



Turning complexity into clarity.  
Powerful, configurable Guava® flow cytometers.

EMD Millipore Corp. is a subsidiary of Merck KGaA, Darmstadt, Germany.



Guava easyCyte™ Flow Cytometers

[Request Demo](#)



## Critical Role and Therapeutic Control of the Lectin Pathway of Complement Activation in an Abortion-Prone Mouse Mating

This information is current as of November 25, 2015.

Marie Petitbarat, Paolo Durigutto, Paolo Macor, Roberta Bulla, Alessandro Palmioli, Anna Bernardi, Maria-Grazia De Simoni, Nathalie Ledee, Gerard Chaouat and Francesco Tedesco

*J Immunol* published online 11 November 2015  
<http://www.jimmunol.org/content/early/2015/11/11/jimmunol.1501361>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2015/11/11/jimmunol.1501361.DCSupplemental.html>

**Subscriptions** Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscriptions>

**Permissions** Submit copyright permission requests at: <http://www.aai.org/ji/copyright.html>

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/cgi/alerts/etoc>

*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
9650 Rockville Pike, Bethesda, MD 20814-3994.  
Copyright © 2015 by The American Association of  
Immunologists, Inc. All rights reserved.  
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Critical Role and Therapeutic Control of the Lectin Pathway of Complement Activation in an Abortion-Prone Mouse Mating

Marie Petitbarat,<sup>\*,1</sup> Paolo Durigutto,<sup>†,1</sup> Paolo Macor,<sup>†</sup> Roberta Bulla,<sup>†</sup> Alessandro Palmioli,<sup>‡</sup> Anna Bernardi,<sup>‡</sup> Maria-Grazia De Simoni,<sup>§</sup> Nathalie Ledee,<sup>\*</sup> Gerard Chaouat,<sup>¶</sup> and Francesco Tedesco<sup>||</sup>

The abortion-prone mating combination CBA/J × DBA/2 has been recognized as a model of preeclampsia, and complement activation has been implicated in the high rate of pregnancy loss observed in CBA/J mice. We have analyzed the implantation sites collected from DBA/2-mated CBA/J mice for the deposition of the complement recognition molecules using CBA/J mated with BALB/c mice as a control group. MBL-A was observed in the implantation sites of CBA/J × DBA/2 combination in the absence of MBL-C and was undetectable in BALB/c-mated CBA/J mice. Conversely, C1q was present in both mating combinations. Searching for other complement components localized at the implantation sites of CBA/J × DBA/2, we found C4 and C3, but we failed to reveal C1r. These data suggest that complement is activated through the lectin pathway and proceeds to completion of the activation sequence as revealed by C9 deposition. MBL-A was detected as early as 3.5 d of pregnancy, and MBL-A deficiency prevented pregnancy loss in the abortion-prone mating combination. The contribution of the terminal complex to miscarriage was supported by the finding that pregnancy failure was largely inhibited by the administration of neutralizing Ab to C5. Treatment of DBA/2-mated CBA/J mice with Polyman2 that binds to MBL-A with high affinity proved to be highly effective in controlling the activation of the lectin pathway and in preventing fetal loss. *The Journal of Immunology*, 2015, 195: 000–000.

Embryo implantation represents a real challenge for the maternal immune system, which is continuously confronted during pregnancy with paternal and sex-specific alloantigens expressed on trophoblasts invading the maternal decidua, as well as with deported fetal and placental cells. This is a unique physiologic condition, which requires adaptation of the immune system to avoid harming the fetoplacental unit and to ensure regular progression of pregnancy. The adaptive and the innate arms of the immune system are both involved in this process and develop strategies to establish a protective envi-

ronment in maternal decidua to prevent fetal demise (1). C is a humoral component of innate immunity, which fulfills protective functions providing a critical defense barrier against infectious agents potentially harmful to the fetus (2, 3). More recent findings have also disclosed the important role of C in placental development in normal pregnancy by promoting tissue remodeling and vascular changes (4). Besides external Ags, an inflammatory state that develops post coitum in the preimplantation phase and during embryo implantation as a result of trophoblast invasion into maternal decidua contributes to activate the C system. This process does not occur in any other tissue or organ under physiologic conditions and leads to the release of activation products that may damage the developing fetus if they were not controlled by C regulatory proteins that are widely distributed in the fetoplacental unit. Unrestrictive C activation may overcome the protective effect of the C regulatory proteins, causing adverse pregnancy outcomes.

Preeclampsia (PE) is one of the pathologic pregnancies in which C has been implicated as part of the systemic and local inflammation associated with this disorder (5). This syndrome that affects 3–5% of pregnancies is an important cause of maternal and perinatal morbidity and mortality, and is characterized by hypertension and proteinuria (6). The idea that C is involved in the development of PE is based on the finding of C activation products in the circulation and in the detection of C deposits in the placenta of these patients. However, the direct contribution of C to the pathogenesis of PE is difficult to establish because PE is a multifactorial clinical condition that manifests with different clinical features suggesting the involvement of various pathogenic factors.

Major progress in our understanding of the role of C in PE has been made using the abortion-prone mating combination CBA/J female × DBA/2 male, characterized by high fetal resorption rate and growth restriction (7, 8). Girardi and colleagues (9) first reported their finding that the angiogenic factors are dysregulated

\*MatriceLAB Innove, Hôpital Saint Louis, 75010 Paris, France; <sup>†</sup>Department of Life Sciences, University of Trieste, 34127 Trieste, Italy; <sup>‡</sup>Department of Chemistry, University of Milan, 20133 Milan, Italy; <sup>§</sup>Dipartimento di Neuroscienze, Istituto di Ricerca e Cura a Carattere Scientifico – Istituto di Ricerche Farmacologiche Mario Negri, 20156 Milan, Italy; <sup>¶</sup>U 976 INSERM, Hôpital Saint Louis, 75010 Paris, France; and <sup>||</sup>Istituto di Ricerca e Cura a Carattere Scientifico – Istituto Auxologico Italiano, 20095 Milan, Italy

<sup>1</sup>M.P. and P.D. contributed equally to this work.

ORCID: 0000-0002-0112-983X (M.P.); 0000-0003-3862-4970 (P.D.); 0000-0003-3079-4019 (P.M.); 0000-0003-1737-5776 (R.B.); 0000-0002-5287-1663 (A.P.); 0000-0002-1258-2007 (A.B.); 0000-0001-6695-5297 (M.-G.D.S.); 0000-0003-2462-0493 (F.T.).

Received for publication June 25, 2015. Accepted for publication October 14, 2015.

This work was supported by grants from the European Network of Excellence on Embryo Implantation Control, U 782 INSERM (France), the Italian Ministry of Health (Ricerca Finalizzata Grants RC 01/09 and RC 08/13), the Italian Ministry of Education, University and Research (Progetti di Ricerca di Interesse Nazionale PRIN MFXE7L\_004), and the Fondazione Casali.

Address correspondence and reprint requests to Prof. Francesco Tedesco, Istituto di Ricerca e Cura a Carattere Scientifico – Istituto Auxologico Italiano, Via Giuseppe Zucchi, 18 20095 Cusano Milanino, Milan, Italy. E-mail address: tedesco@units.it

The online version of this article contains supplemental material.

Abbreviations used in this article: EC, endothelial cell; KO, knockout; PE, preeclampsia.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/\$25.00

in CBA/J × DBA/2 mating with increased levels of soluble vascular-endothelial growth factor receptor and reduced concentration of vascular-endothelial growth factor resulting in defective vascularization and impaired development of the placenta. Subsequently, the same group showed that CBA/J females mated with DBA/2 males share with human PE not only elevated levels of antiangiogenic factors and placental dysfunction, but also proteinuria and glomerular endotheliosis, whereas they failed to detect hypertension, a clinical feature of human PE (10). C has been shown to contribute to the dysregulation of the angiogenic factors resulting in fetal loss and intrauterine growth restriction as documented by C3 deposition at embryo implantation sites and the ability of C inhibitors acting at C3 and C5a levels to prevent embryo damage, as well proteinuria and renal pathologic features of PE in CBA/J × DBA/2 matings (9, 11). Interestingly, Girardi and her group (12) have also shown that C1q-deficient mice present all the features of PE, including hypertension.

We now present data suggesting that C activation occurs in the early phase of embryo implantation and triggers the complex series of events leading to miscarriage in the Ab-independent model of pregnancy loss observed in CBA/J × DBA/2 mating. In addition, we provide evidence that C is activated through the lectin pathway and that selective inhibition of the pathway rescues pregnancies in this mating combination.

## Materials and Methods

### Polymers and recombinant Abs

Polyman2 is a tetravalent pseudotrimannoside Dendron, known also as Dendron 12, which is formed by a tetravalent polyester scaffold, decorated with a mimic of linear 1,2- to 1,6-trimannoside (13), and was previously shown to bind MBL with high affinity (14).

The neutralizing anti-C5 recombinant Ab that recognizes an epitope located in the cleavage site of the C5 convertases on the  $\alpha$ -chain of human C5 shared by rat, mouse, and rabbit C5 (15) was produced from a human single-chain fragment variable fused with hinge-CH2-CH3 regions of IgG1, as previously described (16). This recombinant Ab was previously found to prevent C5 activation in several animal models developed in mice (17, 18) and rats (16, 19–21). An essentially similar procedure was followed for the production of a recombinant Ab to human CD20 reported in a previous publication (22) and used in this study as an unrelated Ab.

Anti-C5 and control recombinant Abs were purified from cell-conditioned medium loaded on Protein A column and eluted with 1M NaCl in PBS. Fractions containing the recombinant molecules were selected by ELISA and checked for purity by NaDodSO<sub>4</sub>-PAGE.

### DNA extraction

DNA was extracted from mice tails (CBA/J × knockout (KO) C57BL/6) using spin columns of a commercial DNA extraction kit (NucleoSpin

Tissue, Macherey-Nagel, Germany) according to the manufacturer's instructions. In brief, tails were incubated at 56°C overnight with lysis buffer solution and proteinase K. Lysis buffer was then added to the solution and the samples were further incubated at 70°C for 10 min. After adding ethanol, the samples were applied to the column and centrifuged. DNA was eluted in water and used for the PCR test.

### Polymerase chain reaction

PCRs were performed using 0.5  $\mu$ g/ml DNA, 0.5  $\mu$ g/ml of each primer, Taq polymerase (0.8 U per 50  $\mu$ l reaction), and a mix containing 10× buffer, 200  $\mu$ M dNTP, DMSO, and 1.5 mM MgCl<sub>2</sub>. The amplification conditions include 1 denaturation cycle (95°C, 2 min), 40 amplification cycles (95°C, 20 s; 60°C, 20 s; 68°C, 45 s), and a final extension cycle (68°C, 5 min). The following primers were used:

MBL-C: left primer, 5'-AGGAGAAAAGGGAGAACCA-3', right primer, 5'-CCTGGGGTCCTGTAGGT-3'.

MBL-A: left primer, 5'-GCTCCTTACTCTAAAGAAACCCTAGT-3', right primer: 5'-TCACCACACACAGAAGGACAG-3'.

The PCR products were analyzed on 10% polyacrylamide gel to identify the MBL-A KO mice.

### Mice

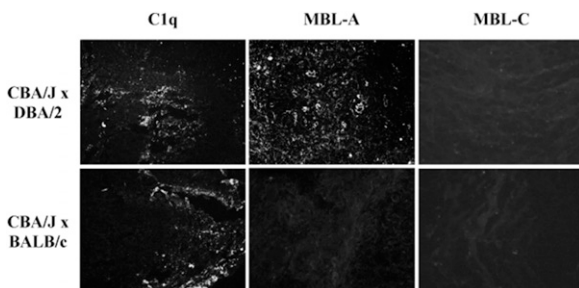
Genetically certified CBA/J females and DBA/2 and BALB/c male mice were all obtained from Charles River/Ifffa Credo (l'Arbresles, France) at the age of 6–8 wk. The mice were housed in the animal facilities of either Clamart, Institut National de la Recherche Agronomique Jouy en Josas, or center Hayem/Hôpital St. Louis (Paris, France) for 15 d and in the case of 6-wk-old mice for 30 d before mating.

### Generation of CBA/J MBL-A null mice

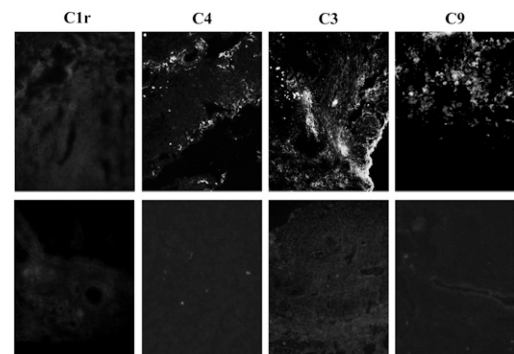
Mice with null mutation in the gene encoding MBL-A were generated using the traditional backcrossing method from MBL-A and MBL-C double-KO breeders generated on a B6 background as described by Shi et al. (23) and kindly provided by Drs. Jensenius and Thiel (Aarhus, Denmark). Offspring of the first-generation backcross matings were genotyped by PCR for MBL-A mutation and then bred back to the same pure strain, thus diluting the contribution of the original genetic background by 50% for each backcross generation. This process was repeated for seven generations, and the MBL-A<sup>-/-</sup> animals used in our experiments were >99% congenic on the CBA/J genome.

### Matings

The mating combinations included (female × male): CBA/J (H-2<sup>k</sup>) × BALB/c (H-2<sup>d</sup>) or CBA/J × DBA/2 (H-2<sup>d</sup>). The latter two combinations both involve minor and major loci allogeneic disparities between males and females. The male strains are MHC identical (H-2<sup>d</sup>) with disparity in only minor loci. The CBA/J × BALB/c is a non-abortion-prone control mating combination, whereas CBA/J × DBA/2 crosses yield abortion-prone pregnancies (7).

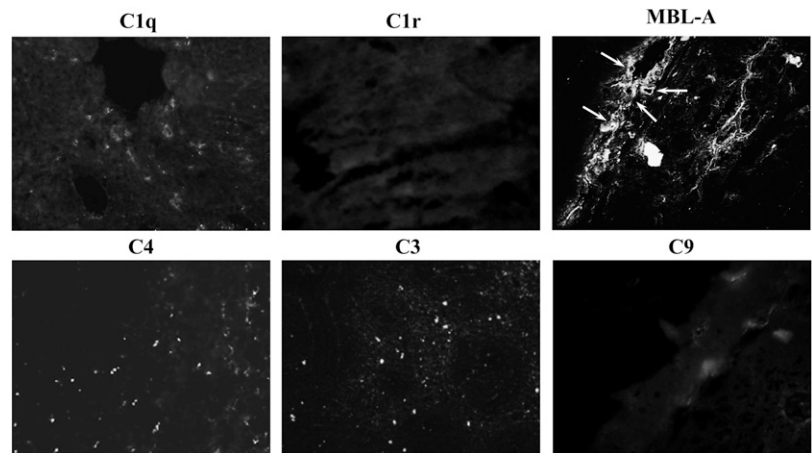


**FIGURE 1.** Detection of C1q, MBL-A, and MBL-C on murine implantation sites. Sections of tissue collected from CBA/J × DBA/2 and CBA/J × BALB/c mating combinations at day 6.5 of pregnancy were examined for the presence of C1q, MBL-A, and MBL-C by immunofluorescence using specific rat mAbs revealed by goat anti-rat IgG labeled with Alexa Fluor 488. Note the selective localization of MBL-A on the implantation sites of CBA/J × DBA/2, whereas C1q is present on sections from both mating combinations. Original magnification ×200.



**FIGURE 2.** Analysis of murine decidua for the presence of C1r, C4, C3, and C9. Implantation sites were obtained from CBA/J mice mated to either DBA/2 or BALB/c male mice at day 6.5 of pregnancy and examined for the localization of the C components by immunofluorescence. Note the positive staining for C4, C3, and C9 in the absence of C1r in CBA/J × DBA/2, whereas all these C components were undetectable in CBA/J × BALB/c mating combination. Original magnification ×200.

**FIGURE 3.** Analysis of early implantation sites for the deposition of C components. Implantation sites were collected from DBA/2-mated CBA/J female mice at day 3.5 of pregnancy and examined for the presence of C components by immunofluorescence. Note the marked staining for MBL-A compared with the mild expression of the other C components. Arrows point to microvessels that are the primary sites of MBL-A deposition. Original magnification  $\times 200$ .



The mice were mated following details in a published procedure (24), and pregnancies were dated from day 0.5 post coitum, the morning of detection of a vaginal plug. The animals were euthanized on 3.5–14 gestation days, and the resorbed fetuses were identified by their small size and necrotic or hemorrhagic appearance compared with normal embryos. Pregnant mice were randomly included in each experimental group, and the results are presented as percentage of fetal loss calculated using the formula  $\%R = Re / (Re + F)$ , where R represents percentage of resorptions as referred to total number of effective implantation sites, Re the number of resorbed embryos, and F the number of viable embryos (25). The experimental procedures were performed in accordance with the rules enforced by the French National Institutes of Health and Medical Research, Université Paris Sud, and Université Paris Diderot and in compliance with the European (86/609/EEC) and French laws.

#### Immunofluorescence analysis

Frozen sections (6  $\mu\text{m}$ ) of implantation sites from 10- to 11-wk-old mice were examined for tissue deposition of C components. MBL-A, MBL-C, C1q, and C4 were detected using rat mAbs (clones 8G6, 14D12, 7H8, and 16D2, respectively; Hycult Biotech, Uden, the Netherlands) followed by Alexa Fluor 488-labeled goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). Rabbit polyclonal Abs to human C1r cross-reacting with mouse C1r (Sigma, Italy) and to mouse C9 (kindly provided by Prof. M. Daha, Leiden, The Netherlands) were used to reveal deposition of C1r and C9 using FITC-labeled goat anti-rabbit IgG (Jackson ImmunoResearch) as secondary Ab. C3 deposits were detected using FITC-conjugated goat F(ab')<sub>2</sub> fragment to mouse C3 (Cappel UK) as previously described (26). All Abs were incubated at room temperature for 1 h to a final concentration of 5  $\mu\text{g}/\text{ml}$ . The slides were examined using a DM2000 fluorescence microscope (Leica, Germany).

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows. The fetal resorption data were analyzed using the Mann–Whitney *U* test and expressed as median and interquartile range for nonparametric variables. The *p* values  $<0.05$  were considered statistically significant.

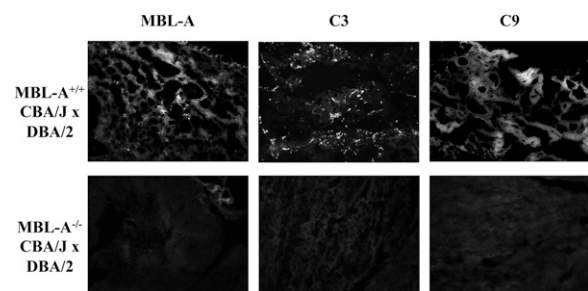
## Results

#### Kinetic analysis of C deposition at embryo implantation site

Previous finding of C3 deposits at embryo–maternal interface by Girardi and colleagues (9) did not clarify the initiator factors responsible for the activation of the C cascade and the pathway involved in this process. To address this issue, we analyzed samples of implantation tissue collected at day 6.5 of pregnancy from both CBA/J  $\times$  DBA/2 and CBA/J  $\times$  BALB/c mating combinations for the presence of the early components of the classical and lectin pathways. As shown in Fig. 1, staining of implantation sites of DBA/2-mated CBA/J female mice showed deposits of C1q and MBL-A, whereas MBL-C was undetectable using an Ab that was previously reported to reveal the presence of this complement component on cerebral ischemic areas (14). The Abs were checked for their specificities by Western blot

analysis and their failure to react with sera from mice with selective complement deficiencies. Unlike MBL-A, C1q was also present in the implantation sites of BALB/c-mated CBA/J mice. We then searched for evidence supporting C activation in the implantation sites of DBA/2-mated CBA/J mice analyzing the tissue for the presence of C components involved in the activation sequence, and we were able to detect C4, C3, and C9, but not C1r (Fig. 2). Failure to observe C1r could not be attributed to the use of an inappropriate Ab because this Ab revealed deposits of C1r on the implantation sites in a mouse model of complement-mediated pregnancy loss induced by an Ab to beta2-GPI (26) (Supplemental Fig. 1).

A strong staining for MBL-A mainly localized on vascular endothelium was already apparent on day 3.5 of pregnancy compared with the negligible or mild expression of the other C components (Fig. 3). Analysis of MBL-A distribution on the implantation site collected in more advanced stage of pregnancy showed colocalization of MBL-A and C1q previously reported to be constitutively expressed by decidual endothelial cells (ECs) and extravillous trophoblasts invading the decidua, suggesting that complement activation exerts its effect on the decidual side (Supplemental Fig. 2). Differences in the staining intensity of C deposits were noticed in various implantation sites. To investigate the contribution of local synthesis of MBL-A to initiate MBL-mediated activation of the lectin pathway, we analyzed MBL-A RNA expression in the implantation sites of DBA/2-mated CBA/J mice by quantitative PCR following a procedure previously reported in detail (24) and failed to detect any signal at different days of gestation (data not shown).



**FIGURE 4.** Analysis of decidua from MBL-A<sup>+/+</sup> and MBL-A<sup>-/-</sup> for complement deposition. Decidual tissues were collected from either MBL-A<sup>+/+</sup> or MBL-A<sup>-/-</sup> CBA/J mice mated to DBA/2 mice on day 14 of pregnancy and analyzed for the localization of MBL-A, C3, and C9. Note the presence of all three C components in MBL-A<sup>+/+</sup> and their absence in MBL-A<sup>-/-</sup> mice. Original magnification  $\times 200$ .

### *MBL-dependent activation of the lectin pathway induces pregnancy loss in CBA/J × DBA/2 mating*

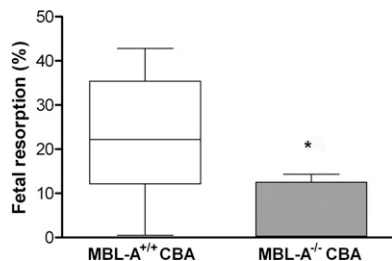
Having found an early deposition of MBL-A in the implantation sites of CBA/J mated to DBA/2 mice, we then investigated the ability of MBL to activate the lectin pathway and to negatively influence the pregnancy outcome. To this end, MBL-A<sup>-/-</sup> mice, generated on a CBA/J background, were mated to DBA/2 male mice, and the implantation sites of pregnant mice at 14 d of pregnancy were examined for the presence of C components. As expected, MBL-A was undetectable in the MBL-A KO mice as opposed to the strong signal visualized in wild-type mice (Fig. 4). We also failed to reveal deposition of C3 and C9 in the MBL-A deficient animal, suggesting that C was activated through the lectin pathway in the CBA/J × DBA/2 mating. Evaluation of pregnancy outcome in pregnant CBA/J showed that MBL-A deficiency was associated with a significant decrease in the median value of fetal resorption from 22 to 0% (Fig. 5). The involvement of the late C components in MBL-mediated C activation was investigated treating pregnant MBL-A<sup>+/+</sup> mice with a neutralizing Ab to C5 that prevented pregnancy loss to a median level 4-fold lower than that observed in mice receiving an unrelated Ab (Fig. 6).

### *Inhibition of lectin pathway activation rescue pregnancy in CBA/J × DBA/2 mating*

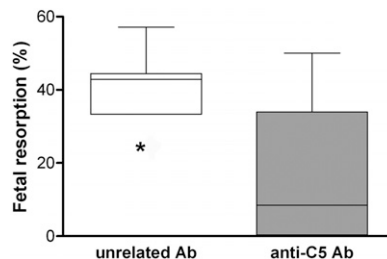
The finding that MBL-A deficiency protected CBA/2 pregnant mice from fetal resorption led us to consider the possibility that the proabortive effect of MBL-A may be controlled by an inhibitor of its functional activity. To this purpose, we used Polyman2, a poly-mannosylated dendrimer (a tetravalent pseudotrimannoside dendron) previously found to bind MBL with high affinity and to reduce MBL-mediated brain ischemic injury (14). Because a substantial amount of MBL-A was already localized in the maternal implantation site at day 3.5 of pregnancy, we decided to treat the animals with Polyman2 administered four times (200 µg/mouse i.p. on alternate day) starting on day 0.5. As shown in Fig. 7, the drug was effective in preventing the fetal resorption rate that dropped from 29% in the untreated mice to 0% in animals receiving Polyman2. In addition, we failed to detect deposition of MBL-A, C3, and C9 in mice treated with the MBL inhibitor (Fig. 8).

## Discussion

Activation of the lectin pathway has been implicated in tissue damage associated with ischemia reperfusion of several organs and tissues including myocardium, gastrointestinal tract, skeletal muscles, and brain (27, 28). The results of this study indicate that



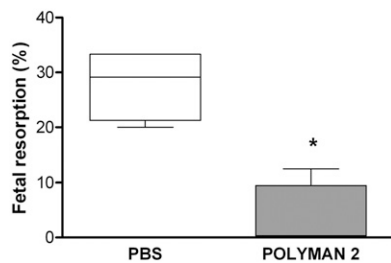
**FIGURE 5.** Deficiency of MBL-A prevents fetal loss in CBA/J × DBA/2 matings. MBL-A<sup>+/+</sup> and MBL-A<sup>-/-</sup> CBA/J female mice were mated to MBL-A<sup>+/+</sup>DBA/2 male mice. Seven to 13 pregnant mice were included in the experimental groups. The total number of implantation versus resorptions in MBL-A<sup>-/-</sup> mice was 53 versus 3 and in MBL-A<sup>+/+</sup> mice was 81 versus 9. Animals were euthanized on day 14 of pregnancy, and the number of resorbed and viable fetuses was counted. The results of percentage of fetal loss are presented as median (horizontal bars) ± interquartile range. \**p* = 0.002.



**FIGURE 6.** Effect of anti-C5 Ab on fetal loss. DBA/2 females mated with CBA/J males received six i.p. injections of either anti-C5 or unrelated Abs (100 µg/400 µl sterile saline) on alternate days starting from the day of vaginal plug detection. Six to 10 mice were in the experimental groups and sacrificed on day 14 of pregnancy. The total number of implantation versus resorptions in the anti-C5-treated mice was 39 versus 6 and in the unrelated Ab treated group was 49 versus 17. The percentages of fetal loss observed in the two groups of mice is presented as median (horizontal bars) ± interquartile range. \**p* = 0.040.

the lectin pathway activation is also involved in the adverse pregnancy outcome observed in DBA/2-mated CBA/J.

Pregnancy loss in CBA/J × DBA/2 mating combination has been shown to be Ab independent and to be mainly mediated by macrophages and NK cells (29), although the contribution of T cells cannot be excluded. A few years ago, Girardi and colleagues (9) identified the C system as an additional player in the CBA/J × DBA/2 model contributing to the high rate of fetal resorption and inducing dysregulation of angiogenic factors. A surprising finding of this study was the strong staining of the implantation site of DBA/2-mated CBA/J pregnant mice for MBL seen as early as 3.5 d of pregnancy. MBL deposition was followed by C3 observed at day 6.5 of pregnancy in agreement with a similar observation of Girardi et al. (9), who also found that C3 appeared at the same time as the proinflammatory cells monocytes and polymorphonuclear leukocytes. Our own observation that cytokines involved in pregnancy loss including IFN-γ, TNF-α, and IL-10 are not expressed earlier than 6.5 d of pregnancy is consistent with this kinetic (30). Taken together, these data suggest that C may be the initiator of the complex series of events mediated by multiple pathogenic factors leading to adverse pregnancy outcome in this murine mating combination. Although this issue was not addressed specifically in this work, the possibility that C acts upstream of the proinflammatory markers is supported by the marked decrease in TNF-α level in a model of anti-phospholipid syndrome established in C5-deficient mice (31) and in rats treated with a neutralizing Ab to C5 undergoing experimental arthritis (18). Despite the early deposition of MBL-A, we believe that C activation triggered by this C component does not affect implantation because the total number of fetuses including both resorbed and live fetuses in MBL<sup>+/+</sup> mice is within the range obtained in MBL-A<sup>-/-</sup> mice. Both C1q and MBL-A were detected at the implantation site of DBA/2-mated CBA/J mice with the only difference that, unlike MBL-A, C1q was also present in CBA/J mated to BALB/c male mice. The persistent localization of C1q in different mating combinations was not surprising because we have previously found that C1q is constitutively expressed by decidual ECs and extravillous trophoblasts, and is used to promote vascular remodeling and trophoblast migration (32, 33). This might explain why C1q-deficient mice, at variance with CBA/J mated with DBA/2, show hypertension during pregnancy. Conversely, the finding that deposits of MBL-A were restricted to the abortion-prone CBA/J mice suggests that this C component may be implicated in the pregnancy loss. Immunofluorescence analysis of CBA/J decida for deposition of MBL-A and MBL-C, the murine

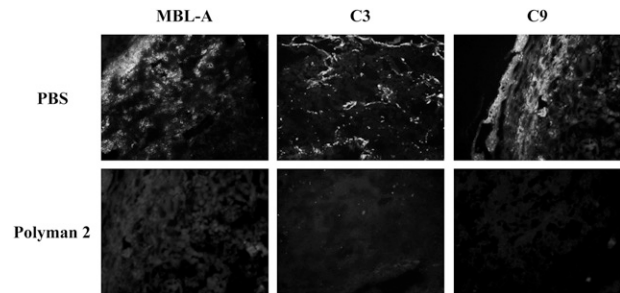


**FIGURE 7.** Effect of Polyman2 on fetal loss. DBA/2 females that mated with CBA/J males received four i.p. injections of either Polyman2 (200  $\mu$ g/400  $\mu$ l PBS) or PBS alone on alternate days starting from the day of vaginal plug detection. Four to seven mice were included in the experimental groups and sacrificed on day 14 of pregnancy. The total number of implantation versus resorptions in the Polyman2-treated mice was 23 versus 1 and in the PBS-treated group was 24 versus 7. The percentages of fetal loss observed in the two groups of mice is presented as median (horizontal bars)  $\pm$  interquartile range. \* $p = 0.002$ .

counterpart of human MBL (34, 35), revealed tissue localization of MBL-A in the absence of detectable MBL-C. This is surprising because MBL-C circulates in the blood of CBA/J mice at higher levels than MBL-A and can more likely interact with ECs, which represent the primary target of this activator of the lectin pathway (36). Moreover, both molecules have been detected on tissues undergoing ischemia-reperfusion injury (14, 37) and on the aortic root in a mouse model of vasculitis (38). A possible explanation for the selective binding of MBL-A is that ECs provide preferential binding site for this C component. Hansen and colleagues (34) observed a marked difference in the carbohydrate specificity of murine MBL-A and MBL-C, and suggested that such a difference may result in preferential binding to different infectious agents. Whether this is also true for ECs from different sources remains to be established. The liver is the main source of MBL-A and MBL-C, suggesting that a substantial portion of MBL-A deposited at the implantation site derives from the circulation (39). The possibility that the protein may also be produced locally, as it is in the kidney (39), can be ruled out by our failure to reveal MBL-A RNA expression in the decidua.

The finding that DBA/2-mated MBL-A<sup>-/-</sup> CBA/J mice showed a marked reduction in miscarriage rates compared with MBL-A<sup>+/+</sup> mice supports a critical role of MBL-A in inducing adverse pregnancy outcome in this mating combination. MBL-A was shown by van der Pol et al. (40) to have a damaging effect independent of C activation in a rat model of renal ischemia injury. This was not the case in the abortion-prone CBA/J mice because deposition of MBL-A in decidual tissue was associated with that of C3 and C9, suggesting activation of the lectin pathway. Failure to detect C1r, despite the persistent presence of C1q, rules out the involvement of the classical pathway of C activation, whereas the presence of C3 is compatible with activation of the alternative pathway. However, this pathway may only contribute to amplify the activation process, although the failure to reveal deposition of C3 and C9 in MBL-A<sup>-/-</sup> mice clearly indicates that the lectin pathway is the major driver of C activation.

The ability of neutralizing recombinant Ab anti-C5 to rescue CBA/J mice from pregnancy loss clearly indicates that the terminal components are involved in the lectin pathway-mediated fetal resorption. The major role in causing fetal demise has been attributed to C5a through the recruitment of monocytes and granulocytes (9), although the deposition of C9 suggests that the terminal complex may also be involved. It is important to note that, despite complement deposition, a proportion of embryos



**FIGURE 8.** Analysis of complement deposition on the decidua of Polyman2-treated DBA/2 females mated with CBA/J males. Sections of implantation sites collected at 14 d of pregnancy were examined for the presence of MBL-A, C3, and C9. Note the total absence of these C components in contrast with the strong staining observed in the decidua of mice receiving PBS. Original magnification  $\times 200$ .

were still alive at day 14 of pregnancy. Although we cannot exclude that some of these embryos would have experienced fetal growth retardation if pregnancy had progressed to term, we are not stating that complement activation is solely responsible for the fetal loss in CBA/J females mated to DBA/2 males. Other factors including proinflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$ , macrophages, and NK cells may contribute to this pathologic process (24). Moreover, the staining intensity of complement deposits differs in various implantation sites and is likely dependent on different expression of triggering factors and complement regulatory proteins.

The data presented in this article that Polyman2 prevents MBL-A binding to the decidua of DBA/2-mated CBA/J mice inhibiting C3 and C9 deposition and rescuing these mice from pregnancy loss further support the important contribution of the lectin pathway of C activation to the adverse pregnancy outcome observed in this mating combination. These findings have important clinical and therapeutic implications because CBA/J  $\times$  DBA/2 mating has been shown to be an animal model of human PE and represents an invaluable tool to validate novel therapies for PE patients. Polyman2 is a tetravalent trimannoside dendron that interacts with MBL and has distinct advantages as a therapeutic inhibitor of C activation. Because of the oligomeric structure, Polyman2 offers multiple binding sites to MBL, which circulates in blood at a relatively low level and is therefore easy to neutralize. Moreover, MBL acts at the initial step of C activation, and its inhibition prevents release of activation products that may cause local damage. Finally, the preparation of Polyman2 is less expensive than the production of neutralizing mAbs and its repeated administration in the course of pregnancy has not been associated with adverse effects.

In conclusion, we have provided data indicating that C activation through the lectin pathway at implantation site in DBA/2-mated CBA/J mice represents an early event leading to fetal resorption in this mating combination. We have also shown that MBL-A is selectively deposited in the decidua of pregnant mice and that MBL-A-deficient mice and animals treated with an inhibitor of MBL-A are rescued from pregnancy loss.

## Acknowledgments

We thank Olivier Sandra, Christophe Auger, and the Institut National de la Recherche Agronomique animal facilities personnel as well as Veronique Parietti and the Centre Hayem animal center facilities for mice housing and matings. We are also grateful to Olivier Sandra and Christophe Auger for helping to generate the MBL null mice on CBA/J background using the speed congenic method.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Hsu, P., and R. K. Nanan. 2014. Innate and adaptive immune interactions at the fetal-maternal interface in healthy human pregnancy and pre-eclampsia. *Front. Immunol.* 5: 125.
- Denny, K. J., T. M. Woodruff, S. M. Taylor, and L. K. Callaway. 2013. Complement in pregnancy: a delicate balance. *Am. J. Reprod. Immunol.* 69: 3–11.
- Girardi, G., Z. Prohászka, R. Bulla, F. Tedesco, and S. Scherjon. 2011. Complement activation in animal and human pregnancies as a model for immunological recognition. *Mol. Immunol.* 48: 1621–1630.
- Bulla, R., F. Bossi, and F. Tedesco. 2012. The complement system at the embryo implantation site: friend or foe? *Front. Immunol.* 3: 55.
- Redman, C. W. 2011. Preeclampsia: a multi-stress disorder. *Rev. Med. Interne* 32 (Suppl. 1): S41–S44.
- Chaiworapongsa, T., P. Chaemsaitong, L. Yeo, and R. Romero. 2014. Preeclampsia part 1: current understanding of its pathophysiology. *Nat. Rev. Nephrol.* 10: 466–480.
- Chaouat, G., N. Kiger, and T. G. Wegmann. 1983. Vaccination against spontaneous abortion in mice. *J. Reprod. Immunol.* 5: 389–392.
- Clark, D. A., M. R. McDermott, and M. R. Szwczuk. 1980. 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: 106–118.
- Girardi, G., D. Yarin, J. M. Thurman, V. M. Holers, and J. E. Salmon. 2006. Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. *J. Exp. Med.* 203: 2165–2175.
- Ahmed, A., J. Singh, Y. Khan, S. V. Seshan, and G. Girardi. 2010. A new mouse model to explore therapies for preeclampsia. *PLoS One* 5: e13663.
- Qing, X., P. B. Redecha, M. A. Burmeister, S. Tomlinson, V. D. D'Agati, R. L. Davison, and J. E. Salmon. 2011. Targeted inhibition of complement activation prevents features of preeclampsia in mice. *Kidney Int.* 79: 331–339.
- Singh, J., A. Ahmed, and G. Girardi. 2011. Role of complement component C1q in the onset of preeclampsia in mice. *Hypertension* 58: 716–724.
- Sattin, S., A. Daghetti, M. Thépaut, A. Berzi, M. Sánchez-Navarro, G. Tabarani, J. Rojo, F. Fieschi, M. Clerici, and A. Bernardi. 2010. Inhibition of DC-SIGN-mediated HIV infection by a linear trimannoside mimic in a tetravalent presentation. *ACS Chem. Biol.* 5: 301–312.
- Orsini, F., P. Villa, S. Parrella, R. Zangari, E. R. Zanier, R. Gesuete, M. Stravalaci, S. Fumagalli, R. Ottria, J. J. Reina, et al. 2012. Targeting mannose-binding lectin confers long-lasting protection with a surprisingly wide therapeutic window in cerebral ischemia. *Circulation* 126: 1484–1494.
- Marzari, R., D. Sblattero, P. Macor, F. Fischetti, R. Gennaro, J. D. Marks, A. Bradbury, and F. Tedesco. 2002. The cleavage site of C5 from man and animals as a common target for neutralizing human monoclonal antibodies: in vitro and in vivo studies. *Eur. J. Immunol.* 32: 2773–2782.
- Fischetti, F., P. Durigutto, V. Pellis, A. Debeus, P. Macor, R. Bulla, F. Bossi, F. Ziller, D. Sblattero, P. Meroni, and F. Tedesco. 2005. Thrombus formation induced by antibodies to beta2-glycoprotein I is complement dependent and requires a priming factor. *Blood* 106: 2340–2346.
- Agostinis, C., S. Biffi, C. Garrovo, P. Durigutto, A. Lorenzon, A. Bek, R. Bulla, C. Grossi, M. O. Borghi, P. Meroni, and F. Tedesco. 2011. In vivo distribution of  $\beta 2$  glycoprotein I under various pathophysiologic conditions. *Blood* 118: 4231–4238.
- Macor, P., P. Durigutto, L. De Maso, C. Garrovo, S. Biffi, A. Cortini, F. Fischetti, D. Sblattero, C. Pitzalis, R. Marzari, and F. Tedesco. 2012. Treatment of experimental arthritis by targeting synovial endothelium with a neutralizing recombinant antibody to C5. *Arthritis Rheum.* 64: 2559–2567.
- Durigutto, P., P. Macor, F. Ziller, L. De Maso, F. Fischetti, R. Marzari, D. Sblattero, and F. Tedesco. 2013. Prevention of arthritis by locally synthesized recombinant antibody neutralizing complement component C5. *PLoS One* 8: e58696.
- Ferrareso, M., P. Macor, M. Valente, M. Della Barbera, F. D'Amelio, O. Borghi, E. Raschi, P. Durigutto, P. Meroni, and F. Tedesco. 2008. Posttransplant ischemia-reperfusion injury in transplanted heart is prevented by a minibody to the fifth component of complement. *Transplantation* 86: 1445–1451.
- Fischetti, F., P. Durigutto, P. Macor, R. Marzari, R. Carretta, and F. Tedesco. 2007. Selective therapeutic control of C5a and the terminal complement complex by anti-C5 single-chain Fv in an experimental model of antigen-induced arthritis in rats. *Arthritis Rheum.* 56: 1187–1197.
- Macor, P., E. Secco, N. Mezzaroba, S. Zorzet, P. Durigutto, T. Gaiotto, L. De Maso, S. Biffi, C. Garrovo, S. Capolla, et al. 2015. Bispecific antibodies targeting tumor-associated antigens and neutralizing complement regulators increase the efficacy of antibody-based immunotherapy in mice. *Leukemia* 29: 406–414.
- Shi, L., K. Takahashi, J. Dundee, S. Shahroor-Karni, S. Thiel, J. C. Jensenius, F. Gad, M. R. Hamblin, K. N. Sastry, and R. A. Ezekowitz. 2004. Mannose-binding lectin-deficient mice are susceptible to infection with *Staphylococcus aureus*. *J. Exp. Med.* 199: 1379–1390.
- Chaouat, G., M. Petitbarat, R. Bulla, S. Dubanchet, K. Valdivia, N. Ledée, T. Steffen, J. C. Jensenius, and F. Tedesco. 2009. Early regulators in abortion and implications for a preeclampsia model. *J. Reprod. Immunol.* 82: 131–140.
- Chaouat, G., A. Assal Meliani, J. Martal, R. Raghupathy, J. F. Elliott, T. Mosmann, and T. G. Wegmann. 1995. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *J. Immunol.* 154: 4261–4268.
- Agostinis, C., P. Durigutto, D. Sblattero, M. O. Borghi, C. Grossi, F. Guida, R. Bulla, P. Macor, F. Pregnolato, P. L. Meroni, and F. Tedesco. 2014. A non-complement-fixing antibody to  $\beta 2$  glycoprotein I as a novel therapy for antiphospholipid syndrome. *Blood* 123: 3478–3487.
- Gorsuch, W. B., E. Chrysanthou, W. J. Schwaeble, and G. L. Stahl. 2012. The complement system in ischemia-reperfusion injuries. *Immunobiology* 217: 1026–1033.
- Orsini, F., D. De Blasio, R. Zangari, E. R. Zanier, and M. G. De Simoni. 2014. Versatility of the complement system in neuroinflammation, neurodegeneration and brain homeostasis. *Front. Cell. Neurosci.* 8: 380.
- Bonney, E. A., and S. A. Brown. 2014. To drive or be driven: the path of a mouse model of recent pregnancy loss. *Reproduction* 147: R153–R167.
- Chaouat, G., N. Rodde, M. Petitbarat, R. Bulla, M. Rahmati, S. Dubanchet, S. Zourbas, I. Bataillon, N. Coqué, B. Henny, et al. 2011. An insight into normal and pathological pregnancies using large-scale microarrays: lessons from microarrays. *J. Reprod. Immunol.* 89: 163–172.
- Berman, J., G. Girardi, and J. E. Salmon. 2005. TNF-alpha is a critical effector and a target for therapy in antiphospholipid antibody-induced pregnancy loss. *J. Immunol.* 174: 485–490.
- Bulla, R., C. Agostinis, F. Bossi, L. Rizzi, A. Debeus, C. Tripodo, O. Radillo, F. De Seta, B. Ghebrehiwet, and F. Tedesco. 2008. Decidual endothelial cells express surface-bound C1q as a molecular bridge between endovascular trophoblast and decidual endothelium. *Mol. Immunol.* 45: 2629–2640.
- Agostinis, C., R. Bulla, C. Tripodo, A. Gismondi, H. Stabile, F. Bossi, C. Guarnotta, C. Garlanda, F. De Seta, P. Spessotto, et al. 2010. An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. *J. Immunol.* 185: 4420–4429.
- Hansen, S., S. Thiel, A. Willis, U. Holmskov, and J. C. Jensenius. 2000. Purification and characterization of two mannan-binding lectins from mouse serum. *J. Immunol.* 164: 2610–2618.
- Ihara, S., A. Takahashi, H. Hatsuse, K. Sumitomo, K. Doi, and M. Kawakami. 1991. Major component of Ra-reactive factor, a complement-activating bactericidal protein, in mouse serum. *J. Immunol.* 146: 1874–1879.
- Liu, H., L. Jensen, S. Hansen, S. V. Petersen, K. Takahashi, A. B. Ezekowitz, F. D. Hansen, J. C. Jensenius, and S. Thiel. 2001. Characterization and quantification of mouse mannan-binding lectins (MBL-A and MBL-C) and study of acute phase responses. *Scand. J. Immunol.* 53: 489–497.
- de Vries, B., S. J. Walter, C. J. Peutz-Kootstra, T. G. Wolfs, L. W. van Heurn, and W. A. Buurman. 2004. The mannose-binding lectin-pathway is involved in complement activation in the course of renal ischemia-reperfusion injury. *Am. J. Pathol.* 165: 1677–1688.
- Nakamura, A., M. Okigaki, N. Miura, C. Suzuki, N. Ohno, F. Kametani, and K. Hamaoka. 2014. Involvement of mannose-binding lectin in the pathogenesis of Kawasaki disease-like murine vasculitis. *Clin. Immunol.* 153: 64–72.
- Wagner, S., N. J. Lynch, W. Walter, W. J. Schwaeble, and M. Loos. 2003. Differential expression of the murine mannose-binding lectins A and C in lymphoid and nonlymphoid organs and tissues. *J. Immunol.* 170: 1462–1465.
- van der Pol, P., N. Schlagwein, D. J. van Gijlswijk, S. P. Berger, A. Roos, I. M. Bajema, H. C. de Boer, J. W. de Fijter, G. L. Stahl, M. R. Daha, and C. van Kooten. 2012. Mannan-binding lectin mediates renal ischemia/reperfusion injury independent of complement activation. *Am. J. Transplant.* 12: 877–887.