Placental ATPase Expression Is a Link Between Multiple Causes of Spontaneous Abortion in Mice 1

Authors: Jaiswal, Mukesh Kumar, Gilman-Sachs, Alice, Chaouat, Gerard, and Beaman, Kenneth D.

Source: Biology of Reproduction, 85(3) : 626-634

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.111.092494

BIOLOGY OF REPRODUCTION **85,** 626–634 (2011)
Published online before print 18 May 2011.
DOI 10.1095/biolreprod.111.092494

Placental ATPase Expression Is a Link Between Multiple Causes of Spontaneous Abortion in Mice[1]

Mukesh Kumar Jaiswal,[3] Alice Gilman-Sachs,[3] Gerard Chaouat,[4] and Kenneth D. Beaman[2,3]

Department of Microbiology and Immunology,[3] Chicago Medical School, Rosalind Franklin University of Medicine and Science, North Chicago, Illinois
U 782 INSERM,[4] Université Paris Sud, and Hôspital Antoine Béclère, Clamart, France

ABSTRACT

The a2 isoform of vacuolar ATPase (ATP6V0A2 referred to as a2V) plays a pivotal role in successful pregnancy and provides a microenvironment to maintain the delicate immunological balance at the feto-maternal interaction. We studied the expression of a2V mRNA in embryos and placenta of abortion-prone (female CBA × male DBA) murine matings or LPS (lipopolysaccharide)-treated mice. The expression of a2V was significantly higher in the placentas of nonabortion-prone (female BALB/c × male BALB/c and female CBA × male BALB/c) matings compared with the abortion-prone (female CBA × male DBA) mating. The expression of a2V was significantly decreased in the placentas treated with LPS in both female CBA × male DBA and female BALB/c × male BALB/c mating combinations with increased *Lif, Il1b,* and *Tnf* expression in the placenta. Decreased expression of a2V in the placenta is directly correlated with high percentages of pregnancy loss in abortion-prone mating (female CBA × male DBA) as well as in LPS-treated animals. The normal expression of placental a2V on Day 16 in the nonabortion-prone matings correlated with higher *Mcp1* (monocyte chemotactic protein 1) gene expression, markedly higher infiltration of M1 and M2 macrophages, and no significant polarization patterns (M1/M2 = 1.2–1.6). However, in the abortion-prone mating, decreased placental a2V expression correlated with significantly lower *Mcp1* gene expression with less infiltration of M1 and M2 macrophages and with polarization patterns skewed to M1 phenotypes (M1/M2 = 3.9–4.2). These data indicate that the higher expression of placental a2V is associated with dynamic infiltration of M1 and M2 macrophages through the induction of *Mcp1* expression. This strengthens our hypothesis that a2V regulates the delicate cytokine and chemokine networks that coordinate the recruitment of macrophages for successful placental development and growth at the feto-maternal interface.

a2V-ATPase, abortion-prone model, macrophages, placenta, placentation, pregnancy, preimplantation embryo, reproductive immunology

INTRODUCTION

The survival of a semiallogeneic fetus in pregnancy depends on adaptations in both the innate and adaptive responses [1, 2]. The maternal immune response is not oblivious to the paternal major histocompatibility complex and other conceptus antigens [3–5]. Feto-maternal tolerance is mediated, at least in part, by the a2 isoform of vacuolar ATPase (ATP6V0A2, referred to as a2V), which is an important molecule expressed at the feto-maternal interface. This protein appears to be one of the first molecules expressed that has important effects on the maternal immune response and is known to be responsible for the survival of a fetal allograft [6]. In early studies, we found that the a2V was uniquely and abundantly expressed in the epididymis, kidney, lung, thymus, and spleen as well as in the tissues surrounding the developing fetus [7–9]. Skinner et al. [10] showed that expression of V-ATPase subunits in the bovine endometrium was crucial for trophoblast invasion and cellular commination during the window of implantation. Additionally, the acidic environment regulated by V-ATPase is important for the development of preimplantation embryo following implantation [11]. Our studies have shown that this molecule has profound effects on cytokine expression and may be a key factor in the early control of the inflammatory process necessary for implantation [6]. Our studies also showed that antibodies to a2V could block the process of implantation [12].

Previously, we have shown that a2V-ATPase can regulate IL1B as well as IL1A without a subsequent increase in TNF secretion, which appears to occur by two different mechanisms. The first is a2V expression on the cell surface, where it functions as an ATPase preventing the danger-associated molecular pattern (DAMP) ATP from activating the inflammasome through the P2X7 channel [13]. ATP binding to P2X7 causes the efflux of potassium ions and the activation of the inflammasome [14]. The second is the cleavage and release of a2NTD (N-terminal portion of a2V) from the a2V polypeptide. This peptide is responsible for the selective increase in transcription and secretion of several different cytokines such as IL1B, IL1A, LIF, IL10, and chemokine (MCP1/CCL2), which are important in maintaining the immune response in pregnancy [6, 15, 16].

The hypothesis that drives this study is based on our previous study where we showed that a2NTD induces MCP1 [16]. It has been suggested by animal models that the secretion of MCP1 amplifies the recruitment of monocytes along with macrophages and other immune effector cells into the reproductive tissues [17–19]. Tissue macrophages can exist in different activation states: either proinflammatory classically activated by interferon-γ, known as M1, or anti-inflammatory alternatively activated by IL13 or IL4, known as M2. The activity of M1 and M2 depend on microenvironmental stimuli. M1- and M2-activated macrophages fulfill different functions through the production of pro- or anti-inflammatory factors [20]. However, it is not clear whether decidual macrophages represent M1 or M2 macrophages [21]. Because of their role in immune regulation, macrophages have multiple functions

[1]Supported by Clinical Immunology, Rosalind Franklin University of Medicine and Science, North Chicago, IL
[2]Correspondence: Kenneth D. Beaman, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, N. Chicago, IL 60064. FAX: 847 775 6506; e-mail: kenneth.beaman@rosalindfranklin.edu

Received: 25 March 2011.
First decision: 19 April 2011.
Accepted: 2 May 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

throughout pregnancy and contribute to local immune tolerance in normal pregnancy, possibly by phagocytosis of apoptotic bodies that can potentially harm the fetus [22]. Temporal expression of anti-inflammatory cytokines is evident at the feto-maternal interface, suggesting an environment capable of facilitating an alternative activation of macrophages [21].

A successful pregnancy relies not only on maternal tolerance to the fetus but also on the unique properties of trophoblast cells. The trophoblast cells are decisive for appropriate materno-fetal interactions. Dysfunction of the trophoblast has been associated not only with spontaneous abortions but also with preeclampsia and fetal growth restriction [23–26]. We have demonstrated that a2V is a crucial molecule at the feto-maternal interface, where maternal immune cells come in close contact with rapidly dividing trophoblast cells that play key roles in the implantation, placentation, and development of the embryo [6]. In the present study, abortion-prone murine models that share features with human recurrent miscarriage and fetal growth restriction [27] were used to study the role of a2V in the development of preimplantation embryos and placentation.

MATERIALS AND METHODS

Mice

Inbred strains of CBA/J (H-2^k) and BALB/c (H-2^d) female mice and DBA/2 (H-2^d) and BALB/c (H-2^d) male mice (8 wk, 18–20 g) were obtained from The Jackson Laboratory and subsequently maintained in Biological Resource Facility of Rosalind Franklin University of Medicine and Science. Mice were usually maintained for 2 wk in the animal facility before use. The housing and handling of experimental animals were in accordance with guidelines of the Institutional Animal Care and Use Committee. Females were caged individually overnight with a proven fertile male for mating. The initiation of pregnancy was marked by the presence of the postcoital vaginal plug (Day 1 of pregnancy).

Embryo Collection

Embryos were recovered from pregnant females of nonabortion-prone (♀ BALB/c × ♂ BALB/c) and abortion-prone (♀ CBA × ♂ DBA) matings. The females were selected for the retrieval of zygotes from their oviducts between 1400 h and 1500 h on the day of the appearance of vaginal plugs, that is, Day 1, ~16 h postcoitus (pc), in a sterile Petri dish with sterile PBS. The 2-cell embryos were recovered from oviducts between 1400 h and 1500 h on the day following the appearance of vaginal plugs, that is, Day 2, ~40 h pc. The compact morula and blastocysts were recovered from the uterus on Day 4 at ~84 and 90 h pc, respectively. The same preimplantation embryos were used for further studies.

Recovery of the Fetus and Placenta at Different Time Points

The fetuses and placentas were collected from pregnant females obtained from nonabortion-prone (♀ BALB/c × ♂ BALB/c and CBA × BALB/c), abortion-prone (♀ CBA × ♂ DBA), LPS (lipopolysaccharide)-treated ♀ BALB/c × ♂ BALB/c, and LPS-treated ♀ CBA × ♂ DBA matings at different days (i.e., Days 8, 12, and 16) of pregnancy. Intraperitoneal (i.p.) injection of 1 or 3 μg LPS from *Salmonella enteritidis* (Sigma Chemicals Co.) in 100 μl sterile PBS was given on Days 8 and 12 of pregnancy for 12 and 16 days, respectively. The recovered tissues were flash frozen and stored at −80°C for RNA exaction or fixed with 4% paraformaldehyde for immunohistochemistry. On Days 12 and 16 of pregnancy, the percentage of resorption was calculated by the following formula: $\%R = Re/(Re + F) \times 100$, where R is the percentage of resorption relative to the total number of effective implantation sites, Re is the number of the resorbed embryos, and F is the number of viable embryos, which was determined as previously described [28].

Real-Time PCR

Embryo. The gene expression of a2V in the different stages preimplantation embryos was analyzed by Taqman gene expression Cell-to-CT kit (Ambion) according to the manufacturer's protocol with slight modifications. A total 25 embryos of each stage were collected, washed, resuspended in 10 μl sterile PBS, and used to prepare cell lysates for RT-PCR. Multiplex real-time PCR was performed for a2V and normalized to the *Gapdh* housekeeping gene by TaqMan analysis using the Applied Biosystems StepOne Real-Time PCR system according to the manufacturer's instructions. The prevalidated Taqman gene expression assays for *Atp6v0a2* (Mm00441848_m1) and *Gapdh* (part number 4352339E) were purchased from Applied Biosystems.

Fetus and placenta. Total cellular RNA was extracted by the Melt Total nucleic acid isolation system (Ambion) according to the manufacturer's protocol. The quantity and integrity of the RNA were confirmed by the ratio at 260 nm to 280 nm and by electrophoresis on 1.5% native agarose gel to visualize of 18S and 28S ribosomal RNA subunits. Samples were stored at −80°C until further use. The cDNA was generated from 1 μg of total cellular RNA by reverse transcription at 50°C for 60 min using the random hexamer primer of transcription first-strand cDNA synthesis kit (Roche Diagnostics GmBH). Multiplex real-time PCR was performed for a2V, *Il1b, Tnf, Il10,* and *Lif* as described above. The real-time PCR was performed using the universal PCR master mix reagent (Applied Biosystems) according to the manufacturer's instructions. The prevalidated Taqman gene expression assays for *Atp6v0a2* (Mm00441848_m1), *Mcp1* (Mm00441242_m1), *Il1b* (Mm00434228_m1), *Tnf* (Mm00443258_m1), *Lif* (Mm00434761_m1), and *Gapdh* (part number 4352339E) were purchased from Applied Biosystems. The experiments were repeated at least three times.

Immunocytochemistry

Embryos were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, treated with 0.1% Triton X-100 and 3% bovine serum albumin (BSA) in PBS for 10 min at room temperature, and placed in a 1:100 dilution (20 μg/ml) of a mouse monoclonal antibody to a2V (named 2C1; Covance) in 1% BSA in PBS (BSA-PBS) overnight at 4°C. After washing with BSA-PBS, the specimens were incubated with secondary antibody, fluorescein isothiocyanate rabbit polyclonal anti-mouse immunoglobulin G (IgG) (DAKO), at a 1:20 dilution in BSA-PBS for 45 min at room temperature. To visualize the nuclei, the embryos were counterstained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). After mounting the specimens on slides with vectashield, the antigen distribution was examined under a Nikon eclipse TE2000-S florescence microscope (Nikon Instrument Inc.).

Immunohistochemistry

The fetuses and placentas were fixed in 4% paraformaldehyde in PBS at 4°C overnight. Tissue was rinsed three times in PBS for 5 min with rocking and infused with 30% sucrose solution at 4°C overnight or until the tissue sank. The tissues were snap frozen in optimum compound temperature (Tissue-Tek) by liquid nitrogen and stored at −80°C until used. Frozen 5-μm sections from frozen tissues were mounted onto saline-coated glass slides (DAKO) and stored at −80°C until used. The DAKO EnVision+ System-HRP was used to stain the frozen sections according to the manufacturer's instructions with slight modification. For primary antibody reaction, sections were incubated in a 1:100 dilution (20 μg/ml) of mouse monoclonal antibody to a2V in 1% BSA-PBS for 1 h. After washing, the sections were incubated with secondary antibody labeled with polymer-HRP anti-mouse IgG1 (DAKO). The 3,3′-diaminobenzidine chromogen was used as substrate for the EnVision+ System-HRP according to the manufacturer's instructions. A mouse IgG1 isotype control (Abcam) was used at the same concentration as the primary antibody. The sections were counterstained with Meyer hematoxylin and mounted in faramount aqueous mounting medium (both from DAKO). The immune-staining of a2V was evaluated in the image generated by light microscopy (Carl Zeiss) and high-resolution camera (Canon G10). The tissue immunostaining results were scored negative if no immunopositive tissue was present. The total score was based on the percentage of stained tissue and immunostaining intensity. The immunostaining index score (ISIS) was calculated according to the method described in Teixeira et al. [29].

Antibodies targeted to the M1 subtype (ITGAX) and M2 subtype (MRC1) [30, 31] were used at 1:100 and 1:25 dilutions, respectively (Abcam). Immunohistochemistry was performed as mentioned above with slight modification. The numbers of macrophages were counted in at least 10 randomly chosen areas per placental section at 40× magnification. Four animals for each mating group were analyzed with six sections per animal.

Statistical Analyses

The results of each experiment were analyzed by one-way ANOVA with the Duncan multiple range test for comparison of the significance level between groups; $P < 0.05$ was considered significant.

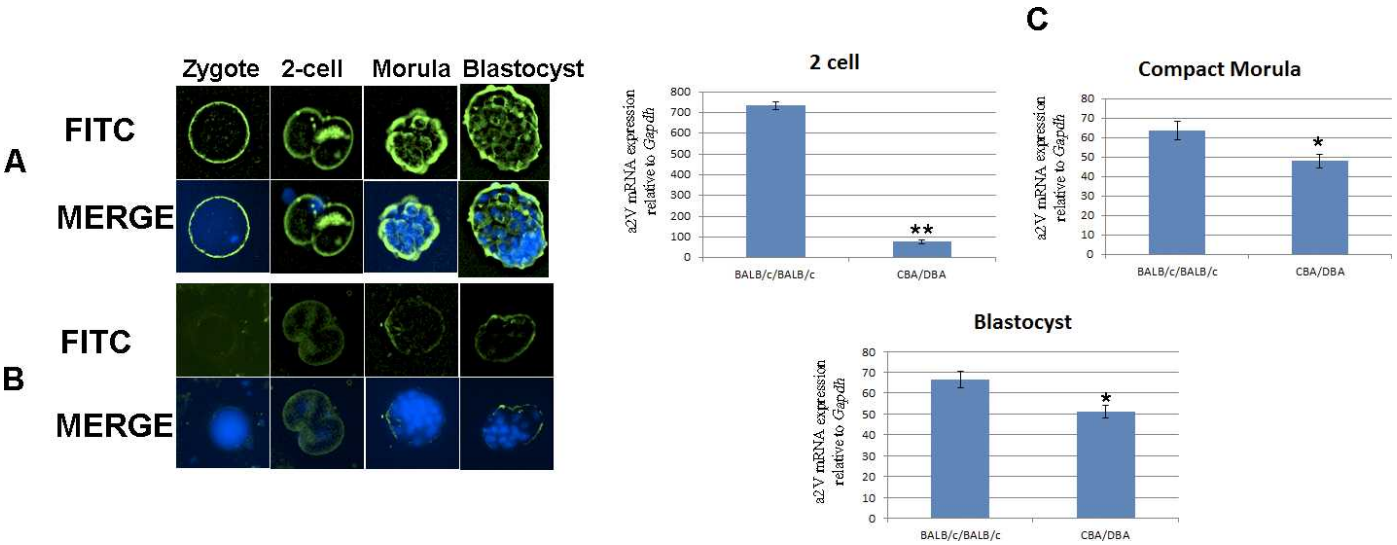

FIG. 1. Immunolocalization of a2V at different stages of preimplantation in embryos recovered from different mating combinations. **A**) Localization of a2V in preimplantation embryos from the nonabortion-prone mating (♀ BALB/c × ♂ BALB/c). **B**) Localization of a2V in preimplantation embryos from abortion-prone mating (♀ CBA × ♂ DBA); magnification ×10. Green indicates a2V; blue, DAPI for nuclear staining. **C**) Analysis of gene expression of a2V in different stages of preimplantation embryos recovered from different mating combinations. Data is expressed in mean ± SEM. *Significant difference vs. BALBc/BALBc; $P < 0.05$. **Significant difference vs. BALBc/BALBc; $P < 0.01$.

RESULTS

Expression of a2V in Preimplantation Embryos

Real-time PCR and immunocytochemistry studies showed that the expression of a2V was significantly lower in the embryos collected from the abortion-prone ♀ CBA × ♂ DBA mating (Fig. 1, B and C) at the different developmental stages of preimplantation compared to the ♀ BALB/c × ♂ BALB/c mating (Fig. 1, A and C). The expression of the a2V gene starts with zygotic gene activation. The highest level of a2V expression was observed in the 2-cell stage collected on Day 2 of pregnancy followed by decrease in the compact morula collected on Day 4 of pregnancy. This level of a2V gene expression was maintained in the blastocyst recovered from the normal mating combination (♀ BALB/c × ♂ BALB/c) (Fig. 1C). Immunocytochemistry studies clearly demonstrated that a2V was predominantly localized on cell surface of zygote and 2-cell embryos. In the blastocyst, a2V expression was highly localized in the trophoblast cells with less being observed in the inner cell mass (Fig. 1A).

Expression of a2V in the Placenta During Different Days of Pregnancy

In the ♀ CBA × ♂ DBA mating as well as mice treated with LPS, the decreased expression of placental a2V directly correlated with the abortion rate (Table 1). The decrease in placental a2V mRNA expression correlates linearly with the percentage of fetal resorption. The percentages of fetal resorption were plotted against the relative mRNA expressions of placental a2V, and linear regression analysis yielded R^2 values of 0.9468 (Day 12) and 0.9162 (Day 16) (Fig. 2, A and B).

The mRNA levels of a2V increased as the placenta developed from Day 12 to 16 in all the mating combinations studied. The gene expression of a2V was higher in the placenta of the nonabortion-prone mating (♀ BALB/c × ♂ BALB/c) (Day 12: 87.46 ± 3.91; Day 16: 99.26 ± 4.23) compared to the abortion-prone mating (♀ CBA × ♂ DBA) (Day 12: 68.14 ± 4.98, $P < 0.035$; Day 16: 82.82 ± 5.37, $P < 0.0495$). The expression of a2V was also significantly higher in placenta of ♀ CBA × ♂ BALB/c (Day 12: 91.92 ± 4.64 ; Day 16: 106.38 ± 8.12) compared to ♀ CBA × ♂ DBA (Table 1).

The mRNA levels of a2V were significantly decreased in the placentas from mice treated with LPS in all the mating combinations tested as compared to their respective controls. However, in LPS-treated ♀ BALB/c × ♂ BALB/c mating combination on Days 12 and 16, a2V expression was less effected than in the LPS-treated ♀ CBA × ♂ DBA mating combination (Table 1). In all the groups, the amount of a2V detected in the placenta appeared to be dependent on the male used in the mating pair rather than the female (Table 1).

The mRNA levels of *Il1b, Tnf,* and *Lif* were significantly increased in the placenta recovered from both LPS-treated ♀ CBA × ♂ DBA and LPS-treated ♀ BALB/c × ♂ BALB/c mating combinations compared to controls. However, significantly higher levels of *Il1b, Tnf,* and *Lif* message were observed in LPS-treated ♀ CBA × ♂ DBA placenta compared to LPS-treated ♀ BALB/c × ♂ BALB/c placenta (Supplemental Fig. S1, A–C; all the supplemental data are available online at www.biolreprod.org). *Il10* mRNA level was not detected in the placenta of any of the groups studied.

Distribution of a2V in the Placenta on Days 12 and 16 of Pregnancy

Because the placenta consists of heterogeneous cell types that undergo dynamic changes to support the fetus development, the spatial expression of a2V protein was examined by immunohistochemistry. Immunohistological staining in placenta recovered from the different mating combinations showed that on Day 12 the density of a2V expression in spongiotrophoblast was reduced in the abortion-prone ♀ CBA × ♂ DBA mating (ISIS = 4.75 ± 0.74; $P \leq 0.0048$) when compared to other nonabortion-prone mating combinations (Table 1 and Fig. 3, C, E, and G), and while a2V expression increased from Day 12 to 16 (ISIS = 4.75 ± 0.74 to 5.93 ± 0.63, respectively), it was significantly ($P \leq 0.031$) lower than

in the nonabortion-prone mating combinations (Table 1 and Fig. 3, D, F, and H). On Day 16, the expression of a2V protein in spongiotrophoblast of ♀ BALB/c × ♂ BALB/c mating (ISIS = 10.80 ± 0.61) was similar to that of ♀ CBA × ♂ BALB/c mating (ISIS = 10.40 ± 0.98) (Table 1 and Fig. 3, D and F).

These findings show that the density of a2V-positive spongiotrophoblasts was higher in the placenta collected from nonabortion-prone matings than the abortion-prone matings. As seen in the mRNA and protein localization results, the male contribution appears to be more important for the expression of a2V: the expression of a2V protein in spongiotrophoblast was much higher when the BALB/c male rather than a DBA male was used in mating with females. A decrease in fetal viability may be associated with the lower density of a2V-positive spongiotrophoblast in the abortion-prone models.

Infiltration of M1 and M2 into the Placenta on Days 12 and 16 of Pregnancy

To further investigate the possible role of differential a2V expression among normal and abortion-prone mating combinations (Table 1), we tested the expression of *Mcp1*/*Ccl2*, which is known to be induced by a2V. The increased expression of a2V in Day 16 placenta in nonabortion-prone matings (Table 1) corresponded with increased gene expression of *Mcp1* compared to the abortion-prone mating (Fig. 4A). The level of expression of *Mcp1* was significantly increased from Day 12 to 16 in the placenta recovered from the nonabortion-prone matings whereas that increase was not observed in the abortion-prone mating. By using immunohistochemistry, we examined the infiltration and polarization of M1, that is, ITGAX$^+$ (Fig. 5), and M2, that is, MRC1$^+$ (Fig. 6), macrophages in the placenta recovered from the different mating combinations. The number of M1 and M2 macrophages was significantly increased from Day 12 to 16 in the placenta recovered from the nonabortion-prone matings compared to the abortion-prone mating (Figs. 4, B and C, 5, and 6). There was a significantly lower expression of a2V in the placenta of abortion-prone matings that corresponded to a significantly lower *Mcp1* expression and decreased infiltration of M1 and M2 macrophages when compared to the normal mating combinations (Fig. 4, B and C). There was no statistically significant polarization pattern of macrophages observed in the nonabortion-prone matings on either Day 12 or 16 (M1/M2 = 1.2–1.6). However, in the abortion-prone mating, the polarization patterns of placental macrophages mainly had M1 phenotypes on Day 16 (M1/M2 = 3.9–4.2).

Expression of a2V in the Fetus During Different Days of Pregnancy

The fetuses collected during different gestation periods from females of nonabortion-prone (♀ BALB/c × ♂ BALB/c and ♀ CBA × ♂ BALB/c) and abortion-prone (♀ CBA × ♂ DBA) mating combinations were used to study the gene expression of a2V, *Il1b, Tnf,* and *Il10* by multiplex real-time PCR. No significant differences in a2V mRNA were observed between syngeneic and allogeneic pregnancies (Supplemental Fig. S2).

Distribution of a2V in the Uterus on Day 8 of Pregnancy

Immunohistochemical analysis was performed to examine the expression and localization of a2V protein in the implanted embryos and uterine decidua of the different mating combinations. On Day 8, a2V was strongly localized to the

TABLE 1. Decrease in relative mRNA expression and ISIS[a] of a2V in placenta correlated with percentage of fetal resorption.[b]

Mating combinations	Relative mRNA expression and [ISIS] of a2V on Day 12	Relative mRNA expression and [ISIS] of a2V on Day 16	Percentage of fetal resorption on Day 12	Percentage of fetal resorption on Day 16
Female BALBc × male BALBc	87.46 ± 3.91 [11.00 ± 0.50]	99.26 ± 4.23 [10.80 ± 0.61]	0.036 ± 0.03	1.95 ± 0.75
Female CBA × male BALBc	91.92 ± 4.64 [8.00 ± 0.71]	106.38 ± 8.12 [10.40 ± 0.98]	0.00 ± 0.00	3.67 ± 1.69
Female CBA × male DBA	68.14 ± 4.98[c] [4.75 ± 0.74[c]]	82.82 ± 5.37[c] [5.93 ± 0.0.63[c]]	33.00 ± 4.20[d]	37.00 ± 3.60[d]
Female BALBc × male BALBc LPS- treated (1 μg LPS i.p.)	78.29 ± 2.95	74.20 ± 3.78[c]	14.10 ± 1.60[d]	45.20 ± 5.84[d]
Female BALBc × male BALBc LPS- treated (3 μg LPS i.p.)	31.45 ± 2.10[d]	59.11 ± 1.3[d]	100.00 ± 0.00[d,e]	100.00 ± 0.00[d,e]
Female CBA × male DBA LPS- treated (3 μg LPS i.p.)	7.30 ± 0.25[d]	38.27 ± 13.73[d]	100.00 ± 0.00[d,e]	100.00 ± 0.00[d,e]

[a] Immunostaining index score (ISIS) = Stained area score (SAS) × Immunostaining intensity score (IIS).
[b] Data is expressed in mean ± SEM.
[c] Significant difference vs. BALBc/BALBc; $P < 0.05$.
[d] Significant difference vs. BALBc/BALBc; $P < 0.01$.
[e] Same on both Day 12 and Day 16.

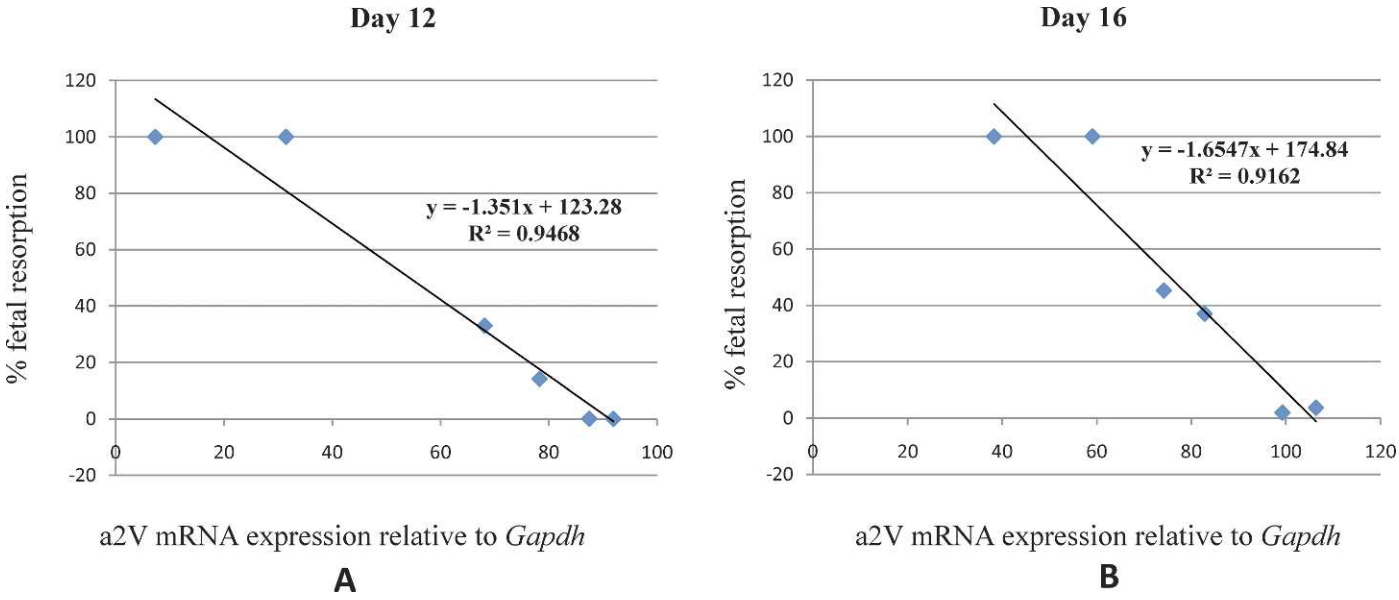

FIG. 2. Linear regression analysis of percent fetal resorption correlates with a2V mRNA expression on Days 12 (**A**) and 16 (**B**) of placenta. $R^2 \geq 0.4$ is statistically significant. Each point shows the mean of four animals.

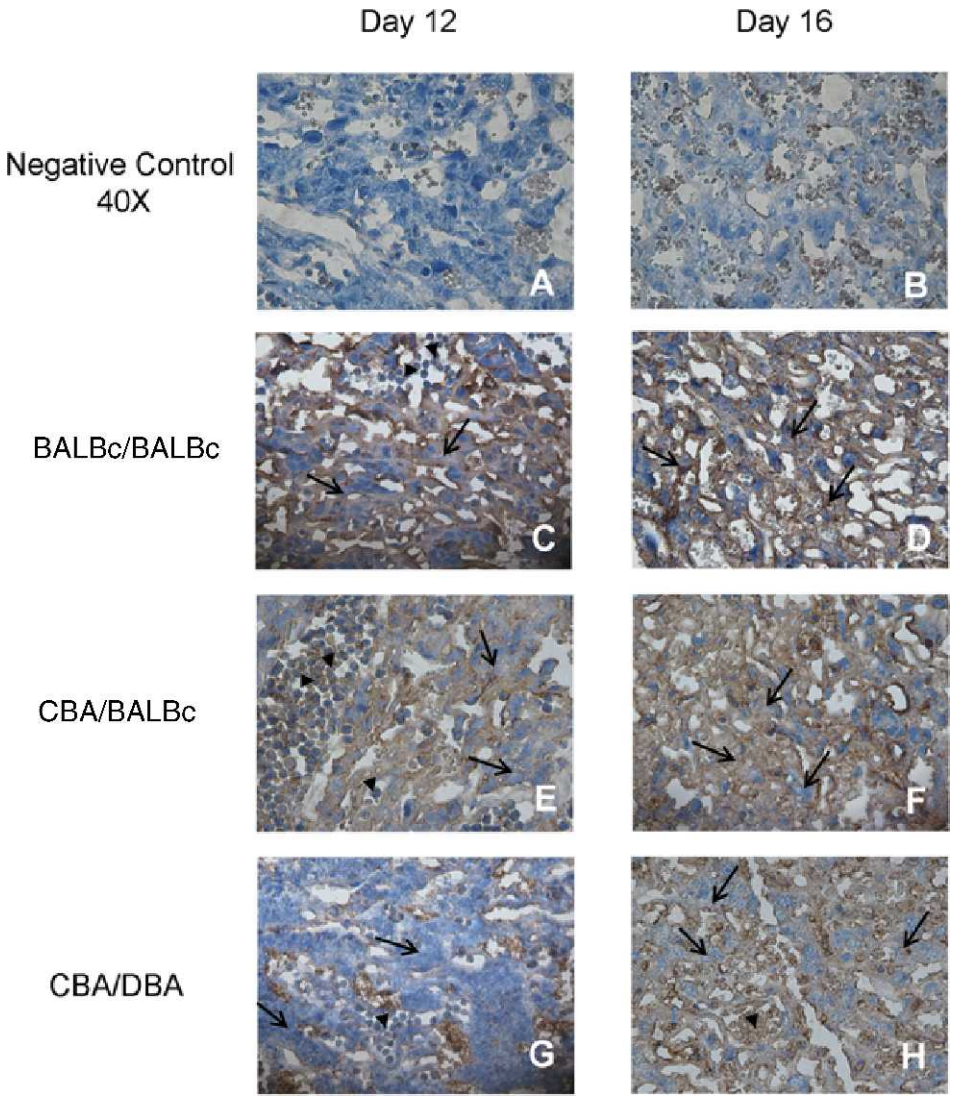

FIG. 3. Distribution of a2V protein in mouse placenta on Days 12 and 16 of pregnancy in different mating combinations. **A**, **B**) Negative controls for Days 12 and 16, respectively. **C**, **E**, and **G**) Localization of a2V in spongiotrophoblast of Day 12 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. **D**, **F**, and **H**) Localization of a2V in spongiotrophoblast of Day 16 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. Magnification ×40; n = 4 for each mating group. Arrow indicates spongiotrophoblast; arrowhead indicates leukocyte.

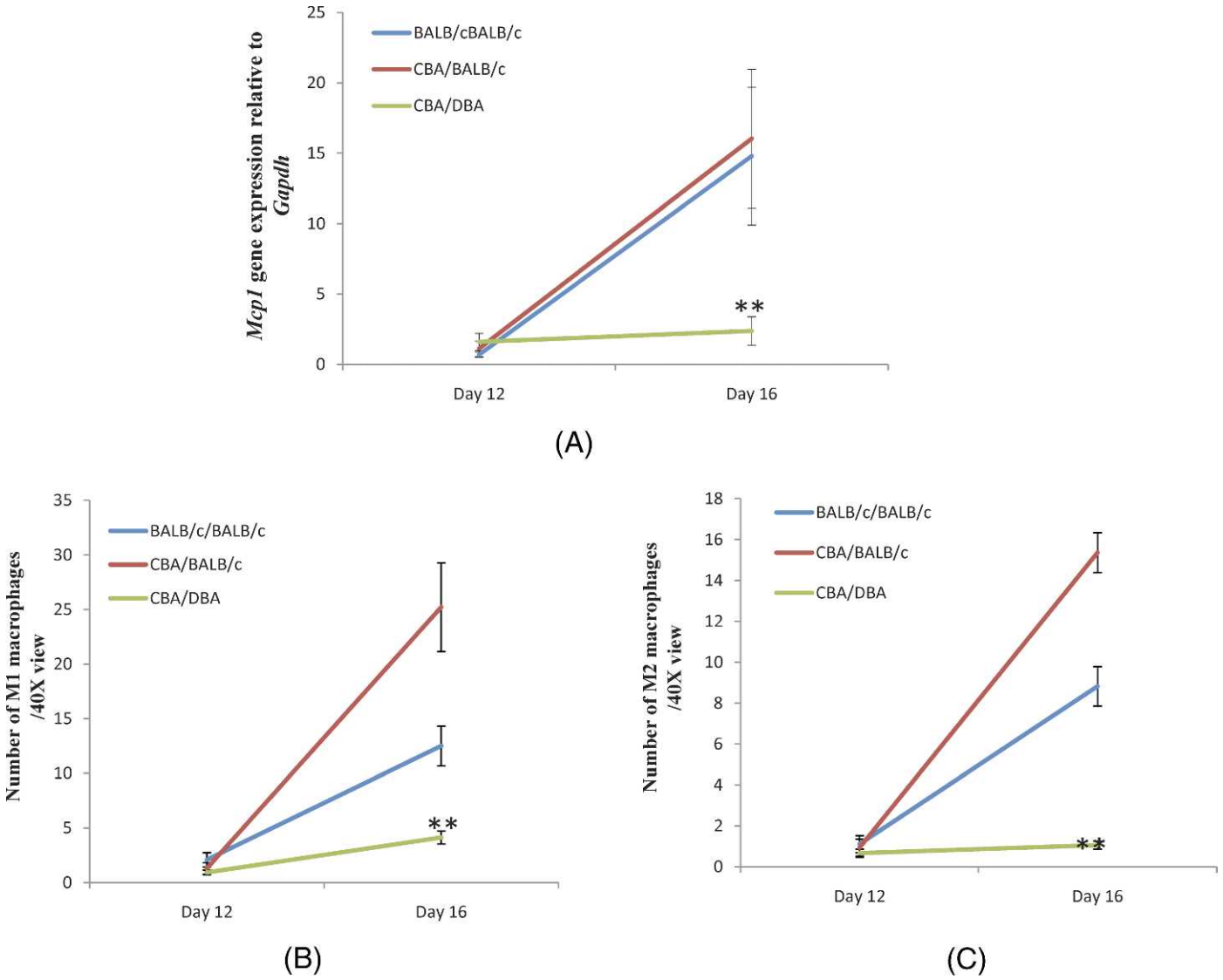

FIG. 4. **A**) Analysis of gene expression of *Mcp1* in placenta recovered from different mating combinations on Days 12 and 16. The gene expression was analyzed by real-time PCR. **B** and **C**) Quantification of M1 and M2 macrophages, respectively, in the placenta from different mating combinations on Days 12 and 16. Data is expressed in mean ± SEM. **Significant difference vs. BALBc/BALBc and CBA/BALB/c; $P < 0.01$; n = 4 for each mating group.

myometrial in all the groups studied (Supplemental Fig. S3, I, J, and K). In all the mating combinations, the a2V protein was abundantly expressed in both stromal and epithelial layers with a diffuse distribution pattern. In contrast, a2V protein was detectable only to a limited extent in primary and secondary decidual cells of all the mating combinations (Supplemental Fig. S3, L, M, and N). The expression of a2V protein was the most intense surrounding the implanted embryos (Supplemental Fig. S3, F, G, and H). This localization of a2V expression may be required to maintain the delicate immunological balance between invading trophoblasts and maternal decidua.

DISCUSSION

In this study, we examined a2V expression in four different mouse models of pregnancy. The first is the syngeneic (nonabortion-prone mating) ♀ BALB/c × ♂ BALB/c model, which yields large litter sizes and has no allogeneic immune response. The second is the allogeneic mating (nonabortion-prone mating) with the ♀ CBA × ♂ BALB/c combination. The third is the abortion-prone model of ♀ CBA × ♂ DBA mating that shares many features with human recurrent miscarriage, fetal growth restriction, and preeclampsia [27, 32, 33]. The fourth is the administration of LPS to induce or amplify the rate of abortion. We examined a2V expression in the developing preimplantation embryos, fetus, and placenta with various cytokines and chemokines known to be important in the maintenance of the immune response in pregnancy.

We began this study by demonstrating that antibodies to a2V has the anticipated effects on pregnancy outcome in mice. In BALB/c females, monoclonal antibody to a2V (2 μg i.p. on Days 3, 4, and 5 of pregnancy) results in 60% abortion rate (Supplemental Fig. S4), which is in agreement with previous finding that the presence of antibody to a2V ablated pregnancy [12]. This finding suggests that changes in a2V concentration may correlate directly with pregnancy outcome. The developing blastocyst differentiates into two distinct cell line inner cell masses that develop into the fetus and trophoblast, which in turn develops into the placenta and external membrane [34].

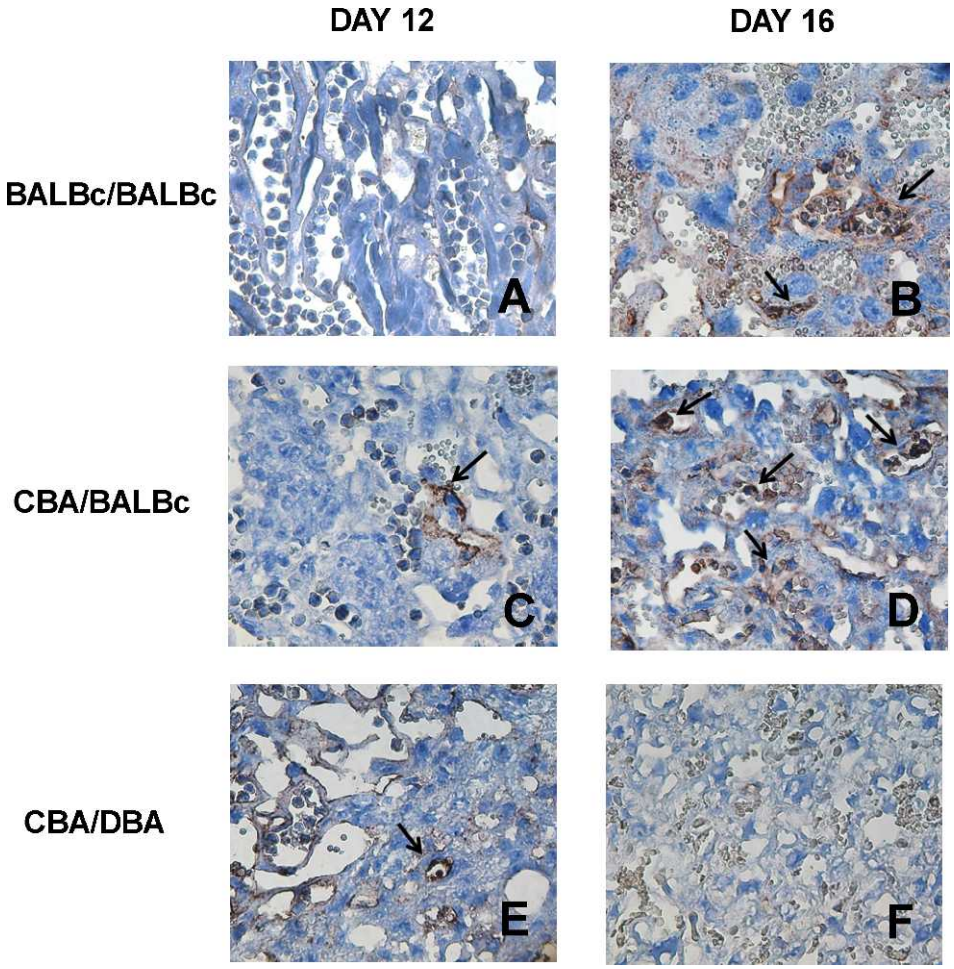

FIG. 5. Distribution of M1 macrophages (black arrow) in mouse placenta on Days 12 and 16 of pregnancy in different mating combinations. **A**, **C**, and **E**) Localization of ITGAX$^+$ (M1) macrophages on Day 12 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. **B**, **D**, and **F**) Localization of ITGAX$^+$ (M1) macrophages on Day 16 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. Magnification ×40; n = 4 for each mating group.

Our studies clearly show that a2V expression starts with zygotic gene activation. The expression of a2V is higher in the trophoblasts of developing blastocysts recovered from the nonabortion-prone ♀ BALB/c × ♂ BALB/c mating combination than the abortion-prone mating combination. Once the blastocyst makes intimate contact with the uterine endometrium, the trophoblast cells invade the endometrium, which begins the process of placentation. A high intensity of invasion of trophoblast is required for the progression of placentation [35]. Any dysregulation in this event results in significant pregnancy loss [36]. A decreased a2V expression in invasive trophoblasts is observed in the abortion-prone mating, which does not affect the process of implantation but may be involved in improper development of the placenta followed by a high percentage of fetal loss. The same pattern of decreased a2V expression is observed in the placenta of Days 12 and 16 recovered from the abortion-prone mating when compared to the normal mating combination studied.

Trophoblast cells, like tumors, display the unique capability to invade their surrounding tissues [35–38]. We have previously reported that a2V is present on the surface of many different types of tumor cells. Various studies have highlighted that a2V promotes tumor invasion and malignancy [39]. Lee et al. [40] showed that a2V expression induces IL10 production in human placenta. Our immunohistochemistry results show that a2V protein is highly expressed in the spongiotrophoblast on both Days 12 and 16, which suggests that this protein may promote the growth and migration of spongiotrophoblast cells by regulating the delicate cytokine and chemokine network for successful pregnancy outcome. Interestingly, a2V has been found at the peripheral layer in both human and murine cytotrophoblasts [41].

The expression of placental a2V is decreased in ♀ CBA × ♂ DBA mating pairs as well as in mice treated with LPS and correlates directly with high abortion rates. The decrease in a2V levels in the spongiotrophoblast of ♀ CBA × ♂ DBA mating suggests that it may cause the dysregulation of maternal immune tolerance to the spongiotrophoblasts, leading to poor pregnancy outcome. In the normal pregnancy models, the higher expression of a2V and specifically the higher density of a2V localization to spongiotrophoblast strongly suggest the importance of a2V in pregnancy. These data clearly demonstrate that a2V may regulate the growth and invasion of spongiotrophoblast as well as maintain the delicate cytokine and chemokine balance that accompanies a successful pregnancy outcome. In this study, we also observe that a2V expression gradually increases in the fetus from Day 8 to 16 in all the mating combinations studied. Collectively, this suggests that the high rates of fetal loss in the abortion-prone matings are due to low placental a2V expression and not fetal a2V expression.

Our data also show that higher expression of a2V in the placenta recovered from normal mating combinations corresponds with higher levels of *Mcp1* expression, while in the abortion-prone mating, lower placental a2V expression corresponds with lower levels of *Mcp1* expression. *Mcp1* is known

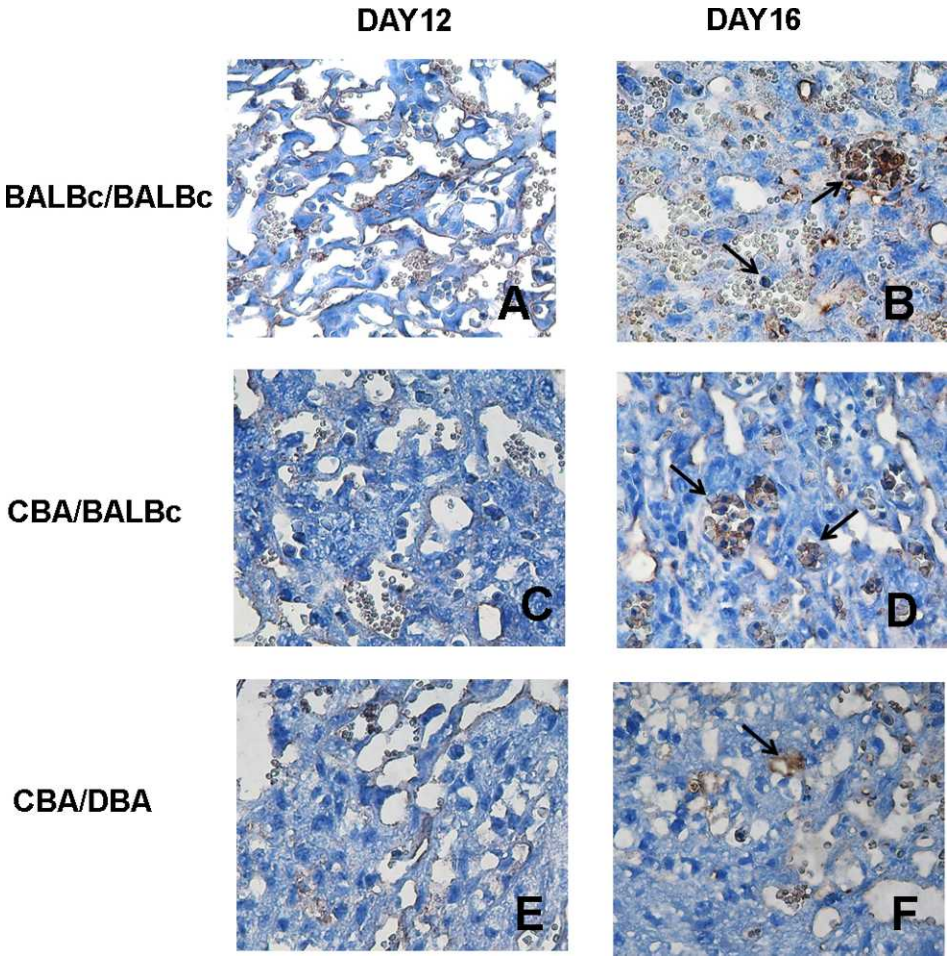

FIG. 6. Distribution of M2 macrophages (black arrow) in mouse placenta on Days 12 and 16 of pregnancy in different mating combinations. **A**, **C**, and **E**) Localization of $MRC1^+$ (M2) macrophages on Day 12 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. **B**, **D**, and **F**) Localization of $MRC1^+$ (M2) macrophages on Day 16 placenta recovered from ♀ BALB/c × ♂ BALB/c, ♀ CBA × ♂ BALB/c, and ♀ CBA × ♂ DBA matings, respectively. Magnification ×40; n = 4 for each mating group.

for coordinating the recruitment of macrophages in reproductive tissues [19]. The quantification of M1 and M2 in this study show that the infiltration of these two types of macrophages is significantly higher in the placenta of normal mating combinations than in the abortion-prone mating. These findings clearly suggest that higher levels of placental a2V trigger *Mcp1* expression, which in turn leads to the infiltration of M1 and M2 macrophages and successful pregnancies. A polarization pattern of M1 and M2 is not observed in the placental macrophages of normal mating combinations. The increased infiltration of both M1 and M2 macrophages suggests a physiological role for both pro- and anti-inflammatory macrophages, and both are required for the healthy growth and development of the placenta. However, in abortion-prone matings, significantly lower expression of *Mcp1* limits the infiltration of both M1 and M2 macrophages; the polarization pattern displays mainly an M1 phenotype. Li et al. [42] reported that MCP-1 promotes tumor growth and invasion by favoring the M2 phenotype. Our previous study [16] showed that a2V was found on the surface of many solid tumors and its cleaved peptide 2NTD led to upregulation of several genes/proteins involved in M2 polarization. So the decreased expression of both a2V and *Mcp1* in the placenta of abortion-prone matings may skew the polarization pattern to an M1 phenotype. This pattern at the microenvironment of the feto-maternal interface appears to induce more inflammatory processes and is deleterious to placental growth and development.

It has been reported that male semen contributes to the events leading to the establishment of maternal tolerance at the outset of pregnancy [43, 44]. We have shown that the a2V expression in preimplantation embryos and placenta are much higher when BALB/c males were used than when DBA male are used. This finding may underscore the importance of the male contribution to a2V expression. It can be postulated that a low paternal contribution of a2V may be responsible for the severe pregnancy complications observed in mothers who suffer from recurrent miscarriages. This finding also suggests that the observed low expression of a2V in ♀ CBA × ♂ DBA mating could be corrected during pregnancy by an increase in a2V from BALB/c males.

The differences in the abortion models in this study are very remarkable. First, we showed that the amount of a2V correlates directly with the number of fetuses seen. Second, in the abortion-prone mating, the decrease in placental a2V resulted in significantly less infiltration of M1 and M2 macrophages, which was associated with an absence of viable embryos. Third, the LPS-treated placentas had the lowest level of a2V expression as well as higher levels of proinflammatory cytokines than normal controls. Taken together, these findings imply that a2V plays a pivotal role in the maintenance of the delicate immunological balance required for successful pregnancies: a2V can regulate part of the invasion of immune cells and maintain the delicate cytokine and chemokine network that attenuates the potentially harmful maternal immune response against the developing conceptus.

REFERENCES

1. Trowsdale J, Betz AG. Mother's little helpers: mechanisms of maternal-fetal tolerance. Nat Immunol 2006; 7:241–246.
2. Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. J Immunol 2007; 178:3345–3351.
3. Tafuri A, Alferink J, Moller P, Hammerling GJ, Arnold B. T cell awareness of paternal alloantigens during pregnancy. Science 1995; 270:630–633.
4. Jiang SP, Vacchio MS. Multiple mechanisms of peripheral T cell tolerance to the fetal ''allograft.'' J Immunol 1998; 160:3086–3090.
5. Zhou M, Mellor AL. Expanded cohorts of maternal CD8+ T-cells specific for paternal MHC class I accumulate during pregnancy. J Reprod Immunol 1998; 40:47–62.
6. Ntrivalas E, Levine R, Kwong C, Gilman-Sachs A, Beaman K. The a2 isoform of vacuolar ATPase is a modulator of implantation and feto-maternal immune tolerance in early pregnancy. J Reprod Immunol 2010; 85:106–111.
7. Kang JA, McBey BA, Angkachatchai V, Croy BA, Beaman KD. Expression of TJ6 during pregnancy. Am J Reprod Immunol 1997; 38:183–188.
8. Lattuada D, Mangioni S, Vigano P, Ntrivalas EI, Rossi M, Palotti F, Carinelli S, Beaman KD, Di Blasio AM. The placental immunomodulatory cytokine regeneration and tolerance factor is also expressed by both human cycling and early pregnant endometrium. Am J Reprod Immunol 2004; 52:224–231.
9. Babichev Y, Tamir A, Park M, Muallem S, Isakov N. Cloning, expression and functional characterization of the putative regeneration and tolerance factor (RTF/TJ6) as a functional vacuolar ATPase proton pump regulatory subunit with a conserved sequence of immunoreceptor tyrosine-based activation motif. Int Immunol 2005; 17:1303–1313.
10. Skinner MA, MacLaren LA, Wildeman, AG. Stage-dependent redistribution of the V-ATPase during bovine implantation. J Histochem Cytochem 1999; 47:1247–1254.
11. Sun-Wada G, Murata Y, Yamamoto A, Kanazawa H, Wada Y, Futai M. Acidic endomembrane organelles are required for mouse postimplantation development. Dev Biol 2000; 228:315–325.
12. Ribbing SL, Hoversland RC, Beaman KD. T-cell suppressor factors play an integral role in preventing fetal rejection. J Reprod Immunol 1988; 14:83–95.
13. Derks RA, Beaman KD. Regeneration and tolerance factor prevents bystander T-cell death associated with human immunodeficiency virus infection. Clin Diagn Lab Immunol 2004; 11:835–840.
14. Kahlenberg JM, Dubyak GR. Mechanisms of caspase-1 activation by P2X7 receptor-mediated K+ release. Am J Physiol Cell Physiol 2004; 286:C1100–C1108.
15. Ntrivalas E, Derks R, Gilman-Sachs A, Kwak-Kim J, Levine R, Beaman KD. Novel role for the N-terminus domain of the a2 isoform of vacuolar ATPase in interleukin-1beta production. Hum Immunol 2007; 68:469–477.
16. Kwong C, Sachs AG, Beaman KD. Tumor-associated a2 vacuolar ATPase acts as a key mediator of cancer-related inflammation by inducing pro-tumorigenic properties in monocytes. J Immunol 2011; 186:1781–1789.
17. Critchley HO, Kelly RW, Lea RG, Drudy TA, Jones RL, Baird DT. Sex steroid regulation of leukocyte traffic in human decidua. Hum Reprod 1996; 11:2257–2262.
18. Jones RL, Kelly RW, Critchley HO. Chemokine and cyclooxygenase-2 expressions in human endometrium coincides with leukocyte accumulation. Hum Reprod 1997; 12:1300–1306.
19. Esplin MS, Romero R, Chaiworapongsa T, Kim YM, Edwin S, Gomez R, Mazor M, Adashi EY. Monocyte chemotactic protein-1 is increased in the amniotic fluid of women who deliver preterm in the presence or absence of intra-amniotic infection. J Matern Fetal Neonatal Med 2005; 17:365–373.
20. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8:958–969.
21. Gustafsson C, Mjosberg J, Matussek A, Geffers R, Matthiesen L, Berg G, Sharma S, Buer J, Ernerudh J. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. PLoS One 3 2008; e2078.
22. Mor G, Abrahams VM. Potential role of macrophages as immunoregulators of pregnancy. Reprod Biol Endocrinol 2003; 1:119.
23. Hustin J, Jauniaux E, Schaaps JP. Histological study of the materno-embryonic interface in spontaneous abortion. Placenta 1990; 11:477–486.
24. Kadyrov M, Kingdom JC, Huppertz B. Divergent trophoblast invasion and apoptosis in placental bed spiral arteries from pregnancies complicated by maternal anemia and early-onset preeclampsia/intrauterine growth restriction. Am J Obstet Gynecol 2006; 194:557–563.
25. de Groot CJ, O'Brien TJ, Taylor RN. Biochemical evidence of impaired trophoblastic invasion of decidual stroma in women destined to have preeclampsia. Am J Obstet Gynecol 1996; 175:24–29.
26. Ball E, Bulmer JN, Ayis S, Lyall F, Robson SC. Late sporadic miscarriage is associated with abnormalities in spiral artery transformation and trophoblast invasion. J Pathol 2006; 208:535–542.
27. Clark DA, Manuel J, Lee L, Chaouat G, Gorczynski RM, Levy GA. Ecology of danger-dependent cytokine-boosted spontaneous abortion in the CBA x DBA/2 mouse model. I. Synergistic effect of LPS and (TNF-alpha + IFN-gamma) on pregnancy loss. Am J Reprod Immunol 2004; 52:370–378.
28. Chavez DJ, McIntyre JA, Colliver JA, Faulk WP. Allogeneic matings and immunization have different effects on nulliparous and multiparous mice. J Immunol 1987; 139:85–88.
29. Teixeira Gomes RC, Verna C, Nader HB, dos Santos Simoes R, Dreyfuss JL, Martins JR, Baracat EC, de Jesus Simoes M, Soares JM Jr. Concentration and distribution of hyaluronic acid in mouse uterus throughout the estrous cycle. Fertil Steril 2009; 92:785–792.
30. Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, Tsuneyama K, Nagai Y, Takatsu K, Urakaze M, Kobayashi M. Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. Diabetes 2009; 58:2574–2582.
31. Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A, Aissat A, Guerre-Millo M, Clément K. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. J Clin Endocrinol Metab 2009; 94:4619–4623.
32. Clark DA, Foerster K, Fung W, He L, Lee L, Mendicino M, Markert UR, Gorczynski RM, Marsden PA, Levy GA. The fgl2 prothrombinase/fibroleukin gene is required for lipopolysaccharide-triggered abortions and for normal mouse reproduction. Mol Hum Reprod 2004; 10:99–108.
33. Redecha P, van Rooijen N, Torry D, Girardi G. Pravastatin prevents miscarriages in mice: role of tissue factor in placental and fetal injury. Blood 2009; 113:4101–4109.
34. Marikawa Y, Alarcon VB. Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. Mol Reprod Dev 2009; 76:1019–1032.
35. Aplin JD. Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. J Cell Sci 1991; 99:681–692.
36. Guvendag Guven ES, Okur H, Beksac MS. Placental fas/fas ligand expression in early pregnancy losses. Am J Reprod Immunol 2008; 60: 1–7.
37. Fitzgerald JS, Poehlmann TG, Schleussner E, Markert UR. Trophoblast invasion: the role of intracellular cytokine signalling via signal transducer and activator of transcription 3 (STAT3). Hum Reprod Update 2008; 14:335–344.
38. Bischof P, Campana A. A putative role for oncogenes in trophoblast invasion? Hum Reprod 2000; 15(suppl 6):51–58.
39. Ntrivalas E, Gilman-Sachs A, Kwak-Kim J, Beaman KD. The N-terminus domain of the a2 isoform of vacuolar ATPase can regulate interleukin-1beta production from mononuclear cells in co-culture with JEG-3 choriocarcinoma cells. Am J Reprod Immunol 2007; 57:201–209.
40. Lee GW, Boomer JS, Gilman-Sachs A, Chedid A, Gudelj L, Rukavina D, Beaman KD. Regeneration and tolerance factor of the human placenta induces IL-10 production. Eur J Immunol 2001; 31:687–691.
41. Rubesa G, Beaman KD, Beer AE, Haller H, Rukavina D. Expression of membrane form of the pregnancy associated protein TJ6 on decidual lymphocytes in the first trimester of pregnancy. J Reprod Immunol 1996; 30:17–27.
42. Li Y, Cai L, Wang H, Wu P, Gu W, Chen Y, Hao H, Tang K, Yi P, Liu M, Miao S, Ye D. Pleiotropic regulation of macrophage polarization and tumorigenesis by formyl peptide receptor-2. Oncogene 2011; (in press). Published online ahead of print 18 April 2011; DOI 10.1038/onc.2011.112.
43. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod 2009; 80:1036–1045.
44. Robertson SA, Sharkey DJ. The role of semen in induction of maternal immune tolerance to pregnancy. Semin Immunol 2001; 13:243–254.

BIOLOGY OF REPRODUCTION **85,** 1285 (2011)
Published online before print 19 October 2011.
DOI 10.1095/biolreprod.111.097048

ADDITIONS AND CORRECTIONS

CORRECTION

Mukesh Kumar Jaiswal, Alice Gilman-Sachs, Gerard Chaouat, and Kenneth D. Beaman. Placental ATPase Expression Is a Link Between Multiple Causes of Spontaneous Abortion in Mice. Biol Reprod 2011; 85:626–634. DOI:10.1095/biolreprod.111.092494

The manuscript referenced above inadvertently is missing the Open Access article footnote within the copyright footnote. The editors regret the error; the correct copyright footnote appears below.

Received: 25 March 2011.
First decision: 19 April 2011.
Accepted: 2 May 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363