2009;7:1737–40.
11. Harris EN, Gharavi AE, Patel SP, Hughes GR. Evaluation of the anti-cardiolipin antibody test: report of an international workshop held 4 April 1986. Clin Exp Immunol. 1987;68(1):215–22.
12. Amengual O, Atsumi T, Khamashta MA, Koike T, Hughes GR. Specificity of ELISA for antibody to beta 2-glycoprotein I in patients with antiphospholipid syndrome. Br J Rheumatol. 1996;35(12):1239–43.
13. Atsumi T, Ieko M, Bertolaccini ML, Ichikawa K, Tsutsumi A, Matsuura E, Koike T. Association of autoantibodies against the phosphatidylserine-prothrombin complex with manifestations of the antiphospholipid syndrome and with the presence of lupus anticoagulant. Arthritis Rheum. 2000;43(9):1982–93.
14. Yamada H, Atsumi T, Kobashi G, Ota C, Kato EH, Tsuruga N, et al. Antiphospholipid antibodies increase the risk of pregnancy-induced hypertension and adverse pregnancy outcomes. J Reprod Immunol. 2009;79(2):188–95.
15. Gladman DD, Ibanez D, Urowitz MB. Systemic lupuserythematosus disease activity index 2000. J Rheumatol. 2002;29:288–91.

Combination therapy with anticoagulants, corticosteroids and intravenous immunoglobulin for women with severe obstetric antiphospholipid syndrome

Sirs,

Obstetric antiphospholipid syndrome (APS) is characterised by recurrent early miscarriages, foetal loss in later pregnancy and maternal morbidity by thrombosis (1). Although the first-choice treatment regimen for women with obstetric APS is anticoagulation therapy with a combination of heparin and low dose aspirin (LDA) (2), 20–30% of cases result in pregnancy failure despite appropriate treatment (2, 3). In addition, among such cases are women with a significantly higher risk of developing recurrent severe gestational complications such as maternal thrombosis, preeclampsia and HELLP syndrome (4-5). Combination therapies with standard antithrombotic therapy for severe APS patients have been anticipated. Immunotherapies such as intravenous immunoglobulin (IVIG) and/or corticosteroids may be good candidates for severe obstetric APS patients (6-8). Here, we describe our experiences treating three severe obstetric APS patients with a combination of anticoagulants, corticosteroids and IVIG.

Table I shows background characteristics and pregnancy outcomes of the three patients. All patients were diagnosed based on the revised Sidney criteria (1). We defined women with "severe APS" as those with (1) severe late gestational complications such as preeclampsia or intrauterine foetal death, or (2) any thrombotic event during or soon after pregnancy despite anticoagulation therapy. Based on recommended protocols, continuous unfractionated heparin injections were initiated after a urine pregnancy test (at around 4 gestational weeks) for all three patients (2, 9, 10). LDA administered before pregnancy was continued after conception and warfarin medication was discontinued after initiating heparin injection. The quantity of injected heparin was adjusted to maintain a plasma heparin concentration above 0.2 U/ml, which is associated with a decreased risk of thrombosis during pregnancy (9, 10). Prednisolone (PSL; 10–20 mg/day) was also initiated upon diagnosis of pregnancy. Further to this, a five-day course of IVIG (400 mg/kg body weight) was initiated soon after confirmation of a foetal heartbeat (FHB) (at around 6–7 gestational weeks), as the rate of early natural pregnancy loss decreases after ultrasound confirmation of FHB (10).

Case 1 suffered from severe preeclampsia with thrombocytopenia and splenic infarction during the postpartum period despite antithrombotic therapy with LDA and heparin.

Case 2 experienced intrauterine foetal deaths (IUFDs) twice. In addition, she experienced a brain infarction during the postpartum period of the first pregnancy. She took no medications during the first pregnancy, and took LDA and corticosteroids during the second pregnancy. Case 3 experienced a thrombotic event after early pregnancy loss despite heparin injection. All three patients were positive for anti-beta 2 glycoprotein I (β2GPI) antibody, anti-cardiolipin (CL) antibody and lupus anticoagulant (LA). All patients had been taking LDA and warfarin while not pregnant. All three patients achieved live births. Case 2 ended in preterm delivery at 32 gestational weeks; however, this was solely attributed to an obstetric complication (*i.e.* placenta previa with increased genital bleeding). All three patients received anticoagulation therapy with LDA and heparin during the intrapartum period given worries of thrombosis rather than bleeding at delivery. No thrombotic or bleeding complications were observed in any of the patients.

Although results from several studies have not supported the use of immunotherapies such as corticosteroids and IVIG for treating APS (2-3), proper management guidelines for severe obstetric APS patients have yet to be established. Once recent study suggested that low dose corticosteroids given during the first trimester in addition to anticoagulation therapy improved pregnancy outcomes in women with severe obstetric APS (8). However, the live birth rate of the series in that study was 61%. We believe that additional IVIG can improve pregnancy outcomes of severe obstetric APS patients. Further studies will be needed to clarify the effectiveness of additional immunotherapies in treating severe obstetric APS patients.

Table I. Background characteristics and pregnancy outcomes.

Case no.	1	2		3
Age (years)	29	35		31
Height (cm), Body weight (kg)	148, 40	162, 46		162, 52
Gravida, Para	G1P1	G2P2		G1P0
Diagnosis	APS	APS		APS
Age of onset (years)	17	24		19
Disorder	DVT	IUFD		Pulmonary embolism
Past pregnancy history	1st	1st	2nd	1st
Age (years)	27	24	29	27
Medication	LDA (81 mg/day) Heparin (10,000 U/day)	None	LDA (81 mg/day)	Heparin (10,000 U/day)
Complication: Mother	Preeclampsia	Postpartum brain infarction	None	Early pregnancy loss, DVT
Complication: Infant	Intact survival	IUFD	IUFD	–
Gestational age at delivery (wks)	29	24	29	7
Birth weight (grams)	984	222	873	–
Titer of aPL antibodies at non-pregnancy period (normal range)				
anti-CL IgG (0–9 U/ml)	19	69		440
anti-β2GPI IgG (0–3.4 U/ml)	22.9	208		218
LA	Positive	Positive		Positive
Pre-conception clinical symptoms	None	None		None
Pre-conception medications	LDA + warfarin	LDA + warfarin		LDA + warfarin
Method of conception	Natural	Natural		Natural
Gestational age at delivery	37w0d	32w2d		37w3d
Indication of delivery	Previous cesarean section	Total placenta previa (Increased genital bleeding)		Increased genital bleeding
Mode of delivery	Cesarean section	Cesarean section		Transvaginal
Birth weight (SD score)	2692 (-0.1SD)	1484 (-1.4SD)		2052 (-1.7SD)
Thrombotic complication	None	None		None

APS: antiphospholipid syndrome; DVT: deep venous thrombosis; LDA: low-dose aspirin; IUFD: intrauterine foetal death; anti-β2GPI: anti-beta 2 glycoprotein I; anti-CL: anti-cardiolipin; LA: lupus anticoagulant; SD: standard deviation.

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References

1. MIYAKIS S, LOCKSHIN MD, ATSUMI T *et al.*: International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 2006; 4: 295-306.
2. ZIAKAS PD, PAVLOU M, VOULGARELIS M: Heparin treatment in antiphospholipid syndrome with recurrent pregnancy loss: a systematic review and meta-analysis. *Obstet Gynecol* 2010; 115: 1256-62.
3. DE JESUS GR, DOS SANTOS FC, OLIVEIRA CS, MENDES-SILVA W, DE JESUS NR, LEVY RA: Management of obstetric antiphospholipid syndrome. *Curr Rheumatol Rep* 2012; 14: 79-86.
4. FISCHER-BETZ R, SPECKER C, BRINKS R, SCHNEIDER M: Pregnancy outcome in patients with antiphospholipid syndrome after cerebral ischaemic events: an observational study. *Lupus* 2012; 21: 1183-9.
5. LE THI THOUONG D, TIEULIE N, COSTEDOAT N *et al.*: The HELLP syndrome in the antiphospholipid syndrome: retrospective study of 16 cases in 15 women. *Ann Rheum Dis* 2005; 64: 273-8.
6. SHERER Y, LEVY Y, SHOENFELD Y: Intravenous immunoglobulin therapy of antiphospholipid syndrome. *Rheumatology* (Oxford) 2000; 39: 421-6.
7. TRIOLO G, FERRANTE A, ACCARDO-PALUMBO A *et al.*: IVIG in APS pregnancy. *Lupus* 2004; 13: 731-5.
8. BRAMHAM K, THOMAS M, NELSON-PIERCY C, KHAMASHTA M, HUNT BJ: First-trimester low-dose prednisolone in refractory antiphospholipid antibody-related pregnancy loss. *Blood* 2011; 117: 6948-51.
9. BATES SM, GREER IA, PABINGER I, SOFAER S, HIRSH J: Venous thromboembolism, thrombophilia, antithrombotic therapy, and pregnancy: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008; 133 (6 Suppl.): 844S-886S.
10. PAPAIOANNOU GI, SYNGELAKI A, MAIZ N, ROSS JA, NICOLAIDES KH: Ultrasonographic prediction of early miscarriage. *Hum Reprod* 2011; 26: 1685-92.

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Helios-positive functional regulatory T cells are decreased in decidua of miscarriage cases with normal fetal chromosomal content

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ABSTRACT

Regulatory (Treg) T cells play essential roles in the maintenance of allogeneic pregnancy in mice and humans. Recent data show that Foxp3 expression occurs in both immunosuppressive Treg and -nonsuppressive effector T (Teff) cells upon activation in humans. Samstein et al. (2012) reported that inducible Treg (iTreg) cells enforce maternal–fetal tolerance in placental mammals. Therefore, we should reanalyze which types of Treg cell play an important role in the maintenance of allogeneic pregnancy. In this study, we studied the frequencies of naïve Treg cells, effector Treg cells, Foxp3$^+$ Teff cells, Helios$^+$ naturally occurring Treg (nTreg) cells, and Helios$^-$ iTreg cells using flow cytometry. The frequencies of effector Treg cells and Foxp3$^+$ Teff cells among CD4$^+$Foxp3$^+$ cells in the decidua of miscarriage cases with a normal embryo karyotype ($n=8$) were significantly lower ($P=0.0105$) and significantly higher ($P=0.0258$) than those in normally progressing pregnancies ($n=11$), respectively. However, these frequencies in miscarriages with an abnormal embryo karyotype ($n=15$) were similar to those in normally progressing pregnancies. The frequencies of these cell populations in the three groups were unchanged in peripheral blood; on the other hand, most of the effector Treg cells in the decidua were Helios$^+$ nTreg cells and these frequencies were significantly higher than those in peripheral blood, while those among effector Treg and naïve Treg cells in the decidua and peripheral blood were similar among the three groups. These data suggest that decreased Helios$^+$ effector nTreg might play an important role in the maintenance of pregnancy in humans.

1. Introduction

Regulatory T cells (Treg) play an important role in the induction and maintenance of tolerance (Sakaguchi et al., 1995; Sakaguchi, 2005). In 2004, Aluvihare et al. and our group first reported that Treg cells might mediate maternal tolerance to the fetus in mice and humans (Aluvihare et al., 2004; Sasaki et al., 2004). Since then, many lines of evidence supporting the importance of Treg cells in the implantation period and the early pregnancy period have been presented, with more detailed characterization of Treg cells in mice (Zenclussen et al., 2005, 2006; Darrasse-Jèze et al., 2006; Kallikourdis et al., 2007; Robertson et al., 2009; Shima et al., 2010; Kahn and Baltimore, 2010; Guerin et al., 2011; Rowe et al., 2011, 2012; Samstein et al., 2012; Yin et al., 2012) and in humans (Tilburgs et al., 2008, 2009; Sasaki et al., 2007; Yang et al., 2008; Jin et al., 2009; Mei et al., 2010; Wang et al., 2010, 2011; Winger and Reed, 2011; Lee et al., 2011; Steinborn et al., 2012).

For example, adoptive transfer of Treg cells purified from normal pregnant mice prevented fetal loss in CBA/J♀ × DBA/2J ♂ mice (Zenclussen et al., 2005; Yin et al.,

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2012), suggesting that fetal antigen-specific Treg cells might play essential roles in the maintenance of allogeneic pregnancy. Seminal plasma plays an important role in the expansion of fetal antigen-specific Treg (Robertson et al., 2009; Guerin et al., 2011). Memory Treg cells that recognize paternal antigens also rapidly increase in a second pregnancy with the same partner compared with the first (Rowe et al., 2012). A decreased Treg cell pool was observed in recurrent miscarriage cases in humans (Yang et al., 2008; Jin et al., 2009; Mei et al., 2010; Wang et al., 2010, 2011; Lee et al., 2011) and a low Treg cell level predicts the risk of miscarriage in cases of unexplained recurrent pregnancy loss (Winger and Reed, 2011). Selective migration of fetus-specific Treg cells from the peripheral blood to the decidua in human pregnancy has also been reported (Tilburgs et al., 2008). We have reported that the population of $CD4^+Foxp3^+$ Treg cells at the decidua basalis in miscarriage with a normal karyotype embryo was significantly lower than in normally progressing pregnancy and in miscarriage with an abnormal embryo, suggesting that dysregulation of maternal tolerance to the fetus might be associated with unknown etiology of miscarriage in humans (Inada et al., 2013).

We considered that Foxp3 is the most reliable marker for Treg cells (Hori et al., 2003), but recent data have shown that $Foxp3^+$ expression in humans occurs in both immunosuppressive Treg cells and non-suppressive and cytokine-producing effector T cells (Walker et al., 2003; Gavin et al., 2006; Allan et al., 2007). Miyara et al. (2009) reported that human $CD4^+Foxp3^+$ cells are classified into $CD4^+CD45RA^+Foxp3^{low}$ naïve Treg cells, $CD4^+CD45RA^-Foxp3^{high}$ effector Treg cells, and $CD4^+CD45RA^-Foxp3^{low}$ effector T (Teff) cells. Importantly, those $Foxp3^+$ Teff cells have no capacity for immunoregulation. $CD4^+CD25^{bright}$ T cells exhibit regulatory function in humans (Beacher-Allan et al., 2001). Miyara et al. (2009) showed that $CD4^+CD25^{bright}$ cells are composed of $CD4^+CD45RA^+Foxp3^{low}$ naïve T cells, $CD4^+CD45RA^-Foxp3^{low}$ Teff cells, and $CD4^+CD45RA^-Foxp3^{high}$ effector Treg cells. $CD4^+CD25^+CD127^-$ cells are believed to be a reliable marker for Treg cells (Seddiki et al., 2006), but Kleinewietfeld et al. (2009) pointed out that some of the $CD4^+CD25^+CD127^-$ cells are Teff cells, which produce IFN-γ, IL-2, and IL-17. These findings suggest that we should re-evaluate which subsets of $Foxp3^+$ cells play an important role in the maintenance of pregnancy.

Treg cells are classified as naturally occurring Treg (nTreg) generated in the thymus and inducible T (iTreg) generated in the periphery (Sakaguchi, 2005). Samstein et al. (2012) reported that conserved noncoding sequence 1 (CNS1), which is essential for iTreg differentiation, is conserved only in placental mammals, and CNS1-deficient female mice showed increased fetal resorption in allogeneic pregnancy. These findings suggest that iTreg induction at the fetomaternal interface might be important for a successful pregnancy in placental mammals.

However, it has still not been reported which subset of Treg cells is important for the maintenance of human pregnancy: naive Treg cells, effector Treg cells, nTregs or iTregs. As such, we have studied the populations of naïve Treg cells, effector Treg cells, $Foxp3^+$ Teff cells, $Helios^+$ nTreg cells, and $Helios^-$ iTreg cells in the decidua or peripheral blood of miscarriage cases with abnormal or normal fetal chromosomal content.

2. Materials and methods

2.1. Cases of normal pregnancy and miscarriage

This study was approved by the Ethics Committee of the University of Toyama. We obtained written informed consent from all the cases. We enrolled 11 subjects with normally progressing pregnancy, 15 with miscarriage and an abnormal embryo karyotype (trisomy [$n=14$] including trisomy8 [$n=2$], trisomy13 [$n=2$], trisomy15 [$n=1$], trisomy16 [$n=3$], trisomy21 [$n=3$], trisomy22 [$n=3$], and translocation [$n=1$]), and 8 with miscarriage and a normal embryo karyotype. Chorionic villi were sampled from miscarriage cells for cytogenetic analysis. Fetal chromosomal karyotyping was performed by conventional G-band staining. Echo sonography was performed every two weeks, and patients were advised to obtain an induced abortion if the fetal heartbeat ceased or was never detected. In women with a normally progressing pregnancy, fetal heartbeat was identified before elective termination. None of the subjects had any risk factors such as genetic abnormalities (neither themselves nor their husband), uterine malformation, thyroid dysfunction or anti-phospholipid antibody syndrome.

The clinical background in these groups is shown in Table 1. The numbers of previous miscarriages in women miscarrying with an abnormal embryo and miscarrying with a normal embryo were significantly higher than in the normal pregnancy group. Gestational weeks at sampling, body mass index (BMI), and frequency of smoking were similar among the three groups.

2.2. Flow cytometry

Decidual mononuclear cells (leukocytes) were purified by the Ficoll Hypaque method after homogenization and filtration through a 32-μm nylon mesh, as previously reported (Saito et al., 1992).

The following monoclonal antibodies (mAbs) were used in this study: anti-CD4 (Per CP-Cy5.5; BD Biosciences, NJ, USA), anti-CD45RA (Biotin; BD Bioscience), and streptavidin labeled with APC-Cy7 (BD Biosciences) as cell surface markers, and anti-Foxp3 (FITC; eBioscience, San Diego, CA, USA) and anti-Helios (Alexa Fluor 647; eBioscience) as intracellular markers. Decidual and peripheral mononuclear cells were first stained with anti-CD4 mAb and anti-CD45RA mAb for 30 min on ice. Cells were washed with phosphate-buffered saline (PBS) three times. Next, APC-Cy7-labeled streptavidin was added and inoculated for 15 min on ice. After washing the cells with PBS three times, they were fixed and permeabilized by incubation for 30 min with fixation/permeabilization buffer (eBioscience), and then stained with anti-Foxp3 and anti-Helios mAb. Flow cytometry analysis was performed on a BD FACScanII (BD Biosciences).

Lymphocytes were gated based on both forward and side scatter parameters (Fig. 1, left). Monocytes,

Table 1
Clinical background in normal pregnancy, miscarriage with an abnormal embryo and miscarriage with a normal embryo.

	Normal pregnancy $n=11$	Miscarriage with an abnormal embryo $n=15$	Miscarriage with a normal embryo $n=8$
Age (year)*	27.5 ± 0.7 (16–39)	37.3 ± 0.3 (29–44)†	32.1 ± 0.8 (22–42)
Gravidities*,**	1.0 ± 0.1 (0–2)	2.2 ± 0.08 (0–4)†	2.5 ± 0.2 (0–5)
Nulliparity	6/11 (54.5%)	10/15 (66.7%)	4/8 (50.0%)
No. of liveborn children*	0.7 ± 0.08 (0–2)	0.3 ± 0.03 (0–1)	0.5 ± 0.07 (0–1)
No. of miscarriages*,***	0 (0–0)	2.5 ± 0.08 (1–5)†††	2.8 ± 0.2 (1–5)††
Stillbirth*	0 (0–0)	0.1 ± 0.02 (0–1)	0 (0–0)
Gestational weeks*	7.5 ± 0.2 (6–10)	7.2 ± 0.09 (5–10)	6.1 ± 0.2 (5–9)
BMI*	20.4 ± 0.2 (17.7–23.1)	21.8 ± 0.2 (17.7–27.1)	20.6 ± 0.4 (18.0–27.7)
Smoker	3/11 (27.2%)	1/15 (6.7%)	0/8 (0%)

* Mean ± SEM (range).
** This pregnancy or miscarriage is not included in gravidities.
*** This miscarriage is included in no. of miscarriages.
† $P<0.05$ vs. normal pregnancy.
†† $P<0.001$ vs. normal pregnancy.
††† $P<0.0001$ vs. normal pregnancy.

granulocytes, and decidual stromal cells were excluded from the setting of a lymphocyte gate as in Fig. 1 (left). After gating on $CD4^+$ cells (Fig. 1, center), the proportions of $CD4^+CD45RA^+Foxp3^{low}$ naïve Treg, $CD4^+CD45RA^-Foxp3^{high}$ effector Treg, and $CD4^+CD45RA^-Foxp3^{low}$ Teff cells were determined among $CD4^+Foxp3^+$ cells (Fig. 1). Isotype-matched fluorochrome-conjugated mice IgG were used as a control. $Foxp3^{low}$ and $Foxp3^{high}$ were classified as follows. In the peripheral blood, some of the $CD4^+$ cells expressed $Foxp3^{low}$ (Fig. 1, upper column). The expression of Foxp3 in $Foxp3^{high}$ cells was brighter than those in $CD4^+Foxp3^{low}$ cells. In the decidua, $CD4^+Foxp3^{high}$ cells formed a cluster, and we classified these as $CD4^+Foxp3^{high}$ cells and $CD4^+Foxp3^{low}$ cells (Fig. 1, center and right).

2.3. Statistical analysis

Statistical analysis was performed using a statistical software package (SAS version 9.1; SAS Institute, USA). Data were analyzed using the Mann–Whitney *U* test. A value of $P<0.05$ was considered statistically significant.

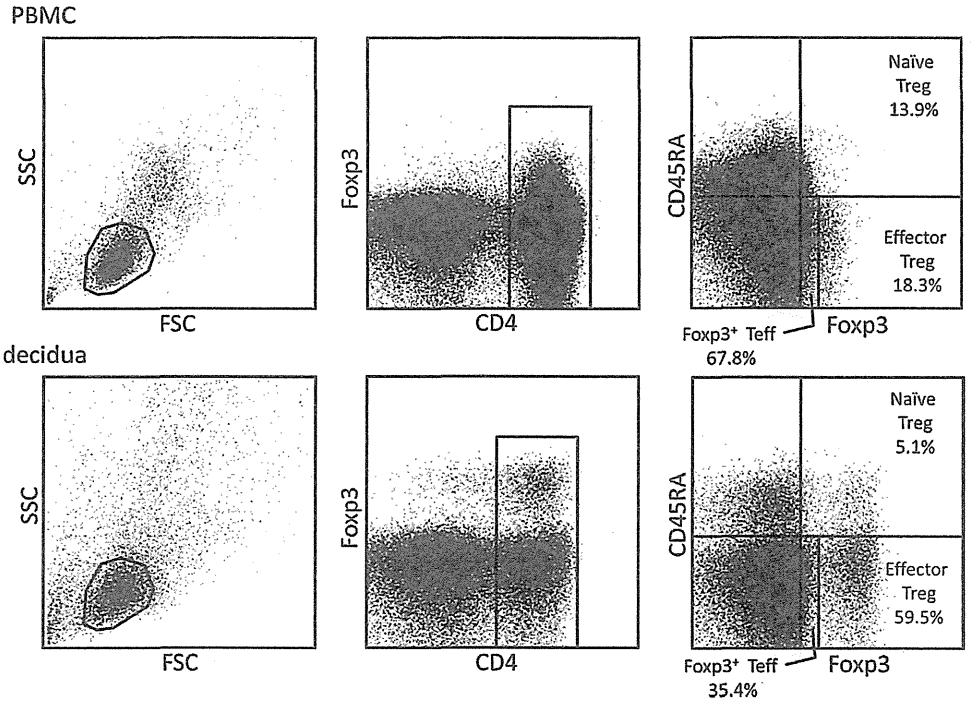

Fig. 1. Gating strategy for the detection of $CD4^+CD45RA^+Foxp3^{low}$ naïve Treg cells, $CD4^+45RA^-Foxp3^{high}$ effector Treg cells, and $CD4^+CD45RA^-Foxp3^{low}$ effector T cells. Lymphocytes in the peripheral blood (upper column) and decidua (lower column) were gated on forward and side scatter parameters. $CD4^+$ T cells in peripheral blood and decidua were classified into $CD45RA^+Foxp3^{low}$ cells, $CD45RA^-Foxp3^{high}$ effector Treg cells, and $CD45RA^-Foxp3^{low}$ Teff cells. The percentages of $CD45RA^+Foxp3^{low}$ cells, $CD45RA^-Foxp3^{high}$ cells, and $CD45RA^-Foxp3^{low}$ cells among $CD4^+Foxp3^+$ T cells are displayed.

3. Results

3.1. Frequencies of Foxp3$^+$ cells and Treg (naïve Treg + effector Treg) cells among CD4$^+$ cells in the decidua

The frequency of decidual Foxp3$^+$ cells among CD4$^+$ cells in miscarriage cases with normal fetal chromosomal content was significantly lower ($P=0.039$) than that in normal pregnancies (Fig. 2A). However, this frequency in miscarriage cases with abnormal chromosomal content was similar to that in normal pregnancies. Next, we measured the frequency of Treg cells (naïve Treg cells + effector Treg cells) among CD4$^+$ T cells. The frequency of the true Treg cell population excluding Foxp3$^+$ Teff cells in the decidua was significantly lower in subjects with miscarriage and a normal embryo karyotype than in those with normal pregnancies, and in subjects with miscarriage and an abnormal embryo karyotype ($P=0.0258$ and $P=0.0389$ respectively; Fig. 2B). We have reanalyzed the frequency of decidual Foxp3$^+$ cells and true Treg cells in subjects with a first pregnancy (○), subjects who have had a previous live birth (●), and the subjects with miscarriage who have had previous miscarriage(s) and no live births (△). There were no significant differences in Foxp3$^+$ cells and true Treg cells among the three groups, although the sample size is small.

3.2. Frequencies of decidual naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells among CD4$^+$Foxp3$^+$ cells

The frequency of decidual effector Treg cells in subjects with miscarriage and a normal embryo karyotype was significantly lower ($P=0.0105$) than that in subjects with normal pregnancies (Fig. 3B). On the other hand, the frequency of decidual Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo karyotype was significantly higher than that in subjects with normal pregnancies and in subjects with miscarriage and an abnormal embryo ($P=0.0258$ and $P=0.0389$ respectively; Fig. 3C). The ratio of effector Treg cells to Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo was significantly lower ($P=0.0106$) than that in subjects with normal pregnancies.

The frequencies of naïve Treg cells were similar among the three groups (Fig. 3A).

3.3. Frequency of naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells among CD4$^+$Foxp3$^+$ cells in the peripheral blood

The frequencies of naïve Treg cells, effector Treg cells and Foxp3$^+$ Teff cells in the peripheral blood were similar among the three groups (Fig. 4A–C). The frequency of effector Treg cells in the peripheral blood in subjects with miscarriage and a normal karyotype embryo appeared to be higher, but the difference did not reach significance (Fig. 4B). Foxp3$^+$ Teff cell population in peripheral blood did not increase in subjects with miscarriage and a normal karyotype embryo. Furthermore, the ratio of effector Treg cells to Foxp3$^+$ Teff cells in subjects with miscarriage and a normal embryo karyotype appeared to be higher, but the difference did not reach significance (Fig. 4D). The frequency of effector Treg cells and Foxp3$^+$ Teff cells was different between decidua and peripheral blood. We have calculated the ratio of the frequency of decidual naïve Treg cells to that of peripheral Treg cells (Fig. 5A), frequency of

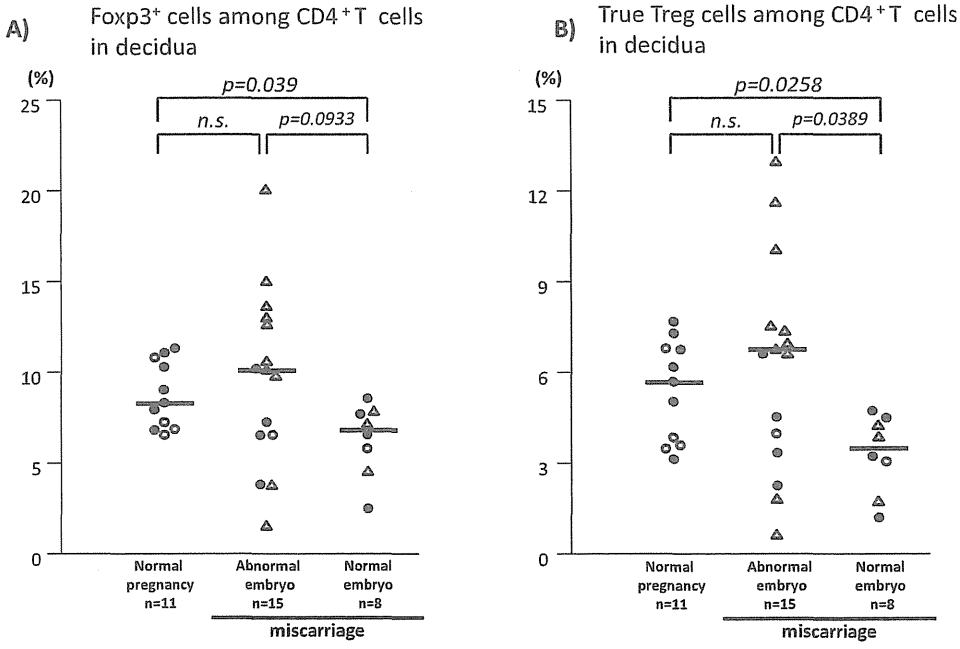

Fig. 2. Frequencies of Foxp3$^+$ cells (A) and true Treg (naïve Treg and effector Treg) cells (B) among CD4$^+$ cells in the decidua of normal pregnancy, miscarriage with abnormal karyotype embryo and miscarriage with normal karyotype embryo. Horizontal bar is the median value. n.s. means not significant. Open circle shows subjects with their first pregnancy. Closed circle shows subjects who have had a previous live birth. Open triangle shows the subjects with miscarriage who have had a previous miscarriage(s) and no live births.

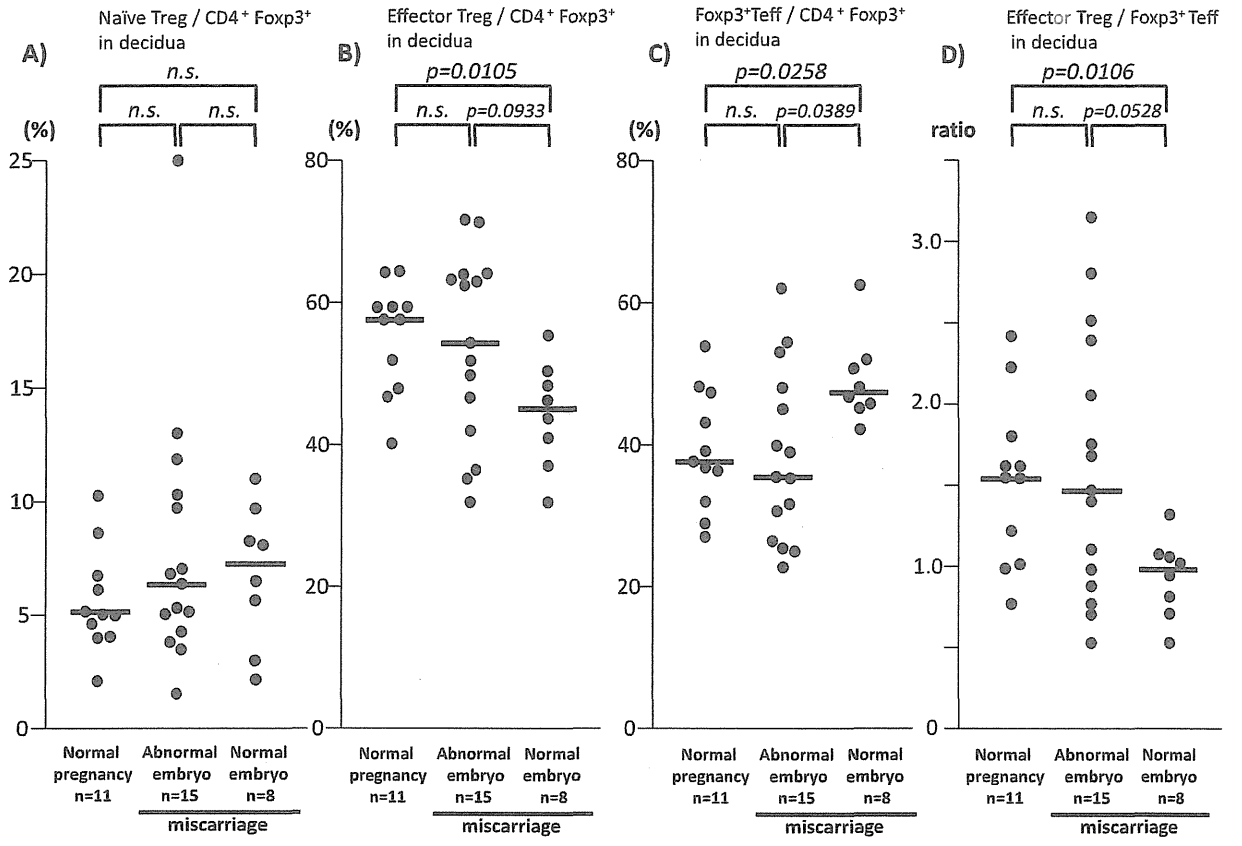

Fig. 3. Frequencies of decidual naïve Treg cells (A), effector Treg cells (B), and Foxp3+ Teff cells (C) among CD4+Foxp3+ cells, and the ratio of effector Treg cells/Foxp3+ Teff cells (D) in normal pregnancy, miscarriage with abnormal karyotype embryo, and miscarriage with normal karyotype embryo. Horizontal bar is the median value. n.s. means not significant.

decidual effector Treg cells to that of the peripheral blood (Fig. 5B), and frequency of decidual Foxp3+ Teff cells to that of the peripheral blood (Fig. 5C). The ratio of effector Treg cells of decidua to peripheral blood in subjects with miscarriage and a normal karyotype embryo was significantly lower than that in subjects with normal pregnancies and in subjects with miscarriage and an abnormal karyotype embryo ($P=0.0128$ and $P=0.0123$ respectively; Fig. 5B). The ratio of Foxp3+ Teff cells of the decidua to those in the peripheral blood was significantly higher ($P=0.0265$) in subjects with miscarriage and a normal karyotype embryo than the ratio in subjects with normal pregnancies, and in subjects with miscarriage and an abnormal karyotype embryo ($P=0.0265$ and $P=0.0066$ respectively; Fig. 5C).

3.4. Frequency of Helios+ effector Treg cells and naïve Treg cells in the decidua and peripheral blood of normal pregnancies, miscarriages with an abnormal karyotype embryo, and miscarriages with a normal karyotype embryo

The frequency of Helios+ Treg cells in the peripheral blood was around 80% of normal pregnancies, miscarriages with an abnormal karyotype embryo, and in miscarriages with a normal karyotype embryo, as previously reported (Thornton et al., 2010; Fig. 6A). The frequencies of Helios+ naïve Treg cells were similar between decidua and peripheral blood in normal pregnancies, miscarriages with abnormal karyotype embryo, and miscarriages with normal karyotype embryo (Fig. 6A). However, the frequency of Helios+ effector Treg cells in the decidua was over 90%, and this frequency in the decidua was significantly higher than in peripheral blood in normal pregnancies ($P=0.02$), miscarriages with abnormal karyotype embryo ($P=0.008$), and miscarriages with a normal karyotype embryo ($P=0.02$) in contrast to the data for naïve Treg cells (Fig. 6B), suggesting the selective migration of effector Treg cells into the decidua from peripheral blood. However, the frequencies of Helios+ cells among effector Treg cells in the decidua and peripheral blood were similar among the three groups. On the other hand, the frequencies of Helios+-naïve Treg cells were similar to those in decidua and peripheral blood (Fig. 6A).

Next, we studied the frequency of Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in the decidua (Table 2). The frequency of decidual Helios+ effector Treg cells among CD4+Foxp3+cells in subjects with miscarriage and a normal embryo was significantly lower compared with that in normal pregnancy subjects ($P=0.0258$). These frequencies in the peripheral blood were similar among the three groups (Table 3).

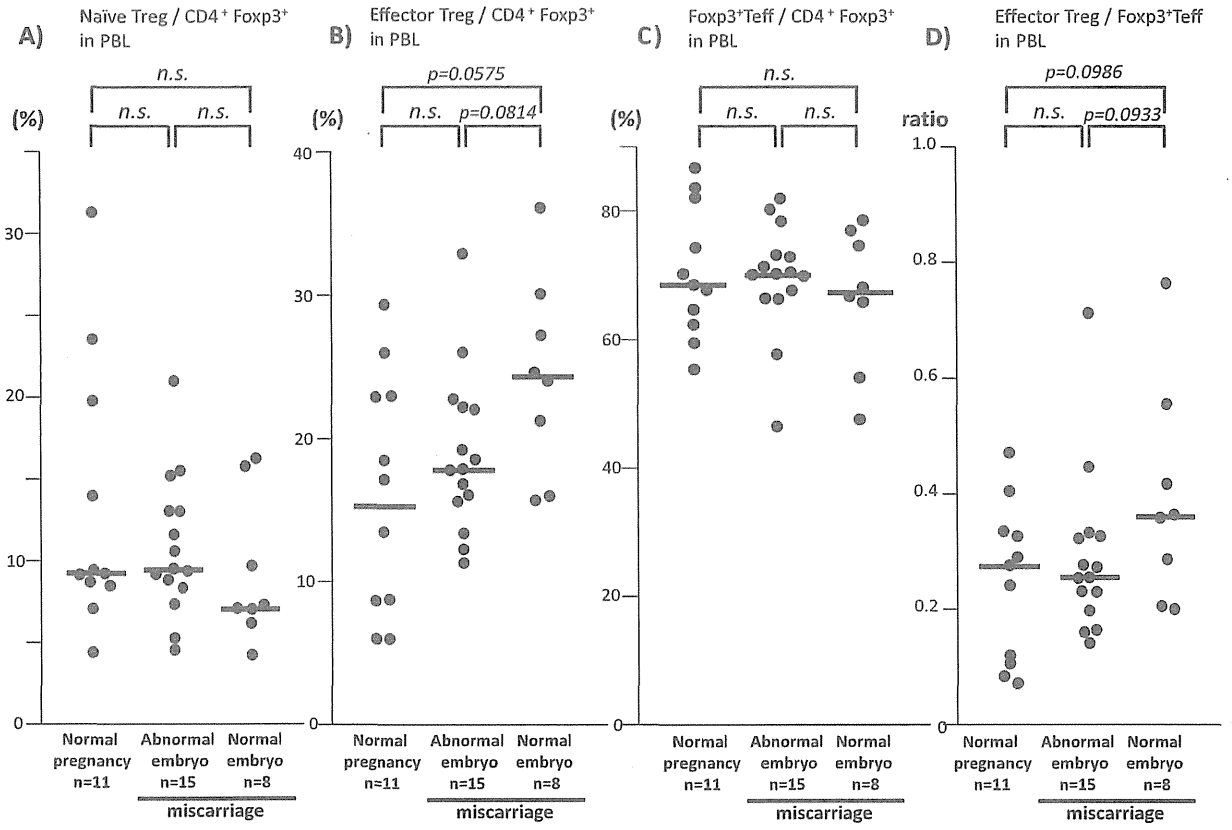

Fig. 4. Frequencies of peripheral blood naïve Treg cells (A), effector Treg cells (B), and Foxp3+ Teff cells (C) among CD4+Foxp3+ cells, and the ratio of effector Treg cells/Foxp3+ Teff cells (D) in normal pregnancy, miscarriage with abnormal karyotype embryo, and miscarriage with normal karyotype embryo. Horizontal bar shows the median value. n.s. means not significant.

Table 2
Frequencies of decidual Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in normal pregnancy, miscarriage with an abnormal embryo and miscarriage with a normal embryo.

	Normal pregnancy $n=11$	Miscarriage with an abnormal embryo $n=15$	Miscarriage with a normal embryo $n=8$
Helios+ naïve Treg cells/CD4+Foxp3+ cells (%)*	4.03 ± 0.20 (0.81–9.08)	6.77 ± 0.37 (1.22–24.07)	5.48 ± 0.35 (1.12–8.53)
Helios− naïve Treg cells/CD4+Foxp3+ cells (%)*	1.53 ± 0.18 (0.49–7.33)	1.13 ± 0.054 (0.23–3.15)	1.14 ± 0.088 (0.54–2.44)
Helios+ effector Treg cells/CD4+Foxp3+ cells (%)*	52.99 ± 0.78 (37.84–64.03)	51.69 ± 0.90 (30.16–71.29)	42.83 ± 0.98 (30.11–54.54)†
Helios− effector Treg cells/CD4+Foxp3+ cells (%)*	2.30 ± 0.17 (0.58–6.81)	2.12 ± 0.18 (0.26–8.74)	1.42 ± 0.12 (0.31–3.47)

* Mean ± SEM (range).
† $P<0.05$ vs. normal pregnancy.

Table 3
Frequencies of peripheral blood Helios-positive or Helios-negative naïve Treg cells and effector Treg cells in normal pregnancy, miscarriage with an abnormal embryo and miscarriage with a normal embryo.

	Normal pregnancy $n=11$	Miscarriage with an abnormal embryo $n=15$	Miscarriage with a normal embryo $n=8$
Helios+ naïve Treg cells/CD4+Foxp3+ cells (%)*	10.41 ± 0.96 (4.77–23.10)	8.09 ± 0.21 (3.09–14.60)	6.74 ± 0.39 (4.10–12.01)
Helios− naïve Treg cells/CD4+Foxp3+ cells (%)*	4.21 ± 0.40 (1.55–8.25)	2.53 ± 0.15 (0–6.8)	2.49 ± 0.19 (0–5.27)
Helios+ effector Treg cells/CD4+Foxp3+ cells (%)*	7.25 ± 0.37 (4.14–11.11)	7.41 ± 0.34 (2.40–20.8)	5.78 ± 0.41 (1.91–10.30)
Helios− effector Treg cells/CD4+Foxp3+ cells (%)*	1.28 ± 0.13 (0.53–2.80)	1.76 ± 0.093 (0–5.08)	0.83 ± 0.099 (0–2.11)

* Mean ± SEM (range).

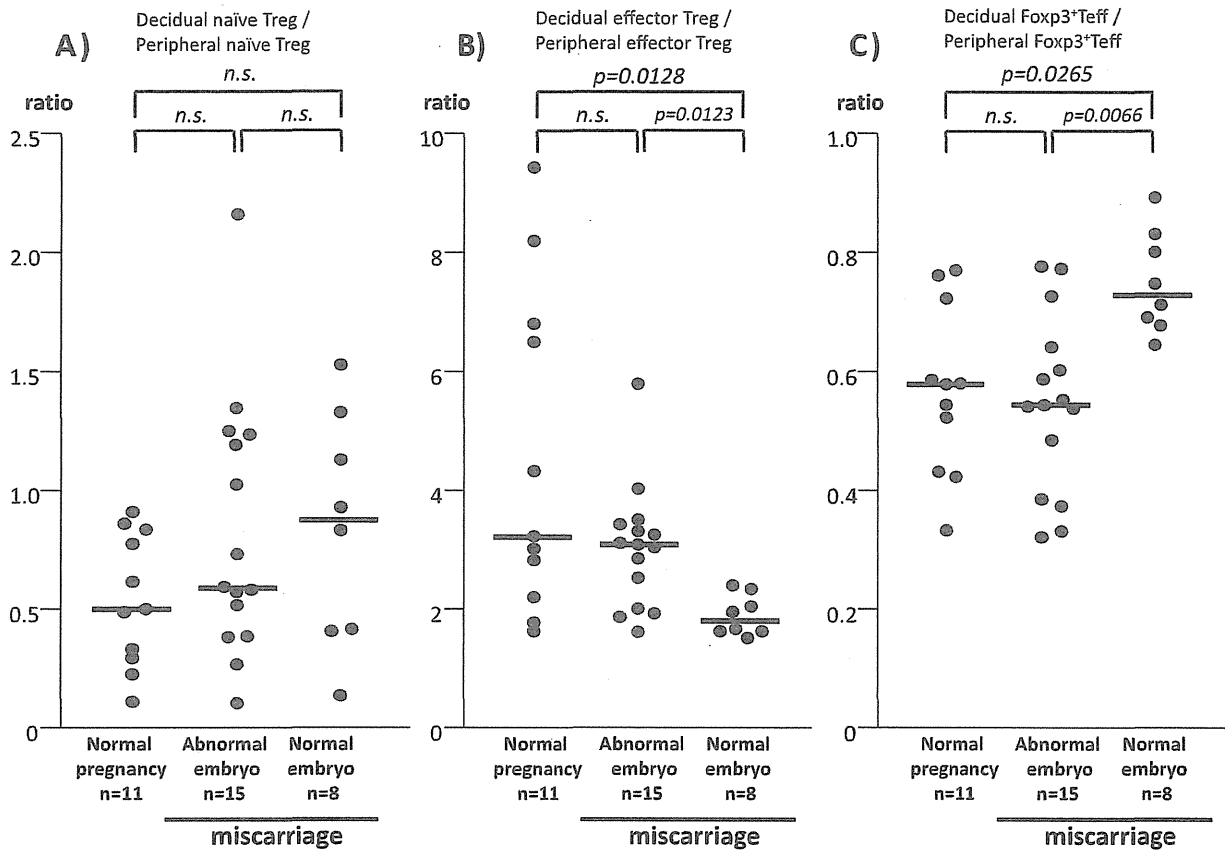

Fig. 5. The ratio of naïve Treg cells in decidua to those in peripheral blood (A), the ratio of effector Treg cells in decidua to those in peripheral blood (B), and the ratio of Foxp3$^+$ Teff cells in decidua to those in peripheral blood (C). Horizontal bar shows the medium value. n.s. means not significant.

4. Discussion

Foxp3 is believed to be a specific marker for Treg cells (Hori et al., 2003), but recent data show that Foxp3 expression transiently occurs in IL-2- and IFN-γ-producing Teff cells in humans (Walker et al., 2003; Gavin et al., 2006; Allan et al., 2007). It is possible that these activated Teff cells might react to fetal antigens presented by antigen-presenting cells (APCs) in the decidua and create harmful inflammation causing demise of the embryo. Many papers have reported that Foxp3$^+$ cells increase in normal pregnancy, but decrease in miscarriage (Sasaki et al., 2004; Yang et al., 2008; Jin et al., 2009; Mei et al., 2010; Wang et al., 2010, 2011; Lee et al., 2011) and preeclampsia (Sasaki et al., 2007; Santner-Nanan et al., 2009). However, there are no reports describing which types of Foxp3$^+$ cell increase or decrease in miscarriage. Foxp3$^+$ cells are classified into effector Treg cells that contribute to immune tolerance, naïve Treg cells that show a weak capacity for immunoregulation, and Teff cells that break immune tolerance. This is the first report that shows that decidual effector Treg cells decreased in miscarriage with a normal karyotype embryo, but not in miscarriage with an abnormal karyotype embryo. Interestingly, Foxp3$^+$ Teff cells in the decidua were increased in miscarriage with a normal karyotype embryo. The immunological environment is controlled by the balance between immunoactivation and immunoregulation. In the miscarriage cases with a normal karyotype embryo, the balance seemed to shift to an immunoactivation-dominant state, and dysregulation of tolerance to the fetus might occur. We compared the percentage of effector Treg cells and Foxp3$^+$ Teff in CD4$^+$Foxp3 cells in decidua and peripheral blood. Our findings suggest that immunological abnormality might be limited in the pregnant uterus in miscarriage with a normal karyotype embryo. Unfortunately, we did not measure the weight of decidual samples; therefore, we could not calculate the numbers of these cells in milligrams of decidua. This is a limitation of our study.

The etiology of recurrent pregnancy loss (RPL) is unknown in 40–60% of cases (Clifford et al., 1994). In a murine model, decreased Treg cells could induce implantation failure (Darrasse-Jèze et al., 2006; Shima et al., 2010) and early pregnancy loss by activation of T cells and NK cells (Aluvihare et al., 2004; Zenclussen et al., 2005; Thaxton et al., 2013), suggesting that immune dysregulation might be one of the etiologies of fetal resorption. These findings suggest that a proportion of sporadic miscarriage cases or RPL cases with a normal fetal karyotype might be associated with the immune etiology of miscarriage in humans. However, further studies are needed to prove this.

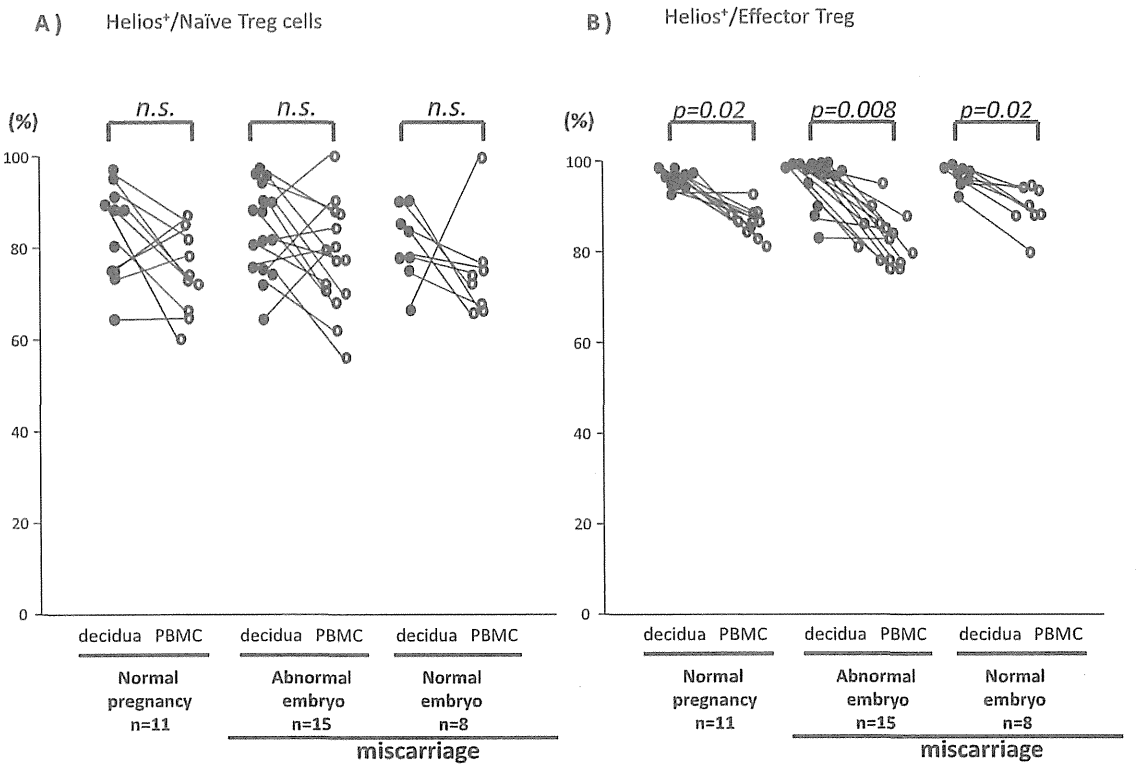

Fig. 6. Helios-positive naïve Treg cells (A) and effector Treg cells (B) in the decidua and peripheral blood of subjects with normal pregnancy, subjects with miscarriage and an abnormal karyotype embryo, and subjects with miscarriage and a normal karyotype embryo. PBMC, peripheral blood mononuclear cells.

Samstein et al. (2012) recently reported that a Foxp3 enhancer, CNS1, is essential for the induction of iTreg cells and that iTreg cells play an essential role in the maintenance of allogeneic pregnancy in placental animals. Thornton et al. (2010) reported that Helios, an Ikaros transcription factor family member, is a specific marker of nTreg cells. Therefore, we analyzed Helios$^+$ nTreg cells and Helios$^-$ iTreg cells among effector Treg cells in the decidua and peripheral blood. Our data first showed that there were very few Helios$^-$ iTreg cells in the decidua of subjects with normal pregnancy and also in that of human subjects with miscarriage, and that the frequency of Helios$^-$ iTreg did not change in miscarriage. Furthermore, the frequency of Helios$^+$ functional nTreg in the decidua was significantly higher than that in peripheral blood in normal pregnancies, miscarriages with an abnormal karyotype embryo and miscarriage with a normal karyotype embryo, but the frequency of Helios$^+$ naïve nTreg cells in the decidua was similar to that in peripheral blood. This finding may be explained by the fact that Helios$^+$ functional nTreg cells selectively accumulated in the pregnant uterus from the periphery. Indeed, selective migration of fetus-specific Treg cells from the peripheral blood to the decidua was reported in human pregnancy (Tilburgs et al., 2008) and murine pregnancy (Kallikourdis et al., 2007). We have already reported that there were decreased numbers of Foxp3$^+$ cells at the decidua basalis were decreased, but not at the decidua parietalis, in subjects with miscarriage and a normal embryo karyotype, showing the failure of the migration of Treg cells at the fetomaternal interface (Inada et al., 2013). The level of effector Treg cells in peripheral blood was slightly elevated in subjects with miscarriages and a normal karyotype embryo (Fig. 4B), which may indirectly support this idea. Indeed, Chen et al. (2013) recently showed that CD44^{high} CD62L^{low} activated memory Treg cells specific for self-antigen were rapidly recruited to the uterus-draining lymph nodes and activated in the first days after embryo implantation. Further studies are necessary to discover whether self-specific memory Treg cells are nTreg or iTreg cells. In summary, Helios$^+$ effector Treg cells were decreased in the decidua of subjects with miscarriage and a normal karyotype embryo. Effector nTreg may play an important role in the maintenance of pregnancy in humans, although Helios is not a strict marker for nTreg (Zabransky et al., 2012).

It is well known that the Treg cell pool is decreased in preeclampsia (Sasaki et al., 2007; Santner-Nanan et al., 2009) and recent data showed that Helios$^-$ iTreg cells, but not Helios$^+$ nTreg cells, were decreased in peripheral blood in preeclampsia (Hsu et al., 2012). iTreg cells may be important for the maintenance of pregnancy at a late stage. Indeed, Rowe et al. (2012) reported that the frequency of Helios$^+$ fetal antigen-specific nTreg cells in mid-gestation (11.5 days post-coitus) was 70%, and this frequency in late gestation (18.5 days post-coitus) was reduced to 40%, suggesting that Helios$^-$ fetal antigen specific iTreg cells expanded in late pregnancy. Neuropilin 1 is expressed on nTreg cells (Weiss et al., 2012; Yadav et al., 2012). We have

tried to study the expression of neuropilin 1 on Treg cells, but the immunostaining of neuropilin 1 was weak; thus, we could not classify Treg cells into nTreg and iTreg cells using neuropilin 1 staining.

Rowe et al. (2012) reported that memory Treg cells that sustain anergy to fetal antigen play a role in the rapid induction of fetomaternal tolerance. Importantly, when Treg cells from female mice were transferred into pregnant mice soon after delivery, they quickly expanded after pregnancy, but when $Foxp3^{-}CD4^{+}$ T cells were transferred into pregnant mice, donor-derived fetal antigen-specific Treg cells did not appear (Rowe et al., 2012). If iTreg cells play an essential role in the maintenance of allogeneic pregnancy, donor-derived fetal antigen-specific iTreg cells should be increased, but $Helios^{+}$-fetal antigen-specific nTreg cells were the major population in mid-gestation (Rowe et al., 2012). Furthermore, when CNS1-deficient female mice were mated with allogeneic male mice, the resorption rate was only 10% (Samstein et al., 2012). More than 50% resorption was observed when total Treg cells were depleted in mice (Shima et al., 2010; Rowe et al., 2012). This finding suggests that not only iTreg cells, but also nTreg cells, might play a role in a successful pregnancy. Further studies are needed on the types of Treg cell that play an important role in successful implantation and pregnancy in mice and humans.

In conclusion, we are, to our knowledge, the first to show that Helios-positive functional Treg cells decreased and $Foxp3^{+}$ Teff cells increased in the decidua of miscarriage cases with normal fetal chromosomal content. These findings suggest that dysregulation of fetomaternal tolerance might be one of the etiologies of miscarriage in humans.

Disclosure

None of the authors has any conflict of interest related to this manuscript.

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References

Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G., Levings, M.K., 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int. Immunol. 19, 345–354.

Aluvihare, V.R., Kallikourdis, M., Betz, A.G., 2004. Regulatory T cells mediate maternal tolerance to the fetus. Nat. Immunol. 5, 266–271.

Beacher-Allan, C., Brown, J.A., Freeman, G.J., Hafler, D.A., 2001. CD4+CD25high regulatory cells in human peripheral blood. J. Immunol. 167, 1245–1253.

Chen, T., Darrasse-Jèze, G., Bergot, A.S., Courau, T., Churlaud, G., Valdivia, K., Strominger, J.L., Ruocco, M.G., Chaouat, G., Klatzmann, D., 2013. Self-specific memory regulatory T cells protect embryos at implantation in mice. J. Immunol. 191, 2273–2281.

Clifford, K., Rai, R., Watson, H., Regan, L., 1994. An informative protocol for the investigation of recurrent miscarriage: preliminary experience of 500 consecutive cases. Hum. Reprod. 9, 1328–1332.

Darrasse-Jèze, G., Klatzmann, D., Charlotte, F., Salomon, B.L., Cohen, J.L., 2006. CD4+CD25+ regulatory/suppressor T cells prevent allogeneic fetus rejection in mice. Immunol. Lett. 102, 106–109.

Gavin, M.A., Torgerson, T.R., Houston, E., DeRoos, P., Ho, W.Y., Stray-Pedersen, A., Ocheltree, E.L., Greenberg, P.D., Ochs, H.D., Rudensky, A.Y., 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. Proc. Natl. Acad. Sci. U.S.A. 103, 6659–6664.

Guerin, L.R., Moldenhauer, L.M., Prins, J.R., Bromfield, J.J., Hayball, J.D., Robertson, S.A., 2011. Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. Biol. Reprod. 85, 397–408.

Hori, S., Nomura, T., Sakaguchi, S., 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299, 1057–1061.

Hsu, P., Santner-Nanan, B., Dahlstrom, J.E., Fadia, M., Chandra, A., Peek, M., Nanan, R., 2012. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. Am. J. Pathol. 181, 2149–2160.

Inada, K., Shima, T., Nakashima, A., Aoki, K., Ito, M., Saito, S., 2013. Characterization of regulatory T cells in decidua of miscarriage cases with abnormal or normal fetal chromosomal content. J. Reprod. Immunol. 97, 104–111.

Jin, L.P., Chen, Q.Y., Zhang, T., Guo, P.F., Li, D.J., 2009. The CD4+CD25 bright regulatory T cells and CTLA-4 expression in peripheral and decidual lymphocytes are down-regulated in human miscarriage. Clin. Immunol. 133, 402–410.

Kahn, D.A., Baltimore, D., 2010. Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. Proc. Natl. Acad. Sci. U.S.A. 107, 9299–9304.

Kallikourdis, M., Andersen, K.G., Welch, K.A., Betz, A.G., 2007. Alloantigen-enhanced accumulation of CCR5+ 'effector' regulatory T cells in the gravid uterus. Proc. Natl. Acad. Sci. U.S.A. 104, 594–599.

Kleinewietfeld, M., Starke, M., Di Mitri, D., Borsellino, G., Battistini, L., Rötzschke, O., Falk, K., 2009. CD49d provides access to untouched human Foxp3+ Treg free of contaminating effector cells. Blood 113, 827–836.

Lee, S.K., Kim, J.Y., Hur, S.E., Kim, C.J., Na, B.J., Lee, M., Gilman-Sachs, A., Kwak-Kim, J., 2011. An imbalance in interleukin-17-producing T and $Foxp3^{+}$ regulatory T cells in women with idiopathic recurrent pregnancy loss. Hum. Reprod. 26, 2964–2971.

Mei, S., Tan, J., Chen, H., Chen, Y., Zhang, J., 2010. Changes of CD4+CD25high regulatory T cells and FOXP3 expression in unexplained recurrent spontaneous abortion patients. Fertil. Steril. 94, 2244–2247.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., Mathian, A., Nakahata, T., Yamaguchi, T., Nomura, T., Ono, M., Amoura, Z., Gorochov, G., Sakaguchi, S., 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 30, 899–911.

Robertson, S.A., Guerin, L.R., Bromfield, J.J., Branson, K.M., Ahlström, A.C., Care, A.S., 2009. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol. Reprod. 80, 1036–1045.

Rowe, J.H., Ertelt, J.M., Aguilera, M.N., Farrar, M.A., Way, S.S., 2011. Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens. Cell Host Microbe 10, 54–64.

Rowe, J.H., Ertelt, J.M., Xin, L., Way, S.S., 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. Nature 490, 102–106.

Saito, S., Nishikawa, K., Morii, T., Narita, N., Enomoto, M., Ichijo, M., 1992. Expression of activation antigens CD69, HLA-DR, interleukin-2 receptor-alpha (IL-2R alpha) and IL-2R beta on T cells of human decidua at an early stage of pregnancy. Immunol. 75, 710–712.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., Toda, M., 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. 155, 1151–1164.

Sakaguchi, S., 2005. Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat. Immunol. 6, 345–352.

Samstein, R.M., Josefowicz, S.Z., Arvey, A., Treuting, P.M., Rudensky, A.Y., 2012. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal–fetal conflict. Cell 150, 29–38.

Santner-Nanan, B., Peek, M.J., Khanam, R., Richarts, L., Zhu, E., Fazekas de St Groth, B., Nanan, R., 2009. Systemic increase in the ratio between Foxp3+ and IL-17-producing CD4+ T cells in healthy pregnancy but not in preeclampsia. J. Immunol. 183, 7023–7030.

Sasaki, Y., Sakai, M., Miyazaki, S., Higuma, S., Shiozaki, A., Saito, S., 2004. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol. Hum. Reprod. 10, 347–353.

Sasaki, Y., Darmochwal-Kolarz, D., Suzuki, D., Sakai, M., Ito, M., Shima, T., Shiozaki, A., Rolinski, J., Saito, S., 2007. Proportion of peripheral blood and decidual CD4(+) CD25(bright) regulatory T cells in pre-eclampsia. Clin. Exp. Immunol. 149, 139–145.

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R., Kelleher, A., Fazekas de St Groth, B., 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J. Exp. Med. 203, 1693–1700.

Shima, T., Sasaki, Y., Itoh, M., Nakashima, A., Ishii, N., Sugamura, K., Saito, S., 2010. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. J. Reprod. Immunol. 85, 121–129.

Steinborn, A., Schmitt, E., Kisielewicz, A., Rechenberg, S., Seissler, N., Mahnke, K., Schaier, M., Zeier, M., Sohn, C., 2012. Pregnancy-associated diseases are characterized by the composition of the systemic regulatory T cell (Treg) pool with distinct subsets of Tregs. Clin. Exp. Immunol. 167, 84–98.

Thaxton, J.E., Nevers, T., Lippe, E.O., Blois, S.M., Saito, S., Sharma, S., 2013. NKG2D blockade inhibits poly(I:C)-triggered fetal loss in wild type but not in IL-1 0-/- mice. J. Immunol. 190, 3639–3647.

Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., Shevach, E.M., 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J. Immunol. 184, 3433–3441.

Tilburgs, T., Roelen, D.L., van der Mast, B.J., de Groot-Swings, G.M., Kleijburg, C., Scherjon, S.A., Claas, F.H., 2008. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. J. Immunol. 180, 5737–5745.

Tilburgs, T., Scherjon, S.A., van der Mast, B.J., Haasnoot, G.W., Versteeg, V.D., Voort-Maarschalk, M., Roelen, D.L., van Rood, J.J., Claas, F.H., 2009. Fetal–maternal HLA-C mismatch is associated with decidual T cell activation and induction of functional T regulatory cells. J. Reprod. Immunol. 82, 148–157.

Walker, M.R., Kasprowicz, D.J., Gersuk, V.H., Benard, A., Van Landeghen, M., Buckner, J.H., Ziegler, S.F., 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25− T cells. J. Clin. Invest. 112, 1437–1443.

Wang, W.J., Hao, C.F., Q. u., Q.L., Wang, X., Qiu, L.H., Lin, Q.D., 2010. The deregulation of regulatory T cells on interleukin-17-producing T helper cells in patients with unexplained early recurrent miscarriage. Hum. Reprod. 25, 2591–2596.

Wang, W.J., Hao, C.F., Lin, Q.D., 2011. Dysregulation of macrophage activation by decidual regulatory T cells in unexplained recurrent miscarriage patients. J. Reprod. Immunol. 92, 97–102.

Weiss, J.M., Bilate, A.M., Gobert, M., Ding, Y., Curotto de Lafaille, M.A., Parkhurst, C.N., Xiong, H., Dolpady, J., Frey, A.B., Ruocco, M.G., Yang, Y., Floess, S., Huehn, J., Oh, S., Li, M.O., Niec, R.E., Rudensky, A.Y., Dustin, M.L., Littman, D.R., Lafaille, J.J., 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. J. Exp. Med. 209, 1723–1742.

Winger, E.E., Reed, J.L., 2011. Low circulating CD4(+) CD25(+) Foxp3(+) T regulatory cell levels predict miscarriage risk in newly pregnant women with a history of failure. Am. J. Reprod. Immunol. 66, 320–328.

Yadav, M., Louvet, C., Davini, D., Gardner, J.M., Martinez-Llordella, M., Bailey-Bucktrout, S., Anthony, B.A., Sverdrup, F.M., Head, R., Kuster, D.J., Ruminski, P., Weiss, D., Von Schack, D., Bluestone, J.A., 2012. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. J. Exp. Med. 209, 1713–1722.

Yang, H., Qiu, L., Chen, G., Ye, Z., Lü, C., Lin, Q., 2008. Proportional change of CD4+CD25+ regulatory T cells in decidua and peripheral blood in unexplained recurrent spontaneous abortion patients. Fertil. Steril. 89, 656–661.

Yin, Y., Han, X., Shi, Q., Zhao, Y., He, Y., 2012. Adoptive transfer of CD4+CD25+ regulatory T cells for prevention and treatment of spontaneous abortion. Eur. J. Obstet. Gynecol. Reprod. Biol. 161, 177–181.

Zabransky, D.J., Nirschl, C.J., Durham, N.M., Park, B.V., Ceccato, C.M., Bruno, T.C., Tam, A.J., Getnet, D., Drake, C.G., 2012. Phenotypic and functional properties of Helios+ regulatory T cells. PLoS ONE 7, e34547.

Zenclussen, A.C., Gerlof, K., Zenclussen, M.L., Sollwedel, A., Bertoja, A.Z., Ritter, T., Kotsch, K., Leber, J., Volk, H.D., 2005. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am. J. Pathol. 166, 811–822.

Zenclussen, A.C., Gerlof, K., Zenclussen, M.L., Ritschel, S., Zambon Bertoja, A., Fest, S., Hontsu, S., Ueha, S., Matsushima, K., Leber, J., Volk, H.D., 2006. Regulatory T cells induce a privileged tolerant microenvironment at the fetal–maternal interface. Eur. J. Immunol. 36, 82–94.

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Human Exosomal Placenta-Associated *miR-517a-3p* Modulates the Expression of *PRKG1* mRNA in Jurkat Cells[1]

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ABSTRACT

During pregnancy, human placenta-associated microRNAs (miRNAs) derived from the miRNA cluster in human chromosome 19 are expressed in villous trophoblasts and secreted into maternal circulation via exosomes; however, little is known about whether circulating placenta-associated miRNAs are transferred into maternal immune cells via exosomes, and modulate expression of target genes in the recipient cells. We employed an in vitro model of trophoblast-immune cell communication using BeWo cells (a human trophoblast cell line) and Jurkat cells (a human leukemic T-cell line) and investigated whether BeWo exosomal placenta-associated miRNAs can suppress expression of target genes in the recipient Jurkat cells. Using this system, we identified *PRKG1* as a target gene of placenta-associated miRNA *miR-517a-3p*. Moreover, we demonstrated that BeWo exosomal *miR-517a-3p* was internalized into Jurkat cells and subsequently suppressed the expression of *PRKG1* in recipient Jurkat cells. Furthermore, using peripheral blood natural killer (NK) cells in vivo, we confirmed that circulating *miR-517a-3p* was delivered into maternal NK cells as it was into Jurkat cells in vitro. Placenta-associated *miR-517a-3p* was incorporated into maternal NK cells in the third trimester, and it was rapidly cleared after delivery. Expression levels of *miR-517a-3p* and its target mRNA *PRKG1* were inversely correlated in NK cells before and after delivery. These in vitro and in vivo results suggest that exosome-mediated transfer of placenta-associated miRNAs and subsequent modulation of their target genes occur in maternal NK cells. The present study provides novel insight into our understanding of placenta-maternal communication.

BeWo, exosome, human placenta, Jurkat, microRNA, natural killer cell, PRKG1, regulatory T cell, villous trophoblast

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INTRODUCTION

Exosomes are nanovesicles (30–100 nm) of multivesicular body origin that can be released from the cell surface by exocytosis [1]. Most cell types are believed to be able to secrete exosomes [2], which can reach bodily fluids, such as plasma [3], urine [4], semen [5], milk [6], and saliva [7]. To date, it is well known that exosomes contain RNAs, lipids, and many kinds of proteins, which are all thought to transfer from the cell of origin to adjacent or distant cells and influence biological functions of the recipient cells [8, 9]. There has been a growing interest in the role of exosomal microRNAs (miRNAs) in cell-cell communication since Valadi et al. [10] demonstrated that miRNAs exist in exosomes. MicroRNAs are small noncoding RNAs approximately 22 nucleotides in length that play a pivotal role in posttranscriptional gene regulation by repressing target mRNA translation by base pairing to the 3′-untranslated region (3′-UTR) [11, 12]. Recent studies have suggested that exosomes have the capacity to shuttle genetic information from cell to cell by transferring miRNAs [13].

MicroRNAs within the human imprinted chromosome 19 miRNA cluster (C19MC), a primate-specific miRNA cluster encompassing 46 miRNAs in the human genome, are expressed exclusively in the placenta (i.e., placenta-associated miRNAs) [14–16]. Although C19MC-associated miRNAs are not detected in other normal adult tissues, they were also identified in some cancer cells [17]. Recent studies have shown that C19MC-associated miRNAs that are packaged within trophoblast-derived exosomes confer viral resistance in recipient cells by the induction of autophagy [18, 19]. However, the biological functions of C19MC-associated miRNAs for the placenta and pregnancy are still not fully understood. We reported previously that placenta-associated miRNAs are expressed in human villous trophoblasts and are secreted into the maternal circulation via exosomes [20]. Exosomal miRNAs are generally considered to be stable in the circulation [21]. This has raised the possibility that exosomal placenta-associated miRNAs may be detected in the circulation during pregnancy [22, 23] and may serve as novel predictive markers [24]. However, there is little information on how exosomal placenta-associated miRNAs participate in cell-cell communication and possibly contribute to the maintenance of pregnancy.

Based on the aforementioned findings of exosomal miRNAs, we hypothesized that circulating placenta-associated miRNAs might be transferred via exosomes from placental trophoblasts into maternal immune cells and repress expression of target genes in the recipient cells. To test our hypothesis, we

initially attempted to search target genes of placenta-associated miRNAs in human peripheral blood immune cells using a DNA microarray analysis. However, this in vivo evaluation proved difficult because these immune cells already contain various miRNAs derived not only from placental cells but also many other cell types [25]. In this study, to avoid the complexity of microarray experiments using circulating cells, we have developed an in vitro model system of trophoblast-immune cell communication using BeWo cells (a human trophoblast cell line) [26] and Jurkat cells (a human leukemic T-cell line) [27]. Using this system, we identified *PRKG1* as a target gene of the placenta-associated miRNA *miR-517a-3p*, via a combination of DNA microarray and in silico analyses. We showed that exosomal *miR-517a-3p* can transfer from BeWo cells into Jurkat cells and modulate the target gene within the recipient cells. Furthermore, we evaluated the uptake of circulating *miR-517a-3p* into immune cells using peripheral blood samples from pregnant women.

MATERIALS AND METHODS

Cell Culture

Jurkat and BeWo cells were purchased from the RIKEN Bioresource Center. JEG3 cells were obtained from the European Collection of Cell Cultures. Jurkat cells were maintained in exosome-free RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Japan Bioserum; 37°C, 5% CO_2); BeWo cells were cultured in exosome-free Ham F-12 medium (Invitrogen) supplemented with 15% FBS (37°C, 5% CO_2); JEG-3 was cultured in exosome-free E-MEM (Wako) supplemented with 10% FBS, 1% nonessential amino acids, and 1 mM pyruvate (37°C, 5% CO_2). Exosome-free medium was prepared by ultracentrifugation at 100 000 × *g* for 12 h at 4°C according to the method of Thery et al. [28].

Isolation of Peripheral Blood Immune Cells from Pregnant Women

Samples of peripheral blood were obtained from full-term pregnant women who gave informed consent (n = 24; gestational age, 36–38 wk). Protocols were approved by the ethics committees of Nippon Medical School and Jichi Medical University. Postdelivery maternal blood samples were also collected 4 days after delivery. Women with significant comorbid medical conditions or concomitant medications that would substantially impact immunologic function were excluded from the study. Peripheral blood mononuclear cells (MNCs) were isolated from heparinized venous blood using Lymphoprep (Axis-Shield PoC AS). Briefly, after the blood was centrifuged at 1490 × *g* for 15 min at 4°C, the pellet was resuspended in PBS containing 1% bovine serum albumin (buffer A). The MNCs were isolated from the suspension according to the manufacturer's instructions, and then were resuspended in buffer A. Natural killer (NK) cells and regulatory T (Treg) cells were isolated from the MNCs using Dynabeads Untouched NK-cell and Dynabeads Regulatory $CD4^+CD25^+$ T-cell kits (Invitrogen), respectively, according to the manufacturer's instructions. Total RNA from the cells was extracted as described below.

Exosome Isolation from Culture Supernatants

Exosomes were isolated from the supernatant of trophoblast cell lines according to the method of Thery et al. [28]. Briefly, culture supernatants were harvested and sequentially centrifuged at 4°C at 300 × *g* for 10 min, 2000 × *g* for 10 min, and 10 000 × *g* for 30 min to eliminate cells, dead cells, and cell debris. The supernatants were filtered through a 0.22-μm filter and then ultracentrifuged at 100 000 × *g* for 70 min at 4°C to pellet exosomes. Exosomal pellets were washed twice in PBS and then resuspended in 200 μl of PBS. The protein content of purified exosomes was determined using a BCA protein assay kit (Pierce).

Exosomes were also prepared from culture supernatants of BeWo cells transfected with Pre-miR-517a (mature *miR-517a-3p* mimic; Applied Biosystems), Pre-miR-1, or Pre-miR Negative Control no. 1 (Pre-miR-NC) to investigate the effect of these miRNAs in the recipient Jurkat cells. BeWo cells were transfected with Pre-miR reagents (30 nM) using Lipofectamine 2000 (Invitrogen) for 4 h. After 48 h of transfection, culture supernatants were collected for exosome isolation. Exosomes from Pre-miR-517a-transfected, Pre-miR-1-transfected, and Pre-miR-NC-transfected BeWo cells were designated *miR-517a-3p*-loaded, *miR-1*-loaded, and miR-NC-loaded exosomes, respectively; exosomes from nontransfected cells were designated wild-type exosomes.

RNA Extraction and Purification

Total RNA within whole cells was extracted using RNAiso reagent (Takara), followed by cleanup with RNeasy Mini Kits (Qiagen), according to the manufacturer's instructions. Total RNA within exosomes was extracted using mirVana miRNA Isolation Kits (Applied Biosystems) according to the manufacturer's instructions.

Real-Time PCR Analysis

Real-time PCR for miRNAs was carried out using TaqMan MicroRNA Assays (Applied Biosystems) in a 7300 Real-Time PCR System (Applied Biosystems) or a 7900 FAST Real-Time PCR System (Applied Biosystems). Briefly, total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The RT products were subsequently subjected to a real-time PCR reaction using TaqMan 2X Universal PCR Master Mix (Applied Biosystems). To quantify mRNA levels, SYBR Premix Ex Taq (Takara Bio) was applied. Total RNA was reverse transcribed with PrimeScript RT reagent Kit (Takara). To quantify mRNA levels, SYBR Premix Ex Taq was applied. To normalize expression levels of miRNAs and mRNAs, *SNORD44* and *GAPDH* were used as endogenous internal controls, respectively. Primers for *miR-512-3p* (assay ID 001823), *miR-517a-3p* (assay ID 002402), *miR-518b* (assay ID 001156), and *miR-1* (assay ID 002222) were from Applied Biosystems. Primers for mRNAs (Eurofins Genomics) were as follows: *ALDH1B1*: forward, CCTGGCTGCGGCTGTGTTCA; reverse, ATGGCGTGTGGCAGGTGACG; *ANP32E*: forward, CCCCGGAGGAGGTGACAGAGT; reverse, AGCCGGGC CAGCGAACTTAG; *DHFR*: forward, CTGCATCGTCGCTGTGTCCCA; reverse, ATTCCTGAGCGGTGGCCAGG; *FAT2*: forward, TCCAGAGTGAG GAAGAGGTA; reverse, TGTGGAGAATTGGGGTATAG; *IGSF5*: forward, CTGGACCCGGCTCCCGGATA; reverse, GAAGGTCGCTGGGCTCCGGA; *PRKG1*: forward, AGGAGCTGAGGCAGCGGGAT; reverse, CAAGGTGC TCGCGCTCTGCT; *TWF1*: forward, GGAGGACAAACAACCATGCTA; reverse, GGGAAGATTGTGACAGCAAGT; and *RSPO3*: forward, TCGGCAGC CAAAACGCCTCC; reverse, TGCACAGCCTCCTTGGCAGC.

Western Blot Analysis

Proteins derived from BeWo cells or Jurkat cells were obtained using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Proteins were separated on 10% SDS-PAGE gels and were transferred onto Sequi-blot PVDF membranes (Bio-Rad). Blots were incubated at 4°C overnight with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Antibodies used in Western blotting were as follows: anti-CD63 monoclonal antibody (mAb; Sanquin), anti-CD81 polyclonal antibody (pAb; Santa Cruz Biotechnology), anti-TSG101 pAb (Sigma-Aldrich), anti-ACTB mAb (Sigma-Aldrich), anti-PRKG1 mAb (Cell Signaling Technologies), and horseradish peroxidase-conjugated secondary antibodies (Pierce). Signals were detected using Immobilon reagent (Millipore) and visualized using an LAS-4000 Lumino image analyzer (Fujifilm).

Immunoelectron Microscopy Analysis

For immunogold labeling of CD63, an mAb (H5C6; provided by Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa) was employed. Exosomes were fixed with 4% paraformaldehyde (Merck) in PBS, and 20 μl of the suspension was placed on 300-mesh nickel grids (Electron Microscopy Sciences) coated with Formvar (Nisshin EM), for 20 min at 22°C. After blocking with 5% normal goat serum (Sigma-Aldrich) in PBS, grids bearing exosomes were incubated with anti-CD63 for 30 min at 37°C. The grids were then incubated with goat anti-mouse immunoglobulin G (IgG) pAb conjugated to 10-nm colloidal gold particles (Jackson Immunoresearch), for 30 min at 37°C. Immunolabeled exosomes on grids were postfixed with 2% glutaraldehyde in PBS for 30 min at 22°C, and then negatively stained with 4% uranyl acetate according to the method of Sakai et al. [29]. Control grids received the same treatment except that the primary antibody was replaced with nonimmune isotype-matched IgG (MOPC-21; Sigma-Aldrich). Samples were observed under a Hitachi H-7650 electron microscope system (Hitachi High-Technologies) operated at 80 kV.

Morphometric analysis of isolated vesicles in electron micrographs was performed. The size of isolated vesicles was measured, and the number of the vesicles labeled with and without colloidal gold particles indicating CD63 was

counted on the micrographs printed at the same magnification (150 000×). A total of 221 vesicles were analyzed.

Microarray Analysis in miR-517a-3p*–Overexpressing Jurkat Cells*

Jurkat cells were transfected with Pre-miR-517a or Pre-miR NC at a final concentration of 40 nM using Lipofectamine 2000. Pre-miR-transfected cells were harvested 48 h after transfection and total RNA was extracted as described above. The integrity of RNA for microarray analysis was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies); samples with RNA integrity number greater than 7 were used. Of the total RNA obtained, 100 ng was used in a labeling reaction using a Low-Input QuickAmp Labeling Kit, One-Color (Agilent Technologies), and the quality and yield of labeled cRNA were evaluated on an Agilent 2100 Bioanalyzer. Gene expression profiling was conducted using an Agilent microarray (Human GE 4×44 K, v2). The resulting signals were normalized to the 75th percentile signal intensity, and processed data were filtered using a 2-fold change threshold with GeneSpring GX software (v. 11.5; Agilent Technologies). The mRNA array data are publicly available (Gene Expression Omnibus accession no. GSE50814; http://www.ncbi.nlm.nih.gov/geo/; accessed September 12, 2013).

MicroRNA Target Prediction

We focused on downregulated mRNAs as potential direct targets of *miR-517a-3p* in the above DNA microarray analysis. The online prediction software MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/; accessed October 1, 2011) was used to search for predicted target genes of *miR-517a-3p*.

Luciferase Assay

To construct a reporter plasmid, we first cloned the 3′-UTR of the human *PRKG1* gene (GenBank accession no. NM_006258) into the pMIR-REPORT vector, in which firefly luciferase expression reports miRNA activity (Applied Biosystems). Genomic DNA from Jurkat cells was isolated using the FastPure DNA Kit (Takara). The 3′-UTR of the *PRKG1* mRNA was then amplified from isolated genomic DNA using the following primers: 5′-CAGG**ACTAGT**TGTATTTCTCTTACCTGCTTCTGCCT-3′ (*Spe*I site underlined) and 5′-GGCA**AAGCTT**GTTACTTATGACAGCTAATAATAGTCAACTGGC-3′ (*Hin*dIII site underlined). After sequence verification by cloning into a pCR-Blunt II-TOPO vector, the *PRKG1* 3′-UTR was cloned into pMIR-REPORT via the *Spe*I and *Hin*dIII restriction sites. This final construct was designated pMIR-PRKG1. To construct a reporter plasmid with a mutated *miR-517a-3p* recognition site of *PRKG1* 3′-UTR (see Fig. 3D), an inverse PCR method was used. The primers used for the inverse PCR were *PRKG1* 3′-UTR mut forward, 5′-ACAGAagactgaaaaattagtacgtaaATTTGTTTGTTTTTTTTGATAAATTGG CATG-3′; and *PRKG1* 3′-UTR mut reverse, 5′-ACAAATttacgtactaatttttcagtctTCTGTGAAAACTCATATATACCTACTATGAGAGGTCTC-3′ (complementary sequence shown in lowercase), and were designed to introduce the mutation. The PCR amplification was carried out using the previously cloned vector pCR-Blunt II-TOPO containing the *PRKG1* 3′-UTR. Plasmid DNA was digested by *Dpn*I. The final construct was confirmed by sequencing using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Amplified DNA was transformed into *Escherichia coli*. After sequence verification, the mutated 3′-UTR sequence was cloned into pMIR-REPORT via *Spe*I and *Hin*dIII restriction sites. The final construct was designated pMIR-PRKG1mt.

Jurkat cells were transfected with pMIR-PRKG1, pMIR-PRKG1mt, or pMIR-cont (empty vector pMiR-REPORT), and the control vector pRL-TK (*Renilla* luciferase expression plasmid), together with Pre-miR-517a or Pre-miR-NC (30 nM), using Lipofectamine 2000 in 24-well plates. Twenty-four hours after transfection, luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Transfer of BeWo Exosomes into Recipient Cells

Jurkat cells were incubated with exosomes isolated from trophoblast cell lines (50 μg/ml) for 24–48 h, and real-time PCR was performed for quantification of miRNA and mRNA as described above. For luciferase assay, Jurkat cells were transfected with pMIR-PRKG1or pMIR-cont, and the control vector pRL-TK. After 24 h of transfection, cells were incubated with BeWo exosomes for another 24 h, and then luciferase assays were performed. Some cells were incubated with BeWo exosomes for 24 h, washed twice with PBS, and then treated with TrypLE Express Enzyme solution (recombinant trypsin-like serine protease [1×] in Dulbecco PBS/1 mM ethylene diamine tetraacetic acid [EDTA]; Gibco) for 10 min at 37°C. The expression levels of *miR-517a-3p* were assayed by real-time PCR.

Isolated peripheral blood immune cells were incubated with BeWo exosomes (50 μg/ml) in exosome-free RPMI 1640 medium supplemented with 10% FBS for 24 h, and the expression levels of *miR-517a-3p* were assayed by real-time PCR.

Statistical Analysis

We conducted all analyses using the SPSS statistical software package (Windows version 20; IBM-SPSS). The significance of between-group differences was assessed using Student *t*-test, ANOVA followed by Tukey test, or Kruskal-Wallis test, and P values <0.05 were considered to indicate statistical significance.

RESULTS

Characterization of Exosomes Derived from BeWo Cells

We first investigated whether BeWo cells could release exosomes into the extracellular space. Because exosomes are known to express CD63, CD81, and TSG101 [3], we examined expression of these molecules in the exosomal fraction isolated from BeWo cell culture medium. As shown in Figure 1A, Western blotting detected all of these molecules in the exosomal fraction, indicating that BeWo cells secrete exosomes into the culture medium. We also confirmed the secretion of exosomes from BeWo cells by immunoelectron microscopy (Fig. 1B). Morphometric analysis of isolated vesicles showed that approximately 80% of the vesicles were positive for CD63. Most of the vesicles were in the 26- to 125-nm range (diameter [mean ± SD], 85 ± 33 nm; Fig. 1C). Moreover, we determined whether the placenta-associated miRNA *miR-517a-3p* exists in exosomes derived from BeWo cells by real-time PCR (Fig. 1D). As expected, *miR-517a-3p* was identified in exosomes secreted from BeWo cells but was not detectable in Jurkat cells.

In Vitro Model of Trophoblast-Immune Cell Communication

To investigate the possibility of functional miRNA transfer from placenta trophoblasts to maternal peripheral blood immune cells via exosomes, we employed an in vitro model system using BeWo and Jurkat cells (Fig. 2A). To confirm whether this system serves as an in vitro model of cell-to-cell communication via exosomes, we first examined transfer of placenta-associated miRNAs to Jurkat cells. Because human placenta expresses placenta-associated miRNAs, such as *miR-512-3p* and *miR-517a-3p*, which are secreted into maternal circulation via exosomes [20, 30], we evaluated the presence of these miRNAs in Jurkat cells after incubation with exosomes released from trophoblast cell lines (BeWo and JEG3 cells) by real-time PCR. When Jurkat cells were incubated with BeWo or JEG3 exosomes for 24 h, *miR-512-3p* and *miR-517a-3p* were detected in the cells (Fig. 2B). In contrast, these miRNAs were not detectable in Jurkat cells treated without exosomes (vehicle). It is likely that miRNAs were transferred into Jurkat cells via trophoblast cell line exosomes.

Target Identification of Placenta-Associated miRNA miR-517a-3p *in Jurkat Cells*

As shown in Figure 2A, a BeWo exosomal miRNA would be expected to bind to a partially complementary sequence (seed sequence) in the 3′-UTR of its target mRNA and suppress translation in recipient Jurkat cells. We next investigated whether BeWo-derived placenta-associated miRNAs function as posttranscriptional gene regulators within

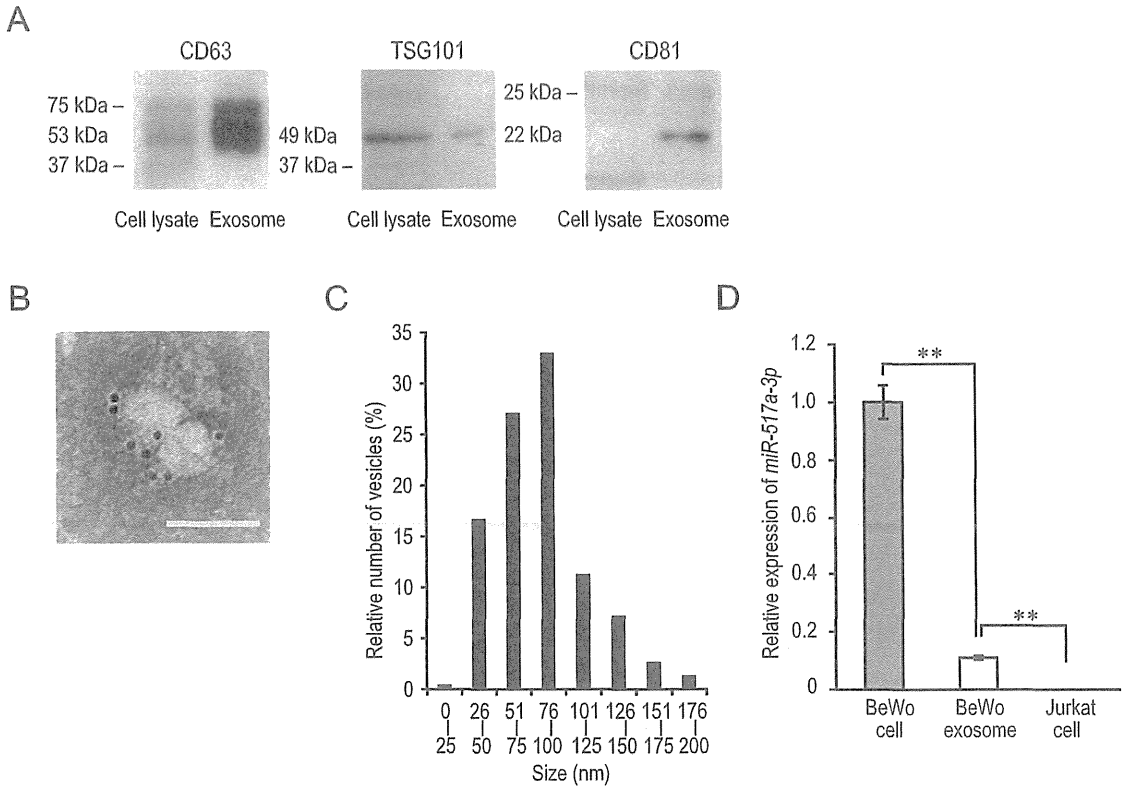

FIG. 1. Characterization of exosomes isolated from culture supernatants of BeWo cells. A) Western blots for exosome markers (CD63, CD81, and TSG101) in BeWo cell lysates (Cell lysate) and exosomal pellets collected from culture supernatants (Exosome). B) Immunoelectron microscopy of BeWo exosomal pellets. Negatively stained exosomes are labeled with 10-nm colloidal gold particles recognizing CD63. Bar = 100 μm. C) Histogram of the number of isolated BeWo vesicles' diameters. The *y* axis shows the relative number of vesicles (%), and the *x* axis shows the vesicle diameter (nm). Vesicles were sorted into 25-nm bins. D) Real-time PCR analysis of *miR-517a-3p* expression in BeWo cells, wild-type exosomes derived from the same cells, and Jurkat cells. Data were normalized to *SNORD44*. Expression in BeWo cells was defined as 1. Data are means ± SD of the results from three independent experiments. Tukey test; $^{**}P < 0.01$.

Jurkat cells after exosome-mediated miRNA transfer. For this purpose, we initially identified candidate target mRNAs of the placenta-associated miRNAs using DNA microarray analysis. In this examination, we focused on *miR-517a-3p* as a representative exosomal placenta-associated miRNA, because this miRNA was detected at high levels in the plasma of pregnant women [20] and in isolated human trophoblast cells [30, 31]. Transfection of Jurkat cells with Pre-miR-517a significantly downregulated 123 genes (Supplemental Table S1, available online at www.biolreprod.org). Among the 123 genes identified, we searched for potential direct targets of *miR-517a-3p* using the online software MicroCosm Targets. Seven genes, *ALDH1B1*, *ANP32E*, *DHFR*, *FAT2*, *IGSF5*, *PRKG1*, and *RSPO3*, had at least one potential *miR-517a-3p*-binding site in their 3′-UTRs.

To confirm downregulated expression of these seven genes, we performed real-time PCR. Among the seven mRNAs selected, *PRKG1* was significantly downregulated in *miR-517a-3p*-overexpressing Jurkat cells (Fig. 3A), whereas expression of *ALDH1B1*, *ANP32E*, *DHFR*, *FAT2*, and *RSPO3* was not significantly altered (data not shown). *IGSF5* expression was undetectable (data not shown). As shown in Figure 3B, PRKG1 protein expression was markedly decreased in Pre-miR-517a-transfected cells compared with control cells. Therefore, we focused on *PRKG1* for *miR-517a-3p* target validation.

We used a luciferase assay to determine whether *PRKG1* is a direct target of *miR-517a-3p*. Overexpression of *miR-517a-3p* significantly decreased luciferase activity in Jurkat cells cotransfected with pMIR-PRKG1 (49% reduction compared with the negative control), but not significantly in cells cotransfected with pMIR-PRKG1mt (Fig. 3C), a reporter plasmid in which the putative *miR-517a-3p* recognition site in the *PRKG1* 3′-UTR is mutated (Fig. 3D). Taken together, these results suggest that *PRKG1* is a target of *miR-517a-3p* in Jurkat cells.

Effect of BeWo Exosomal miR-517a-3p *on* PRKG1 *Expression in Jurkat Cells*

We further investigated whether BeWo exosomal *miR-517a-3p* can modulate *PRKG1* mRNA expression within recipient Jurkat cells. In this examination, we also used *miR-517a-3p*-loaded exosomes collected from culture supernatants of BeWo cells transfected with Pre-miR-517a to enhance the effect of this miRNA in the recipient Jurkat cells because we could not generate C19MC *mir-517a* gene-knockout BeWo cells. As shown in Figure 4A, levels of *miR-517a-3p* were greatly increased in the exosome fraction of cell culture supernatants from Pre-miR-517a-transfected BeWo cells (more than 30-fold greater than that from nontransfected cells).

We quantified exosomal *miR-517a-3p* in Jurkat cells incubated with BeWo exosomes. *miR-517a-3p* was not

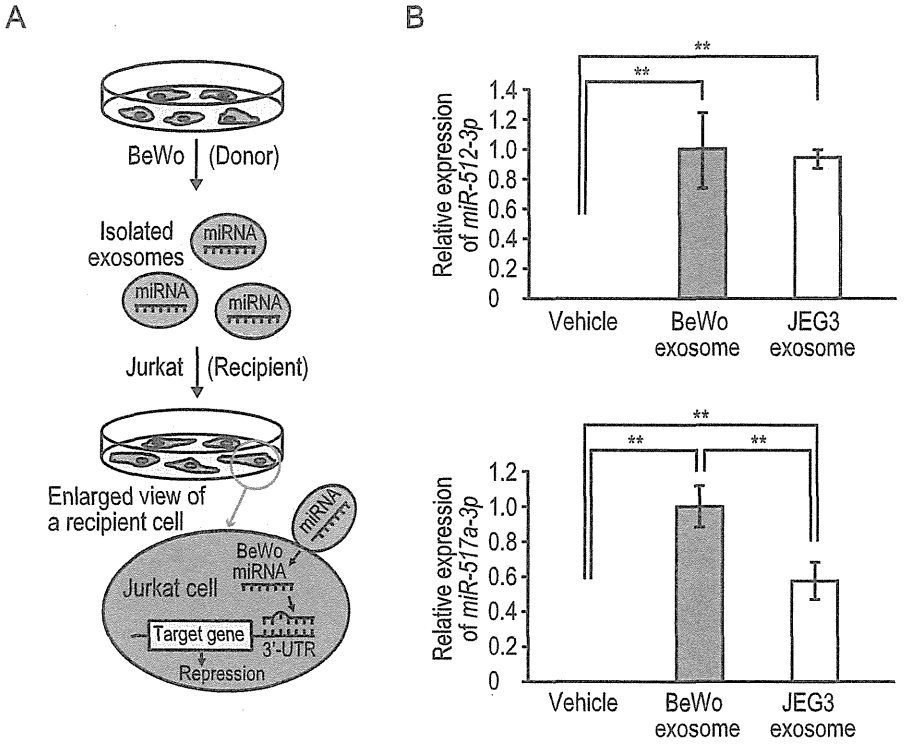

FIG. 2. In vitro model system of trophoblast-immune cell communication. **A**) Scheme of the in vitro transfer system using BeWo cells as donors and Jurkat cells as recipients. Exosomes were collected from culture supernatants of BeWo cells. BeWo exosomes carrying placenta-associated miRNAs (e.g., *miR-517a-3p*) were then added to Jurkat cell culture medium. BeWo exosomal miRNAs were then transferred into Jurkat cells, where they bind to the 3′-UTRs of target genes. **B**) Detection of placenta-associated miRNAs in Jurkat cells treated with wild-type exosomes derived from trophoblast cell lines, BeWo cells, and JEG3 cells. Jurkat cells were incubated in the presence or absence (Vehicle) of exosomes (50 μg/ml) for 24 h. Expression of placenta-associated miRNAs (*miR-512-3p* and *miR-517a-3p*) was analyzed by real-time PCR. Data were normalized to *SNORD44*. Expression in Jurkat cells incubated with BeWo exosomes was defined as 1. Data are means ± SD of the results from three independent experiments. Tukey test; $^{**}P < 0.01$.

detected in Jurkat cells incubated without BeWo cell exosomes (vehicle), and *miR-517a-3p* levels were increased significantly in Jurkat cells treated with BeWo wild-type exosomes (Fig. 4B). Moreover, when Jurkat cells were treated with BeWo *miR-517a-3p*-loaded exosomes, markedly greater levels of *miR-517a-3p* levels were transferred to recipient cells compared with treatment with wild-type exosomes (Fig. 4B). We then investigated whether transferred exosomal *miR-517a-3p* represses endogenous *PRKG1* expression in Jurkat cells. As expected, the expression levels of *PRKG1* were slightly but significantly decreased in Jurkat cells incubated with wild-type exosomes (42% reduction) compared with vehicle-treated cells (Fig. 4C). Further, downregulation of *PRKG1* expression was significantly enhanced by treatment with *miR-517a-3p*-loaded exosomes (70% reduction) compared with vehicle-treated cells (Fig. 4C). In addition, we measured exosomal *miR-517a-3p* in Jurkat cells incubated with BeWo miR-NC-loaded exosomes. No differences were found in the expression levels of *miR-517a-3p* and *PRKG1* between cells treated with miR-NC-loaded exosomes and wild-type exosomes (data not shown).

We also confirmed that exosomal *miR-517a-3p* bound to the 3′-UTR of *PRKG1* in recipient cells by luciferase assay. In pMIR-PRKG1-transfected Jurkat cells, BeWo wild-type exosomes significantly reduced luciferase activity (31% reduction) compared with pMIR-cont-transfected cells; downregulation of *PRKG1* expression was enhanced by incubation with *miR-517a-3p*-loaded exosomes (51% reduction) compared with pMIR-cont-transfected cells (Fig. 4D). Furthermore, we investigated whether remaining cell surface-bound BeWo exosomes affect the expression levels of *miR-517a-3p* that was transferred to Jurkat cells. Jurkat cells were incubated with BeWo exosomes for 24 h, followed by TrypLE digestion (recombinant trypsin-like serine protease-EDTA). These were no differences in the miRNA expression levels between *miR-517a-3p* recipient cells treated with and without TrypLE (Fig. 4E). These results show that BeWo exosomal miRNA *miR-517a-3p* internalized into Jurkat cells and subsequently repressed expression of the target gene *PRKG1* therein.

We used *miR-517a-3p*-loaded exosomes for assessment of the effect of BeWo exosomal *miR-517a-3p* on *PRKG1* in Jurkat cells. An *miR-1* transfection and *TWF1* detection system [32] was also employed to demonstrate that BeWo exosomal miRNAs can transfer to Jurkat cells and suppress target genes in the recipient cells. *TWF1* mRNA is a validated target of *miR-1*, and the repression occurs at the mRNA level [33]. When BeWo *miR-1*-loaded exosomes were incubated with Jurkat cells, *miR-1* was significantly detected in the recipient cells (Fig. 4F). In contrast, *miR-1* was undetectable in Jurkat cells treated with BeWo miR-NC-loaded exosomes (Fig, 4F). As expected, the *miR-1* recipient cells displayed a 55% knockdown of *TWF1* mRNA compared with the miR-NC recipient cells (Fig. 4G). The inverse relationship between *miR-1* expression and the expression level of *TWF1* mRNA provided additional data supporting that BeWo exosomal miRNAs can transfer to Jurkat cells and suppress target genes in the recipient cells.

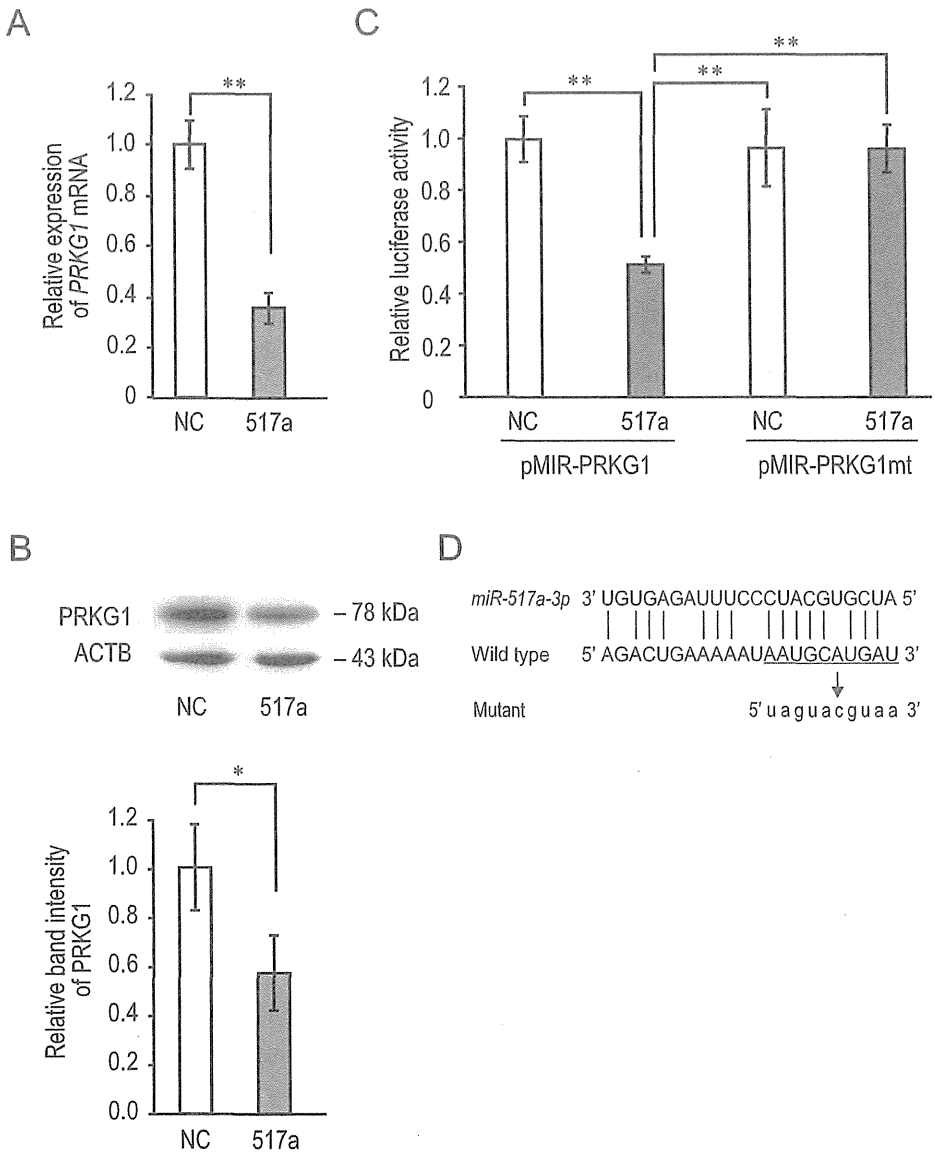

FIG. 3. Validation of *PRKG1* as a *miR-517a-3p* target. **A**) Real-time PCR analysis of candidate *miR-517a-3p* target mRNA *PRKG1* in Jurkat cells. Jurkat cells were transfected with Pre-miR-517a (517a) or Pre-miR-NC (NC), each 40 nM, and cultured for 48 h. Expression of *PRKG1* was examined by real-time PCR. Data were normalized to *GAPDH*. Expression in cells treated with Pre-miR-NC was defined as 1. **B**) Western blots for PRKG1 in Jurkat cells transfected with Pre-miR-517a or Pre-miR-NC (30 μg of total protein per lane). ACTB served as the internal control. Analysis of band intensity is presented as the relative ratio of PRKG1 to ACTB. The relative ratio of PRKG1 in cells treated with Pre-miR-NC was defined as 1. **C**) *PRKG1* 3′-UTR luciferase reporter assay. Reporter vector (pMIR-RPKG1 or pMIR-PRKG1mt) and Pre-miRNA (Pre-miR-517a or Pre-miR-NC [each 30 nM]) were cotransfected into Jurkat cells. *Renilla* luciferase vector pRL-TK was used as the internal control. Luciferase expression levels in cells cotransfected with pMIR-RPKG1 and Pre-miR-NC were defined as 1. **D**) Sequences of mature *miR-517a-3p*, its putative target site in the 3′-UTR of *PRKG1*, and the mutation introduced into the *miR-517a-3p* recognition site of *PRKG1* 3′-UTR in the reporter plasmid. Data are means ± SD of the results from three independent experiments. Student *t*-test (**A** and **B**) or Tukey test (**C**); $^{*}P < 0.05$, $^{**}P < 0.01$.

Detection of Placenta-Associated miRNA miR-517a-3p *in Maternal Immune Cells Isolated from Peripheral Blood of Full-Term Pregnant Women*

As mentioned above, placenta-associated miRNAs are detectable in the maternal circulation during pregnancy [20, 22, 23]. We next investigated whether these miRNAs in the maternal circulation are delivered into immune cells in peripheral blood as they are into Jurkat cells in vitro. For this purpose, we examined whether *miR-517a-3p* and *miR-518b* are present in immune cells isolated from maternal peripheral blood using real-time PCR. These placenta-associated miRNAs

FIG. 4. Transfer of miRNAs into Jurkat cells via BeWo exosomes. **A**) Real-time PCR analysis of *miR-517a-3p* in BeWo wild-type exosomes (Wt-Ex) and *miR-517a-3p*-loaded exosomes (517a-Ex); expression in Wt-Ex was defined as 1. **B**) Detection of transferred *miR-517a-3p* in Jurkat cells cultured with BeWo exosomes. Jurkat cells were treated with vehicle, Wt-Ex, or 517a-Ex (each 50 μg/ml) for 48 h at 37°C. Expression was analyzed by real-time PCR; data were normalized to *SNORD44*. Expression in Jurkat cells incubated with Wt-Ex was defined as 1. **C**) *PRKG1* silencing by exosomal *miR-517a-3p*