Clinical Immunology 203 (2019) 37–44

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

# Pathogenic roles of anti-C1q antibodies in recurrent pregnancy loss

Kazumasa Ohmura[a], Kenji Oku[a,*], Tamao Kitaori[b], Olga Amengual[a], Ryo Hisada[a], Masatoshi Kanda[a], Yuka Shimizu[a], Yuichiro Fujieda[a], Masaru Kato[a], Toshiyuki Bohgaki[a], Tetsuya Horita[a], Shinsuke Yasuda[a], Mayumi Sugiura-Ogasawara[b], Tatsuya Atsumi[a]

[a] Department of Rheumatology, Endocrinology and Nephrology, Hokkaido University Faculty of Medicine and Graduate School of Medicine, Sapporo, Japan
[b] Department of Obstetrics and Gynaecology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan

ARTICLE INFO

*Keywords:*
Anti-C1q autoantibody
Recurrent pregnancy loss
Complement activation

ABSTRACT

Recurrent pregnancy loss (RPL) is often considered idiopathic, however excessive complement activation has been observed in pregnancy related manifestations. Anti-C1q antibodies (anti-C1q) are associated with the activation of complement pathway in lupus patients, while it remains unclear in RPL. Firstly, we showed that both the prevalence and titre of anti-C1q were significantly higher in unexplained RPL than in healthy parous individuals. Secondly, we established the murine model of anti-C1q induced pregnancy loss using a monoclonal anti-mouse C1q antibody, JL-1. In mice treated with JL-1, high ratio of pregnancy loss and fetal growth restriction were frequently observed and complement activation occurred. C5a receptor (C5aR) blockade cancelled these pathogenic changes in mice treated with JL-1. In conclusion, our study reveals an association between the prevalence of anti-C1q and RPL. Additionally, our murine model has indicated that anti-C1q can induce reproductive failure, which might be ameliorated by therapy targeting the C5-C5aR axis.

## 1. Introduction

Recurrent pregnancy loss (RPL) is a heterogeneous clinical condition characterized by the occurrence of two or more failed clinical pregnancies [1]. The causes of RPL include genetic alterations, female genital tract malformations, endocrine diseases and antiphospholipid antibodies. However, up to 50% of RPL cases are of unknown etiology, being termed unexplained RPL [2]. Among those unexplained RPL, autoimmunity would be one of the most plausible pathophysiology, although the autoantigens have not been clearly identified.

In pregnancy, maternal immune system tolerates the semi-allogenic fetus whose tissues are directly exposed to the maternal blood with the potential for attack by the maternal innate and acquired immune systems. Tolerance at the feto-maternal interface in mice is regulated by the local expression of complement regulating proteins, such as CD55 (decay-accelerating factor, DAF), CD59, CD46 (membrane cofactor protein, MCP) and complement receptor 1-like protein y (Crry) and/or some population of regulatory T cell (Treg) induced by exogenous antigens in the periphery [3–6]. Foetal loss occurs when these mechanisms are dysregulated. Excessive complement activation was observed in several murine models, such as the allogeneic murine model, lipopolysaccharide (LPS)-induced miscarriage model, and antibody-mediated foetal loss model [7–11]. The immune regulatory network at the feto-maternal interface is thought to play a key role in preventing infertility, RPL, preeclampsia, foetal growth restriction and premature birth.

Complement system is a component of innate immunity that not only neutralizes infectious agents but also promotes removal of immune complexes and apoptotic cells. In addition, the acquired immune system is collaborating with complement system via activated complement fragments, such as the anaphylatoxins C5a, C3a [12–16]. Plasma levels of complement components are increased and complement deposition in placental tissue is observed in healthy individuals as well as in complicated pregnancies [17–22]. C1q, the sub-component of the C1 protein, is a key molecule in innate immunity that triggers the activation of the classical pathway of the complement system. C1q plays a regulatory role in pregnancy maintenance such as in trophoblast migration, spiral artery remodeling and normal placentation, resulting in foetal survival [23]. Moreover, C1q at the feto-maternal interface is presumed to be involved in preventing pathogen entry through the placenta. Excessive complement activation induces foetal loss in the antibody-mediated pregnancy loss model, due to triggering of abnormal complement activation by antiphospholipid antibodies, which overwhelms the physiological complement regulatory proteins [10,24,25].

* Corresponding author at: Department of Rheumatology, Endocrinology and Nephrology, Hokkaido University Faculty of Medicine and Graduate School of Medicine, N15 W7, Kita-ku, Sapporo, Japan.
*E-mail address:* kenoku@med.hokudai.ac.jp (K. Oku).

https://doi.org/10.1016/j.clim.2019.04.005
Received 4 September 2018; Received in revised form 7 April 2019; Accepted 8 April 2019
Available online 08 April 2019

Autoantibodies, such as antiphospholipid antibodies or antinuclear antibodies, contribute to the pathogenic activation of the complement system [25,26]. Anti-C1q antibodies are present in a large proportion of patients who miscarry during the first trimester of pregnancy, especially in conjunction with antiphospholipid antibodies [27]. Anti-C1q autoantibodies are frequently detected in patients with autoimmune diseases, including hypocomplementemic urticarial vasculitis (HUV; 100%) and proliferative lupus nephritis (30–60%) [28]. Wisnieski et al. reported that the autoantibodies to C1q bind to the common epitope on the collagen like region (CLR) in patients with HUV and lupus patients [29]. Trouw et al. generated mouse anti-mouse C1q monoclonal antibodies and reported that the clone, JL-1, specifically bound to the CLR, showed cross-reactivity with human C1q and amplified the activation of the complement pathway [30]. Administration of JL-1 to naïve mice resulted in glomerular deposition of C1q but not in overt renal disease, however pretreatment with C1q-fixing anti-glomerular basement membrane developed nephritis in mice treated with JL-1. This study suggested that anti-C1q antibodies lead to the development of organ dysfunction in combination with C1q deposition [31].

We hypothesized that anti-C1q autoantibodies lead to reproductive failure via complement activation in patients with unexplained RPL. In this study, we firstly investigated the prevalence of anti-C1q autoantibodies in patients with unexplained RPL. Further, we evaluated the pathophysiological role of anti-C1q antibodies in pregnancy condition using a murine model.

## 2. Patients and methods

### 2.1. Patients

We conducted a retrospective cross-sectional study comprising a total of 134 consecutive RPL patients who visited Nagoya City University Hospital and 27 OAPS patients who attended Hokkaido University Hospital between 2008 and 2013.

Age- and gender-matched fourteen parous connective tissue disease (CTD) patients without historical obstetric/thrombotic complications were enrolled as controls; these comprised ten with rheumatoid arthritis (RA), five with dermatomyositis/polymyositis (DM/PM), four with systemic sclerosis (SSc), four with primary Sjögren's Syndrome (SS) and two with other diseases. Age- and gender-matched twenty six parous healthy controls (HC) were also included in the study. Patients with SLE, mixed connective tissue disease and RA with vasculitis were excluded due to the high prevalence of anti-C1q antibodies in those populations [32]. Plasma was collected from each patient at regular visits to the clinics, after granting of informed consent. Clinical data were obtained at the time of plasma collection. This study was approved by the local Ethics Committee of Hokkaido University Hospital (approval number: 015–0072).

All of the 134 RPL patients had a history of two or more pregnancy loss without OAPS, congenital uterine anomaly or abnormal chromosome in either partner. OAPS was diagnosed according to the Sapporo criteria Sydney revision [33], RA diagnosed according to 2010 ACR/EULAR classification RA criteria [34], SSc according to the 2013 ACR/EULAR SSc criteria [35], DM/PM using the Bohan and Peter criteria [36], and SS based on the 2016 ACR/EULAR classification criteria [37].

### 2.2. Plasma levels of anti-C1q antibodies C1q

Plasma levels of anti-C1q antibodies were measured by enzyme-linked immunosorbent assay (ELISA) (Buhlmann Laboratories AG, Switzerland). A value of > 15 U/mL was defined as positive. In the RPL patients whose plasma were available, C1q levels were measured by ELISA (Assaypro, Missouri, USA).

### 2.3. Mouse experiments

To assess the role of the anti-C1q antibodies in pregnancy loss, we established the murine model of anti-C1q induced pregnancy loss using a monoclonal anti-mouse C1q antibody, JL-1. JL-1 has been well characterized to induce complement activation in mice [30]. All murine experiments were approved by the local Ethics Committee of Hokkaido University Graduate School of Medicine and were performed in accordance with institutional guidelines.

Mice were obtained from CLEA Japan, Inc. A female BALB/c mouse was mated between 8 and 12 weeks old with an isolated BALB/c male mouse. Day 1 of pregnancy was defined as the day on which vaginal plug was detected. On days 8 and 12 of pregnancy, mice were administered, via the tail vein, 100 μL of phosphate-buffered saline (PBS) containing 500 μg/kg JL-1 (HM1096, Hycult Biotech, Uden Netherlands), isotype control IgG2b (500 μg/kg, M077–3, Medical & Biological Laboratories (MBL), Nagoya, Japan), or PBS alone. The dose and frequency of injection of JL-1 determined according to the preliminary experiments as described in Supplementary Fig. S1. On day 16 of pregnancy, mice were sacrificed, their serum collected, uteri dissected, survived fetuses and placentas weighed and placentas harvested for immunohistochemical analysis. The foetal resorption rate was calculated as the ratio of number of resorbed to total embryos. The resorption site resulting from loss of a previous viable fetus was identified. Both serum C1q and C3a levels were measured by ELISA (C1q: HK211, Hycult BIotech, C3a: SEA387Mu, Cloud-Clone Corp, Texas, USA) according to the manufacturer's instruction.

For immunohistochemical examination of C1q, C3 and C4d in placental tissue, the placenta was removed on day 16 of pregnancy, frozen in optimal cutting temperature (O.C.T.) compound and cut into 10 μm sections. The sections were fixed in 4% paraformaldehyde for 7 min at 4 °C. Endogenous peroxidase activity was blocked by incubation in 1% $H_2O_2$ in methanol and incubated for 30 min at room temperature. The treated sections were rinsed with PBS and then incubated for 60 min in PBS containing a monoclonal rat anti-mouse C1q (HM1044, Hycult Biotech), a monoclonal rat anti-mouse C3 (HM1045, Hycult Biotech) at a 1:200 dilution, or in PBS containing a rabbit polyclonal anti-mouse C4d (HP8033, Hycult Biotech) at a 1:50 dilution in antibody diluent (S2022, DAKO, California, USA) for 60 min at room temperature. After washing with PBS, the slides were incubated with a secondary biotinylated anti-rat or anti-rabbit IgG at 1:100 dilution for 30 min at room temperature. Horseradish peroxidase-labeled streptavidin was used to detect the antigen biotinylated antigen (PK4000, Vector Laboratories). Staining was visualized with diaminobenzidine (DAKO) and counterstaining with haematoxylin. The ratio of stained area to total area in 5 high-power fields (HPF) of the decidua was calculated and quantified using the ImageJ software with the colour deconvolution plug-in Java 1.48v (National Institutes of Health, Bethesda, MD, USA).

To assess the role of complement pathway activation in pregnancy loss, in a second set of experiments, we investigated the effect of blockade of the C5a receptor (C5aR) using an anti-C5aR monoclonal antibody (clone 20/70, HM1076, Hycult Biotech). Mice were treated with 100 μL of PBS containing 500 μg/kg anti-C5aR or 500 μg/kg rat IgG2b isotype control (ctrIgG) (MAB0061, R&D Systems, Minnesota, USA) administered via the tail vein 30 min before the first treatment with JL-1 or isotype control IgG2b (M077–3, MBL) on day 8 of pregnancy. On day 16 of pregnancy, mice were sacrificed and their serum collected, uteri dissected, fetuses and placentas weighed and placentas harvested. The serum C3a level was determined and immunohistochemical staining of C1q, C3 and C4d in placental tissue was performed as described earlier.

### 2.4. Statistics

Titres of anti-C1q antibodies presented as medians and interquartile ranges, were compared using Dunn's test for a post hoc analysis. Foetal

resorption ratios, foetal weights, placental weights and serum levels of C1q and C3a were presented as means and standard deviations and were compared using Tukey's honestly significant difference for a post hoc test. These parameters were compared between mice pre-treated with the anti-C5aR and control IgG2b antibodies using the Student's *t*-test. Immunohistochemical staining of C1q, C3 and C4d was compared by one-way analysis of variance (ANOVA) without adjustment for multiple comparisons. Statistical analyses were performed using JMP Pro V.12.0.1 (SAS Institute Inc. Cary, NC, USA) and Graph Pad Software 6.0 (Graph Pad, San Diego, CA, USA). Values of $p < 0.05$ were considered to indicate significance.

## 3. Results

### 3.1. Prevalence and titre of anti-C1q antibodies

The characteristics of patients are shown in Supplementary Table S1. The prevalence (RPL: 47/134; 35% vs. HC: 2/27; 7%, $p < 0.01$, vs. CTD: 3/27; 11%, $p < 0.05$) and the titres of anti-C1q antibodies (RPL: median:12 U/ml, IQR [8.0–21.0] vs. HC: 0 U/ml, IQR [0–1.8], $p < 0.0001$, CTD: 2.1 U/ml, IQR [0–8.1]), $p < 0.001$) were significantly higher in RPL patients than in control groups (HC and CTD) (Fig. 1). The titres of anti-C1q antibodies in OAPS patients (9.8 U/ml, IQR [0–22.0], $p < 0.01$), were significantly higher than those in HC. The prevalence of anti-C1q antibodies was significantly higher in OAPS than those in HC (8/27, 30% vs. 7%, $p < 0.05$). In CTD patients, neither the prevalence nor the titre of anti-C1q antibodies was significantly different from that in HC. We preliminarily evaluated the correlation between titres of anti-C1q and C1q levels of plasma in the RPL patients whose measurements could be performed (Supplementary Fig. S2). In evaluating the complement levels between anti-C1q positive and negative RPL patients, C3 was significantly lower in anti-C1q positive patients compared with anti-C1q negative patients while both CH50 and C4 were not different (Supplementary Fig. S3).

### 3.2. Anti-C1q induced pregnancy loss

Amnion sacs on day 16 of pregnancy are shown in Fig. 2A. The foetal resorption rate, expressed as resorbed embryos/total number of embryos, was significantly higher in mice treated with JL-1 compared with the controls (JL-1 ($n = 10$) vs. mIgG ($n = 6$), 0.44 ± 0.12 vs. 0.16 ± 0.11; JL-1 vs. PBS ($n = 5$), 0.44 ± 0.12 vs. 0.14 ± 0.15%) (Fig. 2B). The mean foetus weight was lower in mice treated with JL-1 compared with the controls (JL-1 ($n = 38$) vs. mIgG ($n = 46$), 322 ± 98 mg vs. 358 ± 70 mg, $p < 0.05$; JL-1 vs. PBS ($n = 39$), 322 ± 98 vs. 381 ± 76 mg, $p < 0.01$), and the mean placenta weight was lower in mice treated with JL-1 compared with the controls (JL-1 ($n = 39$) vs. mIgG (n = 46), 138 ± 14 mg vs. 148 ± 13 mg, $p < 0.01$; JL-1 vs. PBS (n = 39), 138 ± 14 vs. 152 ± 13 mg, $p < 0.0001$) (Fig. 2C, D). The mean serum C3a levels on day 16 of pregnancy were significantly higher in JL-1-treated mice than in the controls (JL-1 ($n = 6$) vs. mIgG ($n = 5$), 1810 ± 532 ng/mL vs. 846 ± 140 ng/mL, p < 0.01; JL-1 vs. PBS ($n = 3$), 1810 ± 532 vs. 819 ± 172 ng/mL, p < 0.01) (Fig. 2E). The mean serum C1q levels in mice treated with JL-1 did not decrease but slightly elevated compare with the control groups, but without statistical significance (JL-1 (n = 6) vs. mIgG (n = 5), 141 ± 27 μg/mL vs. 109 ± 10 μg/mL, $p = 0.07$; JL-1 vs. PBS (n = 3), 141 ± 27 vs. 118 ± 21 μg/mL, $p = 0.32$) (Fig. 2F). The serum anti-C1q monoclonal antibody levels in mice treated with JL-1 were weakly detected, however not be different among these groups.

On day 16 of pregnancy, C1q and C3 deposition in decidua were greater in JL-1 treated mice compared with the controls (Fig. 3). Deposition of C4d (Fig. 3C, F, I) was weak but more extended in mice treated with JL-1 than in those treated with control antibody or PBS. C1q, C3 and C4d were deposited mostly on the decidua or in the labyrinth zone, where active feto-maternal exchange occurs, but not in the junctional zone, of the murine placentae. Deposition of C1q, C3 and C4d was more marked in mice treated with JL-1 than in those treated with mIgG or PBS (JL-1 vs. mIgG, PBS; C1q: 22 ± 8% vs. 8.7 ± 7.2%, 2.3 ± 1.0%, respectively, $p < 0.05$, C3: 25 ± 17% vs. 8.7 ± 6.0%, 6.4 ± 0.23%, respectively p < 0.05, C4d: 17 ± 8.6% vs. 5.6 ± 7.2%, 2.1 ± 1.5%, respectively p < .05).

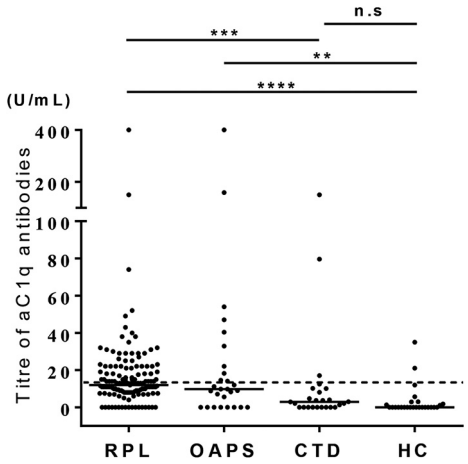


**Fig. 1.** Titres of anti-C1q antibodies in patients with RPL. Anti-C1q (aC1q) antibodies were measured in plasma from RPL patients, OAPS patients, CTD patients and HC by enzyme-linked immunosorbent assay. The titres of aC1q antibodies were higher in RPL and OAPS, but not in CTD, compared to HC. Anti-C1q was more prevalent both in RPL and OAPS compared to HC. The horizontal bars represent the median values of each group. The dotted horizontal line indicated the cut-off value of 15 U/mL recommended by the manufacturer. *P* values were calculated using Dunn's post hoc test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. CTD, connective tissue disease: HC: healthy controls: OAPS obstetric antiphospholipid syndrome: RPL, recurrent pregnancy loss:

### 3.3. C5aR blockade ameliorated pregnancy loss

Mice were intravenously administered with anti-C5aR or IgG2b isotype control (ctrIgG) 30 min before the first treatment with JL-1 or mIgG on day 8 of pregnancy. Representative amnion sacs on day 16 of pregnancy are shown in Fig. 4A. The mean foetal resorption rate was significantly reduced by pre-treatment with anti-C5aR antibody compared with ctrIgG (anti-C5aR ($n = 8$) vs. ctrIgG ($n = 10$), 0.18 ± 0.11 vs 0.40 ± 0.18, $p < 0.01$). The mean foetus and placenta weights of mice treated with JL-1 were significantly different between the two pre-treatment groups (mean foetus weight; anti-C5aR ($n = 54$) vs. ctrIgG ($n = 52$), 393 ± 53 mg vs. 342 ± 62 mg, $p < 0.001$. Mean placenta weight; anti-C5aR (n = 54) vs. ctrIgG (n = 52), 149 ± 17 mg vs. 137 ± 24 mg, p < 0.01). The serum C3a level was also significantly different between the two groups (anti-C5aR ($n = 8$) vs. ctrIgG ($n = 10$), 1072 ± 331 ng/mL vs. 1434 ± 423 ng/mL, $p < 0.05$). Pre-treatment with anti-C5aR ameliorated these pathogenic changes in mice treated with JL-1 (Fig. 4B–E). The mean serum C1q levels in mice treated with JL-1 were not statistically different between the two pre-treatment groups (anti-C5aR (n = 8) vs. ctrIgG (n = 10), 159 ± 58 μg/mL vs 153 ± 39 μg/mL, $p = 0.75$) (Fig. 4F).

C1q, C3 and C4d deposition in decidua was ameliorated by additional blockade of C5aR, but not with statistical differences (Fig. 5). Therefore, blockade of C5aR prevented foetal loss, foetal growth restriction and placental damage, and ameliorated excessive complement activation.

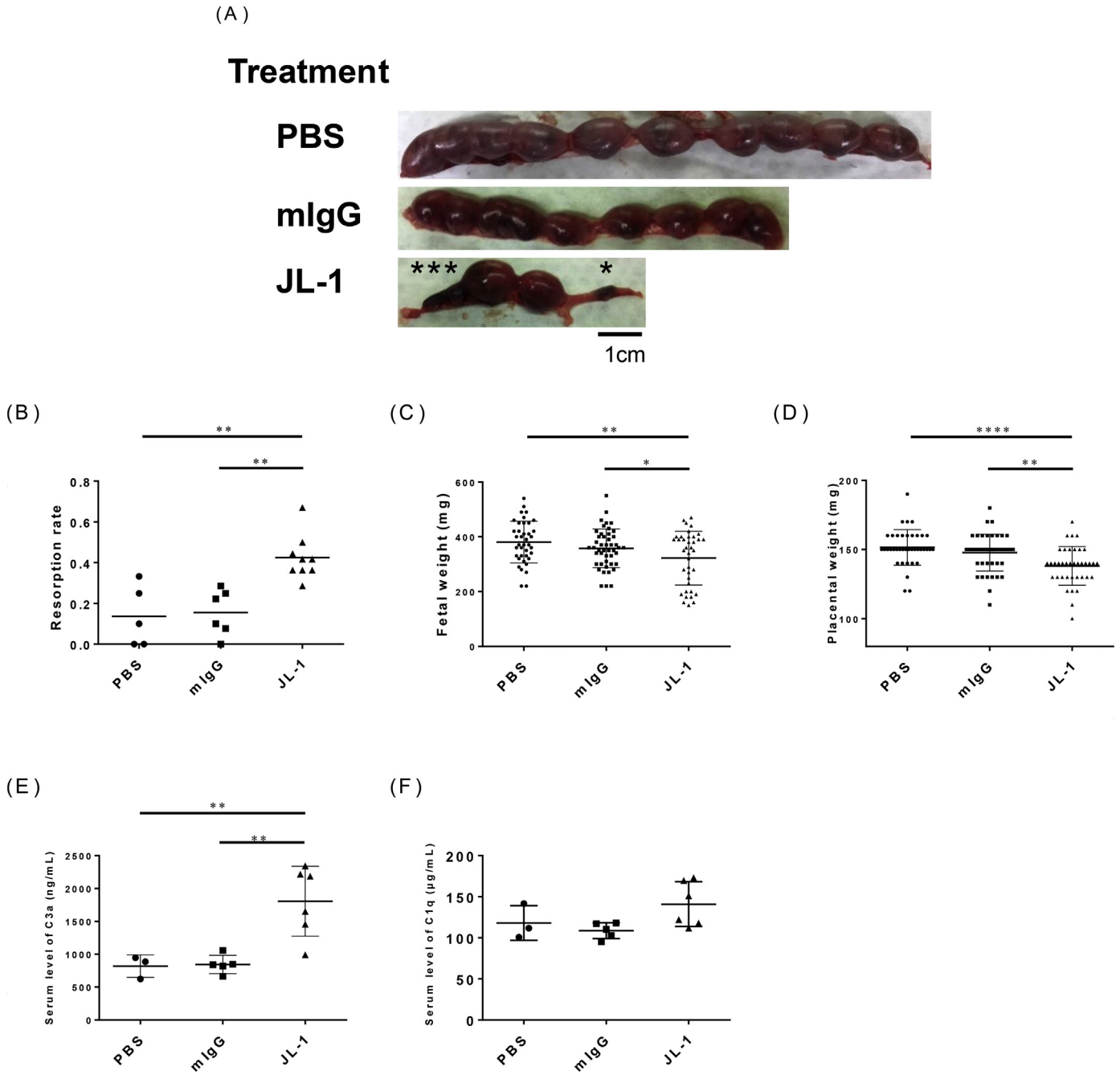


**Fig. 2.** Anti-C1q antibody induced pregnancy loss in mice. Mice were intravenously treated with JL-1 (500 μg/kg), isotype control monoclonal IgG2b (mIgG) or phosphate-buffered saline (PBS) on days 8 and 12 of pregnancy. On day 16 of pregnancy, mice were sacrificed and fetal resorption rates, fetal weight and placental weight were measured. (A) Amnion sacs in uterus appeared high resorption in mice treated with JL-1. (B) (Resorption rate (resorbed embryos/total number of embryos) is showed as a dot among three groups ($n$ = 5–10 mice per group). Resorption rate was higher in mice treated with JL-1). (C) Fetal and (D) placental weight ($n$ = 38–46 fetuses and placentas per group) were lower in mice treated with JL-1. (E) C3a levels were measured in sera from mice at sacrifice on day 16 of pregnancy and were higher in mice treated with JL-1 (n = 3–6 mice per group). (F) C1q levels were measured in sera from mice at sacrifice on day 16 of pregnancy and were not statistically different among 3 groups (n = 3–6 mice per group). Asterisks showed resorption of fetuses. Scale bar represented 1 cm (A). The horizontal bars represented mean values of each group (B). The horizontal bars and whiskers showed means and standard deviations, respectively (C–F). All $p$ values were calculated using Turkey's HSD as a post hoc analysis. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ (B–F).

## 4. Discussion

Our findings suggest that high titres of anti-C1q antibodies enhance placental tissue damage, leading to foetal loss and foetal growth restriction in patients with miscarriage, especially in unexplained RPL or OAPS.

Autoantibodies to C1q and their clinical consequences were first reported in SLE patients and subsequently found to be associated with renal involvement [38]. An increased titre of anti-C1q antibodies is predictive of renal flares in SLE patients [39,40]. However, anti-C1q antibodies are not related with renal involvement in HUV or other autoimmune diseases. Trouw et al. reported that anti-C1q autoantibodies, which react with the collagen-like region of C1q but not to soluble C1q, aggravated glomerulonephritis in patients with immune complex-mediated renal diseases such as lupus nephritis [30]. In pregnancy, C1q is deposited in the placenta with the same distribution

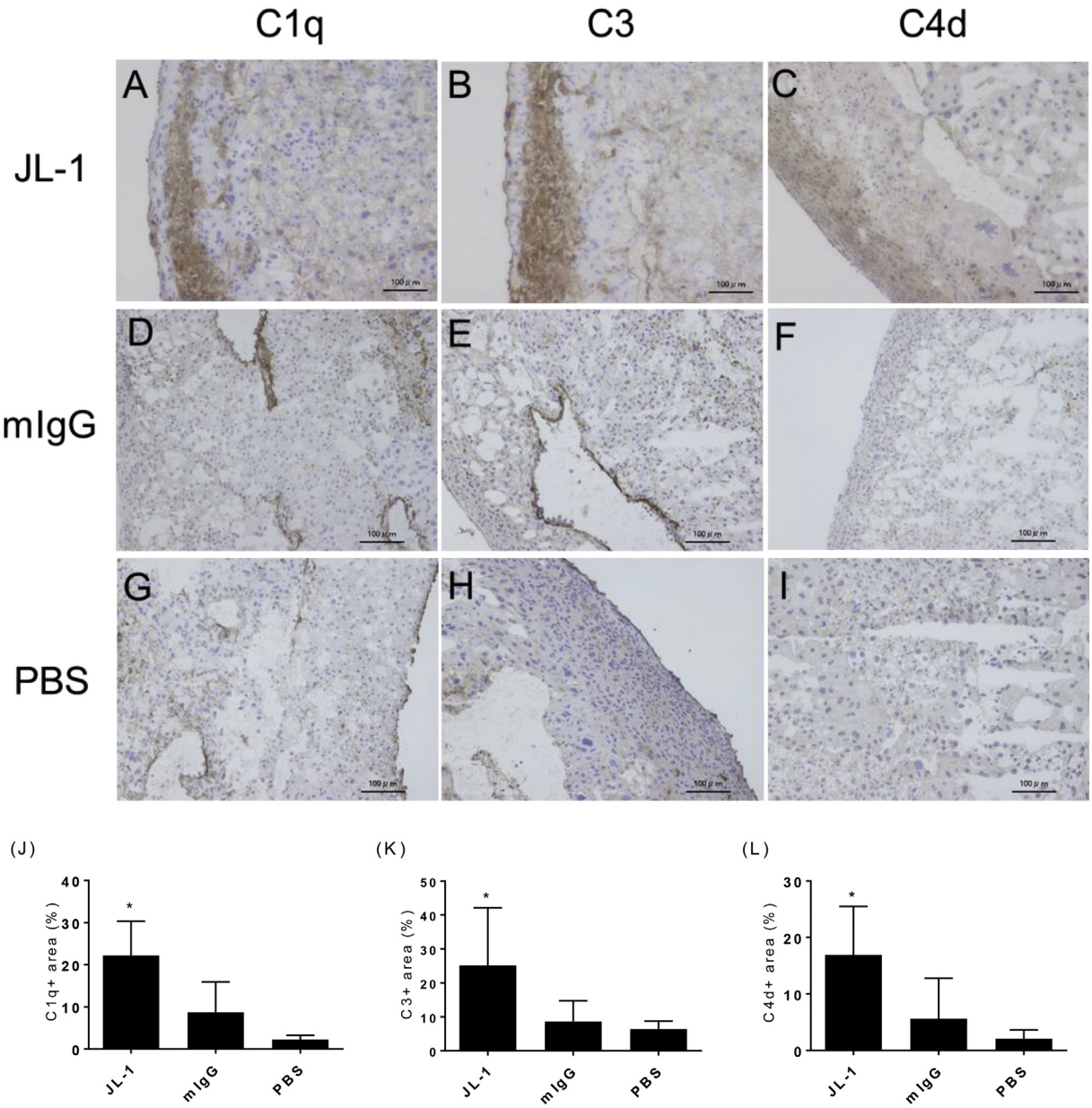


**Fig. 3.** Immunohistochemical analysis of placental tissues from mice on day 16 of pregnancy. Mice were intravenously treated with JL-1 (A-C), isotype control monoclonal IgG2b (mIgG) (D-F) or PBS (G-I) on days 8 and 12 of pregnancy and placentas were obtained on day 16 of pregnancy. Sections were immunohistochemically stained with C1q, C3 or C4d as expressed brown colour. In placental tissue from mice treated with JL-1, C1q (A), C3 (B) and C4d (C) depositions were observed in decidual zone extensively, however not observed from mice treated with mIgG (D, E, F) or PBS (G, H, I). Each complement component was significantly more deposited in mice treated with JL-1 compared with other control groups (J, K, L). The boxes and whiskers showed means and standard deviations, respectively (J-L). All p values were calculated using one-way analysis of variance (ANOVA) without multiple comparison adjustment. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in pre-eclampsia patients and healthy individuals [41]. We previously reported that the titres of the anti-C1q antibodies were significantly higher in refractory APS patients suggesting that anti-C1q antibodies enhance organ dysfunction in RPL [42]. In our cross-sectional study, the titres of anti-C1q antibodies both in RPL and OAPS patients were higher than those in healthy parous individuals, but not in CTD without pregnancy manifestations. Therefore, titres of anti-C1q antibodies may be associated with RPL. However, we did not have a chance to evaluate the temporal change in the titre of anti-C1q antibodies to see whether its titre can fluctuate according to the increase of gestational weeks.

Complement activation has been observed in pregnancy related manifestations such as preeclampsia, foetal loss. In murine models of antibody-mediated foetal loss, human IgG containing antiphospholipid antibodies purified from APS patients' sera activates the complement system via both classical and alternative pathways [10,25,43,44].

C4d is a degradation product of C4 and is used as a biomarker of antibody-mediated rejection in murine models and human [45,46]. C4d represents the classical pathway activation of complement system and C4d bound tissue stays close to the site of activation. In the present study, the deposition of C4d in decidua was greater in mice treated with JL-1 than the controls, indicating that anti-C1q antibodies activate the classical complement pathway.

C5a plays a central role in various antiphospholipid antibody-mediated models of foetal loss. C5a receptors, recruitment of neutrophils and the release of tumor necrosis factor-alpha in the decidua result in foetal injury, which was prevented by the C5a-C5aR blockade

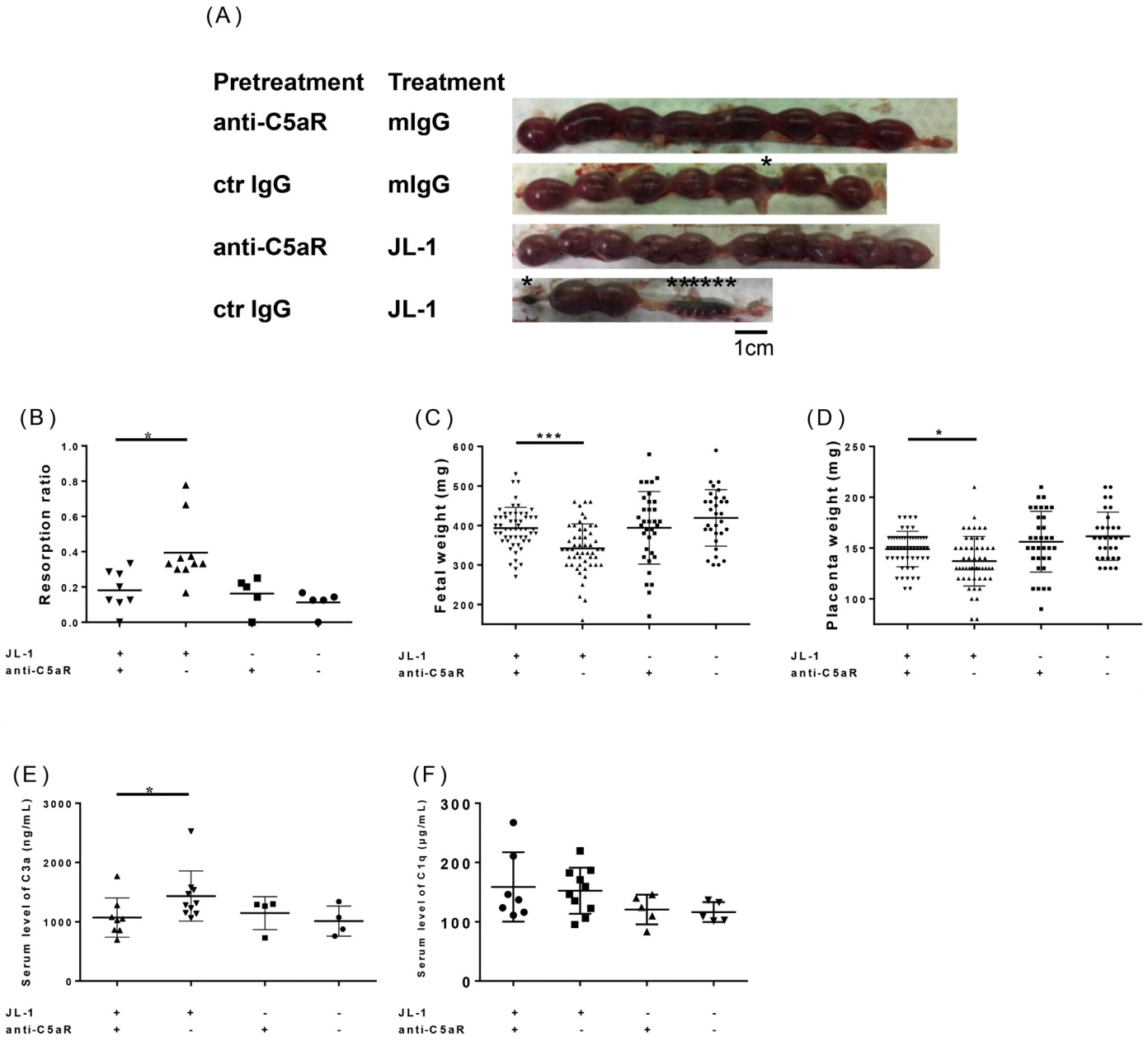


**Fig. 4.** Pre-treatment with anti-C5aR cancelled the pathogenic changes in mice treated with JL-1. Mice were intravenously pre-treated with anti-C5aR (500 μg/kg), isotype control monoclonal IgG2b (control IgG) or phosphate-buffered saline (PBS) 30 min before the first treatment on day 8 of pregnancy. On day 16 of pregnancy, mice were sacrificed and fetal resorption rates, fetal weight and placental weight were measured. (A) Representative amnion sacs in uterus were shown among each group. (B) Each resorption rate (resorbed embryos/total number of embryos) showed as a dot among four groups ($n$ = 5–10 mice per group). Pre-treatment with anti-C5aR protected mice treated with JL-1 from fetal loss. (C, D) Fetal and placental weight ($n$ = 31–54 fetuses and placentas per group) significantly changed to be higher in mice pre-treated with anti-C5aR compared with control IgG group in mice treated with JL-1. (E) C3a levels were measured in sera from mice at sacrifice on day 16 of pregnancy and were neutralized in mice pre-treated with anti-C5aR (n = 5–10 mice per group). (F) C1q levels were measured in sera from mice sacrifice on day 16 of pregnancy. Pre-treatment with anti-C5aR did not change C1q levels in mice treated with JL-1. Asterisks showed resorption of fetuses. Scale bar represented 1 cm (A). The horizontal bars represented mean of each group (B). The bars and whiskers showed means and standard deviations, respectively (C–F). All p values were calculated using Student's $t$-test between mice pre-treated with anti-C5aR and those with control IgG group. *p $<$ 0.05, ***$p$ $<$ 0.001 (B–F).

[10,25,47]. Indeed, in this study, a monoclonal anti-C1q antibody JL-1 induces complement activation in pregnant mice and blockade of the C5a-C5aR axis using an anti-C5aR antibody prevented foetal loss. The pre-treatment with the C5a inhibitor did not affect the serum C1q levels in mice treated with JL-1, which indicated that JL-1 inducing foetal loss did not come from loss of C1q.

This study had some limitations. Our clinical study included only Japanese patients in two university hospitals, indicating relatively small numbers of samples of control groups, and was designed as a retrospective cross-sectional fashion which did not elucidate a causational link. Anti-C1q measured by ELISA were polyclonal and resulted in some outliers in the control groups which could include their non-pathogenic roles of foetal loss. Secondly, we could not evaluate plasma C1q levels and activation of complement, such as plasma levels of complement components or deposition of complement components on placental tissue in this clinical study, due to the absence of samples. In our previous report [42], anti-C1q levels were positively correlated with serum C3a levels in primary APS, but it remains to be unclear whether the patients with high anti-C1q levels display excessive complement activation in RPL. In murine experiments, it was not clear how

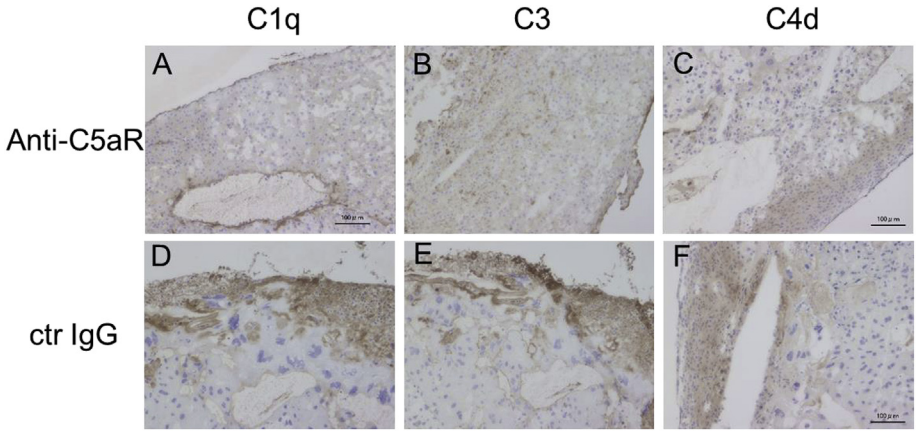


**Fig. 5.** Immunohistochemical findings of placental tissues from mice treated with JL-1 on day 16 of pregnancy. Mice were intravenously pre-treated with anti-C5aR (A-C) or isotype control IgG2b (ctr IgG) (D–F) 30 min before the first treatment with JL-1 on day 8 and placentas were obtained on day 16 of pregnancy. Sections were immunohistochemically stained with C1q, C3 or C4d as expressed brown colour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

much and how circulating anti-C1q monoclonal antibody JL-1 induced pregnancy manifestations compared to those in human, which site of placental tissue JL-1 affected and whether other complement pathways activated or not. JL-1 induced complement activation in the pregnant mice, however it is unclear to what extent immune complexes affected the foetal loss in our murine model. Further investigations are needed to unveil a pathophysiological role of anti-C1q antibodies in RPL.

In conclusion, our study reveals an association between the prevalence and/or titre of anti-C1q antibodies and RPL. Additionally, we have established a novel murine model of antibody-mediated foetal loss using the monoclonal antibody JL-1, which activates the complement system. Our translational research using this murine model has indicated that anti-C1q antibodies can induce reproductive failure, which might be ameliorated by complement regulation therapy targeting the C5-C5aR axis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.04.005.

## Author contributions

The study was conceived and designed by KO, KO, TK and MS. KO and TK contributed to data acquisition and interpretation of the data. KO, MK and YS performed animal experiments. Statistical analysis was performed by KO. KO, KO and TA wrote the manuscript. OA, RH, MK, YS, MK, YF, TB, TH and SY contributed to the critical revision of the article. All authors reviewed and approved the manuscript.

## Disclosure of conflict of interest

The authors declare no conflicts of interests.

## Acknowledgments

This work was supported by grant for The Japan Society for the Promotion of Science, Grant-in-Aid for scientific research (KAKENHI, project number JSPS 16K09885), The Japanese Association for Complement Research, and the SENSHIN Medical Research Foundation.

## References

[1] Practice Committee of the American Society for Reproductive Medicine, Evaluation and treatment of recurrent pregnancy loss: a committee opinion, Fertil. Steril. 98 (2012) 1103–1111.
[2] D.W. Branch, M. Gibson, R.M. Silver, Clinical practice. Recurrent miscarriage, N. Engl. J. Med. 363 (2010) 1740–1747.
[3] C.H. Holmes, K.L. Simpson, S.D. Wainwright, C.G. Tate, J.M. Houlihan, I.H. Sawyer, I.P. Rogers, F.A. Spring, D.J. Anstee, M.J. Tanner, Preferential expression of the complement regulatory protein decay accelerating factor at the fetomaternal interface during human pregnancy, J. Immunol. 144 (1990) 3099–3105.
[4] C.H. Holmes, K.L. Simpson, H. Okada, N. Okada, S.D. Wainwright, D.F. Purcell, J.M. Houlihan, Complement regulatory proteins at the feto-maternal interface during human placental development: distribution of CD59 by comparison with membrane cofactor protein (CD46) and decay accelerating factor (CD55), Eur. J. Immunol. 22 (1992) 1579–1585.
[5] Y.U. Kim, T. Kinoshita, H. Molina, D. Hourcade, T. Seya, L.M. Wagner, V.M. Holers, Mouse complement regulatory protein Crry/p65 uses the specific mechanisms of both human decay-accelerating factor and membrane cofactor protein, J. Exp. Med. 181 (1995) 151–159.
[6] V.R. Aluvihare, M. Kallikourdis, A.G. Betz, Regulatory T cells mediate maternal tolerance to the fetus, Nat. Immunol. 5 (2004) 266–271.
[7] D.A. Clark, M.R. McDermott, M.R. Szewczuk, Impairment of host-versus-graft reaction in pregnant mice. II. Selective suppression of cytotoxic T-cell generation correlates with soluble suppressor activity and with successful allogeneic pregnancy, Cell. Immunol. 52 (1980) 106–118.
[8] M. Petitbarat, P. Durigutto, P. Macor, R. Bulla, A. Palmioli, A. Bernardi, M.G. De Simoni, N. Ledee, G. Chaouat, F. Tedesco, Critical role and therapeutic control of the lectin pathway of complement activation in an abortion-prone mouse mating, J. Immunol. 195 (2015) 5602–5607.
[9] G. Yu, Y. Sun, K. Foerster, J. Manuel, H. Molina, G.A. Levy, R.M. Gorczynski, D.A. Clark, LPS-induced murine abortions require C5 but not C3, and are prevented by upregulating expression of the CD200 tolerance signaling molecule, Am. J. Reprod. Immunol. 60 (2008) 135–140.
[10] V.M. Holers, G. Girardi, L. Mo, J.M. Guthridge, H. Molina, S.S. Pierangeli, R. Espinola, L.E. Xiaowei, D. Mao, C.G. Vialpando, J.E. Salmon, Complement C3 activation is required for antiphospholipid antibody-induced fetal loss, J. Exp. Med. 195 (2002) 211–220.
[11] J.E. Salmon, G. Girardi, V.M. Holers, Activation of complement mediates antiphospholipid antibody-induced pregnancy loss, Lupus. 12 (2003) 535–538.
[12] M.G. Strainic, J. Liu, D. Huang, F. An, P.N. Lalli, N. Muqim, V.S. Shapiro, G.R. Dubyak, P.S. Heeger, M.E. Medof, Locally produced complement fragments C5a and C3a provide both costimulatory and survival signals to naive CD4+ T cells, Immunity. 28 (2008) 425–435.
[13] M.G. Strainic, E.M. Shevach, F. An, F. Lin, M.E. Medof, Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells, Nat. Immunol. 14 (2013) 162–171.
[14] W.H. Kwan, W. van der Touw, E. Paz-Artal, M.O. Li, P.S. Heeger, Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells, J. Exp. Med. 210 (2013) 257–268.
[15] W. van der Touw, P. Cravedi, W.H. Kwan, E. Paz-Artal, M. Merad, P.S. Heeger, Cutting edge: receptors for C3a and C5a modulate stability of alloantigen-reactive induced regulatory T cells, J. Immunol. 190 (2013) 5921–5925.
[16] P. Cravedi, J. Leventhal, P. Lakhani, S.C. Ward, M.J. Donovan, P.S. Heeger, Immune cell-derived C3a and C5a costimulate human T cell alloimmunity, Am. J. Transplant. 13 (2013) 2530–2539.
[17] K. Richani, E. Soto, R. Romero, J. Espinoza, T. Chaiworapongsa, J.K. Nien, S. Edwin, Y.M. Kim, J.S. Hong, M. Mazor, Normal pregnancy is characterized by systemic activation of the complement system, J. Matern. Fetal Neonatal Med. 17 (2005) 239–245.
[18] M.G. Baines, K.G. Millar, P. Mills, Studies of complement levels in normal human pregnancy, Obstet. Gynecol. 43 (1974) 806–810.
[19] W.P. Faulk, R. Jarret, M. Keane, P.M. Johnson, R.J. Boackle, Immunological studies of human placentae: complement components in immature and mature chorionic villi, Clin. Exp. Immunol. 40 (1980) 299–305.
[20] F. Tedesco, O. Radillo, G. Candussi, A. Nazzaro, T.E. Mollnes, D. Pecorari, Immunohistochemical detection of terminal complement complex and S protein in normal and pre-eclamptic placentae, Clin. Exp. Immunol. 80 (1990) 236–240.
[21] K.J. Denny, L.G. Coulthard, R.H. Finnell, L.K. Callaway, S.M. Taylor, T.M. Woodruff, Elevated complement factor C5a in maternal and umbilical cord plasma in preeclampsia, J. Reprod. Immunol. 97 (2013) 211–216.
[22] M. Sugiura-Ogasawara, K. Nozawa, T. Nakanishi, Y. Hattori, Y. Ozaki, Complement as a predictor of further miscarriage in couples with recurrent miscarriages, Hum. Reprod. 21 (2006) 2711–2714.
[23] C. Agostinis, R. Bulla, C. Tripodo, A. Gismondi, H. Stabile, F. Bossi, C. Guarnotta, C. Garlanda, F. De Seta, P. Spessotto, A. Santoni, B. Ghebrehiwet, G. Girardi,

F. Tedesco, An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development, J. Immunol. 185 (2010) 4420–4429.
[24] D.W. Branch, D.J. Dudley, M.D. Mitchell, K.A. Creighton, T.M. Abbott, E.H. Hammond, R.A. Daynes, Immunoglobulin G fractions from patients with antiphospholipid antibodies cause fetal death in BALB/c mice: a model for autoimmune fetal loss, Am. J. Obstet. Gynecol. 163 (1990) 210–216.
[25] G. Girardi, J. Berman, P. Redecha, L. Spruce, J.M. Thurman, D. Kraus, T.J. Hollmann, P. Casali, M.C. Caroll, R.A. Wetsel, J.D. Lambris, V.M. Holers, J.E. Salmon, Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome, J. Clin. Invest. 112 (2003) 1644–1654.
[26] M. Veglia, S. D'Ippolito, R. Marana, F. Di Nicuolo, R. Castellani, V. Bruno, A. Fiorelli, F. Ria, G. Maulucci, M. De Spirito, G. Migliara, G. Scambia, N. Di Simone, Human IgG antinuclear antibodies induce pregnancy loss in mice by increasing immune complex deposition in placental tissue: in vivo study, Am. J. Reprod. Immunol. 74 (2015) 542–552.
[27] I.V. Menzhinskaya, L.V. Van'ko, M.M. Kashentseva, P.A. Kiryushchenkov, G.T. Sukhikh, Incidence of autoantibodies to C1Q complement component in women with miscarriages and autoantibodies to phospholipids and chorionic gonadotropin, Bull. Exp. Biol. Med. 160 (2015) 260–263.
[28] F.J. Beurskens, R.A. van Schaarenburg, L.A. Trouw, C1q, antibodies and anti-C1q autoantibodies, Mol. Immunol. 68 (2015) 6–13.
[29] J.J. Wisnieski, S.M. Jones, Comparison of autoantibodies to the collagen-like region of C1q in hypocomplementemic urticarial vasculitis syndrome and systemic lupus erythematosus, J. Immunol. 148 (1992) 1396–1403.
[30] L.A. Trouw, J.M. Duijs, C. van Kooten, M.R. Daha, Immune deposition of C1q and anti-C1q antibodies in the kidney is dependent on the presence of glomerular IgG, Mol. Immunol. 40 (2003) 595–602.
[31] C. Bigler, M. Schaller, I. Perahud, M. Osthoff, M. Trendelenburg, Autoantibodies against complement C1q specifically target C1q bound on early apoptotic cells, J. Immunol. 183 (2009) 3512–3521.
[32] C.G. Kallenberg, Anti-C1q autoantibodies, Autoimmun. Rev. 7 (2008) 612–615.
[33] S. Miyakis, M.D. Lockshin, T. Atsumi, D.W. Branch, R.L. Brey, R. Cervera, R.H. Derksen, D.E.G. PG, T. Koike, P.L. Meroni, G. Reber, Y. Shoenfeld, A. Tincani, P.G. Vlachoyiannopoulos, S.A. Krilis, International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS), J. Thromb. Haemost. 4 (2006) 295–306.
[34] D. Aletaha, T. Neogi, A.J. Silman, J. Funovits, D.T. Felson, C.O. Bingham III, N.S. Birnbaum, G.R. Burmester, V.P. Bykerk, M.D. Cohen, B. Combe, K.H. Costenbader, M. Dougados, P. Emery, G. Ferraccioli, J.M. Hazes, K. Hobbs, T.W. Huizinga, A. Kavanaugh, J. Kay, T.K. Kvien, T. Laing, P. Mease, H.A. Menard, L.W. Moreland, R.L. Naden, T. Pincus, J.S. Smolen, E. Stanislawska-Biernat, D. Symmons, P.P. Tak, K.S. Upchurch, J. Vencovsky, F. Wolfe, G. Hawker, 2010 rheumatoid arthritis classification criteria: an American College of Rheumatology/European league against rheumatism collaborative initiative, Arthritis Rheum. 62 (2010) 2569–2581.
[35] F. van den Hoogen, D. Khanna, J. Fransen, S.R. Johnson, M. Baron, A. Tyndall, M. Matucci-Cerinic, R.P. Naden, T.A. Medsger Jr., P.E. Carreira, G. Riemekasten, P.J. Clements, C.P. Denton, O. Distler, Y. Allanore, D.E. Furst, A. Gabrielli, M.D. Mayes, J.M. van Laar, J.R. Seibold, L. Czirjak, V.D. Steen, M. Inanc, O. Kowal-Bielecka, U. Muller-Ladner, G. Valentini, D.J. Veale, M.C. Vonk, U.A. Walker, L. Chung, D.H. Collier, M.E. Csuka, B.J. Fessler, S. Guiducci, A. Herrick, V.M. Hsu, S. Jimenez, B. Kahaleh, P.A. Merkel, S. Sierakowski, R.M. Silver, R.W. Simms, J. Varga, J.E. Pope, 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European league against rheumatism collaborative initiative, Arthritis Rheum. 65 (2013) 2737–2747.
[36] A. Bohan, J.B. Peter, Polymyositis and dermatomyositis (first of two parts), N. Engl. J. Med. 292 (1975) 344–347.
[37] C.H. Shiboski, S.C. Shiboski, R. Seror, L.A. Criswell, M. Labetoulle, T.M. Lietman, A. Rasmussen, H. Scofield, C. Vitali, S.J. Bowman, X. Mariette, G. International Sjogren's Syndrome Criteria Working, 2016 American College of Rheumatology/European league against rheumatism classification criteria for primary Sjogren's syndrome: a consensus and data-driven methodology involving three international patient cohorts, Ann. Rheum. Dis. 76 (2017) 9–16.
[38] C. Siegert, M. Daha, M.L. Westedt, E. van der Voort, F. Breedveld, IgG autoantibodies against C1q are correlated with nephritis, hypocomplementemia, and dsDNA antibodies in systemic lupus erythematosus, J. Rheumatol. 18 (1991) 230–234.
[39] A. Matrat, C. Veysseyre-Balter, P. Trolliet, E. Villar, F. Dijoud, J. Bienvenu, N. Fabien, Simultaneous detection of anti-C1q and anti-double stranded DNA autoantibodies in lupus nephritis: predictive value for renal flares, Lupus 20 (2011) 28–34.
[40] O.C. Meyer, P. Nicaise-Roland, N. Cadoudal, S. Grootenboer-Mignot, E. Palazzo, G. Hayem, P. Dieude, S. Chollet-Martin, Anti-C1q antibodies antedate patent active glomerulonephritis in patients with systemic lupus erythematosus, Arthritis Res. Ther. 11 (2009) R87.
[41] D. Sinha, M. Wells, W.P. Faulk, Immunological studies of human placentae: complement components in pre-eclamptic chorionic villi, Clin. Exp. Immunol. 56 (1984) 175–184.
[42] K. Oku, O. Amengual, R. Hisada, K. Ohmura, I. Nakagawa, T. Watanabe, T. Bohgaki, T. Horita, S. Yasuda, T. Atsumi, Autoantibodies against a complement component 1 q subcomponent contribute to complement activation and recurrent thrombosis/pregnancy morbidity in anti-phospholipid syndrome, Rheumatology (Oxford) 55 (2016) 1403–1411.
[43] G. Girardi, P. Redecha, J.E. Salmon, Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation, Nat. Med. 10 (2004) 1222–1226.
[44] J.M. Thurman, D.M. Kraus, G. Girardi, D. Hourcade, H.J. Kang, P.A. Royer, L.M. Mitchell, P.C. Giclas, J. Salmon, G. Gilkeson, V.M. Holers, A novel inhibitor of the alternative complement pathway prevents antiphospholipid antibody-induced pregnancy loss in mice, Mol. Immunol. 42 (2005) 87–97.
[45] D. Cohen, A. Buurma, N.N. Goemaere, G. Girardi, S. le Cessie, S. Scherjon, K.W. Bloemenkamp, E. de Heer, J.A. Bruijn, I.M. Bajema, Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss, J. Pathol. 225 (2011) 502–511.
[46] T. Meuleman, D. Cohen, G.M. Swings, K. Veraar, F.H. Claas, K.W. Bloemenkamp, Increased complement C4d deposition at the maternal-fetal interface in unexplained recurrent miscarriage, J. Reprod. Immunol. 113 (2016) 54–60.
[47] J. Berman, G. Girardi, J.E. Salmon, TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss, J. Immunol. 174 (2005) 485–490.