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CLASSICAL COMPLEMENT ACTIVATION AS A FOOTPRINT FOR MURINE AND HUMAN ANTIPHOSPHOLIPID ANTIBODY-INDUCED FETAL LOSS

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Abstract

INTRODUCTION Recurrent miscarriage, fetal growth restriction and intrauterine fetal death are frequently occurring complications of pregnancy in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS). Murine models show that complement activation plays a pivotal role in antiphospholipid antibodymediated pregnancy morbidity. However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. Given the antibody-mediated nature of SLE and APS in which pregnancy losses are pertinent, we hypothesized that the classical pathway would play a major role in inducing fetal loss.

METHODS To gain more insight into the contribution of different complement pathways to fetal outcome, pregnant C57BL/6 mice and mice deficient in C1q and factor D were injected with antiphospholipid antibodies or normal human IgG. Mice-placentas were subsequently stained with an anti-C4 antibody and anti-normal human IgG to determine presence of classical complement activation and IgG binding. Findings in mice were validated in 88 human placentas from 83 women (SLE and APS cases versus controls), which were immunohistochemically stained for C4d, C1q, properdin and MBL. Staining patterns were compared to pregnancy outcome.

RESULTS In murine placentas of mice pre-treated with antiphospholipid antibodies, increased C4 deposition was observed, which was associated with adverse fetal outcome but not with IgG binding. In humans, diffuse C4d staining at the fetomaternal interface was present almost exclusively in patients with SLE and/or APS ($p < 0.001$) and was related to intrauterine fetal death ($p = 0.03$).

CONCLUSION Our data show that presence of C4d in murine and human placentas is strongly related to adverse fetal outcome in the setting of SLE and APS. The excessive deposition of C4d supports the concept of severe autoantibody-mediated injury at the fetomaternal interface. We suggest C4d as a potential biomarker of autoantibody-mediated fetal loss in SLE and APS.

Introduction

Recurrent miscarriage, fetal growth restriction and intrauterine fetal death (IUFD) are devastating complications of pregnancy that occur 20 to 40 times more often in patients with systemic lupus erythematosus (SLE) and antiphospholipid syndrome (APS) than in healthy pregnant women.[1,2] Presence of circulating antiphospholipid antibodies (aPL) which is a prerequisite for APS and occurs in 40% of SLE patients, is strongly associated with thrombosis and fetal loss.[3]

The increased risk of thrombosis in the presence of aPL suggests that thrombosis of the uteroplacental vasculature could be an important cause of pregnancy morbidity. However, thrombotic lesions are not significant and even not always detectable in placentas of patients with aPL-mediated fetal loss.[4] Recent studies in complement deficient murine models [5-8] suggested that complement activation plays a pivotal role in aPL-mediated fetal loss, shifting the focus more towards inflammation as the primary causative mechanism.[9;10] However, the exact pathways of complement activation and their potential role in human pregnancy are insufficiently understood. We hypothesized that classical complement activation is the main responsible pathway in SLE and APS related pregnancy morbidity, based on an antibody-mediated allo-response at the fetomaternal interface. We therefore investigated the role of classical, alternative and mannose binding lectin (MBL) pathway activation in a murine model of aPL-mediated fetal injury and in human placentas of pregnancies affected by SLE and APS. As a marker for classical pathway activation we used C4d, a marker for classical complement activation.

In transplantation pathology, C4d is widely used as a biomarker to diagnose antibody-mediated allograft rejection.[11] As a degradation product of C4, one of the main components of the classical complement cascade, C4d has the ability to bind covalently to cell surfaces and basement membranes near sites of C4 activation. Covalently bound C4d is anchored to the tissue, and remains attached much longer than the antibodies that originally activated the classical pathway. This makes C4d a highly stable marker, and has led to C4d being referred to as 'a footprint' of antibody-mediated tissue injury.[12]

In a mouse model of aPL-mediated fetal loss we first studied murine placentas with an anti-C4 antibody to determine presence of classical complement activation. Observations in mice were validated by investigating the presence C4d deposits in placentas of patients with SLE and/or APS in relation to pregnancy outcome, compared to healthy and disease controls. In additional studies we confirmed that placental C4d depositions reflect specific involvement of classical pathway activation, and are not derived from lectin or alternative pathway activation.

Materials and methods

MOUSE MODEL OF ANTIPHOSPHOLIPID ANTIBODY-INDUCED FETAL LOSS Adult mice (6-8 weeks), C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA) and mice deficient in C1q and factor D (C1qfDKO) [13] (generously provided by Greg Stahl, Harvard University) were used in all experiments.

On days 8 and 12 of pregnancy, females were treated with I.P. injections of human IgG-containing aPL (aPL-IgG) (10 mg) or normal human IgG (NH-IgG) (10 mg) as previously described.[5;14] APL-IgG were obtained from patients with APS [characterized by high titer aPL antibodies (▸140 GPL units), NH-IgG was obtained from healthy non-autoimmune individuals.

Mice (C57BL/6 and C1qfDKO) were sacrificed on day 8, 2h after aPL-IgG injection (cases, n=5) or NH-IgG injection (controls, n=5), uteri were dissected and deciduas and placentas were harvested. Another group of cases (n=5) and controls (n=5) were euthanized on day 15 of pregnancy to harvest placentas. Resorption sites that result from loss of a previously viable embryo were counted as previously described.[5;14]

For immunohistochemical studies, deciduas from day 8 and placentas from day 15 of pregnancy were fixed in paraformaldehyde 4%, frozen in O.C.T. compound, and cut into 10 m sections. Sections

were stained for C4d with a rat monoclonal anti-C4 antibody (C4 antibody [16D2] (ab11863) Abcam, Cambridge, MA, USA) at a dilution 1/50. This antibody reacts with murine C4, C4b and C4d**.** As a positive control we used a kidney of a mouse injected with FB1 (mouse monoclonal-aPL that activates complement). As a positive control we used a kidney that belonged to a mouse injected with FB1 (mouse monoclonal antiphospholipid antibody from M. Monastier that activates complement). This antibody (specificity, isolation, etc) was described in studies in pregnancy and renal thrombotic microangiopathy. The control mouse received mouse IgG.

An HRP-labeled secondary antibody and DAB as substrate or a FITC-labeled secondary antibody were used to develop the reaction. For fluorescence staining gold antifade reagent with DAPI (Invitrogen, Carisbad, CA, USA) was used.

Day 15 placentas were also stained with anti-human IgG (Sigma-Aldrich, St Louis, MO) at a dilution 1/150.

All animal studies were approved by the institutional Animal Care and Use Committee of the Hospital for Special Surgery or Weill Medical College of Cornell University and York College, City University of New York, New York.

PATIENTS AND PLACENTAS We studied 88 placentas from 83 women who delivered at the Obstetrical Department of the Leiden University Medical Centre (LUMC) between 1995 and 2009. Women were subdivided into three groups: The case-group consisted of 21 patients with a confirmed diagnosis of SLE and/or APS, from which we selected all available placentas (26 placentas of 21 patients). Multiple placentas were available from 4 patients in the case group. For statistical analysis, only the placenta of the first pregnancy was taken into account. All patients in the case group were tested for antiphospholipid antibodies (lupus anticoagulant and anticardiolipin antibodies) according to the most recent guidelines.[15;16]

For a first control group (live-birth controls) we included 40 placentas of pregnancies that resulted in live births in the same period as above. These placentas represented pregnancies ranging from completely normal to relatively complicated, including both maternal and fetal morbidity. None of the patients had preeclampsia or Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome.

For a second control group (IUFD-controls) we included 22 placentas of pregnancies that resulted in IUFD by various causes other than SLE or APS. Fetal losses in this group were mainly caused by fetal chromosomal abnormalities.

An overview of the clinical characteristics of all patients, derived from the case-records of the LUMC, is given in table 1.

All tissue samples were handled in a coded fashion, according to Dutch national ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies).

ROUTINE HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY Placentas were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections were routinely stained with HE. To study complement activation, immunohistochemical staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria), C1q (Dako Cytomation, Denmark), MBL (Sigma-Aldrich Biotechnology) and properdin (primary antibody kindly provided by the department Nephrology, Leiden, the Netherlands). Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections.

C4D Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin.

A tissue sample from a renal biopsy of a patient with humoral rejection with C4d-positive staining served as a positive control.

C1q Endogenous peroxidase activity was blocked. Antigen retrieval was performed in EDTA-TRIS (pH 9.0). A polyclonal rabbit anti-human polyclonal C1q antibody (Dako Cytomation, Denmark) was applied at a dilution of 1:700 in 1%BSA/PBS, and slides were incubated for one hour at 37 C. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Tonsil tissue served as a positive control.

MBL Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). A polyclonal rabbit anti-human MBL antibody (Sigma-Aldrich Biotechnology) was applied at a dilution of 1:250 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. Liver tissue served as a positive control.

PROPERDIN Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0). The slides were subsequently blocked with 5% heat-inactivated normal human serum in 1% bovine serum albumin/phosphate-buffered saline (BSA/PBS) for 45 min at room temperature. A polyclonal rabbit anti-human properdin antibody (kindly provided by the department Nephrology, Leiden, the Netherlands) was applied at a dilution of 1:800 in 1%BSA/PBS overnight. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin. A kidney with membranous nephropathy was used as a positive control.

QUANTIFICATION OF PLACENTAL MORPHOLOGY AND IMMUNOHISTOPATHOLOGY Sections were evaluated by two experienced pathologists who scored the slides blinded to the patients' clinical data. Differences in scorings were resolved by re-reviewing the sections and coming to consensus. Each placenta was scored separately for the presence or absence of histopathological changes associated with decreased uteroplacental perfusion: Acute and/or chronic deciduitis, decidual necrosis, increased syncytial knots, accelerated villous maturity, accellerated maturity, avascular villi, villous infarcts, retroplacental hematomas, intervillous thrombi and decidual vasculopathy.[17;18]

Positivity for immunohistochemical stainings was scored semi-quantitatively. A random area of 1 x 1 cm was selected from each tissue sample for scoring. Staining intensity around syncytiotrophoblast was scored as 0, 1, or 2, with 0 representing the total absence of staining, 1 representing focal positive staining, and 2 representing diffuse staining of all syncytiotrophoblast cell- and basement membranes within this area. Intravillous endothelial staining was scored on a 0, 1, 2 scale, with similar definitions as above.

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test. Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or Kruskal Wallis H one-way analysis (for data not normally distributed). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A p-value less than 0.05 was considered statistically significant.

Results

MOUSE MODEL OF APL-IGG TREATED MICE

C4 DEPOSITION IN APL-IGG TREATED MICE VERSUS CONTROLS

Placentas from the surviving fetuses in aPL -treated mice showed increased C4 deposition in the labyrinth (Figure 1BII, III, IV) when

compared to placentas from NH-IgG treated mice (Figure 1AII, III, IV). The labyrinth is the area of active feto-maternal exchange in the murine placenta, equivalent to chorionic villi in humans.

Specifically, C4 deposition was observed on the trophoblast giant cells (TGC) (Figure 1Bi). These cells are crucial for implantation and invasion of the conceptus into maternal decidua of the uterus. Abnormalities in these cells can cause placental defects and compromised pregnancies [19;20] and can explain the high resorption frequency observed in embryos from aPL-treated mice.[5;14]

C4 deposition in C1q- and factor D-deficient mice

To rule out that C4d positivity was caused by lectin pathway activation we studied pregnancy outcomes in mice that are deficient for complement component C1q and factor D (Figure 1C). Mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies (Figure 1C). No C4 deposition was observed, neither in deciduas nor in placentas from C1qfDKO mice treated with aPL (data not shown).

IgG deposition To study if complement deposition coincidences with aPL-binding in aPL-treated mice with increased fetal loss, we stained for human IgG in day 15 placentas from surviving fetuses. In contrast with the robust C4a deposition observed in these placentas, no IgG staining was found.

Clinical data Patients in all groups were of similar age. In the case group of patients with SLE and/or APS, 15 patients met the American College of Rheumatology criteria for SLE[21], 12 patients met the classification criteria for APS[22], and 6 patients had both SLE and APS (see table 1).

None of the patients in the live-born control group had been tested for aPL. In the IUFD-group all patients with an IUFD of unknown etiology were tested for aPL. None of the patients met the laboratory criteria for APS. Other patient and pregnancy characteristics are shown in table 1.

Histopathological findings in cases versus controls

Retroplacental hematomas and intervillous thrombi were infrequent and not increased either in placentas from patients with SLE and/or APS, compared to both control groups. Villous infarction and accelerated maturity occurred more often in placentas from patients with SLE/APS than in both control groups. Figure 2 shows the incidence of various histopathological findings in all placentas.

Detection of complement components and their association with clinical parameters

C4d When present, C4d showed positivity at the fetomaternal interface, on the maternal side of the syncytiotrophoblast, either in a focal or a diffuse staining pattern. Typical examples of C4d staining patterns are shown in figure 3A-C. A diffuse C4d staining pattern in the placenta was strongly associated with SLE and/or APS ($p < 0.001$) (figure 3M). Within the case group, diffuse C4d staining was associated with IUFD ($p < 0.03$) (figure 3N). Detailed information on C4d staining in the cases versus controls is shown in table 2.

Diffuse C4d staining around all syncytiotrophoblast cell- and basement membranes (figure 3C) was found in 12 placentas: Ten were from patients with SLE and/or APS, of whom 5 had a pregnancy resulting in IUFD, 4 pregnancies were characterized by severe intrauterine growth retardation and/or preeclampsia and one of the patients had an uncomplicated pregnancy, which was her first live born child after 4 late miscarriages. Two of the 12 placentas that were diffusely positive for C4d were from patients from the control group with IUFD: Both patients had an unexplained IUFD. One patient was negative for aPL, the other appeared to have circulating aPL (anticardiolipin IgG antibodies) when she was tested three months after delivery. A diffuse or focal positive C4d staining pattern was never found in the control group with live-born children ($p<0.001$).

Detailed information on focal and absent C4d staining patterns is given in table 2.

C1q C1q was present in both intravillous endothelial cells and around the syncytiotrophoblast cells (Figure 3D-F), and was never completely negative, neither in cases nor controls (data not shown). In cases of diffuse C4d deposition around syncytiotrophoblast cells, C1q clearly co-localized with C4d depositions.

PROPERDIN Properdin was almost uniquely positive in intravillous endothelial cells, and showed minor positivity around syncytiotrophoblast cells in only 2 cases of live birth controls. It did not co-localize with C4d deposition (figure 3G-I). Diffuse properdin staining occurred almost at a similar rate in live born placentas and in placentas in the SLE/APS group (33% and 28% respectively). Properdin positivity was negatively associated with IUFD: In only 2 out of 22 IUFD cases a diffuse staining pattern was observed.

MBL In all cases MBL deposition was absent (figure 3J-L), whereas liver tissue that was used as a positive control was evidently positive. MBL also did not occur in C4d positive placentas, or in placentas of patients with prolonged rupture of membranes.

PATIENTS OF WHICH MULTIPLE PLACENTAS OF SUBSEQUENT PREGNANCIES WERE AVAILABLE Multiple placentas were available from 4 patients in the case group (see table 3)

The first patient was diagnosed with SLE and had four subsequent pregnancies, of which the first ended in an early miscarriage (no tissue available), the second and third ended in intrauterine fetal loss and the fourth was a live birth. For the first fetal loss an explanation or cause was never found, the second fetus died because of congenital heartblock in the presence of maternal anti-SSA and anti-SSB antibodies. In her fourth pregnancy she gave birth to a healthy child. This patient did not have antiphospholipid antibodies at any point in time and her placentas were negative for C4d every time. The next patient had SLE and secondary antiphospholipid syndrome. She had three pregnancies of which two placentas were available. All three pregnancies ended in live births, but with severely growth restricted children and severe maternal preeclampsia during the first and second pregnancy. In the third pregnancy she was treated with aspirin and heparin. Her first placenta was not available, she had diffuse C4d staining in the second, and focal C4d staining in the third placenta. The last two patients had primary antiphospholipid syndrome. One patient had three pregnancies of which two (the first and third pregnancy) ended in intrauterine fetal death, accompanied by severe growth retardation and maternal HELLP syndrome. Both placentas were diffusely positive for C4d. Her second pregnancy, of which no placenta was available, ended in a live birth but with maternal HELLP syndrome complicated by liver infarction. The other patient also had three pregnancies, all of which ended in intrauterine fetal death accompanied by severe fetal growth retardation. Only the last two placentas were available, which were both diffusely positive for C4d.

Discussion

APS and SLE are autoantibody mediated autoimmune diseases in which severe pregnancy morbidity is pertinent. The present study demonstrated that C4d deposits were present in areas of active fetomaternal exchange in the murine placenta of mice pre-treated with human aPL-IgG, and absent in those treated with NH-IgG. C4d deposits in murine placentas were associated with an increased fetal absorption rate (figure 1). Observations in mice were validated in human placentas, where placental C4d deposition was frequently and almost exclusively present around syncytiotrophoblast cells of placentas from patients with SLE and/or APS. Its presence in human placentas was strongly related to adverse pregnancy outcome in SLE and APS patients. In patients without SLE or APS but with IUFDs diffuse C4d staining was rare. C4d staining was always negative in placentas from patients with live births (figure 3, table 2). Our data

support the concept of a severe autoantibody-mediated immune response at the fetomaternal interface, leading to impaired fetal outcome.

To rule out that C4d deposition is a reflection of lectin pathway activation we studied the deposition patterns of MBL. In the mice-experiments, mice deficient in the classical pathway component C1q and the alternative pathway component factor D were protected from aPL-induced fetal injury. No C4 deposition was observed in placentas from these mice. MBL was not detectable in human placentas, and did not co-localize with C4d. These observations indicate that it is very unlikely that the lectin pathway is involved in the pathogenesis of fetal losses induced by aPL.

Direct exposure of trophoblast to the maternal blood puts these cells at risk of being attacked by complement activation products, both in the setting of maternal autoimmune disease as in normal pregnancies. Our data indicate that deposition of early classical pathway component C1q is most likely a non-pathological phenomenon. The observed presence of C1q in placentas of controls could reflect the physiological presence of maternal IgG and IgM. It is not surprising that this usually does not lead to full blown activation of the complement cascade, because of the many complement regulatory mechanisms normally present at the level of the syncytiotrophoblast.[6] Alternatively, it was previously shown that decidual endothelial cells can synthesize C1q and express it on their surface in physiological situations. [23;24] Bulla *et al* showed that deposition of C1q in the placenta was not necessarily associated with the presence of IgG, IgM or C4.[23] The clear association of C4d (a complement split-product more downstream than C1q) with SLE and APS related pregnancy morbidity as presented in this study, may be regarded as evidence of a severe antibody-mediated allo-response, where the complement inhibitory mechanisms are surpassed and fail to suppress the evolving events in the complement cascade.

Evidence from mouse-models suggested that a large part of the aPL-mediated placental damage is caused by amplification of the classical pathway by the alternative pathway.[25] However, we found properdin, a membrane bound marker of alternative pathway activation, in placentas of both patients with SLE and APS as well as in live-birth controls, making no distinction between a good or bad pregnancy outcome. Interestingly, properdin depositions are found within the vessels of the villi, and do not co-localize with C4d deposits at the maternal side of the syncytiotrophoblast. This means that properdin must originate from the fetal complement system[26], and may reflect a general state of fetal distress, rather than being directly related to the C4d depositions.

Although the clinical implications of aPL are well known, testing for their presence remains difficult and only highly specialized laboratories can provide reliable test results.[27;28] C4d stainings are performed in most clinical pathology laboratories and a diffuse C4d staining pattern in the placenta is easy to recognize (figure 3C). We therefore propose that placental C4d staining could be a useful additional tool to further strengthen the diagnosis of aPl-mediated fetal loss, for instance in the work-up of a first late miscarriage or fetal death. This might even have implications for treatment with heparin, as it was shown in animal models that heparin prevents aPL-induced fetal loss by inhibiting complement activation.[29] Alternatively, it would be interesting to explore whether targeted inhibition of complement activation would be beneficial for pregnancy outcome, similar to the findings in mice models.[5;7]

The analogy between pregnancy and transplantation was made as early as 1953, when Peter Medawar introduced the concept of 'the fetal allograft'.[30] In transplantation, humoral allograft rejection has gained much attention since the discovery of C4d.[11] Our data demonstrate that complicated pregnancies of patients with autoimmune diseases such as APS, share several pathofysiological aspects with humoral rejection. Both aPL and donor-specific antibodies to donor-HLA bind at the frontier where cells from the one individual (mother or host) meet the other (fetus or graft).

Interestingly, both in pregnancy and in transplantation we find no histological evidence for the binding of the antibodies themselves, whereas C4d remains attached and is easily detectable

both in mice placentas at day 8 and 15, and in human placentas of various gestational age. In day 15 placentas of aPL-treated mice, we did not find NH-IgG deposition, whereas it was previously published that NH-IgG can be detected in day 8 placentas of similarly treated mice.[31] This shows that these antibodies do bind at the fetomaternal interface initially, but do not remain attached longer than a few days, whereas C4d is detectable throughout the whole pregnancy. From the transplant setting, it is known that this phenomenon can be explained by the relatively weak binding capacity of antibodies, compared to the covalent binding of C4d which anchors to the damaged tissue.[11] In this study a parallel between the humoral rejection and pregnancy is now found in the form of C4d deposition, indicating that C4d might be a more reliable and long lasting marker of placental antibody-mediated tissue injury.

In human placentas, we demonstrated that 62% of patients with SLE/APS have a focal or a diffuse C4d staining pattern. However 38% of these patients do not show any deposition of C4d, while their pregnancy outcomes are also impaired, either by growth restriction, preeclampsia or even fetal death. There are two important explanations for this situation. Firstly, as we take only a small part of the placenta for analysis, C4d positivity present in a focal, patchy pattern through the whole placenta can be missed due to sampling error. In future studies, the effect of more extensive tissue sampling could be investigated. Secondly, it is evident that in any pregnancy, many more than C4d-related causes can lead to IUFD or impaired pregnancy outcome. For instance, anti-SSA or anti-SSB antibodies can cause a congenital heart block and subsequently, fetal death, by travelling through the placenta into the fetal circulation. This is a totally different mechanism for SLE-related IUFD in which no trophoblastic C4d deposition is expected. Furthermore, there is always a chance for fetal chromosomal abnormalities, and in such cases no diffuse C4d deposition is expected as we have illustrated in our IUFD control group.

In conclusion, we have shown that classical complement activation plays a major role in aPL- mediated fetal injury, and that placental C4d deposition is a reflection of classical complement activation.
C4d is strongly associated to impaired fetal outcome, both in a mouse model of aPL- mediated fetal loss and in human pregnancy affected by SLE and APS. Especially in women with a first pregnancy resulting in IUFD, placental C4d staining has potential as a diagnostical tool to detect aPL-mediated fetal loss. Further prospective studies need to confirm if C4d positivity in a previous IUFD or late miscarriage can be considered as a biomarker of a future complicated pregnancy.

FIG 1A-B INCREASED C4 DEPOSITION IN APL-IGG TREATED MICE VERSUS NH-IGG CONTROLS

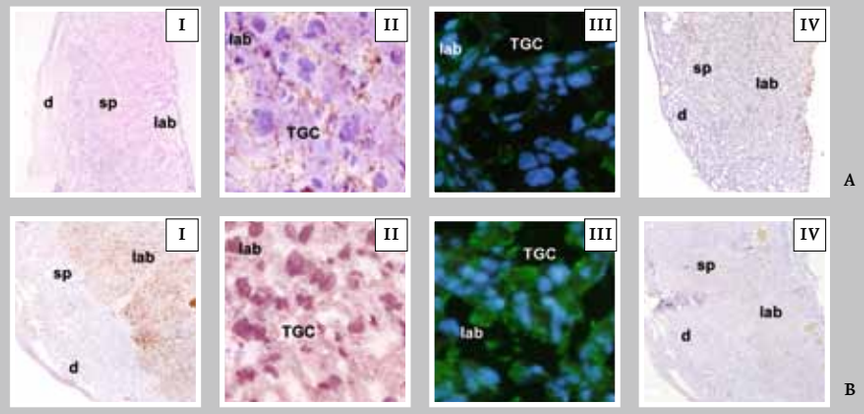


Figure 1BI, II, and III show increased C4 deposition in the labyrinth (lab) compared to figure 1AI, I, and III. The labyrinth is the area of active feto-maternal exchange in the murine placenta. The equivalent to this area in humans is the chorionic villi. C4 deposition was observed on the trophoblast giant cells (TGC) of aPL-IgG treated mice (Figure 1BII and III). In contrast, minimal C4d staining was found in placentas from NH-IgG-treated mice (Figure 1AI, II, III).

FIG 1C

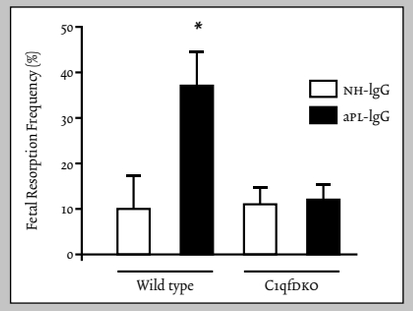
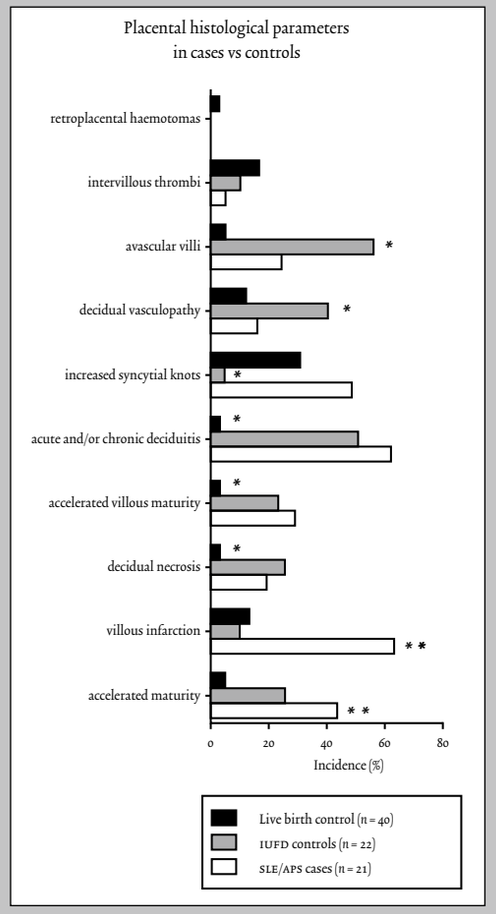


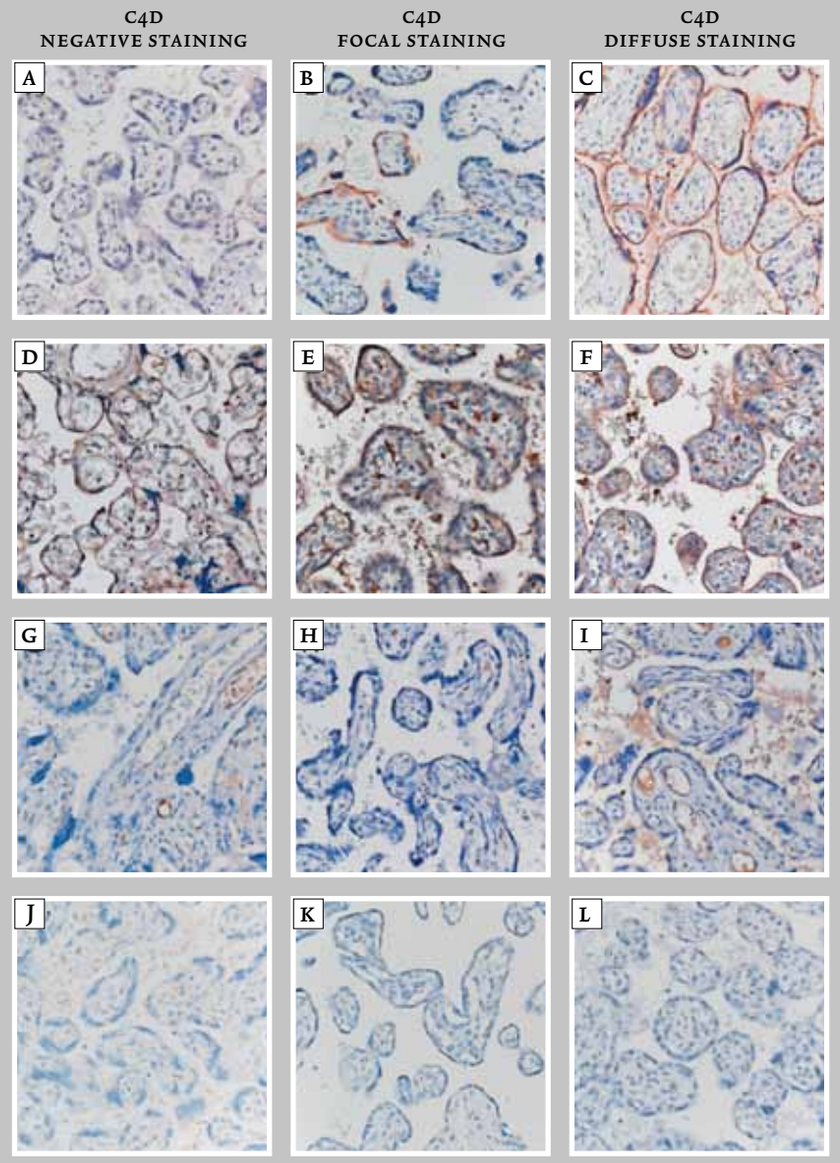
Figure 1C shows fetal resorption frequency in C1q- and factor D-deficient mice. Mice deficient in the classical pathway component C1q and the alternative pathway component factor D are protected from aPL-induced fetal injury. The fetal resorption frequency in these mice was not different from that calculated in NH-IgG treated mice with uneventful pregnancies. No C4 deposition was observed neither in deciduas nor in placentas from C1qfDKO mice treated with antiphospholipid antibodies (data not shown).

FIG 2 BAR GRAPH OF SPECIFIC PLACENTAL HISTOLOGICAL SCORES AS DESCRIBED IN THE RESULT SECTION



This figure gives an indication of the abundance of various lesions in different groups. The differences between groups is represented with (*) indicating a p-value of ◂ 0.05 and (**) indicating a p-value of ◂ 0.01.

FIG 3 **IMMUNOHISTOCHEMICAL STAINING PATTERNS IN HUMAN PLACENTAS**



Panels 3A-L: typical examples of immunohistochemical staining patterns of C4d, C1q, Properdin and MBL of placentas. Vertically the panels are organized in such a way that each column represents the same placenta. Horizontally the different immunohistochemical stainings are shown. The first column represents a C4d negative placenta of a patient with a live birth. The middle column represents a placenta of a patient with an IUFD, with a focal C4d staining pattern. In the third column a placenta of a patient with SLE and secondary APS is shown which is diffusely positive for C4d. The pregnancy was accompanied by severe maternal preeclampsia and severe fetal growth retardation.

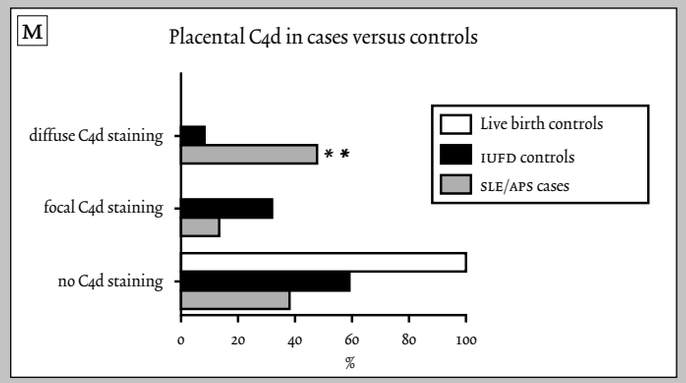
Panels 3A-C: Typical examples of different C4d staining intensities of villous syncytiotrophoblast cell and basement membranes observed in patients with SLE and/or APS compared to controls. Panels demonstrate the different staining intensities by which the placentas were scored: (A) 'no placental C4d staining', (B) 'focal placental C4d staining', and (C) 'diffuse placental C4d staining'. In (C) it is clearly shown that C4d depositions are found on the maternal side of the placental syncytiotrophoblast, and not within the fetal vasculature.

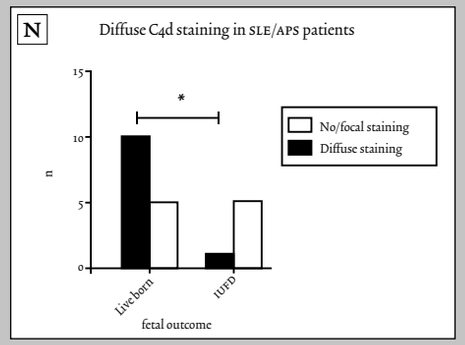
Panels 3D-F: Typical examples of placental C1q staining. Interestingly, C1q was not unique for C4d positive placentas, but was observed frequently in non-C4d positive placentas too. This phenomenon is illustrated in figure (D), where a C4d negative placenta is evidently C1q positive. However, panel (F) shows that C1q does co-localization with C4d in a C4d positive placenta, confirming that C4d originates from classical pathway activity.

Panels 3G-I: Typical examples of properdin staining, in which properdin deposits on endothelial cells of the fetal vessels. Properdin deposition is not corresponding with the sites of C4d deposition, suggesting that classical complement activation does not necessarily lead to properdin deposition – i.e. alternative pathway activation.

Panels J-L: In all cases MBL deposition was absent, further confirming that the MBL pathway does not contribute to the deposition of C4d in the setting of SLE and aPL-mediated fetal loss.

FIG 3





M: Shows the association of diffuse C4d staining with IUFD in SLE/APS cases. The differences between groups is represented with (*) indicating a p-value of ‹0.05 and (**) indicating p-value of ‹ 0.01.

N: Bar graph of C4d staining in SLE/APS cases versus control groups. Figure 3 gives an indication of the abundance of diffuse C4d staining in the SLE/APS case group and the striking absence of diffuse C4d in the live birth controls.

TABLE 1 PATIENT CHARACTERISTICS

	SLE/APS* CASES (N=21)	IUFD** CONTROLS (N=22)	LIVE BIRTH CONTROLS (N=40)
Mean maternal age in years (SD)	29,7(4)	30,5 (6)	31,5 (6)
Mean gravidity (SD)	2,3 (1,5)	2,6 (1,6)	2,2 (0,87)
Mean parity (SD)	0,6 (1,1)	1,3 (1,4)	0,8 (0,8)
Previous live birth (%)	5 (24)	11 (50)	15 (38)
Previous miscarriage or fetal loss (%)	8 (38)	6 (27)	14 (35)
Gestational age at delivery (wk + day) (SD in days)	32 + 6 (4)	25 + 3 (8)	37 + 1 (7)
Delivery at ‹ 24 wk (%)	4 (19)	14 (64)	0
Delivery at 24-38 wk (%)	17 (81)	6 (27)	20 (50)
Delivery at 38-42 (%)	5 (24)	2 (9)	20 (50)
Intrauterine fetal death (%)	6(29)	22(100)	0
Fetal distress (%)	6(29)	0	11(27)
Mean birth weight (grams) (SD)	1639,3 (1060)	901,3 (1203)	2534,5 (981)
Placental weight (grams) (SD)	306,8 (173)	230,2 (205)	472,9 (139)
Heparin therapy during pregnancy (%)	8 (38)	0	0
Comorbidity			
SLE (%)	15 (71)	0	0
SLE and APS (seconday APS) (%)	6 (28)	0	0
APS, no SLE (primary APS) (%)	6 (28)	0	0
Lupus anticoagulant (%)	8 (38)	0	0
Anticardiolipin antibodies, IgG (%)	10 (48)	0	0
Preeclampsia (%)	5 (24)	0	0
HELLP syndrome (%)	2 (10)	0	0
Normal pregnancy and delivery (%)	2 (10)	0	14 (35)
Unexplained intrauterine fetal death (%)	0	5 (23)	0
Infant congenital abnormalities (%)	0	12 (55)	3 (8)

* Systemic Lupus Erythematosus and antiphospholipid syndrome

** Intrauterine fetal death

TABLE 2 C4D AND PROPERDIN IN SLE AND APS PLACENTAS VERSUS CONTROLS

	SLE/APS CASES* (N=21)	IUFD** CONTROLS (N=22)	LIVE BORN CONTROLS (N=40)	P-VALUE SLE/APS VS CONTROLS
No C4d deposition (%)	8 (38)	13 (59)	40(100)	
Focal C4d deposition (%)	3 (14)	7 (32)	0	
Diffuse C4d deposition (%)	10 (48)	2 (9)	0	P < 0.001
No properdin deposition (%)	8 (38)	19 (86)	17 (43)	
Focal properdin deposition (%)	6 (29)	2 (9)	12 (30)	
Diffuse properdin deposition (%)	7 (33)	1 (5)	11 (28)	P < 0.918

** Systemic Lupus Erythematosus and APS

* IUFD control group

TABLE 3 PATIENTS WITH MULTIPLE PLACENTAS

CASE NR	PATIENT DIAGNOSIS	GRAVIDITY	C4D STAINING	FETAL OUTCOME	WEEKS & DAYS
1	SLE*	G1	no tissue available	early miscarriage	< 10
		G2	no C4d staining	IUFD*** of unknown etiology	18
		G3	no C4d staining	IUFD, congenital heart block in the presence of anti-SSA and anti-SSB antibodies	31
		G4	no C4d staining	Live birth	38 + 1
2	SLE + APS**	G1	no tissue available	Live birth, IUGR†	37 + 2
		G2	diffuse C4d staining	Live birth, IUGR and severe Preeclampsia	38 + 4
		G3	focal C4d staining	Live birth, IUGR	38 + 3
3	Primary APS	G1	diffuse C4d staining	IUFD, severe IUGR and maternal HELLP ‡ syndrome.	23 + 2
		G2	no tissue available	live birth, HELLP syndrome and maternal liver infarction	37
		G3	diffuse C4d staining	IUFD, severe HELLP syndrome	28 + 2
4	Primary APS	G2	no tissue available	IUFD of unknown etiology	21 + 4
		G3	diffuse C4d staining	IUFD, severe IUGR	18 + 1
		G4	diffuse C4d staining	IUFD, severe IUGR	26 + 1

* Systemic lupus erythematosus

** Antiphospholipid syndrome

*** Intrauterine fetal death

† Intrauterine growth retardation

‡ Hemolysis Elevated Liver enzymes and Low Platelets

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